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Nadia Gasmi. In-depth cellular and molecular characterization of neuroinflammation in epilepsy and its potential resolution by the use of mesenchymal stem cells. *Neurons and Cognition* [q-bio.NC]. Université de Lyon, 2020. English. NNT : 2020LYSE1025 . tel-03266996

HAL Id: tel-03266996

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N° d'ordre NNT : **2020LYSE1025**

THESE de DOCTORAT DE L'UNIVERSITE DE LYON
opérée au sein de
l'Université Claude Bernard Lyon 1

École Doctorale N° 476
Neurosciences et Cognition

Spécialité de doctorat : Neurosciences

Soutenue publiquement le 3 février 2020, par :

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**IN-DEPTH CELLULAR AND MOLECULAR CHARACTERIZATION OF
NEUROINFLAMMATION IN EPILEPSY AND ITS POTENTIAL
RESOLUTION BY THE USE OF MESENCHYMAL STEM CELLS**

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Epilepsy Institute - Europe

THIS PHD WORK WAS CONDUCTED AT THE 'CENTRE
DE RECHERCHE EN NEUROSCIENCES DE LYON'
(CRNL), IN THE EPILEPSY INSTITUTE IDÉE.

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THIS WORK WAS FUNDED BY A GRANT FROM THE
FONDATION POUR LA RECHERCHE MÉDICALE.



“Success is not final, failure is not fatal: it is the courage to continue that counts.”

~ Winston Churchill

ACKNOWLEDGEMENTS

These pages of acknowledgements mark the end of my doctoral years, and I would not have reached this moment without all the people listed below who have participated in the successful completion of this thesis and to whom I would like to express my deepest gratitude.

First, I would like to thank the Fondation pour la Recherche Médicale for giving me the opportunity to conduct this doctoral work by providing financial support (FRM grant number ECO20160736074 to NG).

I would like to acknowledge Dr. Annamaria VEZZANI and Dr. Emmanuel PINTEAUX for the great honor they have accorded me by agreeing to review this thesis. I sincerely thank you for the time you devoted for reviewing my work, for the great interest you have shown, for the constructive feedback on the experimental results and for your great kindness.

I also express my gratitude to Pr. Anne DIDIER and Dr. Nadia SOUSSI-YANICOSTAS for accepting to evaluate my work and being members of my PhD defense jury. Thank you for letting my defense be an enjoyable moment, and for your valuable comments and suggestions.

The rest of these acknowledgements will be written in French.

Je tiens à remercier très sincèrement l'ensemble des personnes qui, par leurs connaissances, leurs compétences et leurs qualités humaines ont contribué à l'aboutissement de ce travail de recherche.

Je commencerai par remercier mes directeurs de thèse, le Dr. Laurent BEZIN et le Pr. Sylvain RHEIMS, pour la confiance qu'ils m'ont témoignée en acceptant la direction scientifique de ces travaux. Bien que constamment sollicités et chargés de très nombreuses responsabilités, je vous remercie tous les deux d'avoir su vous rendre disponibles pour mon travail de thèse. J'espère qu'au travers de ce manuscrit vous trouverez une partie des efforts que j'ai fournis pour être à la hauteur de la confiance que vous m'avez accordée.

Sylvain, merci de m'avoir accueillie au sein de l'équipe TIGER et pour ta constante bienveillance à mon égard. Je te remercie également d'avoir contribué au bien déroulé de mes années de thèse, ainsi que pour tes conseils et avis scientifiques avisés. Enfin, merci de m'avoir permis d'assister à différents congrès et d'y représenter l'équipe.

Laurent, merci de m'avoir guidée toutes ces années, depuis mon arrivée pour un stage dans le laboratoire il y a déjà cinq ans jusqu'à la fin de cette thèse. Je ne pensais sincèrement pas que ce mail envoyé en octobre 2014 pour une demande de stage de Master 1 au sein de l'équipe TIGER me mènerait jusqu'ici ! Merci de m'avoir encadrée depuis lors et prodigué une formation à la recherche de qualité, ainsi que pour toutes les connaissances scientifiques et techniques dont tu m'as fait profiter. Cela a été un plaisir et un véritable honneur d'être ton étudiante. Merci pour la confiance que tu m'as accordée sur les projets que j'ai pu mener et

tes précieux conseils. Merci pour tes encouragements constants à toujours faire mieux, et à ne pas seulement essayer parce que « essayer, c'est échouer ! ». Et enfin, merci pour ta bienveillance quotidienne avec ceux qui t'entourent, pour ton oreille attentive et pour ton écoute, toujours sans jugement. Tu donnes tout son sens à la citation qui dit : « Ce que nous faisons pour nous même disparaît avec nous. Ce que nous faisons pour les autres et le monde est immortel et demeure. » Merci de faire autant pour les autres.

L'ensemble de cette thèse n'aurait pu aboutir sans l'implication de plusieurs membres de l'équipe TIGER. Je remercie en particulier Béatrice GEORGES, Wanda GRABON et Amor BELMEGUENAI et Sandrine BOUVARD pour leur grande contribution aux expérimentations dont les résultats sont présentés dans ce manuscrit. Tout d'abord, un énorme merci à toi BÉA pour avoir été là dès le départ et pendant toutes ces années, pour ton aide expérimentale, mais aussi pour ta bienveillance et ta patience au quotidien. Amor, merci pour l'apprentissage de l'électrophysiologie, pour le temps passé sur les expérimentations du projet « cellules souches », pour ta patience et ta disponibilité. Enfin, un très grand merci à toi Wanda pour ton investissement dans ce même projet, ta rigueur et ton intérêt permanent, ainsi que pour ta bonne humeur, ta grande gentillesse et les formidables jeux de mots expérimentaux. Cela a été un réel plaisir de travailler avec toi cette dernière année. Enfin, je te remercie également Sandrine pour ton aide dans les expérimentations durant la première partie de ma thèse et ta gentillesse.

Je tiens également à exprimer ma reconnaissance envers Rachel Shadi ALARY, Gwenndal DUTENDAS, Théo ELIA et Jérémy FOURIÉ qui, au cours leur passage dans l'équipe pendant quelques semaines à quelques mois, ont participé à l'acquisition de résultats ayant contribué à cette thèse.

Au-delà de la science, les membres de l'équipe TIGER au sens large forment une famille dont on est heureux de faire partie au quotidien. Merci de m'avoir si bien accueillie il y a déjà cinq ans pour un stage de première année de master, et de m'avoir permis de rester et d'apprendre à vos côtés depuis lors. Merci à vous tous, Amor, Béatrice, Frédérique, Hayet, Jacques, Laurent, Michaël, Nadine, Victor, Sandrine, Selena, Sylvain, Thomas, Wanda. Merci pour votre bienveillance, votre générosité, pour les blagues, les rires, les moments conviviaux partagés, et pour votre bonne humeur constante. Plus que jamais dans ces derniers mois de thèse, je suis convaincue que le travail de recherche est loin d'être un travail solitaire et qu'il représente une véritable aventure humaine. Je conclurai sur mes années au sein de l'équipe en citant William Faulkner qui a dit : « vous ne pouvez pas nager vers de nouveaux horizons avant d'avoir le courage de perdre de vue le rivage ». Je m'éloigne à présent de ce rivage qu'a été l'équipe TIGER ces cinq dernières années en espérant trouver dans la suite de mon avenir professionnel de toutes aussi belles personnes que vous l'êtes.

Je tiens également à remercier toutes les personnes avec qui j'ai eu la chance de travailler ces dernières années, en commençant par le Centre d'Imagerie Quantitative Lyon Est (CIQLE), avec en particulier Denis RESSNIKOFF, Batoule SMATI, Bruno CHAPUIS et Annabelle BOUCHARDON. Merci pour votre aide sur les outils de microscopie que j'ai utilisés

tout au long de ces années, votre disponibilité constante et votre patience. Je remercie également Anne RUIZ avec qui j'ai eu l'occasion de faire quelques expérimentations, pour son temps, son expertise et sa gentillesse.

Je remercie le Pr. Marc GUÉNOT, qui nous a donné l'accès aux pièces résequées des patients opérés à l'hôpital neurologique et neurochirurgical Pierre Wertheimer de Lyon et le Dr. Nathalie STREICHENBERGER de nous avoir donné accès aux conclusions anatomo-pathologiques.

J'exprime ma gratitude envers le Dr. Etienne AUDINAT et le Dr. Romain MARIGNIER pour avoir pris le temps d'évaluer mon travail en acceptant de faire partie de mon Comité de Suivi Individuel Doctoral.

Je tiens également à témoigner toute ma reconnaissance aux membres du corps enseignant de l'équipe pédagogique de Neurosciences de l'Université Claude Bernard Lyon 1 que j'ai eu le plaisir de côtoyer ces trois dernières années. Merci de m'avoir offert l'opportunité de découvrir l'enseignement et la pédagogie au travers de cette expérience extrêmement enrichissante.

Enfin, je remercie de façon plus large les membres de l'Institut des Épilepsies et l'ensemble des membres du Centre de Recherche en Neurosciences de Lyon que j'ai eu la chance de côtoyer durant ces quelques années, surtout depuis l'ouverture du Neurocampus.

Au terme de ce parcours, je remercie enfin celles et ceux qui me sont chers et que j'ai quelque peu délaissés durant ces derniers mois pour achever cette thèse. Parce qu'une thèse ce n'est pas seulement du travail de laboratoire, c'est aussi trois ans de vie avec des hauts et des bas, je voudrais très sincèrement remercier mes proches, famille et amis qui, de près ou de loin, m'ont accompagnée par leurs attentions et encouragements tout au long de ces années. Peut-être parce que le rendu de ce manuscrit est proche de la période des fêtes et des vœux de fin d'années, je profite de cette occasion pour leur écrire ici à quel point je suis heureuse qu'ils soient présents dans ma vie.

Je commencerai par remercier très profondément Aurélie, Baptiste, Inès, Lise, Mathilde, Maxime et Rémi. Merci d'avoir été là, merci pour les rires, les jeux, l'écoute, le soutien et les longues discussions. Merci pour les voyages, les week-end, les sorties et tout le reste. Merci d'être vous tout simplement. Indubitablement, ces dernières années n'auraient pas été les mêmes sans vous.

Baptiste, Inès, un merci particulier pour avoir partagé durant cette dernière période de thèse les sessions rédactions et les pauses café inhérentes à ces sessions. Finir au même moment nos thèses respectives a grandement contribué à l'achèvement de ce travail.

Merci à Lisa et Samantha, avec qui j'ai eu le plaisir de partager le bureau 106, et qui depuis sont devenues des amies précieuses que même la distance et les chemins différents n'ont pas éloigné.

Merci à tous les autres amis et camarades doctorants de la communauté des « Neurosciences et Cognition » que j'ai eu plaisir à côtoyer ces dernières années et qui n'ont fait qu'enrichir positivement mon expérience de thèse.

Et enfin merci à tous les autres non cités individuellement ici qui, parfois même de loin, sont toujours là quand il le faut.

Pour finir, un *merci* ne suffit pas pour témoigner toute ma gratitude envers mes parents et mes sœurs, Inès et Sonia, pour avoir toujours été présents, avoir grandement participé à ma réussite et pour m'avoir toujours encouragée à aller au bout de ce que j'entreprenais. Cette thèse est aussi la vôtre. Merci de croire en moi dans tout ce que je réalise et de m'avoir toujours témoigné votre soutien infaillible et inconditionnel.

RÉSUMÉ

Malgré la diversité d'antiépileptiques disponibles ayant pour but la réduction de l'excitabilité neuronale, près d'un tiers des patients souffrant d'épilepsie du lobe temporal demeurent résistants aux traitements. La neuroinflammation est aujourd'hui considérée comme une composante majeure contribuant non seulement à la mise en place des crises d'épilepsie et leur perpétuation, mais également aux troubles cognitifs et psycho-affectifs associés à la pathologie. Dans ce travail de thèse, nous avons dans un premier temps cherché à caractériser cette inflammation dans des pièces réséquées de patients épileptiques, avant de l'étudier dans des modèles animaux, des phases les plus précoces de l'épileptogénèse faisant suite à l'agression cérébrale aux phases plus tardives de l'épilepsie chronique. Sur la base des marqueurs prototypiques de l'inflammation que nous avons mesurés, nous avons mis en évidence que l'inflammation peut être inexistante au sein du foyer épileptique du patient, mais que lorsqu'elle est présente, elle est dite de bas bruit, remettant ainsi en cause le dogme invoquant la neuroinflammation comme condition *sine qua non* associée à la symptomatologie de l'épilepsie. De surcroît, l'accès par les modèles animaux de la maladie à des phases précoces de la transformation du tissu sain en tissu épileptique (épileptogénèse) nous a permis de montrer que l'ampleur de la neuroinflammation mesurée pendant la phase chronique de l'épilepsie n'a de commune mesure avec l'inflammation explosive présente pendant les phases précoces de l'épileptogénèse. Nous avons par ailleurs évalué la contribution des cellules gliales parenchymateuses, à savoir les astrocytes et la microglie, au statut neuroinflammatoire en détectant in situ l'ARNm de l'IL-1 β considéré comme le marqueur prototypique de la réponse inflammatoire. Nous avons ainsi montré que, dans notre modèle, la microglie serait le principal producteur de molécules pro-inflammatoires dans la phase aigüe post-agression cérébrale, les astrocytes y participant également mais dans une bien moindre mesure.

La question posée ensuite a été celle de l'origine cellulaire de cette neuroinflammation cérébrale. De nombreux travaux de la littérature ont montré le rôle manifeste de cellules immunitaires périphériques, les monocytes, qui infiltrent le parenchyme cérébral dans de nombreuses pathologies neurologiques et contribuent à la production de molécules inflammatoires. Le devenir de ces cellules dans le cerveau épileptique n'a été que peu exploré jusqu'à présent de par l'absence de marqueurs fiables permettant de les suivre dans le temps chez le rat. Nous avons identifié au cours de cette thèse deux marqueurs spécifiques, les chaînes de sulfates d'héparane ainsi que le CD68, nous ayant permis de suivre l'infiltration de

ces monocytes, leur transdifférenciation en monocytes-macrophages morphologiquement similaires aux cellules microgliales activées, ainsi que leur intégration au long cours dans le réseau microglial.

Nous avons ensuite cherché à moduler cette neuroinflammation par l'injection précoce de cellules souches mésenchymateuses (CSM) après l'agression cérébrale pro-épileptogène, ces cellules ayant été utilisées dans de nombreux modèles de maladies neurologiques pour leurs propriétés anti-inflammatoires et neuroprotectrices. Outre la comparaison d'une administration unique à celle d'une administration répétée intervenant à la suite de l'agression cérébrale, l'aspect novateur de notre projet résidait dans la voie intranasale choisie pour administrer les CSM dans notre modèle animal d'épilepsie. De façon surprenante, aucun effet sur la neuroinflammation moléculaire précoce n'a été mesuré. Néanmoins, il a été observé des modifications de la réponse inflammatoire cellulaire avec notamment une transdifférenciation plus marquée des monocytes infiltrants ayant conduit à une augmentation de leur volume cellulaire. Par ailleurs, au niveau fonctionnel, nous avons évalué la potentialisation à long-terme (LTP) des neurones pyramidaux de la couche CA1 de l'hippocampe, qui est un mécanisme sous-tendant les processus moléculaires et cellulaires de l'apprentissage. Nous avons montré que l'injection des CSM a permis de prévenir l'altération de la LTP, sans toutefois montrer d'effet additionnel de l'injection répétée de CSM par rapport à l'injection unique. Ces résultats ouvrent ainsi de nouveaux espoirs thérapeutiques pour les patients épileptiques souffrant de troubles cognitifs.

Mots clés : Inflammation, épilepsie du lobe temporal, cellules souches mésenchymateuses, intranasale, cytokines, monocytes, microglie

SUMMARY

Despite the diversity of anti-epileptic drugs available to reduce neuronal excitability, nearly one-third of patients with temporal lobe epilepsy (TLE) remain resistant to treatment. Accumulating evidence supports a role for neuroinflammation as a primary driver of epileptogenesis occurring after brain insults, as a self-perpetuating factor of epileptic seizure activity, but also as a major contributor to epilepsy co-morbidities such as cognitive dysfunctions and mental-health conditions. This thesis first attempted to characterize inflammation in the resected hippocampus of TLE patients, before studying it in rats during the chronic phase of epilepsy following status epilepticus (SE) or during the acute phase after brain insults. Based on the quantitation of prototypical inflammatory gene transcripts that we have measured, we have shown that some, not all, TLE patients may present with a hippocampal inflammatory status that is likely to correspond to low-grade inflammation, thus challenging the dogma invoking neuroinflammation as a prerequisite to the symptomatology of epilepsy. In addition, access to early phases of the disease in animal models has allowed us to show that the extent of neuroinflammation measured during the chronic phase of epilepsy has no common measure with the explosive inflammation present during the early phases of epileptogenesis. We evaluated the contribution of parenchymal glial cells, astrocytes and microglia, to inflammatory status by detecting *in situ* the mRNA of IL-1 β considered as the prototypic marker of the inflammatory response. We have shown that, in our model, microglia is the main producer of pro-inflammatory molecules in the acute phase after brain-insult, the astrocytes also participating, but to a much lesser extent.

The corollary of this first part was to define the cellular origin of this cerebral neuroinflammation. Numerous studies in the literature have shown the well-known role of peripheral monocytes, which infiltrate the brain parenchyma in many neurological diseases and contribute to the production of inflammatory molecules. The fate of these cells in the epileptic brain has been little explored so far due to the lack of reliable markers to follow them over time in rats. In this thesis, we identified two specific markers, heparan sulfate chains and CD68, which allowed us to monitor the infiltration of these monocytes, their transdifferentiation into monocyte-macrophages morphologically similar to activated microglial cells, as well as their long-term integration into the microglial network.

We then sought to modulate this neuroinflammation by early injection of mesenchymal stem cells (MSCs) after pro-epileptogenic brain damage, these cells having been used in many models of neurological diseases for their anti-inflammatory and neuroprotective

properties. In addition to comparing a single administration to a repeated administration of MSCs following the brain insult, the innovative aspect of our project was the intranasal route chosen to administer MSCs in our animal model of epilepsy. Surprisingly, no effect on early molecular neuroinflammation was measured. Nevertheless, changes in the cellular inflammatory response have been observed, including more pronounced transdifferentiation of infiltrating monocytes, leading to an increase in their cellular volume. In addition, at the functional level, we evaluated the long-term potentiation (LTP) of the hippocampal CA1 pyramidal neurons, which is a mechanism underlying the molecular and cellular processes of learning. We have shown that the injection of MSCs has prevented the alteration of LTP, but without any difference between the groups receiving single *versus* repeated MSCs administration. Hence, these results open up new therapeutic hopes for epileptic patients with cognitive disorders.

Key words: Inflammation, temporal lobe epilepsy, mesenchymal stem cells, intranasal, cytokines, monocytes, microglia

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ABBREVIATIONS

DNA	Desoxyribonucleic acid
AEDs	Anti epileptic drugs
ANOVA	Analysis of variance
APC	Antigen-presenting cell
ASDs	Anti seizure drugs
BBB	Blood brain barrier
BDNF	Brain-derived neurotrophic factor
blFP	Bilateral Fluid percussion
BMSCs	Bone marrow-derived mesenchymal stem cells
CA	Cornu Ammonis
CCL	C-C motif chemokine ligand
CCR	C-C motif chemokine receptor
CD	Cluster of differentiation
CNS	Central nervous system
COX	Cyclooxygenase
CSF	Cerebro-spinal fluid
CX3CL1	Chemokine (C-X3-C) motif ligand 1, fractalkine
CXCL	Chemokine (C-X-C) motif ligand
CXCR	Chemokine (C-X-C) motif receptor
DAMPs	Damage-associated molecular pattern
EC	Endothelial cells
EEG	Electroencephalography
ELISA	Enzyme-linked immunosorbent assay
ESCs	Embryonic stem cells
FACS	Fluorescence-activated cell sorting
GABA	Gamma-aminobutyric acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDNF	Glial cell-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
HGF	Hepatocyte growth factor
HIF-1α	Hypoxia-inducible factor 1-alpha
HMGB1	High mobility group box 1
HS	Hippocampal sclerosis
HSCs	Heparan Sulfate Chains
Iba-1	Ionised calcium binding adaptator molecule 1
IC	Intracranial
ICAM	Intracellular adhesion molecule
ICV	Intracerebroventricular

IFNγ	Interferon γ
IGF-1	Insulin-like growth factor-1
IHC	Immunohistochemistry
IL1β	Interleukine 1 beta
IL10	Interleukine 10
IL6	Interleukine 6
IL8	Interleukine 8
IL1R	Interleukine-1 receptor
IL1RA	Interleukine-1 receptor antagonist
ILAE	International league against epilepsy
IN	Intranasal
iNOS	Inducible nitric oxide synthase
iPSC	Induced Pluripotent Stem Cells
ISCT	International society for cellular therapy
ISH	<i>In situ</i> hybridization
ITGAM	Integrin alpha M
IV	Intravenous
LPS	Lipopolysaccharide
MCP-1	Monocyte chemoattractant protein-1
MIP-1α	Macrophage inflammatory protein-1 alpha
MMP	Metalloproteinase
mRNA	Messenger ribonucleic acid
MSCs	Mesenchymal stem cells
MWM	Morris water maze
ND	Not detectable
NF-κB	Nuclear factor kappa B
NGF	Nerve growth factor
NK	Natural killer
NMDA	N-methyl-D-aspartate
NSAIDs	Nonsteroidal anti-inflammatory drugs
NT-3	Neurotrophin-3
PAMPs	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PET	Positron emission tomography
PFA	Paraformaldehyde
PGE-2	Prostaglandin E2
Pilo-SE	Pilocarpine-induced <i>status epilepticus</i>
PTE	Post-traumatic epilepsy
RANTES	Regulated upon activation normal T-cell expressed and secreted
ROS	Reactive oxygen species
RT-qPCR	Reverse transcriptase-polymerase chain reaction

SDF-1	Stromal derived factor-1
SE	<i>Status epilepticus</i>
SEM	Standard error of the mean
TBI	Traumatic brain injury
TGF-β1	Transforming growth factor B1
TIMP	Tissue inhibitors of metalloproteinases
TLE	Temporal lobe epilepsy
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
TSG-6	Tissue necrosis factor alpha induced protein 6
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

CHAPTER 1

INTRODUCTION

GENERAL INTRODUCTION

Epilepsy is one of the most common neurological disease characterized by abnormal neural activity leading to seizures that are associated with multiple cognitive and neuropsychiatric comorbidities. Such a complex disease is not limited to a single form and may involve different clinical conditions with different symptoms, diagnostic criteria and therapeutic strategies. The etiology of these epilepsies may be either genetic, acquired or unknown. Acquired epilepsy, also known as symptomatic epilepsy, occurs as a result of a severe brain injury such as stroke, traumatic brain injury, *status epilepticus* or febrile seizure. After the initial insult, the brain is reshaped asymptotically during a latent phase of the illness, called epileptogenesis, which can last months to years. Following this phase, chronic epilepsy appears with the first spontaneous and recurrent seizures and their associated symptoms.

Temporal lobe epilepsy (TLE) is the most frequently diagnosed form of epilepsy. TLE are related to profound structural changes that affect the hippocampus, a structure of the limbic system involved in memory processes. These changes include neuronal loss and reactive gliosis. Other brain regions such as the amygdala, the site of emotions, may undergo changes during the progression of the disease, alterations correlated with the severity of psychiatric disorders. The pathophysiology of TLE also involves immune cells, including peripheral monocytes, which infiltrate the brain parenchyma and the diseased areas of the brain although their role is still being debated. Nowadays, is not well established whether these cells transdifferentiate an integrate the brain parenchyma, act in the brain and then leave or die in the brain.

Therapeutically, TLE is managed by anti-seizures drugs, which are effective for two out of three patients. However, the remaining one-third of patients do not respond to these treatments and are referred to as drug-resistant. For these patients, surgical resection of the epileptogenic focus may be considered although the procedure is not always successful, and some patients remain epileptic. Therefore, there is an urgent need to develop new treatments and novel alternative therapeutic strategies to act at all stages of the disease, from the earliest phases of epileptogenesis to the chronic phase.

To develop further antiepileptogenic, antiepileptic, or disease-modifying treatments, specific therapeutic targets needed to be identified. A considerable number of investigations have shown that brain inflammation, also referred to as neuroinflammation, has a major role in the development and maintenance of epilepsy. The study of neuroinflammation as a potential therapeutic target has also gained considerable attention in recent decades. The neuroinflammatory processes include the release of pro-inflammatory cytokines and chemokines and the activation of microglial cells and astrocytes. The infiltration of peripheral immune cells called leukocytes, including monocytes, also participates in early epileptogenesis processes. Experimental models of TLE have revealed that these monocytes highly infiltrate the brain parenchyma after a pro-epileptogenic insult. The challenge now lies in the long-term follow-up of these monocytes to determine their fate into the brain. Altogether, these neuroinflammatory components represent potential targets for effective novel therapeutics.

The use of stem cells in cell therapy has seen a rapid and increasing advance over the past fifty years. Stem cells are undifferentiated cells that are defined by their ability to self-renew and to differentiate into various cell types. These cells are found from the earliest stages of embryogenesis to adulthood, where they are present in different tissues and engaged in specific differentiation pathways. In regenerative medicine, stem cells are increasingly used for their widely described trophic and immunomodulatory properties. Mesenchymal stem cells (MSC) are one of the most broadly used types of stem cells and are characterized by their high self-renewal ability and their immunomodulatory and tissue repair properties, making them promising for a wide clinical use. MSC-based therapy has gained considerable attention due to the multilineage differentiation potential of MSC as well as their high amount of secreted factors (immunomodulators, anti-apoptotic, angiogenic, chemo-attractants, anti-scarring and growth factors) that can act in a paracrine or endocrine way. In cell therapy, their advantages over other sources of stem cells are related to: 1. their relatively easy access; 2. their possible use in autologous as well as allogeneic transplantation protocols because of their low immunogenicity; and 3. their proven effect in numerous diseases, including neurological disease. MSC have been widely reported as cells able to stimulate or participate to the regeneration of diverse tissues and organs, including the central nervous system. In epilepsy, these stem cells have already been used in a handful of clinical trials and

have shown promising effects. In preclinical models of epilepsy, the use of MSCs has shown effects on the duration and frequency of seizures, microglial and astrocyte activation, neuronal loss or synaptic neuroplasticity. Yet, many questions remain to be answered, particularly regarding the effects of MSCs on neuroinflammation, on cognition and learning ability as well as on neuropsychiatric disorders. The route of stem cell administration also appears to be an important factor in the success or failure of the cell therapy protocol. Indeed, numerous studies in which cells were injected intravenously or intraperitoneally have shown that MSCs remain trapped in peripheral tissues and never reach the brain.

Despite considerable breakthroughs in the management and treatment of epileptic patients, many questions remain unsolved. The underlying unknown mechanisms of epileptogenesis at the cellular and molecular level, as well as the more precise role of neuroinflammation from a brain insult to the onset of seizures are not fully understood. The further question to be addressed is how this neuroinflammation contributes to the heterogeneity of clinical outcomes in epileptic patients. Moreover, it is not clear to what extent glial cells and peripheral immune cells that infiltrates the brain will contribute to these neuroinflammatory processes due to the lack of reliable markers for the tracking of the different cell population over time. Finally, innovative non-pharmacological therapies addressing the burden that heavy treatments can represent for epileptic patients need to be developed. Non-invasive cell therapy by intranasal administration seems to be a promising avenue that requires preclinical study to determine its effects on neuroinflammation and pathological processes.

Altogether, these elements are at the basis of this doctoral research work, whose aims were:

- To provide an in-depth characterization of cellular and molecular inflammation in the hippocampus during epilepsy in TLE patients, and from epileptogenesis to chronic epilepsy in experimental models;
- To identify two specific markers, heparan sulfate chains and CD68, allowing to monitor the infiltration of monocytes after SE and their transdifferentiation into monocyte-macrophages morphologically similar to activated microglial cells;
- To compare brain inflammation after *status epilepticus* in Sprague-Dawley rats from two vendors;

- And to define whether TLE can be prevented, reversed or alleviated by mesenchymal stem cell therapy by studying the effect of intranasally injected MSCs after SE on the acute inflammatory response and on neuroplasticity.

The current work was conducted in four successive studies using different methodology to first study the neuroinflammation in epileptogenesis and epilepsy at the cellular and molecular level, in human and animals; and then to deepen the research on mesenchymal stem cells as a promising therapeutic tool targeting neuroinflammation by testing their effects *in vivo* after intranasal administration in a model of epilepsy.

After a review of the literature concerning epilepsy, inflammation and mesenchymal stem cells as a cell therapy tool, each study will be reported with a separate introduction, methods, results and discussion section. A general discussion will establish the link between the four studies and will be followed by a conclusion highlighting the perspectives and ongoing studies that follow this work.

STATE OF THE ART

I. Temporal Lobe Epilepsy (TLE)

Epilepsy is the third most common neurological disorder, after Alzheimer disease and dementia, affecting about 65 million people worldwide. This chronic/lifelong medical condition is defined as a central nervous system disorder which affects the neuronal activity, causing unprovoked seizures and associated with behavioral comorbidities (**Box 1**) (Fisher et al., 2014). Epilepsy was first described during the antiquity in Babylonian texts (Magiorkinis et al., 2010). At that time, popular belief was that seizures were of supernatural origin and that they were induced by the gods. Hippocrate was the first to claim that epilepsy was a disease of the body and denied that seizures were divinely provoked (Rektor et al., 2013).

The impact of this disability is a major health problem insofar as the social and professional life of these patients can be severely affected by the unpredictable occurrence of seizures as well as by the behavioral outcomes. An epileptic seizure is triggered in the brain by an abnormal excessive or synchronous neuronal activity, and, on a symptomatic level, leads to objective or subjective transient behavioral changes (Devinsky et al., 2018). The term epileptic focus refers to the place where abnormal neural activity will arise from. As described by the International League Against Epilepsy, this epileptic focus will determine in which way seizures are likely to occur: when it is located in one or more specific brain region, the resulting seizure is defined as focal, while generalized seizures are the result of the whole-brain distribution of abnormal neural activity (Devinsky et al., 2018). Generalized seizures are generally bilateral and are characterized as seizures with a single epileptic focus. Symptomatically, generalized seizures can be tonic-clonic, myoclonic, tonic, clonic, atonic or absence seizures. Focal

BOX 1 | DEFINITION (Fisher, 2014)

Operational clinical definition of **epilepsy** by the International League Against Epilepsy (ILAE):

“**Epilepsy** is a disease of the brain defined by **any of the following conditions**:

- At least two unprovoked (or reflex) seizures occurring > 24 hours apart;
- One unprovoked (or reflex) seizure and a probability of further seizures similar to the general recurrence risk (at least 60%) after two unprovoked seizures, occurring over the next 10 years;
- Diagnosis of an epilepsy syndrome.”

seizures usually arise from a single hemisphere and the symptomatic manifestation of the seizure will depend on the function of the brain area where the seizure is generated (Devinsky et al., 2018).

Therefore, there is no such thing as a single type of epilepsy, but rather several different types of epilepsy. The etiology of these epilepsies is wide ranging and most of the time unknown for many patients. Among the recognized causes involved in the onset of epilepsy are genetic mutations, autoimmune or infectious diseases, or even brain insults such as traumatic brain injury or stroke. Epilepsy triggered in adulthood are usually acquired. Mesial temporal lobe epilepsy (TLE) is the most commonly diagnosed form of acquired epilepsy in adults. After the initial insult, epileptogenesis corresponds to the seizure-free latent phase during which healthy brain tissue is turned into epileptic tissue, leading ultimately to epileptic seizures. This phase may last from a few months to several years.

Epilepsy is diagnosed on the basis of the occurrence of seizures in conjunction with electroencephalographic (EEG) and imaging data. The first line treatment is anti-epileptic drugs (AEDs), also called anti-seizure drugs (ASDs), of which more than 20 drugs are routinely used and approved by the US Food and Drug Administration as well as by the European Medicines Agency (Banerjee et al., 2009; Devinsky et al., 2018). However, the available therapies are primarily symptom-driven and fail to address the underlying disease processes. Among all epileptic patients, nearly 70% respond correctly to existing treatments, although complaining of severe mind-numbing side effects. Unfortunately, the remaining one-third of epileptic patients are unresponsive to available therapies.

Quality of life among these drug-resistant patients with epilepsy is highly impacted since they also suffer depression and anxiety, a sensitivity to mental health issues that is commensurately increased or worsen by the number of antiepileptic drugs used (Brooks-Kayal et al., 2013; Eddy et al., 2011; Ortinski and Meador, 2004; Park and Kwon, 2008; Stephen et al., 2017). **Figure 1** resumes the effects of drug-resistant epilepsy on the quality of life of patients. Epilepsy surgery, which consist on the resection of the epileptic focus or foci, is the only effective way to permanently control seizures, but only some patients are likely to undergo such a resection procedure. Moreover, this surgery is not always effective since one in three patients who have been operated will experience persistent epileptic seizures after

the procedure (Harroud et al., 2012). In 2005, a working group commissioned by the ILAE worked, among other things, on the most appropriate terminology to define patients who no longer have seizures after their resection surgery, and their recommendations were adopted in 2013 by the ILAE Executive Committee (Fisher et al., 2014). The terms “remission” and “cure” were excluded, the first because it implied a suspension of the disease rather than an absence of disease, the second because it suggested that these patients would no longer have seizures, while it was well-documented by epidemiological studies that with a history of epilepsy, the risk of having new seizures at later ages is higher than that of the rest of the population. Hence the term “resolution” was adopted: when the epilepsy is “resolved”, the patient is no longer epileptic, but this does not ensure that the epilepsy will not return. Epilepsy is now considered as “resolved” when the patient has been seizure-free for the most recent 10 years and off medications for the most recent 5 years, which predicts future freedom from seizures in a high percentage of cases (Fisher et al., 2014).

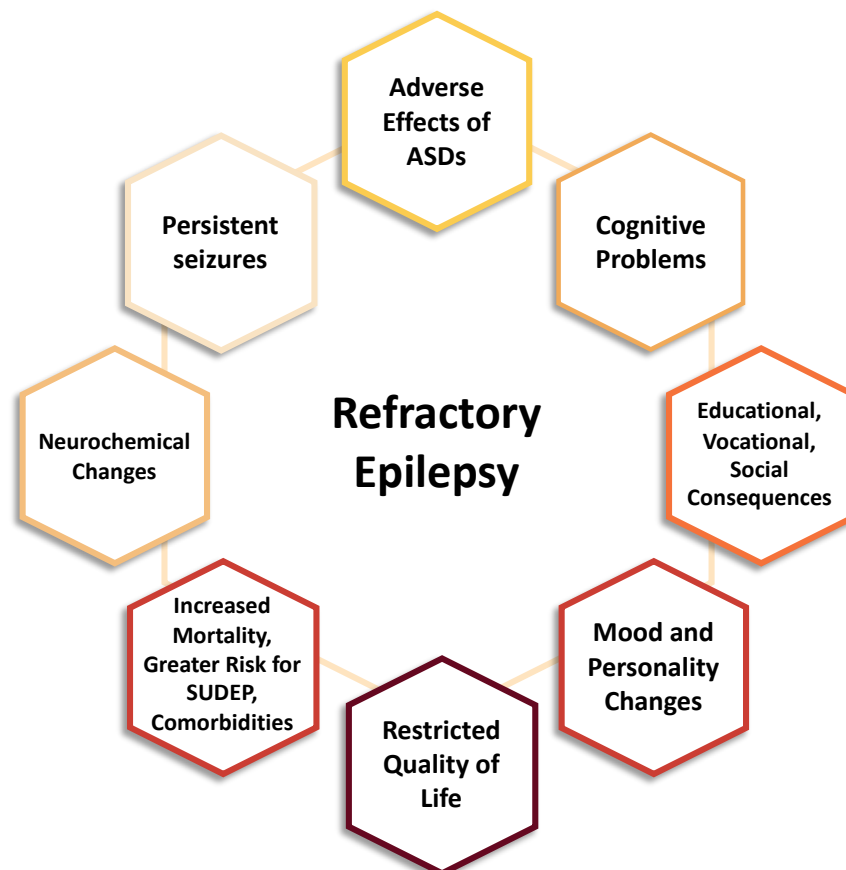


Figure 1. Effect of refractory epilepsy on patients' quality of life. “The hexagons depict the impact of recurrent seizures on the quality of life of patients with refractory epilepsy. Refractory epilepsy is associated with increased morbidity and mortality, serious psychosocial consequences, cognitive problems, and reduced quality of life.” Adapted from Tang et al., 2017.

Epilepsy accounts for a significant proportion of the world's disease burden, with each year five million people diagnosed with the disease. The estimated proportion of the general population with active epilepsy (i.e patients with continuing seizures or with need treatment) at a given time is between 4 and 10 per 1,000 people (World Health Organization, www.who.int) with an equivalent ratio between female and male. The meta-analysis carried out in 2017 by Fiest established, by conducting research on all the epidemiological articles on epilepsy published between 1985 and 2013, that the prevalence of active epilepsy was 6.38 per 1,000 and did not vary by age group or sex (Fiest et al., 2017). It is noteworthy that the incidence of the disease is quite different between high-income and low-income countries, with an estimated new case rate of 49 per 100,000 inhabitants in high-income countries compared to 139 per 100,000 in low-income countries (World Health Organization, www.who.int). In addition, nearly 80% of people with epilepsy today live in low-income countries. These strong differences may be attributed in part to the higher risk of endemic conditions (such as malaria or central nervous system infections, more common in tropical areas) in these countries that may be a precursor to the development of epilepsy, to the higher incidence of road traffic injuries, or to the fact that prevention and care services are less easily available.

Societal impact of epilepsy is mainly related to the severity of the disease, and whether the frequency of seizures in addition to the behavioral, psychological and social co-morbidities makes it possible to pursue a normal social and professional life. According to the World Health Organization, the risk of premature death in people with epilepsy is up to three times higher than for the general population and life expectancy of epileptic patients depends mainly on their responsiveness to treatment and the country they live in. Indeed, some of the causes of premature death of epileptic patients living in low-income countries such as falls, drownings, burns or prolonged seizures are generally prevented in high-income countries.

1. Etiology and symptomatology

The question concerning the origin of epilepsy is often relevant to the subsequent choice of treatment. As mentioned above, etiologies are classified into three broad categories: genetic epilepsy, symptomatic epilepsy acquired as a result of structural or metabolic modification, and epilepsy of unknown cause. In patients for whom recurrent epileptic seizures are not congenital but appears later in life, seizures occur commonly after a brain insult. Mesial temporal lobe epilepsy (TLE) is the most commonly diagnosed form of acquired epilepsy, usually associated with a seizure onset in the hippocampal formation (Engel, 2001). After an acute brain injury, not all patients develop epilepsy, but there are various risk factors and underlying epileptogenic processes that may or may not lead to the development of epilepsy in the long term. These risk factors and epileptogenic processes are described in **Figure 2** (Klein et al., 2018). Temporal lobe epilepsy also has the highest number of drug-resistant patients. While approximately 1 in 3 patients of epileptic is drug-resistant, this number rises to or exceeds two thirds in TLE patients (Schmidt and Löscher, 2005). The drug-resistance has been defined by the ILAE in 2010 as the “failure of adequate trials of at least two tolerated and appropriately chosen and used ASDs to achieve sustained seizure freedom” (Kwan et al., 2010). Several possible underlying factors have been suggested to explain this drug-resistance, but the vast majority of hypotheses remain uncertain today (Tang et al., 2017). **Figure 3** resumes the major hypotheses well-reviewed by Tang and colleagues in 2017.

TLE diagnosed most of the time in adulthood occurs frequently after an acute brain injury such as cerebrovascular accident, infections, traumatic brain injury (TBI) or status epilepticus (SE) (Klein et al., 2018). Among the epileptogenic brain insults, status epilepticus (SE) is an abnormally prolonged or recurrent epileptic seizure that is a serious, life-threatening medical emergency (Wang and Chen, 2018). Convulsive SE in human, especially in children, is a neurological emergency that will lead in 13%-74% to the development of chronic epilepsy (Raspall-Chaure et al., 2006). When SE occurs during childhood, it is often associated with hippocampal injury and mesial temporal sclerosis, as well as neurological, cognitive, and behavioral impairments.

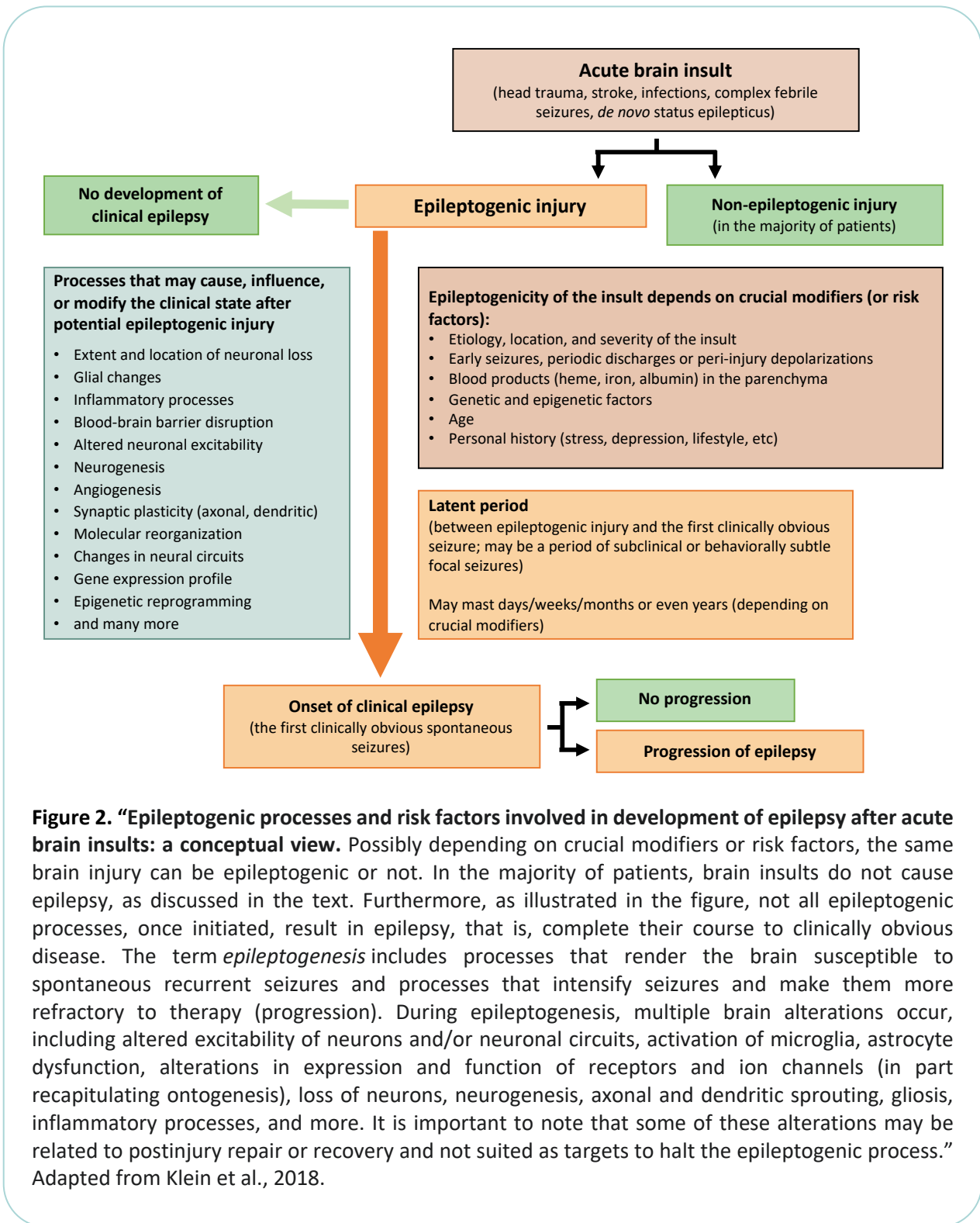


Figure 2. “Epileptogenic processes and risk factors involved in development of epilepsy after acute brain insults: a conceptual view. Possibly depending on crucial modifiers or risk factors, the same brain injury can be epileptogenic or not. In the majority of patients, brain insults do not cause epilepsy, as discussed in the text. Furthermore, as illustrated in the figure, not all epileptogenic processes, once initiated, result in epilepsy, that is, complete their course to clinically obvious disease. The term *epileptogenesis* includes processes that render the brain susceptible to spontaneous recurrent seizures and processes that intensify seizures and make them more refractory to therapy (progression). During epileptogenesis, multiple brain alterations occur, including altered excitability of neurons and/or neuronal circuits, activation of microglia, astrocyte dysfunction, alterations in expression and function of receptors and ion channels (in part recapitulating ontogenesis), loss of neurons, neurogenesis, axonal and dendritic sprouting, gliosis, inflammatory processes, and more. It is important to note that some of these alterations may be related to postinjury repair or recovery and not suited as targets to halt the epileptogenic process.” Adapted from Klein et al., 2018.

Epileptogenic brain insults are followed by a latent period, which may last months to years, before chronic recurrent seizures start. The multitude of structural and cellular mechanisms that convert the hippocampal formation to become chronically hyperexcitable after transient insult to the brain are summarized under the term *epileptogenesis*. In TLE, the epileptic focus is located within a circumscribed area of the mesial temporal lobe, often including, in addition to the hippocampus, the entorhinal cortex and amygdala. The main symptom of TLE is recurrent and spontaneous seizures as a chronic condition. Epileptic seizures are caused by sudden and highly synchronized electrical discharges of neurons arising in the temporal lobe, which disrupt normal brain function from a few seconds to several hours in the so-called postictal phase. Potential routes of seizure propagation are described in **Figure 4** (Löscher et al., 2008). Given the location of the epileptic foci in the limbic system, symptomatology of TLE includes well-defined comorbidities such as cognitive and psychiatric disorders, including depression, anxiety, panic disorder, mood disorder and other mental health disorder (Hermann et al., 2008; Kandratavicius et al., 2012). The hurdle lies in the fact that these cognitive and behavioral disorders are often worsened by the anti-seizure drugs which can exert detrimental psychopathological side-effects that are not fully understood (Cavanna et al., 2010; Eddy et al., 2011).

Drug-resistance in epilepsy: multiple hypotheses, few answers (from Tang et al., 2017)

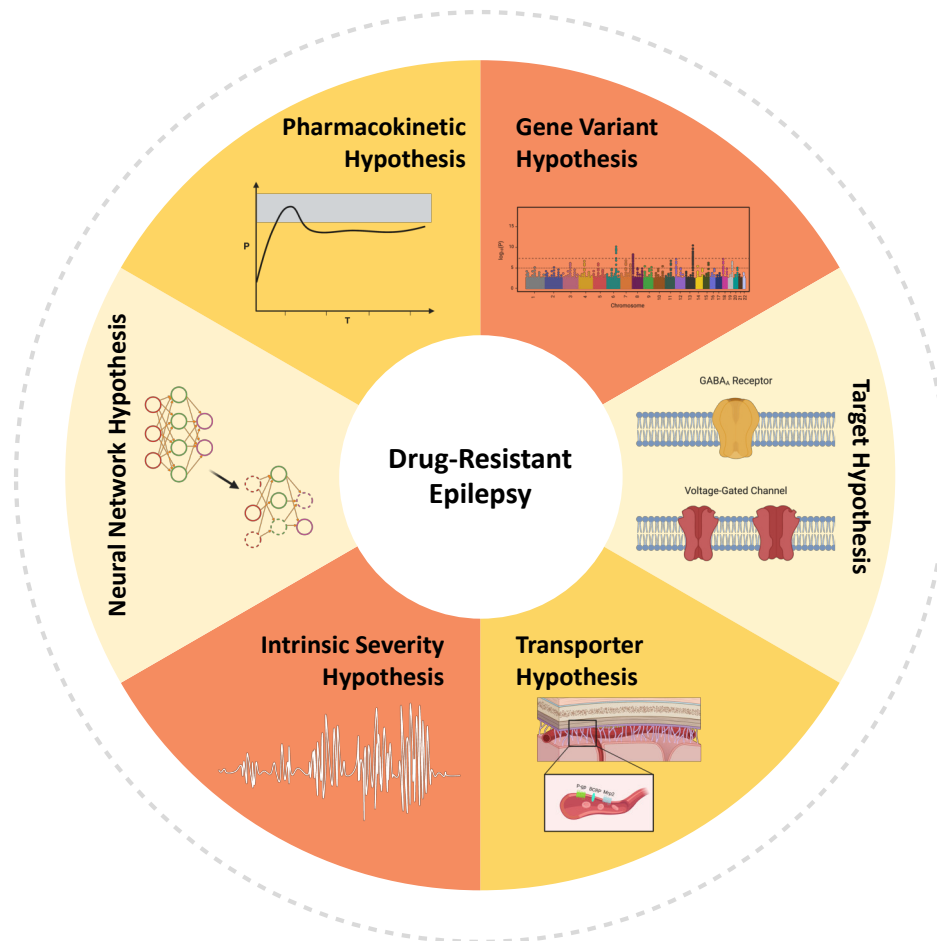


Figure 3. “Overview of proposed hypotheses for possible underlying mechanism(s) of antiseizure drug (ASD) resistance. (1) The gene variant hypothesis states that variations in genes associated with ASD pharmacokinetics and pharmacodynamics cause inherent pharmacoresistance. These genes include metabolic enzymes, ion channels, and certain neurotransmitter receptors that are targets for ASDs. (2) The target hypothesis postulates that alterations in the properties of ASD targets, such as changes in voltage-gated ion channels and neurotransmitter receptors (e.g., GABA_A receptor), result in decreased drug sensitivity and thus lead to refractoriness. (3) The transporter hypothesis states that overexpression of ASD efflux transporters at the blood–brain barrier in epilepsy leads to decreased ASD brain uptake and thus ASD resistance. (4) The intrinsic severity hypothesis proposes that common neurobiological factors contribute to both epilepsy severity and pharmacoresistance. (5) The neuronal network hypothesis states that seizure-induced degeneration and remodeling of the neural network suppresses the brain’s seizure control system and restricts ASDs from accessing neuronal targets. (6) The pharmacokinetic hypothesis proposes that overexpression of drug efflux transporters in peripheral organs decreases ASD plasma levels, thereby reducing the amount of ASD available to enter the brain and reach the epileptic focus.” Adapted from Tang et al., 2017.

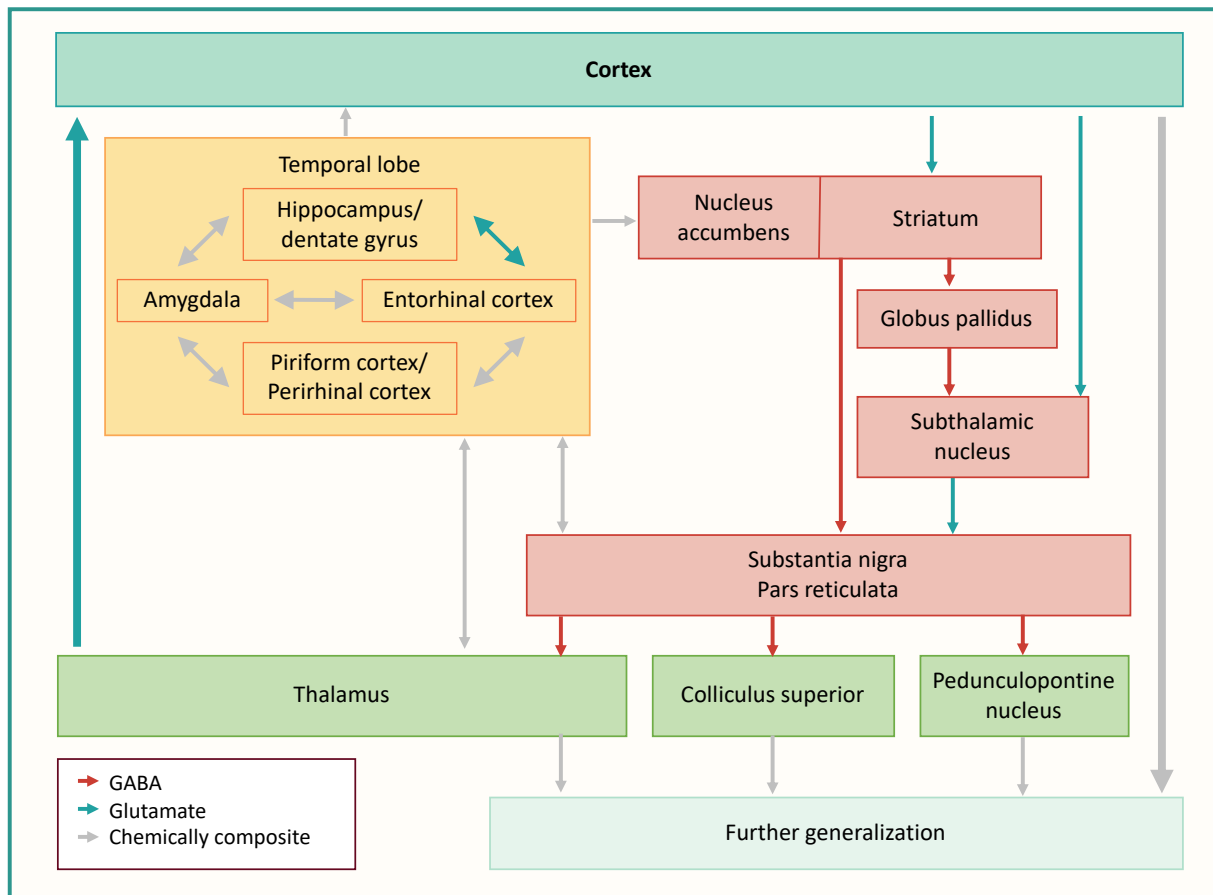


Figure 4. “Potential routes of seizure propagation of partial (limbic) and secondarily generalized seizures in temporal lobe epilepsy (TLE) and suitable anatomical targets for therapeutic interventions. In TLE and other types of epilepsy, seizure activity does not spread randomly throughout the brain but rather is generated and propagated by specific anatomical routes. At least in part, the spread of seizures follows pathways that are also utilized in normal movements. Although seizures can be initiated experimentally by a large number of neuronal manipulations, the behavioral alterations associated with different means of seizure induction are often remarkably similar, suggesting that certain propagation pathways might function as common denominators for the development of certain types of epileptic seizures, independent of the specific pathological condition involved in their induction. The same is true for clinical epilepsy, where different types of brain insults can lead to the same type of epilepsy, for example, TLE. In TLE, seizures emanate from the temporal lobe, most often from the hippocampus, entorhinal cortex or amygdala, which therefore form targets for cell- or gene-based therapies aimed at suppressing seizure initiation. Several regions within the temporal lobe, including the hippocampus and dentate gyrus, the entorhinal cortex, the amygdala and the piriform and perirhinal cortices, form an initiating, epileptic circuit. Based on experimental evidence from the kindling and other models of TLE, the piriform cortex is critically involved in the amplification and propagation of paroxysmal activity emanating from the amygdala or hippocampus. Propagation of seizure activity from the temporal lobe to the cortex, basal ganglia, thalamic nuclei and midbrain/brain stem nuclei results in further generalization of seizures.” Adapted from Löscher et al., 2008.

2. Physiopathology

TLE is one of the most devastating form of epilepsy in humans because of its resistance to anti-seizure drugs (ASDs) and its associated cognitive and behavioral comorbidities. Surgical procedures for TLE and post-mortem tissue samples allow the collection of resected epileptogenic tissue and offer the opportunity to study the anatomical changes associated with the onset of epilepsy in humans. However, some caution should be used regarding the conclusions provided by these tissues. The tissues collected are not representative of the entire spectrum of epileptic patients and only represent few patients with different clinical pictures. Furthermore, it is difficult to distinguish in these tissues the lesions triggered by seizures from those attributable to the initial brain injury. Besides, the unavailability of tissues from healthy or non-epileptic subjects is an additional obstacle to the analysis of resected tissues from epileptic patients.

An atrophy of the hippocampus is often symptomatic of refractory TLE and is the hallmark of a hippocampal sclerosis (HS) (Engel, 2001). This HS is the most frequent pathological substrate of drug-resistant TLE and can be identified by medical imagery. In many cases, hippocampal sclerosis is unilateral. Histologically, hippocampal sclerosis commonly found in TLE patients is defined by a selective loss of hippocampal neurons and a concomitant gliosis (Blümcke et al., 2013; Thom, 2014). This segmental neuronal loss is associated with the disturbance of the excitatory and inhibitory balance of the remaining cells, which is at the origin of the epileptic focus (Sloviter, 1996). Neuronal loss particularly affects some specific populations such as the interneurons of the hilus and the pyramidal cells of both the CA1 and CA3 layers of the hippocampus, while the neurons of the dentate gyrus (DG) and the subiculum are rather preserved (de Lanerolle et al., 2003). A dispersion of the granular cells is also observed (Freiman et al., 2011). The role of neuronal loss in the development of hyperexcitability and seizure generating circuits is not clearly established. Conflicting results have been documented with some evidence indicating that seizures do not cause neuronal loss (Thom et al., 2005) while others have reported that the total number of neuronal cells in the hippocampus is negatively correlated with the seizure frequency, indicating an association between occurrence of epileptic seizures throughout life and neuronal loss (Lopim et al., 2016). Granular cell axons, also called mossy fibers, also appears to be a histopathological feature in the pathogenesis of epilepsy. The abnormal mossy fiber sprouting into the dentate

gyrus inner molecular layer has been reported to be involved in seizure generation and hippocampal excitability (Cavarsan et al., 2018; Godale and Danzer, 2018) (**Figure 5**). The “mossy fiber sprouting hypothesis”, firstly described in 90’s (Cavazos and Sutula, 1990; McNamara, 1994), argues that the increased excitability of dentate gyrus’ granular cells is the result of a pathological rearrangement of the neuronal circuits on which the excitatory granular cells innervate themselves and form recurrent excitatory circuits (**Figure 5**) (Cavarsan et al., 2018). This hypothesis is supported by numerous findings in TLE patients and in preclinical models.

Experimental models of epilepsy, particularly that induced by *status epilepticus* which results in hippocampal sclerosis (HS), have shown that HS is defined by selective neuronal loss as previously described, astrogliosis and inflammation (Cavarsan et al., 2018; Covolán and Mello, 2000; Vezzani et al., 2008). Hippocampal histopathological features undergo remodeling within the hours to days following the initial insult and include cellular apoptosis (Henshall and Meldrum, 2012), abnormal neurogenesis (Dudek, 2004; Parent et al., 1997) and the production of ectopic granule cells (Dudek, 2004). As for the synaptic reorganization and granule cell dispersion, they appear lately and may coincide with the onset of spontaneous seizures (Mathern et al., 1993).

Glial scar, resulting from astrogliosis and microgliosis, is also another characteristic of the sclerosed hippocampus in the epileptic patient. Astrogliosis is a further major feature of epileptic foci found in up to 90% of surgically resected epileptic hippocampus (Thom et al., 2002). Astrocytes are central nervous system cells in contact with blood vessels and neurons, which were originally described for their supportive structural role in the brain. Subsequently, it was established that these astrocytes fulfilled much more complex functions, essential for maintaining cerebral homeostasis. Astrocytes are abundantly found in the areas that are the most severely affected by neuronal loss (de Lanerolle et al., 2010; Losi et al., 2012). In the epileptic hippocampus, these astrocytes are different from those found in healthy tissues through their morphology with an enlarged cellular body and through their functions. *In vitro* studies showed that reactive astrocytes synthesize in culture neurotrophic and pro-synaptogenic factors involved in axonal growth, suggesting that when they are located on the lesion site they would contribute to the development of hyperexcitable neuronal networks by supporting potential aberrant neosynaptogenesis (Kim et al., 2017). In addition, these

astrocytes would have a reduced ability to recapture extracellular glutamate (de Lanerolle and Lee, 2005). Recently it has also been proposed that astrocytes were involved in epileptogenesis processes as well as in recurrent spontaneous seizures since they are involved in the modulation of NMDA neuronal receptors (Clasadonte et al., 2013).

Astrocytes are not the only glial cells involved in the pathophysiology of epilepsy, since microglia also play a significant role. Microglial cells, considered as the immune cells of the brain, also have a considerable increase in their density in the most damaged areas of the hippocampus. In the epileptic brain, microglia participate to the release of inflammatory cytokines, to neuronal hyperexcitability and to neurodegeneration (Hiragi et al., 2018). A recent study compared in TLE patients' tissues the basal profiles of microglia in regions of neuronal loss and gliosis with those of microglia in others brain regions where lower neuronal loss was reported (Morin-Brureau et al., 2018). They showed that in sclerotic areas associated with high loss of neurons, microglia have an amoeboid rather than ramified shape. They also suggested that the microglial phenotype is changed after seizures to be able to produce specific pro-inflammatory cytokines such as IL1 β (Morin-Brureau et al., 2018).

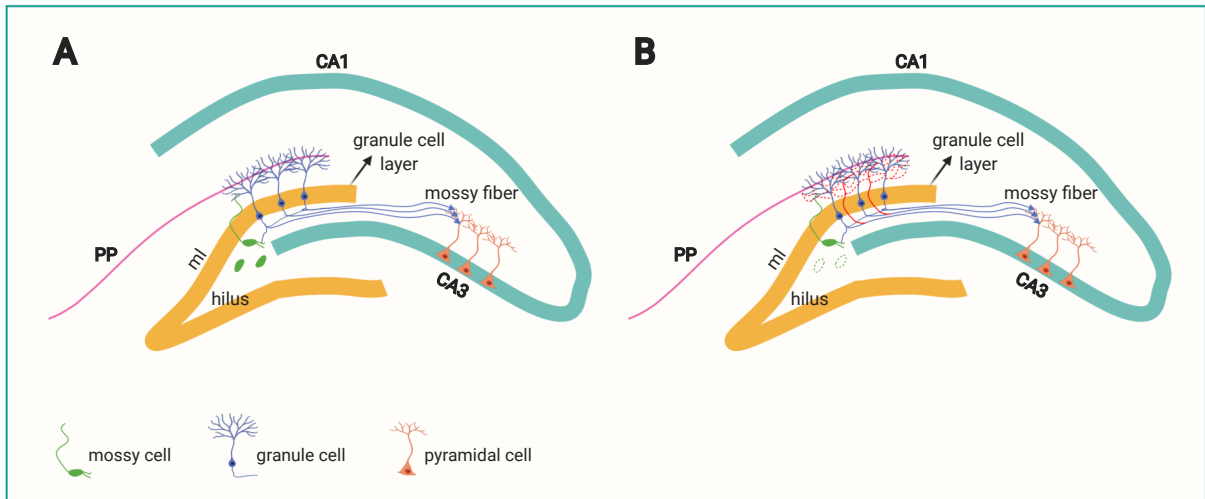


Figure 5. “Hippocampal formation in the normal and epileptic brain. (A) The dentate gyrus granule cell layer is densely packed with small diameter cell bodies named granule cells. Just above the granule cell layer is the molecular layer (ml) that is considered cell-free, as it contains the apical dendrites of granule cells. The outer molecular layer receives entorhinal cortex information via a perforant pathway (PP). Granule cell axons, named mossy fibers, extend to the hilus with projection to the mossy cells and CA3 pyramidal neurons. The mossy cell axons project to contralateral granule cell dendrites in the inner molecular layer. **(B)** In the epileptic hippocampus, with the loss of mossy fibers target in the hilus, the granule cell axons sprout and extensively innervate the dentate inner molecular layer of the hippocampus, a phenomenon called mossy fiber sprouting, illustrated in red.” Adapted from Cavarsan et al., 2018.

Hence, physiopathology of epilepsy is currently studied to define the underlying processes causing the drug-resistance and how to counteract it. The involvement of inflammatory mediators in the development and perpetuation of epilepsy is now widely acknowledged; but the question of whether the inflammation is the cause, or the consequence of epileptogenesis and epilepsy is still a matter of debate. Thus, in order to understand the molecular and cellular mechanisms associated with epileptogenesis and chronic phase of epilepsy and further investigate new treatments, numerous animal models of epilepsy have been developed.

BOX 2 | KEY TERMS (adapted from Devinsky et al., 2018)**Epileptogenesis**

A multifactorial process that underlies the development and extension of brain tissue that generates spontaneous seizures.

Focal seizure

A seizure that originate in one or more localized parts of the brain.

Generalized seizure

A seizure that originates from widespread regions on both hemisphere of the brain.

Hippocampal sclerosis

Scarring and neuronal loss in the hippocampus. This pathology is commonly found in patients with temporal lobe epilepsy.

Ictogenesis

The dynamic changes responsible for seizure onset, progression and termination and for the transition from the interictal state into seizures.

Interictal state

The period between seizures.

Myoclonic seizure

A seizure accompanied by rapid, involuntary muscle twitches.

Reactive gliosis

Hypertrophy and proliferation of glial cells (including astrocytes and microglial cells) in response to central nervous system injury or increased neuronal activity).

Status epilepticus (SE)

Abnormally long seizures that occur in individuals with or without epilepsy. The seizures can be convulsive or non-convulsive.

Tonic seizure

A seizure accompanied by sustained muscle contraction.

Tonic-clonic seizure

A seizure characterized by initial muscle contraction (tonic phase), usually causing the patient to fall, followed by rhythmic muscle jerks (clonic phase).

3. Animal models

Comprehension of the complex molecular and cellular mechanisms underlying epileptogenesis and seizure generation in an attempt to develop effective epilepsy therapy requires the use of appropriate animal models that can replicate human pathology in a physiologically and reproducible relevant manner. The model of choice will depend on the type of epilepsy to be induced, the symptomatology to be studied, the question of interest, and convenience. An accurate TLE model must reproduce the classic pattern of epilepsy development, namely the initial epileptogenic insult, followed by a latent period that leads to chronic hyperexcitability and by a subsequent period of chronic recurrent and spontaneous seizures. Seizures are a combination of electrical and behavioral events that can induce chemical, molecular, and anatomic alterations (Becker, 2018; Kandratavicius et al., 2014; Sharma et al., 2007; Yin et al., 2013). TLE models may thus reproduce many of the pathophysiological changes found in epileptic patients, including:

- The loss of gamma-aminobutyric acid (GABAergic) interneurons;

- In the hippocampus, the loss of excitatory granular cells of the dentate gyrus and pyramidal cells of the *Cornu Ammonis*;
- The aberrant neural circuits formation (mossy fiber sprouting);
- The modification of neuronal receptors and ion channels;
- The network hyperexcitability caused by a modification of the excitation/inhibition balance;
- The presence of a hippocampal sclerosis.

In addition to modelling seizures, animal models of epilepsy must also replicate epilepsy comorbidities, i.e. behavioral, cognitive and psychiatric symptoms of TLE, that are a part of the global symptomatology of the disease (Löscher, 2017).

In order to characterize the underlying mechanisms of TLE, many induction techniques have been developed using chemoconvulsants, electrical or sound stimuli, hyperthermia, genetic modifications, traumatic brain injury, hypoxia model for newborn animals and tetanus toxins (Kandratavicius et al., 2014; Lévesque et al., 2016). *Status epilepticus* (SE) is one of the most established model of TLE. SE in humans is an acute convulsive state with many repeated seizures in a prolonged period of time (more than 5 minutes) (Lowenstein and Alldredge, 1998). In animals, SE induction can be done by pharmacological treatments or intracerebral stimulation. Among the pharmacological agents that trigger SE, the most common are pilocarpine, an agonist of muscarinic cholinergic receptors, and kainic acid, specific for glutamate "kainate" receptors. Another way to induce SE is electrical stimulation. Electric induction of SE is achieved by high-frequency stimulation of the ventral hippocampus, the perforating pathway, or the amygdala during several minutes (Kandratavicius et al., 2014; Löscher, 2017).

It is to note that rodents are not the only animals used as models to investigate epilepsy. Many other non-mammalian models have been developed in recent years, such as models on zebrafish, fruit fly or planaria (Brenet et al., 2019; Johan Arief et al., 2018).

3.1. Chemoconvulsants

Spontaneous recurrent seizures have for long been generated by the use of kainic acid and pilocarpine (Leite et al., 2002). These models intend to mimic TLE in a similar way to humans: 1) initial precipitant injury afflicting the hippocampus and/or the temporal lobe; 2) a latent period, called epileptogenesis, between the injury and the occurrence of spontaneous seizures; 3) chronic manifestation of spontaneous seizures (electrical and behavioral events) within the following weeks after the chemoconvulsant injection and histopathological changes associated with TLE. Convulsive SE in human is a neurological emergency that can lead to the development of chronic epilepsy (Scott, 2014). Hence, animal models of SE are crucial to assess whether can lead to inappropriate neuronal reorganization, epileptogenesis and cognitive dysfunction.

Historically, kainic acid (KA) was one of the first compounds used to model TLE in rodents (Sharma et al., 2007). This L-glutamate analog, injected systemically or intracerebrally, can induce SE and cause neuronal depolarization and seizures, preferentially in the hippocampus (Nadler et al., 1978). This model is associated with recurrent seizures and a hippocampal sclerosis correlating remarkably with histopathological features in humans (Raedt et al., 2009). KA cause generally hippocampus-restricted injuries, which distinguishes it from pilocarpine that causes lesions in neocortical areas (Sharma et al., 2007). This makes pilocarpine a very valuable chemoconvulsant considering that extrahippocampal areas are also altered in human TLE (Bonilha et al., 2010).

Pilocarpine is a muscarinic acetylcholine receptor agonist, usually injected systemically or intracerebrally, which triggers seizures that developed into a limbic SE (Cavalheiro, 1995; Curia et al., 2008; Furtado et al., 2002). SE initiation by pilocarpine is thought to be a result of the hyperactivation of cholinergic system, but the continuation of seizure activity is likely to be caused by a glutamatergic mechanism (Reddy and Kuruba, 2013). In this model, pilocarpine is injected intraperitoneally and the SE stopped between 30 min and 3 hours later, depending on the age of the animal, by benzodiazepine administration. Subsequently, structural damage and spontaneous seizures thrives, in a similar way to those of human complex partial seizures (Cavalheiro, 1995). Pilocarpine-induced SE leads to massive neuronal damage observed as early as 24h to 72h post-SE. Cell death have also been reported in different brains areas,

namely the olfactory cortex, the amygdaloid complex, the thalamus, the neocortex, the substantia nigra and the hippocampus (Reddy and Kuruba, 2013). It has been shown that in this model, antiseizures drugs (ASDs) used for humans to abort seizures are also effective in stopping spontaneous seizures in animals (Leite and Cavalheiro, 1995). Besides this analogous response to ASDs, the pathophysiological bases associated with disease development such as neural networks and neurochemical modification are similar between the pilocarpine model and TLE in humans. The pilocarpine model is associated with prolonged seizures and neurodegeneration and hence represent paradigms of refractory epilepsy (Reddy and Kuruba, 2013). This model also reproduces the cognitive deficits observed in epileptic patients, deficits correlated with loss of granular cells in the hippocampus (Leite et al., 1990; Pauli et al., 2006).

Other types of chemoconvulsants are commonly used in animals, but more as a model of acute seizure than as a model of epilepsy (Löscher, 2017).

3.2. Post-traumatic epilepsy

Traumatic brain injury (TBI) is one of the leading etiologies for symptomatic epilepsies in adults. Post-traumatic epilepsy (PTE) accounts for 20% of all symptomatic epilepsy and describes the situation in which recurrent spontaneous seizures are experienced more than a week after a TBI (Pitkänen and McIntosh, 2006). PTE that develop after TBI have similar clinical characteristics to TLE that develop after SE: the initial injury caused by trauma is followed by a latent period without clinical manifestation, after which the first spontaneous recurrent seizures appear (Glushakov et al., 2016). However, the underlying mechanisms leading to the development of epilepsy are not fully understood. At the experimental level, PTE models are often associated with long latent periods and low final yield of animals that actually develop epilepsy (Kandratavicius et al., 2014). A large number of TBI animal models exist due to the great heterogeneity of trauma in humans, although no model perfectly replicates their clinical pictures (Ostergard et al., 2016). One of the most commonly used models to simulate PTE is the lateral fluid-percussion injury model (LFP) (Kharatishvili et al., 2006). In this model, a single episode of severe LFP is sufficient to induce epilepsy that may progress from fronto-parietal epilepsy to mesial TLE as frequently reported in human PTE (D'Ambrosio et al., 2005; Kharatishvili et al., 2006). The LFP model also present the neuropathological correlates of mesial TLE such as hippocampal neuronal loss and mossy fiber sprouting (Kharatishvili et al.,

2006). Further research is needed to determine the common underlying mechanisms shared by TBI, SE or other types of brain insult leading to the development of symptomatic epilepsy.

3.3. Electrical induction of epilepsy

SE induction can also be achieved by continuous electrical stimulation of limbic structures. There are various types of inductions depending on the stimulated limbic structure, which may be the amygdala, the piriform cortex or the hippocampus. High-frequency stimulation of the ventral hippocampus for one hour is effective, for example, to induce SE in many rats (Rolston et al., 2011). Intermittent stimulation models were then developed in which SE is induced by stimulation of excitatory pathways, the most common protocol being the stimulation of the perforant pathway (Sloviter et al., 2007). This model is known to reliably reproduce the hippocampal pathology resulting from epileptogenic and seizure-induced brain damage (Kienzler et al., 2009; Sloviter et al., 2007).

The kindling model is also a widely used model to reproduce the pathophysiology of TLE. In this model, animals undergo daily electrical stimulation in seizure-prone areas of the brain administered through stereotaxically implanted cerebral electrodes. Kindling is a gradual process in which electrical stimulation, initially resulting in brief low-frequency electrographic post-discharges without behavioral response, progresses with repeated electrical stimulation over several days into long high-frequency post-discharges with a high convulsive response. After several days of kindling, the repeated stimulations lower the local seizure threshold and animals start to experience complex seizures with secondary generalizations (Rolston et al., 2011).

II. Neuroinflammation: cells and molecular markers

Classic peripheral inflammation is a process usually described by 4 criteria: heat, redness, pain and swelling. The criteria are the product of the immune system's response at the site of infection or traumatic shock. During the initiation of an inflammatory event, immune cells (innate or adaptative) are accumulated and proliferate at the inflammatory site, and neutrophils and macrophages are recruited. Hence, classic inflammation is a multicellular process, involving numerous immune cells which cause environmental changes.

Brain inflammation is different from the rest of the body due to the presence of the blood brain barrier (BBB). The central nervous system has long been considered as immunologically privileged. However, there is considerable research now demonstrating that the immune system is not only necessary for normal brain homeostatic functions, but is also involved in the pathophysiological processes of many, if not all, neurological diseases (Brambilla, 2019; Ransohoff and Engelhardt, 2012).

The neuroimmune system includes not only physical barriers, but also different brain cell types and inflammatory molecules involved in the brain protection from foreign pathogens and infections (Louveau et al., 2015). Neuroinflammation is a complex multicellular process that, like its peripheral counterpart, will cause vascular and molecular changes in the environment to respond to and to resolve an infection or a lesion that has occurred in the CNS. Under normal conditions, the action of neuroinflammation is transient and beneficial and prevents the spread of a pathogen/infection. However, in some cases, it can become chronic and detrimental, which nowadays makes it a key element of neurodegenerative and neuropsychiatric pathologies.

Although the term *neuroinflammation* is currently used as this inflammation only born near neurons, we now know that peripheral inflammation is also related to neuroinflammation and that peripheral blood-borne inflammation can trigger a neuroinflammatory phenomenon, just as purely cerebral neuroinflammation can modify the panel of peripheral inflammatory markers found in the serum. The term neuroinflammation also includes the intervention of peripheral immune cells since the leukocyte infiltration occurring after a blood-brain barrier disruption is now well-established.

Neuroinflammation has emerged during the past few decades as an underlying cause of epileptogenic and seizure perpetuating factor. Pro-epileptogenic brain injury lead to the release of cytokines and chemokines and it is postulated that focal or systemic unregulated inflammatory processes result in aberrant neuronal connectivity and hyperexcitable neuronal network, which mediate the onset on epilepsy. In addition to the biological pathways involved in epilepsy, neuroinflammation also plays an important role in the whole clinical picture. A number of study analyses in both human and experimental models have examined the relationship between the inflammatory status (in brain parenchyma or in peripheral blood) and epilepsy (from epileptogenesis to ictogenesis and comorbidities during chronic phase). Numerous results obtained in experimental models have highlighted the dichotomous role of inflammatory processes in the CNS, showing that they can be either protective, thus constituting an adaptive and beneficial endogenous response, or deleterious as a direct or indirect cause of neuronal dysfunction (Nguyen et al., 2002). Cytokines are overexpressed in a wide range of epilepsy types and may lead to increased excitability. Thus, neuroinflammation processes are said to be involved not only in the genesis of spontaneous seizures but also in their perpetuation (Lehtimäki et al., 2007; Vezzani et al., 2011), the seizure reported to be caused by cytokines themselves but also by the degradation products released as a result of cell death processes. In addition to this role in epileptic seizures, abnormally high levels of pro-inflammatory cytokines interfere with synaptic transmission, neuronal excitability and long-term potentiation (McAfoose and Baune, 2009; Vezzani and Viviani, 2015; Yirmiya and Goshen, 2011), can lead to neurodegeneration (Allan and Rothwell, 2001) and affect learning and memory processes.

In epilepsy physiopathology, neuroinflammation also refers to leukocyte infiltration which results in an ion influx and serum protein extravasation that may lead to seizure activity. Long-term changes in excitability may also be triggered by the abnormal activation of distinct signaling pathways, such as nuclear factor- κ B (NF κ B), which can be activated by neuroinflammatory molecules.

Research on neuroinflammation in epilepsy refers mainly to the innate immune system rather than the adaptive one. The innate immunity enables to limit the extent of the lesion/infection and to amplify the inflammatory reaction via mediators such as cytokines or chemokines. Adaptive immunity, mainly represented by lymphocytes, specifically targets

pathogens and eliminates them with killer lymphocytes. In addition, the main characteristic of adaptive immunity is also to create immunological memory after encountering a specific pathogen to improve the efficiency of the inflammatory response during a subsequent encounter with the pathogen. Adaptive immunity also has a role in the pathophysiology of epilepsy since it is known that both antibody-mediated mechanisms and cytotoxic T cells may be involved (Vezzani et al., 2016). Besides, it has been reported that some seizure-related disorders may involve an autoimmune component and autoantibodies (Vezzani et al., 2016). In this manuscript we will focus more closely on innate immunity, while keeping in mind that adaptive immunity is also part of the overall clinical picture found in epileptic patients.

1. Cells involved in the brain inflammatory response and their functional role

Since neuroinflammation appears to be an important component of epilepsy and epileptogenesis, it is important to explore its cellular supports. We will here review what is known about the relative contribution of the different types of brain cells (microglia, astrocytes, neurons, pericytes, endothelial cells), as well as peripheral immune cells to the neuroinflammation.

1.1. Resident brain cells

1.1.1. Resident microglial cells

Microglial cells are the resident mononuclear phagocytes of the central nervous system with two main functional aspects: immune defense and maintenance of normal CNS functions (Nimmerjahn et al., 2005; Sousa et al., 2017; Tremblay et al., 2011; Ziv and Schwartz, 2008). These cells were originally observed by Franz Nissl in the late nineteenth century, who distinguished them from other cells according to the rod shape of their nuclei and described them as reactive glial elements with migratory, phagocytic and proliferative potential (Ginhoux et al., 2010, 2013). Officially discovered by Cajal in 1913 and his student Del-Rio Hortega in 1919, microglia are currently accepted as self-renewing cells with a unique embryonic origin (Ginhoux et al., 2010). In 1927, Pio Del-Rio Hortega suggested, according to histological observations, that the microglia cells (initially called Hortega cells) were distinct from other glial cells. Later on, he suggested that these cells would derive from circulating

mononuclear cells and could shift from a quiescent state with long ramifications to an activated state where they acquire an amoeboid form. Hence, defining the origin of resident microglial cells in the CNS has been a longstanding issue of debate and multiple schools of thought have emerged. It took several years to clarify the origin of microglial cells that do not derive, unlike neurons and astrocytes, from neural stem cells. Microglial cells derive from yolk sac-primitive macrophages, which colonize the CNS during embryonic development in two waves, one on embryonic day 9.5 (E9.5) and another on E14.5 (**Figure 6**). At this second time point, the final progenitor population is established (Ginhoux and Prinz, 2015; Ginhoux et al., 2010). At the time of infiltration, the myeloid precursors of the microglial cells exhibit a round amoeboid shape with little or no extension. Once the BBB is completely closed and the brain parenchyma isolated from the blood compartment, these precursors differentiate and take their quiescent form with thin extensions. Within the first two postnatal weeks, approximately 95% of the microglial population is formed (Augusto-Oliveira et al., 2019).

In the adult central nervous system, microglial cells represent approximately 10% of all the glial cells (Augusto-Oliveira et al., 2019). The role of these microglial quiescent cells is to constantly scan the entire cerebral parenchyma to detect any pathogens or lesions. In this quiescent state, microglial cells arbor thin ramifications, have limited mobility and a low renewal rate. The function of microglial cells in the healthy brain has gained increasing interest in recent years (Augusto-Oliveira et al., 2019; Tremblay et al., 2011). Their function in the normal brain includes actions that occur during development or in the adult brain, such as phagocytosis of extraneuronal synapses or of apoptotic newborn neurons, regulation of microglia-synapse interactions, regulation of neuronal activity through interaction with the synapse/astrocyte complex that forms the tripartite synapse and reorganization of neuronal circuits (Tremblay et al., 2011). Although microglial cells have a macrophagic origin, they present a low level of CD45 expression (antigen common to all leukocytes) in the healthy brain and a very low expression on their surface of ligands and/or receptors essential for the classic immune functions of macrophages (cytotoxic and pro-inflammatory function).

In the pathological brain, lesions or pathogens detection is achieved through the expression by microglia of Pattern-Recognition Receptors (PRRs), which are divided into two classes of receptors (ElAli and Rivest, 2016):

- Damage-Associated Molecular Patterns (DAMPs) receptors that recognize the molecular pattern associated with damage
- Pathogen-Associated Molecular Patterns (PAMPs) receptors that recognize molecular patterns associated with pathogens. This family of receptors includes Toll-Like Receptors (TLR) which, once activated, triggers a cascade of intracellular events leading to the production of cytokines and chemokines, and promotes cell proliferation.

When PRRs bind one of the specific recognized patterns, it leads to an intracellular cascade that activates transcription factors (such as NF- κ B) involved in the activation of microglial cells and the initiation of the inflammatory response. These activation processes allow the microglia to acquire a different morphology. Thereafter, microglia transiently shift from a quiescent state with thin ramifications to an activated state with an amoeboid morphology in order to increase its mobility and migrate as quickly as possible to the site of the lesion (Russo and McGavern, 2016). These reactive microglia detected in the suffering brain have many common features with macrophages since activation processes include not only morphological changes affecting cellular bodies and extensions but also the activation of many genes, including the differentiation markers specific to macrophages (Ladeby et al., 2005).

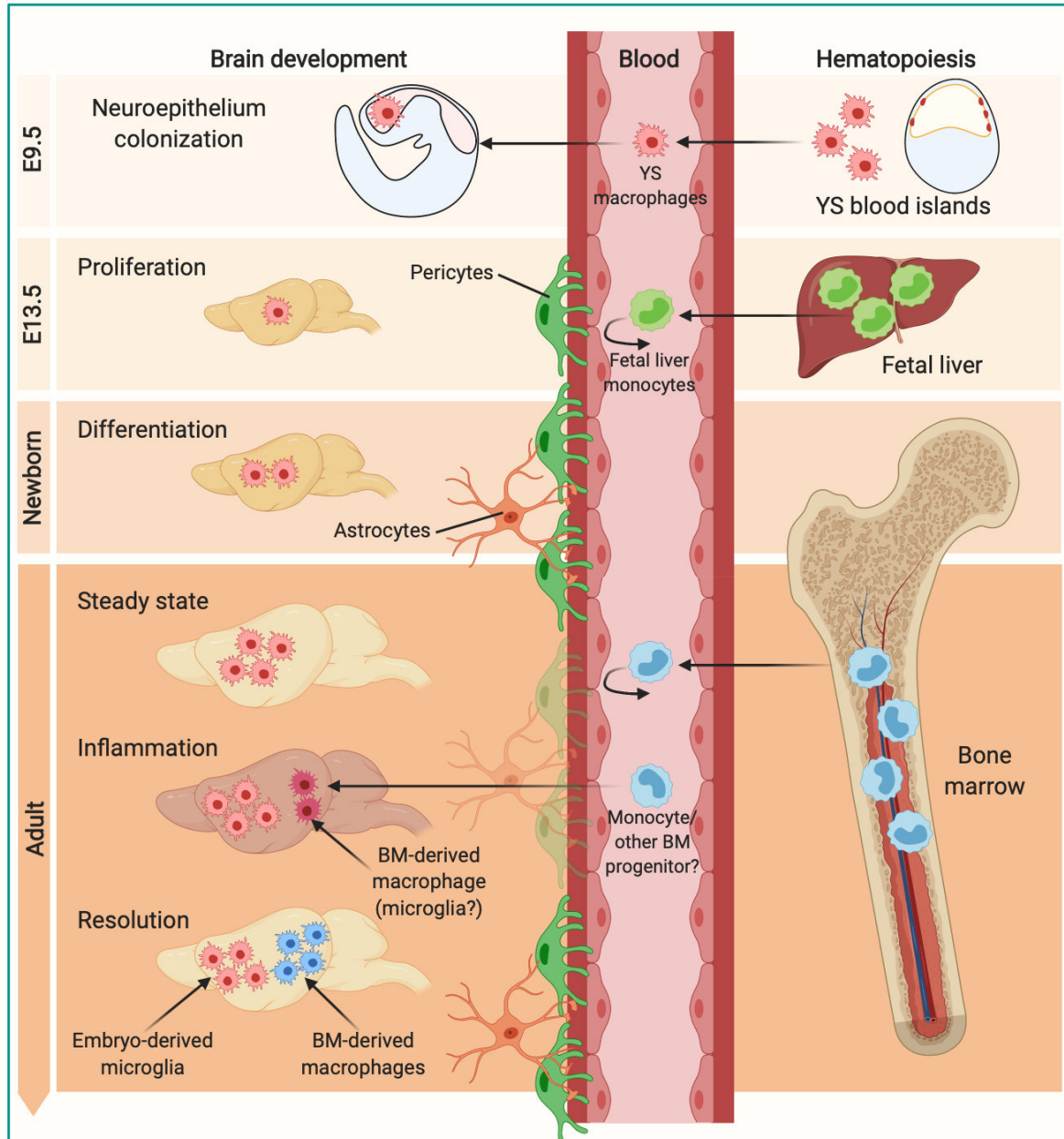


Figure 6. Microglial ontogeny in the developing brain. “Primitive macrophages generated in the yolk sac (YS) blood islands around E8.0 spread into the embryos at the onset of blood circulation established around E8.5 and colonize the neuroepithelium from E9.0/E9.5, giving rise to embryonic microglia. In parallel, definitive hematopoiesis arises in the aorta–gonad–mesonephros and gives rise to progenitors that colonize the fetal liver (FL) from E10.5. The blood–brain barrier starts to form from E13.5 and may isolate the developing brain from the contribution of FL and, later, of bone marrow (BM) hematopoiesis. Embryonic microglial cells expand, colonize the whole CNS, and will maintain themselves until adulthood via local proliferation during late gestation and postnatal development, as well as in the injured adult brain in reaction to inflammation. Nevertheless, under certain inflammatory conditions found, for example, after BM transplantation, the recruitment of BM-derived progenitors can supplement the microglial population to some extent.” Adapted from Ginhoux and Prinz, 2015.

Over the past two decades, a number of studies have addressed the issue of microglia phenotype and it is now well-established that microglia have a wide range of phenotype and go far beyond a steady state (Dubbelaar et al., 2018). Phenotypic polarization was first described in the peripheral macrophage population before being documented in the microglial cell population. Nevertheless, considering the common origin of these two populations, it is not surprising to find similar phenotypic modulations. Thus, microglia do not constitute homogeneous cell populations and have been shown to exhibit at least two distinct molecular phenotypes (**Figure 7**) (Nakagawa and Chiba, 2015; Salvi et al., 2017):

- The M1 phenotype, corresponding to the pro-inflammatory state in which microglial cells will release pro-inflammatory cytokines and chemokines such as interleukin-1 β (IL1 β), IL6, IL12, IL17, IL18, IL23, tumor necrosis factor- α (TNF α), interferon- γ (IFN γ), nitric oxide and monocyte chemoattractant protein 1 (MCP1, also called CCL2) (Dubbelaar et al., 2018; Nakagawa and Chiba, 2015; Subramaniam and Federoff, 2017). This state is supported by the presence of IFN γ and TNF α in the local environment. M1 phenotype is characterized by pro-inflammatory functions that serve as the first line of defense. M1 microglia hinder CNS repair and expands tissue damage leading to chronic neurodegeneration after brain insults. In this state of polarization, microglia appear to exhibit phenotypic markers such as inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), MHC-II, and CD86 (cluster of differentiation marker 86) and express other compounds including reactive oxygen species (ROS), reactive nitrogen species, and prostaglandin E2 (Subramaniam and Federoff, 2017). These coordinated processes are intended to cleanse foreign pathogens and polarize T-cells to trigger an adaptive immune response.
- M2 phenotype, corresponding to the anti-inflammatory state in which microglial cells will release anti-inflammatory cytokines, extracellular matrix proteins, glucocorticoids, as well as neurotrophic and angiogenic factors, and leads to a decrease in nitric oxide (NO) release and promotes neurorestorative processes (Subramaniam and Federoff, 2017). The M2 activation state of the microglia parallel that of the macrophages and was initially identified on the basis of mannose receptor expression. Since then, a set of specific markers of the M2

phenotype have been identified such as arginase 1 (Arg 1), Ym1 a heparin-binding lectin, FIZZ1, which promotes deposition of extracellular matrix, and CD206, another mannose receptor (Cherry et al., 2014; Dubbelaar et al., 2018; Subramaniam and Federoff, 2017). The anti-inflammatory cytokines IL4, IL10 and IL13 are both potential inducers of the M2 phenotype when present in the medium, and molecules produced by the microglial cells themselves. The M2 phenotype is more heterogeneous than the M1 phenotype and has relatively different subtypes. The M2 phenotype appears to be subdivided into M2a, M2b and M2c phenotypes, each associated with more specific functions and phenotypes (Cherry et al., 2014). This has been documented by observations that stimulation by different cytokines leads to different expression of cytokines and chemokines, different receptor profiles and different functions (Mantovani et al., 2006). Nevertheless, these subtypes have in common that they are capable of alleviating, repairing or protecting the body from pro-inflammatory consequences.

It is now well known that these M1 and M2 phenotypes have particular subtypes whose markers and secretions will be modified by the environmental niches in which the cells are located. Such a polarization is now considered as beneficial depending on the time-window when it occurs following brain insults: microglia exhibiting a M1 phenotype are needed in the acute phase following brain injuries to remove cell debris and toxic molecules released by dying cells. However, maintaining M1 phenotype in the long term is considered deleterious to the development of tissue repair processes, justifying the idea that a switch from M1 to M2 phenotype may prove to be beneficial when therapeutically guided in the setting of diseases for which an inflammatory component is associated.

The microglia can shift from an M1 phenotype to an M2 phenotype when exposed to IL10, beta interferons or *peroxisome proliferator-activated receptor gamma* (PPAR γ) agonists (Subramaniam and Federoff, 2017). Nevertheless, in a given environmental niche, not all microglial cells will have the same phenotype at the same time and different subpopulations may express different phenotypes due to a concomitant expression of M1- and M2-related factors, resulting in mixed M1/M2 phenotypes (Morganti et al., 2016; Orihuela et al., 2016; Subramaniam and Federoff, 2017).

The notion of morphological transformation associated with the activation of resident microglial cells has however been challenged. Historically, histological staining has revealed 3 types of microglia shape: the quiescent shape with thin extensions, the amoeboid shape and an intermediate shape with thicker and shorter ramifications combined with an enlarged cell body (Streit et al., 1999). However, after considering for a long time that the cells of amoeboid shapes observed after a brain insult were only activated microglia, it has been suggested that it could be either perivascular cells and/or infiltrating leukocytes (monocytes/macrophages or neutrophils). Thus, the hypertrophied shape characterized by the strong expression of the CD11b immunocompetent cell marker (significantly less expressed in the quiescent microglia) would correspond either to the transformation of the quiescent microglial cells, or to the differentiation of the invasive immune cells, or would be a combination of these two processes. In addition to their morphological changes and phenotypic changes, microglial cells are able to proliferate after a brain insult.

In addition to inducing phenotype changes, it has been shown that neuroinflammation causes the priming of microglia, which is the process of sensitizing microglial cells, making them more reactive during a second stimulus. The problem is that neuroinflammation induces increased sensitization of the microglia, leading to a strong elevation in the release of pro-inflammatory cytokines by the microglia (Frank et al., 2007). This priming state has been shown to be responsible for increased neurodegeneration during neuroinflammation (Cunningham, 2013; Murray et al., 2012; Perry and Teeling, 2013). In diseases where a state of chronic brain inflammation is present, even at low grade, an overabundance of inflammatory cytokines leads to microglial polarization towards the M1 phenotype. The M1 microglia then in turn releases further inflammatory cytokines, triggering a cycle that induces inflammation and sustains the M1 state (Cherry et al., 2014).

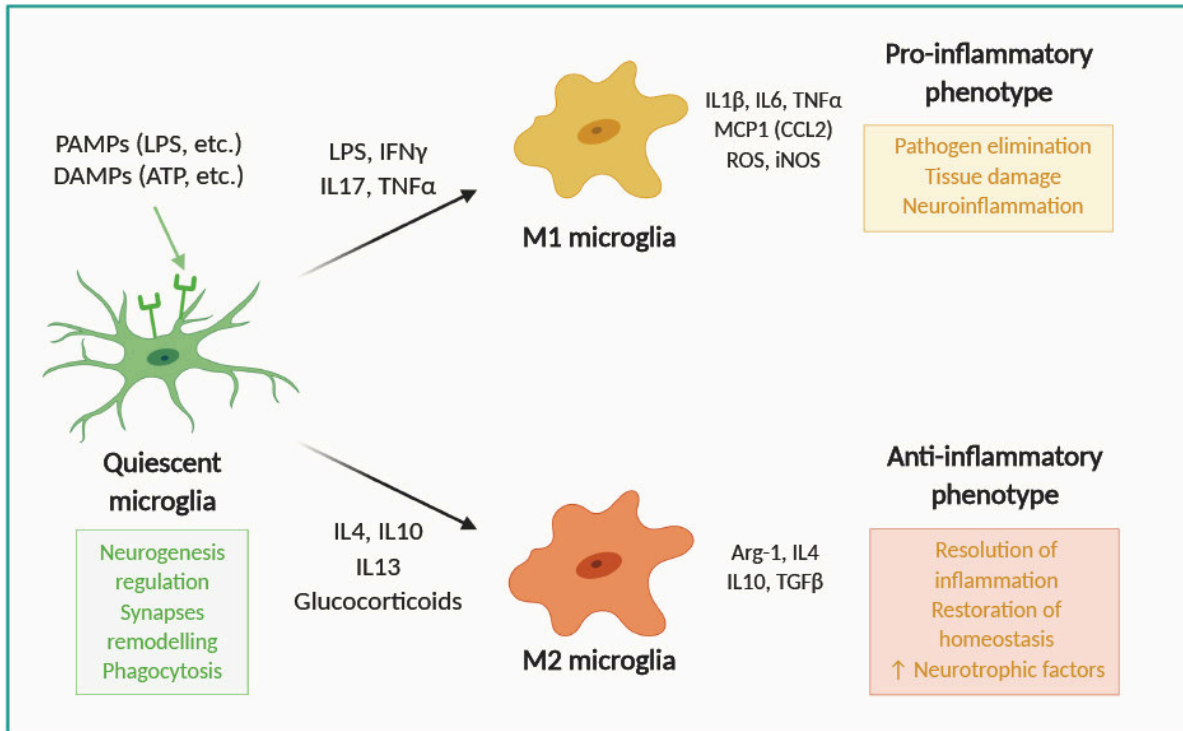


Figure 7. M1/M2 polarization of microglia and their immunoregulatory functions. In physiological conditions, patrolling quiescent microglia regulate CNS homeostasis. Resting microglial cells are stimulated with PAMPs or DAMPs via TLR or ATP receptors. In the presence of LPS and IFN γ , microglial cells polarize to M1 phenotype and produce pro-inflammatory cytokines/mediators including IL1 β , IL6, TNF α , CCL2, ROS, and NO. In contrast, IL4, IL10 and IL13 induce alternative activation of microglia to M2 phenotype which down-regulates M1 functions by the release of anti-inflammatory cytokine such as IL4 or IL10. Adapted from Nakagawa & Chiba (2014) and Salvi et al. (2017).

The role of microglia in epilepsy has been widely studied. Research in animal models has shown that microglial activation, in parallel with astrocyte activation, will contribute to epileptogenesis processes after SE, although this microglial activation would be model-dependent (Benson et al., 2015). In epileptic patients, microglial activation states have also been reported (Morin-Brureau et al., 2018). Nevertheless, in all these studies, in both humans and animals, activated microglia showed high phenotypic heterogeneity, making it difficult to draw conclusions about their pro- or antiepileptic role (Hiragi et al., 2018). It has been suggested that short-term activation would be beneficial (Mirrione et al., 2010; Vinet et al., 2012) whereas chronic microglia activation is deleterious in the pathogenesis of epilepsy. Overall, in the epileptic brain, it seems that microglia play an important role in the secretion of inflammatory molecules, in the phagocytosis of cellular debris, in the protection of neurons

from damage and are able to extend their processes towards the damaged cells (Hiragi et al., 2018).

During microglial activation, data from the literature reported that an outer mitochondrial membrane protein, called translocator protein (18 kDa, TSPO), would be overexpressed (Liu et al., 2014). This has led to the development of a radiotracer, the [¹¹C]PK111P5 that would bind to TSPO and allow, by using positron emission tomography (PET), imaging of brain regions subject to high levels of inflammation in the epileptic brain (Butler et al., 2016).

1.1.2. Astrocytes

The role of astrocytes in the BBB during neuroinflammatory processes has been the focus of numerous studies due to the importance of astrocyte-endothelial interaction in the healthy CNS. These cells represent the largest glial population (Sofroniew and Vinters, 2010). In the homeostatic brain, astrocytes support neurons, and more particularly synapses, communicate with surveilling microglia and myelinating oligodendrocytes, and are in permanent connection with neighbouring astrocytes through gap junctions. They also form a protective barrier that supplements the BBB to limit the infiltration of immune peripheral cells into the brain (Alvarez et al., 2013). In addition to their close interaction with endothelial cells of the BBB, astrocytes are also sensitive to environmental damage and pathogens through the presence of PRR on their surface (Sofroniew and Vinters, 2010). Astrocytic activation is called astrogliosis and allows a specific inflammatory reaction to protect and repair the affected tissue. When activated, astrocytes increase their proliferation and over-express the Glial Fibrillary Acidic Protein (GFAP). During the inflammatory reaction, astrocytes will also release pro-inflammatory molecules such as TNF α or NO that will sustain this neuroinflammation. This release is under the control of the transcription factor NF κ B whose inactivation reduces the production of pro-inflammatory cytokines and increases tissue regeneration (Brambilla et al., 2005; Haenold et al., 2014).

Astrocytes have very important roles since they are also involved in the recapture of potassium (K⁺) ions and glutamate in the perisynaptic space as well as in the release of various neuroactive substances, such as glutamate. Their role as energy reservoirs makes them indispensable for neuronal metabolism. Their role in restoring extracellular concentrations of

excitatory amino acids, which are neurotoxic in high concentrations, is essential for a good glutamate/GABA balance (Bellot-Saez et al., 2017; Coulter and Steinhauser, 2015).

Astrocytes are highly connected and can modulate the excitability of neurons by altering the concentration of potassium ions in the extracellular environment, a process called K⁺ clearance (Bellot-Saez et al., 2017). Their role as a potassium buffer is essential in any situation where the extracellular K⁺ level rises abruptly. The efficient removal of K⁺ from the extracellular space is essential for the maintenance of brain homeostasis and likely limits the network's hyperexcitability during normal brain function, as disturbances in K⁺ clearance have been linked to disease states, including epilepsy. Previous studies have shown that elevated K⁺ concentration in the extracellular microenvironment may be linked to spontaneous epileptic seizure activity in the absence of external stimuli (Fröhlich et al., 2008; Traynelis and Dingledine, 1988). Astrocyte-mediated buffering of potassium generally serves to maintain a homeostatic K⁺ extracellular concentration, supporting normal neuronal electrical activities (Coulter and Steinhauser, 2015). The study of samples from patients with drug-resistant temporal lobe epilepsy as well as epilepsy models showed alterations in the expression, localization, and function of astroglial K⁺ and water channels. Furthermore, dysfunction of glutamate transporters and of the astrocyte glutamate converting enzyme, glutamine synthetase, was observed in epileptic tissues (Losi et al., 2012; Steinhäuser and Seifert, 2012). In addition, it has recently been reported that down-regulation or dysfunction of K⁺ uptake channels in astrocytes triggers tonic-clonic seizures (Du et al., 2018; Mukai et al., 2018a).

Astrocytes are also involved in neuroinflammatory processes in epilepsy since it has been shown that hippocampal astrocytes become reactive (astrogliosis) and secrete inflammatory cytokines, chemokines and their receptors (e. g. Toll-like receptors) (Aronica et al., 2012). The epileptogenic glial scar hypothesis claims that reactive astrocytes would release trophic factors that lead to axonal sprouting, synapse formation and hyperexcitability (Crespel et al., 2002). Studies have measured an increase in glial fibrillary acid protein, a molecule associated with hypertrophy and astrocyte proliferation, in epileptogenic tissues (Pitkänen and Sutula, 2002). The clearance of neurotransmitters by reactive astrocytes is reduced due to the altered expression of glutamate transporters, which may promote convulsive activity (Seifert et al., 2006). In addition, reactive astrocytes no longer adequately regulate extracellular potassium concentrations, resulting in a decrease in the firing threshold of

neurons, thereby triggering seizures (de Lanerolle et al., 2010). Astrogliosis is therefore an important component of the pathology of epilepsy in the hippocampus and a process contributing to epileptogenesis (Tian et al., 2005).

1.1.3. Pericytes

Brain pericytes are macrophage-like cells with smooth muscle properties that are found around the endothelial cells of blood vessels and are mainly involved in the regulation of capillary function (Jayaraj et al., 2019). However, their localization confers on them a crucial role in the regulation of inflammation at the neurovascular unit. A particular interest has been directed at these cells to determine their role as regulators of inflammatory molecules produced peripherally by circulating leukocytes or secreted within the brain parenchyma by neurons and glial cells.

Pericytes are not only present in the brain, and their physiological role has been defined mainly in peripheral tissues, where they regulate the function of endothelial cells and ensure the integrity of the endothelial wall (Rucker et al., 2000; Thomas, 1999). They also play a regulatory role in controlling blood flow through their contractile properties, which are of great interest to capillaries lacking smooth muscular cells (Rucker et al., 2000; Thomas, 1999). Brain pericytes have been reported to have a wide range of immunoregulatory properties, including responding to and expressing a multitude of inflammatory molecules, presenting antigen, and displaying phagocytic ability (Rustenhoven et al., 2017). Their perivascular location provides them with an ideal location to control multiple aspects of the CNS immune response, including leukocyte extravasation, inflammation-induced BBB disruption, spread of peripheral and central inflammation, polarization of inflammatory cells in the BBB or parenchyma of the brain and adaptive immunity (Rustenhoven et al., 2017).

Surprisingly, it has recently been defined that some pericytes are actually progenitors of mesenchymal stem cells located near vessels and distributed throughout the body, to be rapidly mobilized in their environment if necessary and to differentiate into microglial cells or macrophages (Caplan, 2008).

1.1.4. Endothelial cells

Endothelial cells (EC) are one of the components of the neurovascular unit. These cells are bound together by tight-junction proteins. The EC are bordered by basal lamina composed of laminin, type IV collagen, fibronectin, heparin sulfate and other extracellular molecules (Jayaraj et al., 2019). The onset of neuroinflammation induces changes in the vascular system and more particularly in the BBB. As a result, there is an increase in BBB permeability allowing peripheral immune cells to infiltrate the brain. This change in permeability is the consequence of the increased level of pro-inflammatory cytokines such as IL1 β , IL6 or TNF α . The BBB's properties are also modified by chemokines such as CCL19 which will promote the adhesion of T cells to brain endothelial cells or CXCL12 which is known to reduce T-cell infiltration (Engelhardt, 2010).

The BBB breakdown following an epileptogenic brain insult has been suggested as a possible etiologic mechanism in epileptogenesis. During the chronic phase, the increased permeability of the blood-brain barrier may also be involved in the perpetuation of the disease and the genesis of the seizures (Marchi et al., 2012; Oby and Janigro, 2006; van Vliet et al., 2007). The relationship between pilocarpine-induced SE and BBB integrity has been investigated in a recent study by Mendes and colleagues (2019) in which they sought to determine the time course of the BBB opening and its subsequent recovery during the acute phase (Mendes et al., 2019). Their results show that within the first 30 minutes after SE, BBB becomes permeable to micromolecules and reaches its peak permeability to macromolecules 5 hours later. They also reported that the permeability to macromolecules is restored 24 hours following pilocarpine-induced SE, but the leakage of micromolecules persist longer. Their results highlight that BBB dysfunction during the acute phase following pilocarpine-induced SE is a process that takes place within a particular time window and is an inherent component of the pathogenesis of epilepsy.

1.2. Peripheral cells and their molecular mechanisms of infiltration into the CNS

1.2.1. Leukocytes

Leukocytes, or white blood cells, are the main cellular components of the peripheral inflammatory and immune response that protect against infection and assist in the repair of

damaged tissue. The leukocyte family includes granulocytes (neutrophils, eosinophils and basophils), monocytes and lymphocytes (T cells and B cells).

The BBB is responsible for regulating the migration of cells and blood molecules into the brain parenchyma. Brain injuries such as infections, head injuries or prolonged seizures can alter the properties of the BBB causing an increase in membrane permeability and thus promoting the extravasation of peripheral immune cells or molecules that, under non-pathological conditions, would not cross it. A key mechanism of inflammatory response is the migration of circulating immune cells to the injured brain, facilitated by BBB disruption, increased endothelial adhesion molecule expression and inflammatory molecules release by brain cells, as well as endothelial cells. Cytokines contribute to the inflammatory response of the BBB by increasing the expression of metalloproteases, selectins and adhesion molecules, as well as chemokines and their receptors in the endothelial cells of the cerebrovascular unit. These molecules are involved in the recruitment of leukocytes from the blood system by interacting with integrin molecules on their surface, promoting their adhesion and their subsequent entry into the perivascular space, the cerebrospinal fluid and the brain parenchyma (Ransohoff et al., 2003).

Invading leukocytes are well-known contributing to early-stage of neuroinflammation after BBB breakdown, but their role in the resolution of neuroinflammation and further brain repair has for long been debated and has been discussed in many studies with conflicting results. It has been proposed that the interaction between leukocytes and vascular endothelial cells in a murine TLE model modulates recurrent and spontaneous seizures (Fabene et al., 2008). Among leukocytes, the two cell populations that will mainly infiltrate the parenchyma after a brain injury by crossing the blood-brain barrier are monocytes and neutrophils. We will focus here in more detail on the monocyte population.

1.2.2. Monocytes/macrophages

Disruption of the BBB has been reported in numerous pathologies of the CNS, including neuroinflammatory disorders. Blood monocytes are recruited to inflamed tissues following the release of chemoattractant chemokines. Among the chemokines released into the brain after a brain insult, MCP1 (Monocyte Chemoattractant Protein 1, or CCL2) and MIP1 α (Macrophage Inflammatory Protein 1 α , or CCL3) will contribute to the attraction and

domiciliation of these circulating immune cells, and more particularly monocytes/macrophages, in which the presence of chemokine receptors on their surface will enable them to cross the blood-brain barrier (BBB) by extravasation and to integrate the brain parenchyma (Fabene et al., 2010; London et al., 2013). Previous research conducted in the laboratory (Navarro, 2007) has shown that the hippocampal infiltration of monocytes after pilocarpine-induced *status epilepticus* is a part of the inflammatory response in the hippocampus that precedes the neurodegenerative processes and is concomitant with the production of pro-inflammatory cytokines and microglia activation. These results are consistent with published data showing that infiltrating monocytes that invade the hippocampus significantly contribute to pathogenesis (Fabene et al., 2008; Ravizza et al., 2008; Tian et al., 2017; Varvel et al., 2016; Zattoni et al., 2011). At their infiltration site, these monocytes/macrophages would then undergo microglial transdifferentiation, i.e. cytological and phenotypic differentiation similar to the state of the activated residential microglial cells. However, because they do not belong to the residential microglial cell contingent, these cells are referred to as "monocyte-macrophages" (mo-M Φ) (London et al., 2013). The role of this monocyte infiltration is still debated: some studies suggest that it would be protective since preventing it leads to an increase in the severity of epilepsy symptoms (Zattoni et al., 2011), while others argue that these infiltrating monocytes would be deleterious (Tian et al., 2017; Varvel et al., 2016).

The contribution of infiltrating monocytes to the production of inflammatory molecules was also evaluated and conflicting results are at odds. Varvel and colleagues (2016) showed by FACS and cell sorting analysis that IL1 β expression is more important in microglial cells than in monocytes (Varvel et al., 2016). Conversely, the study of Vinet *et al.* (2016) revealed that infiltrating myeloid cells are more involved in neuroinflammation than microglial cells 24 and 96h after SE, and express a broader range of inflammatory molecules such as IL1 β and IFN γ or matrix metalloproteinase involved in the BBB leakage following SE (Vinet et al., 2016).

However, at this stage, the role of mo-M Φ on the excitability of nearby neurons remains uncertain, especially since studies in which they can be distinguished from residential microglial cells once their transdifferentiation has been completed are rare, due to the following reasons:

- The lack of specific markers for tracking infiltrating cells over long timespans are not universal or transposable to different species and/or disease models;
- Transgenic models are not always easy to develop, especially in rats, and have a cost that is not always affordable by laboratories;
- And finally, it cannot be excluded that some of the cells may lose during the transdifferentiation process the markers used to initially identify them, making it impossible to track some subpopulations of transdifferentiated cells.

1.3. Physiopathological consequences of the removal of residential microglia and infiltrating monocytes

It has become clear that microglia play a protective role in non-pathological conditions, but that after a severe brain injury and a persistent neuroinflammatory state, these cells may remain abnormally activated, helping to maintain a chronic and harmful neuroinflammatory response. Likewise, the substantial role of infiltrating monocytes in neuroinflammatory processes following a severe brain attack is no longer to be demonstrated. There are contradictory results in the literature: some studies support a positive role for both cell populations while others report a more deleterious role, depending largely on the disease being investigated, the models used, the time frame chosen, and the analysis performed to determine location of cells. Nevertheless, there is agreement among all these studies that deregulation of either cell population by its environmental niche and by the factors to which these cells are exposed can have significant consequences on physiopathological processes. This observation has led many studies to seek to inhibit the action of these cells or to suppress them completely (Han et al., 2017). Mirrione et al. (2010) showed that depletion of microglial cells in the hippocampus didn't lead to changes in acute seizure sensitivity suggesting that microglia are not responsible for disease development. In addition, in absence of microglia, the stimulation with lipopolysaccharide (LPS) before the acute seizure induction triggered caused greater seizure activity and increased mortality, suggesting that microglial activation processes after brain aggression could have a protective function during SE (Mirrione et al., 2010). In a mouse model of extensive neuronal loss, Rice and colleagues (2017), studied the effects of the elimination and repopulation of microglial cells by inhibition of the colony-stimulating factor 1 receptor (CSF1R), a receptor involved in the survival of microglial cells.

They showed that the elimination of microglial cells for a short time and their repopulation led to a reduction in the expression of inflammatory molecules, an increase in the density of dendritic spines and restored the behavioral deficits initially induced by neuronal loss (Rice et al., 2017).

The role of “activated” microglia in the epileptic brain has also been studied by using minocycline to inhibit microglial activation (Hiragi et al., 2018). Studies have shown that activated microglia have a neurodegenerative role after induction of seizures, and that the suppression of microglial cells after SE has resulted in a reduction in the number of spontaneous recurrent seizures as well as in the duration and severity of seizures (Abraham et al., 2012; Heo et al., 2006). However, these results should be taken with caution since it is known that minocycline can also act on non-microglial cells and has been shown to have neuroprotective effects on neurons in cultures (Huang et al., 2010) and anti-inflammatory effects on astrocytes and oligodendrocytes (Hiragi et al., 2018; Möller et al., 2016). In addition, minocycline limits the infiltration of immune cells into the CNS and their activation.

Regarding infiltrating monocytes/macrophages, several attempts have been made to deplete this cell population and to investigate the effect of their non-recruitment into the brain parenchyma after an insult. Literature has emerged that offers contradictory findings about the role of monocyte infiltration. In a model of experimental autoimmune encephalitis (EAE), myeloablation and subsequently, the suppression of monocyte recruitment in the CNS has blocked the progression of EAE (Ajami et al., 2011). This view is supported by Makinde et al. (2017), who found that monocyte depletion in a model of traumatic brain injury led to a significant improvement in brain edema, motor coordination and working memory and abolished neutrophil infiltration into the brain (Makinde et al., 2017). To study the role of microglia and infiltrating monocytes in cognitive deficits induced by brain radiation therapy, a very recent study sought to deplete microglial cells after irradiation of the entire brain (Feng et al., 2019). Subsequently, they showed that circulating monocytes infiltrate the brain and replace the microglial cell pool, and that these monocytes-macrophages had reduced aberrant phagocytic activity of synapses compared to activated microglia, resulting in prevention of memory deficits (Feng et al., 2019).

In epilepsy, Zattoni et al. (2011) demonstrated in a model of TLE after intrahippocampal kainic acid injection that the depletion of peripheral macrophages by clodronate liposome treatment resulted in a reduction of the granular cell layer thickness, suggesting that the presence of monocytes/macrophages may promote granular cell survival. Waltl (2018) observed in a model of encephalitis-induced epilepsy that monocytes depletion induced a reduction in the number of seizures. However, the development of hippocampal damage was not prevented or reduced (Waltl et al., 2018). Varvel et al. (2016) on the other hand, observed that the inhibition of the recruitment of chemokine receptor 2 (CCR2⁺) monocytes reduced SE-induced BBB damage and provided significant neuroprotection.

2. Prototypic molecular markers of brain inflammatory response and their functional role

The actors of neuroinflammation are diverse and include both different types of cell populations and inflammatory molecules such as cytokines and chemokines. Each of these markers will play a specific role in the initiation of neuroinflammatory response within the brain. Cytokines provide cells with the ability to communicate and orchestrate complex multicellular behavior. There is an emerging understanding of the role that cytokines play in normal homeostatic tissue function and how dysregulation of these cytokine networks is associated with pathological conditions (Becher et al., 2017).

The most extensively studied molecular mediators of inflammation are cytokines, which are small soluble proteins (< 60 kDa) involved not only in the inflammatory response, but also in cell-growth, cell activation and differentiation processes. The large cytokine family includes interleukins (IL), tumor necrosis factors (TNF), interferon (IFN), chemokines and transforming growth factors (TGF).

Cytokines are produced by a wide range of cell types including resident brain cells such as glial cells (microglia and astrocytes), neurons, endothelial cells as well as peripheral immune cells such as granulocytes, macrophages and lymphocytes (Alyu and Dikmen, 2017; Boulanger, 2009; Vezzani et al., 2011). They act by autocrine (when the cell itself binds the cytokine that is released), paracrine (cytokine acts locally on adjacent cells) or more occasionally by endocrine (on distant cells) signaling. They are biologically active at very low concentrations, by binding to specific high affinity membrane receptors. They constitute an interdependent

network of mediators that mutually influence their synthesis through positive and negative feedback circuits (Cavaillon and Haeffner-Cavaillon, 1993). Their purpose is to induce, control or inhibit the intensity and duration of the immune response. Cytokines are divided into two categories:

- The pro-inflammatory cytokines, which will sustain the inflammation and will promote the switch of microglial and immune cells phenotype toward an M1 phenotype. The most studied pro-inflammatory cytokines are IL1 β , IL6, TNF α and IFN γ ;
- The anti-inflammatory cytokines that will facilitate the switch toward M2 phenotype. The most studied are IL4, IL10 and IL13.

Cytokines operate via their binding to specific receptors that trigger specific signaling pathways leading to the production of new cytokines by the cell. For example, IL1 β by binding to its receptor IL1R will activate many signaling pathways associated with Mitogen-Associated Protein Kinase (MAPK). One pathway involving the p38 MAPK will result in the production of IL8 and IL6, which are themselves pro-inflammatory molecules (Jung et al., 2002). More than 35 pro-inflammatory cytokines have been identified to date, many of them belonging to the IL-1 family (**Box 3**).

Chemokines are molecules with chemotactic properties that induce the migration of immune or non-immune cells and play a role in maintaining CNS homeostatic functions. They are divided into four sub-families, based on the positioning of the conserved cysteine residues in their amino acid chain: CXC, CC, CX3C and XC (Zlotnik and Yoshie, 2000). Chemokines are small secreted cytokines (8-14 kDa) involved in many physiological processes as well as inflammatory processes. Chemokines are able, on the one hand, to promote the recruitment of immune cells and, on the other hand, to trigger signaling pathways such as those involved in the modulation of synaptic activity (Cerri et al., 2017). In the brain, chemokines have receptors on microglial cells, astrocytes, oligodendrocytes and neurons. In physiological conditions, previous investigations showed that chemokines are secreted by neurons as well as by perivascular macrophages and glial cells, suggesting a role in the regulation of cerebral homeostasis by acting as neurotransmitters (Miller et al., 2008; Rostène et al., 2011).

Box 3 | FOCUS ON THE IL1 FAMILY

IL1 was the first interleukin to be identified in the 1940s. There are three main cytokines in the IL1 family: two agonists, IL1 α and IL1 β and one natural antagonist, IL-1ra (Dinarello, 1996). Interleukin-1 β is the most studied pro-inflammatory cytokine in neurological diseases with a neuroinflammatory component. This cytokine is known to act on brain cells (neurons, astrocytes) due to the presence of its specific IL1R1 receptor on the surface of these cells. IL1R1 is a Toll-like receptor whose activation will trigger different signaling cascades that will lead to gene expression and release of factors involved in both promoting cell survival and toxicity (Allan et al., 2005; Fogal and Hewett, 2008; Weber et al., 2010). The beneficial or deleterious mechanisms of IL1 β are very difficult to study *in vivo* since this cytokine exerts complex actions that depend on the cell type on which it will bind, on the concentration of IL1 β present in the environment and on the duration of exposure (Pinteaux et al., 2009). Although all microbial components, such as LPS, are able to stimulate its production, IL1 β itself is able to stimulate its own synthesis and secretion. This retroactive circuit allows the response to IL-1 β to be amplified by autocrine or paracrine signaling. In the CNS, IL1 β is mainly synthesized by microglial cells, perivascular macrophages and astrocytes (Vitkovic et al., 2000). While IL1 cytokines are constitutively expressed at very low levels in the human CNS, they are often elevated in the brain under certain pathological states such as during an active seizure, hypoxic injury, and during the process of an infection (Kobylarek et al., 2019; Youn et al., 2013). IL1 β has been shown to negatively affect dendritic structure and synaptic plasticity and has also been reported to be involved in the exacerbation of excitotoxic insult (Allan et al., 2005; Fogal and Hewett, 2008). However, the effect of interleukin 1 is not always deleterious since this cytokine is also essential in learning and memory processes, with an important role in long-term potentiation mechanisms (Yirmiya and Goshen, 2011).

Other molecules such as cyclooxygenase (COX) 1 and 2 also play a role in the neuroinflammation. It has been shown that the COX 2 pathway appears to have an anti-inflammatory role, particularly in neurological diseases such as epilepsy (Vezzani et al., 2019).

Thus, even if the primary goal of neuroinflammation is to provide an effective response against CNS damage, the system can overreact, and chronic inflammation can result in numerous detrimental alterations in the brain environment. It is now well established that a chronic state of neuroinflammation is associated with significant synaptic dysfunction. For example, IL-1 β , when expressed chronically, will lead to a decrease in synaptic connection (Mishra et al., 2012). The microglia, which normally plays a protective role when inactive (Vinet et al., 2012), will lead to a decrease in synaptic efficiency in its activated state (Culbert et al., 2006). Similarly, astrocytes will see their role in the tripartite synapse reduced (Faissner et al., 2010) under the influence of TNF, which will disrupt the astrocyte calcium cascade

(Köller et al., 2001). Neuroinflammation will also decrease neurogenesis under the effect of pro-inflammatory cytokines such as IL6 and TNF α . These cytokines will induce an increase of neuronal precursor death and an inhibition of differentiation of these precursors, thus significantly decreasing the renewal rate of the neuronal population (Liu and Hwang, 2005).

III. Neuroinflammation: a key player in the pathophysiology of epilepsy?

1. Immunological features of seizures pathogenesis

1.1. Role and characterization of inflammation in epilepsy

Inflammation has been evidenced as an important factor in the pathophysiology of seizure generation and in neurobehavioral comorbidities (Mazarati et al., 2017; Paudel et al., 2018; Shimada et al., 2014; Vezzani et al., 2011; Wilcox and Vezzani, 2014). Elevated levels of inflammatory mediators have been measured in the serum of patients with various neurological disorders such as head injuries, multiple sclerosis or neurodegenerative diseases such as Alzheimer's or Parkinson's disease (Hemmer et al., 2015; Perry, 2004), but also in different forms of epilepsy (de Vries et al., 2016). Access to surgically resected parts in TLE patients allowed the assessment of inflammatory status within the epileptic focus itself. Several studies evaluated the expression levels of some mediators of inflammation in resected hippocampus of TLE patients. All these studies revealed a particularly high pro-inflammatory state compared to that measured in hippocampus from control post-mortem cases. The interleukin 1 (IL1) family is the most widely studied family in neuroinflammation studies and includes IL1 α , IL1 β and their receptor IL1R1. For IL1 α , its tissue expression level is higher in the hippocampus of patients with TLE than in control cases (Kan et al., 2012; Sheng et al., 1994), whether or not hippocampal sclerosis is present (Kan et al., 2012). For IL1 β , its level of tissue expression is also higher in the hippocampus of patients with TLE than in control cases (Fiala et al., 2013), the result having been found in another study only in TLE patients with hippocampal sclerosis (Ravizza et al., 2008). A significantly higher expression level of a wide range of cytokines and chemokines in the hippocampus of TLE patients has also been demonstrated, namely IL1Ra, IFN α , IL5, IL7, IL10, IL13, IL22, IL25, IL27, MCP1 (CCL2), MIP1 α (CCL3), MIP1 β (CCL4), RANTES (CCL5), CCL19 and CCL22 (Kan et al., 2012; de Vries et al., 2016). Higher levels of COX-2 and TGF- β have also been observed (Das et al., 2012). These results, although very supportive of the hypothesis that there is a pro-inflammatory state in the hippocampus in which the epileptic focus is most commonly located in refractory TLE, present a major limitation, namely the control cases to which they are compared. Indeed, the "control" tissues used are collected at the forensic institute from people with no history of

epilepsy or from tumors or post-traumatic surgical samples. In addition, when mentioned, sampling times range from 4 hours to 20.5 hours post-mortem, which is significantly longer than surgical sampling of TLE patients where specimens are usually immediately managed, either by freezing in the ice (Das et al., 2012; Kan et al., 2012; Omran et al., 2012) or by fixation (Das et al., 2012; Fiala et al., 2013; Ravizza et al., 2008; Sheng et al., 1994).

IL1 β

The role of IL1 β in the genesis and perpetuation of seizures has been extensively studied (Rijkers et al., 2009). During generalized tonic-clonic seizures, no changes has been reported in the IL1 β plasma levels of epileptic patients, whereas it has been shown that the CSF IL1 β level is increased after seizure with a significant positive correlation with the duration and frequency of seizures. It has been shown that high levels of IL1 β would exacerbate the intensity of seizures and lower their induction threshold (Dubé et al., 2005). Very recently, it has been shown on resected parts of TLE patients that IL1 β , by interacting with its IL1R1 receptor, could trigger a seizure, by reducing GABAergic inhibitory neurotransmission (Roseti et al., 2015).

IL6

In experimental studies of epilepsy, it has been established that after a KA-induced SE, IL6 mRNA is overexpressed in the hippocampus, the amygdala and the meninges (Lehtimäki et al., 2003). However, literature has emerged that offers contradictory findings about the role of IL6 in inflammatory processes and its functional role remain much debated today. Considered as a pro-convulsive cytokine by some studies (Kalueff et al., 2004), its neuroprotective role has also been documented (Penkowa et al., 2001). IL6 synthesis can also be induced, in a similar way to a second messenger, by the presence of other pro-inflammatory cytokines in the environment of the producing cells (Benveniste, 1992). Experimental studies have followed the evolution of IL6 production in response to trauma and inflammatory aggression. They showed that IL6 was released rapidly, with initial detection within 1 hour after the insult and a peak concentration between 2 and 8 hours after (Morganti-Kossmann et al., 2007).

TNF α

TNF α appears to play a dichotomous role in epilepsy. In a transgenic mouse model where TNF α was overexpressed by astrocytes, it was reported that seizures were shorter than in mice lacking TNF α (Balosso et al., 2005). In contrast, other studies have reported that transgenic mice in which TNF α was overexpressed by neurons throughout the brain developed seizures and died prematurely (Probert et al., 1995). These effects could be explained, on the one hand, by the types of receptors on which TNF α acts (Balosso et al., 2005), and on the other hand, by the fact that TNF α is thought to act in a concentration-dependent manner (Li et al., 2011).

1.2. Central and peripheric markers of inflammation in epilepsy

Despite the tight regulation of inflammation in the brain under normal conditions, a peripheral immune response can induce brain inflammation and exacerbate neurodegeneration (Perry et al., 2007). In humans, numerous investigations have focused on the link between the peripheral level of inflammatory markers and the severity of epilepsy. Others research aimed to investigate whether peripheral cytokine could be used as a predictive marker of the seizure onset since serum and CSF are much more easily accessible than brain tissue collected only from epileptic patients who are eligible for resection surgery.

Outcomes from different studies are not always consistent regarding the values obtained for the same cytokine/chemokine in serum. Uludag and colleagues (Uludag et al., 2013) showed that serum IL-1ra levels in TLE patients were lower than in the normal population, while other studies showed no difference (Lehtimäki et al., 2007; Liimatainen et al., 2009). In contrast to the findings obtained in brain tissues of TLE patients, serum IL-1 levels were not significantly higher than those of healthy controls (Hulkkonen et al., 2004; Lehtimäki et al., 2007; Liimatainen et al., 2009; Nowak et al., 2011). For IL6, serum levels were reported to be significantly higher in TLE patients in five studies (Alapirtti et al., 2009; Lehtimäki et al., 2011; Liimatainen et al., 2009; Nowak et al., 2011; Uludag et al., 2013) when two others showed no differences with the controls (Hulkkonen et al., 2004; Lehtimäki et al., 2007). A retrospective study performed on more than a thousand epileptic patients highlighted that

the level of IL1RA, IL6, IL8, IFN λ 2 and IL17a in the serum and CSF during the interictal period is positively correlated to the seizure severity of epileptic patients (Wang et al., 2015).

Studies that measured inflammatory mediators in brain tissue and/or in the cerebrospinal fluid or serum of epileptic patients were well documented in a meta-analysis conducted by de Vries *et al.* in 2016. In this review, the authors discuss the differences in expression of inflammatory markers between the periphery and CNS tissues. They suggest that the differences observed between CSF and tissues could be related to the fact that the expression of certain markers, such as IL6, may be seen as general markers attesting on the local and systemic activation of the immune system, when chemokine expression may be more a consequence of focal cerebral ischemia (de Vries et al., 2016). They also pointed out that several studies comparing inflammatory levels in the CSF and brain tissue have encountered significant differences, even when looking within the same patient. The question raised by the measurements of inflammatory mediators in serum or CSF during interictal periods is related to the uncertain origin of this increase in cytokine level. Indeed, the measurement of such factors does not enable to establish whether this inflammation is related only to cerebral inflammatory processes or whether it is the result of postictal peripheral muscular recovery or of the activation of circulating immune cells (de Vries et al., 2016). Hence, these elements hinder the identification of reliable peripheral markers for measuring brain inflammation and for potentially predicting seizure onset.

2. Role of inflammation during the development of epilepsy (i.e. epileptogenesis)

A large majority of studies that have investigated the links between neuroinflammation and epilepsy have not focused on the chronic phase of epilepsy, when seizures are recurrent and spontaneous, but rather on the epileptogenesis phase, during which a healthy brain is transformed into an epileptic brain following a severe brain injury (such as SE). In humans, this time is difficult to assess since it may last from a few months to several years and to date there is no biomarker that can reliably predict the development of epilepsy. These elements justify why epileptogenesis has been almost exclusively addressed using animal models of the pathology. As described in the previous chapter, numerous animal models have been developed in rodents. The pilocarpine-induced SE model has been

extensively studied because of its similarity in the pathophysiological profile observed in animals with that of TLE in humans (Curia et al., 2008; Scorza et al., 2009). Studies conducted in the late 1990s and early 2000s established that all models inducing chronic epilepsy were associated with a period of strong and transient inflammatory response, often limited to the acute phase following severe brain damage, and involved the pro-inflammatory cytokines IL1 β , IL6 and TNF α (Rana and Musto, 2018; Vezzani et al., 2011). The cascade of events that would lead from the initial aggression to the ultimate development of epilepsy is described in **Figure 8** (Vezzani, 2014). Cytokines released in the CNS during the epileptogenesis phase will be involved in different mechanisms leading to the development of epilepsy. IL1 β , IL6, TNF α and IFN γ have been shown to be implicated in brain hyperexcitability, in the alteration of glutamatergic transmission, in BBB disruption and in the potentiation of the deleterious effect of reactive oxygen species (Klein et al., 2018; Kobylarek et al., 2019; Rana and Musto, 2018).

Various other mediators of inflammation are believed to be involved in the processes that lead to the transformation of healthy brain tissue into epileptic tissue. Prostaglandins, derivatives of arachidonic acid produced by constitutively expressed (COX1) or inducible (COX2) cyclooxygenases, are secreted mainly by astrocytes and microglial cells. When prostaglandin E2 stimulates its receptor present on the surface of astrocytes, it leads to an increase in glutamate release inducing hyperexcitability and neuronal death (Rana and Musto, 2018; Shimada et al., 2014). Nevertheless, despite the fact that targeting the prostaglandin synthesis pathway appears to be a promising target, studies that have sought to inhibit the expression of COX2 have reported conflicting effects due to the involvement of this enzyme in physiological processes (Rana and Musto, 2018).

Other inflammatory cascades involving molecular mediators such as interferons, transforming growth factor TGF- β , vasoactive endothelial growth factor (VEGF), platelet-activating factor, matrix metalloproteinases such as MMP-9, or Toll-like receptors are implicated in signaling pathways that have been reported to play a role in epileptogenesis-related processes such as neurodegeneration, modulation of neurogenesis and synaptic plasticity, and regulation of BBB permeability (Rana and Musto, 2018; Ravizza et al., 2011; Vezzani et al., 2011).

When talking about the role of inflammation during epileptogenesis, reference is made not only to molecular inflammation but also to the cellular changes that take place in response

to the production of these molecular mediators. Indeed, it is known that IL1 β , IL6 and TNF α will be responsible for some cellular effects by acting notably on mechanisms involved in the increase of BBB permeability, thus facilitating the increased penetration of these same mediators coming from the periphery. Brain vascular cells possess receptors for inflammatory mediators whose activation has been reported to lead to the production of chemokines, nitric oxide and prostaglandins that are believed to further impair the integrity of BBB (Rana and Musto, 2018; Vezzani et al., 2011). Infiltrating immune cells, especially monocytes, are also thought to play a role in the inflammatory processes leading to the development of epilepsy, although their exact role is not clear, as discussed in section II.1.2. Finally, the activation of both microglial cells and astrocyte cells after a pro-epileptogenic brain insult and the subsequent formation glial scar also appear to be involved in the processes of epileptogenesis.

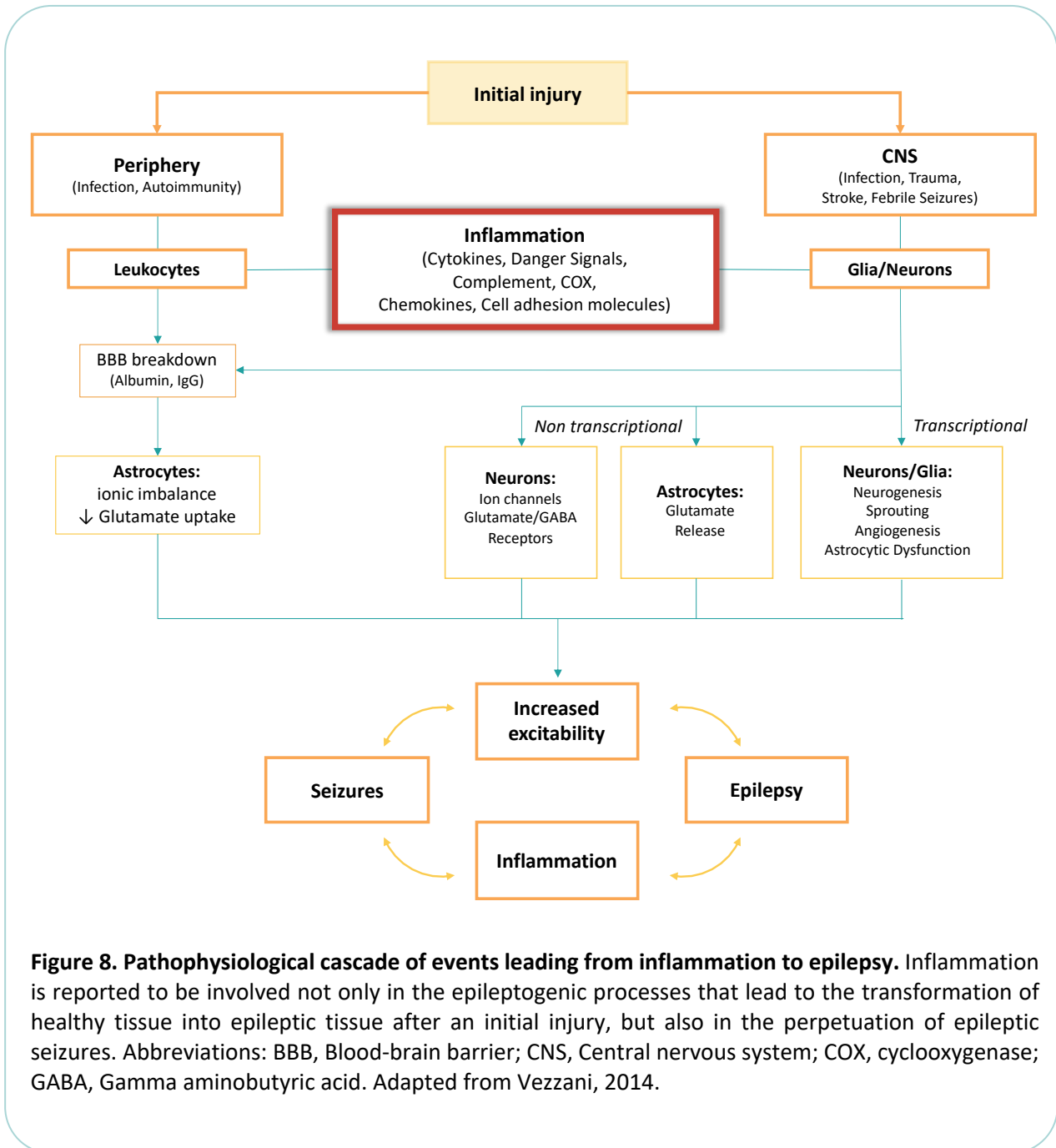


Figure 8. Pathophysiological cascade of events leading from inflammation to epilepsy. Inflammation is reported to be involved not only in the epileptogenic processes that lead to the transformation of healthy tissue into epileptic tissue after an initial injury, but also in the perpetuation of epileptic seizures. Abbreviations: BBB, Blood-brain barrier; CNS, Central nervous system; COX, cyclooxygenase; GABA, Gamma aminobutyric acid. Adapted from Vezzani, 2014.

3. Resolving inflammation in epilepsy: pharmacological approaches and functional consequences

The acute inflammatory response is a self-resolving protective mechanism, allowing tissue repair and pathogen removal to restore homeostasis. It must be highly regulated and effective. The resolution of inflammation has long been considered as a passive process (Serhan et al., 2007). However, the identification of endogenous bioactive mediators revealed

that this process is active and highly regulated. It controls immune cells through the reduction of pro-inflammatory factor production and the increase of anti-inflammatory factors and activates the phagocytosis and apoptosis processes needed to restore homeostasis (Serhan et al., 2015; Sugimoto et al., 2016). Therefore, many investigations have focused on targeting this inflammation and attempting to resolve it. The resolution of inflammation process has been defined as the “molecular and cellular events that ultimately assure tissue repair and regain of physiological function” (Ortega-Gómez et al., 2013). Since this endogenous process is not always sufficient to halt the development or perpetuation of diseases, the purpose of the multiple therapeutic approaches has been to modulate the deleterious action of the major inflammatory actors in order to support the overall resolution of inflammation.

Uncovering the relationship between epilepsy and inflammation have led to the emergence of new therapeutic strategies designed to counteract or alleviate the perpetuation of the disease (Vezzani et al., 2015). Debate continues about the best strategies for the management of inflammation in epilepsy. Since inflammatory pathways have been identified as a key factor in the development of epileptic seizure, a considerable amount of research has been conducted to counter the release of specific cytokines, in particular IL1 β . It has been shown that the use of the natural IL1 receptor antagonist (IL1RA), which is opposed to the action of IL1 β , or the use of an inhibitor of the IL1 β precursor cleavage enzyme, resulted in a decrease in the SE-induced neuronal loss (Noe et al., 2013). Furthermore, the *in vitro* use of a human recombinant IL-1 β receptor antagonist, named Anakinra, on isolated guinea pig brain in which epileptiform activity was provoked by bicuculline, has shown promising results in terminating seizures (Librizzi et al., 2012). Systemic inflammation has also been targeted to prevent the development of epilepsy. In a 2009 study, Marchi et al. showed in rodents that intravenous administration of an IL1 receptor antagonist reduced the incidence of SE and the SE-induced BBB damage (Marchi et al., 2009). Unfortunately, these pharmacological blockade of IL1 β or other cytokines were not effective in totally counteracting epileptogenic processes and the onset of epilepsy (Noe et al., 2013). These results suggest that other neurobiological mechanisms than just the IL1 β pathway contribute to the epileptogenic process.

In clinical practice, anti-inflammatory drugs have also been used in patients who do not respond to conventional ASDs. Anakinra, the human recombinant IL-1 β receptor antagonist, has been used in children with febrile infection-related epilepsy syndrome (FIRES)

(Dilena et al., 2019; Kenney-Jung et al., 2016), as well as in adolescents with drug-resistant epilepsy (Jyonouchi and Geng, 2016; Vezzani et al., 2019). A case report showed that the use of Anakinra and canakinumab, a monoclonal antibody anti-IL1 β , induced in a female epileptic patient resistant to any anti-epileptic drug a quite impressive clinical response, with almost complete resolution of clinical seizures associated with a significant improvement in her quality of life (DeSena et al., 2018). However, questions have been raised about the safety of prolonged use of Anakinra, especially at high doses. This has been evaluated in other inflammatory diseases that can be treated with this antagonist. Patients with rheumatoid arthritis, a condition in which high levels of IL1 are produced in the articular synovium, may also be managed by Anakinra. The long-term safety assessment of the treatment has shown that the use of high doses of anakinra in these patients increases the risk of serious infections since the antagonist is likely to interfere with the endogenous immune response to pathogens (Cabral et al., 2016; Salliot et al., 2009).

In pilot studies in patients with epilepsy, other treatments with monoclonal antibodies directed against TNF α or IL6 have been tested and have resulted in a reduction in the frequency of seizures in some patients (Lagarde et al., 2016), although sometimes associated with severe infections as side effects (Jun et al., 2018). The cyclooxygenase (COX)-2 signaling pathway has also been targeted by anti-inflammatory treatments nonsteroidal anti-inflammatory drugs (NSAIDs) such as Ibuprofen and Aspirin, demonstrating or not significant effects on reducing the number of seizures (Godfred et al., 2013; Radu et al., 2017; van Stuijvenberg et al., 1998; Vezzani et al., 2019). NSAIDs are commonly used in clinics for their anti-inflammatory, analgesic and antipyretic role. In the brain, NSAIDs have a broad spectrum of action since they are likely to reduce the inflammatory response more globally by acting on multiple neuronal and non-neuronal targets at the neurovascular unit or at the glial parenchyma level (Radu et al., 2017). Among their effects, it has been reported that they lead to a reduction in neuronal firing, a modulation of leukocyte-mediated inflammation and a decrease in neutrophil migration. Nevertheless, as discussed by some authors of these studies (Kenney-Jung et al., 2016), caution should be exercised in the conclusions of pilot investigations since patients often received several treatments at the same time, and the specific effect of each is difficult to determine.

All these therapeutic approaches to modulate inflammation have been tried in patients with epilepsy. But in patients at risk of developing acquired epilepsy, i.e. after a pro-epileptogenic injury, pharmacological treatments can also be dispensed. As a first line of defence, for brain insults such as head trauma or stroke, anti-seizure drugs are administered. Nevertheless, the administration of anticonvulsants following acute brain insults has so far failed to prevent late onset of epilepsy. It has been shown that After a traumatic brain injury, prophylaxis with conventional ASDs, such as phenytoin, carbamazepine and valproate benzodiazepines, were ineffective in reducing or preventing the development of epilepsy (Webster et al., 2017). The results of these clinical trials are sometimes difficult to interpret because the non-development of epilepsy is not easily imputable to the use of the drug, since many risk factors are involved. Numerous preclinical studies with the use of agents targeting inflammation signaling pathways during epileptogenesis following SE or TBI have also been tested, some showing promising results by modifying the disease course, but none completely preventing the onset of epilepsy (Clossen and Reddy, 2017; Pitkänen and Lukasiuk, 2011). As Teresa Ravizza and Annamaria Vezzani wrote in 2018, it is necessary to 1. understand more deeply the dynamic changes in neuroinflammation during the epileptogenesis phase in order to determine the best window for therapeutic intervention and 2. better distinguish physiological inflammation from pathological inflammation in order to not interfere in endogenous repair processes by administering exogenous treatment (Ravizza and Vezzani, 2018).

Anti-epileptic pharmacological approaches in humans are mainly aimed at reducing the number of seizures, a legitimate purpose considering the impact of seizures on the individual's life, especially when they are high in frequency. However, therapeutic decisions rarely include the management of cognitive and psychiatric aspects, comorbidities that are often associated with temporal lobe epilepsy. Many anti-epileptic treatments even have adverse effects that worsen psychiatric symptomatology or further increase cognitive impairment with side effects such as depression, aggression, irritable mood, paranoid ideations, anxiety, hallucinations, attentional problems or slower cognitive processing speed (Eddy et al., 2011; Ortinski and Meador, 2004; Park and Kwon, 2008; Stephen et al., 2017). These disorders are not harmless and have a strong impact on the daily lives of epileptic patients, sometimes with life-threatening consequences. Therefore, it is now necessary to develop therapies that will address as many symptoms as possible, with the minimum number of side effects.

Nowadays, the development of personalized medicine has contributed to a more global reflection on the use of treatment targeting inflammation. Recent studies have questioned the relevance of the non-targeted use of these anti-inflammatory treatments, i.e. without having identified in advance which patients might be receptive to them (Terrone et al., 2019). In addition, the intervention window could also have an important role in the efficiency of the treatment applied (Löscher, 2019). Novel therapies may focus on either epileptogenesis, during which the morphological and functional changes lead to epilepsy after a first brain insult, or ictogenesis, the processes that initiate, spread and amplify seizures in the epileptic brain. Based on this paradigm and the limitations of conventional treatments, refractory epilepsy can be a potential candidate for alternative treatments. Such treatments may be used to directly target the epileptic foci or the seizure generation pathways. Löscher and colleagues proposed in 2008 an explanatory diagram of the potential time points for therapeutic intervention along with the outcomes that could be measured (Löscher et al., 2008). This diagram is presented in **Figure 9**. Among the non-pharmacological alternative treatments that may be used as anti-epileptogenic or disease-modifying, cell therapy has an increasingly prominent place in clinical and preclinical research, which we will discuss in greater detail in the following section.

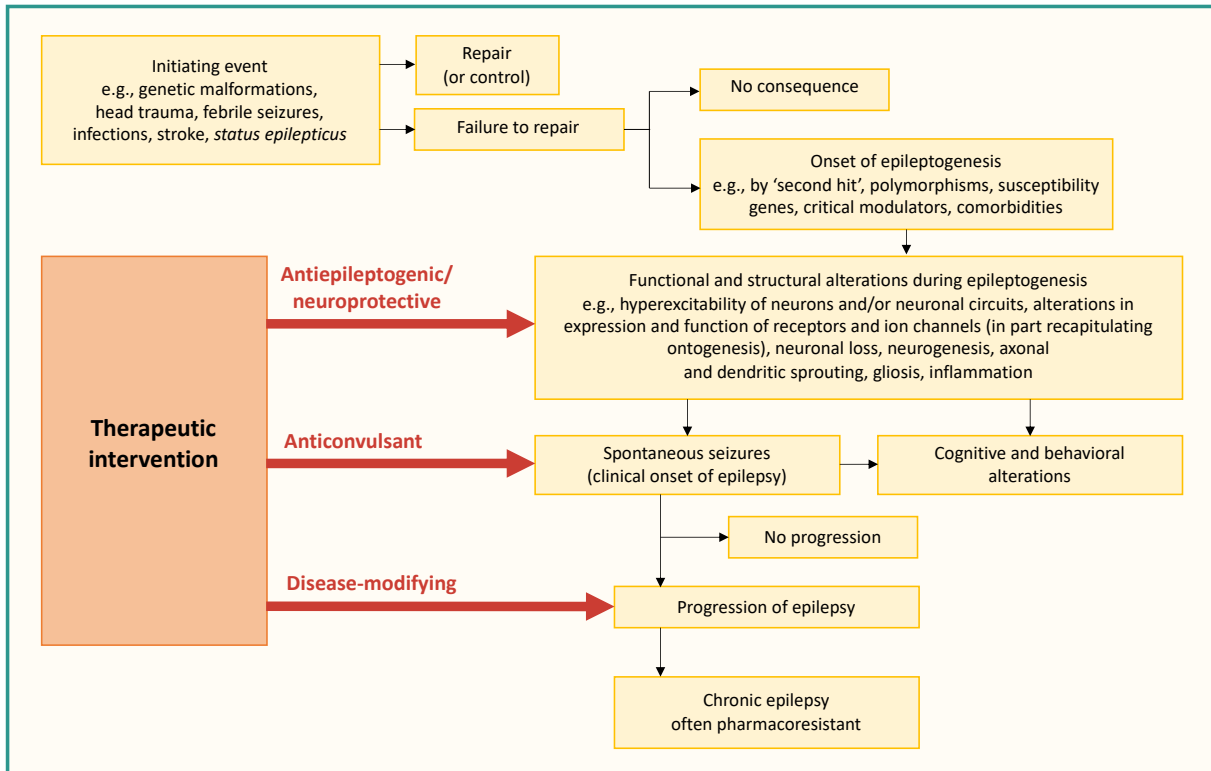


Figure 9. “Steps in the development and progression of temporal lobe epilepsy and possible therapeutic interventions. The term epileptogenesis includes processes that take place before the first spontaneous seizure occurs to render the epileptic brain susceptible to spontaneous recurrent seizures and processes that intensify seizures and make them more refractory to therapy (progression). The concept illustrated in the figure is based on both experimental and clinical data.” Antiepileptogenic approaches are strategies designed to be applied at the initial brain injury to prevent/reduce deleterious processes of brain transformation from healthy tissue to epileptic tissue. Anti-convulsive therapies aim to directly address recurrent and spontaneous seizures and alleviate associated behavioural disorders. Finally, disease-modifying therapies are designed to delay the progression of the disease to more serious forms associated with total non-response to medications. Adapted from Löscher et al., 2008.

IV. Mesenchymal stem cells: the new *trendy* strategy for treating neurological diseases

The past fifty years have seen increasingly rapid advances in the field of stem cell research and their use in cell therapy. Stem cells are undifferentiated cells defined by their abilities to self-renew and give rise to various types of differentiated cells, depending on their potency (**Box 4**). The self-renewal property is achieved by a symmetric cell division into two identical cells while the differentiation occur by asymmetric division giving rise to two different cells, an undifferentiated one to maintain the pool of stem cells, and a cell engaged into a specialized differentiation path. Stem cells are also classified according to their origin (**Figure 10**, Pelegrine and Aloise, 2018):

- Embryonic stem cells (ES cells), found during the early stages of the embryonic development, are totipotent or pluripotent: totipotent ES cells are found within the zygote and the morula (from 2 to 16 cells) and have the highest differentiation potential since they can give rise to any embryonic cell as well as extra-embryonic cells. Pluripotent ES cells are then found in the blastocyst and can differentiate into any of the three germ layers (endoderm, mesoderm and ectoderm);
- Fetal stem cells are multipotent cells found in one of the three germ layers during the fetal development;
- Adult stem cells are post-natal multipotent or unipotent cells present in several tissues, already engaged in a specific differentiation pathway.

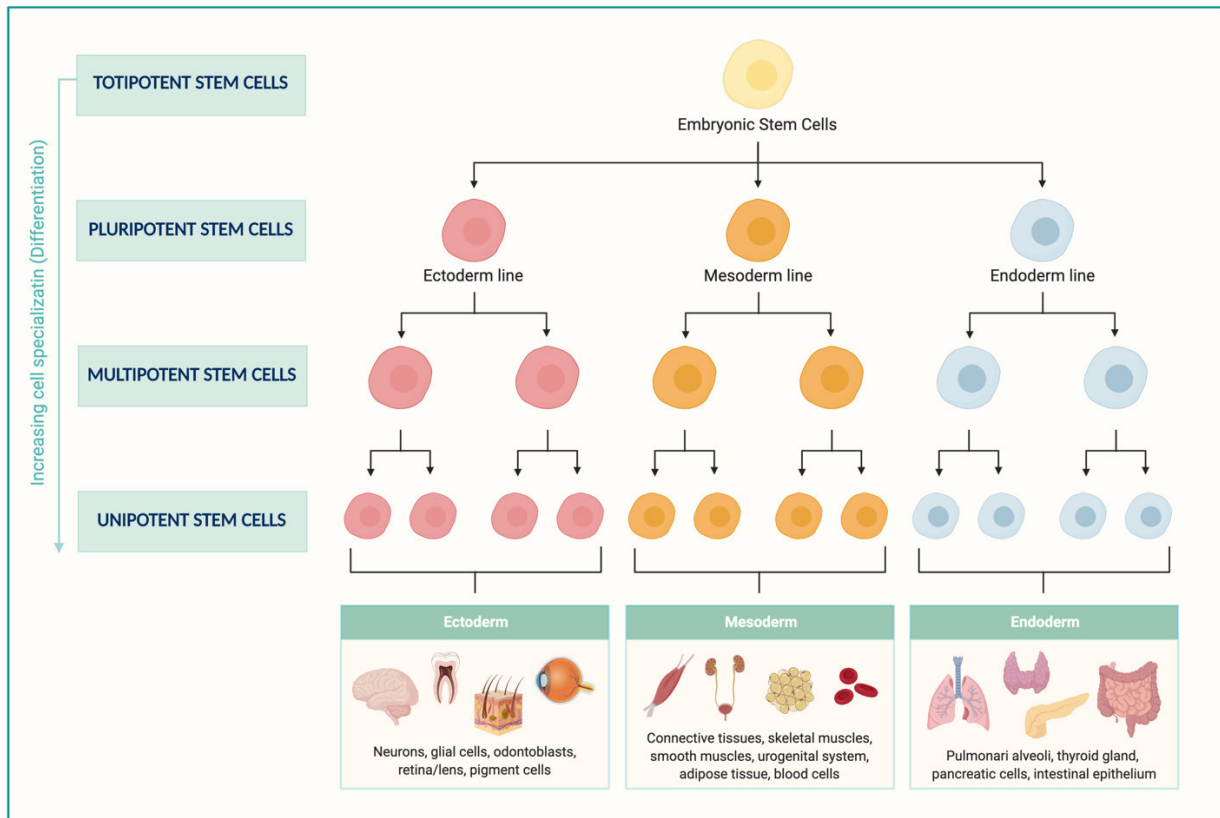


Figure 10. Stem cell potency. Embryonic stem cells are totipotent during the early stage of embryonic development, meaning that they have the potential to give rise to an entire person. As an embryo develops, cells engage in specific differentiation pathways belonging to one of the three lineages (endoderm, mesoderm or ectoderm), and are said pluripotent. MSCs are multipotent stem cells that arise from mesodermal lineage and that have the potential to differentiate into osteocytes, chondrocytes and adipocytes *in vitro* and *in vivo*, as well as in neurons, hepatocytes or pancreatic islet cells *in vitro* under specific conditions. Adapted from Pelegrine and Aloise, 2018.

The common origin of all human cells has led to the idea that different incurable diseases might be treated by the application of stem cells as a therapeutic agent in regenerative medicine, as these cells can provide trophic support or even replace dying cells with new ones (Sykova and Forostyak, 2013).

Cell-based therapies offer several advantages over gene therapy for treating neurological diseases. Unlike other peripheral tissues, the CNS has a very restricted ability to self-repair due to the impossibility for mature neurons to regenerate, despite the presence of neural stem cells in the hippocampus or in the sub ventricular zone. Several attempts have been made to replace destroyed neurons or glial cells after injury using stem cells (George et al., 2019; Krabbe et al., 2005; Neirinckx et al., 2013; Scuteri et al., 2011; Takeda and Xu, 2015; Urrutia et al., 2019). For long, it has been thought that to be successful, the grafts of stem cells

and/or their different derivatives in the injured brain areas must not only survive for long periods of time but also need to migrate correctly to the appropriate sites, integrate, and establish the correct types of synaptic connections with the host brain. Emerging research area seeks to dissociate the presence of stem cells in the damaged tissues from their therapeutic effects caused by their production of molecular mediators. Therefore, it is important to differentiate studies where stem cells have been used for their differentiation abilities and for replacement of pre-existing cells from studies in which their paracrine abilities are harnessed.

One of the most used adult stem cells in preclinical research and in clinical trials are mesenchymal stem cells (MSCs). MSCs were first tested as a cellular pharmaceutical agent in human subjects in 1995 by Hilard Lazarus (Lazarus et al., 1995). The regenerative capabilities of MSCs as well as their potential to produce trophic and anti-inflammatory molecules have made them a therapeutically promising tool of the future, hence high expectations are placed in these MSCs in the field of translational medicine (Wei et al., 2013).

BOX 4 | STEM CELL POTENCY / TYPES AND CLASSIFICATION

Potency of stem cell refers to the capacity to differentiate into specialized cells types and be able to give rise to any mature cell type.

Totipotent/Omnipotent stem cells

Can give rise to any of the 220 embryonic cell types as well as extra-embryonic cells (i.e. placenta).

Pluripotent stem cells

Can self-renew and give rise to all cell types in an organism (but not placenta) originating from the 3 germ layers: endoderm, mesoderm and ectoderm.

Multipotent stem cells

Can self-renew and give rise to a limited number of cell types in a particular lineage.

Oligopotent stem cells

Can only self-renew and give rise to only a few different cell types.

Unipotent stem cells

Can only self-renew and give rise only to their own types of cells.

1. Overview of principal mesenchymal stem cells features

1.1. Sources, phenotype and differentiation pathway

MSCs have been firstly identified and characterized in 1968 by Alexander Friedenstein (Friedenstein et al., 1968) that was the first to differentiate them from hematopoietic stem cells (Chamberlain et al., 2007). He was also the first to observe that these cells, when cultured *in vitro*, were forming cell colonies of fibroblastic morphology that were adherent to plastic and initially named them Colony-Forming Unit-Fibroblast (CFU-F). These cells were officially named as Mesenchymal Stem Cells more than 25 years ago to represent a class of cells from human and mammalian bone marrow and periosteum that could be isolated and expanded in culture while maintaining their *in vitro* capacity to be induced to form a variety of mesodermal phenotypes and tissues (Caplan, 1991). Subsequently, the self-renewal capacity of MSCs was demonstrated first *in vitro* in a study describing their division over several generations without any change in morphology or loss of their differentiation potential (Bruder et al., 1997), and then *in vivo* ten years later (Sacchetti et al., 2007).

In 2006, the International Society for Cell Therapy (ISCT) defined the following minimum criteria for these non-hematopoietic cells that are MSCs (Dominici et al., 2006; Samsonraj et al., 2015):

- Adherence to plastic in standard culture conditions;
- Multipotent *in vitro* differentiation potential into osteocytes/osteoblasts (demonstrated by staining with Alizarin Red or von Kossa staining), chondrocytes/chondroblasts (demonstrated by staining with Alcian blue or immunohistochemical staining for collagen type II), and adipocytes (demonstrated by staining with Oil Red O);
- $\geq 95\%$ of the MSC population measured by flow cytometry must express the specific surface antigen: CD105/Endoglin, CD90/Thy1 and CD73/5'-Nucleosidase (**Table 1**);
- Lack of expression (or $\leq 2\%$ of MSC population measure by flow cytometry) of: CD45, CD34, CD14 or CD11b, CD79 α or CD19 and HLA-DR surface molecules (**Table 1**).

The above criteria applied only for human at first and were defined to standardize the cell preparations and allow for a comparison of scientific studies among laboratories (Dominici et al., 2006). Negative markers identified by the ISCT are used to exclude certain type of cells, while positive markers allow to identify with certainty the surface antigens that are absent from most hematopoietic cells (**Table 1**). The absence of a single specific marker to identify these cells limits the purity of MSCs isolated by the detection of a combination of positive and negative markers. This heterogeneity can increase variability in experimental studies, decrease the differentiation potential, and contribute to conflicting data in the literature. These stem cells are multipotent, meaning that they are able to differentiate into mature cells of mesodermal lineages (**Figure 11**). Transdifferentiation of MSC into cells of non-mesodermal origin such as neurons, hepatocytes or pancreatic islet cells has also been reported *in vitro* under specific culture conditions and stimuli (Uccelli et al., 2008).

MSC are immune evasive due to their very low expression of histocompatibility complex class I molecules, known to cause immunogenic reaction after engraftment (Ankrum et al., 2014). Moreover, MSCs don't express on their surface co-stimulatory antigens such as CD40, CD80 and CD86, which are known to activate T-cells (Mukai et al., 2018b). Therefore, these cells are frequently referred to as "universally donor cells", allowing to consider allogeneic sources of MSCs over autologous MSCs while avoiding the use of immunosuppressive drugs and the risk of graft-versus-host disease (Chamberlain et al., 2007; Vanikar et al., 2014). Many companies are now developing stem cells banks where all the cells are cultivated according to standardized protocols, then providing commercially available MSC. This represents a growing economic market for industries involved in healthcare.

The clinical trials database (clinicaltrials.gov) currently (retrieved in November 2019) lists 1008 studies (735 when excluding those of unknown status) in which MSC were used for all types of clinical conditions. These trials aim to evaluate the biomedical potential of MSCs in bone and cartilage repair, in the treatment of cardiovascular, hepatic, renal, pulmonary and neurological diseases, as well as on immune-related pathologies such as graft-versus-host disease (Squillaro et al., 2016).

TABLE 1 | NEGATIVE AND POSITIVE MSC MARKERS AS PROPOSED BY THE INTERNATIONAL SOCIETY FOR CELL THERAPY (Adapted from Schachtele et al., R&D System)

NEGATIVE MSC MARKERS	CD34	Specific to primitive hematopoietic cells and endothelial cells
	CD45	Specific to leukocytes
	CD11b & CD14	Specific to monocytes and macrophages
	CD79 α & CD19 α	Specific to B cells
	HLA Class II	Specific to antigen presenting cells and lymphocytes
POSITIVE MSC MARKERS	CD73	Catalyzes production of extracellular adenosine from AMP
	CD90	Wound repair, cell-cell and cell-matrix interactions
	CD105	Vascular homeostasis; modulation of TGF β functions

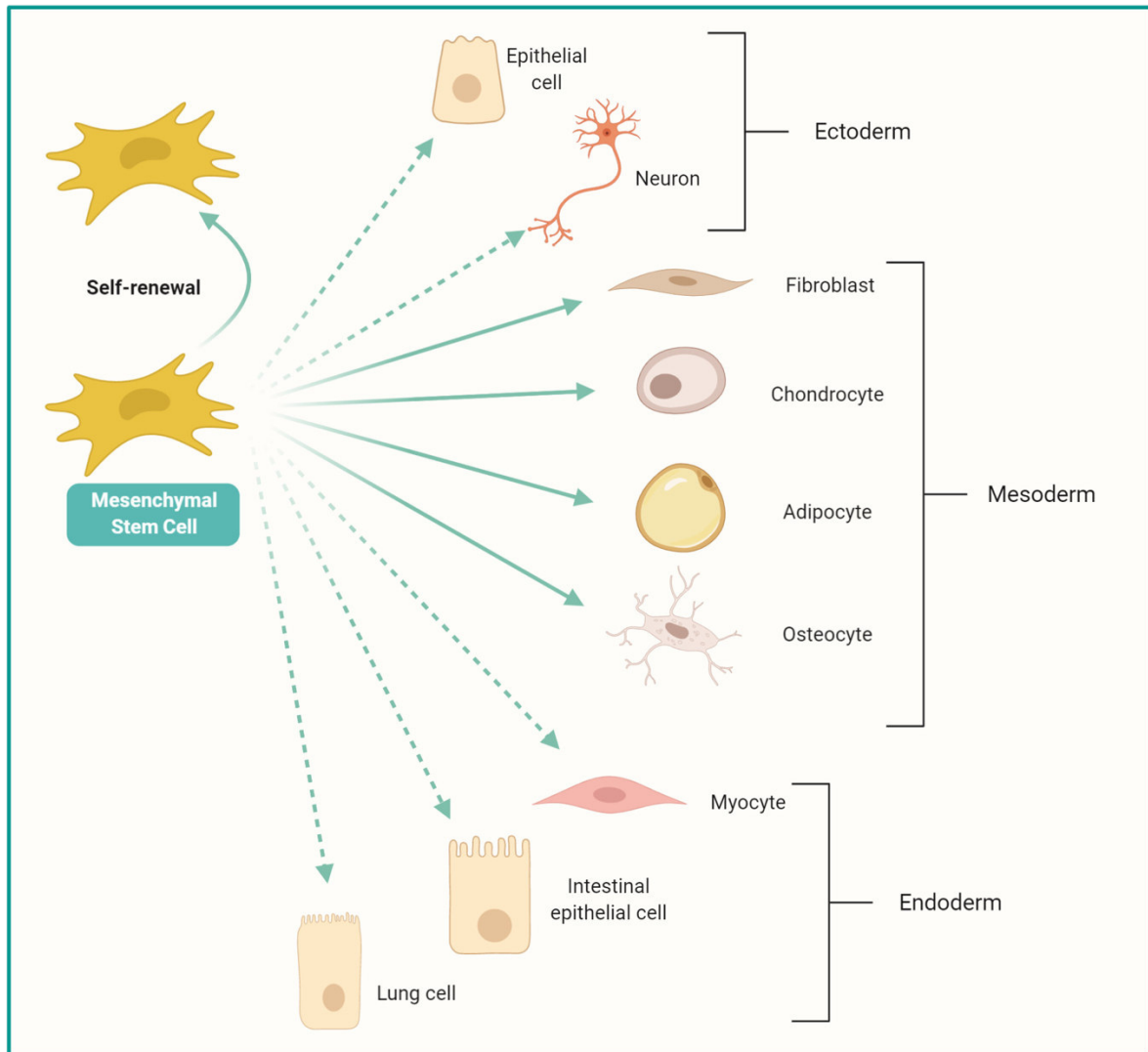
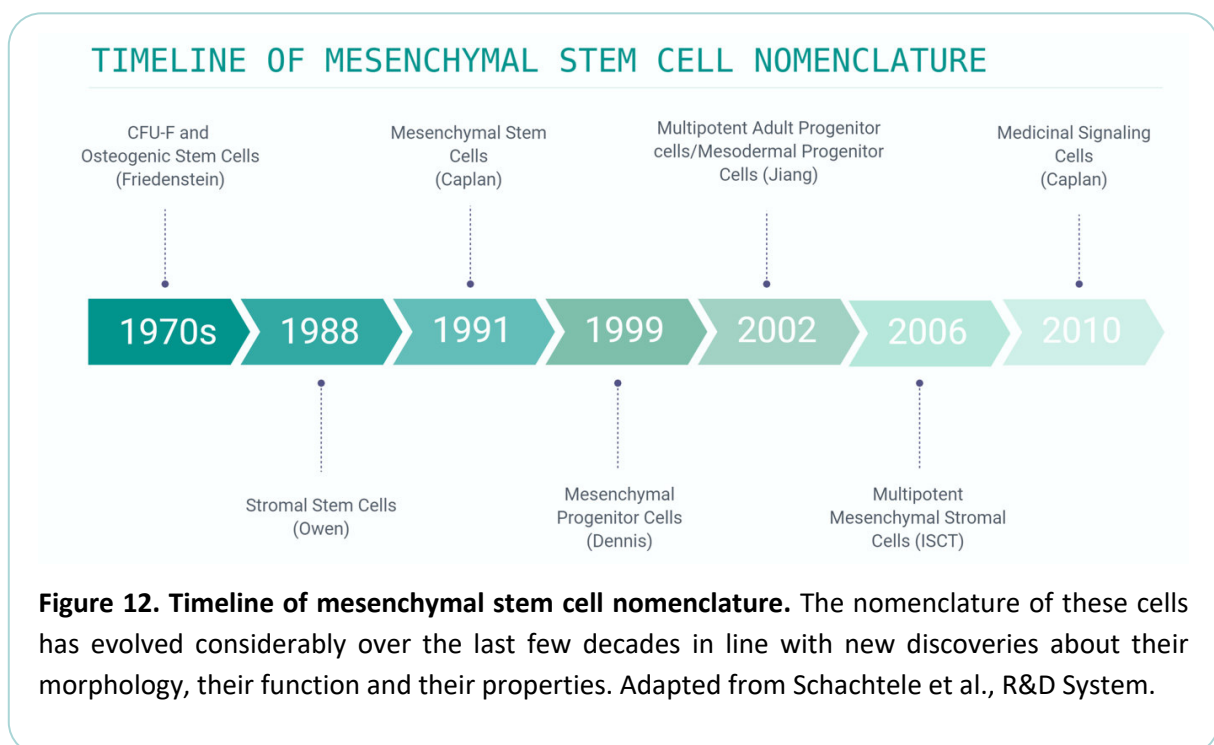


Figure 11. Multipotency properties of MSCs. The filled arrows correspond to the classical differentiation pathways of MSCs into cells of the mesodermic lineage. The dotted arrows show the differentiation paths that MSCs can follow when they are provided with the appropriate factors in their environment. These potential differentiation pathways have mainly been shown *in vitro*. Adapted from Uccelli et al., 2008.

The terminology used to define these stem cells has evolved between their first identification in 1968 and now. MSCs were originally described by Friedenstein as colony forming unit fibroblasts (CFU-F) and osteogenic stem cells, a name that Owen and colleagues have replaced by “Stromal Stem Cells”, which refers to the location of these cells that can be found in the stroma rather than the hematopoietic compartment (Bianco et al., 2008). In 1991, Caplan proposed the name Mesenchymal Stem Cells to emphasize the differentiation potential of these cells as well as their self-renewal properties (Caplan, 1991). Since, a range of names have been used to designate these cells (**Figure 12**): “Mesenchymal Progenitor Cells”

was suggested by Dennis et al. in 1999 who considered MSCs more as progenitor as stem cells *per se* (Dennis et al., 1999). Then, the names “Multipotent Adult Progenitor” or “Mesodermal Progenitor Cells” were raised by Jiang, referring to their intrinsic properties of multipotency, or differentiation abilities into all the cells of mesodermal lineage. The ISCT then proposed in 2006 the term “multipotent mesenchymal stromal cells” since no direct evidence demonstrated that MSCs are able to self-renew and differentiate *in vivo*. The most recent name suggestion was issued in 2010 and reiterated in 2017 by Caplan, who strongly recommends that the name of MSCs should be replaced by "medicinal signaling cell", in order to highlight that the main therapeutic advantage of these stem cells lies mainly in their ability to secrete immunomodulating and trophic bioactive molecules rather than in their ability to integrate tissue and to differentiate directly to induce tissue regeneration (Caplan, 2010, 2017). For the rest of this thesis, the term MSCs for “Mesenchymal Stem Cells” will be retained, but it must be remembered that the debate concerning their denomination is still ongoing and is constantly evolving as the precise functions and mechanisms of these cells are uncovered.



Tissue source of MSC

According to the minimal criteria established by the ISCT, MSCs are not restricted to bone marrow but can be isolated from a wide range of tissues. The first studies were performed using MSCs isolated from bone marrow. But MSCs only account for 0.001 to 0.01% of the bone marrow nucleated cells, which represents a 10-fold lower number of cells than hematopoietic cells (Shi et al., 2010). Therefore, other sources of MSCs have been considered and similar cells were isolated from a variety of fetal and adult human tissues. Initially considered as identical and equipotent, MSCs from tissues other than bone marrow actually display differences in terms of their plastic abilities, which can be ascribed to the tissue origin and/or to the procedures used for their isolation (Vezzani et al., 2018). MSC-like populations have since been harvested from autologous and allogeneic sources, including placenta, amniotic fluid (In 't Anker et al., 2003), umbilical cord, cord blood (Mareschi et al., 2001), Wharthon's Jelly (Davies et al., 2017), adipose tissue (Zuk et al., 2001), dental pulp (Shi and Gronthos, 2003), peripheral adult blood (He et al., 2007), lung (Lama et al., 2007), synovial fluids (Fan et al., 2009), muscle (Jackson et al., 2013) and brain (Kang et al., 2010). This wide distribution in all parts of the body has highlighted a potential role for MSCs in tissue repair and regeneration over the lifespan.

MSCs obtained from different sources differ in their biological characteristics (Elahi et al., 2016). It has been established that variation in their surface protein profile, their multilineage differentiation and/or their secretion of paracrine factors may influence their various clinical applications (Davies et al., 2017; Maleki et al., 2014). Hence, the translation of MSC-based therapy has been hindered by the heterogeneity of the isolated cells, as well as the lack of standardized methods for their characterization.

In bone marrow, MSCs have a supportive function for the hematopoiesis, contribute to the maturation of hematopoietic stem cells and increase their proliferation (Saleh et al., 2015). However, the self-renewal potential of MSCs may also vary and is difficult to demonstrate *in vivo* since markers defined by ISCT to identify MSCs have been characterized *in vitro*, and thus their expression may only be falsely determined by culture conditions (da Silva Meirelles et al., 2009).

Phenotypic identification

To specifically identify MSCs, phenotypic screening assays are performed in which the presence of specific surface markers is investigated as well as their differentiation potential into their classically admitted tri-lineage pathways that is chondrocytes, osteocytes and adipocytes. The most frequently used methods for verifying MSC phenotype are listed in **Table 2**, as well as the advantages and disadvantages of each method. Marker expression analysis is most commonly performed by flow cytometry and immunocytochemistry, firstly because of the simplicity of these techniques, and secondly because they enable the evaluation of individual cell profiles. Alternatively, techniques like Western Blot, protein array or RT-qPCR to assess quickly the average marker expression for an entire cell population.

For use in preclinical studies and in the veterinarian clinic, MSCs can be harvested from different species (mice, pigs, sheep, goats, horses, dogs). No minimal criteria for the identification of MSCs in non-humans have been established so far, despite their increasing use in preclinical models where they are being tested for translational cell therapy in humans. Indeed, while all MSCs display plastic adherence and tri-lineage differentiation, not all express the same panel of surface markers that has been described for human MSCs. When the cells are purchased from a supplier, all characterization and identification tests are normally performed and are then provided with the data sheet.

METHOD	TYPE OF VERIFICATION	ADVANTAGES	DISADVANTAGES
Flow Cytometry	Phenotypic	<ul style="list-style-type: none"> ▪ Reveals the phenotype of individual cells ▪ Can assess multiple markers simultaneously ▪ Can be used to isolate specific populations (when equipped with a cell sorter) ▪ High sensitivity ▪ High speed analysis ▪ Can use gating to remove dead cells from analysis 	<ul style="list-style-type: none"> ▪ Requires expensive equipment ▪ Requires skilled operator ▪ Potential cross-reactivity when using several antibodies ▪ Potential autofluorescence ▪ Cell sorting by FACS can be time consuming and expensive

(Continued on next page)

METHOD	TYPE OF VERIFICATION	ADVANTAGES	DISADVANTAGES
Immunohisto-fluorescence / Immunohisto-chemistry	Phenotypic	<ul style="list-style-type: none"> Reveals localization of marker proteins Can assess multiple markers simultaneously More efficient than WB analysis Can use live or fixed cells 	<ul style="list-style-type: none"> Requires specialized equipment Potential for cross-reactivity when using multiple antibodies Potential autofluorescence Photobleaching More time consuming than flow cytometry
Western Blot (WB)	Phenotypic	<ul style="list-style-type: none"> High sensitivity and specificity 	<ul style="list-style-type: none"> Does not reveal heterogeneity of cell population Low throughput
Protein Array	Phenotypic	<ul style="list-style-type: none"> Analyze several markers simultaneously High sensitivity and specificity More efficient and cheaper than individual WB Ideal for screening cell populations 	<ul style="list-style-type: none"> Does not reveal heterogeneity of cell population Low throughput
RT-qPCR	Phenotypic	<ul style="list-style-type: none"> Detects early changes in marker expression induced by differentiation Analyze several markers simultaneously High sensitivity and specificity 	<ul style="list-style-type: none"> Yields the average marker expression of a population Does not reveal heterogeneity of cell population
Induction of differentiation	Functional	<ul style="list-style-type: none"> Does not rely on MSC markers Not dependent on tissue or species type 	<ul style="list-style-type: none"> Time consuming Cannot recover MSCs

Table 2. Methods for MSC verification and characterization. The characterization of MSCs is performed by phenotypic verification methods (flow cytometry, immunohistochemistry, Western Blot, protein array, RT-qPCR) to assess the presence or absence of specific surface markers or by functional verification aimed at inducing the differentiation of these stem cells into osteocytes, chondrocytes or adipocytes. From Schachtele et al., R&D System.

Ethical considerations

Unlike embryonic or fetal stem cell therapy which faces many ethical roadblocks, the use of MSCs is socially more acceptable due to their adult origin. Besides, one of the other major hurdle of embryonic/fetal stem cell therapy is the risk of tumor formation due to their pluripotency, risk that is less significant with multipotent MSCs. But although MSCs represent a population of stem cells that has already been evaluated for non-toxicity in clinical and preclinical studies, the risk of tumorigenesis is not null either and must be kept in mind and taken into account when applying these cells to patients.

1.2. Mechanisms of actions

The broad therapeutic potential of MSCs for self-renewal, differentiation as well as their advantages over other stem cell types discussed in the previous paragraph have led many research groups to go and study the mechanisms of action of these cells. Initially, their therapeutic use *in vivo* focused on their potential for differentiation to replace pathological or defective cells. However, in many of these studies, it was shown that little or no cells were actually present in the target tissue, notwithstanding the therapeutic effects observed. It has become well-known today that MSCs actions relies more on their paracrine effects through the production of biologically active molecules exerting beneficial effects on other cell types than on direct differentiation into specific cells. As Lindolfo da Silva Meirelles mentioned in his 2009 review, “this shifts a paradigm centered on differentiation to a view in which MSCs can be therapeutic even if they do not engraft or differentiate into tissue-specific cells”. The main paracrine functions exerted by MSCs secreted factors are trophic (i.e. anti-apoptotic, supportive and angiogenic), immunomodulatory, anti-scarring and chemoattractant (da Silva Meirelles et al., 2009, **Table 3**). We will mention here some of the factors secreted by MSCs as well as some of their actions, but it should be noted that this list is not exhaustive and is increasing daily. Moreover, the range of effects of these molecules is still not fully known because: 1. a large amount of data on MSCs mechanisms of actions have been obtained with cultured cells *in vitro*, and therefore cannot be reliably transposed to *in vivo* paradigm, especially considering the extent to which the environmental niche can modify the functions/potential of MSCs; 2. variations in differentiation potential (da Silva Meirelles, 2006) and gene expression (Panepucci et al., 2004) of MSCs exist, especially depending on their

sources. Nevertheless, some of the key functions of MSCs appear to be shared by all and constitute the underlying basis for their use in many stem cell-based therapy in regenerative medicine (Samsonraj et al., 2017).

Effects	Molecules
Anti-apoptotic	VEGF, HGF, IGF-1, Stanniocalcin-1, TGF β , bFGF, GM-CSF
Immunomodulatory	PGE2, TGF β , HGF, mpCCL2, IDO, iNOS, HLA-G5, LIF
Anti-scarring	bFGF, HGF
Supportive	SCF, LIF, IL6, M-CSF, SDF-1, Angiopoietin-1
Angiogenic	bFGF, VEGF, PlGF, MCP1, IL6, Extracellular matrix protein
Chemoattractant	CCL2 (MCP1), CCL3 (MIP1 α), CCL4 (MIP1 β), CCL5 (RANTES), CCL7 (MCP3), CCL20 (MIP3 α), CCL26 (Eotaxin-3), CX3CL1 (Fractalkine), CXCL5 (ENA-78), CXCL11 (i-TAC), CXCL1 (GRO α), CXCL2 (GRO β), CXCL8 (IL8), CCL10 (IP-10), CXCL12 (SDF-1)

Table 3. Trophic and immunomodulatory factors secreted by cultured MSCs. *In vitro* studies have shown that MSCs have the ability to secrete multiple factors exerting anti-apoptotic, immunomodulatory, anti-scarring, supportive, angiogenic and chemoattractant effects. From da Silva Meirelles, 2009.

1.2.1. MSC properties in tissue repair and recovery

In adult mammals, severe trauma causes a spontaneous repair process that, in most cases, does not ensure the integrity of the tissue. The *in vivo* transplantation of MSCs has highlighted their restorative properties. MSCs are able to create a regenerative microenvironment by releasing anti-apoptotic, anti-scarring, angiogenic and mitotic factors (Caplan and Correa, 2011; da Silva Meirelles et al., 2009). The trophic functions of MSCs are related to their functional potential to provide a restorative environment through intercellular contact and/or paracrine secretion of broad range of bioactive molecules that promote the immune modulation of inflammatory cells involved in tissue repair such as macrophages, T-cells, B-cells, dendritic cells and T-regs cells (Caplan and Correa, 2011). Trophic factors identified as being produced by MSCs are growth factors, cytokines, chemokines, morphogens, extracellular vesicles and glycosaminoglycans (Amable et al., 2014; Caplan and Correa, 2011).

Their ability to differentiate into cells of mesodermal origin (osteocytes, chondrocytes, adipocytes) allows them to contribute to the repair process by replacing cells that have been altered (Phinney and Prockop, 2007). In addition, they could also differentiate into cells of non-mesodermal origin such as hepatocytes, neural cells and epithelial cells when subjected to appropriate environmental conditions (Uccelli et al., 2008).

1.2.2. Immunomodulatory properties

MSCs are clinically of interest for their potential to regenerate tissues as well as their ability to modulate the immune system. The anti-inflammatory properties of MSCs are commonly reported in the literature as a key factor contributing to their use as therapeutic tools, particularly in a wide range of diseases with an inflammatory component. The mechanisms underlying their immunomodulatory properties are not fully understood but may involve cell-to-cell contact and secreted molecules. Immunomodulation is defined as the modification of the immune system, either by enhancement (i.e. immunopotentialization strategy) or by suppression of the immune responses. It can be achieved by different immunomodulators, including monoclonal antibodies, cytokines or glucocorticoids. MSCs can interact with cells of the innate or adaptive immune system. These immunoregulatory properties have been widely described *in vitro* and *in vivo* (Ma et al., 2014). It is to note that the criteria defined by ISCT to identify MSCs do not include the range of immunomodulatory factors secreted by MSCs since it is highly dependent on their sources (**Table 4**, Castro-Manrreza and Montesinos, 2015).

It has been shown *in vitro* that MSC can inhibit T cell and B cell proliferation (Glennie et al., 2005), as well as dendritic cell proliferation (Corcione et al., 2006). Moreover, it has been shown that MSC-immune regulation is achieved by upregulating the numbers of regulatory T cells which actively suppress effector T cell functions (Gonzalez-Rey et al., 2009). Besides, MSC are able to secrete, constitutively or by interaction with target cells, factors such as indoleamine 2,3-dioxygenase (IDO), VEGF, IL-10, VEGF, RANTES/CCL5, prostaglandin E2, nitric oxide (NO), IL-6, HGF, CCL1/MCP1, TGF β 1, leukemia inhibitory factor (LIF) (Kyurkchiev et al., 2014; Uccelli et al., 2008).

MSC Source	Molecules involved in MSC immunosuppression
Bone marrow	IDO, TGF β ₁ , HGF, IL10, HLA-G, PDL-1, PGE ₂
Adipose tissue	IDO, TGF β ₁ , HGF, IL10, PGE ₂
Placenta	IDO, TGF β ₁ , IL10, HLA-G, PDL-1
Umbilical cord blood	IDO, TGF β ₁ , HGF, HLA-G, PDL-1, PGE ₂
Umbilical cord	IDO, TGF β ₁ , HGF, IL10, HLA-G, PDL-1, PGE ₂
Wharton's Jelly	IDO, TGF β ₁ , HGF, IL10, HLA-G, PGE ₂

Table 4. MSCs immunoregulatory molecules depends on their sources. MSCs can be found in various body tissues such as bone marrow, adipose tissue, placenta, umbilical cord blood, umbilical cord or Wharton's Jelly. Depending on their origin, MSCs will be able to produce a range of different immunomodulatory factors. From Castro-Manrreza, 2015.

Due to their immunomodulatory and trophic properties, MSCs are considered to have broader benefits in cell-based regenerative medicine than other types of stem cells. In addition, it has been reported that MSCs may display immunosuppressive properties only after exposure to inflammatory cytokines and/or activated T-cells, this process being called licensing or priming. For example, it has been shown that chemokine-induced migration is enhanced when MSCs are primed 24 hours before the migration assay with TNF α but not IL1 β (Mukai et al., 2018b; Ponte et al., 2007). In addition, in culture conditions, it has been described that MSCs can be attracted by the presence of chemokine such as MCP1, MIP1 and IL8 (Chulpanova et al., 2018).

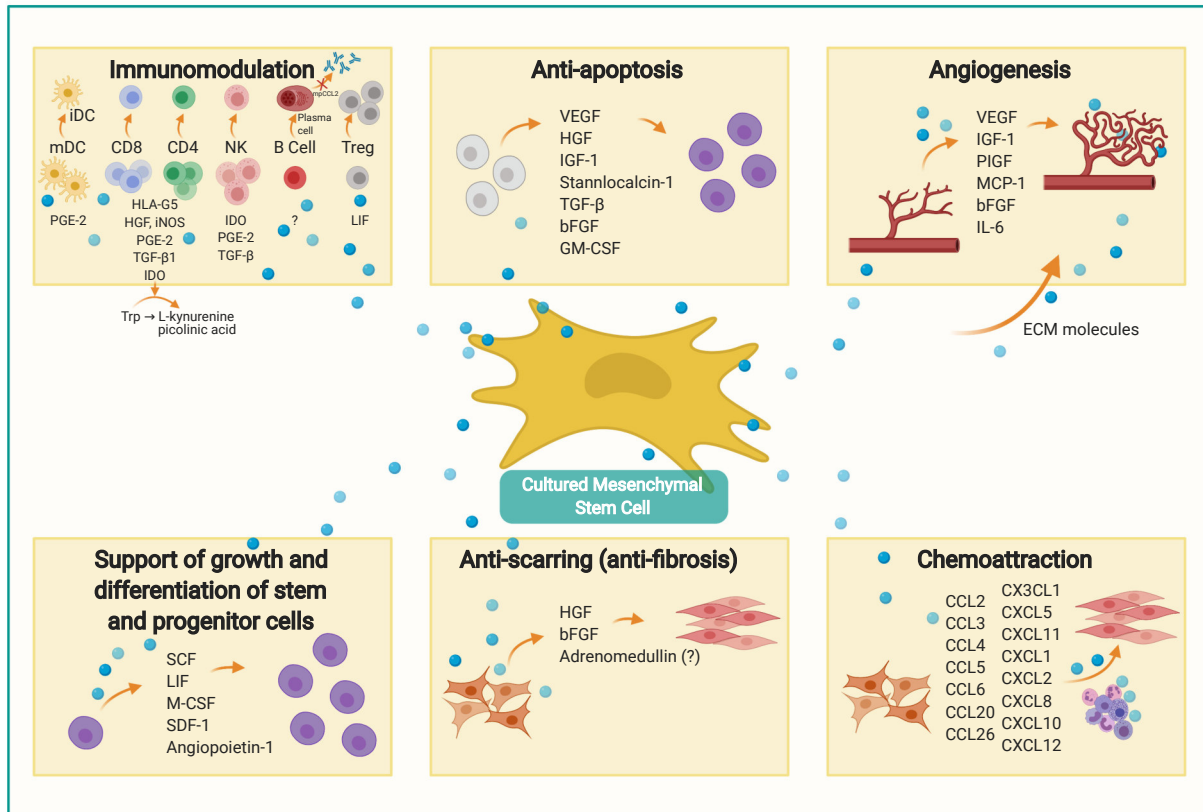


Figure 13. Summary of all paracrine effects of cultured MSCs. “The secretion of a broad range of bioactive molecules is now believed to be the main mechanism by which MSCs achieve their therapeutic effect and it can be divided into six main categories: immunomodulation, anti-apoptosis, angiogenesis, support of the growth and differentiation of local stem and progenitor cells, anti-scarring and chemoattraction. Although the number of molecules known to mediate the paracrine action of MSCs increases every day, several factors that have been shown to be secreted by cultured MSC are depicted here for illustrative purposes. The immunomodulatory effects of MSCs consist of inhibition of the proliferation of CD8+ and CD4+ T lymphocytes and natural killer (NK) cells, suppression of immunoglobulin production by plasma cells, inhibition of maturation of dendritic cells (DCs) and stimulation of the proliferation of regulatory T cells. The secretion of PGE-2, HLA-G5, HGF, iNOS, IDO, TGF-β, LIF and IL-10 contributes to this effect. MSCs also limit apoptosis, and the principal bioactive molecules responsible for this are VEGF, HGF, IGF-1, stanniocalcin-1, TGF-β and GM-CSF. In addition, MSCs stimulate local angiogenesis by secretion of extracellular matrix molecules, VEGF, IGF-1, PIGF, MCP-1, bFGF and IL-6, and also stimulate mitosis of tissue-intrinsic progenitor or stem cells by secretion of SCF, LIF, M-CSF, SDF-1 and angiopoietin-1. Moreover, HGF and bFGF (and, possibly, adrenomedullin) produced by MSCs contribute to inhibition of scarring caused by ischemia. Finally, a group of at least 15 chemokines produced by MSCs can elicit leukocyte migration to the injured area, which is important in normal tissue maintenance.” Adapted from da Silva Meirelles et al., 2009.

1.3. MSC homing in tissue

The interest in therapeutic use of stem cells has led to the consideration of non-invasive administration techniques by systemic infusion rather than local transplantation. However, the challenge to overcome when using such transplantation is the impossibility to target these cells to a specific tissue located far from the graft site. This raised the questions of how stem cells will reach the tissue of interest and then integrate into this tissue to exert their therapeutic actions.

The “homing” process is defined in the literature as the “arrest of MSCs within the vasculature of a tissue followed by transmigration across the endothelium” (Karp and Leng Teo, 2009; Leibacher and Henschler, 2016). The molecular processes underlying stem cells homing have been extensively investigated over the past decades and facilitated the development of MSC-based therapies for clinical purposes. In most studies, MSCs are administered systemically. One of the most interesting features of MSCs that makes them a very promising tool for treating different types of diseases is their ability to preferentially migrate to injured sites, as showed in many animal models (Karp and Leng Teo, 2009; Ullah et al., 2019). In damaged tissues, there is commonly a release of cytokines, chemokines and growth factors, molecules for which MSCs have receptors. Their ability to migrate to affected sites is achieved by the activation of these specific receptors and by chemoattraction. It has been shown in an *in vitro* study that MSCs migrate in a dose-dependent manner in response to growth factors and chemokines (RANTES, SDF-1 and macrophage-derived chemokine), with a stronger chemotactic response to growth factors (PDGF-AB, IGF-1, EGF, HGF) (Ponte et al., 2007).

Of what we know today, the complex sequence of events involved in stem cells homing are almost identical to those of leukocytes. The process of MSC homing into damaged tissues can be divided into five steps (**Figure 14**): tethering and rolling, activation, arrest, transmigration or diapedesis, and migration and enlodgement (Karp and Leng Teo, 2009; Marquez-Curtis and Janowska-Wieczorek, 2013; Nitzsche et al., 2017; Ullah et al., 2019). The initial tethering process is facilitated by selectins expressed by endothelial cells and for which MSCs have receptors such as CD44, galectin and CD24 that has been identified as a potential P-selectin ligand (Bailey et al., 2009; Sackstein et al., 2008). Then, the activation step is

facilitated by G protein-coupled chemokine receptors, generally in response to inflammatory signals. MSCs express numerous receptors to chemokines such as CXCR4, CXCR7 and CCR2 (Ullah et al., 2019). A wide range of other receptors, including CCR1, CCR4, CCR7, CCR9, CCR10, CXCR5 and CXCR6 are also present at the surface of MSC, although their precise role remains unclear (Ahmadian kia et al., 2011; Honczarenko et al., 2006). Corresponding ligands to these receptors, such as stromal cell-derived factor (SDF)-1, MCP3 and MCP1, are commonly upregulated at the site of tissue injury. These ligand-receptor interactions, as well as chemotactic bioactive lipids, modulate cell-cell contact between MSCs and endothelial cells. The third step, arrest, is supported by integrins. MSC express the $\alpha 4\beta 1$ integrin very late antigen (VLA)-4, and endothelial cells express the corresponding ligand, vascular cell adhesion molecule (VAM)-1. The onset of inflammation in injured tissue causes the release of cytokines which upregulated VCAM-1 and activates VLA-4, leading to initial arrest of MSC on the endothelium surface. The transmigration process or diapedesis is enabled by the expression by MSCs of extracellular matrix-degrading enzymes, matrix metalloproteinase (MMP)-2 and MMP9, membrane type (MT)1-MMP that promote the extravasation by breaking down the endothelial basement membrane and the endothelial cells tight junctions. This process is also helped by the expression of remodeling enzyme, namely the tissue inhibitors of metalloproteinases (TIMPs). The expression of TIMPs is induced by inflammatory cytokines, which serve as a signal for migration into damaged tissue (Ries et al., 2007; Ullah et al., 2019). It has been suggested that the expression of TIMP2 facilitates transmigration by playing a role in the maturation of MMP2 from its proenzyme form to its active form (Ullah et al., 2019; Will et al., 1996). Finally, the last step corresponds to the migration through the interstitium to the wound site. MSCs will migrate and advance in the parenchyma in response to factors such as the growth factors platelet-derived growth factor (PDGF)-AB and insulin-like growth factor (IGF)-1, and to a lesser extent, the chemokines RANTES, macrophage-derived chemokine (MDC) and SDF-1 (Ponte et al., 2007; Ullah et al., 2019).

It should be noted that when MSCs are injected in healthy subjects, i.e. in a nonwounded homeostatic host, it has been shown that they are rapidly cleared from the circulation and initially become entrapped within the lungs (Kidd et al., 2009; Rustad and Gurtner, 2012), this being explained by the fact that the above-mentioned factors produced at the suffering sites are absent in healthy subjects.

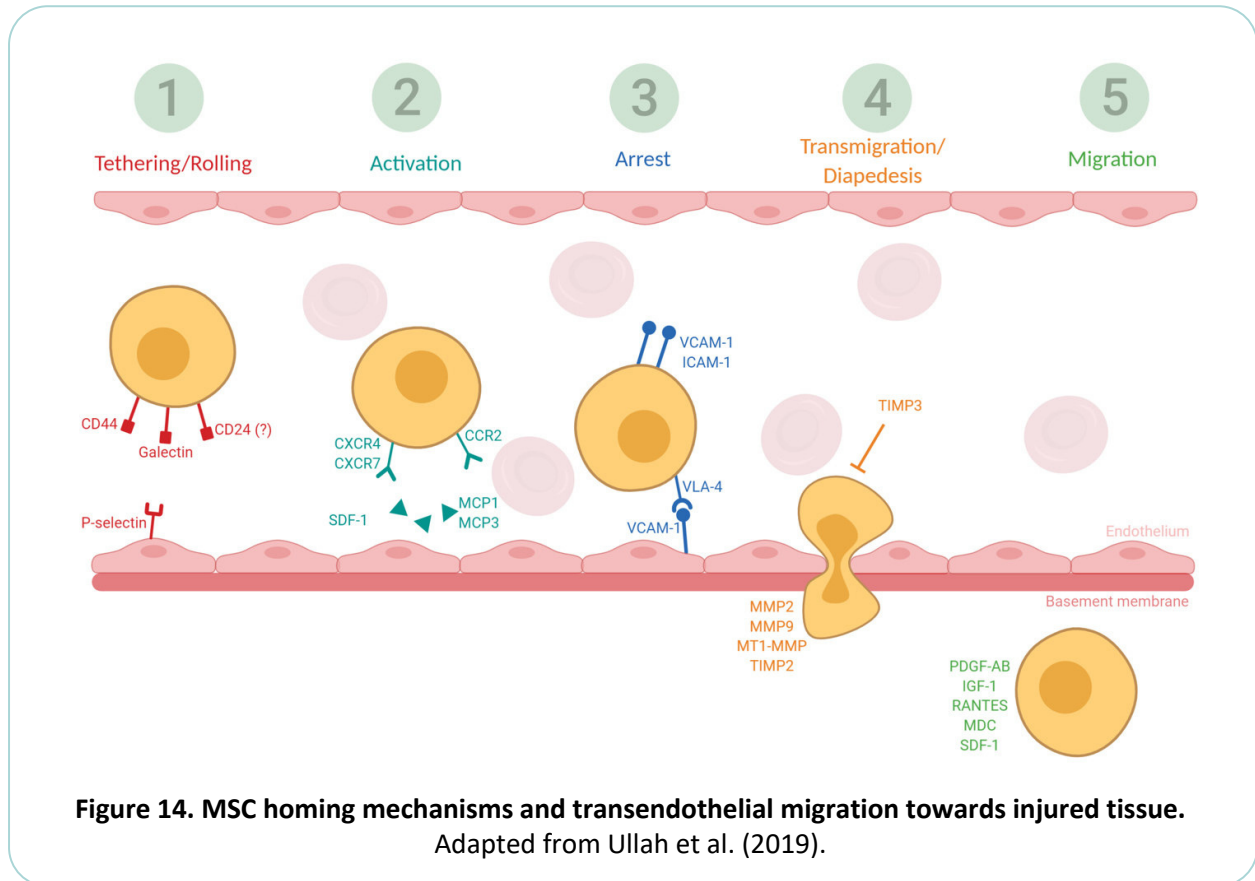


Figure 14. MSC homing mechanisms and transendothelial migration towards injured tissue.
Adapted from Ullah et al. (2019).

2. The use of MSC in cell therapy: clinical applications in brain diseases

MSC were first tested as a cellular pharmaceutical agent in humans in 1995 (Galipeau and Sensébé, 2018; Lazarus et al., 1995) and have since become the most clinically studied experimental cell therapy platform worldwide. The enthusiasm for these clinical trials is driven by the ease of access of these cells, which have a high proliferation capacity and that can be collected from different types of tissues in healthy volunteers. Due to their well-characterized immunomodulatory properties *in vitro*, MSC-based therapy holds considerable potential for the treatment of diseases that include an immunological aspect in their pathophysiology. However, it is important to note that MSCs from different sources may differ in their immunomodulation abilities (Mattar and Bieback, 2015). In addition, paracrine secretion from MSC offer broad clinical potential by also regulating angiogenesis, apoptosis, oxidative stress, cell-differentiation and extra-cellular matrix composition (Liang et al., 2014). There are two types of stem cell transplantation in cell therapy depending on the cell donor: autologous or allogeneic (**Box 5**). The success of MSCs lies in their low immunogenicity, which makes

allogeneic transplants more easily conceivable than for other stem cell types (Ankrum et al., 2014).

Numerous studies in recent years have sought to treat neurological diseases with MSCs. Indeed, the literature with the use of these cells is increasing in diseases such as stroke, traumatic brain injury, neurodegenerative diseases such as Parkinson's disease or Alzheimer's disease, amyotrophic lateral sclerosis, Huntington's disease or epilepsy (Adami et al., 2014; Joyce et al., 2010; Laroni et al., 2015; Mashkouri et al., 2016; Mukai et al., 2018b; Volkman and Offen, 2017). However, the exact mechanism by which MSCs exert their function remains uncertain, as several mechanisms have been proposed, such as neuroprotection by secretion of neurotrophic factors, induction of neurogenesis, or modulation of inflammation (Baez-Jurado et al., 2019; Gneccchi et al., 2016). Since the majority of these diseases have a complex etiology, it appears that the multiple beneficial roles of MSCs are able to target different aspects of the diseases.

Homing processes of MSCs within the CNS are accomplished as for all other organs, as described in Part III.1.3, and preferentially migrate to brain areas where inflammatory molecules are expressed. Stem cells represent an effective strategy to treat brain injury, but the precise mechanisms underlying stem cell therapy remain elusive due to the lack of appropriate cell tracking technology. In addition, the cell type, timing, dosage and route of administration as well as the safety and biocompatibility of the tracker agents must all be considered. The delivery methods that have been tested for stem cell transplantation in neurological diseases are the intracerebral or intracerebroventricular transplantation, intraperitoneal, intraarterial, intravenous or intranasal administration, each method presenting its advantages and disadvantages.

BOX 5 | STEM CELL TRANSPLANTATION

There are 3 types of transplants, depending on the source of stem cells.

Autologous stem cell transplant

The patients' own stem cells are harvested, thawed and re injected in the same patient.

Allogeneic stem cell transplant

The stem cells do not come from the patient but from a donor.

Syngeneic stem cell transplant

Special kind of allogeneic transplants that can only happen when donor and receiver are identical twins.

MSCs have demonstrated in both *in vitro* and *in vivo* studies their ability to modulate the activity and polarization of brain cells such as astrocytes, microglia, monocytes-macrophages or neurons and reciprocally, brain cells are able to modulate the activity of MSCs (Cho et al., 2014; Chu et al., 2008; Jose et al., 2014; Lv et al., 2016; Mukhamedshina et al., 2019; Papazian et al., 2018; Pluchino and Cossetti, 2013; Rahmat et al., 2013; Uccelli et al., 2011; Zanier et al., 2014). The use of MSCs in a spinal cord injury model has suggested various actions of MSCs on brain cells, as shown in **Figure 15** (Mukhamedshina et al., 2019). Even if these actions are not totally translatable to all brain pathologies and must be adapted to the different pathophysiological processes this schematic diagram gives an idea of the potential effects that MSCs may have in their environment. However, this diagram suggests that MSCs can only have an effect when they are present in the immediate surroundings of the affected sites. It is important to note that MSCs, due to their paracrine and endocrine activity described in the previous sections, are also able to operate far from the graft location.

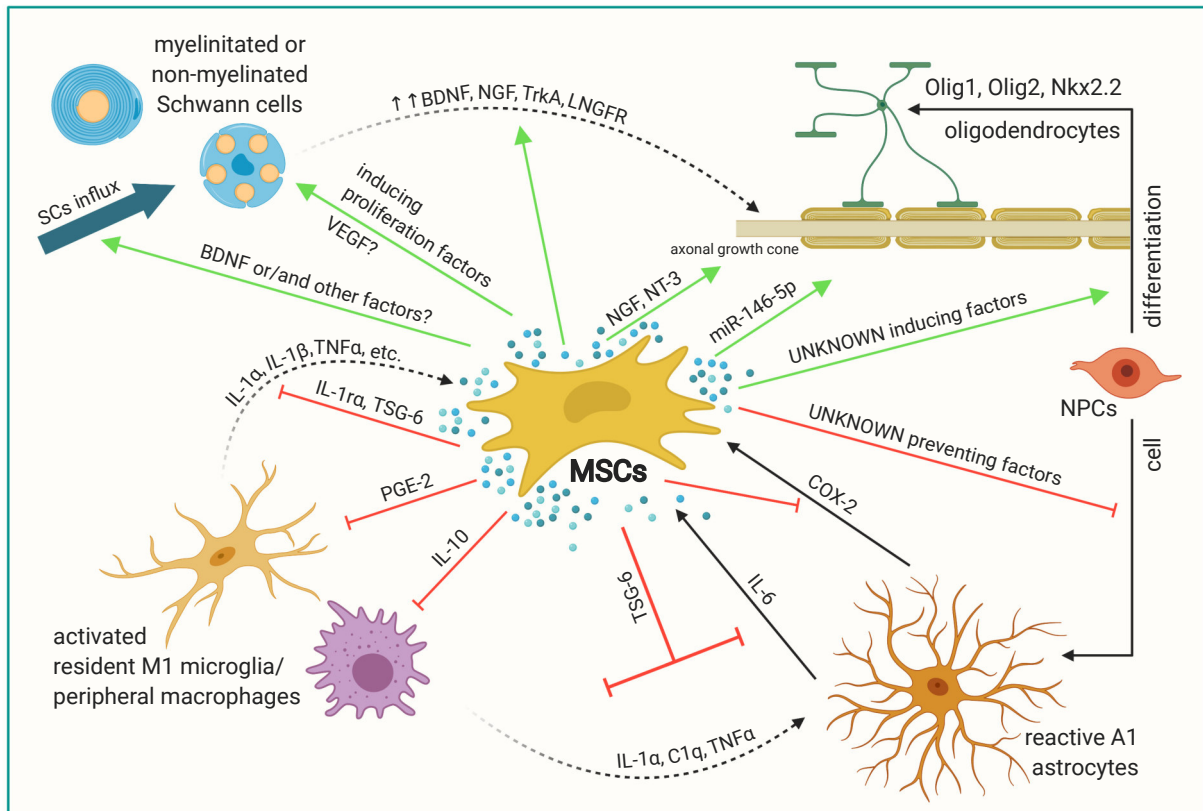


Figure 15. “Schematic diagram for some effects of mesenchymal stem cells (MSCs) on the neuronal microenvironment in the area of spinal cord injury (SCI). Activated resident microglia and peripheral macrophages attracted in the area of SCI produce proinflammatory cytokines such as interleukin (IL)-1 α , IL-1 β , tumor necrosis factor (TNF)- α , C1q and activate A1 astrocytes and MSCs. In response to these stimuli and probably other signals, the MSCs start to secrete anti-inflammatory factors such as IL-1ra, TNF stimulated gene-6 (TSG-6), prostaglandin E2 (PGE2), and IL-10, modulate the microglia/macrophages phenotype toward the anti-inflammatory M2 one and reduce the reactivity of astrocytes. The MSCs induce neural progenitor cells differentiation into oligodendrocytes and prevent differentiation into astrocytes. They facilitate myelination and axon growth by producing miR-146-5p and neurotrophic factors, lead not only to influx of Schwann cells in the area of SCI, but promote an increased expression of brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF) and its high- and low-affinity receptors (TrkA and LNGFR) in these cells. COX-2: Cyclooxygenase-2; NT-3: neurotrophin-3.” Adapted from Mukhamedshina et al., 2019.

2.1. Engraftment techniques and tracking of MSC in the CNS

2.1.1. Technical considerations: route of administration, time window, and dose

The biggest challenges in cellular therapy protocols relate to technical considerations such as the choice of cell delivery method, the therapeutic intervention window and the dose of cells needed to observe clinical outcomes. MSC-based therapy studies raised numerous questions about the best way to administer these cells to ensure their effective delivery to the affected areas and/or to maximize their effect, even if they are paracrine. It is known that successful recruitment of MSCs from the systemic circulation to the injured site is facilitated by chemotactic stimuli. However, depending on their initial location, and the concentration of chemotactic signals to which they are exposed, the response of the cells will vary.

The most commonly used route of administration is intravenous. Nevertheless, with this delivery route it has been shown that cells, instead of being found in areas in need, are distributed in the lungs, spleen, liver, kidney, lymph nodes or thymus (Kurtz, 2008; Leibacher and Henschler, 2016). Transplantation into the lungs, and into the other organs mentioned above, is an extremely rapid process, since the cells can already be detected a few seconds or minutes after an intravenous grafting (Schrepfer et al., 2007). The most likely reason for the entrapment in the lungs is a combination of mechanical and physiological conditions and may be related to the small size of the capillaries, the large capillary network and the strong adhesion properties of MSCs (Kurtz, 2008). In addition, pulmonary entrapment can cause undesirable side effects such as embolism, especially in small animal models.

The intranasal route of administration has emerged in recent years as a potential alternative route to target the brain, due to the proximity of the nasal cavity to the cerebral regions (Danielyan et al., 2009, 2014; Dhuria et al., 2010; Li et al., 2015; Lochhead and Thorne, 2012). The interest of this pathway also lies in its ability to bypass cell engraftment into the lungs or other peripheral organs. In addition, one of the major advantages is the non-invasive nature of this technique, which allow to avoid surgical transplantation. Besides being less invasive, and potentially safer and more cost-effective, this technique can be more easily used for multiple injections and avoids the inflammation associated with more invasive methods and potential peripheral side effects. This therapeutic delivery technique was first used in the early 2000s in studies showing that it is possible to use the intranasal route to deliver protein

directly to the brain (Born et al., 2002; Hanson and Frey, 2008; Kern et al., 1999). This route was then tested with cell administration (Danielyan et al., 2009). In this study, fluorescent MSCs were administered in the nasal cavity and a few hours later, they were observed in the olfactory bulb, hippocampus, thalamus and cerebral cortex (Danielyan et al., 2009). The intranasal method of delivery has proved promising outcomes in a variety of neurological disease (Li et al., 2015), including Parkinson disease (Danielyan et al., 2011, 2014), Alzheimer disease, ischemic stroke (Wei et al., 2013), hypoxic-ischemic brain injury (Donega et al., 2013, 2014; van Velthoven et al., 2010), in the animal model of multiple sclerosis i.e. experimental autoimmune encephalomyelitis (Wu, 2013) or in brain tumors (Balyasnikova et al., 2014).

The question of the routes used by cells to reach brain regions of interest was then raised. Although the exact migration pathways from the nasal cavity to the brain are not clear, different pathways have been suggested and described for drug delivery to the CNS, and it is likely that intranasally injected stem cells will also follow these routes. The different pathways from the nasal cavity to the CNS would involve the olfactory and trigeminal nerves, the vascular system, the cerebrospinal fluid and the lymphatic system (Dhuria et al., 2010):

1. The first possible pathway for stem cells migration is the olfactory nerve pathways in the upper part of the nasal cavity, where olfactory receptor neurons are interspersed between supporting cells, microvillar cells and basal cells. It is likely that intranasally delivered cells bypass the BBB and access the brain regions by passing through the cribriform plate of the ethmoid bone that separates the nasal cavity from the olfactory bulbs (Danielyan et al., 2009; Dhuria et al., 2010; Li et al., 2015; Lochhead and Thorne, 2012). From then on, the cells would migrate to the neuroinflammatory areas in which many cytokines and chemokines are released (**Figure 16**, Danielyan et al., 2009).
2. The second pathway that connects the nasal passages to the CNS involves the trigeminal nerve, which innervates the respiratory and olfactory epithelium of the nasal passages and penetrates the CNS in the pons. As Dhuria et al (2009) wrote, a unique feature of the trigeminal nerve is that it enters the brain through the respiratory epithelium of the nasal passages in two places: (1) by the anterior lacerated foramen near the pons and (2) by the cribriform plate near the olfactory

bulbs, creating entry points into the caudal and rostral areas of the brain after intranasal administration (**Figure 16**, Danieylyan et al., 2009).

3. The nasal mucosa is highly blood-supplied and receives its blood from the branches of the maxillary, ophthalmic and facial arteries, which originate in the carotid artery. Historically, the intranasal route of administration has been used to administer drugs to the systemic circulation by absorption into the capillary blood vessels underlying the nasal mucosa (Dhuria et al., 2010). It is therefore possible to assume that intranasally administered cells can enter the bloodstream and be delivered to the suffering brain regions where they would cross the BBB.
4. Finally, the last pathways that drugs and cells can follow are those connecting the subarachnoid space containing CSF and the nasal lymphatic pathways. To access brain regions of interest, cells would pass from the nasal cavity to the CSF then to the interstitial spaces of the brain and perivascular spaces to be distributed (Dhuria et al., 2010; Li et al., 2015).

The question of the best therapeutic intervention window is then raised after the choice of the best route of MSC administration. This time window will be highly dependent on the disease for which MSCs are used and the underlying pathophysiology of this illness. MSCs are used and the underlying pathophysiology of this illness. MSCs have been used either during the acute phase following a brain injury, a phase in which strong toxicity and/or inflammation is present at the wounded site, or during the chronic phase. The therapeutic actions of MSCs in each of these cases will differ and will strongly depend on the cellular and molecular environment to which they will be exposed. The corollary aspect of the time window issue is the number of stem cell administrations that must be carried out. Only experiments with single or repeated injections of MSCs at different stages of the diseases will provide answers to these questions.

Finally, the answers regarding the optimal injection dose of MSCs remain unclear. This will depend strongly on the route of administration chosen and the target tissues, taking into account the possible loss of cells that may remain trapped in the lungs or other peripheral organs. In animal models, beyond the dose, the injection volume will also be important, depending on the delivery route adopted. For the intranasal route, for example, volumes used in preclinical studies range from 10 to 200 μ L depending on the animal species.

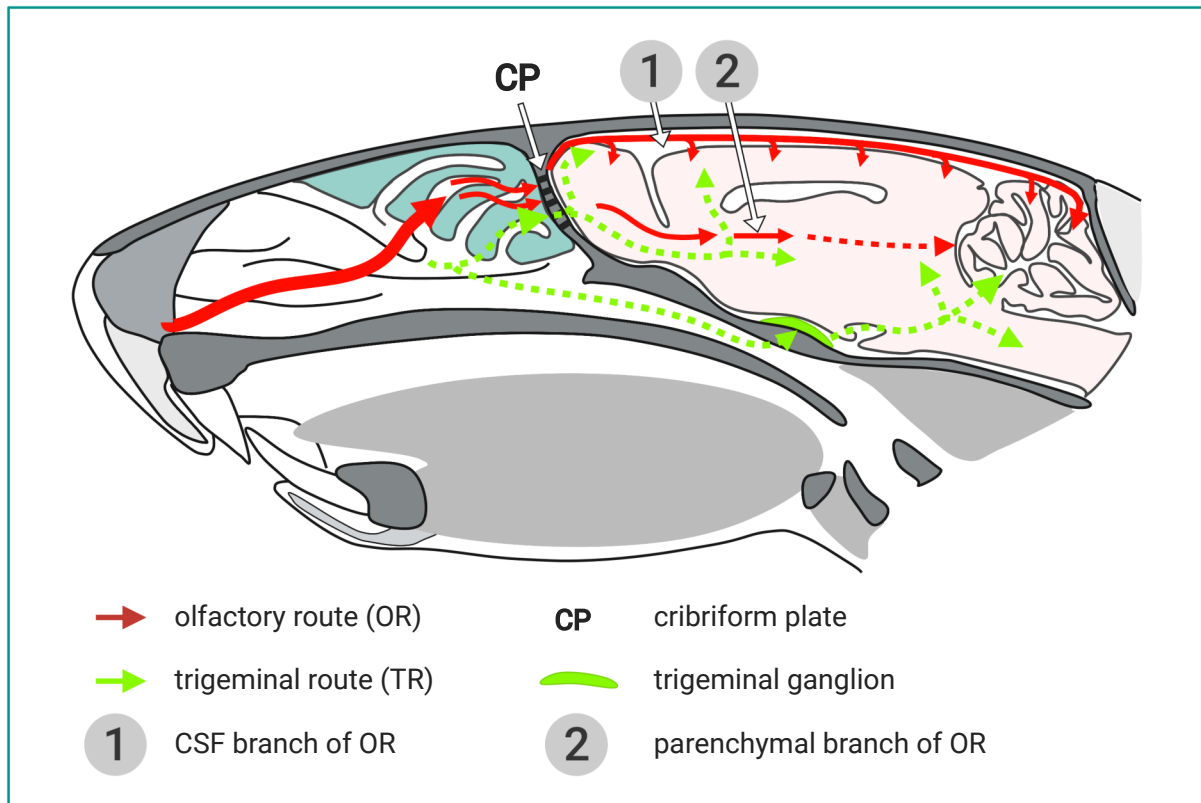


Figure 16. Schematic drawing of two routes of IN delivery of cells to the brain. After crossing the cribriform plate (CP), the olfactory route (OR, red arrows) divides into two branches: (1) the CSF branch and (2) the parenchyma branch. The trigeminal route consists also of at least two branches, one of which crosses the cribriform plate into the brain parenchymal where it diverges to the rostral and caudal parts of the brain. The second branch projects from the nasal mucosa to the trigeminal ganglion, where the exogenously applied cells are further distributed to the forebrain, olfactory bulb and caudal brain areas including the brainstem and the cerebellum. Adapted from Danielyan et al., 2009.

2.1.2. Tracking of MSC

Cell tracking methods are needed to evaluate the fate of transplanted cells in preclinical and clinical studies. Pre- and post-mortem tracking approaches for transplanted cells have been developed in recent years, each with varying robustness and complexity.

The simplest and most commonly employed strategy is the use of fluorescent MSCs, which enable their identification under a microscope and after tissue collections. The tagging of stem cells can be achieved with a wide range of labels such as green fluorescent protein (GFP), fluorescent lipophilic cationic indocarbocyanine dye (DiI), bromodeoxyuridine (BrdU), or PKH26, a fluorescent lipophilic dye solution that marks the cellular membranes (Vaegler et al., 2014).

Another frequently used technique for clinical and preclinical *in vivo* imaging of stem cells is superparamagnetic iron oxid (SPIO) nanoparticles method, because of their ability to generate a very high MRI signal (Sohni and Verfaillie, 2013; Wang et al., 2013). After tracking the cells by MRI, the tissues can be collected and stained with Prussian blue to histologically observe the stem cell transplant sites and the potential cellular modifications they have triggered in their environmental niche. This approach has been used in a lithium chloride-pilocarpine induced TLE model where ultras-small superparamagnetic iron oxid (USPIO)-labelled MSCs were injected in the brain lateral ventricle (Long et al., 2015). Using this method, they were able to follow the cells up to 14 days after transplantation.

Nevertheless, the effect of iron nanoparticle on MSC phenotype and on their intrinsic properties has yet to be defined (Drela et al., 2019). A further limitation to the use of nanoparticles is that a sufficient number of them must be phagocytosed by stem cells. It has previously been shown that MSCs are phagocytic in nature and that they can be labeled *in vivo* directly by intravenous injection of Ferumoxytol, the only intravenous FDA-approved SPIO nanoparticles (Khurana et al., 2013; Liu et al., 2016). Ferumoxytol is originally an iron supplement for the treatment of iron deficiency. In the study of Khurana (2013), MSCs were then harvested 48 hours after the i.v. injection of Ferumoxytol, and a significant concentration of iron was measured. However, this cell-tracking method has limitations, as described by Bulte (2013), this first one being based on the fact that *in vivo*, MSCs are not the only phagocytic cells and that an equally large number of Ferumoxytol-labeled macrophages would be observed, making the use of SPIOs non-discriminating for tracking purposes. *Ex vivo* Ferumoxytol labelling of MSCs also has its limitations insofar as cell expansion protocols to obtain a sufficient number of SCMs for injection can induce dilution of the iron tag below cellular MRI detection levels (Bulte, 2013; Liu et al., 2016).

Besides, despite the fact that the phagocytic capacity of SPIO nanoparticles by MSCs has been demonstrated *in vivo* and *ex vivo* (Addicott et al., 2011; Khurana et al., 2013; Liu et al., 2016), it is well established that not all cells have the same uptake ratio for nanoparticles. Therefore, even considering that the cells are selected by flow cytometry and cell sorting, it cannot be excluded that the phagocytic selected MSCs constitute a subpopulation of all MSCs.

Recent studies are still seeking to develop the most appropriate techniques for MSC tracking after transplantation. The combination of fluorescence and magnetic nanoparticles may offer an interesting alternative solution for MSC tracking.

2.2. Fate of MSC into the CNS

One of the questions that has emerged from all studies with *in vivo* administration of MSC concern the fate of the stem cells once they reach the target tissue. Depending on the route of administration of MSCs, their fate will not be the same. Many studies have shown that stem cells injected intravenously remain trapped in the lungs, spleen and lymph nodes (Acosta et al., 2015; Eggenhofer et al., 2012; Fischer et al., 2009; Liu et al., 2013; Rustad and Gurtner, 2012; Xu et al., 2019). Targeting peripheral tissues is sometimes easier by injecting cells directly into the targeted tissue. However, for neurological diseases where cell therapy is desired to act on the brain, it is more complicated to consider injections directly into the brain parenchyma. The fate of stem cells after transplantation is now being investigated in numerous studies and several hypotheses have been proposed. The first is related to the differentiation potential of MSCs. While research in peripheral tissues, such as heart or kidney tissue, has shown that MSCs are able to differentiate and fully integrate the target tissue to replace defective cells, such results are less common in brain diseases (D'souza et al., 2015). The majority of investigations that showed a differentiation of MSCs into neuron- or astrocyte-like cells used the intracerebroventricular or intrahippocampal injection route, but it is unclear whether those differentiated cells are fully functional and able to integrate the cerebral network (George et al., 2019; Wislet-Gendebien et al., 2005).

Another possible fate of MSCs may involve their presence in peripheral tissues and/or bloodstream. MSCs would thus have a therapeutic action on the targeted structures thanks to their paracrine and endocrine properties, but also by modifying the immune cells' response. Ultimately, after a time that remains rather unknown today, the MSCs would be phagocytated by macrophages and or sent to the spleen to be degraded.

3. MSC in epilepsy

Due to their broad-spectrum mechanism of action, as well as their above-mentioned immunomodulatory and neuroprotective properties, stem cells represent a new therapeutic hope for patients with drug-resistant epilepsy (Löscher et al., 2008; Shetty, 2011). To be successful and to overcome the pharmacoresistance in mTLE patients, therapeutic strategy needs an integrated vision encompassing the basic features of intractable epilepsies (Agadi and Shetty, 2015; Yasuhara et al., 2017). Stem cells have been tested to either counteract the development of chronic epilepsy after SE or to treat chronic epilepsy once it is established. With the aim of substituting damaged hippocampal neurons, different types of stem cells have been used such as neural cells or induced Pluripotent Stem Cells (iPSCs), stem cells derived from skin or blood cells that have been reprogrammed back into an embryonic-like pluripotent state that enables the development of an unlimited source of any type of human cell (Yasuhara et al., 2017). Neural cells that have been tested so far include hippocampal precursor cells (Rao et al., 2007; Shetty et al., 2005), neural stem cells (Shetty, 2011; Waldau et al., 2010) or GABA-positive neural precursors (Baraban et al., 2009; Cunningham et al., 2014; Hunt et al., 2013; Southwell et al., 2014). The aim of all these studies was to modify altered circuitry, increase inhibitory neurotransmission in epileptic foci by replacing lost GABA-ergic interneurons, or promote the differentiation of neural stem cells into healthy astrocytes releasing anticonvulsant-proteins and/or trophic factors (Agadi and Shetty, 2015). Numerous studies have shown in epilepsy models that the transplantation of exogenous neural stem cells leads to promising results in the reduction of abnormal electrical activity, normalization of abnormal spouting of mossy fibers and preservation of GABA-ergic inhibitory interneurons (Jing et al., 2009; Maisano et al., 2012; Shetty, 2014; Waldau et al., 2010). But while the transplantation of these exogenous NSCs is a potential strategy to improve currently incurable neurological conditions, there are several obstacles to its implementation including tumorigenic, immunological and ethical problems (Kaneko et al., 2011).

The use of MSCs for epilepsy has been considered for the same reasons as for all other neurological diseases in which they have been used, including their multiple differentiation potential, their immunomodulatory properties and trophic effects, and their ability to improve function in many neurological diseases (Agadi and Shetty, 2015). Few studies have used mesenchymal stem cells in models of epilepsy. In these studies, the efficacy of MSCs to

counteract or modulate the effect of SE when administered in the early phase post-SE or during the chronic phase was tested.

In a recent investigation, Salem et al. (2018) evaluated the effect of MSC injected intravenously or bilaterally directly into the hippocampus 22 days after pilocarpine-induced SE in rats. Results indicated that 15 days after transplantation, MSC resulted in a decrease in certain inflammatory markers in the hippocampus such as IL1 β or TNF α , and a reduction in excitatory amino acid neurotransmitters such as glutamate and aspartate compared to the SE-untreated group (Salem et al., 2018).

In the study of Voulgari-Kokota (2012), the neuroprotective effects of CD11b-, Sca1+, CD44+ MSCs isolated from mouse bone marrow were first examined in a cell culture model. They used a co-culture system in which mouse cortical neurons were cultivated with the MSC and then exposed to N-methyl-d-aspartate (NMDA). In a control culture of neurons, this exposure leads to excitotoxicity due to an influx of calcium triggered by the NMDA receptor (NMDAR). However, co-culture of cortical neurons with MSCs prior to exposure to NMDA protected the neurons from excitotoxic cell death. Neuroprotection was also evidenced when neurons were incubated with the MSC-conditioned medium for 24 hours prior to treatment with NMDA, suggesting that soluble factors secreted by MSCs are responsible for neuroprotection against NMDA. The next step in this study was to elucidate whether MSCs are capable of having a similar effect *in vivo* to promote neuroprotection in a mouse model where excitotoxicity is induced by kainic acid administration (Voulgari-Kokota et al., 2012). Intravenous administration of MSC-EGFP 24 hours after induction of SE reduced neuronal damage, hypertrophy of GFAP+ astrocytes and activation of Iba-1+ microglia in the hippocampus. Since the intravenously administered MSCs did not graft into the injured hippocampus, it has been suggested that soluble factors produced by MSCs provided neuroprotection. These results are in line with the idea that the therapeutic benefits of MSCs do not depend on their transplantation and integration into the affected organ.

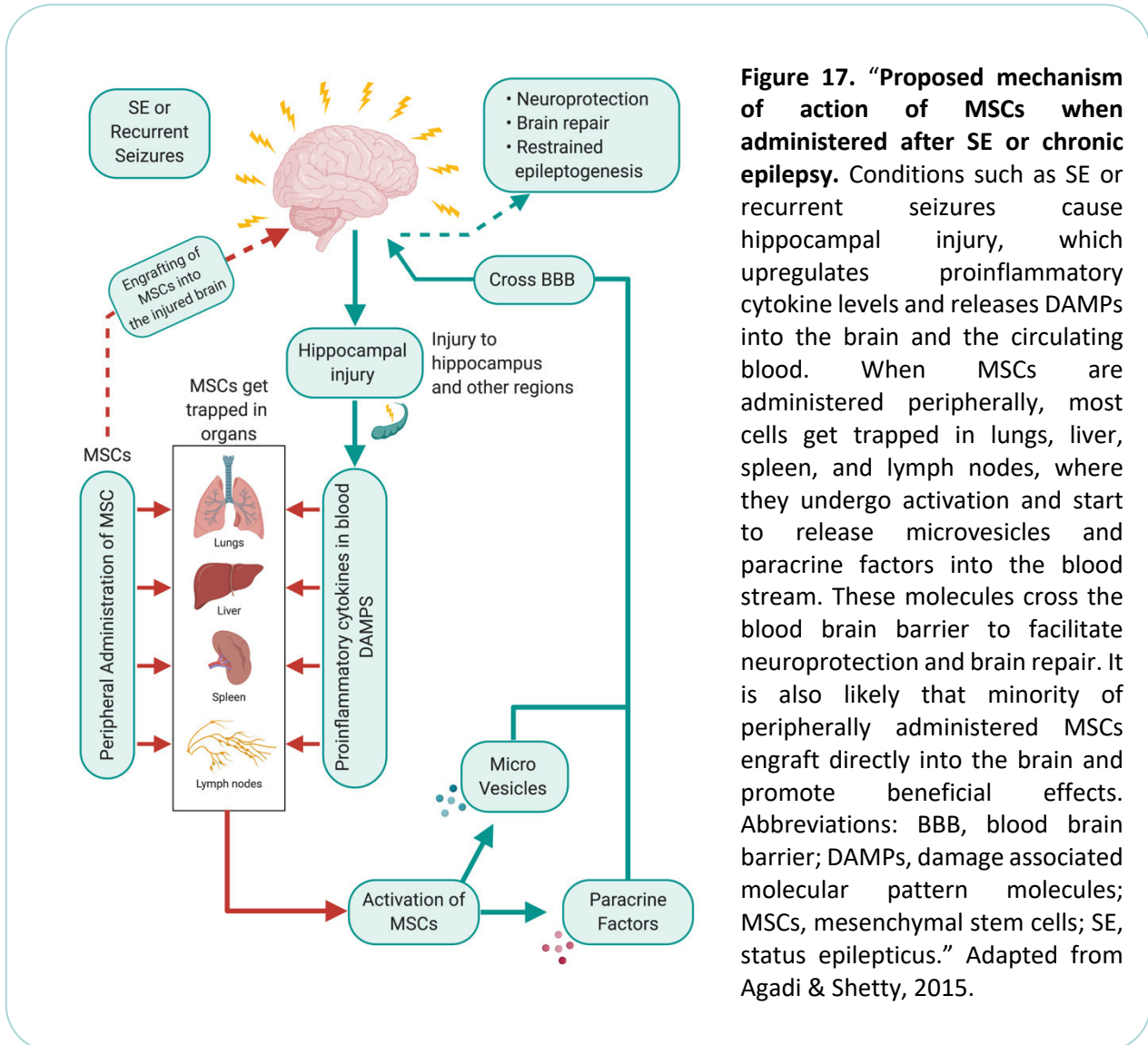
A study by Shetty et al announced in its 2015 review with findings presented at the 12th Annual meeting of International Society for Stem Cell Research has not yet resulted in an associated publication. In this study the effects of intraperitoneal administration of human bone-marrow derived MSC one hour after kainic acid-induced SE were evaluated. The results that were announced in the 2015 review were that MSCs induced considerable protection of

key neurons, reduced loss of GABAergic interneurons, normalized pro-inflammatory cytokine levels, reduced myeloperoxidase concentration and increased expression of genes encoding anti-inflammatory cytokines in the hippocampus.

Another study sought to determine the effect of MSCs on seizures (Abdanipour et al., 2011). SE was induced by pilocarpine and MSCs were injected 24 to 36 hours after the first seizures, which occurred within 6-7 days after SE. Monitoring of spontaneous recurrent seizures (SRS) during the subsequent three weeks showed that rats receiving MSCs had a reduction in behavioral SRS of approximately 66% compared to rats receiving PBS after the SE (Abdanipour et al., 2011).

Collectively, above-mentioned studies seem to indicate that inhibition of NMDAR subunit expression and glutamate-induced calcium fluxes by soluble factors produced by MSCs probably mediate neuroprotection and reduced development of chronic epilepsy after MSCs administration. It should be noted that several studies have shown that transplantation of infused MSCs into the damaged brain is not a prerequisite for functional recovery. On the contrary, a global modification of the immune system by these cells through potent anti-inflammatory effects and possibly other trophic effects are sufficient to allow neuroprotection and modification of the disease (Agadi and Shetty, 2015; Uccelli and Prockop, 2010). The potential mechanisms by which MSCs are thought to provide beneficial effects when administered after SE or in chronic epilepsy are illustrated in **Figure 17** (Agadi and Shetty, 2015).

Other studies have sought to elucidate the effect of genetically modified MSCs. In the study of Li et al (2009), human MSCs designed to release adenosine were tested in a mouse model of SE to assess their effect on seizures. Adenosine is known to be an endogenous anticonvulsant with an effect on drug-resistant epilepsy (Gouder et al., 2003). Adenosine-engineered MSCs were grafted intrahippocampally 24 hours after SE and then SRS were evaluated 3 weeks after grafting, showing a reduction in frequency and duration compared to sham rats. This investigation suggests that MSCs are potentially valuable as drug carriers that deliver drugs over prolonged periods of time in the epileptic brain (Li et al., 2009).



Clinical trials using mesenchymal stem cells in patients with epilepsy have already been conducted in recent years, with promising results reported. In a study conducted in 2017 by Hlebokazov, 10 patients with drug-resistant epilepsy received a therapy with anti-seizures drugs (ASDs) combined with a single intravenous administration of undifferentiated autologous MSCs at a dose of 1×10^6 cells/kg, followed 5-7 days later by a single intrathecal injection of neuro-induced autologous MSCs at a target dose of 0.1×10^6 cells/kg. Their results show that in the group of patients who received MSCs, 3 were seizure-free for a year or more, and were therefore considered to be in remission, and 5 others became responders to ASDs, compared to only 2 out of 12 patients in the group not treated with MSCs (Hlebokazov et al., 2017). In a subsequent study in 2019 (Slobina et al., 2019), 6 patients with pharmaco-resistant

epilepsy received, after stereotactic irradiation for 2-3 months, a single intravenous injection of undifferentiated autologous MSCs at a dose of 1×10^6 cells/kg, followed by an intrathecal injection of neurally induced autologous MSCs at a target dose of 0.1×10^6 cells/kg. Of the 6 patients who received stereotactic radiosurgery followed by MSCs therapy, 2 were in remission, and 3 became responsive to ASDs and had an improved EEG (Slobina et al., 2019). Nevertheless, despite the promising results of this study, it is difficult to attribute the results solely to the effect of MSCs as they were combined with other treatments. In another study conducted in 2018 by Milczarek, 4 children with drug-resistant epilepsy received combined therapy consisting of an autologous bone marrow nucleated cells transplantation (intrathecal in cerebrospinal fluid and intravenous) followed by four rounds of intrathecal bone marrow mesenchymal stem cells transplantation every three months. A total of four intravenous and 20 cerebrospinal fluid intrathecal transplants were performed. No side effects were reported in their study, and administration of MSCs resulted in neurological and cognitive improvement in all patients, evaluated by tests assessing psychomotor and psychological neurodevelopment. Finally, cellular therapy also led to a reduction in epileptic activity and seizure frequency (Milczarek et al., 2018).

Although these results are promising, many questions remain unanswered, and require innovative research to determine the best conditions of administration and to develop less burdensome treatments for epileptic patients that will pave the way for clinical translation.

V. Research questions and objectives

This research seeks to address the following questions:

1. How extensive is the neuroinflammation in the hippocampus of epileptic patients? Could it be considered as high? Does the explosive inflammatory level following pro-epileptogenic brain insult always lead to the development of epilepsy?
2. How to follow the infiltration of monocytes invading the brain parenchyma after a brain insult such as *status epilepticus* (SE) and what are their fate?
3. Is the inflammatory level in other areas of the brain after SE the same as in the hippocampus? Is inflammation always associated with high glial reactivity and significant neurodegeneration?
4. Can the acute post-SE inflammation be modulated using an innovative therapeutic tool, mesenchymal stem cells (MSCs), when MSCs are injected intranasally? Are MSCs able to counteract or reduce the severity of epilepsy and associated disorders?

To answer these questions, the objectives were:

1. In a first study:
 - To define the inflammatory status in resected hippocampus of epileptic patients at the molecular level;
 - To study the inflammation in animal models of pilocarpine-induced SE, from the earliest phase of epileptogenesis to the chronic phase of epilepsy;
 - To establish which type of glial cell participates most in the production of inflammatory molecules during the acute phase of epileptogenesis;
 - To assess whether the onset of epilepsy as a result of pro-epileptogenic brain injury is necessarily associated with high inflammation during the acute phase.
2. In a second study:
 - To investigate the infiltration and transdifferentiation kinetics of circulating monocytes in the cerebral parenchyma after SE;

- To ascertain whether two specific markers, CD68 and heparan sulfate chains, are suitable markers for monitoring infiltration and fate of infiltrating monocytes after SE.
3. In a third study:
- To define the inflammatory status in three other brain regions affected by SE, namely, the ventral limbic region, the dorsal thalamus and the neocortex.
 - To evaluate the level of inflammation and neurodegeneration after SE in Sprague-Dawley (SD) rats from Charles Rivers Laboratories and to compare it with our data on Harlan Laboratories SD rats used so far.
4. In a fourth study:
- To determine the effect of intranasal administration of MSCs on post-SE acute inflammation at the cellular and molecular level;
 - To investigate whether MSCs can prevent cognitive impairment after SE by studying the mechanism underlying the learning and memory processes, i.e. long-term potentiation.

LIST OF STUDIES

This PhD work is divided in the following studies:

Study 1.....113

Low grade inflammation in the epileptic hippocampus contrasts with explosive inflammation occurring in the acute phase following epileptogenic brain insults

Study 2.....171

Extravasating monocytes harboring heparan sulfate chains in adult rats can transdifferentiate into monocyte-macrophages, integrate resident microglia network, and maintain CD68 expression

Study 3.....200

Comparison of the inflammatory response to pilocarpine-induced *status epilepticus* in Sprague-Dawley rats from different origins

Study 4.....245

Intranasal mesenchymal stem cells treatment does not resolve but fine-tunes the inflammatory response following *status epilepticus* and prevents long-term potentiation alterations

CHAPTER 2

Study 1

INSIGHTS INTO THE INFLAMMATORY STATUS OF RESECTED HIPPOCAMPUS
OF EPILEPTIC PATIENTS AND CONTRIBUTION OF ANIMAL MODELS

Low grade inflammation in the epileptic hippocampus contrasts with explosive inflammation occurring in the acute phase following epileptogenic brain insults

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ABSTRACT

The role of neuroinflammation is now commonly accepted as a trigger and self-sustaining factor in epileptic seizure activity and as a major factor in the epileptogenesis that occurs after brain injuries. However, to date, there is still a lack of robust data, acquired identically and reliably from tissues either surgically resected from TLE patients or dissected in animal models, to answer the question of whether the inflammatory state of the hippocampus differs between TLE patients on the one hand, and between epilepsy and epileptogenesis on the other hand. Here, using calibrated RT and qPCR, we show that the expression of neuro-inflammatory markers is highly variable in the hippocampus of TLE patients, and is not necessarily associated with a high frequency of seizures. We further report from animal models that inflammation measured during the chronic phase of epilepsy has no common measure with the explosive inflammation that occurs after brain injuries induced either by status epilepticus (SE) or by bilateral fluid percussion (LFP), a model of moderate traumatic brain injury, whether neuroinflammation is followed by real epileptogenesis, as following SE or not, as after LFP. Finally, we show that microglial cells are the most important contributors to the early production of IL1 β after SE. Thus, our results provide strong evidence that: 1. some, but not all, TLE patients present with an inflammatory state in the hippocampus that is likely to be of low-grade, associated with the presence of both neurodegenerative processes and astrogliosis/microgliosis, suggesting that neuroinflammation alone cannot explain ictogenesis; 2. the explosive neuroinflammation that occurs early after brain insults may be important, but not sufficient, to trigger epileptogenesis.

1 | INTRODUCTION

Mesial temporal lobe epilepsy (TLE) is the most commonly diagnosed form of epilepsy that occurs frequently after an acute brain injury such as cerebrovascular accident, infections, traumatic brain injury (TBI) or status epilepticus (SE) (Klein et al., 2018). An atrophy of the hippocampus is often symptomatic of TLE and is the hallmark of a hippocampal sclerosis (Engel, 2001). The use of anti-seizure drugs (ASDs) remains the most widely used therapeutic approach but only protects against seizures in one in four cases in TLE patients, leaving nearly 75% of the patients with pharmaco-resistant epilepsies (Schmidt and Löscher, 2005). This statement strengthens the need of new therapeutics and hence the required understanding of the underlying physiopathological processes of epilepsy.

Considerable research attention has been directed towards a role for neuroinflammation as a primary driver of epileptogenesis occurring after brain insults and as a self-perpetuating factor of epileptic seizure activity (Rana and Musto, 2018; Terrone et al., 2019; Vezzani et al., 2011, 2013). The most widely studied molecular mediators of inflammation are cytokines. The broader cytokine family also includes chemokines, molecules with chemotactic properties that induce migration of immune or non-immune cells that play a role in maintaining central nervous system (CNS) homeostasis (Cerri et al., 2017). In physiological conditions, cytokines are released by immune cells such as granulocytes, macrophages and lymphocytes as well as by other cell types such as endothelial cells (Legido and Katsetos, 2014). In the brain, studies have also shown an important contribution of glial cells (astrocytes and microglia) and neurons in the production of pro-inflammatory cytokines (Boulanger, 2009; Vezzani et al., 2008, 2011). Numerous results obtained in preclinical models of neurological diseases, particularly epilepsy, have highlighted the dichotomous role of inflammatory reactions in the CNS, showing that they can be either protective, constituting an adaptive and beneficial endogenous response, or deleterious as a direct or indirect cause of neuronal dysfunction (Nguyen et al., 2002). Seizure-induced brain inflammation is long-lasting and may persist for days (Butler et al., 2016; Frigerio et al., 2018; Ravizza et al., 2008), indicating a failure of endogenous anti-inflammatory control mechanisms.

All preclinical models of chronic epilepsy showed a transient inflammatory reaction period, often limited to the acute phase following severe brain damage, involving inflammatory mediators such as the pro-inflammatory cytokines IL1 β , IL6 and TNF α (Vezzani

et al., 2011), chemokines, cyclo-oxygenases and prostanoids, damage-associated molecular patterns, toll-like receptors and complement pathway (Klein et al., 2018). Neuroinflammation processes are involved not only in the genesis of spontaneous seizures but also in their perpetuation (Lehtimäki et al., 2007; Vezzani et al., 2011), the seizures being caused by cytokines themselves but also by the degradation products released as a result of cell death processes. Besides, high levels of circulating IL1 β exacerbate the severity of seizures and lower their induction threshold (Dubé et al., 2005).

Elevated concentrations of inflammatory markers have been measured in cerebrospinal fluid and serum of patients that suffered various epileptogenic brain insults (Klein et al., 2018), but also in different forms of epilepsy (Lorigados Pedre et al., 2013; de Vries et al., 2016). Access to surgically resected tissue in TLE patients allowed evaluation of inflammatory status within the epileptic focus. Studies in human brain tissue evaluated the expression levels of certain inflammation markers in resected hippocampus of TLE patients (Klein et al., 2018; Leal et al., 2017; de Vries et al., 2016). They all revealed a particularly high pro-inflammatory state in the hippocampus of TLE patients. However, all these studies, even if they present comparisons with non-epileptic tissue, suffer from the absence of control tissues collected under conditions similar to those of operated TLE patients. Control tissues are often autopsy specimen from people with no history of epilepsy or brain-related disease and who died without associated brain damage. Furthermore, when mentioned, sampling times range from 4 to 20.5 hours post-mortem, which is significantly longer than surgical collection of tissue from TLE patients, with samples usually managed immediately, either by freezing (Aalbers et al., 2014; Das et al., 2012; Kan et al., 2012; Omran et al., 2012; Strauss and Elisevich, 2016) or by fixation (Aalbers et al., 2014; Das et al., 2012; Fiala et al., 2013; Leal et al., 2017; Ravizza et al., 2008; Sheng et al., 1994).

It must be recognized that a large number of studies have been carried out over the past 2 decades, which have allowed breakthroughs in the mechanistic hypotheses of epileptogenesis and ictiogenesis, in particular those involving neuroinflammation (Klein et al., 2018; Vezzani et al., 2011, 2019; van Vliet et al., 2018). Unfortunately, for the vast majority of these studies, the gene markers of inflammation were measured at the level of mRNAs or proteins, by methods today recognized as very little, if at all, quantitative. Recently, in a model of mice whose epilepsy developed after SE induced by the intrahippocampal administration

of kainic acid, a quantitative evaluation of inflammation was performed during epileptogenesis until epilepsy onset (Frigerio et al., 2018). In our study, after demonstrating that mRNAs of three housekeeping genes were rapidly degraded in the minutes / hours following the surgical resection of the hippocampus if the resected tissues were not immediately frozen in liquid nitrogen, the first objective was to evaluate, using calibrated reverse transcription and quantitative PCR, the dispersion of the mRNA levels of prototypical inflammatory markers measured in 22 patients who underwent surgical resection of the hippocampus. Then, in the absence of human control tissue, we used two models of TLE in rats, which allowed us not only to assess the time course of the inflammatory response during epileptogenesis and in the long term after the onset of epilepsy, but also the basal levels of inflammatory markers in the hippocampus. Quantitative RNAscope in situ hybridization studies have been performed to identify cells that express IL1 β gene throughout this time course. Finally, we investigated whether the absence of epileptogenesis in rats subjected to moderate bilateral traumatic brain injury (TBI) was due to a less inflammatory response than that observed after SE.

2 | MATERIAL AND METHODS

Study Design

Study 1. Impact of delayed cryopreservation in the processing of human brain samples on mRNA levels of housekeeping genes (HSG) determined by RT-qPCR. Three groups have been constituted. In the first group, samples used for RT-qPCR (n=13) were immediately frozen in liquid nitrogen after resection. In the second group, freezing of the samples (n=10) was delayed, as explained below. After quantification of 3 genes unrelated to the inflammatory cascade, the third group of samples (n=9) has been processed as for the first one.

Study 2. Evaluating transcript levels of inflammatory markers in the resected hippocampus of mTLE patients using RT-qPCR. In this experiment, samples corresponding to the first and third groups mentioned just above have been selected (n=22).

Study 3. Contribution of blood cells from capillaries to the levels of inflammatory markers measured in the hippocampus of juvenile (J) rats. Status epilepticus (SE) was induced at postnatal day (P) 42 (P42) by pilocarpine (Pilo-SE), and both rats subjected to SE and control rats were killed 7 hours after SE. At termination time, brains were collected from rats that

were transcardially perfused with saline (control rats: n=5; SE rats: n=4) or not (control rats: n=5; SE rats: n=5).

Study 4. Evaluation of gene expression at transcript level in the rat hippocampus during epileptogenesis and chronic epilepsy. Pilo-SE was induced in weanlings (W) at P21 or juvenile (J) rats at P42. Hippocampus of rats were dissected after transcardial perfusion of NaCl and the inflammatory profile was evaluated by RT-qPCR. Analysis was performed in rats sacrificed at different time points after SE: during epileptogenesis, that is at 7 hours (W, n=7 ; J, n=6), 1 day (W, n=8; J, n= 6), 9 days (W, n=10 ; J, n=7) post-SE, and once chronic epilepsy was developed in all rats, i.e. 7 weeks post-SE (W, n=8 ; J, n=8). Brains of control rats were also collected; however, to reduce the number of animals used, some time points have been pooled: W rats (7h and 1 day: n=5 ; 9 days: n=5 ; 7 weeks: n=6) and J rats (7h and 1-9 days: n=6 ; 7 weeks: n=6).

Study 5. Astroglial and microglial activations evaluated using GFAP- and ITGAM-immunofluorescent detections, respectively, in the rat hippocampus at 1 day (W, n=4 ; J, n=5), 9 days (W, n=6 ; J, n=7) and 7 weeks post-SE (W, n=6 ; J, n=7), induced in W and J rats, and in respective controls (W, n=3 for 1-9 days, n=5 for 7 weeks; J, n=5 for both 1-9 days and 7 weeks).

Study 6. Distribution and quantitation of IL-1 β transcript were evaluated using RNAscope[®]-based quantitative *in situ* hybridization in the hippocampus of 5 patients with mTLE (3 with high and 2 with low tissue levels of IL-1 β mRNA determined by RT-qPCR) and of rats subjected to Pilo-SE at P42 and sacrificed 7 hours (n=3), 1 day (n=5), 9 days (n=3) and 7 weeks (n=3) post-SE and in respective controls (7h and 1-9 days: n=2; 7 weeks: n=2).

Study 7. Comparison between peaks of inflammatory response to Pilo-SE and mild-to-moderate traumatic brain injury (TBI). For TBI, transcript levels of inflammatory markers have been measured from a RNA bank obtained from rats subjected or not to a bilateral fluid percussion (bLFP) (controls, n=4; bLFP, n=5), used in a previous published study (Ogier et al., 2017). For Pilo-SE, data used are those obtained in Experiment 4 at time point “7 hours post-SE”.

Patients

The cases in this study were obtained from the neurological and neurosurgical hospital Pierre Wertheimer in Lyon between 2009 and 2012. A total of 32 mTLE surgical hippocampus were obtained from patients undergoing standard corticoamygdalo-hippocampectomy for refractory epilepsy. The first group of patients included 6 males (15-56 years) and 7 females (15-51 years); the second included 7 males (19-50 years) and 3 females (17-37 years); the third included 4 males (14-49 years) and 5 females (12-42 years). Pre-operative informed consent was obtained for the use of resected brain tissue. The detailed clinical data for each specimen are listed in Table 1 and Table S1. Briefly, hippocampi were resected *en bloc*, rinsed for 1 min in ice-cold saline and cut in 3 equal parts perpendicularly to the longitudinal axis from the head to the body: the first part was immediately frozen in liquid nitrogen for 22 samples and then stored at -80°C or immersed into an ice-cold RNAlater® solution for 45 to 90 min before freezing in liquid nitrogen; the second part was fixed for 72 hours in an ice-cold 4% paraformaldehyde solution, immediately after resection (n=22) or after a 45-90 min delay (n=10), cryoprotected into an ice-cold 30% sucrose solution prepared in 0.1M phosphate buffer, frozen at -40°C in isopentane and then stored at -80°C; and the third part was used for routine histopathological evaluation.

Animals

All animal procedures were in compliance with the guidelines of the European Union (directive 2010-63), taken in the French law (decree 2013/118) regulating animal experimentation, and have been approved by the ethical committee of the Claude Bernard Lyon 1 University (protocol # BH-2008-11). We used a tissue collection bank generated by TIGER team in 2009-2012. Briefly, male Sprague-Dawley rats (Harlan, Italy) were used in these experiments. They were housed in a temperature-controlled room ($23 \pm 1^\circ\text{C}$) under diurnal lighting conditions (lights on from 6 a.m to 6 p.m). Pups arrived at 15 day-old and were maintained in groups of 10 with their foster mother until P21. Beyond that age, rats were maintained in groups of 5 in 1,800 cm² plastic cages, with free access to food and water. After SE, rats were maintained in individual cages and weighed daily until they gained weight. Until sacrifice, epileptic rats were housed alone and control rats were housed in groups of 5 in standard cages.

Pilocarpine-induced *status epilepticus* (SE). SE was induced by pilocarpine, injected at day 21 or 42. To prevent peripheral cholinergic side effects, scopolamine methylnitrate (1 mg/kg in

saline, s.c.; Sigma-Aldrich) was administered 30 min before pilocarpine hydrochloride (25 mg/kg at P21 and 350 mg/kg at P42, in saline, i.p.; Sigma-Aldrich). For P21 rat pups, lithium chloride (127 mg/kg in saline, i.p.; Sigma-Aldrich) was injected 18 hours before scopolamine. After 30 min of continuous behavioral SE at P21 and 2 hours at P42, 10 mg/kg diazepam (i.p.; Valium; Roche®) was injected, followed, 90 min later for P21 and 60 min later for P42, by a second injection of 5 mg/kg diazepam to terminate behavioral seizures. Control rats received systematically corresponding injections of saline solution. The animals were then sacrificed at various time points: 7 hours, 1 day, 9 days and 7 weeks after SE.

Animal care after Pilo-SE. Control and treated rats were weighted every day during the first two weeks following Pilo-SE, and then every week until termination of the experiment. Daily abdominal massages were performed twice a day during the first week to activate intestinal motility, which was disrupted following Pilo-SE.

Onset of handling-induced seizures. Electroencephalographic recordings were excluded to determine epilepsy onset due to preliminary experiments that showed that the sole implantation of screws into the skull induced significant and lasting inflammation over time in the cortex and, to a lesser extent, in the hippocampus. As a result, epilepsy onset was determined according to clinical criteria. Therefore, animals were tested for the occurrence of handling-induced seizures (HIS) three times a day between the 1st and the 5th week post-SE. HIS were triggered by restraining rats for 10 seconds at the level of the chest with gentle pressure. Animals were declared as “epileptic” (EPI) once they developed HIS on 2 consecutive trials. By the end of the 5th week post-SE, all rats were considered as EPI.

Mild-to-moderate bilateral fluid percussion (bLFP). We used our total RNA library built from rats used in a previously published study (Ogier et al., 2017). Briefly, bLFP was induced in rats at 9 weeks, subjected to 4-mm diameter craniotomies centered at -3 mm from bregma and \pm 3.8 mm left/right of the sagittal suture and to a peak pressure and pulse duration of 2.25 ± 0.08 ATM and 22 ± 2 ms, respectively, thus modeling mild-to-moderate severity. Under these conditions, rats did not develop epilepsy as monitored by handling-induced seizures (see above) up to 10 weeks of observation post-bLFP. For this study we used total RNAs extracted from the hippocampus of rats sacrificed 7 hours post-bLFP (n=5) and controls (rats subjected to surgery only; n=4) and stored at -80°C.

Ex Vivo Procedures

All rats were deeply anesthetized with a lethal dose of pentobarbital (100 mg/kg; Dolethal) before being sacrificed. Hippocampus were rapidly microdissected, frozen in liquid nitrogen, and stored at -80°C. For immunochemistry analysis, animals were transcardially perfused (30 mL/min) with 4% paraformaldehyde in 0.1 M phosphate buffer. After cryoprotection in 30% sucrose, brains were frozen at -40°C in isopentane and stored at -80°C.

RNA extraction and quantification of transcript level variations by reverse transcriptase real-time polymerase chain reaction (RT-qPCR). Brain structures frozen in liquid nitrogen were crushed using Tissue-Lyser (Qiagen®) in 250 µL of ultrapure RNase-free water (Eurobio). Nucleic acids were extracted by adding 750 µL Tri-Reagent LS (TS120, Euromedex) and 200 µL chloroform (VWR®). After precipitation with isopropanol (I-9516, Sigma-Aldrich®), washing in 75% ethanol (VWR) and drying, total nucleic acids were resuspended in 50 µL ultrapure water and treated with DNase I (Turbo DNA Free® kit; AM1907, Ambion®) to eliminate any trace of possible genomic DNA contamination. The purified total RNAs were then washed using the RNeasy® minikit (Qiagen®) kit. After elution, the total RNA concentration was determined for each sample on BioDrop® µLite. The quality of total RNAs was verified on microgel chips using LabChip® 90 (Caliper), which provides an RNA Integrity Number (RIN) value by analyzing the integrity of two ribosomal RNAs (18S and 28S) predominantly present in all tissue RNA extracts. All selected samples had a RIN value greater than 7.0, and were stored at -80°C until use. Total tissue RNAs (480 ng) were reverse transcribed to complementary DNA (cDNA) using both oligo dT and random primers with PrimeScript RT Reagent Kit (Takara) according to manufacturer's instructions, in a total volume of 10 µL. In RT reaction, 300 000 copies of a synthetic external non-homologous poly(A) standard messenger RNA (SmRNA; A. Morales and L. Bezin, patent WO2004.092414) were added to normalize the RT step (Sanchez et al., 2009, PNAS). cDNA was diluted 1:13 with nuclease free Eurobio water and stored at -20°C until further use. Each cDNA of interest was amplified using 5 µL of the diluted RT reaction by the "real-time" quantitative polymerase chain reaction (PCR) technique, using the Rotor-Gene Q thermocycler (Qiagen®), the SYBR Green Rotor-Gene PCR kit (Qiagen®) and oligonucleotide primers specific to the targeted cDNA. The sequences of the specific forward and reverse primer pairs were constructed using the Primer-BLAST tool or using the "Universal Probe Library" software (Roche Diagnostics). Sequences of the different primer pairs used are listed

in Supplementary Table 7 for humans and Supplementary Table 8 for rats. The number of copies of each targeted cDNA contained in 5 μ L of the diluted RT reaction was quantified using a calibration curve based on cascade dilutions of a solution containing a known number of cDNA copies.

Pro-inflammatory (PI-I), anti-inflammatory (AI-I), inflammation cell (IC-I) and housekeeping gene (HSKG-I) indexes were calculated for each series of individuals to be compared using a specific set of genes: IL1 β , IL6, TNF α , MCP1 and MIP1 α for PI-I; IL4, IL10 and IL13 for AI-I; ITGAM and GFAP for IC-I; DMD, HPRT1 and GAPDH for HSKG-I. For each individual, the number of copies of each transcript has been expressed in percent of the averaged number of copies measured in the whole considered population of individuals. Once each transcript is expressed in percent, an index is calculated by adding the percent of each transcript involved in the composition of the index and expressed in arbitrary units (A.U.). Each time that an index is presented, the groups of individuals constituting the population is specified.

Tissue processing for histological procedures. Cryostat-cut (40 μ m thick) sections from mTLE patient tissue samples or from rat samples were transferred into a cryopreservative solution composed of 19.5 mM NaH₂PO₄·2H₂O, 19.2 mM NaOH, 30% (v/v) glycerol and 30% (v/v) ethyleneglycol and stored at -25°C.

Immunohistochemistry. Free-floating sections (40 μ m thick) from paraformaldehyde-fixed tissue were incubated with a rabbit polyclonal anti-GFAP antibody (1:1000; AB5804; Chemicon) to label astrocytes and a mouse monoclonal anti-ITGAM (1:1000; CBL1512Z, Chemicon) to detect microglia and immunocompetent cells. For fluorescent dual immunolabeling, sections were incubated with an Alexa-Fluor-488-conjugated donkey anti-rabbit IgG antibody (1:1000; A-21206; Molecular Probes) and with an Alexa-Fluor-647-conjugated donkey anti-mouse IgG antibody (1:1000; A-31571; Molecular Probes). Sections were then mounted on SuperFrost Plus slides and coverglassed with Prolong Diamond Antifade reagent (Molecular Probes). The GFAP and ITGAM immunostained slides were observed using a Carl Zeiss Axio Scan.Z1 Digital Slide Scanner with a resolution of x40. Images were then imported into Adobe Photoshop CS6 13.0 (Adobe Systems) for further editing.

In Situ Hybridization using RNAscope®. Probes were designed by ACD (Advanced Cell Diagnostics, Newark, New Jersey) to hybridize to IL1 β , ITGAM and GFAP mRNA molecules with species specificity (*Homo sapiens* -Hs- probes for humans; *Rattus Norvegicus* -Rn- for rats).

The RNAscope® Multiplex Fluorescent Reagent Kit v2 (Cat. 323100) and the hybridization oven (HybEZ Oven) were also obtained from ACD. The RNAscope® assay was performed as described by the supplier. Briefly, the staining protocol included five steps: pretreatment with protease, hybridization of target probes, amplification of the signal, detection of the signal and mounting of the slides.

Selected tissue sections of resected hippocampus from mTLE patients or selected rat tissue section including the hippocampus were removed from cryoprotectant solution and rinsed in phosphate-buffered saline (PBS) three times. RNAscope® assays were performed on tissue mounted on SuperFrost slides. Sections went through treatment with Protease III solution during 30 minutes at 40°C. Three different probes were then used to localize mRNAs of IL1 β (Hs-IL1 β , Cat. 310361; Rn-IL1 β , Cat. 314011), ITGAM (Hs-ITGAM, Cat. 555091-C3; Rn-ITGAM, Cat. 300031-C3) and GFAP (Hs-GFAP, Cat. 311801-C2; Rn-GFAP, Cat. 407881-C2). Sections subsequently passed through amplification steps followed by fluorescent labeling in Opal 520, Opal 570 and Opal 690 (NEL810001KT, PerkinElmer) at 1:1000 dilution with amplification diluent. Sections were then counterstained with DAPI and coverglassed with Prolong Diamond Antifade reagent (Molecular Probes). Slides were observed using a TCS SP5X confocal microscopy system (Leica). All sections were analyzed under identical conditions of photomultiplier gain, offset and pinhole aperture, allowing the comparison of fluorescence intensity between regions of interest. Then, for each of the hybridized probe, ImageJ software was used to measure areas of fluorescence using thresholding procedure.

Data and statistical analysis.

GraphPad Prism (v.7) software was used to statistically analyze data. Majority of data are expressed as mean \pm SEM of the different variables analyzed. Transcript levels are also expressed using box-and-whisker plots to illustrate the distribution of the considered cohort. Statistical significance for within-group comparisons was calculated by one-way or two-way analysis of variance (ANOVA) with Bonferroni or Tukey's *post hoc* test. The p value of 0.05 defined the significance cut-off. Correlations were assessed using Spearman's rank correlation test.

3 | RESULTS

Human brain tissues with delayed cryopreservation are not suitable controls for transcriptomic studies

In this study, 32 patients were included, whose main clinical characteristics are summarized in Supplementary Table 1. Ideally, surgically resected brain tissues should be frozen at a very low temperature immediately after collection, so as to preserve the molecules to be measured. These conditions were those observed for Patient Groups 1 and 3 (G1 and G3), whose resected brain tissues were frozen less than 5 minutes after neurosurgical removal. For logistic reasons, it was temporarily decided to delay the freezing procedure of the resected tissues from Patient Group 2 (G2). To this end, tissues were transferred into ice-cold RNALater® immediately after their resection, then given to the research staff in charge of freezing them in liquid nitrogen back to the laboratory within a period ranging between 45 and 90 minutes. RNALater® has been developed to preserve RNA integrity even if samples are stored for days to weeks at 4°C after collection either before freezing or direct extraction of total RNAs (Florell et al., 2001). We first compared the mRNA levels of three housekeeping genes (HSKG = GAPDH, HPRT1 and DMD) between G1 (P01-P17, n=13) and G2 (P18-P29, n=10). A very large decrease was observed in G2 (7.2-fold less than G1 for DMD: p=0.0022; 4.7-fold less than G1 for GAPDH: p=0.0371; 14-fold less than G1 for HPRT1: p=0.0008, Fig. S1). By switching back to the first freezing protocol (i.e. freezing immediately after resection) for G3 (P40-P49, n=9), the average values for the 3 housekeeping genes were closer to that obtained for G1 (Fig. S1). All of the samples used in this study had RIN values >7, attesting that all RNA samples were of excellent quality, according to the integrity of 18S and 28S ribosomal RNAs. Overall, these results indicate that delayed cryopreservation protocol caused alteration of the transcript levels in human resected hippocampi, precluding the use of autopsy/post-mortem tissue as valid controls, in particular to determine reference / basal levels of neuroinflammatory markers. For the rest of the study, values of patient group 2 were excluded.

Inflammation in the hippocampus of mTLE patients is highly variable

Twenty-two fresh frozen surgically resected hippocampi of mTLE patients (P01-P17 and P40-49 from G1 and G3; 12F, 10M, 31 ± 14 years) were subjected to gene-specific transcript quantification for a set of 11 inflammatory markers including pro-inflammatory (IL1 β , IL6,

TNF α , IFN γ) and anti-inflammatory (IL4, IL10, IL13) cytokines, chemokines (MCP1, MIP1 α) and cell markers (microglia/macrophages: ITGAM, astrocytes: GFAP). For each patient, all the above-mentioned gene transcripts could be detected and then quantified, except those of IFN γ , IL4 and IL13 that were not detected in any of the 22 samples, even when using several primer pairs designed in different parts of the corresponding cDNAs. Individual cDNA value for each marker was expressed in percent of the calculated average (n=22) value (Fig. 1). IL6 and MCP1 had the highest and lowest interindividual variability, respectively.

Not all the lowest values are observed in the same patient, nor are the highest values (Table S2). For example, patient P41 who had the lowest values for TNF α , MCP1 and MIP1 α did not have the lowest values for IL1 β and IL6. Similarly, patient P49, who had the highest values for TNF α and IL6, did not have the highest values for IL1 β , MCP1 and MIP1 α (Tables S2 and S3). Therefore, to provide a general overview of the inflammatory status for each patient, we calculated a pro-inflammatory index and an inflammation cell index that integrate for each patient the average normalized expression of each individual cytokine/chemokine or each individual cell marker, respectively. Patients P41 and P49 had the lowest (56 A.U.) and the greatest (1,730 A.U.) pro-inflammatory index, respectively (Fig. 2A-B), corresponding to a ~31-fold difference. It is to note that the pro-inflammatory index does not correlate well with the inflammation cell index (Fig. 2C), as pointed out for patient P47 (see arrows, Fig. 2A-C).

Even if the greatest value for the anti-inflammatory cytokine IL10 was observed in patient P49 (Table S3) who had the greatest value for pro-inflammatory index (Fig. 2A), IL10 did not correlate with the pro-inflammatory index (compare Table S3 with Fig. 2A).

To our knowledge, in order to normalize RT-PCR data, all prior studies used one or a combination of housekeeping genes considered as invariant between samples. We previously stressed the fact that high variability was also found in housekeeping genes (Fig. S1). We calculated a housekeeping gene index integrating DMD, GAPDH and HPRT1, which confirmed the high variability in the pool of the three housekeeping genes between patients, e.g. a 22-fold difference between patients 5 and 42 (Fig. 3A). We show that the housekeeping gene variability did not fit with that of the pro-inflammatory index (Fig. 2A and Fig. 3A). Hence, if housekeeping genes had been used to normalize the RT reaction, this would have led to biased results (Fig. 3B).

We next investigated whether the variation of the pro-inflammatory index was associated with relevant clinical features. The six patients with the greatest pro-inflammatory index (P07, P10, P13, P42, P44, P49) all had neuronal loss (Table S4), but neuronal loss was not systematically associated with a high pro-inflammatory and inflammation cell index values (compare Table S4 and Fig. 2A-B). The pro-inflammatory index (P-I, in A.U.) did not correlate either with the age (in year) at epilepsy onset ($P-I = 14.95 \times \text{age} + 342.3$; $r^2=0.11939$) nor with the duration (in year) of epilepsy ($P-I = -0.017 \times \text{duration} + 503.5$; $r^2 = 0.00002$). While reports on seizure frequency before surgery were lacking for most patients, data available for 5/22 patients provide indication that rare seizures (P07, 1 seizure per month) and frequent seizures (P15, 2 seizures per week) were associated with high (1,132 A.U.) and low (228 A.U.) pro-inflammatory index values, respectively. Finally, the extent of the pro-inflammatory and inflammation cell indexes were not associated with any given anti-epileptic drug treatment (compare Table S1 and Fig. 2A-B).

Overall, our results show that some, but not all patients with TLE subjected to surgery, had a substantial level of inflammation within the resected hippocampus. At this stage, the absence of adequate human control tissues did not allow us to know if the inflammation observed was at low or very high level. In order to provide answers to this question, the rest of this study was conducted on preclinical models in order to have access to valid control tissues and to compare the inflammatory level during chronic epilepsy to that reported during epileptogenesis (Frigerio et al., 2018; Vezzani et al., 2019).

Circulating inflammatory markers do not contribute significantly to the quantitation performed in whole brain extracts

Resected hippocampi from epileptic patients contain blood tissue; it was thus essential to ascertain whether the presence of blood could be a hindrance to the evaluation of brain parenchyma inflammatory status. We used rats subjected to pilocarpine-induced *status epilepticus* (SE) to evaluate the potential contribution of blood into the measures performed in brain tissue. Transcripts levels of IL1 β , IL6, TNF α , MCP1, MIP1 α and ITGAM were compared between rats devoid of blood tissue following transcatheter perfusion of sodium chloride (NaCl, 0.9%) and rats that were not subjected to perfusion (Table S5). The study was conducted in juvenile rats 7h after SE induction (perfused rats: SE-NaCl; not perfused: SE-blood) and in their respective controls (perfused rats: CTRL-NaCl; not perfused rats: CTRL-blood). Results are

expressed as the percentage of the mean transcript level value measured in CTRL-NaCl group. Except for TNF α , where a significant difference is observed between the two groups of controls ($p < 0.01$), the inflammatory expression profiles are identical with or without transcatheter perfusion of NaCl, showing that the level of inflammatory molecules into brain vessels remains marginal, indicating that most inflammatory molecules measured in whole brain extracts originated more from brain parenchyma than blood.

Model-specific differences in post-SE microgliosis and astrogliosis

All but one patient with TLE presented with hippocampal sclerosis and among these patients, the extent of neuronal loss and reactive gliosis was highly variable (Table S4). Therefore, to model the heterogeneity of patients with TLE, we used two well-known rat models presenting various extents of neuronal degeneration. The first model used consisted of juvenile P42 rats subjected to pilocarpine-induced SE, characterized by extensive neuronal degeneration in the hippocampus, the piriform cortex, the amygdala and the insular agranular cortex (Nadam et al., 2007; Sanchez et al., 2009; Voutsinos-Porche et al., 2004). By contrast, the second model used consisted of weaned P21 rats subjected to lithium-pilocarpine-induced SE, characterized by minimal or not detectable neuronal loss once adults (Cilio et al., 2003; Cross and Cavazos, 2007).

In these two models, characterization of SE-induced reactive gliosis in the rat hippocampus was performed histologically during epileptogenesis (1 day and 9 days post-SE) and during the chronic phase of epilepsy (7 weeks post-SE), by double-labeling immunofluorescence targeting GFAP and ITGAM (CD11b) to evaluate astroglial and microglial/macrophage reactivity, respectively (Fig. S2). Before induction of SE, astrocytes and microglia showed low GFAP and ITGAM signal, respectively. High reactivity of both GFAP and ITGAM was observed at 1 day and 9 days post-SE in rats subjected to juvenile SE, and, to a lesser extent for rats subjected to SE at weaning. These histological results are in line with those obtained for the corresponding transcripts measured by RT-qPCR, the induction of GFAP and ITGAM in the hippocampus of rats subjected to SE at P42 being greater during epileptogenesis to that of rats subjected to SE at P21 (Fig. S3 A-B). These histological results are in line with those obtained for the corresponding transcripts measured by RT-qPCR, the induction of GFAP and ITGAM in the hippocampus of rats subjected to SE at P42 being greater during epileptogenesis than that of rats subjected to SE at P21 (Fig. S3 A-B). In addition, as previously reported in rats

following pilocarpine-induced SE (Ravizza et al., 2008), numerous circulating macrophages, as identified as ITGAM⁺ round-shaped cells, infiltrate the brain parenchyma beyond 7h and until 2 days post-SE, the peak of extravasation being observed 24h post-SE (data not shown here, but extensively presented in study #2). During the chronic phase of epilepsy, at 7 weeks after SE, GFAP and ITGAM mRNA levels decreased markedly in the hippocampus, and GFAP transcript remained higher than controls only in rats subjected to SE at P42 (Fig. S3 A-B). When considering the overall markers of reactive gliosis (GFAP and ITGAM mRNAs), the inflammation cell index was always greater in rats subjected to SE at P42 compared to P21, both during epileptogenesis and the chronic phase of epilepsy (Fig. S3 C).

Modeling of TLE in rats suggests that some patients may have basal inflammatory levels in the hippocampus

As highlighted above, no control hippocampal tissues collected under similar conditions to those of TLE patients were available to compare levels of inflammation measured in the resected hippocampi of TLE patients to reference / baseline values. In this context, animal models of TLE presented above have provided all their added value in that epileptic rats can be compared to control rats for which samples were obtained under perfectly identical conditions and, in addition, very similar to those of surgically resected tissues of TLE patients, i.e. with quasi immediate freezing following tissue collection.

We quantified in the hippocampus of epileptic rats (SD rats EPI-W and EPI-J) the transcripts level of the same panel of inflammatory mediators that were studied in TLE patients and values were compared to that of control rat group (CTRL) (Fig. 4). The inflammatory levels of control rats (sacrificed at the same time as the epileptic animals, i.e. 7 weeks post-SE) whose SE was induced at weaning (CTRL-W) and juvenile (CTRL-J) stages were not statistically different, hence the two control groups were pooled in a same control group (CTRL). In any of the rat samples, IL6 was not detected.

For rats that developed epilepsy after SE induced at weaning (EPI-W), statistical analyses revealed that, except for IL13 ($p=0.0384$) and GFAP ($p=0.0043$), there was no significant difference between the dispersion of the CTRL group and the EPI-W group, indicating that when the SE is induced in weaned rats, the inflammation does not differ substantially from healthy rats. In contrast, for rats that developed epilepsy after SE induced at juvenile stage (EPI-J), we show a significant difference between CTRL group and EPI-J group for IL1 β

($p=0.0002$), MCP1 ($p<0.0001$), MIP1 α ($p<0.0001$), IL13 ($p=0.0234$), ITGAM ($p=0.0039$) and GFAP ($p<0.0001$) (Fig. 4). We also demonstrate that EPI-J group is significantly different from EPI-W group for IL1 β ($p=0.0005$), MCP1 ($p=0.0005$), MIP1 α ($p<0.0001$) and GFAP ($p=0.0034$) (Fig. 4). No differences in expression of TNF α , IL4 and IL10 were found between epileptic groups and control group as well as within epileptic groups. When considering the pro-inflammatory index and the inflammation cell index, a strong difference was confirmed between the two models of epilepsy (Fig. 5), highlighting a significant difference between controls and EPI-W rats for the inflammatory cell index but not for the pro-inflammatory index. On average, the pro-inflammatory index and the inflammatory cell index increased at most 1.96 times and 1.90 times, respectively, in epileptic rats compared to control rats (Fig. 5). All together, our preclinical data indicate that depending on the epilepsy model used, epilepsy can be associated or not with an induction of pro-inflammatory cytokines and chemokines (Fig. 5A), but is constantly associated with an induction of inflammation cell index (Fig. 5B) resulting from an induction of astroglial GFAP (Fig. 4).

In the absence of reference values for samples from TLE patients, we undertook a translational approach by facing off the data obtained in rats with those of patients, based on the dispersion of normalized values obtained for the different inflammatory markers. The total variability observed in rats, including both the control group and the two epileptic rat groups, covered between 49% and 93% of the variability observed in patients, with: 76% for IL1 β , 49% for TNF α , 93% for MCP1, 87% for MIP1 α , 87% for IL10, 59% for ITGAM, 69% for GFAP, 50% for the pro-inflammatory index and 47% for the inflammation cell index. In addition, for each of the transcripts, the lowest normalized values were always observed in patients, and thus lower than the lowest values measured in control rats.

Inflammation is of low grade in chronic epilepsy compared to explosive inflammation during epileptogenesis

The ~ two-fold increase in the pro-inflammatory index in rats with epilepsy developed after SE induced at the juvenile age (EPI-J) (Fig. 5) raised the issue of whether this increase was greater or lesser than that occurring after SE itself, as already questioned using undisputable quantitative procedures following kainic acid-induced SE (Frigerio et al., 2019). To this end, inflammatory levels in our two models of TLE were investigated during epileptogenesis and compared to those measured during chronic epilepsy. Transcript levels of inflammatory and

anti-inflammatory markers were quantified in the hippocampus of rats during epileptogenesis (at 7 hours, 1 day, 9 days) and during epilepsy (7 weeks) after the onset of SE induced at P21 (SE-W, light blue bars) or P42 (SE-J, dark blue bars). The results are presented for each pro-inflammatory (Fig. S4) and anti-inflammatory (Fig. S5) markers, as well as for the corresponding pro-inflammatory and anti-inflammatory indexes (Fig. S6). They reveal that the induction peak occurred between 7 hours and 1 day after SE for both epileptic models. The comparison of the peak values of pro-inflammatory markers determined during epileptogenesis with the values measured during the chronic phase of epilepsy (7 weeks post-SE) reveals that the difference between these two values ranged between 0.46-fold (TNF α) and 740-fold (MCP1) for rats subjected to SE at weaning (P21) and between 8-fold (TNF α) and 781-fold (MCP1) for rats subjected to SE at the juvenile stage (P42) (Table 1). Hence, the pro-inflammatory index measured at the peak during epileptogenesis was 17.77- and 23.95-fold greater than that measured in the chronic phase of epilepsy, for SE induced at P21 and P42, respectively (Table 1).

Quantitative RNAScope® *in situ* hybridization confirms data obtained by RT-qPCR

Data on transcript levels acquired so far in this study were obtained by RT-qPCR. They indicate wide variations for most of the studied inflammation markers in the hippocampus both between patients and between different groups of rats, especially for the latter between the epileptogenesis period and the chronic phase of epilepsy. In order to rule out any hypothesis that the observed variations could be the result of random degradation of mRNAs during the extraction and purification phases of total RNAs, RT-qPCR data for IL1 β were compared to those obtained on fixed brain sections by the RNAScope® technology, which is a highly quantitative *in situ* hybridization (ISH) method. IL1 β was selected for this comparison because it is one of the most studied inflammation markers in the context of neuroinflammation. Finally, to carry out this comparison, we selected 5 patients for whom the IL1 β -cDNA copy numbers were either low (P15 and P45), intermediate (P43 and P44) or high (P42), and the different time points studied by RT-qPCR during epileptogenesis and the chronic phase of epilepsy in rats subjected to Pilo-SE at the juvenile (P42) stage. In sections of the hippocampus resected from TLE patients, the density of IL1 β -mRNA signal (magenta dots) was greater in patient P42 compared to patient P15 (Fig. S7A), as expected, and the surface area occupied by IL1 β -mRNA signal in the 5 selected patients correlated significantly with the corresponding

IL1 β -cDNA copy numbers quantified by RT-qPCR (Fig. 6A). In rats subjected to SE at P42, IL1 β -mRNA signal was quantified in the dentate gyrus where it was greater than other regions of the hippocampus, including CA1 area. The density of IL1 β -mRNA signal, measured at 7h, 1 day, 9 days and 7 weeks post-SE, was greater at 7 hours post-SE (Fig. S8 and Fig. 6 B1) and significantly correlated with IL1 β -cDNA copy numbers quantified by RT-qPCR in different sets of animals (Fig. 6 B2).

Microglial cells seem to produce most of the IL1 β during the acute phase

Previous studies using immunohistochemical procedures reported that IL1 β was expressed mainly by astrocytes and more rarely by microglial cells/macrophages in the hippocampus following pilocarpine-induced SE and self-sustained limbic SE (Ravizza et al., 2008), and exclusively by astrocytes following kainic acid-induced SE (Frigerio et al., 2018). To determine to which extent microglial cells or astrocytes were each involved in the production of IL1 β -mRNA, we used multiplex detection of IL1 β , ITGAM and GFAP transcripts using RNAscope[®] ISH. We could not combine IL1 β -mRNA ISH with immunohistofluorescent detection of GFAP and ITGAM because antigens recognized by the different antibodies tested were altered by the permeabilization and fixation procedures in RNAscope[®] protocols. In patient P42, who had the greatest IL1 β -cDNA copy number, IL1 β -mRNA signal (magenta dots) was located in cells bearing morphological features of glial cells (Fig. S7A). However, the paucity of ITGAM-mRNA and GFAP-mRNA signals at the location of IL1 β -mRNA signal precluded the identification of IL1 β -mRNA signal as being of astroglial or microglial origin (Fig. S7B-C). In rats, the only time point it was possible to identify the glial cells expressing IL1 β -mRNA was 7 hours post-SE. Cells with a large and packed IL1 β -mRNA signal appeared to be ramified microglial cells, as identified by the presence of ITGAM-mRNA signal in the core of the IL1 β -mRNA signal (Fig. 6 C-D). At this time point, numerous astrocytes also expressed IL1 β -mRNA, but at weaker levels compared to ITGAM⁺ cells, as depicted by the small surface area occupied by IL1 β -mRNA signal with the dense signal corresponding to GFAP-mRNA (Fig. 6E-F).

Heterogeneous inflammation after different epileptogenic brain insults

SE is a severe epileptogenic condition in rats, but other brain insults such as traumatic brain injury (TBI) are known to induce an inflammatory response and lead to epilepsy (Klein et al., 2017). However, only TBI of severe intensity are known to cause epilepsy, while mild to moderate TBI, even if they cause cognitive deficits, are not considered as epileptogenic

conditions. In this context, we determined whether moderate severity bilateral fluid percussion (bLFP) induced a lower inflammatory response in the hippocampus than SE. For this purpose, we performed measurements at 7h post-bLFP, which corresponds to the apparent peak of inflammation (Ogier et al., 2017), coinciding with the one observed after Pilo-SE.

Pro-inflammatory, anti-inflammatory and inflammation cell indexes were calculated (Fig. 7). The individual quantitation of cDNA copy number for each of the markers included in the indexes are provided in Figures S9 and S10. Globally, the bLFP model showed an inflammatory response at least equal (pro-inflammatory index, Fig. 7A) or greater (inflammatory cell index, Fig. 7C) to the SE-J model, while the anti-inflammatory index following bLFP was lower than the one observed SE-J model (Fig. 7B). Overall, these data support that the inflammatory response in the bLFP model is equal to or greater than that measured following SE, at least at the time of the apparent peak of inflammation.

4 | DISCUSSION

The current study reports that inflammation in resected hippocampus of patients with TLE presents with a high interindividual variability. Our intriguing result showing that short-term delay of resected tissue processing leads to large decrease of targeted RNAs, even those of housekeeping genes, precluded the possibility of using post-mortem tissues to estimate baseline transcript levels in non-epileptic tissues, and then to evaluate the inflammatory status in the resected hippocampus of TLE patients. To overcome this problem, we used as an alternative rats that developed epilepsy after SE induced during weaning and juvenile stages, which have the advantage of having access to healthy controls. We show that the data obtained in epileptic rats model a large part of the variability observed in patients. In addition, in the chronic phase of epilepsy, the levels of selected neuroinflammatory markers measured in the hippocampus varied between values ranging from 0.87 to 9.55 times those of controls. We also showed that inflammation during the chronic phase, when present, is of low grade compared to that measured after an epileptogenic brain insult, and that not all epileptogenic insults associated with high neuroinflammatory response necessarily leads to epilepsy. Finally, we demonstrated that microglial cells are the main contributors to the production of interleukin-1 β during the acute phase after SE.

Methodological considerations

In our study, we chose to evaluate gene expression at the transcript level rather than at the protein level, because the method mostly used to quantify RNA (calibrated RT and real-time PCR) is much more quantitative than those used for proteins quantification (Western Blot and Elisa). Indeed, the two most common methods for protein quantification depend on the availability of validated antibodies for each of the targeted genes. In addition, we showed in this study that a given protein (GFAP) did not show the same tissue distribution pattern when detected with two distinct specific antibodies. By contrast, when considering RNAs, even if amplification of given cDNAs by PCR requires different primer pairs between humans and rats, it remains highly specific to the corresponding mRNA. Furthermore, PCR is quantitative as soon as it is performed on a real-time thermocycler and a calibration curve is used, giving access to the number of cDNA copies detected. To generate the cDNAs to be amplified by PCR, we have chosen a method that allows us to calibrate reverse transcription using a synthetic and exogenous poly-A RNA (SmRNA) (WO20040404092414) (Fares et al., 2013; Nadam et al., 2007; Ogier et al., 2017; Sanchez et al., 2009). This contrasts with the selection of one or more endogenous genes so-called housekeeping genes, considered as internal controls and, *de facto*, as being *a priori* invariant in all studies that use this kind of standardization. Our methodological approach is all the more justified when considering our results showing that three mostly used housekeeping genes greatly vary between patients.

When several mRNAs of inflammation markers are quantified, and some vary upward while others vary downward, one of the major difficulties is to define whether the overall level of inflammation has increased or decreased. For this reason, we have established three indexes to report "global" pro-inflammatory, anti-inflammatory and glial activation states, based on a number of mRNAs for which we also provide individual quantifications. To generate these indexes, we have chosen prototypical pro-inflammatory cytokines and chemokines IL1 β , IL6, TNF α , MCP1 and MIP1 α involved in epilepsy physiopathology (Aronica et al., 2017; Cerri et al., 2017; Vezzani et al., 2011), anti-inflammatory cytokines IL4, IL10 and IL13 whose expression is increased in various neurological pathologies (Gadani et al., 2012; Lobo-Silva et al., 2016; Mori et al., 2016), and finally GFAP and ITGAM, which are respective markers of astrogliosis and microgliosis, both involved in epilepsy (Devinsky et al., 2013).

Post-mortem tissues

Inflammation has for years been considered as a key contributor to the pathophysiology of epilepsy (Vezzani et al., 2019), which encouraged several studies to investigate the level of neuroinflammation in the epileptic brain. One of the commonalities between most of these studies, regardless of the quantification methodology employed, has been the use of post-mortem tissue obtained from autopsy non-epileptic control subjects to compare with values measured in specimen of epileptic patients. Although not epileptic, some individuals suffered from other neurological conditions such as brain tumor or had experienced traumatic injuries, raising concerns about the inflammatory status of these samples in comparison with healthy tissues. Another issue is the delay of processing of these post-mortem tissues, ranging from 4 to 20.5 hours (Aalbers et al., 2014; Das et al., 2012; Fiala et al., 2013; Kan et al., 2012; Leal et al., 2017; Omran et al., 2012; Ravizza et al., 2008; Sheng et al., 1994; Strauss and Elisevich, 2016). While earlier studies have shown that RNA can remain substantially intact, even for long periods of time after death (Fitzpatrick et al., 2013; Heinrich et al., 2007; Preece et al., 2003), other studies have reported that post-mortem interval should be controlled in human and animal models (Catts et al., 2005), especially for mRNA profiling studies (Ferreira et al., 2018; Vennemann and Koppelkamm, 2010). In addition, a recent study has provided evidence that data obtained for miRNAs extracted from resected tissues of epileptic patients were different to those of post-mortem tissues from epileptic patients (Roncon et al., 2017). A further concern not raised so far about the use of post-mortem human tissues in general is related to the heterogeneity of the individuals included in a study. Thus, adding over this heterogeneity the variability related to the uncontrolled degradation of the mRNAs only adds uncertainty to the data produced.

Our results obtained on hippocampal tissues from 10 epileptic patients showed that a short (45-90 min) delay in the processing of a sample, although handled according to standard procedures, resulted in substantial decrease in three housekeeping gene transcript levels. This may reflect a specific degradation of mRNAs, not detected by the widely used reference method based on the integrity of two very abundant ribosomal RNAs. For all these above-mentioned reasons, it appeared to us that the use of post-mortem tissues should be avoided to define basal inflammatory levels in the hippocampus.

Inflammatory levels during epilepsy

While several studies have reported increased inflammation in the resected hippocampus of TLE patients when compared to post-mortem controls (Fiala et al., 2013; Kan et al., 2012; Omran et al., 2012; Ravizza et al., 2008; de Vries et al., 2016), greater IL1 β and IL6 levels were measured in resected hippocampus of non-epileptic patients compared to TLE patients (Strauss and Elisevich, 2016). These conflicting data fuel the debate about whether substantial inflammation is present in the epileptic focus (Aalbers et al., 2014). On our side, we do not have any reference values for basal mRNA levels of the targeted cytokines and chemokines due to the absence of appropriate control tissues. However, we show that the variations of the mRNA levels are highly variable in resected hippocampus of TLE patients, the lowest variation observed being for MCP1, with a ratio of 11 between the lowest and the highest measured value. These variations did not correlate with gender, age, duration of epilepsy, and treatments. Our choice to model temporal lobe epilepsy in rats after SE induced by pilocarpine provided us the possibility to have controls giving access to baseline values for the different markers of inflammation measured in the hippocampus of TLE patients. In addition, the induction of SE in young rats at weaning (P21) and in young juvenile rats (P42) allowed us to model not only TLE epilepsy with massive lesions in cortico-limbic and thalamic areas (P42), but also TLE epilepsy with very discrete neuronal losses (P21) (Cilio et al., 2003; Cross and Cavazos, 2007; Nadam et al., 2007; Sanchez et al., 2009; Voutsinos-Porche et al., 2004). When taking the pro-inflammatory and anti-inflammatory index, or the individual mRNAs constituting these indices, our results show that once epilepsy is developed, rats whose SE was triggered at P21 are not distinguishable from controls, unlike rats whose SE was induced at P42. Given the variability observed between rats, including both controls and epileptic rats, which almost overlaps the variability observed between TLE patients, our results indicate that epilepsy can be active regardless of the inflammatory status, at least as represented by the selected genes. These results are partly consistent with a recent study showing that mRNA levels of IL1 β and TNF α remain elevated in the hippocampus of epileptic mice after kainic acid-induced SE, although they are not statistically different from controls (Frigerio et al., 2018).

Seizure-induced inflammation & inflammation-induced seizure

Another important question is whether there is a strong link between inflammation in the hippocampus and the recurrence of seizures. In this study, although data on seizure frequency

have only been obtained in 5 patients, they do not indicate a positive correlation between pro-inflammatory cytokine levels in the hippocampus and seizure frequency. In humans, for understandable reasons, no reliable data are available to link levels of inflammatory markers in the hippocampus to the time interval between its surgical resection and the last seizure. However, a PET study evaluating microglial activation in a TLE patient showed that it was greater 36 hours after the last seizure compared to a seizure-free period (Butler et al., 2016). It cannot thus be excluded that patients with the highest inflammatory levels are those who experienced the most recent seizures.

Since our preliminary studies showed that the sole implantation of screws into the skull induced long lasting brain inflammation, not only in the underneath cortex, but also in the hippocampus (data not shown), a limitation of our study is that we were not able to monitor the EEG activity of the rats in order to measure the time interval between the last seizure and death. Indeed, inflammation is now commonly accepted as having an important role to play in perpetuating seizures during the chronic phase, since seizures themselves are responsible for the release of inflammatory molecules, and inflammation contributes to changes in excitability thresholds (Vezzani and Viviani, 2015; Vezzani et al., 2019). However, in a few studies that have used amygdala kindling as a model of temporal lobe epilepsy to control each single tonic-clonic seizure by electrical stimulation of the amygdala, no increase in inflammatory mRNAs was found after seizures (Aalbers et al., 2014; Yi et al., 2004). The lack of extensive cell loss in this model (Tuunanen and Pitkänen, 2000) was proposed as an explanation to the lack of inflammatory response after induced seizures in kindled rats. This might also apply in our study for rats that developed active epilepsy following SE induced at weaning (P21), which had discrete cell loss only and an inflammatory status in the hippocampus similar to that of control rats. The fact that on average these rats do not have a higher level of inflammation than controls cannot be due to a lack of synchronization of spontaneous recurrent seizures in this rat population. Indeed, when animals develop active epilepsy in the presence of extensive neuronal loss, such as after SE induced at juvenile stage or older, lack of seizure synchronization is likely not less, and yet inflammation is significantly higher than controls, mainly due to greater IL1 β gene activation, as previously reported (Ravizza et al., 2008). While prior studies have shown that inflammation in TLE patients was not greater in the presence of hippocampal sclerosis (Aalbers et al., 2014; Kan et al., 2012),

we provide evidence that patients who had the highest values of pro-inflammatory index (P07, P10, P13, P44 and P49) were those with the greatest scores of neuronal loss and reactive gliosis. Thus, all these data support the hypothesis that the inflammatory status of the hippocampus in TLE patients and in animal models of the disease would be proportional to the extent of neuronal lesions.

Role of a peak of inflammation

One of the major added values of our study is to have been able to quantify with the same methodology the mRNAs of some prototypical markers of inflammation, both in the TLE patients and in rats in different models of epileptogenic conditions. In addition to providing us with valuable controls to establish baseline levels of inflammation, the advantage of the animal model is to give us access to the entire period of epileptogenesis following a brain injury, which is of course impossible in humans for ethical reasons.

Very few studies have reported variations in mRNA levels of prototypic markers of inflammation during epileptogenesis up to epilepsy onset, with a method as quantitative as RT-qPCR. Recently, changes in IL1 β and TNF α mRNA levels were quantified in the mouse hippocampus in a model of epilepsy triggered by SE induced by intrahippocampal administration of kainic acid. Although the onset of epilepsy in this model was rapid (<7 days), quantification was limited to the first week post-SE and showed that the apparent peak of inflammation was between 2h and 72h, with values at 7 days still very high but not statistically different from that of controls (Frigerio et al., 2018). Maximum increases, corrected by 3 reference genes, were in the range of 15 to 50-fold compared to controls for IL1 β and TNF α , respectively. In our study, after induction of SE by pilocarpine, the maximum increases reported, without the use of reference genes thanks to the use of an external calibrator, were of an order of magnitude equivalent to that of the intrahippocampal kainate model in mice, ranging from 6 to 55-fold relative to controls for TNF α and IL1 β , respectively. However, in the two SE models that we used, the apparent peaks were much more transient (between 7h and 24h post-SE) than those observed in mice. Furthermore, the comparison of SE induced at P21 and P42 highlighted that the peak of the pro-inflammatory index was higher at P42 than at P21, with a slower return to baseline, suggesting that a higher and longer exposure to inflammatory molecules might partly explain the massive neurodegenerative processes observed when SE is induced at P42. Contrary to the intrahippocampal kainate model in mice,

close to baseline values were recovered more rapidly, as early as 24 hours after SE for the majority of inflammatory markers in rats subjected to SE at P21. Finally, it is now clear that the inflammatory status of epileptic rats, 7 weeks after SE induced at P42, is of low-grade compared to the explosive inflammation measured in the first hours to days following SE.

Neuroinflammation is one of the commonalities shared by numerous epileptogenic insults tested in animal models so far (Klein et al., 2018). Several of the signaling pathways by which inflammatory molecules modify neuronal excitability by acting on glia and neurons and then induce epileptic seizures are common to both conventional animal models of acquired epilepsy such as electrically and chemical-induced SE and to animal models of traumatic brain injury (Webster et al., 2017). We have previously shown that the apparent peak of mRNAs of prototypic markers of inflammation was around 10h post-TBI (Ogier et al., 2017). In this study, we took advantage of the availability of total RNAs extracted from the hippocampus of rats subjected to bilateral TBI to quantify these mRNAs in parallel to those measured after SE so that the peaks could be compared between the three conditions, i.e. SE induced at P21, SE induced at P42 and moderate TBI. We show that the levels of prototypic markers of inflammation after moderate TBI are at least as high, if not higher, than those after SE. It is now clearly established that inflammatory processes are a major component of epileptogenesis (Klein et al., 2018; Vezzani et al., 2011, 2019; van Vliet et al., 2018). However, the fact that we have shown that TBI rats do not develop clinical signs of epilepsy despite high inflammatory reactivity supports the hypothesis that inflammatory processes are essential for the development of epilepsy, but are not sufficient on their own, even in a context of brain injury.

Which brain cells contribute the most to neuroinflammation?

Of the three cytokines studied (IL1 β , IL6 and TNF α), IL1 β is the one that was still at a level above controls in rats that developed epilepsy after SE induced at P42. In order to know which cells express IL1 β , we had initially opted for double immunohistological labeling, but obtaining radically different results with the different anti-IL1 β antibodies tested (none of which have been validated) led us towards quantitative RNAscope[®] *in situ* hybridization. In accordance with RT-qPCR results, maximum signal was observed 7h post-SE, and was clearly located in cells expressing *Itgam*-mRNA, with a morphology resembling that of activated microglial cells. Very small amounts of IL1 β -mRNA were also detected in astrocytes at 7h post-SE. Due to the

rapid decrease in IL1 β -mRNA levels following SE, the dispersion of the corresponding signal did not make it possible to identify whether IL1 β -mRNA was expressed in microglial cells or astrocytes. According to the results of previous studies, even if IL1 β -immunopositive cells have been shown to resemble activated microglial cells in the hours following SE induced by intrahippocampic kainic acid in rats (Vezzani et al., 1999), IL1 β has been shown to be expressed in the hippocampus mainly by astrocytes at all phases of epileptogenesis and once epilepsy has developed in rats after pilocarpine-induced SE or after self-sustained SE (Ravizza et al., 2008) or at epilepsy onset in mice subjected to SE after intrahippocampic kainic acid administration (Frigerio et al., 2018).

While studies in mice after pilocarpine-induced SE indicate that myeloid infiltrates (essentially macrophages) are responsible for the majority of the pro-inflammatory cytokines measured in brain tissue in the acute phase (24h-96h) post-SE (Varvel et al., 2016; Vinet et al., 2016), our data acquired in rats subjected to pilocarpine-induced SE show on the one hand that the inflammatory peak occurred 7h post-SE, at a time when no myeloid infiltrate is detected, whereas when these are present between 24 and 48 hours post-SE, mRNA levels of pro-inflammatory cytokines are dramatically decreasing. In addition, the detection of IL1 β by RNAscope® *in situ* hybridization did not make it possible to demonstrate, 24 hours post-SE, a stronger signal in of round-shaped cells, resembling infiltrating macrophages. Therefore, if our *in situ* quantification methods are correct, one must consider that either the contribution of macrophages to brain inflammation following SE is radically different between rat and mouse models, or that the rather long procedures needed for separating microglial cells from macrophages by FACS in mice differently affected the turnover of cytokine mRNAs, leading to the differences observed between the two populations of cells.

Translational relevance of the study

Even if our study does not include control brain tissue, the hippocampi resected from TLE patients, treated exactly like dissected tissues from epileptic and control rats, made it possible to acquire, probably for the first time, data acquired in rats that it is possible to transpose to epileptic patients. Thus, our data show that the inflammation in the hippocampus of TLE patients is very variable, some may have values probably close to basal values, while others have much higher inflammatory levels, but nevertheless contained in view of the explosive inflammation measured in the acute phase following a brain injury.

At this stage, the question that arises is that of how to apprehend the level of cerebral inflammation in a non-invasive way, and, in the best of cases, from peripheral biomarkers (Vezzani et al., 2019). Among the cytokines studied, IL6 is the pro-inflammatory cytokine for which serum levels were found to be significantly elevated in epileptic patients (de Vries et al., 2016), including TLE (Alapirtti et al., 2009; Lehtimäki et al., 2011; Liimatainen et al., 2009; Nowak et al., 2011; Uludag et al., 2013), Lennox-Gastaud syndrome and electrical SE in sleep (Lehtimäki et al., 2011). Monitoring brain inflammation from the periphery is an important issue since one year after surgery, serum values of IL1 β and IL6 were significantly higher in patients with remaining seizures compared to seizure-free patients (Lorigados Pedre et al., 2013). Inflammation in epilepsy is also known to play a prominent role in neurobehavioral comorbidities (Paudel et al., 2018) and low-grade inflammation has been shown to predict persistence of depressive symptoms (Zalli et al., 2016). In addition, the chronic presence of pro-inflammatory cytokines above a certain threshold has been reported to have a deleterious effect on hippocampal function (Yirmiya and Goshen, 2011) and adult hippocampus neurogenesis (Hueston et al., 2018). It is however unclear at what thresholds IL1 becomes harmful, since low levels of IL1 are necessary for memory formation (Yirmiya and Goshen, 2011).

Given the high inflammatory values in some patients and the effect that these molecules can have on the symptomatology of epilepsy (Terrone et al., 2017; Vezzani et al., 2011), it is natural to consider that all of these neuroinflammatory pathways and associated biomarkers may represent new therapeutic targets for seizure control (van Vliet et al., 2018). In addition to technological developments which will make it possible to identify the most relevant biomarkers of active brain inflammation in epileptic patients, future studies will have to provide all possible correlations between recurrence of seizures, comorbidities associated with epilepsy and both peripheral and central inflammation, to develop the most appropriate disease-modifying treatments, targeting the most relevant signaling pathways associated with neuroinflammation.

Limitations of the study

One of the major limitations of our study is that we were unable to measure the EEG activity of rats, by fear of inducing significant inflammatory levels at the hippocampal level, which could themselves have modified the course of the disease.

Inflammation is looked at here through the prototypic cytokines and chemokines most studied in epilepsy (Terrone et al., 2017; Vezzani et al., 2011), but cannot be limited to these only (de Vries et al., 2016). In addition, the whole family of eicosonoids, metabolic derivatives of arachidonic acid, play a major role in inflammatory signaling and are largely overlooked in this study, while their deregulation has clearly been identified in epilepsy (Vezzani et al., 2019).

Targeting mRNAs by RT-qPCR is certainly one of the most accessible methods of measuring gene expression in the most quantitative and reliable way possible. However, variations in the corresponding proteins would have certainly provided more relevant information on the most active signaling pathways in epileptic tissue. Easier access to high-throughput proteomics should help solve this issue, at least in part. It remains that we had trouble identifying the cells expressing cytokines at all stages of the development of the disease in animal models and on the resected hippocampi of TLE patients. Such identification of cells expressing the proteins of interest will depend on the development of more specific and better validated antibodies.

More research needs to be undertaken to better understand the link existing between brain inflammatory markers and ictogenesis. Our study provides important evidence for the presence of inflammatory processes in epilepsy, represented by three of the most studied prototypic cytokines. It is clear that IL1 β is the most over-expressed cytokine in epileptic rats that developed the disease following SE induced at the juvenile stage. However, IL1 β levels during the active phase of epilepsy are much lower than those reached acutely in response to proepileptogenic brain insults. However, recurrence of seizures may not be systematically associated with inflammatory processes involving IL1 β . It does not mean that these cases of epilepsy are independent of other inflammatory processes, which will have to be identified and which could involve molecules of the arachidonic acid metabolite family, for example.

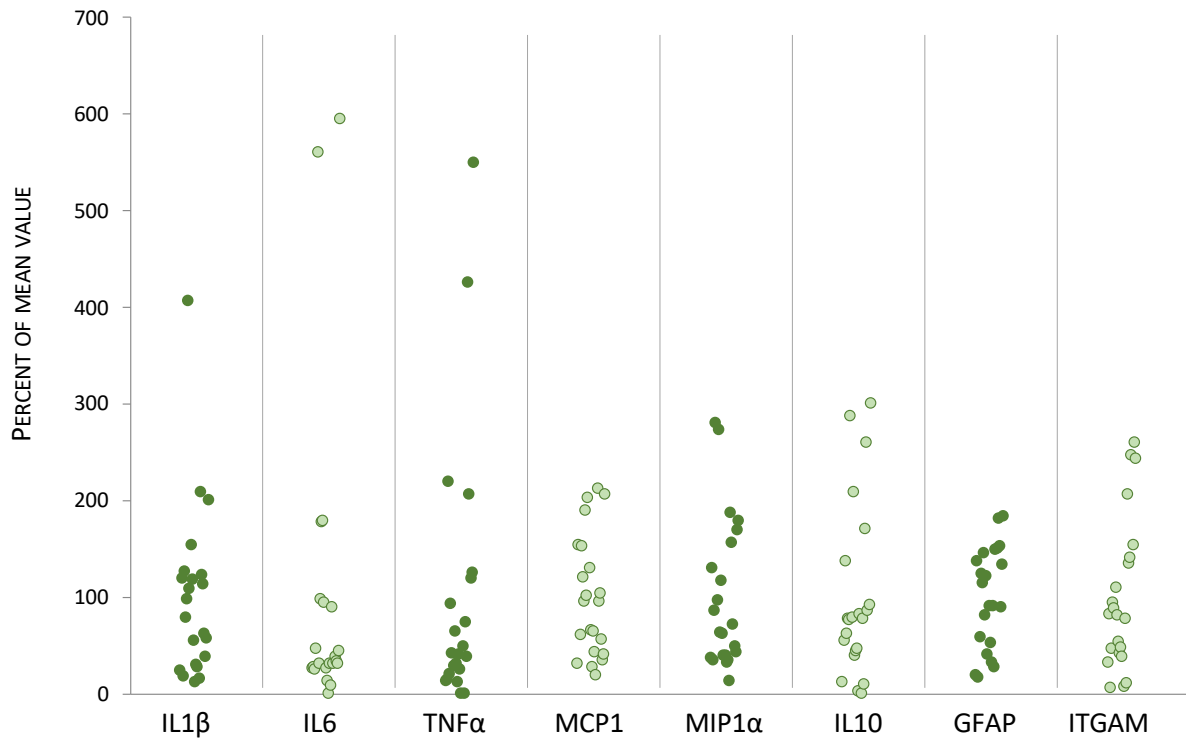


Figure 1. Patients with TLE are heterogeneously distributed regarding the molecular and cellular markers of inflammation measured in the hippocampus. Transcript level of pro-inflammatory cytokines (IL1 β , IL6, TNF α), chemokines (MCP1, MIP1 α), anti-inflammatory cytokine IL10, and cellular markers (GFAP for astrocytes, ITGAM for microglia/macrophages) were measured in resected hippocampus of TLE patients (n=22). Each point represents a patient, and individual values are expressed in percent of the mean value for each marker.

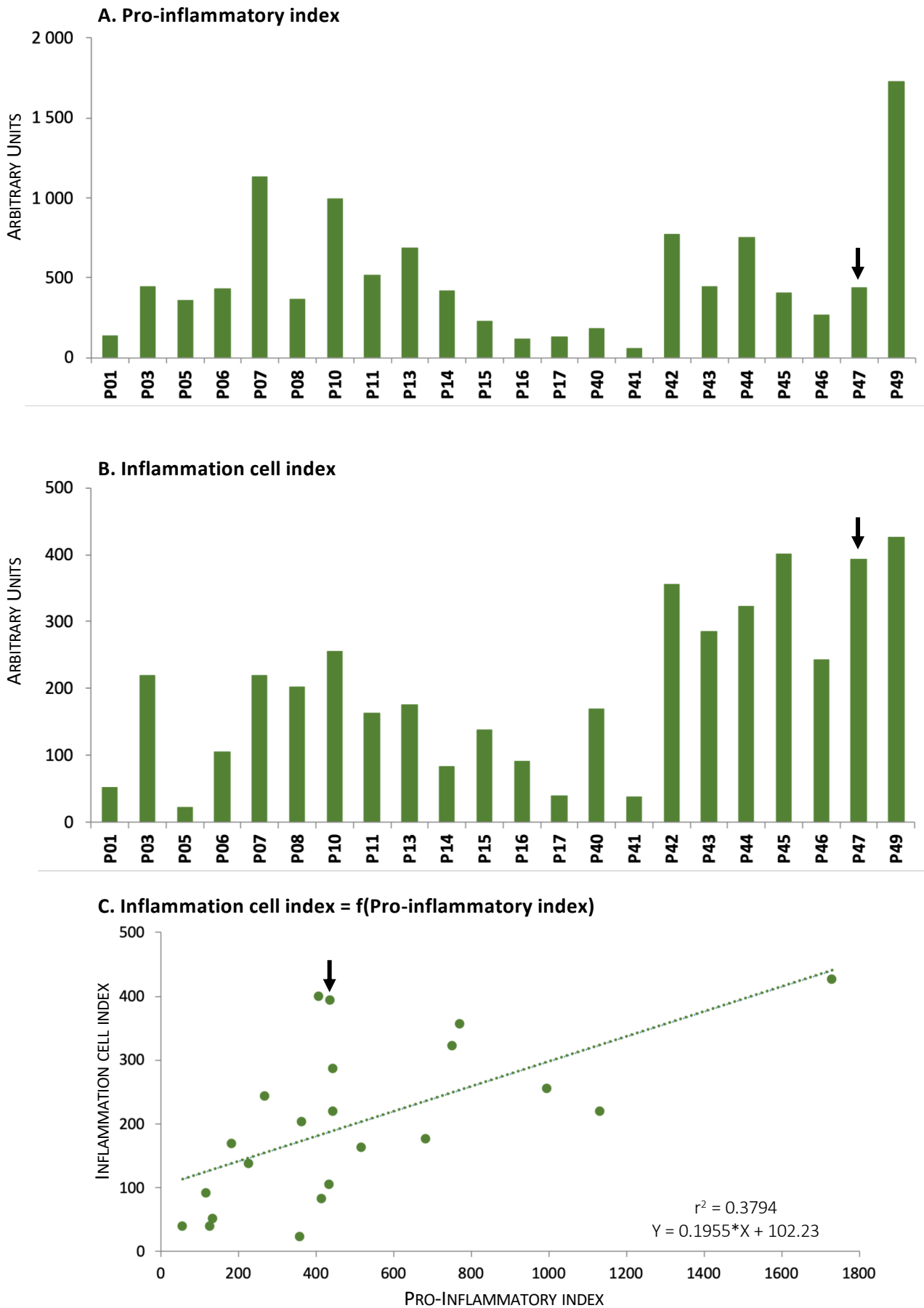


Figure 2. Individual inflammatory indexes in the hippocampus of TLE patients. Distribution of the values of pro-inflammatory index (A), and inflammation cell index (B) in resected hippocampus of TLE patients (n=22). Indexes were calculated from transcript levels as described in the methods section. (C) Scatter plot between inflammation cell index value and pro-inflammatory index value shown in (A) and (B). Data are significantly correlated and fitted by a linear regression, $p < 0.0023$.

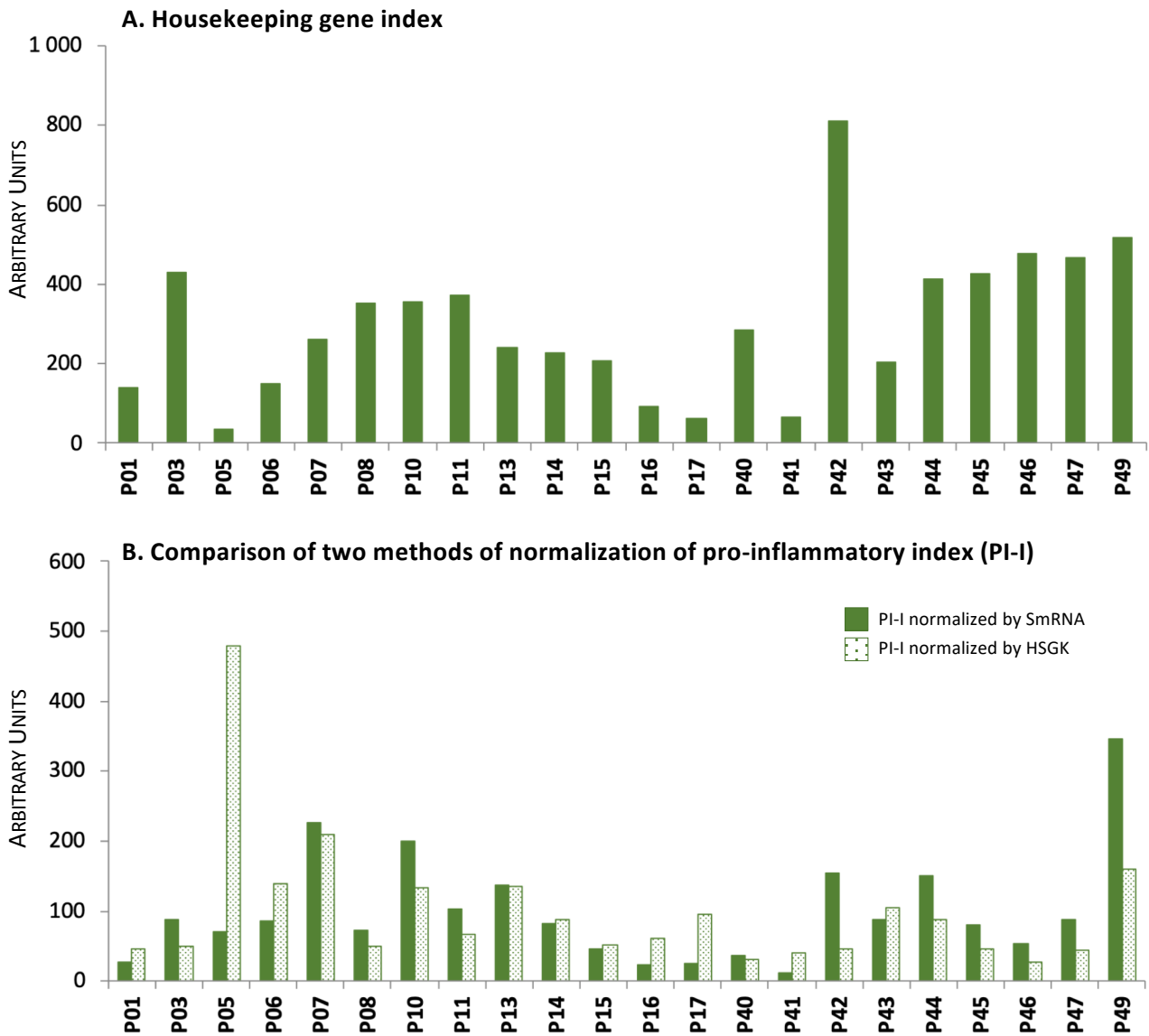


Figure 3. The normalization techniques used in RT-PCR can modify the results. (A) Housekeeping gene (HSKG) index in resected hippocampus of each TLE patient (n=22). Index was calculated by integrating transcript levels of DMD, GAPDH and HPRT1 housekeeping genes. **(B)** Comparison of the pro-inflammatory index values for each patient after unbiased normalization with SmRNA (filled green bars) or after normalization with housekeeping genes (dotted green bars).

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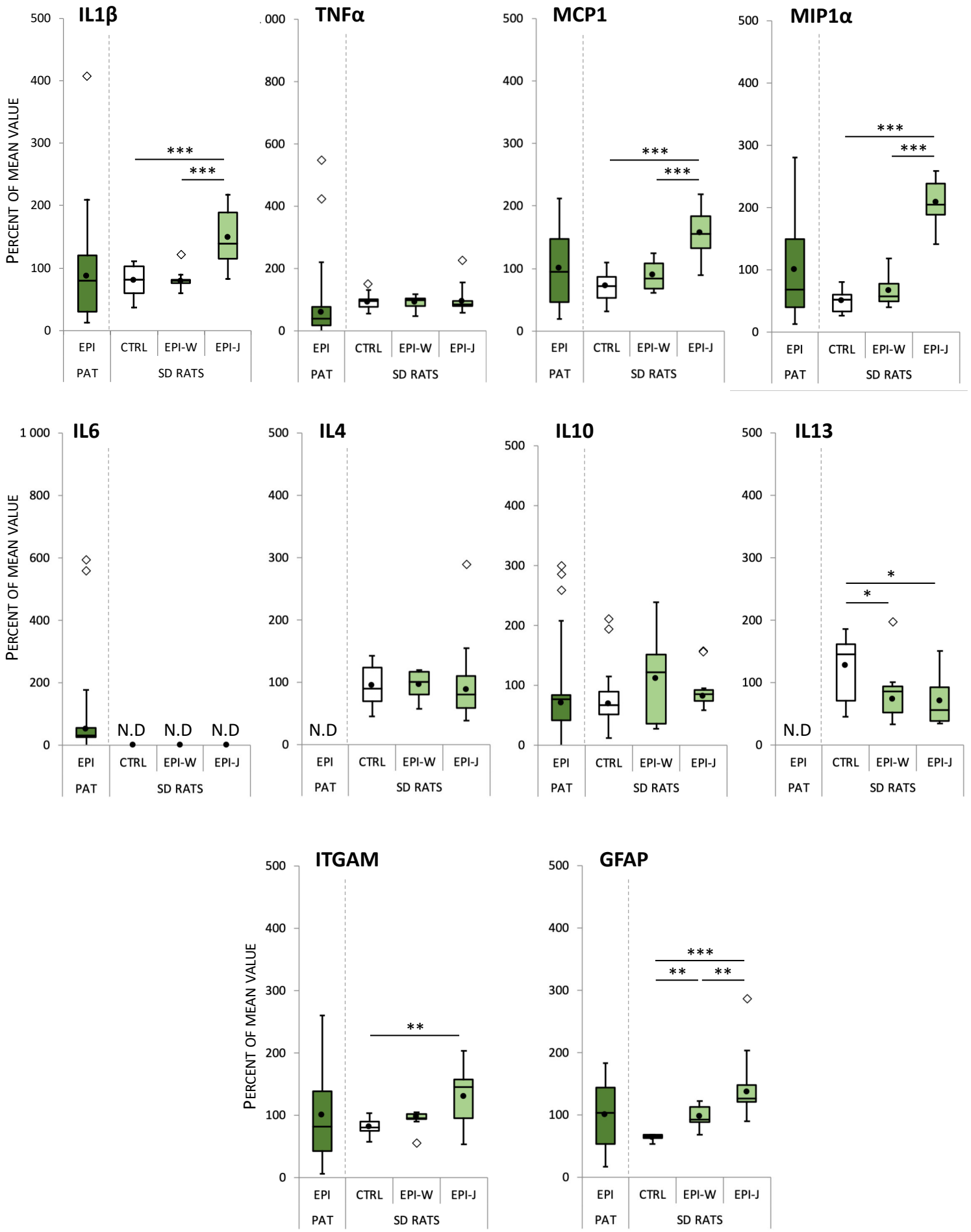


Figure 4. Heterogeneous distribution of inflammatory markers in the hippocampus of TLE patients can be modeled by the combination of two complementary models of TLE in rats. Distribution of the values of pro-inflammatory cytokines (IL1 β , TNF α , IL6), chemokines (MCP1, MIP1 α), anti-inflammatory cytokines (IL4, IL10, IL13), and cellular markers (ITGAM, GFAP) in TLE patients (EPI-PAT) as well as in Sprague Dawley (SD) rats at the epileptic stage (7 weeks post-SE) following Pilo-SE induced at weaning (EPI-W, n=8) or at the juvenile stage (EPI-J, n=8) and in control SD rats (CTRL, n=12). Box-and-whisker plots model the distribution of each value around the median. Mean is represented by circles. Outliers are represented by diamonds. Tukey's *post-hoc* analysis following one-way ANOVA: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Abbreviations: N.D., not detected.

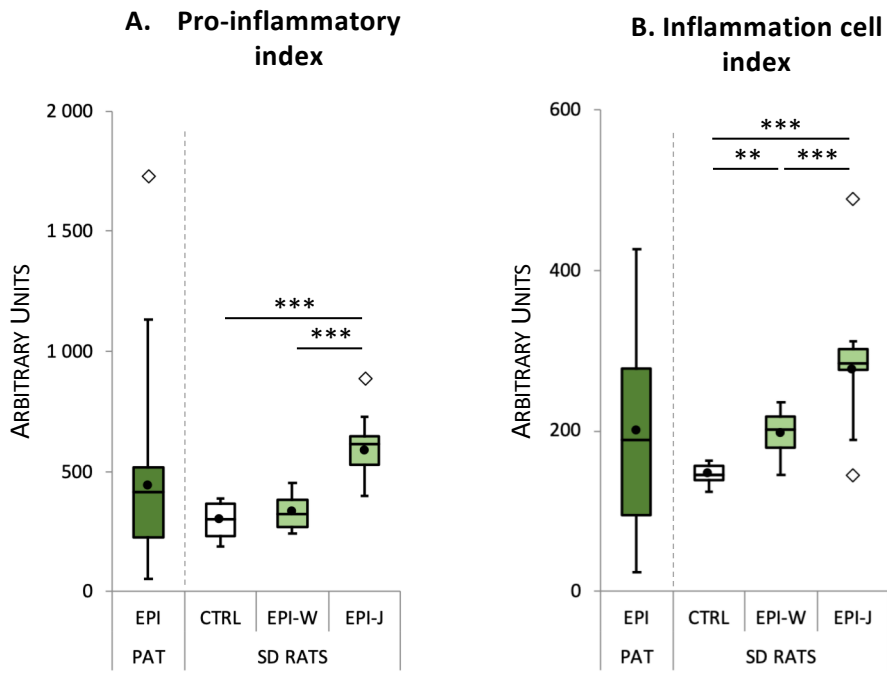


Figure 5. Indexes of inflammation in resected hippocampus of TLE patients and in epileptic rats. Pro-inflammatory **(A)** and inflammation cell **(B)** indexes in TLE patients (EPI-PAT) as well as in Sprague Dawley (SD) rats at the epileptic stage (7 weeks post-SE) following Pilo-SE induced at weaning (EPI-W, n=8) or at the juvenile stage (EPI-J, n=8) and in control SD rats (CTRL, n=12). Indexes were calculated from transcript levels as described in the methods section. Results are expressed as in Fig. 4. Tukey's *post-hoc* analysis following one-way ANOVA: ** $p < 0.01$, *** $p < 0.001$.

Table 1. Fold-changes in inflammatory markers between epileptogenesis and epilepsy in rats

	SE induced at P21 (weaning stage)				SE induced at P42 (juvenile stage)					
	Peak epileptogenesis	Value epilepsy 7W post-SE	Fold difference (Peak vs 7W)	<i>p</i> value (<i>t</i> -test)	Peak epileptogenesis	Value epilepsy 7W post-SE	Fold difference (Peak vs 7W)	<i>p</i> value (<i>t</i> -test)		
Molecular markers	copy number	copy number			copy number	copy number				
IL1β	5 849	232	25	<i>0.0075</i>	**	3 812	415	9	<i>0.0148</i>	*
IL6	2 480	0	-	<i>0.0360</i>	*	21 797	0	-	<i>0.0004</i>	***
TNFα	75	34	0.46	<i>0.0309</i>	*	337	42	8	<i><0.0001</i>	***
IFNγ	57	8	7	<i>0.0318</i>	*	61	5	12	<i><0.0001</i>	***
MCP1	1 488 688	2 012	740	<i>0.0001</i>	***	2 774 798	3 554	781	<i><0.0001</i>	***
MIP1α	403 597	3 552	114	<i>0.0020</i>	**	685 572	11 097	62	<i>0.0011</i>	**
Index										
Pro-inflammatory	1 480	83	17.77	<i>0.0029</i>	**	2 877	120	23.95	<i>0.0001</i>	***

For each molecular marker included in the pro-inflammatory index (i.e IL1β, IL6, TNFα, IFNγ, MCP1, MIP1α), the highest value of cDNA copy number measured during the epileptogenesis phase was compared to the value measured during the chronic phase of epilepsy (7 weeks post-SE) in rats whose SE was induced at P21 or at P42. Fold difference between epileptogenesis and epilepsy was calculated. Statistical difference was determined with Student's *t*-test: * *p*<0.05, ** *p*<0.01, *** *p*<0.001.

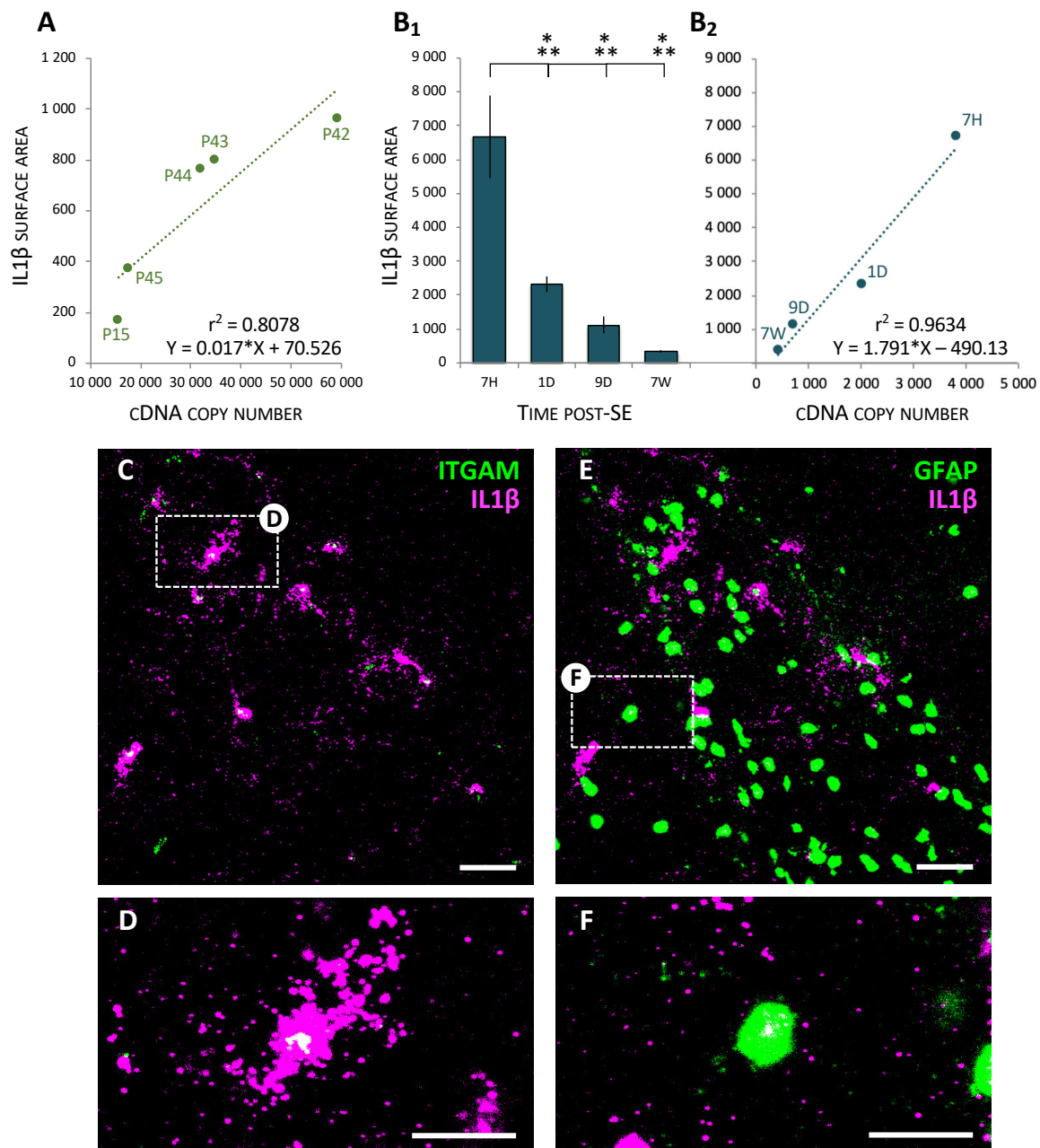


Figure 6. RNAscope[®] ISH of IL1 β -mRNA confirms RT-qPCR data and reveals in rats subjected to Pilo-SE that IL1 β -mRNA is mainly expressed by microglia at the peak of inflammation. (A) Scatter plot between IL1 β cDNA copy number measured by RT-qPCR in the hippocampus of TLE patients and the surface area occupied by IL1 β -transcript signal in sections processed by RNAscope[®] ISH. Data are obtained from the 5 patients, whose resected hippocampi were split in two parts, one reserved for RT-qPCR, the other one for histology. Data are significantly correlated and fitted by a linear regression, $p < 0.0381$. **(B)** Quantitation of the surface area occupied by IL1 β -transcript signal in the granule cell layer of the dentate gyrus of rat brain sections processed by RNAscope[®] ISH (B1). Sections were selected at Bregma -4.16 mm from rats sacrificed during epileptogenesis (7 hours, 1 day, 9 days after SE) or during chronic epilepsy (7 weeks after SE). Statistical analyses showed significant differences between the IL1 β surface area measured 7h post SE ($n=4$) and all the other time points (1D: $n=5$; 9D: $n=3$; 7W: $n=5$). Scatter plot (Fig. B2) between the average IL1 β cDNA copy number determined by RT-qPCR in the hippocampus of rats sacrificed at the same time points as in B1 (Fig. S4) and the average surface area occupied by IL1 β -transcript signal measured in sections processed by RNAscope[®] ISH (Fig. B1). Data are significantly correlated and fitted by a linear regression, $p < 0.0194$. **(C-F)** Triple *in situ* hybridization of IL1 β together with ITGAM **(D-F)** and GFAP **(E-G)** transcripts using RNAscope[®] technology, in the dentate gyrus of rat hippocampus 7 hours (peak of inflammation) after pilocarpine-induced SE at 42 days. To facilitate the visualization of IL1 β in microglia and astrocytes, we used two colors providing the best contrasts, and thus assigned magenta to IL1 β and green either to ITGAM (microglia) or GFAP (astrocytes). Colocation is displayed in white when magenta and green are superimposed. In this area, the largest amount of IL1 β transcript is colocalized with ITGAM+ cells. Confocal microscope images are magnified at 63X. Scale bars: C-E: 50 μ m; D-F: 25 μ m.

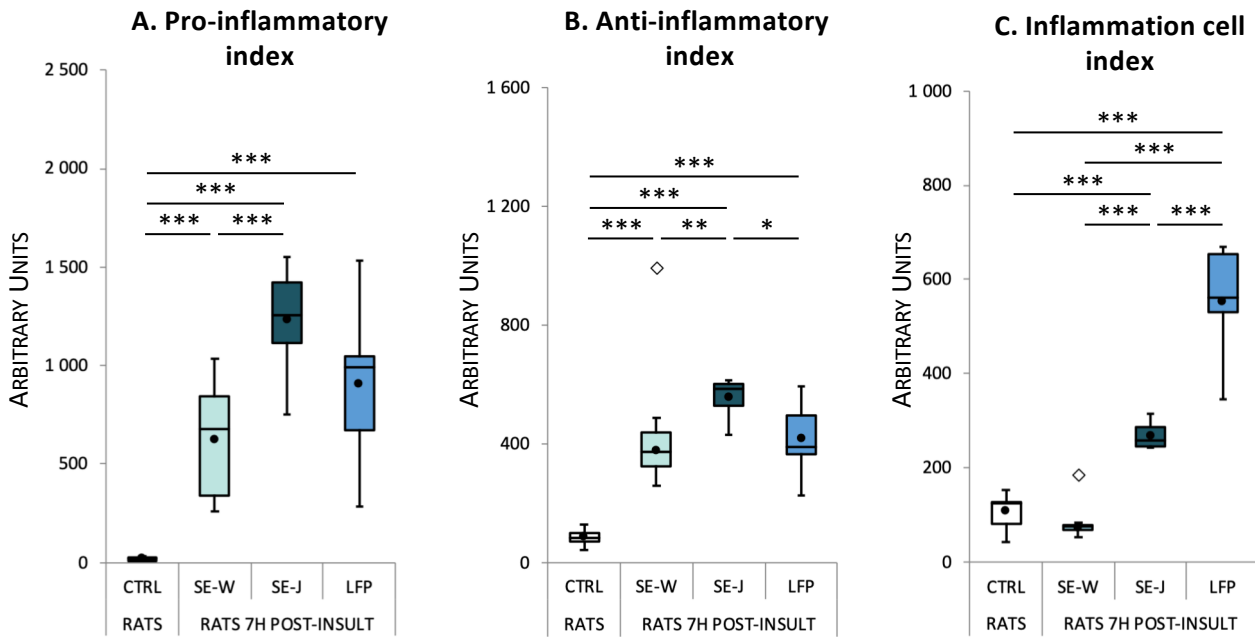


Figure 7. Comparison of peaks of inflammatory responses in rats following different brain insults. Pro-inflammatory (A), anti-inflammatory (B), and inflammation cell (C) indexes measured in the rat hippocampus 7h after Pilo-SE induced at weaning (SE-W, n=7) or at juvenile stage (SE-J, n=6), or after bilateral fluid percussion head injury at 9 weeks (bLFP, n=4) and in control rats (CTRL). Control rats have been included at both weaning (n=10) and juvenile (P42; n=6) and adult (9 weeks, n=4) stages, with data pooled together after ensuring for no statistical difference between these stages. Indexes were calculated from transcript levels as described in the methods section. Results are expressed as in Fig. 4. Tukey's *post-hoc* analysis following one-way ANOVA: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

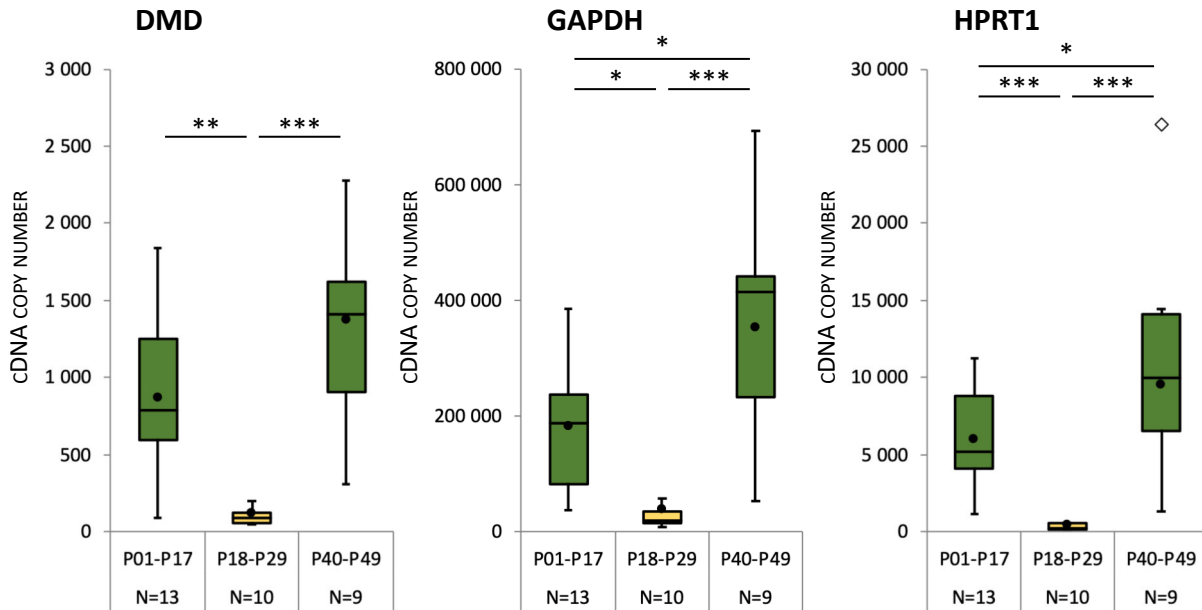


Figure S1. Delayed cryopreservation of human brain tissue significantly alters transcript levels of housekeeping genes. Hippocampus from 3 groups of TLE patients were resected surgically and frozen in liquid nitrogen during the 5 minutes (Group 1: P01-P17; Group 3: P40-P49; green box and whisker plots) or 45 to 90 minutes (Group 2; P18-P29 group, yellow box and whiskers plot) after resection. Transcript levels of three housekeeping genes (DMD, GAPDH and HPRT1) were quantified. Box-and-whisker plots model the distribution of each value around the median of the cDNA copy number measured by RT-qPCR. Mean is represented by circles. Outliers are represented by diamonds. Tukey's *post-hoc* analysis following one-way ANOVA: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

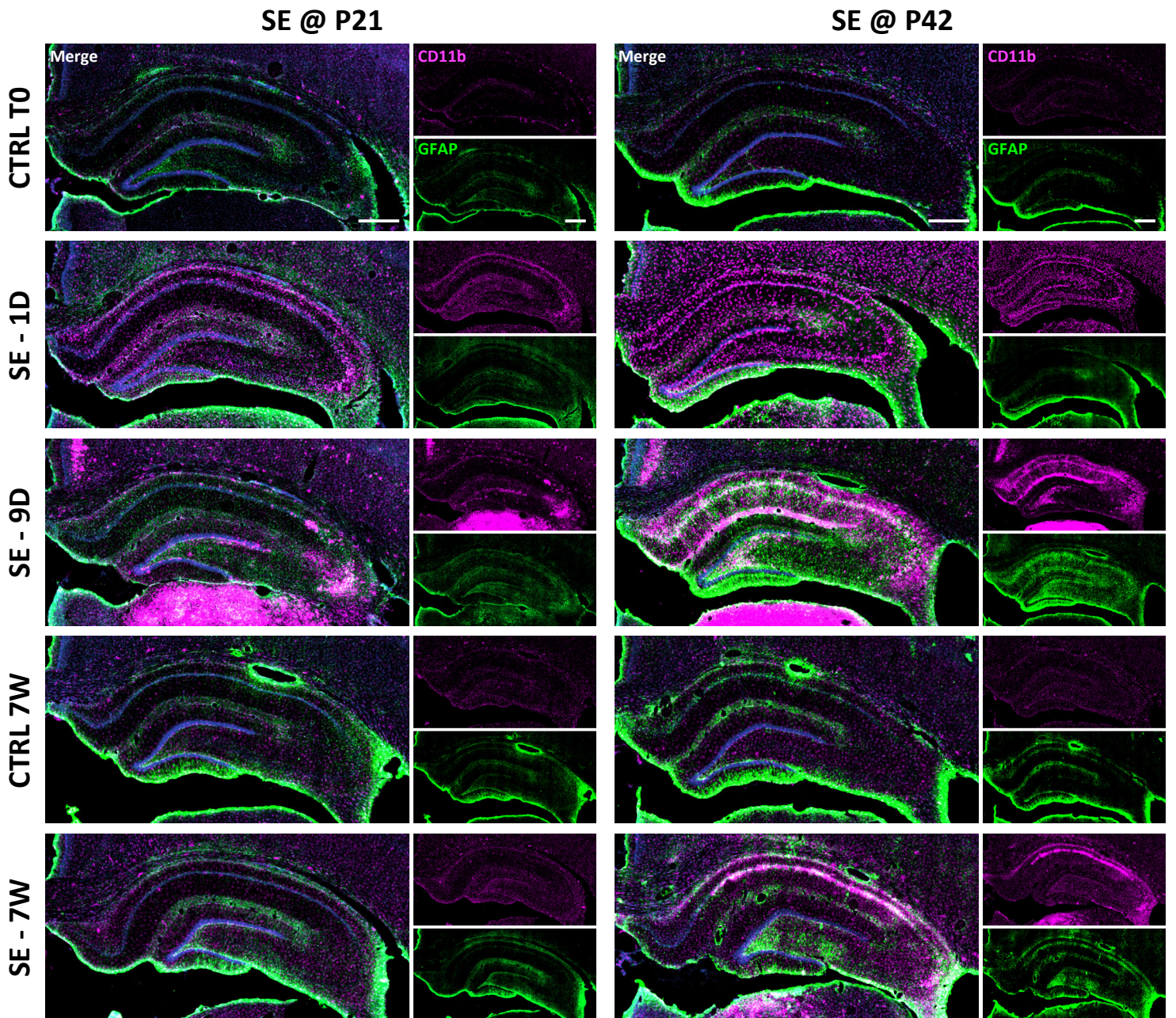


Figure S2. Evolution of glial cell activation in the hippocampus after pilocarpine induced-SE. Immunofluorescence detection was performed in the rat hippocampus using specific antibodies directed against ITGAM (CD11b) for microglia/macrophages (magenta) and GFAP for astrocytes (green). Nuclei were counterstained with DAPI. Different stages of epileptogenesis (SE-1D: 1-day post-SE; SE-9D: 9 days post-SE) or chronic epilepsy (SE-7W: 7 weeks post-SE) after pilocarpine-induced SE induced at weaning (P21) or at juvenile age (P42) are compared to their respective controls. Scale bar: 500 μm .

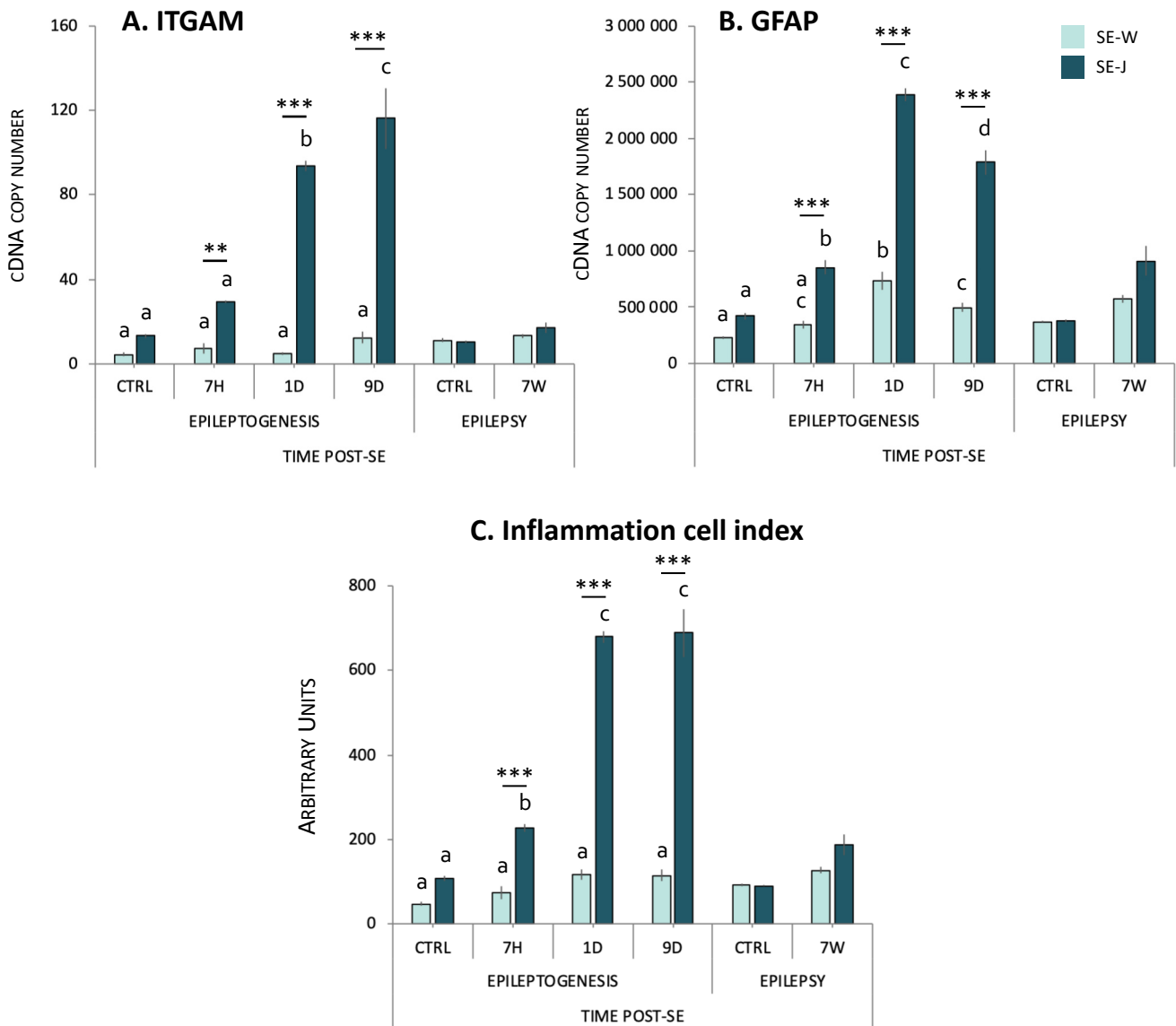


Figure S3. Expression of cell markers (ITGAM and GFAP) after pilocarpine-induced SE. Transcript values of ITGAM (A) and GFAP (B) and inflammation cell index (C) in Sprague-Dawley (SD) rats are given during epileptogenesis, i.e. at 7 hours (SE-W, n=7; SE-J, n=6), 1 day (SE-W, n=8; SE-J, n=6), 9 days (SE-W, n=10; SE-J, n=7) post-SE and once epilepsy is chronically installed, i.e. 7 weeks post-SE (SE-W, n=8; SE-J, n=8) compared to respective controls. When comparing two bars within a same model, the difference is considered as statistically significant ($p < 0.05$) when letters (a, b, c, d) above the bars are different (a-b; a-c; a-d; b-c; b-d; c-d). Asterisks indicate statistical significance between the two models (SE induced at weaning or juvenile stage) at a same post-SE time. The statistical analysis here only represents significant differences during epileptogenesis. Bonferroni *post-hoc* analysis following two-way ANOVA: * $p < 0.05$, *** $p < 0.001$. Abbreviations: SE-W, SE induced at weaning; SE-J, SE induced at juvenile stage.

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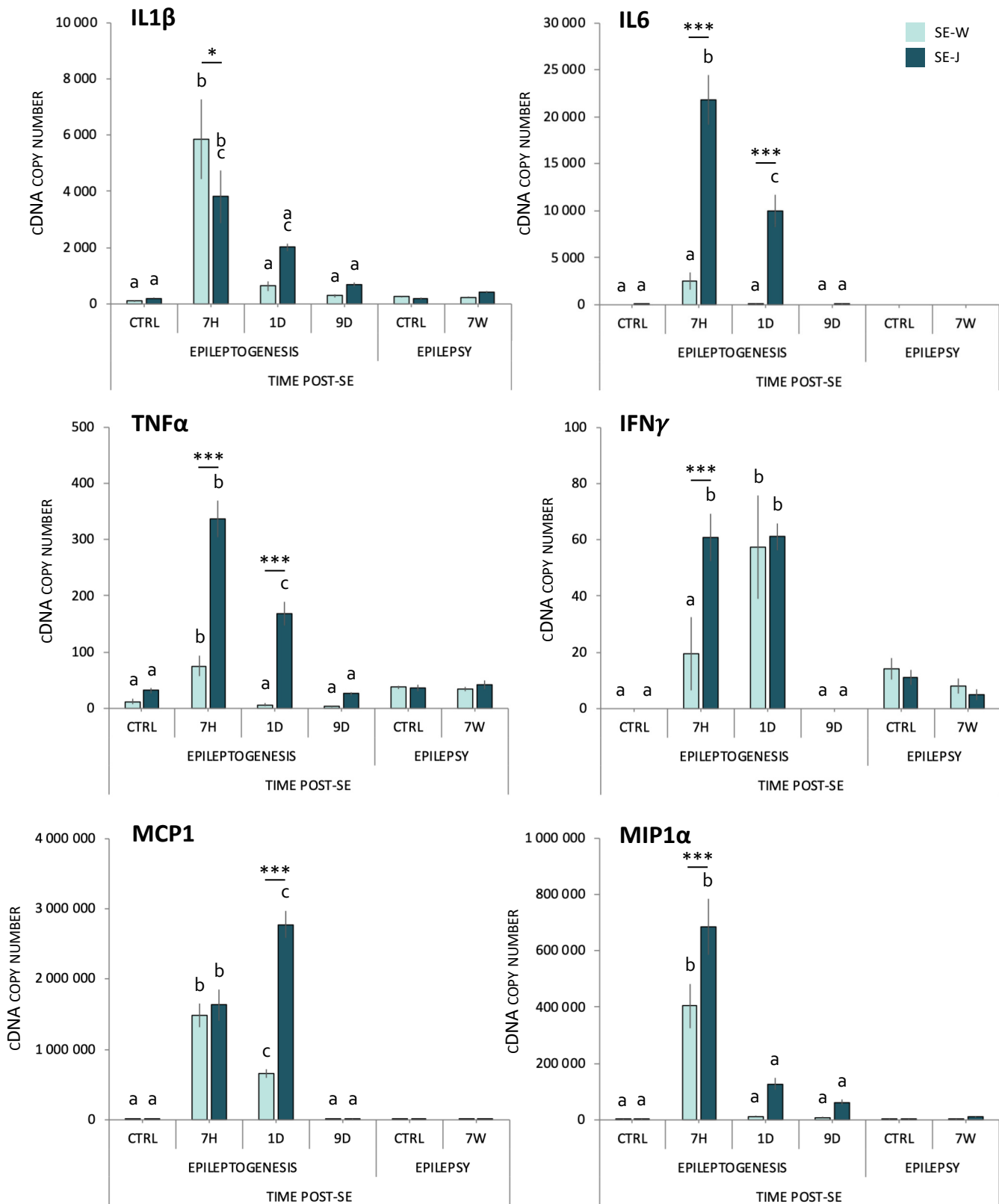


Figure S4. Transcript levels of pro-inflammatory cytokines and chemokines after pilocarpine-induced SE. Transcript values of pro-inflammatory cytokines (IL1 β , TNF α , IL6, IFN γ) and chemokines (MCP1, MIP1 α), during epileptogenesis, i.e. at 7 hours (SE-W, n=7; SE-J, n=6), 1 day (SE-W, n=8; SE-J, n=6), 9 days (SE-W, n=10; SE-J, n=7) post-SE and once epilepsy is chronically installed, i.e. 7 weeks post-SE (SE-W, n=8; SE-J, n=8) compared to respective weaned and juvenile control rats. When comparing two bars within a same model, the difference is considered as statistically significant ($p < 0.05$) when letters (a, b, c) above the bars are different (a-b; a-c; b-c). Asterisks indicate statistical significance between the two models (SE induced at weaning or juvenile stage) at a same post-SE time. The statistical analysis here only represents significant differences during epileptogenesis. Bonferroni *post-hoc* analysis following two-way ANOVA: * $p < 0.05$, *** $p < 0.001$. Abbreviations: SE-W, SE induced at weaning; SE-J, SE in induced at juvenile stage. 154

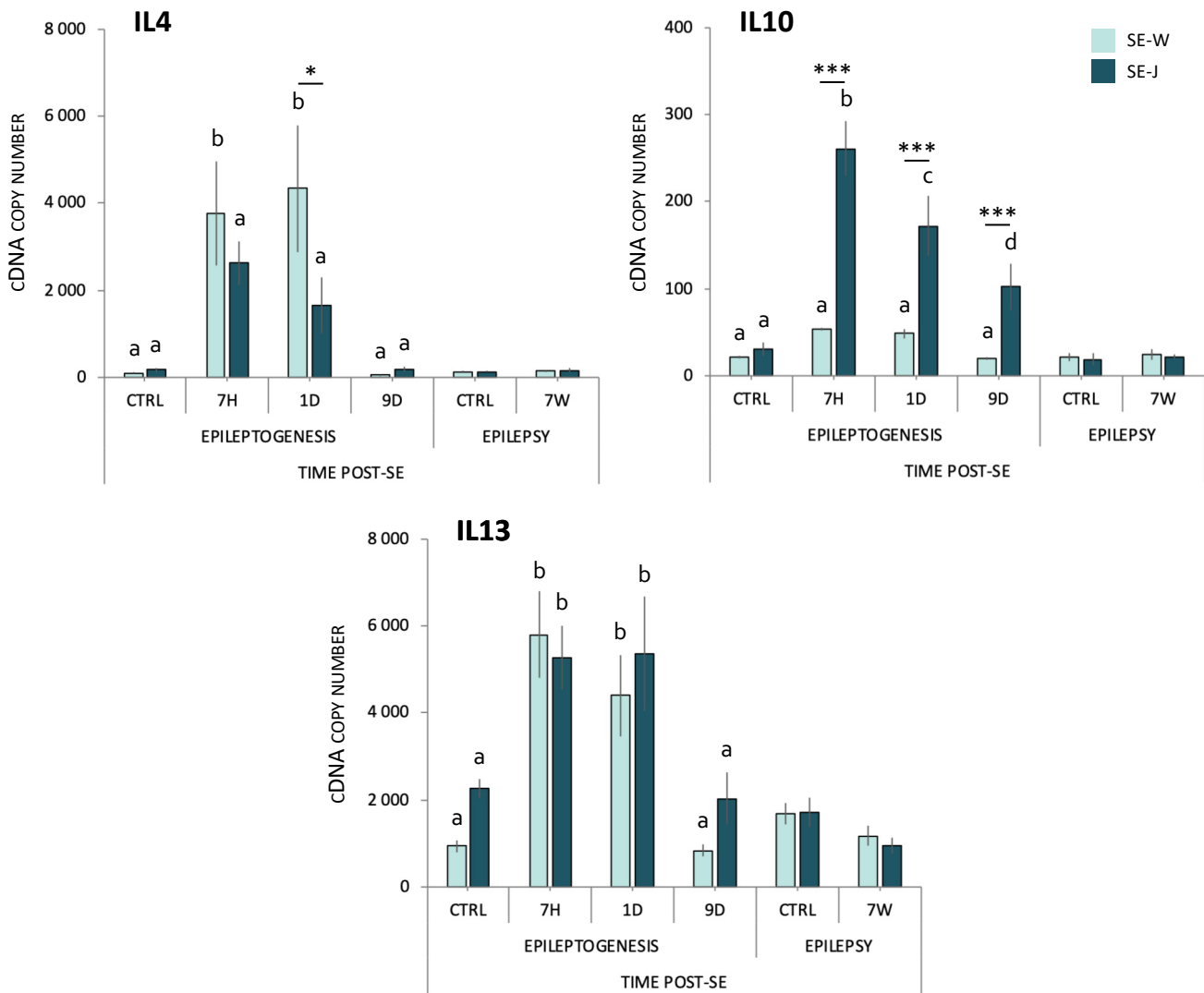


Figure S5. Transcript levels of anti-inflammatory cytokines after pilocarpine-induced SE. Transcript values of anti-inflammatory cytokines (IL4, IL10, IL13), during epileptogenesis, i.e. at 7 hours (SE-W, n=7; SE-J, n=6), 1 day (SE-W, n=8; SE-J, n=6), 9 days (SE-W, n=10; SE-J, n=7) post-SE and once epilepsy is chronically installed, i.e. 7 weeks post-SE (SE-W, n=8; SE-J, n=8) compared to respective weaned and juvenile control rats. When comparing two bars within a same model, the difference is considered as statistically significant ($p < 0.05$) when letters (a, b, c, d) above the bars are different (a-b; a-c; a-d; b-c; b-d; c-d). Asterisks indicate statistical significance between the two models (SE induced at weaning or juvenile stage) at a same post-SE time. The statistical analysis here only represents significant differences during epileptogenesis. Bonferroni *post-hoc* analysis following two-way ANOVA: * $p < 0.05$, *** $p < 0.001$. Abbreviations: SE-W, SE induced at weaning; SE-J, SE induced at juvenile stage.

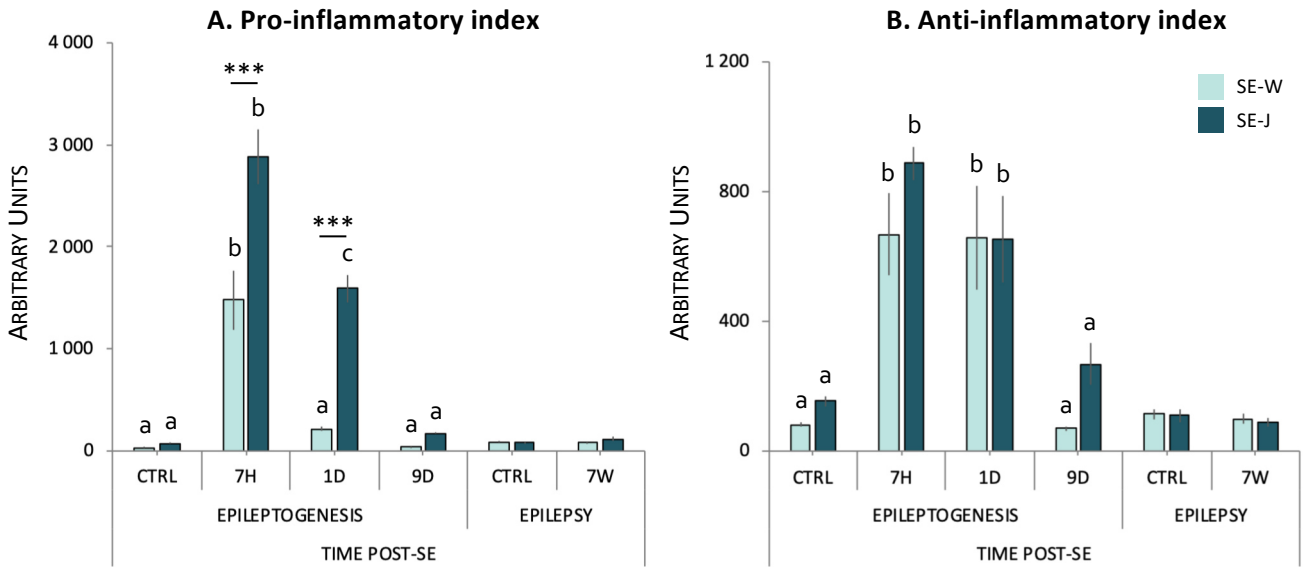


Figure S6. Inflammation during epilepsy is of low-grade compared to that during epileptogenesis. Pro-inflammatory **(A)** and anti-inflammatory **(B)** indexes in Sprague-Dawley (SD) rats were calculated during epileptogenesis, i.e. at 7 hours (SE-W, n=7; SE-J, n=6), 1 day (SE-W, n=8; SE-J, n=6), 9 days (SE-W, n=10; SE-J, n=7) post-SE and once epilepsy is chronically installed, i.e. 7 weeks post-SE (SE-W, n=8; SE-J, n=8) compared to respective weaned and juvenile control rats. When comparing two bars within a same model, the difference is considered as statistically significant ($p < 0.05$) when letters (a, b, c) above the bars are different (a-b; a-c; b-c). Asterisks indicate statistical significance between the two models (SE induced at weaning or juvenile stage) at a same post-SE time. The statistical analysis here only represents significant differences during epileptogenesis. Bonferroni *post-hoc* analysis following two-way ANOVA: * $p < 0.05$, *** $p < 0.001$. Abbreviations: SE-W, SE induced at weaning; SE-J, SE induced at juvenile stage.

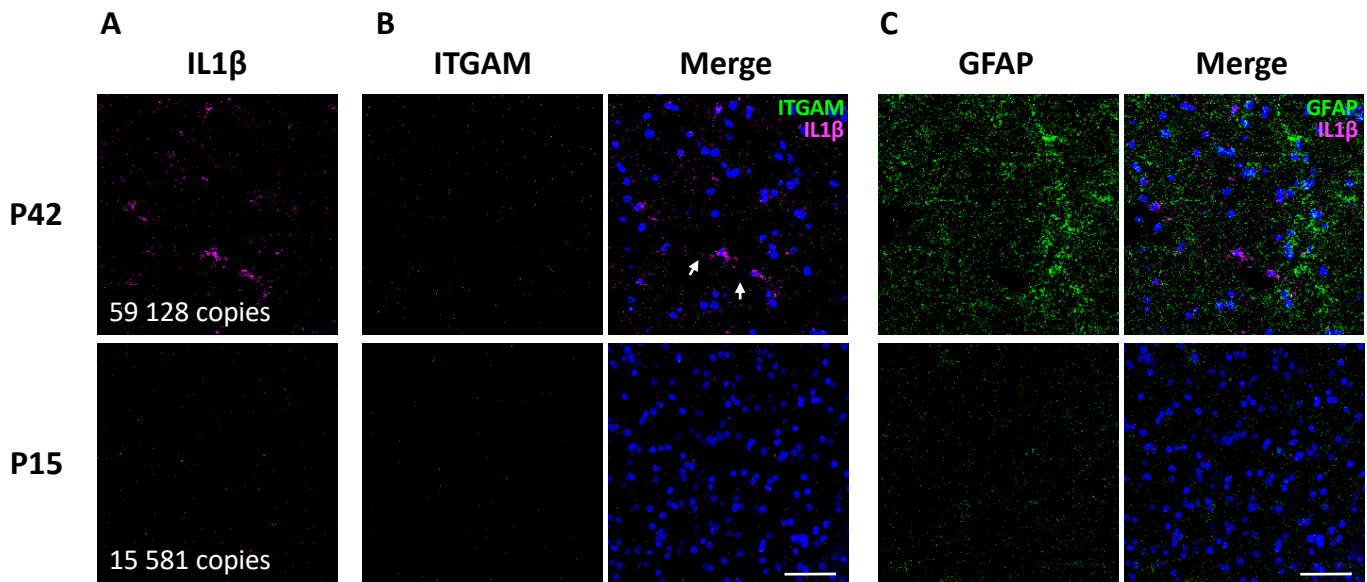


Figure S7. RNAscope® ISH of IL1 β -mRNA in resected hippocampus from TLE patients corroborates data obtained in the same hippocampus by RT-qPCR. RNAscope® ISH of IL1 β -mRNA (A, magenta) was detected together with ITGAM (CD11b)-mRNA (B, green) or GFAP-mRNA (C, green) in the resected hippocampus of TLE patients. Two patients are represented (P15 and P42) and the respective IL1 β cDNA copy numbers measured by RT-qPCR are provided. As shown by the white arrows, IL1 β -mRNA appears to be located in cells bearing morphological features of glial cells. Scale bar: 50 μ m.

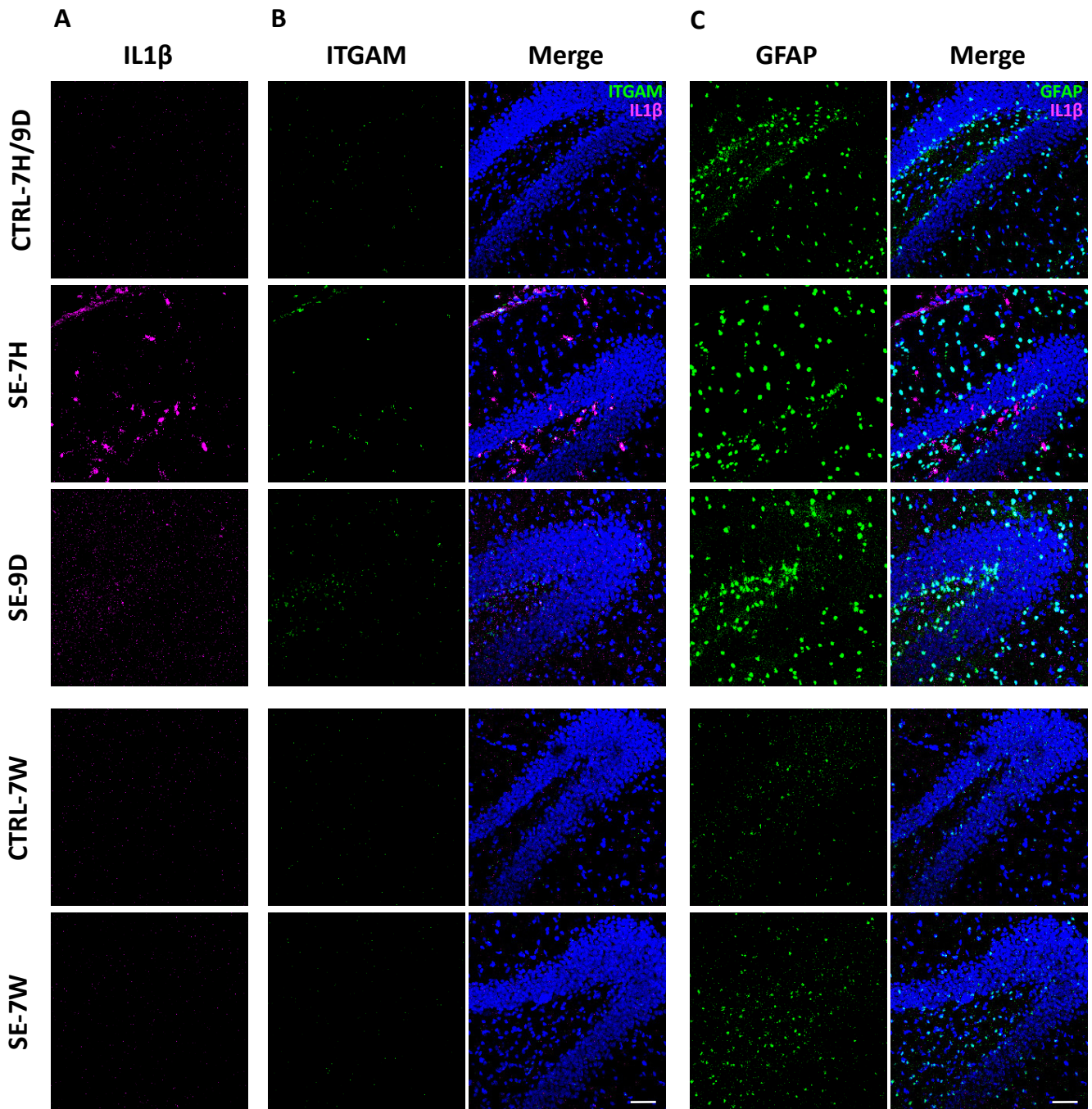


Figure S8. RNAscope® ISH of IL1 β , ITGAM and GFAP transcripts in the dentate gyrus of rats after pilocarpine-induced SE at 42 days. Triple ISH of IL1 β (A), ITGAM (CD11b) (B) and GFAP (C) transcripts using RNAscope® technology is depicted in the rat dentate gyrus. Nuclei were counterstained with DAPI. Different stage of epileptogenesis (SE-7H: 7 hours post-SE; SE-9D: 9 days post-SE) or chronic epilepsy (SE-7W: 7 weeks post-SE) after pilocarpine-induced SE at juvenile age (P42) are compared to their respective controls (CTRL 7H/9D and CTRL 7W). Scale bar: 50 μ m.

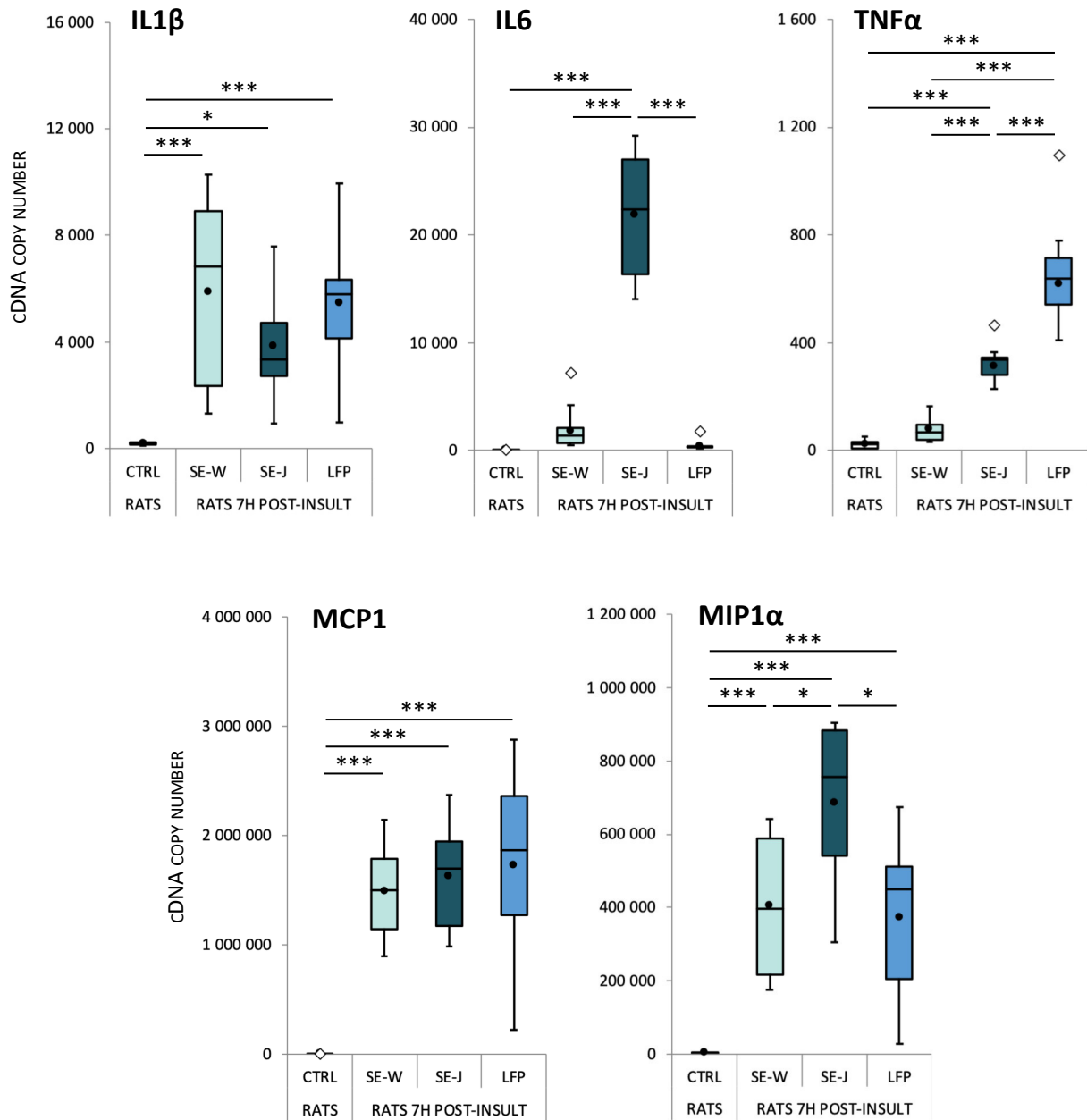


Figure S9. Transcript levels of pro-inflammatory cytokines and chemokines following different epileptogenic brain insults. Distribution of cDNA copy numbers of pro-inflammatory cytokines (IL1 β , TNF α , IL6) and chemokines (MCP1, MIP1 α) measured 7h post-insult in pilocarpine-induced SE rats at weaning (SE-W, n=7) or at juvenile stage (SE-J, n=6) or after bilateral fluid percussion head injury at 9 weeks (LFP, n=4) and in respective control rats (CTRL). Control rats have been included at both weaning (n=10) and juvenile (n=6) stages and at 9 weeks (n=4), with data pooled together when no statistical difference between stages is obtained. Box-and-whisker plots model the distribution of each index value around the median. Mean is represented by circles. Outliers are represented by diamonds. Tukey's *post-hoc* analysis following one-way ANOVA: * $p < 0.05$, *** $p < 0.001$.

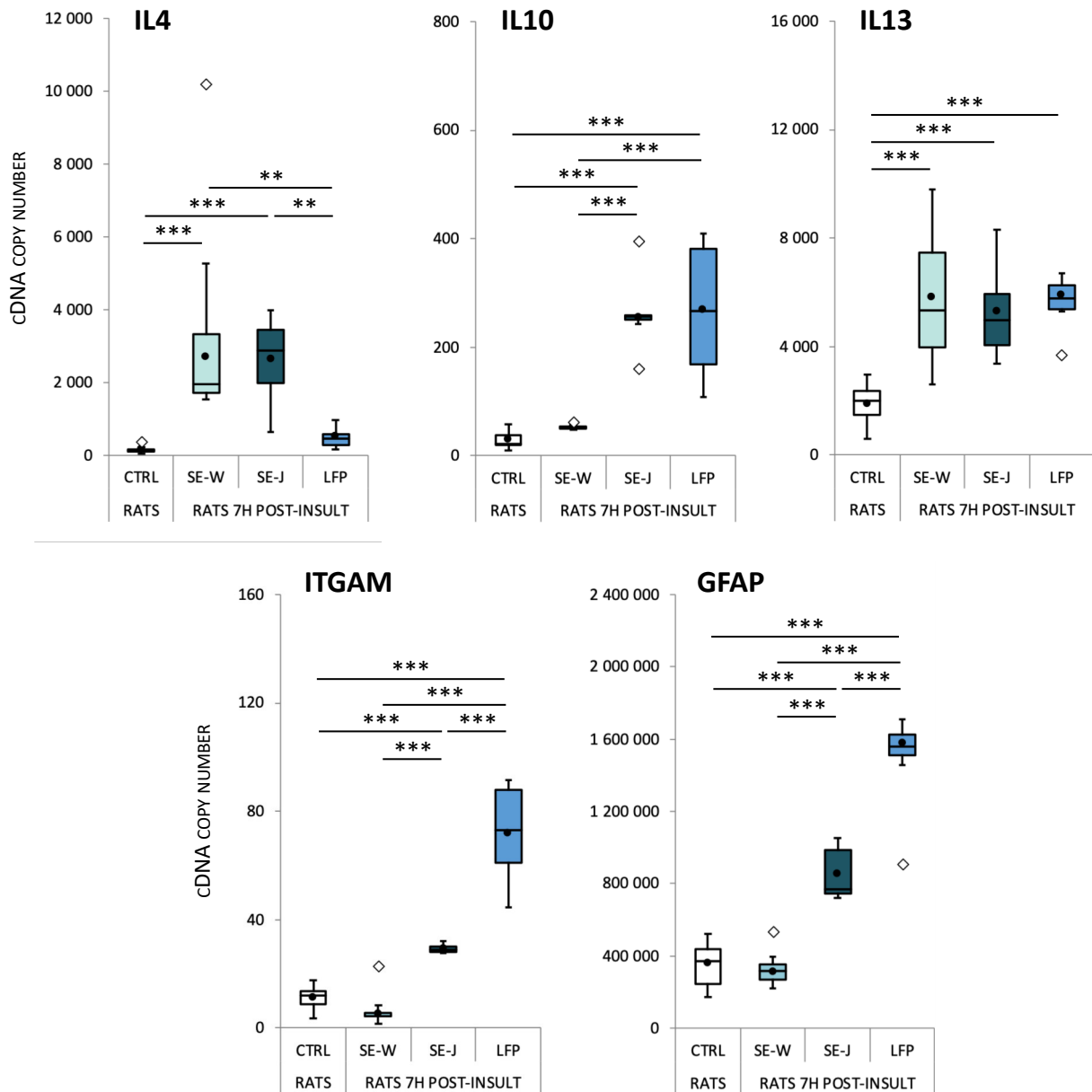


Figure S10. Transcript levels of anti-inflammatory cytokines and cell markers following different epileptogenic brain insults. Distribution of cDNA copy numbers of anti-inflammatory cytokines (IL4, IL10, IL13) and cell markers (ITGAM and GFAP) measured 7h post-insult in pilocarpine-induced SE rats at weaning (SE-W, n=7) or at juvenile stage (SE-J, n=6) or after bilateral fluid percussion head injury at 9 weeks (LFP, n=4) and in respective control rats (CTRL). Control rats have been included at both weaning (n=10) and juvenile (n=6) stages and at 9 weeks (n=4), with data pooled together after ensuring for no statistical difference between the stages. Box-and-whisker plots model the distribution of each index value around the median. Mean is represented by circles. Outliers are represented by diamonds. Tukey's *post-hoc* analysis following one-way ANOVA: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

ID	Gender	Age at surgery (years)	Duration of epilepsy (years)	AEDs	Seizure frequency	MRI	Pathology report
P01	F	51	36	OXC, URB, VGB	-	N	RG
P03	F	23	22	CBZ, TPM	Several/week	rHS	RG
P05	M	56	44	CLZ, CBZ, PHT, URB,	-	lHS	NL,RG,AB
P06	M	27	23	CBZ, LTG, PGB	-	lHS	RG,AB
P07	M	36	26	CBZ, LTG, URB	1/month	rHS	NL,O,RG
P08	F	15	13	LEV, OXC	2/month	lHS	-
P10	M	42	33	CBZ, LEV	-	lHS + tumor lesion	NL
P11	F	15	4	CBZ	1/month to 2/day	rHS	NL,O,RG
P13	M	49	16	LCS, LTG, PGB	-	rHS	NL,RG
P14	F	22	21	GBP, LTG	-	rHS	NL,RG
P15	M	15	12	LCS, LEV	2/week	rHS	-
P16	F	42	27	OXC, ZNS	-	lHS	NL
P17	F	29	21	LTG, OXC, PGB, TPM	-	rHS	NL,RG
P18	M	19	8	LEV, URB, ZNS	-	-	-
P19	F	17	9	OXC, TPM	-	lHS	NL,O,RG
P20	M	36	5	CBZ, URB, VPA	-	N	RG
P21	M	26	11	LTG	-	rHS	-
P22	M	19	8	LEV	-	rHS	NL,RG
P25	M	28	8	CBZ, URB	-	rHS	NL
P26	M	39	24	CBZ, PB, TPM	-	lHS	NL,RG
P27	F	37	35	CBZ, LTG, URB	-	lHS	NL,RG,AB
P28	M	50	47	CBZ, LCS, LEV, TPM	-	lHS	NL,RG
P29	F	36	27	CBZ, LEV, URB	-	lHS	-
P40	M	14	13	CBZ, LCS	-	lHS	NL,RG
P41	F	16	6	LEV, TPM	-	rHS	NL,RG
P42	M	21	17	CLZ, PHT, TPM	-	lHS	NL,RG
P43	F	26	11	LTG	-	bilateral HS	RG
P44	F	42	39	CBZ, LEV, PB	-	lHS	NL,RG,AB
P45	F	36	11	CBZ	-	lHS	NL,RG
P46	F	12	8	CBZ	-	lHS	RG
P47	M	49	31	LTG, ZNS	-	lHS	NL,RG
P49	M	35	7	CBZ, LEV	-	rHS	NL,AB

Table S1. Clinical characteristics of TLE patients from group 1 (G1: P01-P17), group 2 (G2: P18-P29) and group 3 (G3: P40-P49). Data not shown in the table were not available in the patients' medical record. Abbreviations: N: normal; HS: *hippocampal sclerosis*; l: left; r: right; AB: amyloid bodies; NL: neuronal loss; O: oedema; RG: reactive gliosis. Anti-epileptic drugs (AEDs): CLZ: clonazepam; CBZ: carbamazepine; GBP: gabapentin; LEV: levetiracetam; LCS: lacosamide; LTG: lamotrigine; OXC: oxcarbazepine; PB: phenobarbital; PHT: phenytoin; PGB: pregabalin; TPM: topiramate; URB: urbanil; VGB: vigabatrin; VPA: valproate; ZNS: zonisamide.

cDNA copies number in hippocampus of epileptic patients

	Mean \pm SEM	Min	Max	P41	P49
IL1β	28 245 \pm 5 388	3 474	114 957	7 833	56 674
IL6	948 \pm 327	0	5 634	75	5 634
TNFα	3 727 \pm 1 115	0	20 485	0	20 485
MCP1	201 347 \pm 26 979	37 289	425 797	37 289	415 250
MIP1α	99 659 \pm 16 512	13 683	278 864	13 683	178 990
IL10	425 \pm 82	0	1274	0	1 274
GFAP	4 274 122 \pm 481 786	736 298	7 849 435	1 190 100	7 849 435
ITGAM	1 661 \pm 279	99	4 314	181	4 024

Table S2. Number of cDNA copies (mean \pm SEM) after reverse transcription in the resected hippocampus of patients with refractory epilepsy (n = 22). P41 and P49 patients have been chosen for exemplification, as they present with the lowest and the highest values of the pro-inflammatory index (Figure 2), respectively.

Patient ID	IL1 β	IL6	TNF α	MCP1	MIP1 α	IL10	GFAP	ITGAM
P01	24	26	14	31	38	11	19	32
P03	120	27	15	153	130	55	137	82
P05	18	25	220	61	34	137	17	6
P06	127	46	21	153	87	61	58	47
P07	80	559	93	120	280	77	124	95
P08	98	31	43	95	97	77	115	88
P10	407	98	29	189	273	287	146	109
P11	109	177	65	101	64	78	82	81
P13	154	179	32	202	117	208	122	54
P14	118	93	12	130	62	39	42	41
P15	55	26	41	66	40	44	91	47
P16	12	13	26	27	40	46	53	39
P17	30	0	0	64	32	2	32	7
P40	28	30	49	42	34	82	91	78
P41	16	8	0	19	14	0	28	11
P42	209	89	75	211	188	77	150	206
P43	123	31	38	95	156	9	151	135
P44	113	37	425	104	72	171	182	140
P45	62	33	207	56	49	260	153	247
P46	39	31	119	34	44	85	89	153
P47	58	43	126	41	170	92	133	260
P49	201	594	550	206	180	300	184	242

Table S3. Individual values of TLE patients for molecular and cellular markers of inflammation measured in the hippocampus. Transcript level of pro-inflammatory cytokines (IL1 β , IL6, TNF α), chemokines (MCP1, MIP1 α), anti-inflammatory cytokine IL10, and cell markers (GFAP, ITGAM) were measured in resected hippocampus of TLE patients (n=22). Individual values are expressed in percent of the mean value for each marker.

ID	Gender	MRI	Neuronal loss	Reactive gliosis
P01	F	N	+	+
P03	F	rHS	-	+
P05	M	lHS	++	++
P06	M	lHS	+	+
P07	M	rHS	+++	+++
P08	F	lHS	N.A.	N.A.
P10	M	lHS + tumor lesion	++	++
P11	F	rHS	+	+
P13	M	rHS	+++	+++
P14	F	rHS	+++	+++
P15	M	rHS	N.A.	N.A.
P16	F	lHS	++	++
P17	F	rHS	+++	+++
P40	M	lHS	+++	++
P41	F	rHS	+++	+++
P42	M	lHS	+++	++
P43	F	bilateral HS	++	++
P44	F	lHS	+++	+++
P45	F	lHS	+++	+++
P46	F	lHS	++	++
P47	M	lHS	++	++
P49	M	rHS	+++	++

Table S4. Scoring of neuronal loss and reactive gliosis in the resected hippocampus of TLE patients. Abbreviations: -: not present; +: mild; ++: moderate; +++: severe; N.A.: pathology report not available.

Transcript levels in rat hippocampal tissue (percent of CTRL-NaCl)						
	IL1 β	IL6	TNF α	MCP1	MIP1 α	ITGAM
CTRL-NaCl	100 \pm 17	100 \pm 15	100 \pm 13	100 \pm 55	100 \pm 8	100 \pm 8
CTRL-blood	140 \pm 14	65 \pm 16	153 \pm 8	70 \pm 8	90 \pm 5	99 \pm 6
	NS	NS	<i>p</i> <0.01	NS	NS	NS
SE-NaCl	2 197 \pm 794	23 720 \pm 10 375	345 \pm 100	131 243 \pm 23 148	1 151 \pm 159	527 \pm 66
SE-blood	1 855 \pm 757	24 127 \pm 13 099	255 \pm 32	124 747 \pm 19 724	885 \pm 155	617 \pm 89
	NS	NS	NS	NS	NS	NS

Table S5. Blood cells do not contribute significantly to the inflammatory markers detected in brain. Transcript level quantitation was performed in the hippocampus of control rats or epileptic rats 7h after SE, after transcardial perfusion of 0.9% NaCl or not. Brains were dissected immediately after death (CTRL-blood: n=5 ; SE-blood: n=5; CTRL-NaCl: n=5 ; SE-NaCl: n=4). NS: statistically not significant.

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	P21 - Weaning				P42 - Juvenile				Pool P21/P42	
	Epileptogenesis		Epilepsy		Epileptogenesis		Epilepsy		Epilepsy	
	7H/1D/9D		7W		7H/1D/9D		7W		7W	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
IL1b	106	13	247	28	199	16	195	28	221	21
IL6	0	0	0	0	15	5	0	0	0	0
TNFA	12	5	37	2	32	5	36	6	37	3
IFNg	0	0	14	4	0	0	11	3	13	2
MCP1	844	111	1 733	178	1 980	350	1 496	292	1 614	167
MIP1a	2 189	291	3 093	411	2 767	351	2 336	342	2 714	279
IL4	92	17	132	21	168	45	135	18	133	13
IL10	21	1	22	5	31	7	19	6	20	4
IL13	940	137	1 678	248	2 253	219	1 704	331	1 691	197
ITGAM	5	1	11	1	13	1	10	1	11	1
GFAP	228 476	8 908	372 334	9413	421 669	27720	380 725	14148	376 529	8199

Table S6. Number of cDNA copies (mean ± SEM) in control rat hippocampus after reverse transcription of total RNA.

cDNA	Primer pairs – <i>Homo sapiens sapiens</i>	Product sizes (bp)	GenBank ID#
DMD	F-CCTCCACTCGTACCCACACT R-TCCCAGCAAGTTGTTTGAGTC	89	NM_004015/16/17/ 18.2
GAPDH	F-AGCCACATCGCTCAGACAC R-GCCCAATACGACCAAATCC	66	NM_002046.3
HPRT1	F-TGACCTTGATTTATTTTCATACC R-CGAGCAAGACGTTTCAGTCCT	102	NM_000194.2
IL1β	F-TACCTGTCCTGCGTGTGAA R-TCTTTGGGTAATTTTGGGATCT	76	NM_000576.2
IL6	F-CAGGAGCCCAGCTATGAACT R-AGCAGGCAACACCAGGAG	85	NM_000600.3
TNFα	F-CAGCCTTCTCCTTCCTGAT R-GCCAGAGGGCTGATTAGAGA	123	NM_000594.2
IFNγ	F-GGCATTTTGAAGAATTGGAAAG R-TTTGGATGCTCTGGTCATCTT	112	NM_000619.2
MCP1	F-AGTCTCTGCCGCCCTTCT R-GTGACTGGGGCATTGATTG	93	NM_002982.3
MIP1α	F-TGCAACCAGTTCTCTGCATC R-AATCTGCCGGGAGGTGTA	75	NM_002983.2
IL4	Qiagen®, Cat. #330001 PPH00565B	93	NM_000583.3
IL10	F-AGGACTTTAAGGGTTACCTGGGTTG R-TTGATGTCTGGGTCTTGGTTCT	103	NM_000572.3
IL13	Qiagen®, Cat. #330001 PPH00688F	78	NM_002188.2
GFAP	F-AGAGGGACAATCTGGCACA R-CAGCCTCAGTTGGTTTCAT	71	NM_002055.4
ITGAM	F-GGCATCCGCAAAGTGGTA R-GGATCTTAAAGGCATTCTTTCG	70	NM_000632.3

Table S7. Primer sequences – *Homo sapiens sapiens*

cDNA	Primer sequences – <i>Rattus norvegicus</i>	Product sizes (bp)	GenBank ID#
IL1 β	F-TGTGATGAAAGACGGCACAC R-CTTCTTCTTTGGGTATTGTTTGG	70	NM_031512.2
IL6	F-CCCTTCAGGAACAGCTATGAA R-ACAACATCAGTCCCAAGAAGG	74	NM_012589.1
TNF α	F-TGAACTTCGGGGTGATCG R-GGGCTTGCTACTCGAGTTTT	122	NM_012675.3
IFN γ	F-TTTTGCAGCTCTGCCTCAT R-AGCATCCATGCTACTTGAGTTAAA	107	NM_138880.2
MCP1	F-CGGCTGGAGA AACTACAAGAGA R-TCTCTTGAGCTTGGTGACAAATA	78	NM_031530.1
MIP1 α	F-TCCACGAAAATTCATTGCTG R-AGATCTGCCGTTTCTCTTG	92	NM_013025.2
IL4	F-GTAGAGGTGTCAGCGGTCTG R-TTCAGTGTTGTGAGCGTGGA	70	NM_201270.1
IL10	F-AGTGGAGCAGGTGAAGAATGA R-TCATGGCCTTG TAGACACCTT	62	NM_012854.2
IL13	F-AGTCCTGGCTCTCGCTTG R-GATGTGGATCTCCGCACTG	63	NM_053828.1
GFAP	F-ACATCGAGATCGCCACCTAC R-GGATCTGGAGTTGGAGAAA	90	NM_017009.2
ITGAM	F-ACTCTGATGCCTCCCTTGG R-TCCTGGACACGTTGTTCTCA	72	NM_012711.1

Table S8. Primer sequences – *Rattus Norvegicus*

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BOX 6 | HIGHLIGHTS OF STUDY 1

- *Post-mortem* tissues are not appropriate controls when comparing mRNA values of inflammatory marker;
- Not all TLE patients have high levels of inflammatory mediators in their hippocampus;
- Inflammation during chronic epilepsy is low grade compared to the explosive inflammation occurring after *status epilepticus* (SE);
- At a cellular level, microglial cells contribute to a greater extent than astrocytes to the production of interleukin-1 β after pilocarpine-induced SE;
- High inflammation after an epileptogenic insult does not necessarily lead to the development of epilepsy.

CHAPTER 3

Study 2

HOW TO FOLLOW EXTRAVASATING MONOCYTES INTO THE RAT BRAIN
AFTER *STATUS EPILEPTICUS*?

Extravasating monocytes harboring heparan sulfate chains in rats can transdifferentiate into monocyte-macrophages, integrate resident microglia network and maintain CD68 expression

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ABSTRACT

Under certain pathological circumstances associated with inflammation, in particular following epileptogenic conditions, e.g. *status epilepticus* (SE), circulating monocytes have been shown to extravasate into the CNS parenchyma. However, it is still unknown whether these cells are temporary present in the infiltrated areas of the brain or whether they become integrated in the local microglia network. Our study was devoted to provide strong evidence that extravasating monocytes transdifferentiate into reactive microglial-like cells and integrate the resident microglial network in the limbic regions of adult rats following pilocarpine-induced SE (Pilo-SE). We observed that resident microglial cells started being highly activated within the hours following Pilo-SE, showing a bushy morphology with highly ramified processes by day 1. We also found that numerous monocytes harboring heparan sulfate chains (HSCs) extravasated within the days after Pilo-SE (apparent peak observed at 24h) in the hippocampus in which highly activated microglia phenotype was displayed from day 3 to day 9 after Pilo-SE. We also provided strong evidence that extravasating monocytes transdifferentiated into cells that exhibited morphological features of highly activated microglial-like cells. In addition, CD68 expression within the brain parenchyma was identified as a specific marker of monocytes, not only at the time of extravasation, but also in the long term during both epileptogenesis and the chronic phase of epilepsy, once monocytes transdifferentiated into microglial-like cells. In addition, HSCs were present on monocyte cell surface at the most during the first two days after SE, likely because of their degradation by heparanase, an endo- β -D-glucuronidase which specifically cleaves extracellular and cell surface heparan sulphates (HS) at intrachain sites. Heparanase expression by monocyte infiltrates and neurons may facilitate degradation of HSCs to ease migration of monocytes until their final location. This study clearly demonstrates that extravasating monocytes,

instead of being transiently present in the brain parenchyma following Pilo-SE, can reside definitively in the brain tissue after they transdifferentiate into monocyte-macrophages and integrate resident microglia networks. CD68, identified as a permanent marker of monocyte-macrophages, enables to distinguish them from resident microglial cells and offers new opportunities to generate transgenic rats to specifically address the function that these cells play in pathological conditions such as epilepsy.

Key words: epilepsy, microglia, monocytes/macrophages, CD68, heparan sulfate chains, heparanase, inflammation

1 | INTRODUCTION

The origin of microglia in the central nervous system (CNS) has been a longstanding issue of debate. Microglial cells arise early during development from progenitors that give rise to primitive yolk sac-derived macrophages invading the neuroepithelium at embryonic day 9.5 where they persist in the adult brain as resident microglia (Ginhoux et al., 2010). Under healthy conditions, resident microglial cells are sustained only through self-renewal from the CNS pool. Microglia are involved in processes that maintain cerebral homeostasis such as clearance of accumulated metabolic products, neuronal proliferation and differentiation, synaptogenesis and synaptic structures in the adult brain, or synaptic pruning during development (Ginhoux and Prinz, 2015; Ginhoux et al., 2010). After brain insult such as *status epilepticus* (SE), microglia become activated and contribute to an inflammatory response that, depending on the context, can be either detrimental for surrounding cells, including neurons and astrocytes, or can support CNS repair and remodeling. Brain injuries are also known to impair the properties of the blood-brain barrier (BBB), causing an increase in membrane permeability and thus promoting the extravasation of peripheral immune cells and inflammatory molecules. Although it is well established that invading leukocytes are part of the early-stage neuroinflammatory responses following BBB breakdown, their precise role in the resolution of neuroinflammation is still uncertain and controversial (London et al., 2013).

In epilepsy, clinical and preclinical data have shown that these infiltrating cells are involved in the pathogenesis of the disease (Broekaart et al., 2018; Fabene et al., 2013; Ravizza et al., 2008; Tian et al., 2017; Varvel et al., 2016; Walzl et al., 2018; Zattoni et al., 2011). But while some studies suggest that infiltrating leukocytes would be protective since preventing their infiltration leads to an increase in the severity of epilepsy symptoms (Zattoni et al., 2011), others argue that these infiltrating monocytes would be deleterious (Tian et al., 2017; Varvel et al., 2016). Among leukocytes, monocytes are one of the major cell types recruited to inflamed tissues following brain damage (Ravizza et al., 2008) and involved in the release of chemoattractant chemokines (Fabene et al., 2013; London et al., 2013). However, the fate of these cells in the epileptic brain is still a matter of debate, since it is unclear whether these cells are temporarily present in the infiltrated areas of the brain or whether they become part of the microglia network over the long-term. While the identification of the microglial cells and infiltrating monocytes as two distinct cell populations is feasible in mice by detecting a

combination of markers such as CCR2, CX3CR1 and Ly6C (Greter et al., 2015; London et al., 2013; Tian et al., 2017; Varvel et al., 2016; Vinet et al., 2016; Zattoni et al., 2011), in rats, the tracking of these cells is more challenging since the use of the above-mentioned markers has been inconclusive so far and no other reliable markers have been identified. In mouse models of brain injury, studies have demonstrated that monocytes invading the brain are able to transdifferentiate and integrate the microglial cell network (Djukic et al., 2006; Feng et al., 2019), while other investigations indicated that they do not persist in brain tissue (Ajami et al., 2011). The identification of specific markers is today essential to know the fate and function of these cells in preclinical models in rats. The aim being to use these markers to specifically target in these cells and over time the expression of transgenes coding for proteins allowing either to track them over the long term, through the expression of Green Fluorescent Protein for example, or to activate or inhibit them pharmacologically, or finally to induce their death for example.

The first purpose of this study was to identify a specific marker of monocyte infiltration in a rat model of epilepsy. We first evaluated after pilocarpine-induced SE the expression of CD68 (ED-1), a member of the lysosomal/endosomal-associated membrane glycoprotein (LAMP) family, classically used in human and mouse tissues as a marker of activated microglia, monocytes or macrophages (Broekaart et al., 2018; Hiragi et al., 2018). In our model, the use of this marker allowed us to follow the morphological evolution of monocytes and their transdifferentiation into monocytes-macrophages displaying a microglia-like morphology, from the earliest stages after SE to the chronic phase of epilepsy.

We then sought to determine which processes were involved in the migration of monocytes into the brain parenchyma once they cross the BBB. It is well known that leukocytes express heparan sulfate chains (HSCs) on their surface and that HSCs can modulate monocyte migration through the cerebral endothelium (Floris et al., 2003). The role of these HSCs is known to ensure structural integrity as well as to serve as storage reservoirs for growth factors and chemokines (Rabenstein, 2002). In oncology, HSCs have demonstrated roles in tumor cell proliferation, tumor angiogenesis and metastasis (Knelson et al., 2014), and HSC degradation by heparanase, a β -D-endoglucuronidase that degrades HSCs, is thought to play a role in the migration of malignant cells (Chen et al., 2017; Mayfosh et al., 2019). Thus, since monocytes express HSCs on their surface, the latter may hinder their progression within the brain tissue. We therefore investigated, using a specific peptide that binds to these HSCs,

whether all infiltrating monocytes express HSCs on their surface and whether the presence of these HSCs persists over time.

2 | MATERIAL AND METHODS

Animals

All animal procedures were in compliance with the guidelines of the European Union (directive 2010-63), taken in the French law (decree 2013/118) regulating animal experimentation, and have been approved by the ethical committee of the Claude Bernard Lyon 1 University (protocol # BH-2008-11). We used a tissue collection bank generated by TIGER team in 2009-2012. Briefly, male Sprague-Dawley rats (Harlan, France) were used in these experiments. They were housed in a temperature-controlled room ($23 \pm 1^\circ\text{C}$) under diurnal lighting conditions (lights on from 6 a.m to 6 p.m). Pups arrived at 15 day-old and were maintained in groups of 10 with their foster mother until P21. Beyond that age, rats were maintained in groups of 5 in 1,800 cm² plastic cages, with free access to food and water. After SE, rats were maintained in individual cages and weighed daily until they gained weight. Until sacrifice, epileptic rats were housed alone and control rats were housed in groups of 5 in standard cages.

Pilocarpine-induced status epilepticus (SE)

SE was induced by pilocarpine injected at day 42. To prevent peripheral cholinergic side effects, scopolamine methylnitrate (1 mg/kg in saline, s.c.; Sigma-Aldrich) was administered 30 min before pilocarpine hydrochloride (350 mg/kg, in saline, i.p.; Sigma-Aldrich). After 2 hours of continuous behavioral SE, 10 mg/kg diazepam (i.p.; Valium; Roche®) was injected, followed, 60 min later, by a second injection of 5 mg/kg diazepam to terminate behavioral seizures. Control rats received systematically corresponding injections of saline solution. The animals were then sacrificed at various time points after SE for molecular and cellular analysis.

Animal care after SE

Control and treated rats were weighted every day during the first two weeks following SE, and then every week until termination of the experiment. Daily abdominal massages were performed twice a day during the first week to activate intestinal motility, which was disrupted following SE. All efforts were made to minimize pain or discomfort of the animals used.

Onset of handling-induced seizures

Electroencephalographic recordings were excluded to determine epilepsy onset due to preliminary experiments that showed that the sole implantation of screws into the skull induced significant and lasting inflammation over time in the cortex and, to a lesser extent, in the hippocampus. As a result, epilepsy onset was determined according to clinical criteria. Therefore, animals were tested for the occurrence of handling-induced seizures (HIS) three times a day between the 1st and the 5th week post-SE. HIS were triggered by restraining rats for 10 seconds at the level of the chest with gentle pressure. Animals were declared as “epileptic” (EPI) once they developed HIS on 2 consecutive trials. By the end of the 5th week post-SE, all rats were considered as EPI.

Monocytes/macrophages depletion and fluorescent labelling

Animals received clodronate liposomes intraperitoneally at the dosage of 1 mL per 100g body weight. Clodronate was administered as a single dose at 3 days before pilocarpine-induced SE. Liposomes containing clodronate were purchased from Liposoma B.V. (Amsterdam, The Netherlands), with a concentration of clodronate of 5 mg/mL. Following treatment, rats were observed for adverse effects. Then, rats underwent pilocarpine-induced SE and received 6 hours after the onset of SE an injection of fluoresbrite YG carboxylate microsphere (0.5 µm diameter) via the tail vein ($9,1 \times 10^{10}$ particles per rat).

Ex Vivo Procedures

All rats were deeply anesthetized with a lethal dose of pentobarbital (100 mg/kg; Dolethal) before being sacrificed. Hippocampus were rapidly microdissected, frozen in liquid nitrogen, and stored at -80°C. For immunochemistry analysis, animals were transcardially perfused (30 mL/min) with 4% paraformaldehyde in 0.1 M phosphate buffer. After cryoprotection in 30% sucrose, brains were frozen at -40°C in isopentane and stored at -80°C.

RNA extraction and quantification of transcript level variations by reverse transcriptase real-time polymerase chain reaction (RT-qPCR)

Brain structures frozen in liquid nitrogen were crushed using Tissue-Lyser (Qiagen®) in 250 µL of ultrapure RNase-free water (Eurobio). Nucleic acids were extracted by adding 750 µL Tri-Reagent LS (TS120, Euromedex) and 200 µL chloroform (VWR®). After precipitation with isopropanol (I-9516, Sigma-Aldrich®), washing in 75% ethanol (VWR) and drying, total nucleic acids were resuspended in 50 µL ultrapure water and treated with DNase I (Turbo DNA Free®

kit; AM1907, Ambion®) to eliminate any trace of possible genomic DNA contamination. The purified total RNAs were then washed using the RNeasy® minikit (Qiagen®) kit. After elution, the total RNA concentration was determined for each sample on BioDrop® µLite. The quality of total RNAs was verified on microgel chips using LabChip® 90 (Caliper), which provides an RNA Integrity Number (RIN) value by analyzing the integrity of two ribosomal RNAs (18S and 28S) predominantly present in all tissue RNA extracts. All selected samples had a RIN value greater than 7.0, and were stored at -80°C until use. Total tissue RNAs (480 ng) were reverse transcribed to complementary DNA (cDNA) using both oligo dT and random primers with PrimeScript RT Reagent Kit (Takara) according to manufacturer's instructions, in a total volume of 10 µL. In RT reaction, 300 000 copies of a synthetic external non-homologous poly(A) standard messenger RNA (SmRNA; A. Morales and L. Bezin, patent WO2004.092414) were added to normalize the RT step (Sanchez et al., 2009). cDNA was diluted 1:13 with nuclease free Eurobio water and stored at -20°C until further use. Each cDNA of interest was amplified using 5 µL of the diluted RT reaction by the "real-time" quantitative polymerase chain reaction (PCR) technique, using the Rotor-Gene Q thermocycler (Qiagen®), the SYBR Green Rotor-Gene PCR kit (Qiagen®) and oligonucleotide primers specific to the targeted cDNA. The sequences of the specific forward and reverse primer pairs were constructed using the Primer-BLAST tool or using the "Universal Probe Library" software (Roche Diagnostics). Sequences of the different primer pairs used are: CD68 (GenBank NM_001031638.1) Forward-5' CTTTCTCCAGCAATTCACCTG 3', Reverse-5' ACTGGCGCAAGAGAAGCA 3' (99 bp), heparanase (GenBank NM_022605) Forward-5' CAA TGA TAT TTG CGG GTC TG 3', Reverse-5' TGC GTT TTG GAA AGC TGA CT 3' (415 bp). The number of copies of each targeted cDNA contained in 5 µL of the diluted RT reaction was quantified using a calibration curve based on cascade dilutions of a solution containing a known number of cDNA copies.

Production of a rabbit polyclonal anti-rat heparanase antibody

A polyclonal immuno-affinity purified antibody directed against rat heparanase protein (GenBank NP_072127) has been produced (Covalab, Lyon, France) after immunization of a rabbit with two 13/14-residue peptides located within heparan sulfate binding domains of the rat heparanase protein (Hulett et al., 1999). The sequences of these peptides are ¹⁴⁹YQREKNSTYSRS¹⁶¹ and ²⁷³RSFLKAGGEVIDS²⁸⁴, both preceded by an additional cystein residue.

Western Blot analysis of immunoreactive heparanase

For each age studied, 50 µg of proteins extracted from the hippocampus of each animal were pooled. Equal amounts of pooled proteins (40 µg) were separated on 12% SDS/PAGE gels and then transferred onto nitrocellulose membranes by standard procedures. The membranes were blocked by incubation in 5% skim milk in TBS buffer containing 0.1% Tween 20 (TBS-T) at room temperature for 1 hour. All washes were performed in TBS buffer. The membranes were successively incubated in avidin and biotin solutions (Avidin Biotin blocking kit; Vector) according to manufacturer's instructions. Then, the membranes were sequentially incubated with a rabbit anti-heparanase polyclonal antibody (Covalab) diluted at 1/100 in TBS-T containing 1% skim milk (overnight at 4°C), with a biotinylated goat anti-rabbit IgG (AP187B; Chemicon) diluted at 1/10000 in TBS-T containing 1% skim milk (2 hours at room temperature), with avidin biotin peroxidase complex (Vectastain Elite ABC kit; Vector) diluted at 1/1500 in TBS (1 hour at room temperature), and finally reacted with a 3',3'-diaminobenzidine (DAB) solution (Vector). After intensive washes in tap-water, membranes were digitalized and analyzed for quantification with an image analysis system (Visilog 6.3; Noesis). For each band, the integrated optical density (OD) and the surface area (S; pixels) were measured. The index of protein level was calculated by multiplying OD with S, and expressed in arbitrary units (A.U.).

Tissue processing for histological procedures

Cryostat-cut (40 µm thick) sections from rat samples were transferred into a cryopreservative solution composed of 19.5 mM NaH₂PO₄·2H₂O, 19.2 mM NaOH, 30% (v/v) glycerol and 30% (v/v) ethyleneglycol and stored at -25°C.

Colorimetric immunohistochemistry

Free-floating sections (40 µm thick) from paraformaldehyde-fixed tissue were incubated with a mouse monoclonal anti-CD11b/ITGAM (1:1000; CBL1512Z, Chemicon) to detect microglia and immunocompetent cells. After washes, sections were then incubated with a biotinylated donkey antibody raised against mouse IgG antibody (1:1000; 715-065-151, Jackson ImmunoResearch). After washes, sections were incubated with avidin biotin peroxidase (1:1000; Vectastain Elite ABC kit, Vector) and reacted with 0.4 mM 3',3'-diaminobenzidine (DAB, Sigma Fast). Sections were then mounted, dehydrated and coverglassed in DPX (Fluka).

Production of a biotinylated peptide, PepHep, for the detection of heparan sulfate chains

To detect heparan sulfate chains (HSC), a peptide corresponding to the binding domain sequence of heparanase to HSC has been selected and produced (Covalab, Lyon, France). The sequences of this peptide is ¹⁴⁹YQREKNSTYSRS¹⁶¹, preceded by an additional cystein residue and a biotin.

Fluorescent immunohistochemistry

Free-floating sections (40 µm thick) from paraformaldehyde-fixed tissue were incubated with a goat polyclonal anti-Iba-1 antibody (1:500; AB5076, Abcam) to detect microglia, a mouse monoclonal anti-CD11b/ITGAM (1:1000; CBL1512Z, Chemicon) to detect microglia and immunocompetent cells, a mouse monoclonal anti-CD68 antibody (1:1000; MCA341GA, Bio-rad), with a mouse monoclonal anti-NeuN antibody (1:1000; MAB-377, Chemicon) to detect neurons and PepHep (1:100; Covalab®) to detect heparan sulfate chains. After washes, sections were incubated with one or a combination of the following secondary antibody: Alexa-Fluor-405-conjugated Streptavidin (1:500; S-32351; Molecular Probes), Alexa-Fluor-488-conjugated donkey anti-goat IgG antibody (1:750; A-11055; Molecular Probes), or Alexa-Fluor-647-conjugated donkey anti-mouse IgG antibody (1:1000; A-31571; Molecular Probes). Sections were then mounted on SuperFrost Plus slides and coverglassed with Prolong Diamond Antifade reagent (Molecular Probes). The immunostained slides were captured using a Carl Zeiss Axio Scan.Z1 Digital Slide Scanner with a resolution of x40. Images were then processed on with ZEN Imaging software (Zeiss) for further editing.

Data and statistical analysis

GraphPad Prism (v.7) software was used to statistically analyze data. Data are expressed as mean ± SEM of the different variables analyzed. Statistical significance for within-group comparisons was calculated by one-way analysis of variance (ANOVA) with Tukey's *post hoc* test. The p value of 0.05 defined the significance cut-off.

3 | RESULTS AND DISCUSSION

CD11b marker was significantly more expressed after SE on cells displaying different morphologies

The first objective of our study was to investigate the histological changes of microglial cells after a pilocarpine-induced SE. The reactivity of microglial cells was addressed by immunohistochemical detection of CD11b (Fig. 1). Of note, other peripheral immune cells such as monocytes also express this marker. In control rats, microglial cells appear in high numbers in the dentate gyrus of the hippocampus, with thin, long and very dense extensions around a small cellular body (CTRL, Fig. 1). At 7 hours post-SE, microglial cells exhibited a larger cell body with shorter and thicker expanded extensions (SE-7H, Fig. 1). This state of activation was then followed, 24h after SE, by an intense CD11b signal within cells exhibiting a bushy morphology or with hyper-ramified extensions. At this stage, small round-shaped cells were also detected (SE-24H, Fig. 1), but were no more visible by day 3 post-SE and beyond. At 9 days post-SE, CD11b labelling was still pronounced, but the majority of microglial cells had recovered a morphology similar to that of controls, except in some brain regions exhibiting a very dense signal, evocating a microglial scar, not only in the hippocampus, but also in the dorsal thalamus and the ventral limbic region which includes the insular agranular cortex, the piriform cortex and the amygdala (SE-9D, Fig. 1).

Iba-1 was detected only in resident microglial cells

CD11b-positive round-shaped cells observed at 1 days post-SE (Fig.1) could be either resident microglial cells that adopt an amoeboid shape while moving through the brain tissue, and/or immunocompetent cells coming from the periphery. To answer this question, we verified whether round-shaped cells were found to express the specific microglial cell marker Iba-1 (Fig. 2). In control brains, resident microglial cells were in their quiescent state, with a morphology identical to that described with CD11b marker in these same tissues, i.e. showing fine extensions with a small cell body (CTRL, Fig. 2). At 24 hours post-SE, Iba-1 positive cells increased in size and acquired a more bushy morphology with smaller but thicker extensions (SE-24H, Fig. 2). However, in contrast to tissues labeled with CD11b, no round-shaped Iba-1-positive cells were observed. This result suggests that the CD11b-positive round-shaped cells observed 24 hours post-SE (Fig. 1), were infiltrating leukocytes.

Expression of prototypical markers of inflammation after pilocarpine-induced SE was assessed in Study 1. The mRNA levels of inflammatory markers were measured in the rat hippocampus during the epileptogenesis (7 hours, 1 day, 9 days post-SE), and during the chronic phase (7 weeks post-SE). Among the markers investigated, we quantified the levels of the chemokines MCP1 and MIP1 α , two chemokines known to promote the attraction of immune cells to inflamed tissues. Our results showed that the expression of these chemokines is significantly modulated in the early times post-SE: as early as 7h post-SE, the cDNA copy numbers of MCP1 and MIP1 α increased, respectively, by 824-fold or 247-fold compared to controls. These results have oriented the pursuit of our study towards the identification of a specific marker that might identify CD11b⁺ round-shaped cells as monocytes that infiltrated the brain parenchyma. If so, this specific marker could make it possible to track these cells over time to determine their fate.

CD68 labeling progressed from round-shaped cells in the early stages post-SE to cells bearing morphological features of microglial cells in the later stages of epileptogenesis

We then tested whether CD68 could be a potential specific marker for infiltrating monocytes. Classically, this marker is used in immunohistochemical investigations in humans and mice for the detection of monocytes or activated microglial cells. Here we observed that CD68 was not detected in the parenchyma of brain sections from control Sprague-Dawley rats. One day after SE, CD68 labeling performed on sections adjacent to those used for CD11b staining showed a large number of small round-shaped cells positive for CD11b and CD68 (SE-1D, Fig. 3 A, B, C). These cells were observed in the cerebral parenchyma, but also in blood vessels, in the light of the capillaries and across, suggesting extravasation events, both observed with the CD11b (Fig. 3A, B, D) and with the CD68 (Fig. 3 A, C, E). This result strongly supports the hypothesis that all CD11b round-shaped cells detected 1 day post-SE are infiltrating monocytes. Indeed, cell quantifications performed over the entire surface of the dorsal hippocampus demonstrated no significant difference between CD11b-positive and CD68-positive round-shaped cells (Fig. 3F). Nine days after SE, no more round-shaped cells were visible (SE-9D, Fig. 3A). Instead, CD68⁺ cells had morphological features similar to that of microglial cells. At 7 weeks post-SE, CD11b-positive cell morphology in the hippocampus was similar to that observed in control rats. It should be noted that CD68 signal is less intense and less homogeneously distributed than CD11b across the cell bodies and extensions. Apart from the

hippocampus, CD68 labeling throughout the brain showed that the CD68+ round-shaped cells observed at 1-day post-SE were located in the same brain areas as those displaying an intense CD11b signal at 9 days post-SE, i.e. the hippocampus, dorsal thalamus and ventral limbic region. Finally, quantification of CD68 transcript levels in the rat hippocampus showed a marked increase in CD68 expression at 1-day post-SE, when the peak of CD68+ round-shaped cells in the hippocampus was observed (Fig. 3H).

Peak expression of CD68 transcript measured at 9 days indicates transdifferentiation and cell enlargement

Considering our results showing that CD68+ round-shaped cells were present at a maximum number 1-day post-SE, and that this number decreased thereafter replaced by CD68+ cells with extensions, we subsequently wondered how the mRNA level of this gene varied from the earliest phases of epileptogenesis to the chronic phase. Hence, in addition to the hippocampus, the transcript level of CD68 expression was measured in the VLR, the dorsal thalamus and the neocortex (Fig. 4A). In each of these structures, peak expression of CD68 is observed at 9 days, showing a very strong induction exceeding 30-fold, 29-fold, 15-fold and 11-fold that of control rats in the hippocampus, VLR, dorsal thalamus and neocortex, respectively.

In order to define whether *cd68* gene induction could be related to more than just immune cell infiltration and could originate from activation of resident microglial cells, we investigated by immunohistofluorescence whether CD68 colocalized with Iba-1. We previously showed that 24 hours after pilocarpine-induced SE, no round-shaped cells were positive for Iba-1, in contrast to CD11b and CD68 (Figs. 1 and 2). The immunofluorescent double detection of Iba-1 and CD68 revealed no colocalization at 7 and 24 hours post-SE, CD68 being only present in round-shaped cells, while the Iba-1 marker only detected microglial cells with a bushy morphology and short prolongations (images not shown). However, 9 days after SE, CD68 colocalized with Iba-1 in non-ovoid cells, as observed in the dorsal thalamus of rats (Fig. 4B). This result suggests that the CD68 + monocytes which infiltrated the cerebral parenchyma approximately 1 day after SE have started transdifferentiation processes towards a phenotype whose morphological characteristics are close to that of resident microglial cells.

This transdifferentiation process would lead many cells to acquire the Iba-1 phenotype, with the possibility of integrating the network of resident microglial cells. Hence, CD68 expression

peak at 9 days post-SE might support the differentiation of infiltrating CD68+ monocytes into monocytes-macrophages with morphological features of microglial cells, as evidenced by the expression of Iba-1 in a large majority of CD68+ cells.

Infiltrating monocytes transdifferentiate into monocytes-macrophages bearing morphological feature of microglial cells

In order to follow the fate of peripheral monocytes up to their final site in the brain after SE, we decided to tag them by phagocytosis of fluorescent YG nanoparticles (FYG) injected by the tail vein. The problem which has been encountered is that of a rapid domiciliation of the FYG + monocytes towards the peripheral lymphoid organs (data not shown), making them then no longer available at all to re-populate the blood compartment in order to colonize the brain tissue following an SE. To work around this problem, we used a depletion approach of circulating monocytes/macrophages 3 days prior to the induction of SE by pilocarpine, followed by the injection of fluorescent nanoparticles.

Selective depletion of macrophages is a widely accepted approach to study their functional aspects *in vivo*. The selective removal of macrophages by intravenous injection of clodronate liposomes has been described previously (Van Rooijen and Sanders, 1994). After systemic injection, liposomes are phagocytosed and digested by macrophages, the phospholipid bilayers of the liposomes are disrupted under the influence of lysosomal phospholipases and clodronate released intracellularly induces apoptosis, thus causing 90% depletion of macrophages within 24–72 h (Biewenga et al., 1995; van Rooijen et al., 1996, 1997). Free clodronate has an extremely short half-life, so non-phagocytic cells are not affected (van Rooijen et al., 1996). Because macrophages are present in almost all organs of the body and have important immunoregulatory functions, the liposome depletion method is widely used. Organ-specific depletion of macrophages is possible, depending on the route of administration of the liposomes. Administration of clodronate liposome intraperitoneally depletes all monocyte/macrophages present in the spleen, liver, bone marrow and bloodstream. The subsequent injection of FYG into the tail vein, concomitant with the release of new monocytes / macrophages resulting from the maturation of hematopoietic stem cells, leads to phagocytosis of FYG by monocytes, then available for any suffering tissue, including the brain especially after SE. In our protocol, FYG were injected 6 hours after pilocarpine-induced SE. Immunofluorescent detection of CD11b and FYG was performed at 1 day, 3 days

and 6 days post-SE in the hilus and CA1 layer of the hippocampus (Fig 5). We observed that at 1 day post-SE, CD11b+ round-shaped cells located in the blood vessels, adjacent to the vascular wall, were also positive for FYG (Figs. 5A1 and 5A2). At 3 days post-SE, fluorescent nanoparticles were found in the hilus of the hippocampus in CD11b-positive cells that display, at that time point, a morphology similar to microglial cells (Figs. 5B1 and 5B2). At 6 days post-SE, FYGs were still observed only in cells whose morphology corresponded to those of activated microglial cells. Due to the low number of dots detected (Fig. 5 C1, C2), the possibility cannot be ruled out that resident microglial cells have phagocytosed FYG + monocytes, and subsequently kept some of the FYG molecules. This hypothesis is however not the one that we will retain because labeling carried out with an antibody directed against Iba-1 did not make it possible to observe FYG in Iba-1 positive cells. It is therefore very likely that at 6 days, the CD11b+ cells labeled with FYG are therefore monocyte-macrophages resulting from the transdifferentiation of monocytes. In this case, the lower number of dots per cell could be explained either by the significant increase in cell volume, making that the probability to detect them in the same confocal plane was lower, or that certain nanoparticles were released or lost during the transdifferentiation process. At the time of these studies, CD68 had not yet been identified as a potential specific marker for monocyte-macrophages. Today we no longer have brain sections available to show that the FYGs are well located in CD68+ cells. A similar study will be carried out very soon, which will provide additional elements of response on the potential presence of FYG in highly transdifferentiated CD68 cells. Finally, due to the emergence of adverse effects that threatened the welfare of the animals, this experiment could not be conducted beyond 6 days post-SE.

Heparan sulfate chains (HSCs) of infiltrating monocytes are rapidly lost after SE to facilitate the migration of monocytes through the brain tissue

The processes of leukocyte infiltration into the cerebral parenchyma following cerebral aggression are well described in the literature. Among the elements involved in cell trafficking, the heparan sulfate chains (HSCs) present in the endothelial cells of blood vessels are known to be involved in the migration of monocytes across the cerebral endothelium (Floris et al., 2003), while expression of heparan sulfate chains is recognized on the surface of leukocytes (Parish, 2006). The HSCs were detected in the dentate gyrus of the hippocampus by a biotinylated peptide, called PepHep, described in the method section (Fig. 6). Fluorescent dual

labeling using PepHep an antibody directed against CD68 on rat brain sections provided evidence that HSCs were predominantly located on CD68 positive round-shaped cells (Fig. 6A). Nevertheless, it should be noted that some cells appear only as either CD68+ or HSCs+ (Fig. 6A). Quantification of cells with HSCs on their surface revealed a peak of presence at 1 day post-SE, whereas at 9 days post-SE, no HSC+ cells could be detected (Fig. 6B). Finally, double labeling of CD68 and HSCs in the whole dorsal hippocampus showed that at 1 day post SE 58% of the CD68-positive cells had HSCs on their surface, while among the cells bearing HSCs, 87% of them colocalized with CD68 (Fig. 6C). These findings thus suggest that the majority of HSCs were present on infiltrating monocytes. The fact that 58% of the infiltrating monocytes possess HSCs and 42% do not suggests either: 1) that there are different monocyte subpopulations, one expressing HSCs and the other not; or 2) that the HSCs have already undergone degradation processes. Given that the presence of HSCs on the surface of leukocytes is reported to have a role in anchoring the cells to the molecules of the surrounding extracellular matrix, it is likely that, in an attempt to migrate to the suffering areas of the brain after SE, monocytes have had their HSCs degraded to facilitate tissue migration.

Heparanase, constitutively expressed by neurons in the hippocampus, is also expressed by infiltrating monocytes, likely to degrade HSCs and promote their migration into the brain parenchyma

The results obtained regarding the presence or absence of HSCs on the surface of monocytes led us to investigate whether the specific degradation enzyme of HSCs was modulated in its expression following SE. Heparanase transcript level was quantified in the hippocampus from epileptogenesis to the chronic phase of epilepsy (Fig. 7A). The expression of heparanase was significantly increased at 1 day post-SE, coinciding with the peak of monocyte infiltration described above (Fig. 7A).

Western blot analysis of immunoreactive heparanase revealed that zymogen and processed active forms of heparanase were specifically detected by the antibody used in the hippocampus 8 hours, 1 day, 2 days and 7 days after pilocarpine-induced SE, as well as in control rats (Fig. 7B). Throughout epileptogenesis, the active form (50 kDa) was more abundant than the zymogen form (65 kDa). The level of the zymogen form was at its lowest value in control rats, increased abruptly 8 hours post-SE, decreased slightly at 1 day post-SE,

then returned to the control level after 2 days. The ratio “active form / zymogen form” thus reached its greatest value 8 hours post-SE (Figure 7B).

Heparanase protein was detected by fluorescent immunolabeling at 8 hours and 1 day post-SE in the pyramidal cell layer of Ammon’s horn (Fig. 7 C, E, G) and the hilus of the hippocampus (Fig. D, F, H). Heparanase was expressed in CA1 pyramidal neurons in controls, as previously reported (Navarro et al., 2008), as well as after SE (Fig. 7 C, E, G), and in round-shaped cells resembling leukocytes infiltrates at 1 day post-SE (Fig. 7H).

Subsequently, heparanase protein was screened at 1 day after pilocarpine-induced SE in the pyramidal layer of CA1 (Fig. 7 I, K, M) and in the hilus (Fig. J, L, N). Previous studies in the laboratory have shown that heparanase was mainly expressed by neurons in the brain throughout lifespan in homeostatic conditions (Navarro et al., 2008). Fluorescent dual labeling using antibodies directed against heparanase and neuronal specific marker NeuN showed that heparanase expression was mainly found in pyramidal neurons of the CA1 layer of the hippocampus (Fig. 7 I, K, M). In addition, double detection of heparanase and CD11b provided evidence that heparanase expression was not restricted to neurons but was also expressed by CD11b+ round-shaped cells which are likely to be infiltrating monocytes (Fig. 7 J, L, N). This result support the hypothesis that neurons and infiltrating monocytes produce heparanase, that both may contribute to degrade HSCs present on the cell surface of monocyte infiltrates, in order to promote their migration towards damaged areas of the brain.

In this study, we have identified CD68 as a specific marker of infiltrating monocytes that enables their differentiation from resident microglial cells in the rat model of pilocarpine-induced SE routinely used in our laboratory. The fate of a number of infiltrating monocytes in the brain after a cerebral aggression such as SE was their long-term integration into the microglial network. Circulating monocytes that infiltrate the brain parenchyma in the acute phase post-SE, would transdifferentiate into monocyte-macrophages, or monocyte-derived microglial cells, maintaining an activated-like phenotype on the long-term. Furthermore, we have highlighted one of the processes that appears to be involved in the tissue migration of infiltrating monocytes after SE and their grafting into the parenchymal microglial network, namely the degradation of heparan sulfate chains by heparanase. The latter would be produced partly by neurons, but mostly by the infiltrating monocytes themselves to prompt

their own migration. Indeed, the transient peak of heparanase expression between 8 hours and 1 day post-SE, overlapped with the time-course of HSC loss and was inversely correlated with the increase in the transdifferentiation processes of the infiltrating monocytes into monocyte-macrophages. However, if the increase in heparanase expression in brain tissue was attributed to the presence of infiltrating monocytes, a decrease in its expression could be ascribed to the disappearance of infiltrating monocytes from brain tissue. This possibility cannot be ruled out, although the presence of the CD68 marker observed histologically in the tissue until late stages suggests that some of the monocytes may die or leave the brain, whereas others remain and become fully integrated into the microglial network. Indeed, it cannot be excluded that other monocytes do not survive in the tissue and are rapidly eliminated by microglial cells or that some monocyte sub-populations may not be detected due to the absence of CD68 expression. The challenge today lies in providing more precise answers to these questions and identifying possible sub-populations of monocytes with characteristics that are currently unknown aimed at facilitating their long-term integration into the brain parenchyma.

Yet, in the light of our findings, we propose the hypothesis illustrated in Figure 8 that: 1) round-shaped monocytes with heparan sulfate chains infiltrate the brain parenchyma with peak observed at 1 day post-SE, 2) concomitantly with their infiltration, the monocytes would start releasing heparanase, supplementing that already produced endogenously by neurons, allowing the degradation of HSCs present at the surface of monocytes, then facilitating their migration towards their definitive target; 3) once HSCs are degraded, the monocytes reduce heparanase expression to initiate their transdifferentiation processes into monocyte-macrophages with morphological features similar to activated microglial cells.

In the late phases of epileptogenesis, certain monocyte-macrophages present in the stratum radiatum, exhibited some intriguing morphological features. Indeed, they appeared perfectly parallel, and consequently aligned on the dendritic processes of the pyramidal neurons of CA1. Such an anatomical proximity might contribute to the modulation that monocyte-macrophages could exert on synaptic activity and neuroplasticity, via the release of cytokines, for example.

To conclude, the identification of CD68 exclusively in monocytes-macrophages in rats offers the unique opportunity to generate transgenic rats that will make easier the functional analysis of these cells in epileptogenic conditions.

ACKNOWLEDGMENTS

We thank Théo Elia for his assistance in confocal microscopy and image analysis.

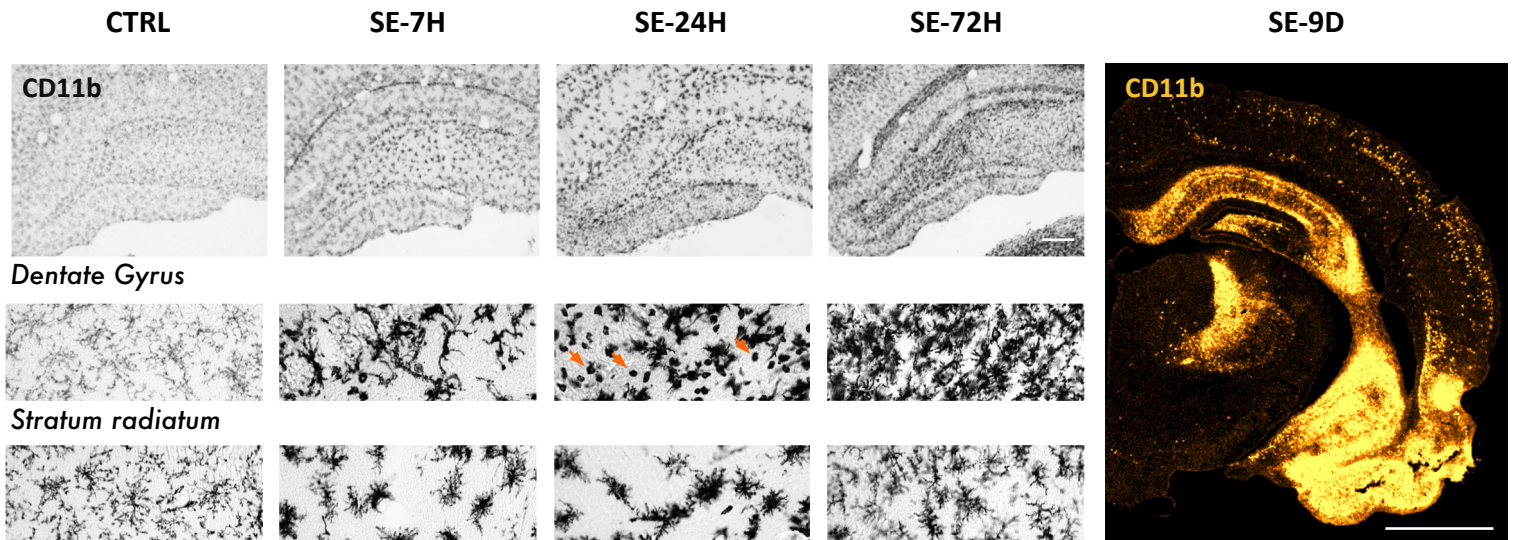


Figure 1. Time-course of CD11b activation after pilocarpine-induced SE. Immunohistochemical or immunohistofluorescence detection was performed in rat brain sections using a specific antibody directed against CD11b for microglia/macrophages. Different stages of epileptogenesis (SE-7H: 7 hours post-SE; SE-24H: 24 hours post-SE; SE-72H: 72 hours post-SE; SE-9D: 9 days post-SE) after pilocarpine-induced SE are compared to controls (CTRL). Orange arrowheads indicate CD11b+ “round-shaped cells”, resembling monocyte infiltrates. Scale bar: CTRL, SE-7H, SE-24H, SE-72H: 200 μm ; SE-9D: 2,000 μm .

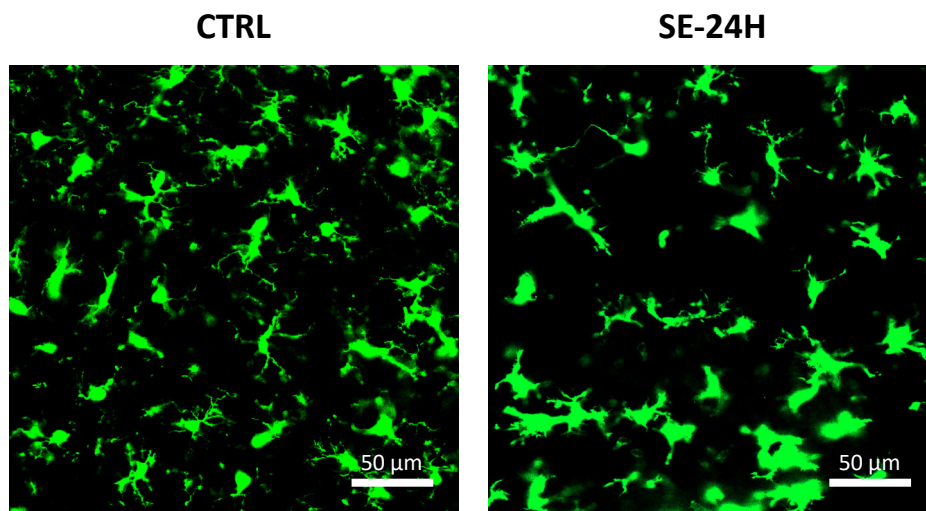


Figure 2. Only resident microglial cells express Iba-1 marker 24 hours after pilocarpine-induced SE. Immunohistofluorescence detection was performed in rat brain sections using a specific antibody directed against Iba-1 for microglia.

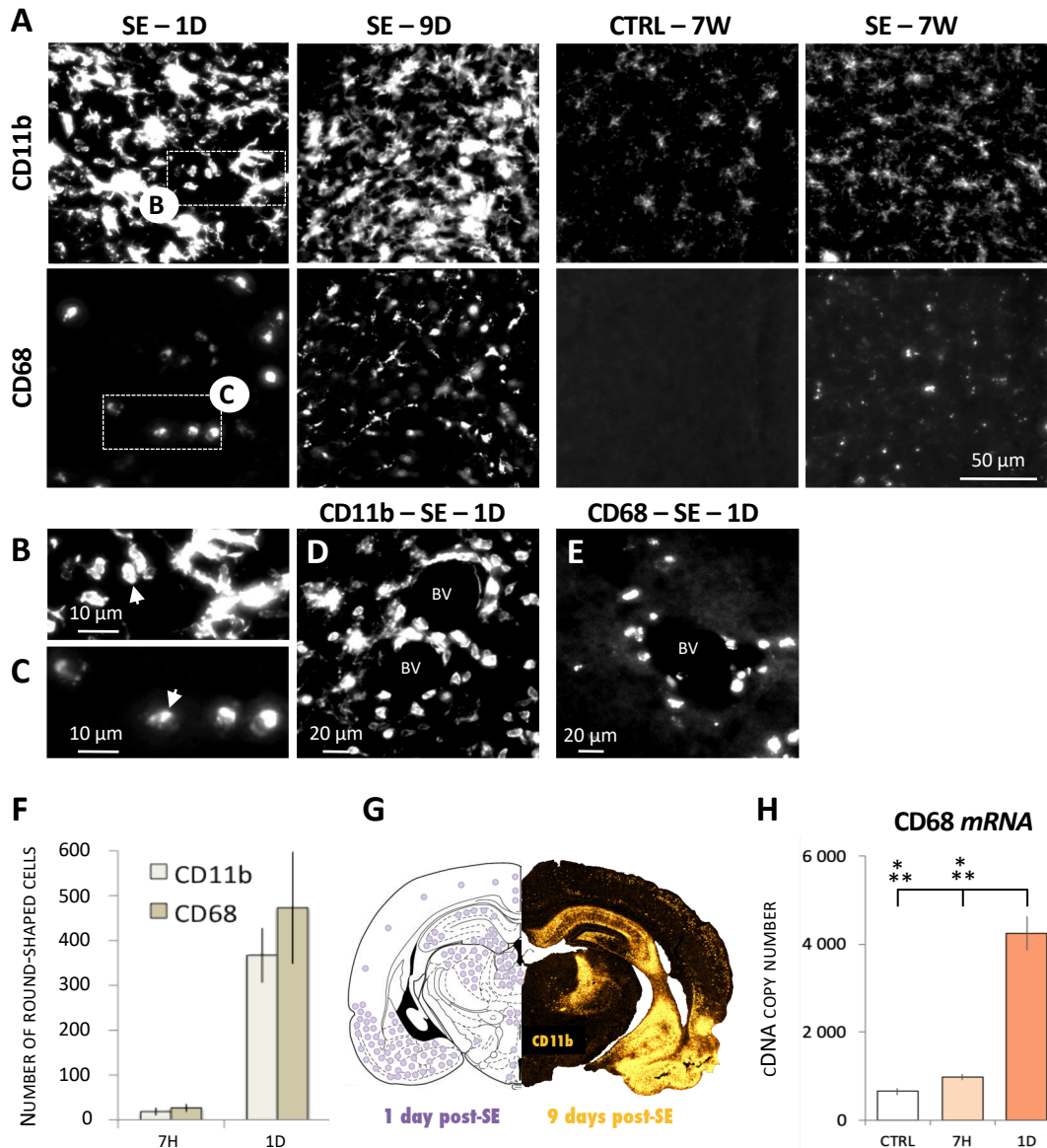


Figure 3. Time-course of monocyte infiltration and transdifferentiation in the hippocampus after pilocarpine induced-SE. Immunofluorescence detection was performed in rat brain sections using specific antibodies directed against CD11b for microglia/macrophages (A-B) and CD68 for monocytes/macrophages (A-C). Different stages of epileptogenesis (SE-1D: 1-day post-SE; SE-9D: 9 days post-SE) or chronic epilepsy (SE-7W: 7 weeks post-SE) after pilocarpine-induced SE are compared to controls. Numerous CD11b+ (D) and CD68+ (E) round-shaped cells are observed in or around the blood vessels at 1 day after pilocarpine-induced SE. (F) Quantification of the number of CD11b+ and CD68+ round-shaped cells on brain sections collected 7 hours post-SE (n=5) and 1-day post-SE (n=5). The number of these cells measured at 1-day post-SE are significantly ($p < 0.001$) higher than those detected at 7h post-SE for both the CD11b and CD68 markers. (G) CD11b+ or CD68+ round-shaped cells detected 24 hours post-SE (purple circles) are localized in brain areas exhibiting intense CD11b-immunolabeling 9 days post-SE. (H) Transcript values of CD68 in the hippocampus are given during epileptogenesis at 7 hours (n=6) and 1 day (n=6) post-SE, compared to controls (n=6). Tukey's *post-hoc* analysis following one-way ANOVA: *** $p < 0.001$. Abbreviation: BV, blood vessels.

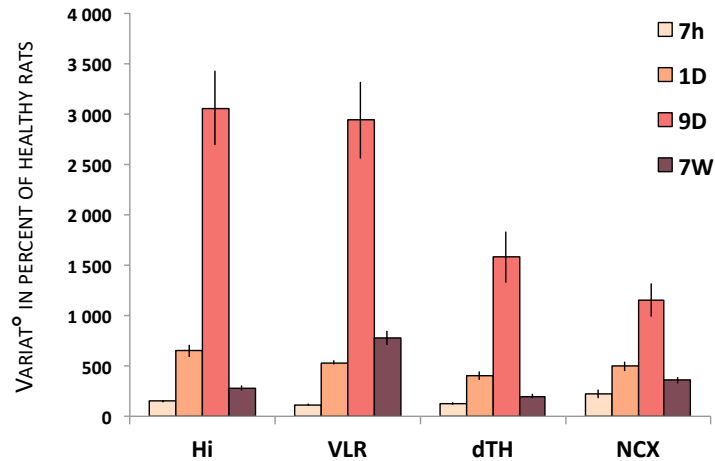
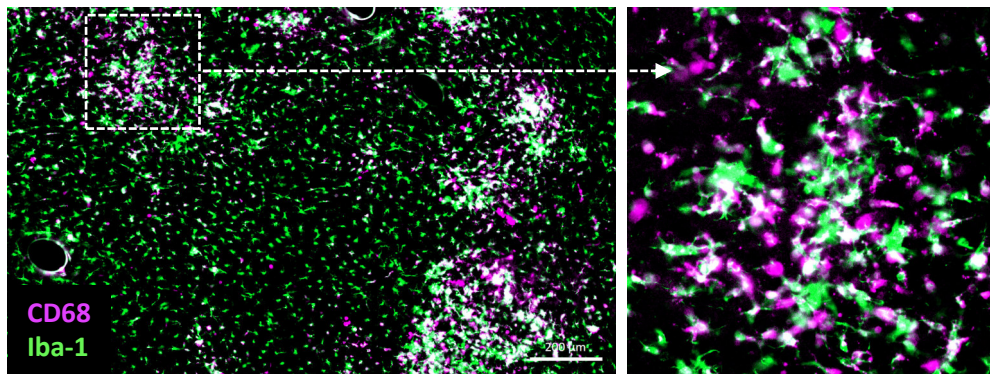
A- CD68 mRNA level from 7h to 7W post-SE**B-** Iba-1 and CD68 double immunofluorescence detection

Figure 4. CD68 expression from epileptogenesis to epilepsy in 4 brain regions colonized by monocyte infiltrates following pilocarpine-induced SE. (A) Transcript levels of CD68 were measured in the hippocampus (Hi), the ventral limbic region (VLR), the dorsal thalamus (dTH) and the neocortex (NCX) of rats at different stages following SE: 7 hours (7H, n=6), 1 day (1D, n=6), 9 days (9D, n=7) and 7 weeks (7W, n=8). Results are expressed as the percentage of variation of respective controls (same controls for 7H, 1D and 9D; n=6; n=6 for 7 weeks) \pm SEM. (B) Representative image of double immunofluorescence detection of microglial-specific Iba-1 marker and of monocyte/macrophage specific CD68 marker in the dorsal thalamus of rats at 9 days post-SE. Immunohistochemical detection was performed in rat brain sections using specific antibodies directed against Iba-1 and CD68. At 9 days post-SE, numerous CD68+ cells express Iba-1, bearing morphological features of microglial cells.

CLODRONATE-INDUCED DEPLETION OF CIRCULATING MONOCYTES PRIOR TO LABELING WITH FLUORESBRITE YG (FYG) CARBOXYLATE MICROSPHERES

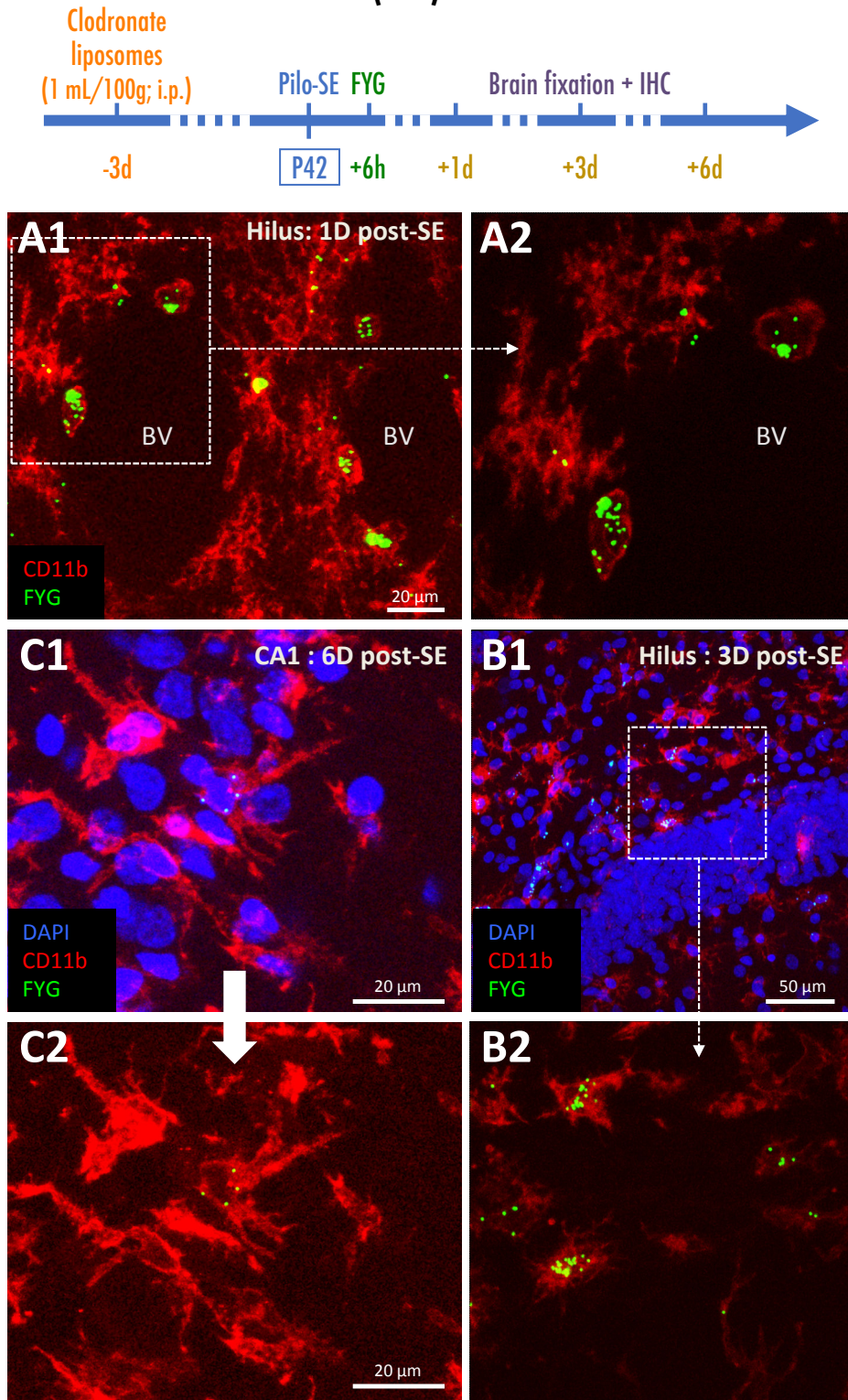


Figure 5. FYG-labeled monocytes infiltrate the hippocampus following Pilo-SE and transdifferentiate into brain monocyte-macrophages bearing morphological features of resident microglial cells. Fluoresbrite YG carboxylate microspheres (0.5 μm diameter) were injected to the tail vein (9.1×10^{10} particles / rat) 6 hours post-SE induced by pilocarpine. No labeled monocyte could be seen in the brain parenchyma unless circulating monocytes were depleted with clodronate liposomes (1 mL/100g; i.p.) administered 3 days prior to SE. Rats were sacrificed 1D, 3D and 6D post-SE. **Figures A-C** illustrate the detection of CD11b (Red) and FYG (green) in numerous circulating monocytes, some of them undergoing extravasation, at 1 day post-SE in the hilus (**A**). FYG were then detected in brain monocyte-macrophages extending processes at 3 days post-SE in the hilus (**B**). Some cells resembling activated microglial cells were also observed in CA1 at 6 days post-SE (**C**). **A2** and **B2** are magnifications of **A1** and **B1**, respectively. **C2** is a representation of **C1** without DAPI-stained nuclei.

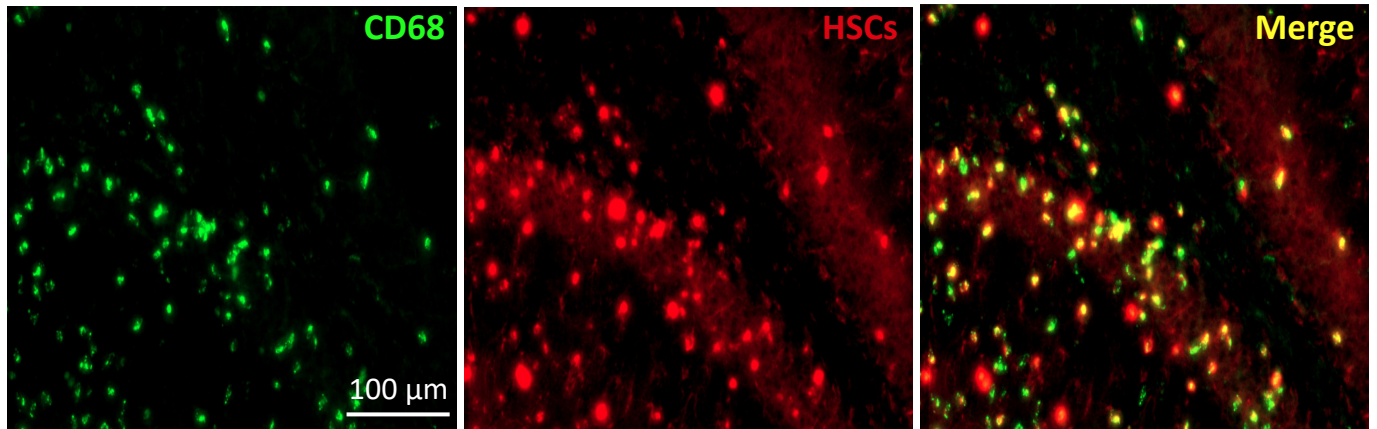
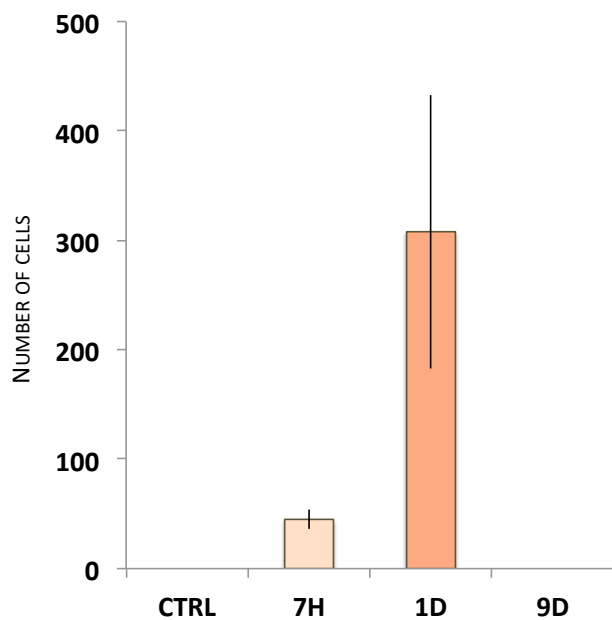
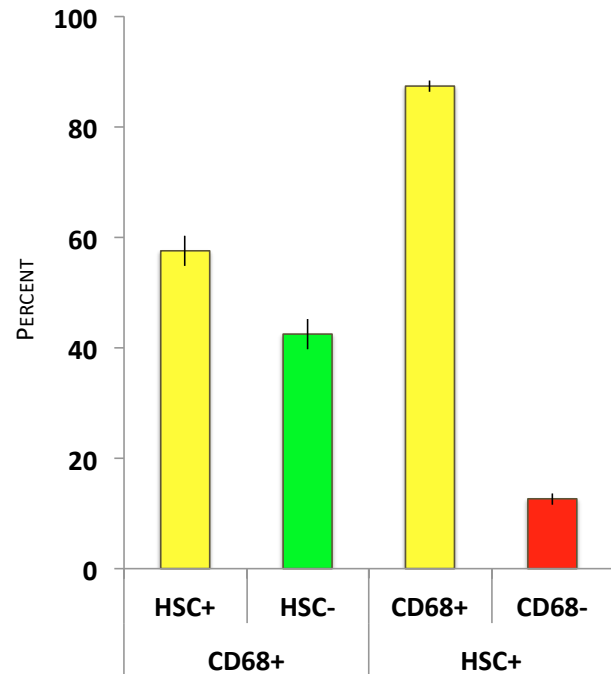
A- Dentate Gyrus: 1 day post-SE**B-** Cells expressing HSCs: 1 day post-SE**C-** Double immunolabeling of CD68 and HSC in the whole dorsal hippocampus: 1 day post-SE

Figure 6. Heparan sulfate chains (HSCs) are lost by CD68-positive round-shaped cells shortly after they infiltrated brain parenchyma following pilocarpine-induced SE (post-SE). (A) Representative image providing evidence that numerous cells with HSCs (red) express CD68 (green) in the hippocampus 24 hours after pilocarpine-induced SE. None were detected in controls. (B) Time-course of cells expressing HSCs in the dorsal hippocampus at 7 hours (n=6), 1 day (n=8), 9 days (n=6) compared to controls (n=3). Three consecutive sections for each rat were selected at IA 4.84 mm according to Paxinos and Watson (1998), the number of cells was counted in the whole dorsal hippocampus of each section and then was averaged for each rat. Results are expressed as the mean number of cells per section \pm SEM. (C) Percentage of cells double labeled for CD68 and HSCs at 1 day post-SE (n=8).

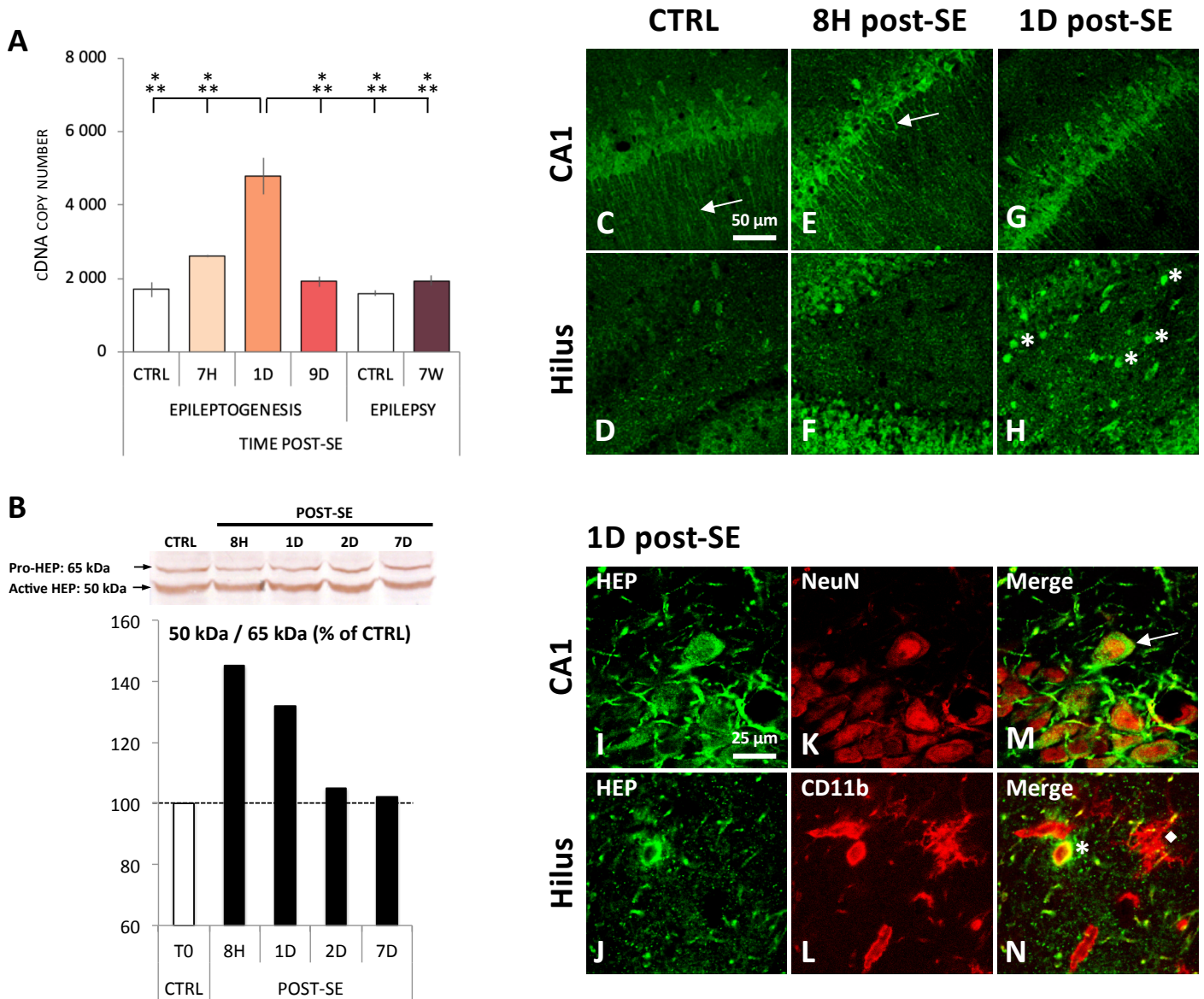


Figure 7. Heparanase expression in the rat hippocampus following pilocarpine-induced SE. (A) Time course of heparanase-mRNA quantified by RT-qPCR in the hippocampus at 7h, 1 day (1D), 9D and 7 weeks (7W) post-SE. (B) Western-blot detection of heparanase protein at different time points following post-SE. The precursor (65 kDa) and the active (50 kDa) forms of are presented, and the 50 kDa / 65 kDa ratio has been calculated. (C-H) Immunoreactivity of heparanase (as revealed by A-488 fluorescence) in CA1 (C,E,G) and the hilus (D, F, H) at t0 (control rats, C-D), 8h (E-F) and 1 day (G-H) post-SE. Arrows indicate heparanase detected in processes of CA1 pyramidal neurons; asterisks are positioned on the right of round-shaped cells resembling leukocyte infiltrates. (I-N) detection of heparanase together with the neuronal marker NeuN (I, K, M) in CA1 or the microglial/macrophage marker CD11b (J, L, N) in the hilus, 1 day post-SE. The arrow points out one neuron with somatic expression of heparanase; the asterisk is positioned at the right of a CD11b-positive round-shaped cell, resembling leukocyte infiltrates compared to the activated resident microglial cells located on the left of the diamond. Abbreviations: CTRL, controls; D, day; HEP, heparanase; SE, *status epilepticus*.

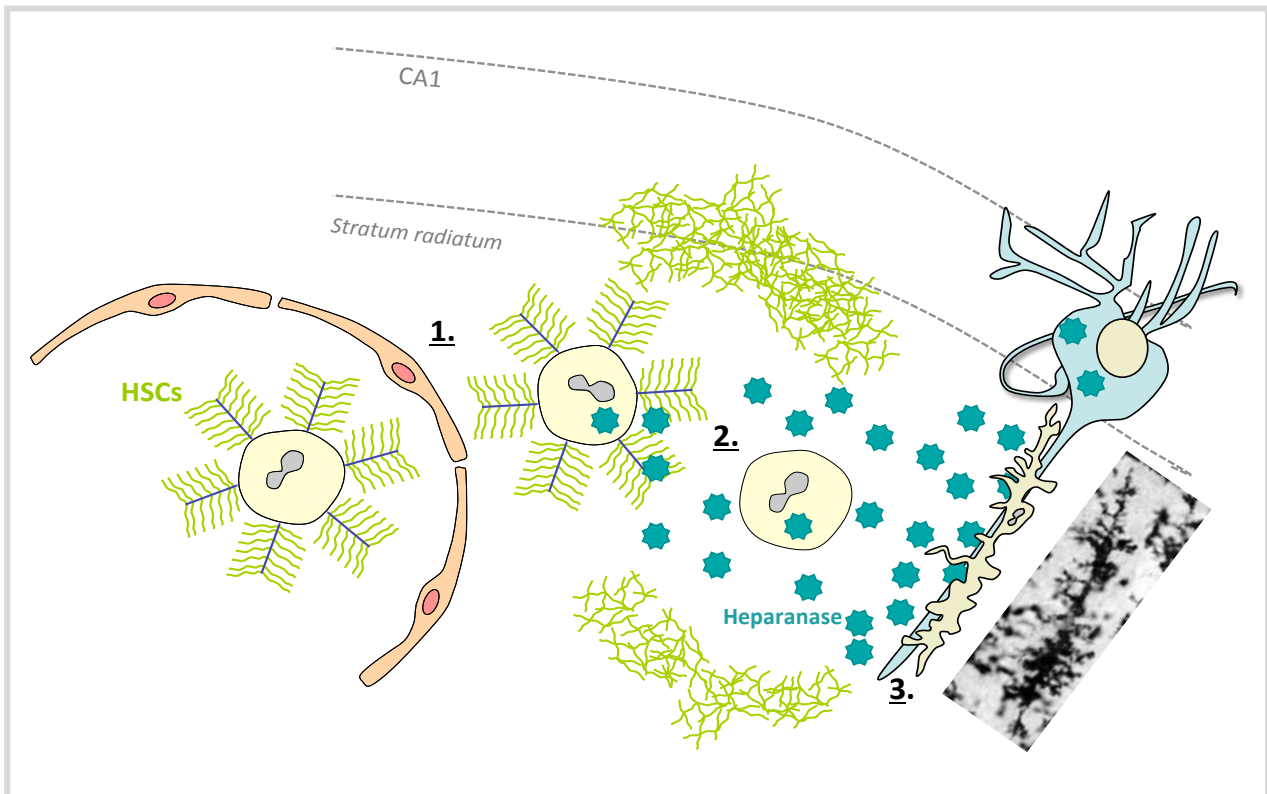


Figure 8. Proposed mechanism of integration of round-shaped monocytes with heparan sulfate chains (HSCs) into the hippocampus after pilocarpine-induced SE. (1) Round-shaped monocytes with HSCs on their surface infiltrate the brain parenchyma shortly after SE, with a maximum infiltration peak observed at 1 day post-SE. (2) Once the cells integrate the brain parenchyma, they contribute to the production of heparanase, a HSC-degrading enzyme that is constitutively produced by neurons. (3) Following the degradation of HSCs by heparanase, the migration of monocytes into the tissue is promoted and the monocytes then begin their transdifferentiation processes into monocyte-macrophages with morphological features of activated microglial cells. Based on morphological features of some CD11b⁺ microglial cells present in the *stratum radiatum* at late stages of epileptogenesis, we propose that they indeed correspond to monocyte-macrophages establishing close anatomical relationships with apical dendrites of pyramidal neurons to modulate synaptic activity and neuronal plasticity, likely mediated by inflammatory signaling molecules.

5 | REFERENCES

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BOX 7 | HIGHLIGHTS OF STUDY 2

- CD68 appears as a specific marker of infiltrating monocytes in the brain parenchyma after pilocarpine-induced SE in rats, allowing differentiation of residential microglial cells from infiltrating monocytes.
- Round-shaped CD68+ monocytes infiltrating the cerebral parenchyma after SE change morphology in the days following SE to initiate transdifferentiation into monocyte-macrophages bearing morphological and phenotypic characteristics of microglial cells.
- CD68+ monocytes are present in the cerebral parenchyma up to 7 weeks post-SE, demonstrating integration of these cells into the microglial cell network.
- Following SE, the migration of monocytes invading the brain tissue would be driven by the degradation of the heparan sulfate chains (HSC) present on their surface by the enzyme heparanase, whose production would mainly be initiated by the monocytes themselves to promote their own tissue migration.

CHAPTER 4

Study 3

DOES THE ORIGIN OF SPRAGUE-DAWLEY RATS MATTER WHEN
STUDYING NEUROINFLAMMATION FOLLOWING PILOCARPINE-
INDUCED STATUS EPILEPTICUS?

Comparison of the inflammatory response to pilocarpine-induced *status epilepticus* in Sprague-Dawley rats from different origins

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ABSTRACT

Neuroinflammation during epileptogenesis and during chronic phase of epilepsy has been extensively studied using outbred Sprague-Dawley (SD) rats. Environmental factors such as breeding conditions in the supplier's animal husbandries and housing conditions in the laboratory, are known to play a role in the phenotype of animals. In addition to environmental factors, genetic variations in outbred animals can randomly fluctuate between the different supplier's colonies. Here, we determined whether the inflammatory profile was similar between SD rats originating from Harlan/Envigo (HAR/ENV) and from Charles River Laboratories (CRL) following pilocarpine-induced SE (Pilo-SE). Pro- and anti-inflammatory mediators, chemokines, and cell markers were quantified by RT-qPCR at different time points, from 7 hours (acute response) to 7 weeks (chronic epilepsy) after SE. Glial cell activation and neurodegeneration were evaluated by immunohistochemistry. While our previous studies focused exclusively on the hippocampus, we explored in a first part the inflammatory response of 3 other brain structures (the ventral limbic region, the dorsal thalamus and the neocortex), known to be differentially affected by neurodegeneration. We show that the severity of neuronal damage is independent of the magnitude of the pro-inflammatory and anti-inflammatory response to Pilo-SE induced in weaning and juvenile rats. In a second part, we compared the hippocampal inflammatory response between CRL and HAR/ENV SD rats. CRL SD rats were found to be much more sensitive to Pilo-SE, that had to be stopped by 1h, instead of 2h in HAR/ENV rats to decrease mortality. Greater neurodegeneration was observed in CRL SD rats compared to HAR/ENV rats, while their pro-inflammatory response was lower and their anti-inflammatory response stronger than that of HAR SD rats. The glial activation was surprisingly more consistent between sub-strains. Overall, our findings highlight that, if not verified, the origin of SD rats used for investigating the pathophysiological mechanisms underlying epilepsy, in particular the neuroinflammatory response to epileptogenic insults, may conduct to misleading conclusions.

1 | INTRODUCTION

Mesial temporal lobe epilepsy (TLE) is the most commonly diagnosed form of human epilepsy (Engel, 2001) in adulthood that occur frequently after an acute brain injury such as cerebrovascular accident, infections, traumatic brain injury or status epilepticus (SE) (Klein et al., 2018). This form of epilepsy also has the highest number of drug-resistant patients, justifying the many experimental studies aimed at finding new therapeutic targets and the necessity to have adequate models. An accurate TLE model must reproduce the classic pattern of epilepsy development, namely the initial epileptogenic insult, followed by a latent period that leads to chronic hyperexcitability and by a subsequent period of chronic recurrent and spontaneous seizures (Kandratavicius et al., 2014; Lévesque et al., 2016; Becker, 2018). One of the most commonly used model of TLE is that induced by pilocarpine, a muscarinic acetylcholine receptor agonist, usually injected systemically or intracerebrally, which triggers seizures that developed into a limbic status epilepticus (SE) (Furtado et al., 2002; Curia et al., 2008). Pilocarpine-induced experimental epilepsy reproduces both the clinical and neuropathological features of human TLE. A large majority of preclinical studies in epilepsy use rodents, and more particularly rats, to model the pathophysiology of epilepsy. While studies conducted in mice use more commonly inbred strains, preclinical investigations conducted in rats are rather performed with outbred strains. A few studies were conducted to investigate the differences in research findings obtained depending on the vendors from whom the rats were purchased or even the breeding location of Wistar or Sprague-Dawley (SD) rats, the two most widely used outbred rats in animal research (West et al., 1993; Oliff et al., 1995; Portelli et al., 2009; Langer et al., 2011; Fitzpatrick et al., 2013; Brandt et al., 2016; Kristensen et al., 2017; Hestehave et al., 2019). Phenotype differences have been observed between outbred SD rats from Harlan Laboratories and Charles River Laboratories (Brandt et al., 2016; Pecoraro et al., 2006; Turnbull and Rivier, 1999). This high intrastrain phenotypic variation is explained by genetic heterogeneity as well as by the environmental conditions under which the animals are bred and maintained (Festing, 1993, 2010; Löscher et al., 2017). In rats experimental models of epilepsy, differences in results were also observed according to the origin of the supplier in parameters such as SE induction sensitivity, recurrent and spontaneous seizures susceptibility, epileptogenesis processes, behavioral alterations, neurodegeneration and hippocampal damage (Brandt et al., 2016; Honndorf et al., 2011; Langer et al., 2011; Löscher et al., 2017; Portelli et al., 2009).

Pilocarpine-induced SE in rats has been used routinely in the laboratory for nearly 15 years (Fabrice Navarro y Garcia, 2007) to assess how neuroinflammation at the molecular and cellular level underlies the development of epilepsy and how it is involved in disease-related disorders. The role of neuroinflammation in the pathophysiology of epilepsy is now well established and have been highlighted by numerous clinical and preclinical studies (Vezzani et al., 2011; de Vries et al., 2016). All of our studies before 2015 have been performed on SD rats originating from Harlan Laboratories (HAR), and then from Envigo (ENV) when Harlan Laboratories merged with Huntingdon Life Sciences in 2015 to become Envigo. In our previous studies performed on the hippocampus, we showed that the inflammatory response peaked 7 hours after SE in SD rats (study #1), and we provided evidence that circulating monocytes infiltrated the brain parenchyma between 8 and 48 hours after SE and then rapidly transdifferentiated into microglial-like brain monocyte-macrophages (mo-MP) that maintain CD68 expression in the very long-term, making it possible to distinguish them from resident microglial cells (study #2).

The role played by mo-MPs is widely debated, with some suggesting that these cells may enhance the deleterious inflammatory response in the acute phase after SE for example (Vinet et al., 2016; Varvel et al., 2016), while others suggest a more beneficial role for monocyte infiltrates on granule cell layer integrity (Zattoni et al., 2011) and seizure burden (Waltl et al., 2018). Recent evidence in mice that infiltrating monocytes and the mo-MPs derived from them are distinct from resident microglial cells and adopt a M2-like phenotype beneficial for post-stroke tissue remodeling (Kronenberg et al., 2018) reopens the debate as to the role these cells may play in models of epilepsy. The question is all the more important since, unlike in mice (Vinet et al., 2016; Varvel et al., 2016), we were unable to show 24 hours after SE in HAR/ENV SD rats that IL1 β -mRNA concentration was higher in cells bearing morphological features of infiltrating monocytes than in resident microglial cells (study #1). Further study of the function that these cells could play in the different phases of the disease requires access to transgenic animals allowing to control specifically in these cells the time from which they will express a fluorescent protein to follow their fate after their infiltration in brain parenchyma on the one hand, in combination with a receptor allowing to control either their activation, their inhibition or their death, on the other hand.

Most of the companies that create transgenic rats on a Sprague-Dawley genetic background mainly use the strain from CRL, with delays being greatly extended when another strain is desired, such as the one from ENV. In order to ensure our choice of rat supplier (ENV versus CRL), we first had to extend beyond the hippocampus, within brain regions more or less affected by neurodegeneration, the characterization of the inflammatory response following pilocarpine-induced SE in ENV SD rats, and, secondly, to verify that similar results were obtained in CRL SD rats, first and foremost in the hippocampus. To meet the first objective, SE was induced in juvenile rats aged of 42 days (P42), because, as in temporal lobe epilepsy in humans, SE-induced neurodegeneration at that age can be either massively observed in the ventral limbic region (that includes the amygdala, the piriform cortex and the insular granular cortex (Sanchez et al., 2009)) and the dorsal thalamus, or barely detectable in the neocortex (Bertram, 2009; Bertram and Scott, 2000; Curia et al., 2008; Sanchez et al., 2009; Saniya et al., 2017; Turski et al., 1983; Voutsinos-Porche et al., 2004). For the second objective, SE was induced in P42 CRL SD rats and the time-course of the inflammatory response was measured at both molecular and cellular level from 7 hours to 7 weeks post-SE, for comparison with that of HAR/ENV SD rats. To our knowledge, this study is the first to highlight differences in the inflammatory response following pilocarpine-induced SE within a same strain originating from two suppliers.

2 | MATERIAL AND METHODS

Study Design

Study 1a. Evaluation of pro-inflammatory, anti-inflammatory and cell inflammation indexes in the ventral limbic region, the dorsal thalamus and the neocortex of HAR rats during epileptogenesis and chronic epilepsy. Pilocarpine-induced SE was induced in weaning (W) at P21 or juvenile (J) rats at P42. The different brain regions were dissected after transcardial perfusion of NaCl and the inflammatory profile was evaluated by RT-qPCR. Analysis was performed in rats sacrificed at different time points after SE: during epileptogenesis, that is at 7 hours (W, n=7 ; J, n=6), 1 day (W, n=8; J, n= 6), 9 days (W, n=10 ; J, n=7) post-SE, and once chronic epilepsy was developed in all rats, i.e. 7 weeks post-SE (W, n=8 ; J, n=8). Brains of control rats were also collected; however, to reduce the number of animals used, some time points have been pooled: W rats (7h and 1 day: n=5 ; 9 days: n=5 ; 7 weeks: n=6) and J rats (7h and 1-9 days: n=6 ; 7 weeks: n=6).

Study 1b. Astroglial and microglial activations evaluated using GFAP- and ITGAM-immunofluorescent detections, respectively, in the brain of HAR/ENV rats at 1 day (W, n=4 ; J, n=5), 9 days (W, n=6 ; J, n=7) and 7 weeks post-SE (W, n=6 ; J, n=7), induced in W and J rats, and in respective controls (W, n=3 for 1-9 days, n=5 for 7 weeks; J, n=5 for both 1-9 days and 7 weeks).

Study 2a. Evaluation of gene expression at transcript level in the rat hippocampus of CRL rats during epileptogenesis and chronic epilepsy. SE was induced in Sprague-Dawley rats from CRL at P42. Hippocampus of rats were dissected after transcardial perfusion of NaCl and the inflammatory profile was evaluated by RT-qPCR. Analysis was performed in rats sacrificed at different time points after SE: during epileptogenesis, that is at 7 hours (n=9), 1 day (n=5), and 9 days (n=5) post-SE, and once chronic epilepsy was developed in all rats, i.e. 7 weeks post-SE (n=8). Due to the many fatal respiratory arrests observed in CRL rats when the SE lasted more than 55-60 min, some rats underwent shorter (45 min) SE. Thus, at 7 hours post-SE we collected brains from CRL rats that underwent a 45 min-SE (7H-SE45: n=5) and a 60 min-SE (7H-SE60: n=4). For the brains collected at 1 day, 9 days and 7 weeks, all CRL rats underwent a 60-minute SE. Brains of control rats were also collected; however, to reduce the number of animals used, some time points have been pooled: 7h and 1-9 days: n=4 ; 7 weeks: n=6.

Study 2b. Astroglial and microglial activations evaluated using GFAP-, ITGAM- and Iba-1-immunodetection in CRL rat hippocampus at 7 hours (n=5), 1 day (n=4), and 9 days (n=3), and in controls (n=3).

Study 3. Neuronal degeneration evaluated in the rat hippocampus of CRL and HAR/ENV rats using NeuN-immunohistochemical or immunofluorescent detections, respectively, at 7 hours (CRL, n=5), 1 day (CRL, n=4 ; HAR/ENV, n=8), and 5 or 9 days (CRL, n=3 ; HAR, n=6) after pilocarpine-induced SE, and in respective controls (CRL, n=3, HAR/ENV, n=3).

Animals

All animal procedures were in compliance with the guidelines of the European Union (directive 2010-63), taken in the French law (decree 2013/118) regulating animal experimentation, and have been approved by the ethical committee of the Claude Bernard Lyon 1 University (protocol # BH-2008-11). Male Sprague-Dawley rats were purchased from Harlan/Envigo Laboratories (HAR/ENV) or Charles River Laboratories (CRL). Data on CRL rats were obtained

from rats purchased in 2018, while data on HAR/ENV rats were obtained from tissue and RNA banks generated by the TIGER team between 2009 and 2012.

The rats were housed in a temperature-controlled room ($23 \pm 1^\circ\text{C}$) under diurnal lighting conditions (lights on from 6 a.m to 6 p.m). Pups arrived at 21 day-old and were maintained in groups of 5 in 1,800 cm² plastic cages, with free access to food and water. After SE, rats were maintained in individual cages and weighed daily until they gained weight. Epileptic rats from 15 days post-SE until sacrifice were housed in groups of 5 in standard cages as well as control rats.

Pilocarpine-induced status epilepticus (SE)

SE in Harlan Laboratories rats

SE was induced by pilocarpine, injected at day 21 or 42. To prevent peripheral cholinergic side effects, scopolamine methylnitrate (1 mg/kg in saline, s.c.; Sigma-Aldrich) was administered 30 min before pilocarpine hydrochloride (25 mg/kg at P21 and 350 mg/kg at P42, in saline, i.p.; Sigma-Aldrich). For P21 rat pups, lithium chloride (127 mg/kg in saline, i.p.; Sigma-Aldrich) was injected 18 hours before scopolamine. After 30 min of continuous behavioral SE at P21 and 2 hours at P42, 10 mg/kg diazepam (i.p.; Valium; Roche®) was injected, followed, 90 min later for P21 and 60 min later for P42, by a second injection of 5 mg/kg diazepam to terminate behavioral seizures. Control rats received systematically corresponding injections of saline solution. The animals were then sacrificed at various time points: 7 hours, 1 day, 9 days and 7 weeks after SE.

SE in Charles River Laboratories rats

SE was induced by pilocarpine injected at day 42, and preceded by scopolamine, as described above. After 45- or 60-minute of continuous behavioral SE, 10 mg/kg diazepam (i.p.; Valium; Roche®) was injected, followed, 60 min later, by a second injection of 5 mg/kg diazepam to terminate behavioral seizures. In CRL rats, tonic-clonic seizures developed from the 60th minute of SE were consistently followed by fatal respiratory arrest; for this reason, diazepam injection was never given beyond this limit. Control rats received systematically corresponding injections of saline solution. The animals were then sacrificed at various time points: 7 hours, 1 day and 9 days after SE.

Animal care after SE

Control and treated rats were weighted every day during the first two weeks following SE, and then every week until termination of the experiment. Daily abdominal massages were performed twice a day during the first week to activate intestinal motility, which was disrupted following SE. All efforts were made to minimize pain or discomfort of the animals used.

Onset of handling-induced seizures

Electroencephalographic recordings were excluded to determine epilepsy onset due to preliminary experiments that showed that the sole implantation of screws into the skull induced significant and lasting inflammation over time in the cortex and, to a lesser extent, in the hippocampus. As a result, epilepsy onset was determined according to clinical criteria. Therefore, animals were tested for the occurrence of handling-induced seizures (HIS) three times a day between the 1st and the 5th week post-SE. HIS were triggered by restraining rats for 10 seconds at the level of the chest with gentle pressure. Animals were declared as “epileptic” (EPI) once they developed HIS on 2 consecutive trials. By the end of the 5th week post-SE, all rats were considered as EPI.

Ex Vivo Procedures

All rats were deeply anesthetized with a lethal dose of pentobarbital (100 mg/kg; Dolethal) before being sacrificed. The different brain regions (hippocampus, VLR, dorsal thalamus and neocortex) were rapidly microdissected, frozen in liquid nitrogen, and stored at -80°C. For immunochemistry analysis, animals were transcardially perfused (30 mL/min) with 4% paraformaldehyde in 0.1 M phosphate buffer. After cryoprotection in 30% sucrose, brains were frozen at -40°C in isopentane and stored at -80°C.

RNA extraction and quantification of transcript level variations by reverse transcriptase real-time polymerase chain reaction (RT-qPCR)

Brain structures frozen in liquid nitrogen were crushed using Tissue-Lyser (Qiagen®) in 250 µL of ultrapure RNase-free water (Eurobio). Nucleic acids were extracted by adding 750 µL Tri-Reagent LS (TS120, Euromedex) and 200 µL chloroform (VWR®). After precipitation with isopropanol (I-9516, Sigma-Aldrich®), washing in 75% ethanol (VWR) and drying, total nucleic acids were resuspended in 50 µL ultrapure water and treated with DNase I (Turbo DNA Free® kit; AM1907, Ambion®) to eliminate any trace of possible genomic DNA contamination. The

purified total RNAs were then washed using the RNeasy[®] minikit (Qiagen[®]) kit. After elution, the total RNA concentration was determined for each sample on BioDrop[®] μ Lite. The quality of total RNAs was verified on microgel chips using LabChip[®] 90 (Caliper), which provides an RNA Integrity Number (RIN) value by analyzing the integrity of two ribosomal RNAs (18S and 28S) predominantly present in all tissue RNA extracts. All selected samples had a RIN value greater than 7.0, and were stored at -80°C until use. Total tissue RNAs (480 ng) were reverse transcribed to complementary DNA (cDNA) using both oligo dT and random primers with PrimeScript RT Reagent Kit (Takara) according to manufacturer's instructions, in a total volume of 10 μ L. In RT reactions, 300 000 copies of a synthetic external non-homologous poly(A) standard messenger RNA (SmRNA; A. Morales and L. Bezin, patent WO2004.092414) were added to normalize the RT step (Sanchez et al., 2009). cDNA was diluted 1:13 with nuclease free Eurobio water and stored at -20°C until further use. Each cDNA of interest was amplified using 5 μ L of the diluted RT reaction by the "real-time" quantitative polymerase chain reaction (PCR) technique, using the Rotor-Gene Q thermocycler (Qiagen[®]), the SYBR Green Rotor-Gene PCR kit (Qiagen[®]) and oligonucleotide primers specific to the targeted cDNA. The sequences of the specific forward and reverse primer pairs were constructed using the Primer-BLAST tool or using the "Universal Probe Library" software (Roche Diagnostics). Sequences of the different primer pairs used are listed in Table S1. The number of copies of each targeted cDNA contained in 5 μ L of the diluted RT reaction was quantified using a calibration curve based on cascade dilutions of a solution containing a known number of cDNA copies.

Pro-inflammatory (PI-I), anti-inflammatory (AI-I) and inflammation cell (IC-I) indexes were calculated for each series of individuals to be compared using a specific set of genes: IL1 β , IL6, TNF α , MCP1 and MIP1 α for PI-I; IL4, IL10 and IL13 for AI-I; ITGAM and GFAP for IC-I. For each individual, the number of copies of each transcript has been expressed in percent of the averaged number of copies measured in the whole considered population of individuals. Once each transcript is expressed in percent, an index is calculated by adding the percent of each transcript involved in the composition of the index and expressed in arbitrary units (A.U.). Each time that an index is presented, the groups of individuals constituting the population is specified.

Tissue processing for histological procedures

Cryostat-cut (40 µm thick) sections from rat samples were transferred into a cryopreservative solution composed of 19.5 mM NaH₂PO₄·2H₂O, 19.2 mM NaOH, 30% (v/v) glycerol and 30% (v/v) ethyleneglycol and stored at -25°C.

Colorimetric immunohistochemistry

Free-floating sections (40 µm thick) from paraformaldehyde-fixed tissue were incubated either with a mouse polyclonal anti-GFAP antibody (1:1000; G3893; Sigma) to label astrocytes, a mouse monoclonal anti-ITGAM (1:1000; CBL1512Z, Chemicon) to detect microglia and immunocompetent cells or a mouse monoclonal anti-NeuN antibody (1:1000; MAB-377, Chemicon) to detect neurons. After washes, sections were then incubated with a biotinylated donkey antibody raised against mouse IgG antibody (1:1000; 715-065-151, Jackson ImmunoResearch). After washes, sections were incubated with avidin biotin peroxidase (1:1000; Vectastain Elite ABC kit, Vector) and reacted with 0.4 mM 3',3-diaminobenzidine (DAB, Sigma Fast). Sections were then mounted, dehydrated and coverglassed in DPX (Fluka). The immunostained slides were captured using a Carl Zeiss Axio Scan.Z1 Digital Slide Scanner with a resolution of x40. Images were then processed on with ZEN Imaging software (Zeiss) for further editing.

Fluorescent immunohistochemistry

Free-floating sections (40 µm thick) from paraformaldehyde-fixed tissue were incubated with a goat polyclonal anti-Iba-1 antibody (1:500; AB5076, Abcam) to detect microglia; with a rabbit polyclonal anti-GFAP antibody (1:1000; AB5804; Chemicon) to label astrocytes and a mouse monoclonal anti-ITGAM (1:1000; CBL1512Z, Chemicon) to detect microglia and immunocompetent cells; or with a mouse monoclonal anti-NeuN antibody (1:1000; MAB-377, Chemicon) to detect neurons. After washes, sections were incubated with a combination of the following secondary antibody: Alexa-Fluor-488-conjugated donkey anti-goat IgG antibody (1:750; A-11055; Molecular Probes), Alexa-Fluor-488-conjugated donkey anti-rabbit IgG antibody (1:1000; A-21206; Molecular Probes) or Alexa-Fluor-647-conjugated donkey anti-mouse IgG antibody (1:1000; A-31571; Molecular Probes). Sections were then mounted on SuperFrost Plus slides and coverglassed with Prolong Diamond Antifade reagent (Molecular Probes). The immunostained slides were captured using a Carl Zeiss Axio Scan.Z1 Digital Slide

Scanner with a resolution of x40. Images were then processed on with ZEN Imaging software (Zeiss) for further editing.

Data and statistical analysis

GraphPad Prism (v.7) software was used to statistically analyze data. Majority of data are expressed as mean \pm SEM of the different variables analyzed. Statistical significance for within-group comparisons was calculated by one-way or two-way analysis of variance (ANOVA) with Bonferroni or Tukey's post hoc test. The p value of 0.05 defined the significance cut-off.

3 | RESULTS

PART 1

Time-course of the inflammatory response in the ventral limbic region (VLR), the dorsal thalamus (dTH) and the neocortex of the Harlan Sprague-Dawley rat are different from that observed in the hippocampus

The first study of this thesis investigated in-depth the extent of the inflammatory response measured in the hippocampus after pilocarpine-induced SE in HAR/ENV rats at 21 (SE-W) or 42 days (SE-J). In order to assess whether the time-course of the inflammatory response was similar in the other brain structures affected in TLE (i.e. the ventral limbic region (VLR), the dorsal thalamus and the neocortex) to that initially reported in the hippocampus, we measured the transcript levels of pro- and anti-inflammatory markers as well as cellular markers. To not overburden the manuscript, here we only report the results of the pro-inflammatory, anti-inflammatory and cellular indexes in each of the structures studied in SD-HAR rats, and not the individual results of each of the quantified genes (Fig. 1-3). The inflammatory indexes of the hippocampus, presented in the first study, are also reported here. It is noteworthy that the values reported here in the hippocampus are different from those mentioned in study #1 because the indexes in this study have been calculated by integrating the values of all investigated brain structures.

For the pro-inflammatory index, which integrates the values of IL1 β , IL6, TNF α , MCP1 and MIP1 α mRNA-levels, the apparent peak has always been observed 7 hours post-SE in all brain structures investigated, except the hippocampus and the VLR of rats that underwent SE at P42, whose values remained at a plateau from 7 hours to 1 day post-SE, the values then decreasing between 1 day and 9 days post-SE to reach low-grade values (Fig. 1). Differences

exist between the different structures and the two models (SE induced at P21 (SE-W) or P42 (SE-J)) in the peak height, but no rule can be established between the two models. The induction peak was higher in the hippocampus of SE-J rats, in contrast to the dorsal thalamus where it was higher in SE-W rats. The inflammatory response was similar in the neocortex between the two models. This would also have been the case for the VLR if the values at 1-day post-SE in SE-W rats had remained at the same level as those measured at 7 hours post-SE. The comparison of the peak values of the pro-inflammatory index determined during epileptogenesis with the values measured in age-matched controls reveals that the increase post-SE reached 98-fold in the hippocampus (Fig. 1A), 65-fold in the VLR (Fig. 1B), 23-fold in the dorsal thalamus (Fig. 1C), and 18-fold in the neocortex (Fig. 1D) for rats subjected to SE at weaning (SE-W) and 77-fold (Fig. 1A), 90-fold (Fig. 1B), 61-fold (Fig. 1C) and 85-fold (Fig. 1D), in the hippocampus, the VLR, the dorsal thalamus and the neocortex, respectively, for rats subjected to SE at the juvenile stage (SE-J).

Concerning the anti-inflammatory index, which integrates the values of IL4, IL10 and IL13 mRNA levels, we observed a strong response between 7 hours and 1-day post-SE, in all investigated structures and both SE-W and SE-J models (Fig. 2). We noted that with the exception of the hippocampus, where the calculated values were almost identical between SE-W and SE-J rats, values were consistently higher 7 hours post-SE in SE-J rats in the VLR, the dorsal thalamus and the neocortex. Finally, the highest values were calculated in the VLR and the lowest in the dorsal thalamus.

Finally, for the cell inflammation index, which reflects glial reactivity and integrates the values of GFAP and ITGAM mRNA levels, we observed during epileptogenesis that, in the hippocampus, the index values were higher in SE-J rats compared to SE-W rats, while, surprisingly, in all other brain structures, the value of the inflammation cell index was greater in SE-W rats compared to SE-J rats (Fig. 3). During the chronic phase, a significant difference between epileptic animals and controls persisted in the hippocampus (Fig. 3A), the VLR (Fig. 3B) and the neocortex (Fig. 3D) in SE-J rats only. Besides, when the level of glial reactivity was relatively low in the hippocampus of SE-W rats (Fig. 3A), it was considerably higher in other structures (Fig. 3B-D).

The profiles of the inflammation cell index from epileptogenesis to epilepsy are consistent with the histological observation performed in rat brain sections during epileptogenesis (1 day

and 9 days post-SE) and during the chronic phase of epilepsy (7 weeks post-SE). Indeed, dual immunofluorescence detection of GFAP and ITGAM (CD11b) has been carried out to evaluate astroglial and microglial/macrophage reactivity, respectively (Fig. 4). As described in Study 1 for microglial and astrocyte activation in the hippocampus, we found that reactive gliosis in other brain areas is also model-specific (Fig. 4). Compared to the low signal observed for GFAP and ITGAM under basal conditions, high reactivity of both of these cell markers was observed at 1 day and 9 days post-SE. At 1-day post-SE the reactivity of ITGAM in the VLR appeared to be higher in SE-W rats, while GFAP signal was much higher in SE-J rats (Fig. 4). Glial reactivity in the dorsal thalamus and the neocortex was more pronounced at 9 days post-SE, with a very intense ITGAM signal in both SE-W and SE-J rats. However, it should be noted that pilocarpine-induced SE can be frequently associated with high tissue degradations and swollen and edematous areas (Curia et al., 2008; Turski et al., 1983), as observed in the VLR, especially in SE-J rats, where tissue integrity was severely damaged. Signs of reactive gliosis persisted at 7 weeks post-SE in SE-J rats only, in the VLR, the dorsal thalamus and the hippocampus.

In this first part, we compared two distinct contexts of epileptogenesis, one associated with strong neuronal degeneration (SE-J), the other with a much more discrete degeneration (SE-W). It appears that the pro-inflammatory response following SE was strong to very strong in the different structures investigated, without any clear link being established between the intensity of the pro-inflammatory response and the level of neurodegeneration. Indeed, for example, while tissue integrity did not appear to be affected in the dorsal thalamus of SE-W rats, the level of the pro-inflammatory response measured there was the highest. This observation is all the more surprising since the anti-inflammatory response was lowest in the SE-W rats. In conclusion, the level of the inflammatory response alone cannot explain the vulnerability of brain tissue to the neuronal hyperexcitability following SE.

PART 2

After evaluating the cellular and molecular inflammatory response in the other brain areas of HAR/ENV rats and thus completing the data in the hippocampus presented in Study 1 of this thesis, we evaluated the time-course of the inflammatory response in Charles River (CRL) rats to establish if both rat sub-strains similarly responded to pilocarpine-induced SE. If so, CRL SD rats could be used instead of HAR/ENV rats to generate transgenic rats aimed at tracking monocyte infiltrates and study the function of brain monocyte-macrophages from epileptogenesis to epilepsy. Indeed, most of the companies that generate transgenic rats use SD rats originating from CRL. We therefore characterized from epileptogenesis to chronic epilepsy the extent of molecular and cellular inflammation in the hippocampus of CRL rats after pilocarpine-induced SE at P42, and then compared it to that of HAR/ENV rats.

CRL SD rats are more vulnerable to pilocarpine-induced SE than HAR/ENV SD rats

In CRL rats, SE was associated with continuous limbic and intermittent generalized convulsive seizures, as for HAR rats. However, as early as 60 minutes of SE, many rats were experiencing tonic seizures associated with fatal respiratory arrest. As a result of this major intersubstrain difference, SE duration was shortened to a maximum of 60 min for CRL rats sacrificed at 7 hours, 1 day, 9 days and 7 weeks post-SE. Besides, in an effort to minimize the mortality, we also tested whether a 45-minute SE could be sufficient to induce a peak of inflammatory response similar to that induced by a 60-minute SE (7H-SE-60); therefore some rats were sacrificed at 7 hours post-SE (7H-SE-45, Fig. 5-8).

SE was induced by an intraperitoneal administration of pilocarpine, given at the dose of 350 mg/kg in rats weighing 190-210 g. If SE was not developed during the first 40 min, a second administration of pilocarpine was given subcutaneously, at the dose of 160 mg/kg. The rate of CRL rats that developed SE (class 5 seizure according to Racine's scale) was 73.3 % (44/60) with a rate of mortality was 6.8% (3/44) in the first hour before diazepam injection, and 2/41 rats died within the following days. The fact that we had to reduce SE duration to a maximum of 60 min shows that CRL rats have a higher sensitivity to pilocarpine than HAR/ENV rats. Differences in sensitivity to pilocarpine in rats from different vendors have already been reported in other studies. Brandt et al. (2016) showed intrastrain differences for the induction of SE in Sprague-Dawley rats purchased from two breeding locations of HAR (Germany and Netherlands) or from CRL in Germany. Using a ramp-up protocol for i.p. injection of

pilocarpine, they showed that HAR rats were more prone to develop SE than CRL rats, among which 34% only developed SE. In addition, they noted that mortality rate was below 10% when SE was stopped after 60-90 minutes by diazepam (Brandt et al., 2016). In a retrospective study, seizure susceptibility following intrahippocampal injection of pilocarpine was compared in male Wistar rats coming from HAR in the Netherlands or from two breeding locations of CRL (Germany and France) and led to the conclusion that, according to their location, CRL rats were more likely to develop seizures than HAR rats (Portelli et al., 2009). Interstrain sensitivity to pharmaceutical agents has long been a concern of scientific research (Kacew and Festing, 1996). Nonetheless, both our study and those above-mentioned also indicate that differences may not only be present between strains but also within the same lineage depending on the vendors or their different breeding locations.

Inflammatory response in CRL rats

Inflammatory response in the hippocampus of CRL rats, based on the calculation of pro-inflammatory, anti-inflammatory and inflammation cell indexes (Fig. 5), was investigated during epileptogenesis (i.e. 7 hours, 1-day and 9 days post-SE) and compared to those measured during chronic epilepsy (i.e. 7 weeks post-SE). To this end, we quantified the transcript levels of the pro-inflammatory (IL1 β , IL6, TNF α , IFN γ , MCP1 and MIP1 α , Fig. 6) and anti-inflammatory cytokines (IL4, IL10, IL13, Fig. 7) as well as cellular markers (ITGAM, GFAP, Fig. 8). Referring to the pro- and anti-inflammatory indexes, we observed that the peak of the inflammatory response is 7 hours post-SE, similar to what we have shown in HAR rats. In addition, we show that the duration of SE (45 min versus 60 min) before the first injection of diazepam significantly influenced the height of the induction peak, with values being significantly higher for a 60-min SE (Fig. 5A-B). Looking at the pro- and anti-inflammatory genes individually, we observed that only TNF α , MIP1 α (Fig. 6) and IL10 (Fig. 7) had statistically higher mRNA levels in rats subjected to a 60 min SE compared to those subjected to a 45 min SE. The mRNA levels of IL1 β , IL6 and IL4 were also higher with a 60 min SE but with too much variability for the differences to be significant with a 45 min SE. During the chronic phase of epilepsy, statistical analysis of the various pro- and anti-inflammatory markers, that included all time points investigated from epileptogenesis to epilepsy, revealed that all markers had returned to the control values. However, it is possible that the magnitude of variation during epileptogenesis may have masked the much smaller differences at 7 weeks between controls

and epileptic rats, particularly for IL1 β , MCP1 and IL4. It cannot be excluded that these differences may be found statistically significant if the values measured at 7 weeks post-SE were compared between epileptic rats and their respective controls, exclusively.

For cellular markers, the highest values were observed 1 day and 9 days post-SE for the inflammation cell index (Fig. 5C) as for the individual genes composing this index, i.e. ITGAM and GFAP (Fig. 8). These molecular results at the mRNA level are in line with histological detections of the corresponding proteins, whose maximum signals appear with a slight delay, which is completely understandable in view of the time needed between transcription and translation (Fig. 9). Astrocyte-specific GFAP, microglia/macrophage-specific ITGAM (CD11b) and microglia-specific Iba-1 markers in the hippocampus following pilocarpine-induced SE reveal the presence of an intense signal, especially in CA1, at 9 days post-SE as a sign of a glial scar formation (Fig. 9). Intriguingly, at 1 day-post-SE, we observed only few ITGAM-positive “round-shaped” cells (not shown), resembling infiltrating monocytes, compared to the numerous cells previously found in HAR/ENV rats (studies # 1 and 2). This may be explained by a slight delay in the time of monocyte extravasation in CRL SD rats, so that the times 1 day and 9 days after SE were either too early or too late, respectively, to visualize the infiltrating monocytes. This may also be due to the fact that the mechanisms necessary for monocyte extravasation, such as chemoattractive signals, were not present at a sufficient level of expression.

The inflammatory response in the hippocampus of CRL rats after a pilocarpine-induced SE is not overlapping with that observed in HAR/ENV rats

To address the question of whether the inflammatory response in CRL rats is similar to that in HAR/ENV rats, we then compared the results of each sub-strain (Fig. 10-13). Data on pro-inflammatory, anti-inflammatory and inflammation cell indexes (Fig. 10), as well as on transcripts levels of pro- (Fig. 11) and anti-inflammatory cytokines (Fig. 12) and on inflammation cell markers (Fig. 13), are juxtaposed to make it easier to compare the profiles of the two rat sub-strains, HAR/ENV (dark blue bars) and CRL (red bars).

When considering the pro- and anti-inflammatory indexes, HAR rats exhibit the highest pro-inflammatory response at 7 hours (Fig. 10A, $p < 0.0001$) and 1 day (Fig. 10A, $p < 0.0001$) post-SE when CRL rats present a greater anti-inflammatory response at 7 hours (Fig. 10B, $p < 0.0001$).

The inflammation cell index does not reveal any difference in glial reactivity between the two rat sub-strains (Fig. 10C).

By looking more carefully at each gene individually, we observed that HAR/ENV SD rats expressed significantly more IL6, IFN γ , MCP1 and MIP1 α at 7 hours and 1-day post-SE than CRL rats (Fig. 11). The only pro-inflammatory marker that was significantly higher in CRL rats than in HAR/ENV rats is TNF α at 7 hours post-SE (Fig. 11, $p < 0.0001$). IL1 β appeared to be expressed identically in both sub-strains. For the anti-inflammatory markers, CRL rats showed higher transcript levels of IL4 and IL10 at the peak of inflammation 7 hours post-SE, when no statistical difference was observed for IL13 (Fig. 12).

Comparison of ITGAM and GFAP mRNA levels in the hippocampus revealed interesting differences between CRL and HAR/ENV rats (Fig. 13). Although inflammation cell indexes in rats from both suppliers were similar, they concealed inverse variations of ITGAM and GFAP: lower levels of ITGAM mRNA found at 1 and 9 days post-SE in CRL rats were compensated by higher levels of GFAP mRNA.

Finally, the fact that we detected only few infiltrating monocytes 24 hours post-SE in the hippocampus of SD rats from CRL could be explained by the very low induction of MCP1 and MIP1 α compared to SD rats from HAR/ENV. This explanation is all the more likely since the comparison of CD68 expression between the two sub-strains clearly shows a significantly lower level of induction in CRL SD rats compared to HAR/ENV rats (Fig. 14), suggesting that monocyte infiltration and transdifferentiation into monocyte-macrophages was much less pronounced in CRL rats than in HAR/ENV rats. It is difficult to know why the induction of chemokines was lower in CRL rats when that of IL1 β , for example, was rather similar. However, it cannot be excluded that the earlier cessation of SE in CRL rats had an effect on chemokine induction. In order to answer this question, we tried to reduce SE duration in HAR/ENV rats to 1 hour. However, the reactivity to diazepam during SE between the two sub-strains rats was too different (inability to stop SE at 1 hour in HAR/ENV rats) to draw any sound conclusions.

Neuronal degeneration is stronger in CRL rats than in HAR rats

Evidence from the literature shows that in rats exposed to pilocarpine-induced SE at 42 days, there is a strong neuronal degeneration in the hippocampus, the piriform cortex, the amygdala and the insular agranular cortex (Nadam et al., 2007; Sanchez et al., 2009; Voutsinos-Porche et al., 2004). Following our previous results regarding inflammation in the hippocampus of CRL rats, we compared neurodegeneration in the days following pilocarpine-induced SE in CRL and HAR/ENV rats. Neuronal damage, evaluated by NeuN-immunodetection, was markedly more severe in the granule cell layer in CRL rats in comparison to HAR rats (Fig. 15). Besides, the two sub-strains presented with clear evidence of equivalent neuronal loss in the hilus.

4 | DISCUSSION

Before starting this discussion, it is important to mention again that what is called "inflammatory response" in this study is limited to the expression of 6 pro-inflammatory genes (IL1 β , IL6, TNF α , IFN γ , MCP1 and MIP1 α), 3 anti-inflammatory genes (IL3, IL10 and IL13) and 2 glial reactivity markers (GFAP and ITGAM). This study does not pretend to describe the complexity of the inflammatory response, which involves much more diverse and numerous molecular mediators, but was limited to the most frequently investigated gene markers.

In this study, the inflammatory response to SE induced in Harlan SD rats was compared, on the one hand, between different brain regions showing different neuronal vulnerabilities, and, on the other hand, between two different ages of SE induction, one associated with high neurodegeneration while the other not. These comparisons allowed us to conclude that the level of inflammatory response assessed at the tissue level cannot alone explain the differences in neuronal vulnerability between the different brain structures investigated. However, we should not exclude the possibility that in brain regions with the highest neuronal vulnerability, there were very localized and small niches in which the inflammatory response could have been much stronger than the one we evaluated from tissue homogenates. If this were the case, at the time of homogenization, the very high concentrations of inflammatory marker mRNAs in these niches could have been strongly diluted by the much lower, or even unchanged, concentrations present in the rest of the tissue, which would have responded little, if at all, to SE.

What is the link between neuroinflammation and post-SE neurodegeneration?

Overall, once epilepsy has developed, hippocampal sclerosis appeared to be more prominent in CRL rats whose neuronal loss and astrogliosis, focused on the CA1 subfield and the granule cell layer of the hippocampus, were stronger than that of HAR/ENV rats.

Insofar as we consider that the higher the inflammatory response, as evidenced by a high level of pro-inflammatory cytokines, the greater the neuronal damage, then any increase in anti-inflammatory processes can be seen as an attempt by the tissue to limit the extent of neurotoxic processes. It is clear that a higher anti-inflammatory response following SE was not sufficient to support greater neuronal survival as evidenced here by the fact that 1) lesions in the VLR were more pronounced and spread than that observed in the hippocampus in juvenile HAR/ENV SD rats, and 2) neuronal loss was greater in the hippocampus of CRL SD rats compared to HAR/ENV SD rats. Thus, as in the case of erythropoietin, a glycoprotein hormone belonging to the superfamily of type I cytokines, whose expression is known to be important in preventing mild brain injury (Sakanaka et al., 1999), but whose induction following SE is not sufficient to promote neuronal survival (Nadam et al., 2007; Sanchez et al., 2009), the induction of anti-inflammatory cytokines may only moderate that of pro-inflammatory cytokines, without inhibiting their action, which may have an important function in removing debris from cells that have not resisted massive post-SE excitotoxicity.

The high presence of IL1 β in the hippocampus has been shown to contribute to neuronal degeneration. Indeed, it has been reported that hippocampal neurons are resistant to bicucullin-induced SE and this surprising resistance is associated with low presence of IL1 β in the tissue (Vezzani et al., 1999). It is thought that the binding of IL1 β to its IL1RI receptor present at the surface of hippocampal neurons would lead to their vulnerability (Ravizza and Vezzani, 2006), likely through the activation of a signaling pathway causing phosphorylation of the NR2B subunit of the NMDA receptor resulting in an increased calcium influx into neurons (Vezzani and Baram, 2007; Viviani et al., 2003). To alleviate the toxicity of IL1 β , the damaged tissues respond by inducing the production of the IL1R1 receptor antagonist so-called IL1Ra. Preliminary data from our laboratory indicate that gene induction of IL1Ra post-SE is obviously not sufficient to counteract the deleterious effects of IL1 β , but it is likely to contribute to its reduction. Based on our results in CRL and HAR/ENV rats expressing the same level of IL1 β , differences in gene expression of IL1R1 and/or IL1Ra, with potentially a higher

IL1R1 and a lower IL1Ra induction in CRL rats, may explain differences in neuronal vulnerability, if it is the case, between the two rat sub-strains. In addition, it has been shown in rats exposed to cerebral ischemia that the presence of TNF α causes an exacerbation of neuronal death (Barone et al., 1997; De Lella Ezcurra et al., 2010; Probert, 2015). The transcript level of TNF α was 5 times higher at 7 hours post-SE in the hippocampus of CRL rats than in that of HAR rats. Thus, it is conceivable that this cytokine also contributes in the more severe neuronal loss found in CRL SD rats.

Intriguingly, microgliosis, as measured by ITGAM transcript levels, was higher in HAR/ENV rats during epileptogenesis. This result is of particular interest because it is associated with a lower induction of CD68 during the same period, which is an argument for less post-SE monocyte infiltrates in CRL rats. This may also partly explain why the degeneration observed in CRL rats was greater than in HAR/ENV rats; indeed, depletion of circulating monocytes, resulting in less monocyte infiltration, has been associated with greater neuronal degeneration (Zattoni et al., 2011).

Consequences of enhanced neuronal loss

Our experimental results show that neuronal atrophy is greater in CRL rats than in HAR rats in the days following SE induction. Numerous studies have shown that hippocampal atrophy developed following SE in rats was associated with impaired memory performance in spatial orientation tests (Bell et al., 2011; Niessen et al., 2005; Saniya et al., 2017; Wu et al., 2018). These studies raise thus the question of whether in a long-term perspective, CRL versus HAR/ENV SD sub-strain differences would be observed in the pathophysiological and symptomatic picture of the disease, with potentially different frequency and severity of seizures and/or severity of memory and behavioral impairments. As reviewed by Löscher et al. (2017), previous investigations have already shown differences in seizure expression and behavioral performance. In a study in which anxious behavior was evaluated in female Wistar rats from Charles River or Harlan, those from CRL showed the highest anxiety-like behavior and the lowest exploratory behavior (Honndorf et al., 2011). Similarly, when comparing SD rats and Wistar rats from CRL, HAR, Janvier and Taconic, Langer et al. (2011) reported that SD rats from CRL markedly differed from other SD sub-strains by being less prone to SE-induced behavioral changes. Besides, in contrast to what our experimental results showed, they also reported that neurodegeneration in CA1, CA3 and in the hilus of CRL SD rats was less

important than in other sub-strains (Langer et al., 2011). In their study, they also showed that all SD rats from the different suppliers developed recurrent and spontaneous seizures following self-sustained SE (SSSE), except rats from CRL that were significantly less sensitive to electrical induction of basolateral amygdala stimulation. If we do confirm that differences in hippocampal damage persist during the chronic phase of epilepsy between SD rats from HAR/ENV and CRL, it will be important to question whether behavioral differences are also observed between the two sub-strains.

How to explain differences observed between SD rats from HAR/ENV and CRL?

The differential results between the two sub-strains of rats may first be explained by an inflammation-related gene polymorphism. These assumptions would require verification by genome-wide association study (GWAS) to detect potential association between the changes in cytokines levels and single nucleotide polymorphisms (SNPs) of each gene. Genetic characterization of more than 4,000 Sprague-Dawley rats from CRL or HAR/ENV was performed in 2018 by Gileta and colleagues. They observed a very high genetic divergence between suppliers, with the "supplier" component accounting for 33.7% of the observed variance (Gileta et al., 2018). They suggested that the differences observed may be related to the fact that “ *Charles River has adhered to their International Genetics Standardization Protocol for more than 25 years, whereas Harlan appears to have focused on maintaining diversity within breeding colonies and may have allowed for a moderate degree of drift between them* ” (Gileta et al., 2018). Genetic polymorphisms of inflammatory genes could explain the different results between CRL and HAR rats that we reported in our study. It has been shown that such variations can be involved in different types of pathologies (Azab et al., 2016; He et al., 2013; Keshavarz et al., 2019; Kwon et al., 2011; Moscovis et al., 2015; Wang et al., 2014). In human epilepsy, polymorphism in the promoter region at position -511 of the *il1b* gene has been shown to be associated with drug-resistant epilepsy in TLE patients with hippocampal sclerosis (Kanemoto et al., 2000, 2003; Vezzani, 2005). Nonetheless, as reviewed by Vezzani in 2005, Heils et al. (2000) did not confirm the results of the above-mentioned study of Kanemoto, suggesting that some polymorphisms may be related to specific ethnicity (Heils et al., 2000).

As reported by Löscher et al. in 2017, multiple confounding variables may influence the experimental outcome of rodent preclinical studies on seizure expression, behavior and drug activities. These variables include parameters such as genetic background, animal source, sanitary status, housing conditions in the supplier's animal husbandries and then in the laboratory, maternal care at the earliest age, shipping conditions to the laboratory, duration of acclimatization before experimentation, age at testing, sex, and handling (Löscher et al., 2017; Manouze et al., 2019). Beyond the genetic background, the breeding conditions at the supplier site from birth to delivery of rats to the laboratory as well as the housing conditions at laboratory may also play a role in the variation of observed results. As reported by Burn et al. (2008), behavior of adult rats can vary depending on the handling procedures or the tail-mark identification. Rats that were tail-marked early in life showed significantly less anxious behavior by staying three times longer in the open arms of the elevated plus maze compared to their unmarked congeners of the same cage. Surprisingly, the same rats also displayed in response to handling a more substantial chromodacryorrhoea, which corresponds to the over-secretion of porphyrin by Harderian glands around the eyes and nose and which is commonly a welfare indicator occurring after exposition to stressors (Burn et al., 2008; Hubrecht and Kirkwood, 2010). Nonetheless, no significant difference in rat anxiety was based on the frequency of cage-cleaning (Burn et al., 2008). In the same study, they also showed that the delivery batch affected the anxiety profiles measured in an elevated-plus maze test. Previous research has been conducted on the effect of environmental enrichment on SE-induced cognitive impairment (Fares et al., 2013; Faverjon et al., 2002; Rutten et al., 2002). For this purpose, the laboratory has developed the Marlau™ cage, a cage designed to increase social interaction, voluntary exercise, entertaining activities and cognitive stimulation through the exploration of maze contained in the cage whose configuration is changed 3 times a week. Housing rats in the Marlau™ cage after SE at weaning was shown to prevent cognitive impairment (Fares et al., 2013). Further research demonstrated that housing in enriched environment drastically reduced amygdala kindling epileptogenesis (Auvergne et al., 2002).

Thus, when examining experimental results of research articles, it is important to remember these variations related not only to the genetic background but also to the breeding conditions, the delivery batch, the handling procedures at the supplier's and to remain critical regarding the reported findings. The present research highlights the need to be aware of the

rat's provenance at the time of experimental design or when reading scientific articles, which can lead to unwanted heterogeneity or replication issues. However, to reproduce human heterogeneity in preclinical studies, it is necessary to include in protocols, if possible, animals from different suppliers/locations, inbred and outbred, and from different genders as well (Festing, 1993; Zucker and Beery, 2010).

Our results obtained show differences in inflammatory response between rats from two different suppliers that may be explained by genetic or environmental disparities. Therefore, the substantial sub-strains differences that we observed between rats from Charles River and Harlan/Envigo laboratories prompted our decision to keep the Harlan/Envigo SD rats for further experimentation and for the creation of transgenic rats in the near future years.

ACKNOWLEDGMENTS

We thank Sandrine Bouvard and Jérémy Fourié for their assistance in experimentations.

PRO-INFLAMMATORY INDEX HARLAN LABORATORIES RATS

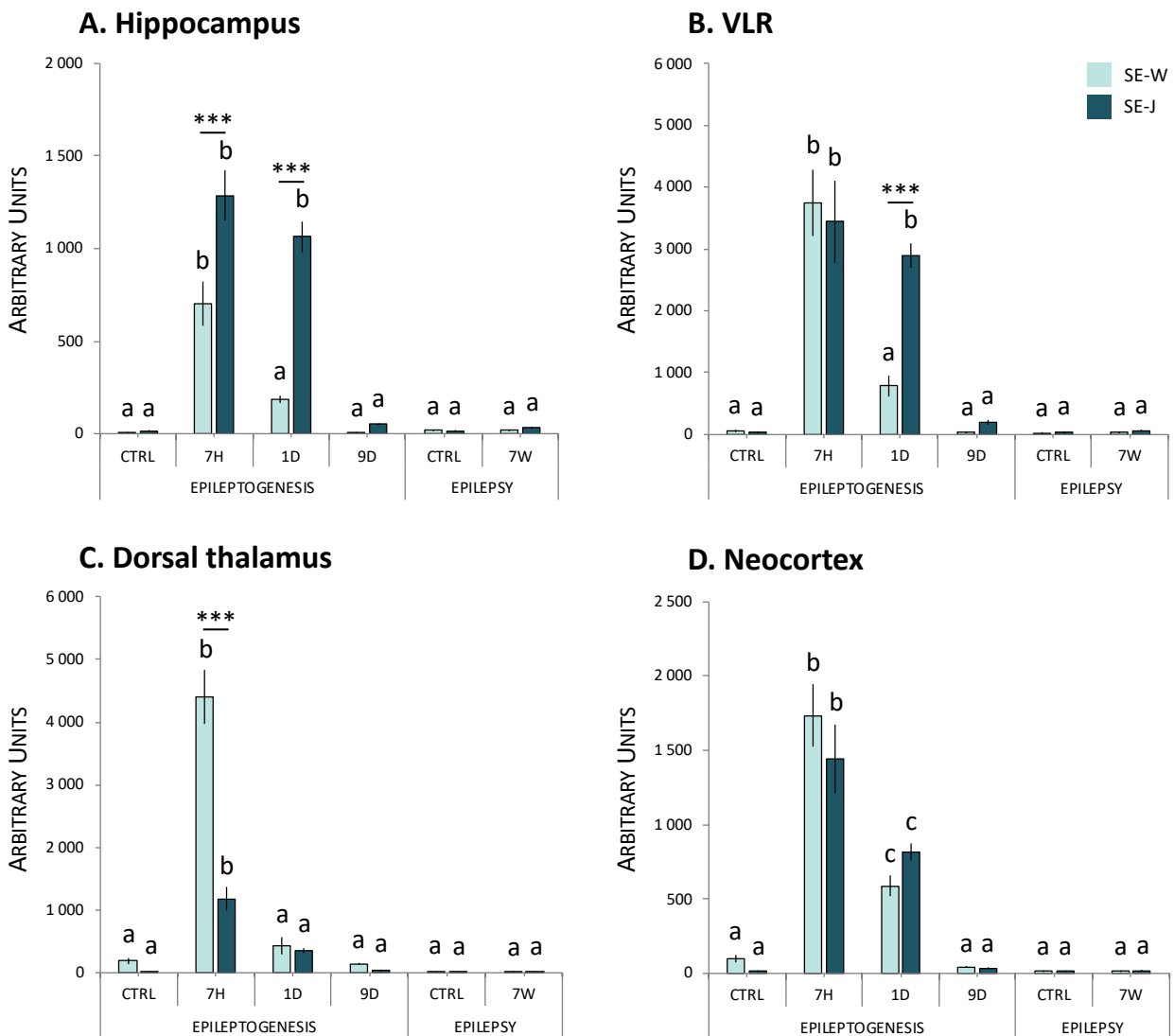


Figure 1. Brain expression of pro-inflammatory cytokines following pilocarpine-induced SE in Sprague-Dawley rats from Harlan Laboratories. Pro-inflammatory index in Sprague-Dawley (SD) rats were calculated in the hippocampus (A), the ventral limbic region (VLR, B), the dorsal thalamus (C) and the neocortex (D) during epileptogenesis, i.e at 7 hours (SE-W, n=7; SE-J, n=6), 1 day (SE-W, n=8; SE-J, n=6), 9 days (SE-W, n=10; SE-J, n=7) post-SE and once epilepsy is chronically installed, i.e. 7 weeks post-SE (SE-W, n=8; SE-J, n=8) compared to respective weaned and juvenile control rats. When comparing two bars within a same model, the difference is considered as statistically significant ($p < 0.05$) when letters (a, b, c) above the bars are different (a-b; a-c; b-c). Asterisks indicate statistical significance between the two models (SE induced at weaning or juvenile stage) at a same post-SE time. Bonferroni *post-hoc* analysis following two-way ANOVA: *** $p < 0.001$. Abbreviations: SE-W, SE induced at weaning; SE-J, SE in induced at juvenile stage.

ANTI-INFLAMMATORY INDEX HARLAN LABORATORIES RATS

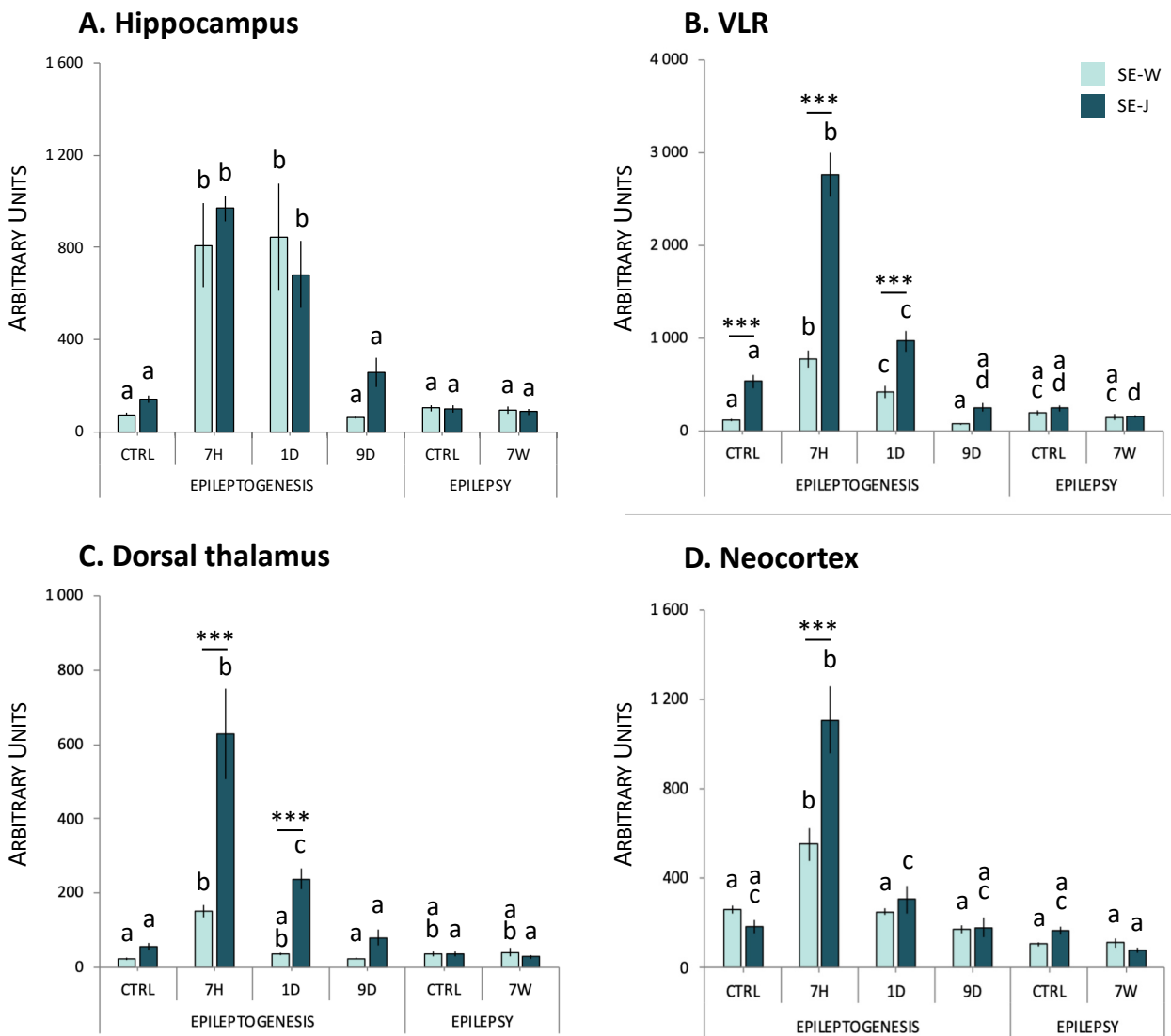


Figure 2. Brain expression of anti-inflammatory cytokines following pilocarpine-induced SE in Sprague-Dawley rats from Harlan Laboratories. As for Figure 1. Bonferroni *post-hoc* analysis following two-way ANOVA: *** $p < 0.001$. Abbreviations: SE-W, SE induced at weaning; SE-J, SE induced at juvenile stage.

INFLAMMATION CELL INDEX HARLAN LABORATORIES RATS

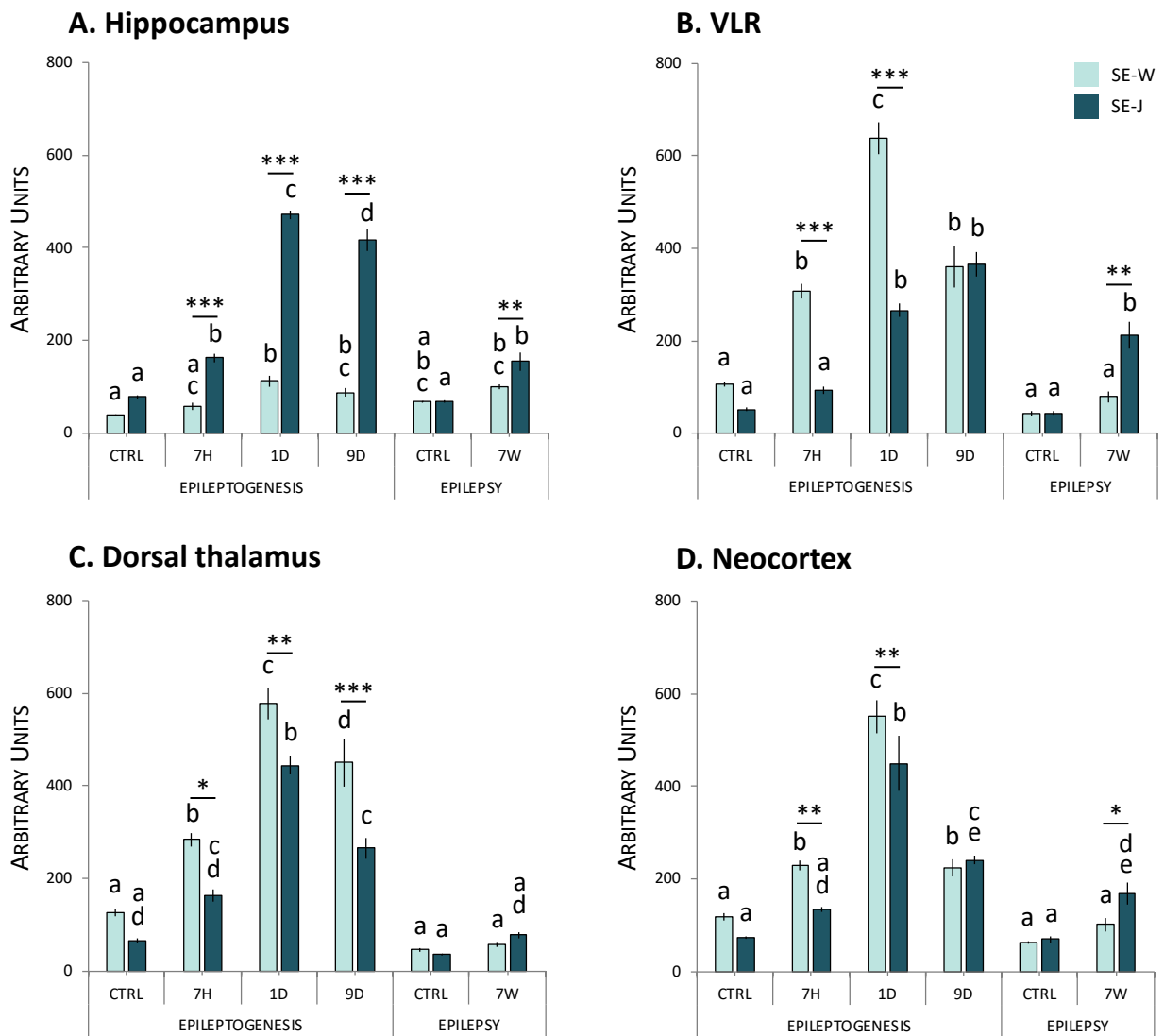


Figure 3. Brain expression of glial cells markers following pilocarpine-induced SE in Sprague-Dawley rats from Harlan Laboratories. As for Figure 1. Bonferroni *post-hoc* analysis following two-way ANOVA: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Abbreviations: SE-W, SE induced at weaning; SE-J, SE induced at juvenile stage.

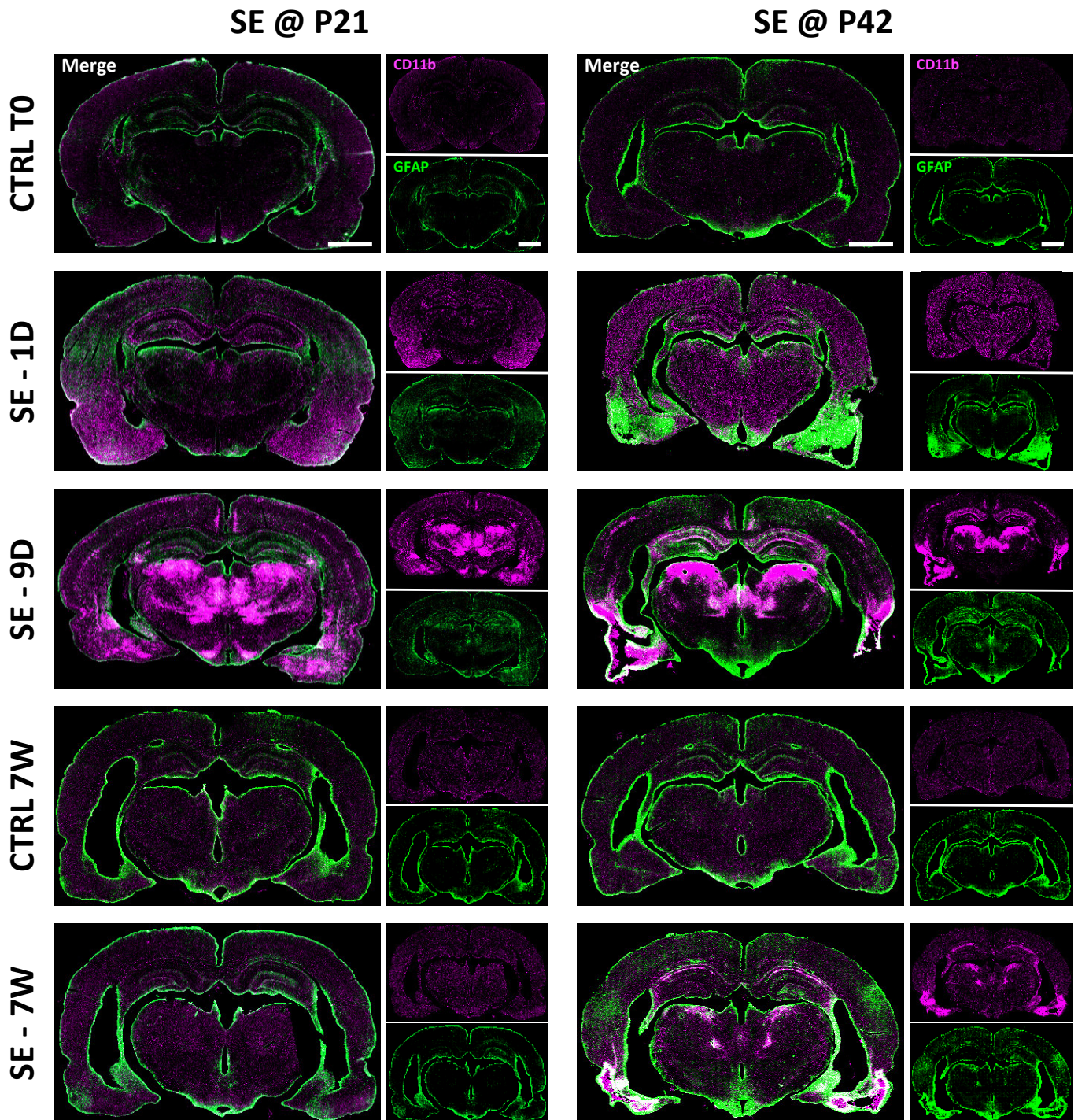


Figure 4. Time-course of glial cell activation in the brain of HAR rats after pilocarpine-induced SE. Immunofluorescence detection was performed in rat brain sections using specific antibodies directed against ITGAM (CD11b) for microglia/macrophages (magenta) and GFAP for astrocytes (green). Two stages of epileptogenesis (SE-1D: 1-day post-SE; SE-9D: 9 days post-SE) and chronic epilepsy (SE-7W: 7 weeks post-SE) after pilocarpine-induced SE triggered at weaning (P21) or at juvenile age (P42) are compared to their respective controls. Scale bar: 2 000 μm .

INFLAMMATORY INDEXES CHARLES RIVER LABORATORIES RATS

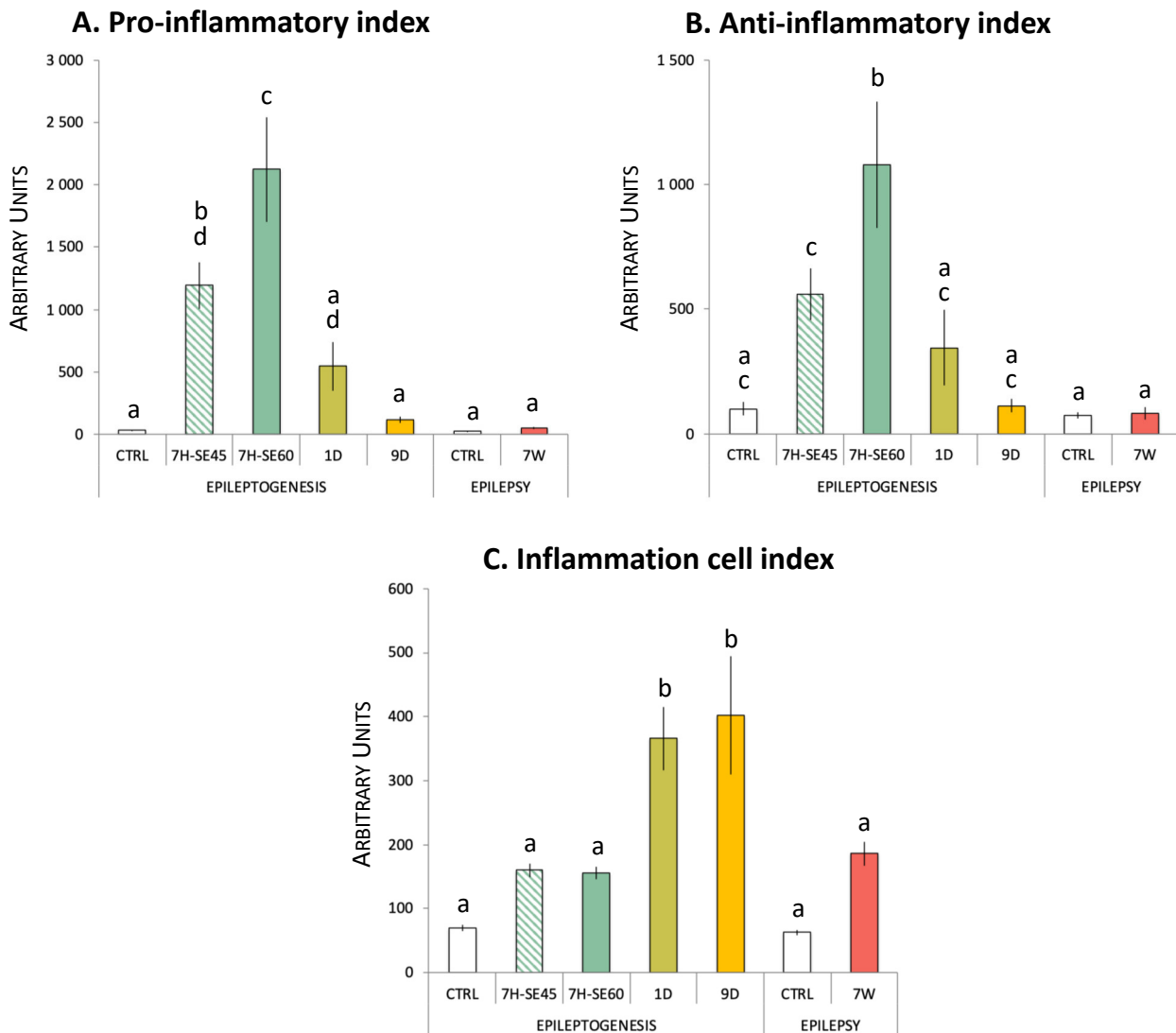


Figure 5. Hippocampal expression of inflammatory markers following pilocarpine-induced SE in Sprague-Dawley rats from Charles River Laboratories. Pro-inflammatory (A), anti-inflammatory (B) and inflammation cell (C) indexes in the hippocampus of Charles River Laboratories (CRL) Sprague-Dawley rats were calculated during epileptogenesis, i.e. at 7 hours (SE45, n=5; SE60, n=4), 1 day (n=5), 9 days (n=5) post-SE and once epilepsy is chronically installed, i.e. 7 weeks post-SE (n=8) compared to respective control rats (CTRL epileptogenesis, n=4 ; CTRL epilepsy, n=6). When comparing two bars, the difference is considered as statistically significant ($p < 0.05$) when letters (a, b, c) above the bars are different (a-b; a-c; a-d; b-c; b-d; c-d). Tukey's *post-hoc* analysis following one-way ANOVA. Abbreviations: SE45, SE duration of 45 min; SE60, SE duration of 60 min.

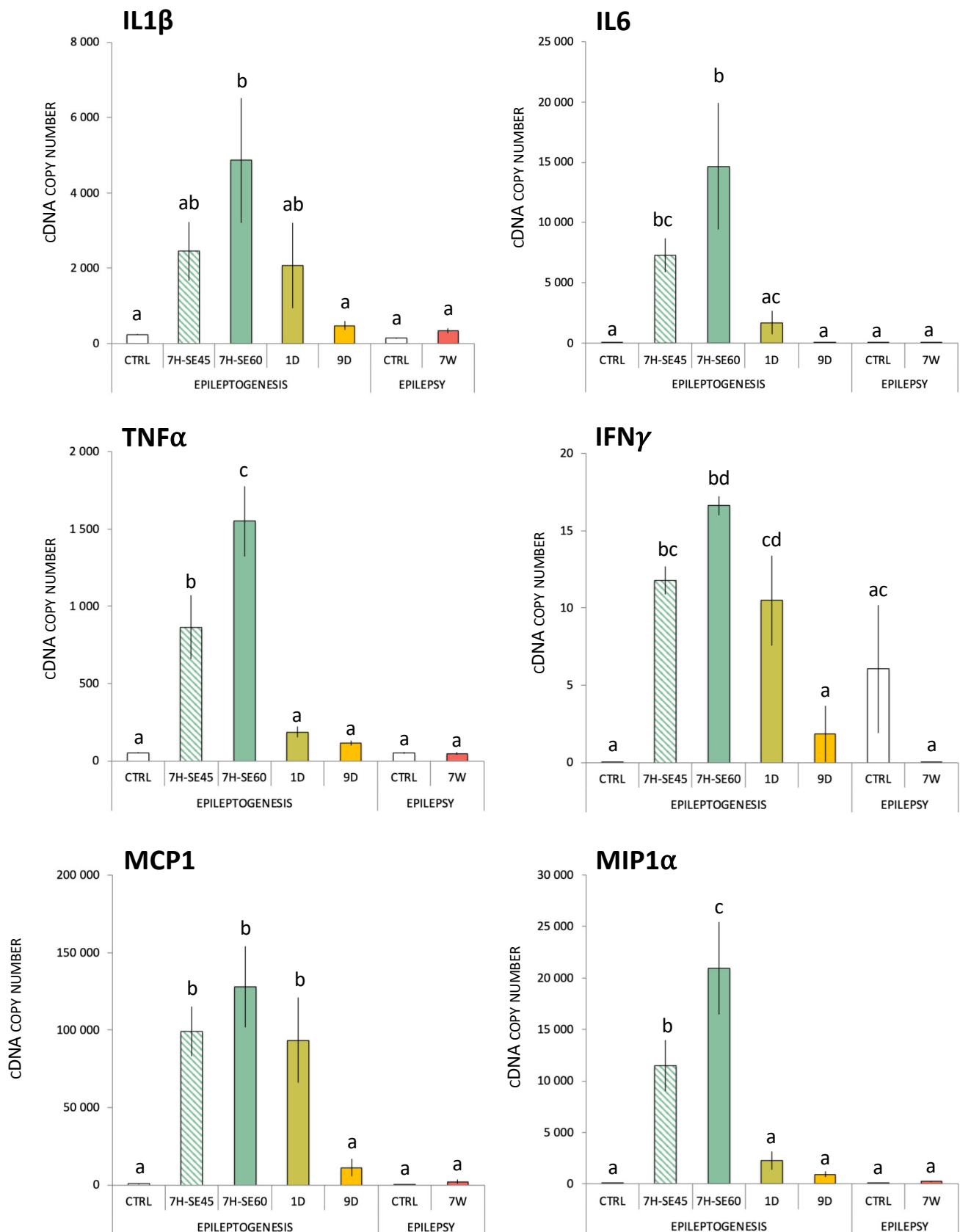


Figure 6. Transcript levels of pro-inflammatory cytokines after pilocarpine-induced SE in the hippocampus of CRL rats. Transcript values of pro-inflammatory cytokines (IL1 β , TNF α , IL6, IFN γ) and chemokines (MCP1, MIP1 α) during epileptogenesis, i.e. at 7 hours (SE45, n=5; SE60, n=4), 1 day (n=5), 9 days (n=5) post-SE and once epilepsy is chronically installed, i.e. 7 weeks post-SE (n=8) compared to respective control rats (CTRL epileptogenesis, n=4; CTRL epilepsy, n=6). When comparing two bars, the difference is considered as statistically significant ($p < 0.05$) when letters (a, b, c) above the bars are different (a-b; a-c; a-d; b-c; b-d; c-d). Tukey's *post-hoc* analysis following one-way ANOVA. Abbreviations: SE45, SE duration of 45 min; SE60, SE duration of 60 min.

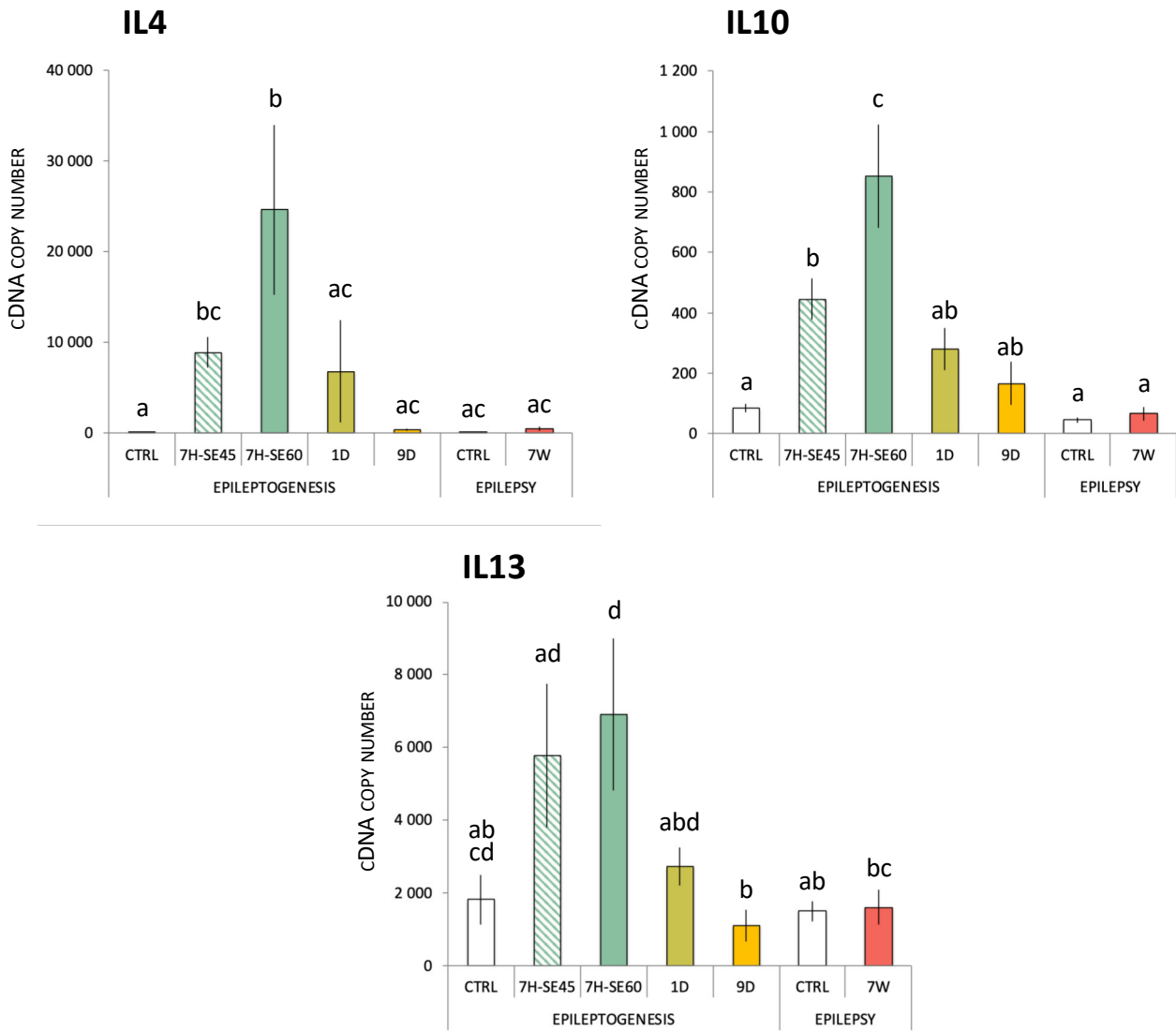


Figure 7. Transcript levels of anti-inflammatory cytokines after pilocarpine-induced SE in the hippocampus of CRL rats. Transcript values of anti-inflammatory cytokines (IL4, IL10, IL13) during epileptogenesis and once epilepsy is chronically installed. As for Figure 5. Tukey's *post-hoc* analysis following one-way ANOVA. Abbreviations: SE45, SE duration of 45 min; SE60, SE duration of 60 min.

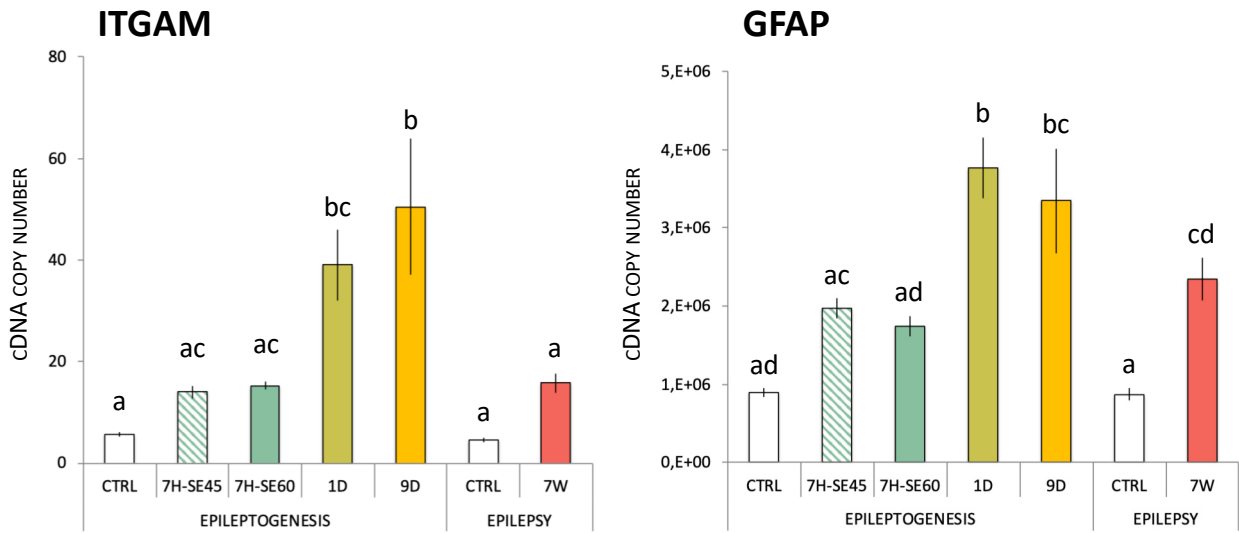


Figure 8. Transcript levels of cell markers after pilocarpine-induced SE in the hippocampus of CRL rats. Transcript values of cell markers (ITGAM for microglia, GFAP for astrocytes) during epileptogenesis and once epilepsy is chronically installed. As for Figure 5. Tukey's *post-hoc* analysis following one-way ANOVA. Abbreviations: SE45, SE duration of 45 min; SE60, SE duration of 60 min.

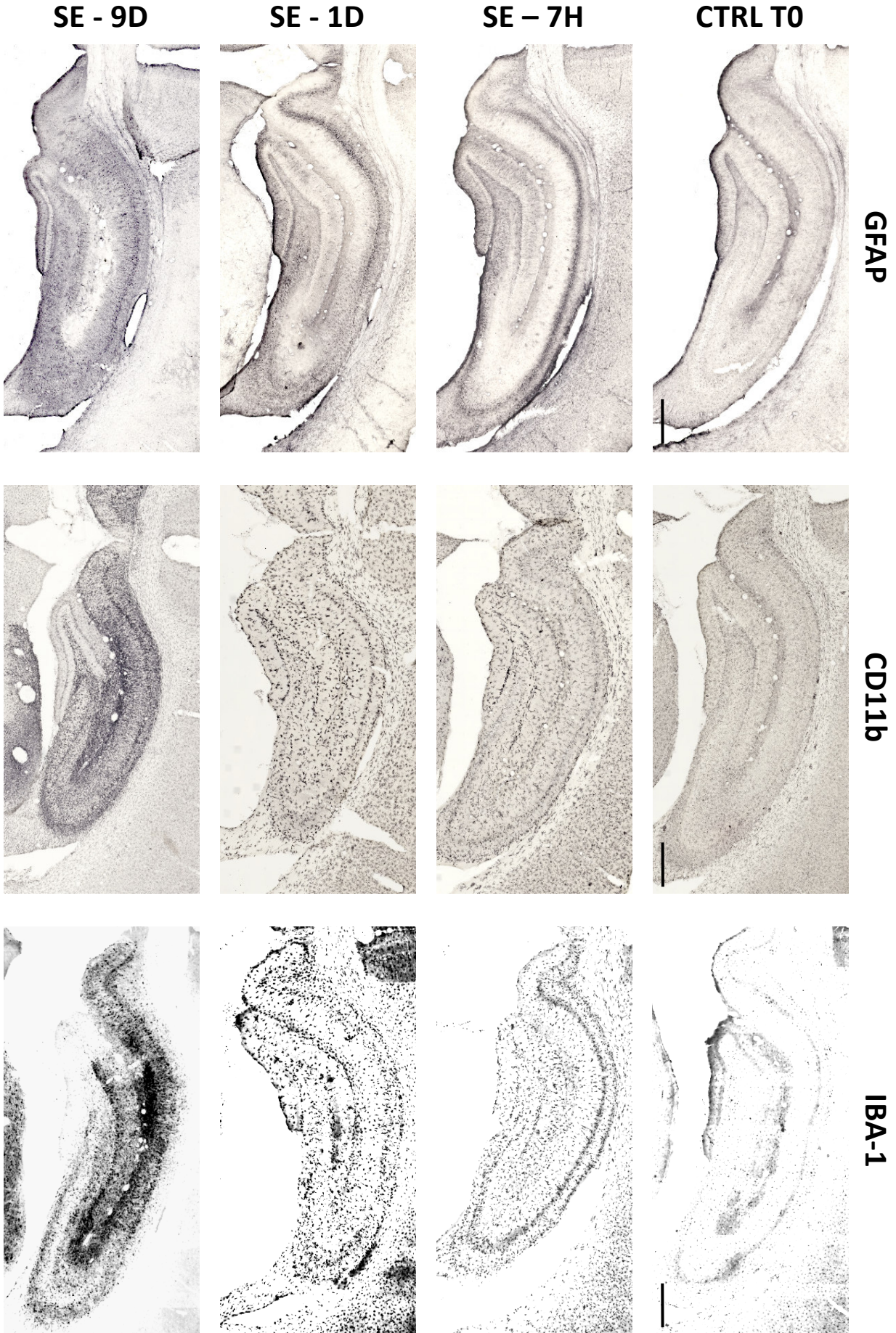


Figure 9. Time-course of glial cell activation during epileptogenesis in the hippocampus of CRL rats after pilocarpine-induced SE. Immunohistochemical or immunohistofluorescence detection was performed in rat brain sections using specific antibodies directed against GFAP for astrocytes, and against CD11b for microglia/macrophages and Iba-1 for resident microglial cells. Three stages of epileptogenesis (SE-7H: 7 hours post-SE; SE-1D: 1 day post-SE; SE-9D: 9 days post-SE) after pilocarpine-induced SE triggered at juvenile age (P42) in CRL rats are compared to controls. Scale bar: 500 μm .



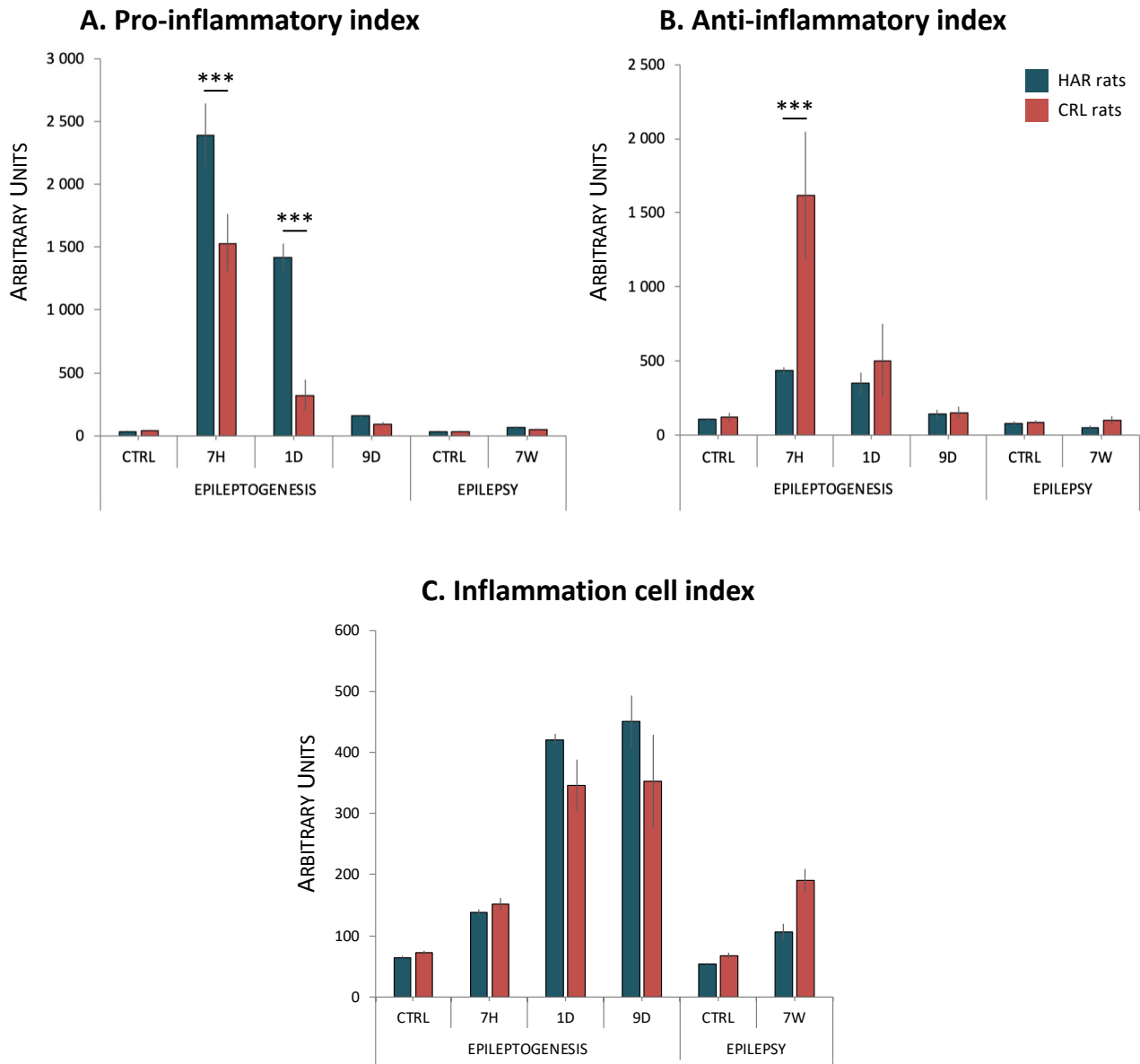


Figure 10. Hippocampal inflammatory response after pilocarpine-induced SE induced at 42 days in Harlan Sprague-Dawley (SD) rats is different from that in Charles River SD rats. Pro-inflammatory (A), anti-inflammatory (B) and inflammation cell (C) indexes in the hippocampus of Harlan Laboratories (HAR) SD rats (blue bars) compared to Charles River Laboratories (CRL) SD rats (red bars) during epileptogenesis, i.e. at 7 hours (HAR, n=6; CRL, n=4), 1 day (HAR, n=6; CRL, n=5), 9 days (HAR, n=7; CRL, n=5) post-SE and once epilepsy is chronically installed, i.e. 7 weeks post-SE (HAR, SE-J, n=8; CRL, n=8) compared to respective controls. When comparing two bars within a same vendor, the difference is considered as statistically significant ($p < 0.05$) when letters (a, b, c, d) above the bars are different (a-b; a-c; a-d; b-c; b-d; c-d). Asterisks indicate statistical significance between the two vendors (HAR or CRL) at a same post-SE time. Bonferroni *post-hoc* analysis following two-way ANOVA: *** $p < 0.001$.

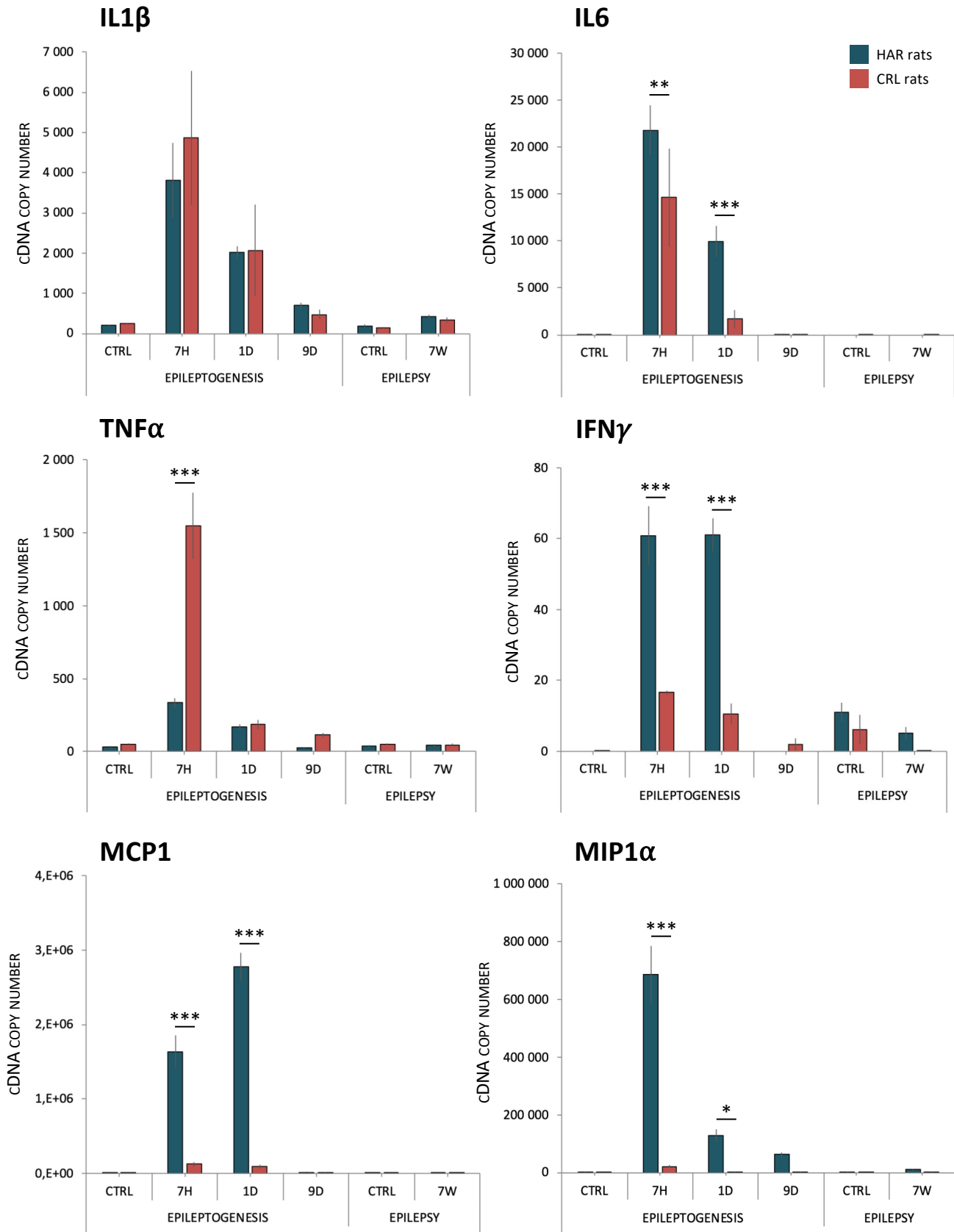


Figure 11. Transcript levels of pro-inflammatory cytokines in the hippocampus after pilocarpine-induced SE induced at 42 days in Harlan Sprague-Dawley (SD) compared to Charles River SD rats.

Transcript values of pro-inflammatory cytokines (IL1 β , TNF α , IL6, IFN γ) and chemokines (MCP1, MIP1 α) in the hippocampus of Harlan Laboratories (HAR) SD rats (blue bars) compared to Charles River Laboratories (CRL) SD rats (red bars) during epileptogenesis, i.e. at 7 hours (HAR, n=6; CRL, n=4), 1 day (HAR, n=6; CRL, n=5), 9 days (HAR, n=7; CRL, n=5) post-SE and once epilepsy is chronically installed, i.e. 7 weeks post-SE (HAR, SE-J, n=8; CRL, n=8) compared to respective controls. When comparing two bars within a same vendor, the difference is considered as statistically significant ($p < 0.05$) when letters (a, b, c, d) above the bars are different (a-b; a-c; a-d; b-c; b-d; c-d). Asterisks indicate statistical significance between the two vendors (HAR or CRL) at a same post-SE time. Bonferroni *post-hoc* analysis following two-way ANOVA: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

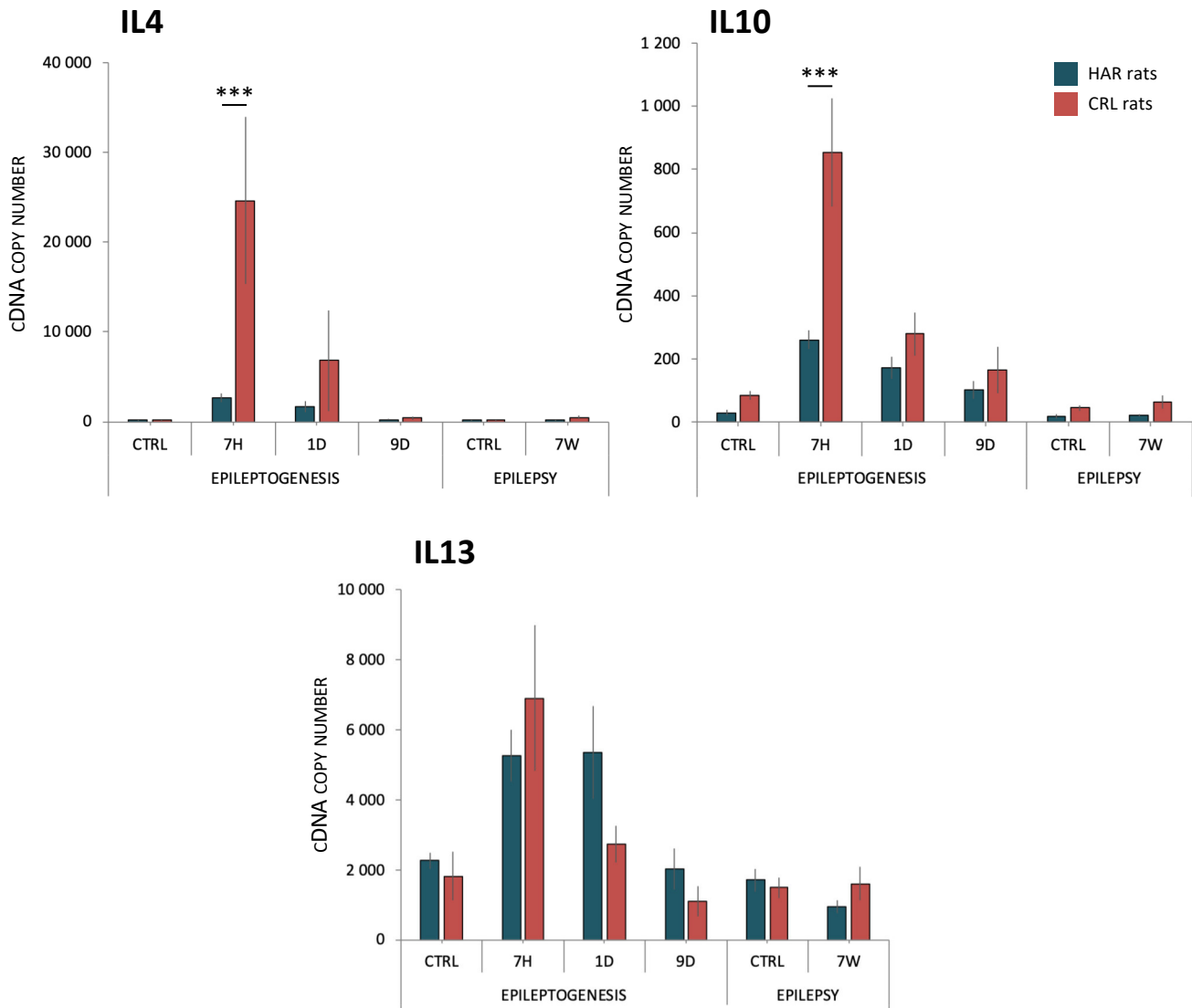


Figure 12. Transcript levels of anti-inflammatory cytokines in the hippocampus after pilocarpine-induced SE induced at 42 days in Harlan Sprague-Dawley (SD) compared to Charles River SD rats. Transcript values of anti-inflammatory cytokines (IL4, IL10, IL13) during epileptogenesis and once epilepsy is chronically installed. As for Figure 9. Bonferroni *post-hoc* analysis following two-way ANOVA: * $p < 0.001$.**

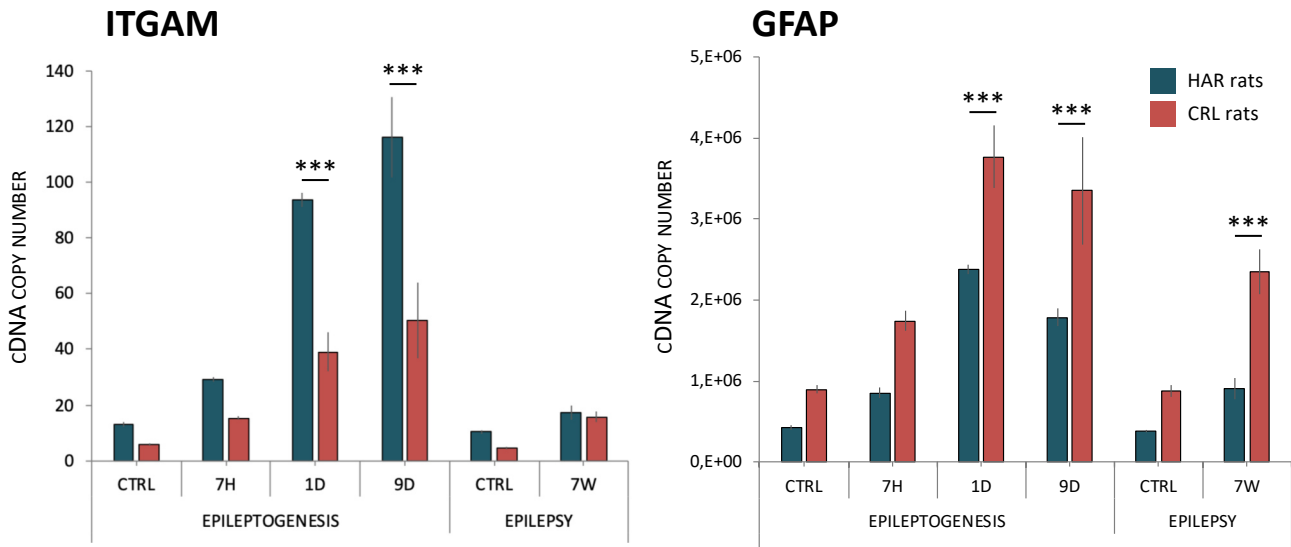


Figure 13. Transcript levels of cell markers in the hippocampus after pilocarpine-induced SE induced at 42 days in Harlan Sprague-Dawley (SD) compared to Charles River SD rats. Transcript values values of cell markers (ITGAM for microglia, GFAP for astrocytes) during epileptogenesis and once epilepsy is chronically installed. As for Figure 9. Bonferroni *post-hoc* analysis following two-way ANOVA: *** $p < 0.001$.

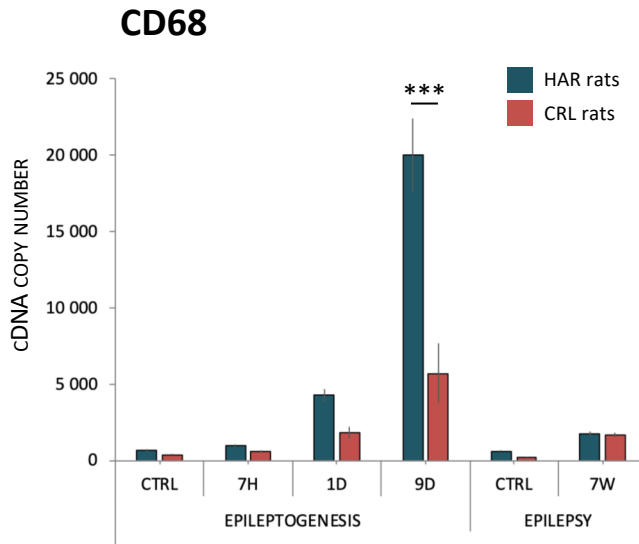
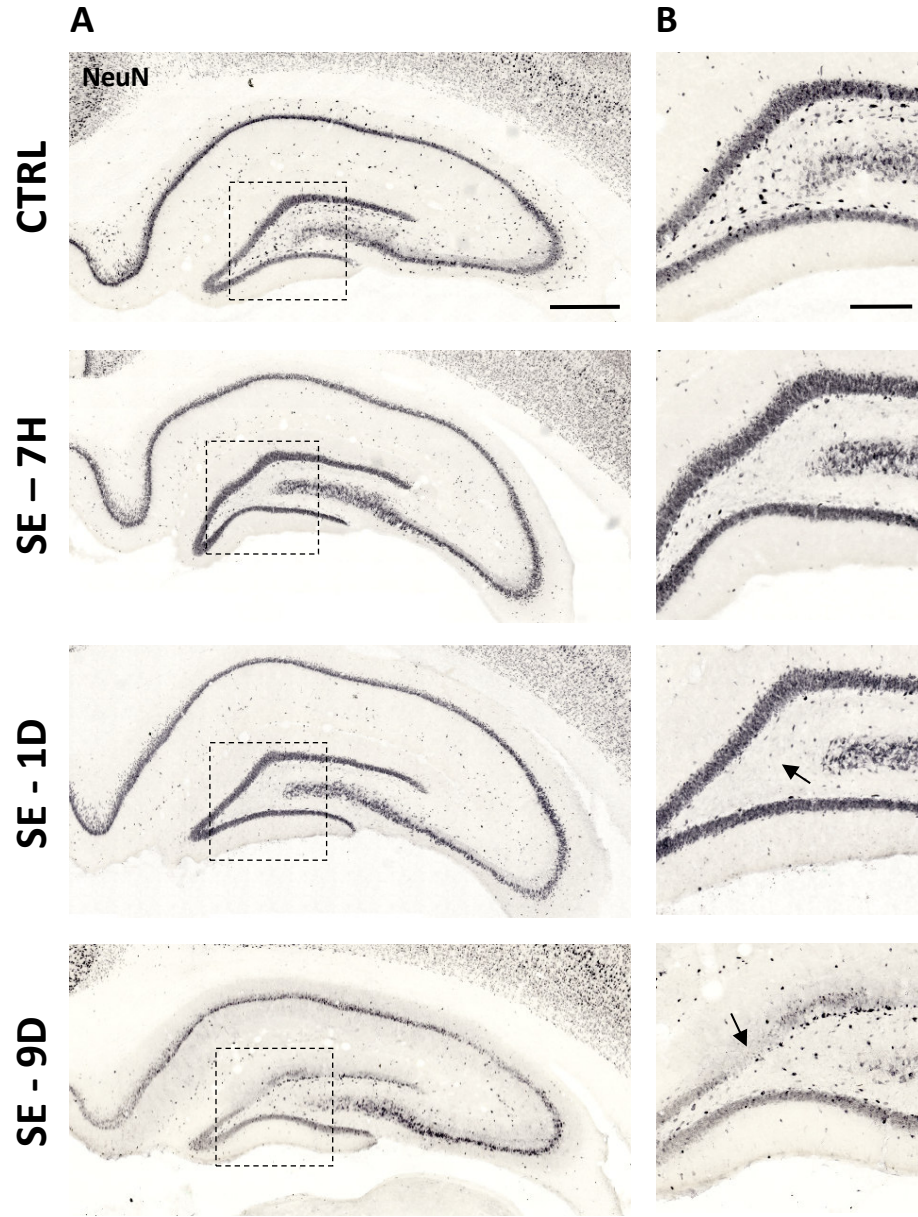


Figure 14. Transcript levels of CD68 in the hippocampus after pilocarpine-induced SE induced at 42 days in Harlan Sprague-Dawley (SD) compared to Charles River SD rats. Transcript values values cell marker of monocyte-macrophages during epileptogenesis and once epilepsy is chronically installed. As for Figure 9. Bonferroni *post-hoc* analysis following two-way ANOVA: *** $p < 0.001$.

Charles River Laboratories rats



Harlan Laboratories rats

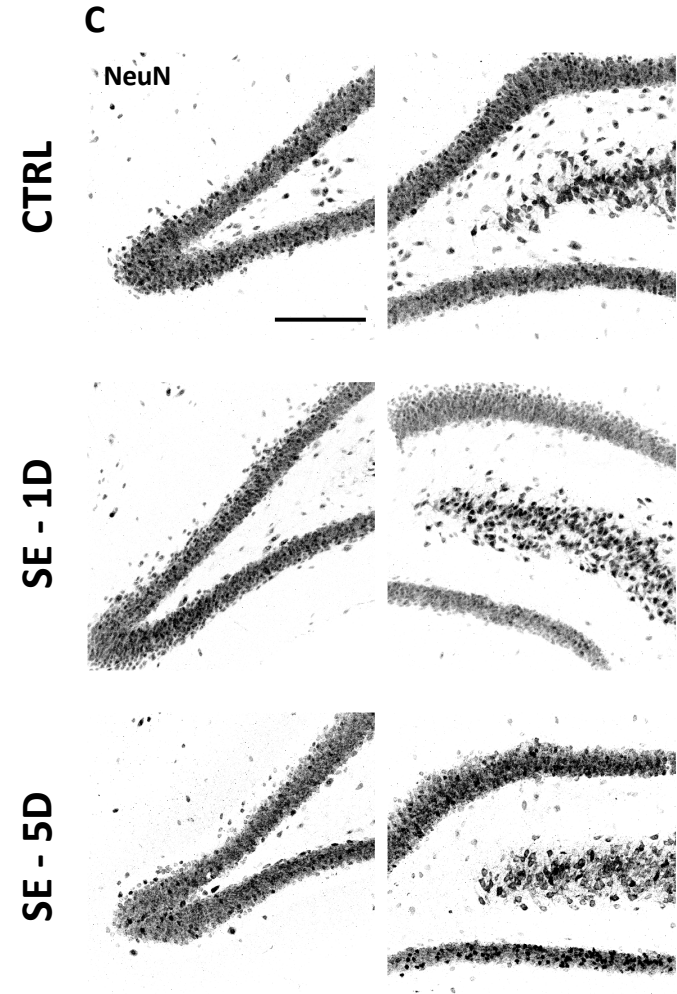


Figure 15. Neurodegeneration following pilocarpine-induced SE is stronger in Sprague-Dawley (SD) rats from Charles River Laboratories than in Harlan SD rats. (A-B) Immunohistochemical detection of NeuN was performed in the hippocampus of CRL rats at different stages of epileptogenesis, i.e. 7 hours (SE-7H), 1 day (SE-1D) and 9 days (SE-9D) after pilocarpine-induced SE induced at juvenile age (P42) and compared to controls (CTRL). A strong reduction in neuronal density is observed in the dentate gyrus, most notably in the granule cell layer and in the subgranular area, as viewed on the magnified image **(B, arrows)**. **(C)** In HAR rats, immunofluorescence detection of NeuN was performed in the hippocampus at three stages of epileptogenesis, i.e. 7 hours (SE-7H), 1 day (SE-1D) and 5 days (SE-5D) after pilocarpine-induced SE triggered at juvenile age (P42) and compared to controls (CTRL). Scale bar: A, 2 000 μm ; B-C, 200 μm .

Table S1. Primer sequences – *Rattus Norvegicus*

cDNA	Primer sequences – <i>Rattus norvegicus</i>	Product sizes (bp)	GenBank ID#
GFAP	F-ACATCGAGATCGCCACCTAC R-GGATCTGGAGGTTGGAGAAA	90	NM_017009.2
IFN γ	F-TTTTGCAGCTCTGCCTCAT R-AGCATCCATGCTACTTGAGTTAAA	107	NM_138880.2
IL1 β	F-TGTGATGAAAGACGGCACAC R-CTTCTTCTTTGGGTATTGTTGG	70	NM_031512.2
IL4	F-GTAGAGGTGTGAGCGGTCTG R-TTCAGTGTGTGAGCGTGGA	70	NM_201270.1
IL6	F-CCCTTCAGGAACAGCTATGAA R-ACAACATCAGTCCAAGAAGG	74	NM_012589.1
IL10	F-AGTGGAGCAGGTGAAGAATGA R-TCATGGCCTTGAGACACCTT	62	NM_012854.2
IL13	F-AGTCCTGGCTCTCGCTTG R-GATGTGGATCTCCGCACTG	63	NM_053828.1
ITGAM	F-ACTCTGATGCCTCCCTTGG R-TCCTGGACACGTTGTTCTCA	72	NM_012711.1
MCP1	F-CGGCTGGAGAACTACAAGAGA R-TCTCTTGAGCTTGGTGACAAATA	78	NM_031530.1
MIP1 α	F-TCCACGAAAATTCATTGCTG R-AGATCTGCCGTTTCTCTTG	92	NM_013025.2
TNF α	F-TGAACTTCGGGGTGATCG R-GGGCTTGCTACTCGAGTTTT	122	NM_012675.3

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BOX 8 | HIGHLIGHTS OF STUDY 3

- In Sprague-Dawley (SD) rats from Harlan/Envigo (HAR/ENV) laboratories, the inflammatory response was different between the 4 vulnerable brain regions tested and was independent of the extent of neuronal damage;
- SD rats from Charles River Laboratories (CRL) had a higher sensitivity to pilocarpine – the chemoconvulsant used to induce *status epilepticus* (SE) – than HAR/ENV SD rats;
- Hippocampal expression of pro-inflammatory and anti-inflammatory cytokines as well as chemokines after SE differed between CRL SD rats and HAR/ENV SD rats;
- At a cellular level, microglial activation was less pronounced in CRL SD rats than in HAR/ENV rats after SE, while astrogliosis and neuronal loss was more prominent in CRL SD rats than HAR/ENV SD rats.
- Decreased microglial activation in CRL SD rats might rely to the fact that monocytes barely infiltrated the hippocampus following SE.

CHAPTER 5

Study 4

MESENCHYMAL STEM CELLS: A DISEASE-MODIFYING TREATMENT TO
PREVENT EXPLOSIVE NEUROINFLAMMATION AND COGNITIVE
IMPAIRMENTS OCCURRING AFTER *STATUS EPILEPTICUS*?

Intranasal mesenchymal stem cells treatment does not resolve but fine-tunes the inflammatory response following *status epilepticus* and prevents long-term potentiation alterations

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ABSTRACT

Acquired epilepsy can occur after a severe epileptogenic brain injury, resulting in a significant impact on the quality of life of patients who develop uncontrolled seizures and debilitating cognitive and behavioral disorders. Temporal lobe epilepsy (TLE) is the most prevalent form of acquired epilepsy, and also accounts for nearly one-third of all patients who remain resistant to anti-seizure drugs and awaiting effective treatment. The last decades have witnessed the emergence of many anti-epileptic treatments intended to relieve seizures. However, therapeutic solutions aimed at preventing epileptogenesis have lag behind. Epileptogenesis is defined as an asymptomatic period following a brain insult during which underlying abnormal brain damage develops within a short or long period of time. Evidence suggests that during this latent phase, neuroinflammation acts as an important component that could be targeted to prevent the development of the disease. The use of mesenchymal stem cells as an innovative therapeutic tool has gained considerable attention in recent years, due to their immunomodulatory and regenerative properties. In our study, MSCs were intranasally administered into juvenile Sprague-Dawley rats after pilocarpine-induced status epilepticus (SE) to investigate their effect on neuroinflammation and on the underlying mechanisms of cognitive impairments. Here we provide evidence that classical alteration of the cellular mechanisms underlying learning and memory in the hippocampus can be counteracted by the intranasal administration of MSCs during the early phase of epileptogenesis. We also show that MSCs: (1) do not alleviate the acute inflammatory response measured 1 day post-SE; (2) do not modify resident microglia activation in rats subjected to SE but, instead, increase the brain territory occupied by monocytes-macrophages and astrocytes at 5 days post-SE. Our findings highlight the therapeutic potential of MSCs to prevent synaptic plasticity alterations when injected early after an epileptogenic brain insult.

Key words: mesenchymal stem cells, intranasal, epileptogenesis, long-term potentiation, inflammation

1 | INTRODUCTION

Temporal lobe epilepsy (TLE) is the most prevalent form of epilepsy, affecting more than 30% of the epileptic patients. Among them, nearly two thirds do not respond to the existing antiepileptic drugs and experience recurrent seizures associated with severe cognitive and neuropsychiatric comorbidities that represent a devastating burden in the life of epileptic patients and their relatives (Fisher et al., 2014; Holmes, 2015; Verrotti et al., 2014). This form of acquired epilepsy occurs following an acute brain insult such as trauma, stroke, hypoxia or infections (Klein et al., 2018). Some of the drug-resistant patients may benefit from surgical resection, but while this procedure is often beneficial, about 20-30% of TLE patients continue to have seizures (Harroud et al., 2012). Over the last decades, many anti-epileptic treatments have emerged to abort seizures. However, in patients with new-onset epilepsy, there is no established treatment to block the progression of the disease and there is no proven solution to prevent the transformation of healthy brain into epileptic brain in people at risk to develop the disease following severe brain injuries (Pitkänen, 2010; Pitkänen and Lukasiuk, 2011). The challenge encountered in many cases lies to the fact that this latent phase is undetectable. To prevent the onset of the disease from the early stages of epileptogenesis, there is now an urgent need to develop safe and effective antiepileptogenic treatments targeting underlying mechanisms of brain alterations that leads to chronic epilepsy. The time window following a brain injury should not be neglected and should be considered as an opportunity to prevent the development of the disease or at least to limit its severity. In preclinical strategies that have been used to change the development of epilepsy, antiepileptogenic treatments or disease-modifying treatments refers to the treatments that are applied after an insult of enough severity and duration to induce epileptogenesis in the absence of any treatment (White and Löscher, 2014). To be effective, these therapies must act on the key mechanisms involved in the pathophysiological processes that cause seizures and cognitive or psychiatric comorbidities.

A promising target for potential antiepileptogenic treatment is neuroinflammation, one of the underpinning mechanisms of epileptogenesis (Pitkänen, 2010; Pitkänen and Lukasiuk, 2011; Rana and Musto, 2018; Vezzani et al., 2019; van Vliet et al., 2018). A number

of studies in both humans and experimental models have examined the relationship between the inflammatory status (in brain parenchyma or in peripheral blood) and epilepsy (from epileptogenesis to ictogenesis). These inflammatory processes are nowadays recognized as having two sides: a protective one that constitutes an adaptive and beneficial endogenous response, and a deleterious one that acts as a direct or indirect cause of dysfunction (Nguyen et al., 2002). Although the precise mechanisms underlying the pro-epileptogenic processes of inflammation are not fully understood, it is known that both inflammatory molecules and peripheral immune cell infiltration are involved in pathogenesis of epilepsy (Cerri et al., 2017; Feng et al., 2019; Li et al., 2011; Varvel et al., 2016; Vezzani et al., 2008, 2013; Zattoni et al., 2011). The enhanced release of cytokines and chemokine, astrogliosis, microgliosis and blood leukocytes infiltration into the brain parenchyma are well-documented processes that occur after an epileptogenic brain injury (Vezzani et al., 2013). A wide range of experimental evidence also reported the role of both cytokines and chemokines on neuromodulatory functions and how they can affect directly or indirectly the excitability thresholds of neurons at cellular and network levels (Vezzani and Viviani, 2015; Vezzani et al., 2013). Therefore, due to its roles in the genesis of acquired epilepsy, inflammation has become the target of many projects that have sought to develop antiepileptogenic or disease-modifying therapies.

Over the past decades, numerous investigations have been conducted using stem cells as a promising therapeutic tool for a wide range of diseases. The clinical interest of these undifferentiated cells, found from the early embryonic stages to adults, lies in their ability to infinitely self-renew and in their potential for tissue regeneration and immunomodulation (Baksh et al., 2004; Brown et al., 2019). Mesenchymal stem cells (MSCs) are multipotent cells present in many adult tissues and capable of differentiating into cells of several lineages, mainly mesodermic (Uccelli et al., 2008). These stem cells, initially identified by Fridenstein in 1968 (Bianco et al., 2008; Caplan, 1991), have become very popular in the field of regenerative medicine and cell therapy due to their high accessibility in adult tissues (especially in bone marrow or adipose tissue), their lack of significant immunogenicity and their well-described anti-inflammatory properties (Brown et al., 2019; Weiss and Dahlke, 2019). This immune-evasiveness represents a key aspect of the use of MSCs since it enables to consider both autologous and allogeneic transplantations (Ankrum et al., 2014).

The immunosuppressive effects of MSCs have fascinated people working on treatment of various inflammatory diseases (Regmi et al., 2019). Within the central nervous system,

MSCs have shown a meaningful potential for clinical use, as they can promote repair and regeneration of injured tissues and support functional recovery in a wide range of neurological diseases (Laroni et al., 2015; Mukai et al., 2018; Volkman and Offen, 2017; Yang and Wernig, 2013). MSCs have also demonstrated impressive results in improving the cellular mechanisms underlying cognitive deficits associated with neurological conditions (Eftekharzadeh et al., 2015; Ge et al., 2018; Kan et al., 2011; Lee et al., 2012; Li et al., 2018; Nasiri et al., 2019; Park et al., 2013; Zappa Villar et al., 2019). Nevertheless, only a few studies have investigated their therapeutic effects in epilepsy. In humans, clinical trials using MSCs to treat epilepsy already showed significant results (Hlebokazov et al., 2017; Milczarek et al., 2018). In animal models, promising results have been reported in reducing inflammation or decreasing seizure severity (Abdanipour et al., 2011; Agadi and Shetty, 2015; Costa-Ferro et al., 2014; Fukumura et al., 2018; Goodarzi et al., 2014; Löscher et al., 2008; Naegele et al., 2010; Roper and Steindler, 2013; Salem et al., 2018; Yasuhara et al., 2017).

Choosing the route of administration of stem cells in cell therapy protocols is of crucial importance. In clinical and preclinical studies in which MSCs were used for CNS disease, the main methods used were by intravenous, intraarterial, intraperitoneal, intrathecal or intracerebroventricular injection. Nevertheless, these delivery methods are more or less invasive and require, especially in patients, more extensive management. In addition, it has been shown that peripheral routes of administration, especially the intravenous route, lead stem cells sequestration in the lungs, spleen or lymphatic nodes (Acosta et al., 2015; Eggenhofer et al., 2012; Liu et al., 2013; Rustad and Gurtner, 2012; Xu et al., 2019). Here, we aimed to develop an innovative way to administrate MSCs intranasally in order to promote brain homing of MSCs and thus try to bypass their peripheral sequestration. This method of cell delivery has been presented as a new non-invasive alternative for cell therapy in neurological diseases (Danielyan et al., 2009, 2014; Dhuria et al., 2010; Li et al., 2015; Lochhead and Thorne, 2012). In addition, the safety of intranasal MSCs administration on the long-term has been previously reported in a model of neonatal brain injury (Donega et al., 2015).

Hence, in the present study, we first investigated whether MSCs directly injected into the nasal cavity through an implanted cannula after pilocarpine-induced status epilepticus (SE) could be detected in four brain areas of interest, i.e. the hippocampus, the ventral limbic region (that includes the piriform cortex, the amygdala and the insular agranular cortex), the

dorsal thalamus and the neocortex. Then, we examined whether MSCs are able to alleviate the acute cellular and molecular mechanisms underlying inflammation that occur during the days after SE. Finally, we studied the effect of MSCs administration during the first week post-SE on long-term potentiation, since synaptic plasticity alterations are known as the underlying mechanism of the cognitive deficits in temporal lobe epilepsy.

2 | MATERIAL AND METHODS

Study design

The study design is illustrated in Figure 1.

Study 1. *In vitro* assessment of the optimal cell culture protocol. Determination of the effect of collagen coating and FBS-containing vs. FBS-free medium on the MSC phenotype.

Study 2. MSC tracking *in situ*. Rats received GFP-expressing MSCs by intranasal injection 6 hours after pilocarpine-induced status epilepticus (SE) and were sacrificed at 24h, 48h and 72h post-SE. EGFP-immunofluorescent detection was performed on brain sections to assess the region in which MSCs migrated as well as the length of time MSCs remained in the brain within the few days after injection.

Study 3. Evaluation of gene expression at transcript level one day after SE in the rat hippocampus, the ventral limbic region, the dorsal thalamus (dTH) and the neocortex (NCX). SE was induced in juvenile rats at P49 and rats received one intranasal injection of MSCs 6 hours post-SE. Hippocampus, VLR, dTH and NCX of rats were dissected after transcardial perfusion of NaCl and the inflammatory profile was evaluated by RT-qPCR. Analysis were performed in rats subjected to SE (SE: n=7), in rats subjected to SE that received intranasal MSCs (SE+MSC: n=6) and in control rats (CTRL: n=5).

Study 4. Effect of intranasal MSC administration on astroglial and microglial activation, as well as monocyte-macrophages infiltration and transdifferentiation evaluated using GFAP-, Iba-1- and CD68-immunofluorescent detections, respectively, in the rat dentate gyrus and the CA1 layer of the hippocampus, the VLR and the dorsal thalamus at 1 day (SE, n=8 ; SE+MSC, n=7) or 5 days (SE, n=6 ; SE+MSC, n=9) post-SE and in control rats (CTRL, n=3).

Study 5. Determination of the effect of a single or repeated intranasal MSC administration on long-term potentiation (LTP) in rats subjected to pilocarpine-induced SE. At P49, 29 rats were subjected to SE. Among the 22 rats that developed SE, 9 rats did not receive MSCs, 6 rats

received one intranasal injection of MSCs 6 hours post-SE, and 7 rats received 4 injections of MSC at 6 hours, 1 day, 4 days and 7 days post-SE. Control rats (n=10) did not receive MSCs. Long-term potentiation (LTP), the cellular mechanisms underlying memory in the hippocampus, was monitored 10-20 days post-SE.

Mesenchymal stem cell (MSC) culture

MSC culture for in vitro study

Bone marrow Sprague-Dawley rat mesenchymal stem cells expressing GFP were purchased from Cyagen® (RASMIX-01101) and expanded from passage 5 to passage 8 using Oricell® Mesenchymal Stem Cell Growth Medium (GUXMX-90011, Cyagen) containing MSC basal medium, 10% MSC-qualified fetal bovine serum (FBS), penicillin-streptomycin and glutamine. At passage 8, to evaluate whether the presence of FBS in the culture medium affected significantly the proliferation of MSCs and their phenotype, we cultured MSCs in the FBS-containing medium Oricell® Mesenchymal Stem Cell Growth Medium (GUXMX-90011, Cyagen) or in a FBS-free medium MesenCult™ Mouse Expansion kit (05513, StemCell Technologies). In addition, the tissue culture treated plates/flasks (Falcon) were coated or not with collagen type I from rat tail (A1048301, Gibco). The required concentration of collagen of 5µg/cm² was diluted in acetic acid and tissue culture plates were coated at room temperature for 1 hour. The collagen excess was then washed with PBS and the plates were used either immediately or stored at 4°C for use within 7 days. MSCs were plated at a density of 25x10³ cells/cm². The medium was changed every 3 days, and the cultures were passaged at a ratio of 1:6 when reaching 70-80% confluence. MSCs were incubated at 37°C in a humidified atmosphere of 5% CO₂. Cells at passage 10 were used for experiments.

MSC for in vivo administration

MSCs at passage 8 were seeded in MesenCult™ Mouse Expansion kit (05513, StemCell Technologies), supplemented with L-glutamine (07100, StemCell). MSCs were plated at a density of 25x10³ cells/cm² and incubated at 37°C in a humidified atmosphere of 5% CO₂. The medium was changed every 3 days, and the cultures were passaged at a ratio of 1:6 when reaching 70-80% confluence. As expanded, to confirm the cellular identity of cultured cells, MSCs were subjected to RT-qPCR to verify that the cells met the specific criteria defined by the International Society for Cellular Therapy (Dominici et al., 2006): positive for CD73, CD90

and CD105 and negative for CD31, CD34 and CD45. Cells at passage 10 were used for experiments.

Animals

All animal procedures were in compliance with the guidelines of the European Union (directive 2010-63), taken in the French law (decree 2013/118) regulating animal experimentation, and have been approved by the ethical committee of the Claude Bernard Lyon 1 University (protocol #12839-2017102515216243). Briefly, male Sprague-Dawley rats (Envigo, Netherlands) were used in these experiments. They were housed in a temperature-controlled room ($23 \pm 1^\circ\text{C}$) under diurnal lighting conditions (lights on from 6 a.m to 6 p.m). Rats arrived at 21 day-old and were maintained in groups of 5 in 1,800 cm² plastic cages, with free access to food and water. After SE, rats were maintained in individual cages and weighed daily until they gained weight. Until sacrifice, epileptic rats were housed alone and control rats were housed in groups of 5 in standard cages.

Intranasal cannula implantation surgery and MSC administration

One week before MSC administration, P42 rats underwent surgery under anesthesia with i.p. injection of ketamine (Imalgene, 80 mg/kg) and xylazine (Rompun, 10 mg/kg) to implant cannula in the nasal cavity. Figure S1 shows the position of the implanted intranasal cannulas allowing the cells to be injected directly onto the olfactory mucosa. Age-matched control sham-operated rats underwent anesthesia and incision only. At the day of MSC administration, rats were subjected to anesthesia with 4% isoflurane in an induction chamber before 10 μL of hyaluronidase (100U, Sigma-Aldrich, St. Louis, MO) treatment was applied to each nostril 30 minutes prior to the administration of MSCs to increase the permeability of the nasal mucosa, its use having shown a significant increase in the number of MSCs crossing the olfactory epithelium to integrate brain regions (Danielyan et al., 2009). For the rats that received four injections of MSCs, hyaluronidase was applied only for the two first ones (6 hours and 1 day post-SE). Each administration consisted of $1 \cdot 10^6$ MSCs per nostril (i.e. $2 \cdot 10^6$ MSC per rat) diluted in 10 μL of saline injected intranasally through the cannula.

Pilocarpine-induced *status epilepticus* (SE)

SE was induced by pilocarpine injected at day 49. To prevent peripheral cholinergic side effects, scopolamine methylnitrate (1 mg/kg in saline, s.c.; Sigma-Aldrich) was administered 30 min before pilocarpine hydrochloride (350 mg/kg, in saline, i.p.; Sigma-Aldrich). After 2

hours of continuous behavioral SE, 10 mg/kg diazepam (i.p.; Valium; Roche®) was injected, followed, 60 min later, by a second injection of 5 mg/kg diazepam to terminate behavioral seizures. Control rats received systematically corresponding injections of saline solution. The animals were then sacrificed at various time points for electrophysiological recordings (LTP assessment) or for molecular and cellular analysis.

Animal care after SE

Control and treated rats were weighted every day during the first two weeks following SE, and then every week until termination of the experiment. Daily abdominal massages were performed twice a day during the first week to activate intestinal motility, which was disrupted following SE.

Onset of handling-induced seizures

Electroencephalographic recordings were excluded to determine epilepsy onset due to preliminary experiments that showed that the sole implantation of screws into the skull induced significant and lasting inflammation over time in the cortex and, to a lesser extent, in the hippocampus. As a result, epilepsy onset was determined according to clinical criteria. Therefore, animals used for electrophysiological recordings were tested for the occurrence of handling-induced seizures (HIS) three times a day until termination of the experiment. HIS were triggered by restraining rats for 10 seconds at the level of the chest with gentle pressure. Animals were declared as “epileptic” once they developed HIS on 2 consecutive trials.

Ex vivo procedures

All rats were deeply anesthetized with a lethal dose of pentobarbital (100 mg/kg; Dolethal) before being sacrificed. For RT-qPCR analysis, hippocampus, ventral limbic region, dorsal thalamus and neocortex were rapidly microdissected, frozen in liquid nitrogen, and stored at -80°C. For immunohistochemistry analysis, animals were transcardially perfused (30 mL/min) with 4% paraformaldehyde in 0.1 M phosphate buffer. After cryoprotection in 30% sucrose, brains were frozen at -40°C in isopentane and stored at -80°C.

RNA extraction and quantification of transcript level variations by reverse transcriptase real-time polymerase chain reaction (RT-qPCR)

MSCs nucleic acids were extracted by adding 750 µL Tri-Reagent (TR118, Euromedex) and 200 µL chloroform (VWR®). Brain structures frozen in liquid nitrogen were crushed using Tissue-Lyser (Qiagen®) in 250 µL of ultrapure RNase-free water (Eurobio) before nucleic acids were

extracted by adding 750 μ L Tri-Reagent LS (TS120, Euromedex) and 200 μ L chloroform (VWR[®]). For both MSCs and brain structures, after precipitation with isopropanol (I-9516, Sigma-Aldrich[®]), washing in 75% ethanol (VWR) and drying, total nucleic acids were resuspended in 50 μ L ultrapure water and treated with DNase I (Turbo DNA Free[®] kit; AM1907, Ambion[®]) to eliminate any trace of possible genomic DNA contamination. The purified total RNAs were then washed using the RNeasy[®] minikit (Qiagen[®]) kit. After elution, the total RNA concentration was determined for each sample on BioDrop[®] μ Lite. All RNA extracts were stored at -80°C until use.

Total RNAs (480 ng) were reverse transcribed to complementary DNA (cDNA) using both oligo dT and random primers with PrimeScript RT Reagent Kit (Takara) according to manufacturer's instructions, in a total volume of 10 μ L. In RT reaction, 300 000 copies of a synthetic external non-homologous poly(A) standard messenger RNA (SmRNA; A. Morales and L. Bezin, patent WO2004.092414) were added to normalize the RT step (Sanchez et al., 2009). cDNA was diluted 1:13 with nuclease free Eurobio water and stored at -20°C until further use. Each cDNA of interest was amplified using 5 μ L of the diluted RT reaction by the "real-time" quantitative polymerase chain reaction (PCR) technique, using the Rotor-Gene Q thermocycler (Qiagen[®]), the SYBR Green Rotor-Gene PCR kit (Qiagen[®]) and oligonucleotide primers specific to the targeted cDNA. The sequences of the specific forward and reverse primer pairs were constructed using the Primer-BLAST tool or using the "Universal Probe Library" software (Roche Diagnostics). Sequences of the different primer pairs used for MSCs and for rat brain tissue are listed in Table S1 and Table S2, respectively. The number of copies of each targeted cDNA contained in 5 μ L of the diluted RT reaction was quantified using a calibration curve based on cascade dilutions of a solution containing a known number of cDNA copies.

Pro-inflammatory (PI-I), anti-inflammatory (AI-I) and inflammation cell (IC-I) indexes were calculated for each series of individuals to be compared using a specific set of genes: IL1 β , IL6, TNF α , MCP1 and MIP1 α for PI-I; IL4, IL10 and IL13 for AI-I; ITGAM and GFAP for IC-I. For each individual, the number of copies of each transcript has been expressed in percent of the averaged number of copies measured in the whole considered population of individuals. Once each transcript is expressed in percent, an index is calculated by adding the percent of each transcript involved in the composition of the index and expressed in arbitrary units (A.U.). Each time that an index is presented, the groups of individuals constituting the population is specified.

Tissue processing for histological procedures

Cryostat-cut (40 µm thick) sections from rat samples were transferred into a cryopreservative solution composed of 19.5 mM NaH₂PO₄·2H₂O, 19.2 mM NaOH, 30% (v/v) glycerol and 30% (v/v) ethyleneglycol and stored at -25°C.

Immunohistochemistry

We have previously reported (Fig. S2) that the use of a single anti-GFAP antibody was not optimal for labelling all the astrocytes. Therefore, dual GFAP immunolabeling in free-floating sections (40 µm thick) from paraformaldehyde-fixed tissue was performed with a rabbit polyclonal anti-GFAP antibody (1:1000; AB5804, Chemicon) and a mouse monoclonal anti-GFAP antibody (1:1000; G3893, Chemicon). For detection of microglia and monocytes-macrophages, free-floating sections were incubated with a goat polyclonal anti-Iba-1 antibody (1:500; AB5076, Abcam) and a mouse monoclonal anti-CD68 antibody (1:1000; MCA341GA, Bio-rad). Finally, for detection of GFP-expressing MSCs, free-floating sections were incubated with a chicken polyclonal anti-GFP antibody (1:1000; 600-901-215, Rockland) or a mouse monoclonal anti-GFP antibody (1:1500; 66002-1, Proteintech).

For fluorescent dual immunolabeling of GFAP, sections were incubated with an Alexa-Fluor-488-conjugated donkey anti-rabbit IgG antibody (1:1000; A-21206; Molecular Probes) and to an Alexa-Fluor-647-conjugated donkey anti-mouse IgG antibody (1:1000; A-31571; Molecular Probes). For fluorescent dual immunolabeling of Iba-1 and CD68, sections were incubated with an Alexa-Fluor-488-conjugated donkey anti-goat IgG antibody (1:750; A-11055; Molecular Probes) and an Alexa-Fluor-647-conjugated donkey anti-mouse IgG antibody (1:1000; A-31571; Molecular Probes). For fluorescent dual immunolabeling of GFP, sections were incubated with an Alexa-Fluor-633-conjugated goat anti-chicken IgG antibody (1:1000; A-21103; Molecular Probes) or an Alexa-Fluor-633-conjugated goat anti-mouse IgG antibody (1:500; A-21052; Molecular Probes).

Sections were then mounted on SuperFrost Plus slides and coverglassed with Prolong Diamond Antifade reagent (Molecular Probes). Slides were observed using a LSM 800 confocal microscopy system (Zeiss) with ZEN Imaging software (Zeiss). All sections were analyzed under identical conditions of photomultiplier gain, offset and pinhole aperture, allowing the comparison of fluorescence intensity between regions of interest. Then, for each of the markers, ImageJ software was used to measure areas of fluorescence using thresholding

procedure. The quantifications of the immunofluorescent surface were performed on stacks of 12 images taken over a thickness of 11.36 μm with a step of 1,03 μm .

Electrophysiology

Slices preparation

Transverse hippocampal slices were prepared from postnatal day 10-20 Sprague-Dawley rats. Animals were anesthetized using isoflurane and sacrificed by decapitation. As described in Fares et al. (2013), brain was quickly extracted and cooled with ice-cold standard artificial cerebrospinal fluid (ACSF) composed of the following (in mM): 124 NaCl, 5 KCl, 1.25 $\text{Na}_2\text{H-PO}_4$, 2 MgSO_4 , 2 CaCl_2 , 26 NaHCO_3 and 10 D-Glucose, saturated with 95% O_2 and 5% CO_2 . Hippocampi were dissected out and 370 μm -thick transversal slices were prepared using a vibratome (VT1000S; Leica) equipped with a ceramic blade. The slices were then incubated in ACSF at room temperature for at least 1h before transfer to the recording chamber. The ACSF perfused during the recording was supplemented with 100 μM picrotoxin to block GABA_A receptors.

Electrophysiological recordings

Whole-cell patch-clamp recordings were obtained from CA1 pyramidal neurons in current clamp mode at -70mV with a patch pipette (3-5 M Ω) containing the following drugs (Sigma): 120 mM potassium gluconate, 20 mM KCl, 0.2 mM EGTA, 2 mM MgCl_2 , 10 mM HEPES, 4 mM Na_2ATP , 0.3 mM Tris-GTP and 14 mM phosphocreatine (pH 7.3, adjusted with KOH). Hippocampal CA1 pyramidal neurons were visualized with a Zeiss Axioskop 2 equipped with a x40 objective, using infrared video microscopy and differential interference contrast optics. Series resistance (typically 15-25 M Ω) was monitored throughout each experiment; cells with more than 20% change in series resistance were excluded from analysis.

Whole-cell patch clamp recordings were performed using an Axopatch-200B amplifier (Molecular Devices) at the sampling rate of 10 kHz and filtered at 5 kHz. Data were recorded and analyzed using a Digidata 1440A interface and pClamp 10 software (Molecular Devices). Capillary glass microelectrodes filled with ACSF and connected to an isolator (Iso-Flex, AMPI) were used to stimulate presynaptic axons in the stratum radiatum layer of the hippocampus (120–150 μm away from the soma). Stimulation at 0.05 Hz was used to establish baseline synaptic responses. The stimulation strength was set to evoke excitatory postsynaptic potentials (EPSPs) between 5 and 8 mV.

Once a steady baseline level of EPSPs was recorded for 10 to 20 min, long-term potentiation (LTP) was induced using the theta burst pairing (TBP) protocol, which was delivered within 20 min after whole-cell formation to prevent the washout of LTP induction. Back propagating action potentials were elicited by direct somatic current injection (1 ms, 1-2 nA). The standard TBP protocol consists of EPSPs paired with a single back propagating action potential (bp-AP) timed so that the bp-AP (approximately 15 ms delay) occurred at the peak of the EPSPs as measured in the soma. A single burst contained five pairs delivered at 100 Hz and ten bursts were delivered at 5 Hz per sweep. Three sweeps were delivered at 10 s intervals for a total of 30 bursts (150 bp-AP-EPSP pairs). Electrophysiological data were analyzed using pClamp 10 and Igor pro software (WaveMetrics).

Data and statistical analysis

GraphPad Prism (v.7) software was used to statistically analyze data. Majority of data are expressed as mean \pm SEM of the different variables analyzed. Statistical significance for transcripts levels was calculated by one-way analysis of variance (ANOVA) with Tukey's *post hoc* test. Differences in the surface area occupied by GFAP, Iba-1 and CD68 cells were tested using two-way ANOVA. Statistical significance for number of Iba-1-positive cells among CD68-positive cells was calculated by one-way analysis of variance (ANOVA) with Sidak *post hoc* test. Differences in amplitude of LTP was tested using two-way analysis of variance (ANOVA) followed by *post-hoc* Mann-Whitney U test. The p value of 0.05 defined the significance cut-off.

3 | RESULTS

MSCs grown in FBS-free medium exhibit an identical phenotype to MSCs cultured in FBS-containing medium

We first sought to determine the most reproducible MSC culture conditions, *i.e.* the conditions in which we excluded any element that could induce intrinsic variability in the MSC phenotype between the different experiments of this study. In cell culture media, serum is a commonly used supplement, allowing to provide, among others, macromolecules, nutrients and growth factors to the growing cells. Fetal bovine serum (FBS) is the most frequently used in cell culture protocols, although its composition is ill-defined and highly affected by various factors such as the method of blood collection and processing, the donor diet and country of origin, and the inherent biological differences between donors (Gottipamula et al., 2013). Previous investigations have shown that the presence of FBS in cell cultures can be a source of variability in the results obtained (Gottipamula et al., 2013; Stein, 2007). In addition, the ethical concerns about the animal origin of the FBS and therefore its unsteady supply is a major problem for its regular use over long periods of time (Gstraunthaler, 2003). Considerable efforts towards a standardization of cell culture protocol have been made these last decades by developing chemically defined serum-free media formulations. Hence, we performed an *in vitro* comparison of cell culture conditions by growing MSCs in FBS-containing or FBS-free medium. The phenotypic characteristics of MSCs were confirmed using RT-qPCR by measuring the transcripts of specific positive (CD29, CD44, CD49a, CD90 and CD105) and negative MSC markers (CD11b, CD31, CD34, CD45 and CD117), as established by the ISCT (Dominici et al., 2006). For positive gene expression, statistical differences were only observed for CD44 and CD49a between the conditions in which MSCs were grown with or without FBS (Fig. 2). Negative genes were not detected in RT-qPCR under any cell culture conditions, except for CD34 with a low number of cDNA copies detected.

A further investigation was conducted to evaluate the impact of the presence or absence of an extracellular matrix, here a collagen coating, on the culture surface on the MSC phenotype, as well as the effect of this coating on the adhesion properties of MSCs. No change was observed in the adhesion properties or cell expansion of stem cells due to the presence or absence of collagen I (observational data). As depicted in Figure 2, the cell phenotype of MSCs was not substantially modified as well in presence of collagen I, except for CD34 which had a significantly higher expression with collagen in the FBS-containing medium. Overall, these

results indicate that FBS and collagen coating can be precluded from the cell culture protocol with no risk of phenotypic modifications of MSCs. Therefore, for the rest of the *in vivo* study, the cells were cultured in the FBS-free medium.

MSCs are not detectable *in situ* after intranasal administration post-SE

Inflammation-induced mobilization of MSCs is a well-established mechanism by which MSCs will migrate towards the suffering areas where an inflammatory environment is usually present and then home into sites of wounding (Karp and Leng Teo, 2009; Ponte et al., 2007; Rustad and Gurtner, 2012; Spaeth et al., 2012; Ullah et al., 2019). Studies 1 and 3 presented in this thesis have shown a strong inflammatory response within the first 24 hours after pilocarpine-induced SE in the hippocampus (Hi), VLR, dorsal thalamus (dTH) and neocortex (NCX). Hence, we sought to determine whether MSCs injected 6 hours post-SE through the nose migrated preferentially into these areas. Several experiments have been carried out to detect over time the presence of MSCs. Rat brains were collected at 24h, 48h and 72 hours post-SE. Considering that the MSCs used in this study are GFP-positive, brain sections of rats were observed directly under confocal microscopy to investigate the intrinsic green fluorescence of the cells, with or without amplification with different anti-EGFP antibodies. Surprisingly, nasally applied MSCs were not detected neither in the hippocampus, nor in the ventral limbic region, the thalamus or the neocortex when observed in brain sections selected at coronal planes between IA+6.70 and IA+3.70 mm according to Paxinos and Watson (Paxinos, G. and Watson, C., 1998).

MSCs do not resolve the explosive SE-induced inflammation

To determine whether one injection of MSCs 6 hours after SE elicit an immunomodulatory response and lead to a containment of the explosive inflammation observed 24 hours post-SE, we next assessed the transcript levels of pro- and anti-inflammatory cytokines, chemokines and cellular markers in the hippocampus (Fig. 3, Table S3), the ventral limbic region (VLR, Fig. 4, Table S4), the dorsal thalamus (Fig. 5, Table S5) and the neocortex (Fig. 6, Table S6). These particular regions were investigated because it is known that in TLE, seizures emanate from the temporal lobe, generally from the hippocampus, the entorhinal cortex or the amygdala, then diffuse to the rest of the limbic system, passing through the piriform and perirhinal cortices and then spread to, among other regions, the cortex and the thalamic nuclei (Löscher et al., 2008). Therefore, these regions constitute valuable targets for cell

therapy aiming at suppressing seizure generation. Moreover, as we have shown in studies 1 and 3 of this thesis, we know that in these brain regions, very high levels of inflammatory molecules are released within the first 24 hours after the SE. Here, we quantified the transcripts levels of a set of 11 inflammatory markers including pro-inflammatory cytokines (IL1 β , TNF α , IL6), chemokines (MCP1, MIP1 α), interleukin-1 receptor IL1R, anti-inflammatory cytokines (IL4, IL10, IL13) and cell markers (ITGAM for microglia, GFAP for astrocytes). In all structures, high significant differences were observed between control group (CTRL) and SE or SE+MSC group, for almost all investigated genes (Fig. 3-6, hashtags indicates statistical significance when comparing to CTRL group). When considering the differences between rats subjected to SE, in the hippocampus, statistical analyses revealed that except for GFAP where the transcript level was significantly higher in rats that received MSCs ($p=0.0024$), there were no statistical differences between the SE group and the SE+MSC group (Fig. 3). In the VLR, significant differences between the two groups of SE rats were observed for IL1R whose expression was increased in the SE+MSC group ($p=0.0023$), while, unexpectedly, both anti-inflammatory cytokines IL4 and IL13 had their expression decreased after MSCs administration ($p=0.0403$ and $p=0.0445$, respectively, Fig. 4). In the dorsal thalamus, only MIP1 α -mRNA was increased in the SE+MSC group ($p=0.0360$, Fig.5). Finally, in the neocortex, no significant differences were observed for all genes (Fig. 6). The inflammatory indexes, i.e. the pro-inflammatory, anti-inflammatory and inflammation cell indexes, reflect these findings, and except for the inflammation cell index in the hippocampus where a significant difference resulting from an induction of astroglial GFAP is observed between the SE and SE+MSC group, no other difference is detected, regardless of the brain structure considered (Fig. 3-6). Overall, these findings indicate that MSCs did not substantially alleviate the acute inflammatory response observed 24h post-SE. Intriguingly, the expression profiles reported here are inconsistent with the existing knowledge of an anti-inflammatory role of MSCs.

MSCs exacerbate astrogliosis and monocytes-macrophages (mo-M Φ) transdifferentiation

In studies 1 and 3 presented in this thesis, we showed that microglial and astrocyte reactivity at 1 day and 9 days were very high within the four structures investigated here (i.e. Hi, VLR, dTH and NCX). In addition, it is now well-documented that after a brain injury such as SE, the integrity of the blood-brain barrier – usually impermeable to many molecules, toxins and cells from the periphery due to inter-endothelial tight junctions – is compromised and become

permeable, especially to infiltrating immune cells (Rana and Musto, 2018). In study 2, we identified CD68 as a specific marker of infiltrating monocytes and established that infiltration of these cells occurred within the first 24 hours after SE, with a characteristic round-shaped morphology at that time. We then showed that transdifferentiation processes into monocyte-macrophages bearing microglia-like morphology occurred in the following days and that CD68 marker colocalized, at 9 days post-SE, with the Iba-1 marker, specific to residential microglia, in a substantial number of cells, suggesting that some cells acquired a microglial phenotype. To evaluate the effects of MSCs on early glial activation and immune cell infiltration during epileptogenesis in the most vulnerable brain region, brain sections of rats subjected to SE that received or not MSCs were stained at 1 day and 5 days post-SE for GFAP (Fig. 7), Iba-1 and CD68 and stained surface area were quantified (Fig. 8, 10, 11, 12). Induction of SE by pilocarpine leads to a high tissue degradation frequently associated with swollen and edematous areas (Curia, 2008). Therefore, in the ventral limbic region and in the dorsal thalamus where tissue integrity was severely damaged, it should be noted that the surface areas of GFAP, CD68 and Iba-1 were quantified in the surviving areas. In the hippocampus, dentate gyrus and CA1 layer were observed and evaluated separately.

As described in the section method, two antibodies directed against GFAP were used here to ensure the detection of all surface area occupied by astrocytes. Image observation and surface area quantification of GFAP were ascertained on superimposed images of the two antibodies. Clusters of GFAP+ reactive astrocytes (Fig. 7) appeared in all regions of interest at 5 days post-SE in the group that received MSCs. SE groups (1 day and 5 days post-SE) as well as the SE+MSC group at 1 post-SE did not differ from the control group in all brain regions. Quantification of the GFAP-Immunolabelled surface area confirmed that the area covered by astrocytes at 5 days post-SE was significantly higher in the groups of rats that received stem cells (Fig. 7B-7D). Iba-1 labelling was significantly higher at 5 days compared to 1-day post-SE, although no significant difference was found between the SE and SE+MSC groups, regardless of the brain area (Fig. 8A-B, 10A-B, 11A-B, 12A-B). For CD68, histological observations have revealed a phenotypic shift. CD68+ cells, that were round-shaped at 1-day post-SE, exhibited an enlarged cell body with thick extensions at 5 days post-SE (Fig. 8A, 10A, 11A, 12A). The quantification of the CD68-immunolabelled surface area did not reveal any difference at 1-day post-SE between SE rats and SE+MSC rats (Fig. 8C, 10C, 11C, 12C). At 5 days post-SE, stem cell

transplantation resulted in a robust and significant increase in the surface area occupied by infiltrating monocytes in the CA1 layer of the hippocampus (Fig. 10C).

To determine whether the presence of MSCs could play a role in the transdifferentiation processes of CD68+ infiltrating monocytes into monocytes-macrophages (mo-MΦ) with a microglia-like phenotype, we quantified the number of CD68+ cells for which Iba-1 was co-expressed. Figure 9 provides the percentage of CD68+/Iba-1+ and CD68+/Iba-1- cells in the dentate gyrus of rat brain sections from SE and SE+ MSC groups at 1- and 5-days post-SE. This hippocampal subregion presented at 1-day post-SE with a significantly higher number of CD68+ cells that were negative for Iba-1 (93% for the SE group; 89% for the SE+MSC group). At 5 days, we found that the percentage of Iba-1+ cells increased to 34% in the SE group, and 47% in the SE+MSC group. In the SE+MSC group, no statistical difference was observed between the percentage of CD68 positive or negative cells for Iba-1 (Fig.9). Overall, these results suggest that the significant increase in Iba-1 labelling at 5 days post-SE compared to 1 day post-SE reflects, on the one hand, the activation of microglial cells in response to pilocarpine-induced SE, and on the other hand, the transdifferentiation of the infiltrating monocytes into mo-MΦ with microglia-like morphology, as demonstrated by the overlays of CD68 and Iba-1 labelling at 5 days post-SE.

To complete these histological results, we quantified by RT-qPCR the transcript levels of CD68, as well as ITGAL and CD14 in the rat brains 24 hours post-SE (Fig. 13). ITGAL, also known as CD11a, has recently been identified as a specific marker very strongly expressed by peripheral immune cells and absent from residential microglia, thus allowing, like CD68, to distinguish monocyte-derived macrophages from microglia (Shukla et al., 2019). CD14 is also a monocyte/macrophage marker but less exclusive than CD68 and ITGAL since its expression is found in tissue macrophages and perivascular cells, and to a lesser extent, on the surface of neutrophils (Guillemin and Brew, 2004; Navarro, 2007). This marker was previously used to discriminate in the brain parenchymal monocytes-macrophages from residential microglial cells, however, its expression decreases over the long term, making it difficult to assess the fate of these cells (Navarro, 2007). Figure 13 show that increased levels of CD68, ITGAL and CD64 were measured in rats that received MSCs, although these increases were not always significant in the different brain areas due to variability. The increase in CD68 and ITGAL

expression was similar, with strong correlations between the two genes observed for the different brain areas (Hi, $r^2= 0.86$; VLR, $r^2= 0.83$; dTH, $r^2= 0.70$; Ncx, $r^2= 0.82$).

MSCs prevent long-term potentiation alterations after SE

Cognitive deficits are commonly reported disorders in patients with epilepsy and prior experimental studies have shown that these cognitive impairments are also observed in rats subjected to pilocarpine-induced SE after a latent period (Curia et al., 2008). SE has been shown to alter molecular and cellular processes underlying these deficits, especially synaptic plasticity (Postnikova et al., 2017). To analyze the effects of nasally applied MSCs on synaptic transmission, we examined their effect on EPSPs, which is the underlying cellular mechanism of information coding. Synaptic gain change, which reflects the strength of neuronal responsiveness to synaptic activity, can result from modifications in properties of synaptic transmission such as long-term potentiation (LTP). In hippocampus, LTP is a well-established general candidate mechanism for learning and memory. By using electrophysiological stimulation of CA1 pyramidal neurons, we first investigated how SE impacted the EPSPs level (Fig. 14A). We show that LTP monitored in SE rats (121 ± 7 %, $n=14$, $p=0.009$; Fig. 14A) is significantly reduced compared to healthy CTRL rats ($p<0.001$; Fig. 14A-E), indicating that LTP was severely impaired in rats subjected to pilocarpine-induced SE. We then explored the effect of either a single injection of MSCs 6 hours post-SE or a repeated administration at 6 hours, 1 day, 4 and 7 days post-SE on the synaptic functions (Fig. 14B-C). Compared with baseline data, i.e before theta burst pairing (TBP) stimulation, the SE+1MSC group showed a significant increase in the EPSP amplitude (196 ± 11 %, $n=14$, $p<0.001$; Fig. 14B). Besides, when compared to untreated SE rats, we show that exposure to SE followed by one injection of MSCs induced a significant increase in the LTP amplitude ($p<0.001$; Fig. 14B-E). Treatment with four injections of MSCs resulted as well in a statistically significant increase in the EPSPs amplitude (240 ± 21 %, $n=11$, $p<0.001$; Fig. 14C) and was significantly higher than that of the SE group ($p<0.001$; Fig. 14C-E). Finally, when comparing MSC-treated rat groups (SE+1 x MSC, $n=14$; SE+ 4 x MSC, $n=11$) with control rats (CTRL, $n=17$), our data demonstrate that the magnitude of LTP was similar (SE+ 1 x MSC, $p=0.108$; Fig 14D-E) or higher (SE+ 4 x MSC, $p=0.007$; Fig 14D-E) than CTRL group. These results suggest that intranasal MSCs administration could induce a significant increase in excitatory synaptic transmission. Altogether, our findings indicate that intranasal MSC therapy within hours after the induction

of SE can counteract the alteration of the cellular mechanisms underlying learning and memory in the hippocampus.

4 | DISCUSSION

The current study shows that intranasally applied MSCs improve mechanisms underlying cognitive function that are altered in a model of pilocarpine-induced SE. We show that LTP measured in the hippocampus is facilitated with one administration of MSCs during the acute phase of epileptogenesis (i.e. 6 hours post-SE), with no significant difference measured with the control healthy group. Most interestingly, after four intranasal administrations of MSCs (6 hours, 1 day, 4 days and 7 days after the onset of SE), we observe that MSCs not only prevent synaptic function alterations, but also outreach the LTP measured in control healthy group. Moreover, we show that this modulation of synaptic function occurs independently from the alleviation of the explosive inflammatory response that follows pilocarpine-induced SE, or from the physical presence of MSCs into injured brain areas. Furthermore, we demonstrate that the use of MSCs is associated with an exacerbation of astrocyte response 5 days after SE. In addition, the presence of MSCs appears to facilitate the transdifferentiation of infiltrating monocytes into brain monocyte-macrophages with microglial (Iba-1 expression) characteristics and their integration into the microglial network. Finally, our findings indicate that the intranasal administration represents an efficient route for stem cell transplantation targeting epileptogenesis processes after an epileptogenic brain insult.

Because the purpose of this type of preclinical study is to lead the way towards the development of new therapies for humans, it is important to consider the conditions under which stem cells are produced. For the translational use of MSCs in humans, cell culture protocols must comply with good cell culture practice, requiring that the constitution of each component must be known, constant over time and non-harmful to humans. Clinical applications are now focusing on the large-scale production of high quality, safe and reproducible cells that cannot be generated with the use serum and undefined supplements during the manufacturing process. Therefore, serum of animal origin should be prohibited as its composition is poorly defined and may induce uncontrolled variability in protocols that must be carried out over long periods of time, precluding the comparison of the treatment effects from one patient to another. MSCs used in clinical trials are usually cultured in FBS-

containing medium but may therefore constitute a threat to the communication of transmissible spongiform encephalopathy and bovine spongiform encephalopathy to recipient patients (Gottipamula et al., 2013). The use of human serum, human platelet lysate or human cord blood serum was one way to overcome this problem (Gottipamula et al., 2013; Witzeneder et al., 2013). Although these alternatives have been extensively tested in MSC culture with very promising results (Gottipamula et al., 2013), the concerns regarding lot variability, undefined composition and higher risk of contamination should be kept in mind. The demonstration that the lack of serum in the culture medium had no consequence neither on the phenotype nor on the proliferation and adhesion properties of MSCs used in our study allowed us to reproduce identical growing conditions throughout the whole duration of the study and across the various experiments.

Why were MSCs not found in brain tissue?

In our study, we show that effects of MSCs in brain tissue can be measured without the physical presence of stem cells on brain tissue. We used the nasal route to target the central nervous system, hypothesizing that the closer to the brain MSCs are administered, the more efficiently they can reach the damaged brain areas. The aim was also to bypass their potential sequestration in lungs associated with intravenous administration (Fischer et al., 2009). MSCs are thought to migrate towards the lesion sites throughout the chemokines secreted by glial cells and neurons in the injured brain areas (Liu et al., 2013). *In vitro* studies that we previously performed showed that MSCs used here express high levels of chemokine receptor CCR1, which binds, among others, the chemokine MIP1 α that is strongly upregulated during the acute phase of inflammation between 7 and 24 hours post SE, as demonstrated in study 1 and 3. Nevertheless, we reported that intranasally-delivered MSCs, tracked through their expression of GFP, were not found in the brain within 3 days after injection. We are not the first to report the absence of MSC grafting within the brain after their transplantation in a model of epilepsy (Voulgari-Kokota et al., 2012). The intriguing absence of MSCs in regions expressing high levels of chemokines following SE, whereas beneficial effects have been observed, might be attributed to the fact, even if rather unlikely, that we may have not looked for them in the right brain regions and at the right time. At the time we searched for brain presence of MSCs, they might not have yet migrated to the most caudal brain areas, i.e. the areas of interest that include the hippocampus, the ventral limbic region and the dorsal

thalamus. However, if MSCs use during their extravasation chemokine signalling pathways similar to those involved in leukocyte infiltration, they should have been massively detected around 24 hours post-SE, as are the very numerous monocytes that infiltrate the vulnerable areas of the brain following pilocarpine-induced SE. In studies where MSCs were injected intranasally, it has been shown that they could accumulate in the olfactory bulb rather than in the hippocampus, amygdala, cerebellum, brain stem or spinal cord (Danielyan et al., 2009, 2011, 2014). In addition, these same studies showed that a fraction of the transplanted cells survived for at least 4.5 months in the CNS. Further studies are thus needed to look for MSCs presence in more rostral brain regions and over longer periods of time after transplantation.

One of the questions that has emerged from all studies with *in vivo* administration of MSCs concern the fate of the stem cells in the target tissue. It is unlikely that stem cells differentiated at such an early stage into brain cells such as neurons, astrocytes or microglia. One possible explanation for not observing an engraftment of MSCs in the injured areas after SE may be related to their low survival rate. Different factors such as excessive oxidative stress, acute immune response, or highly inflammatory or hypoxic microenvironments at sites of injury are known to reduce survival and engraftment of MSCs into the brain (Chang et al., 2013; Regmi et al., 2019). Further research on the migration route of MSCs, their distribution and survival into the brain parenchyma after intranasal administration is still needed to address these concerns.

MSCs do not exhibit anti-inflammatory effects in brain areas prone to lesions after SE

The anti-inflammatory effects of MSCs are commonly reported in the literature as a key factor justifying their use as therapeutic tools, particularly in a wide range of diseases with an inflammatory component. Surprisingly, we observed no significant alleviation of the inflammatory response observed 24h after pilocarpine-induced SE, i.e. 18h after MSCs intranasal transplantation. Considering that the peak of mRNAs of inflammatory markers in our model is observed 7 hours post-SE and that the first application of MSCs was performed 6 hours post-SE, the time window may be too short to act on this acute release of inflammatory cytokines. It can be assumed that an earlier administration of MSCs (within the couple hours following SE) might have contained the inflammatory response in a more effective way.

Since acute inflammation may represent a necessary tissue adaptation over a short period of time following severe aggression to remove cell debris and toxic molecules released by dying cells (Russo and McGavern, 2016), MSCs may have preserved it by maintaining temporarily microglia and monocyte-macrophages into a M1 phenotype. Later, they may have targeted their action towards a change in polarization of brain microglial cells and monocyte-macrophages, favoring the expression of an anti-inflammatory M2 phenotype, involved in tissue repair.. Some previous *in vitro* and *in vivo* work supports the hypothesis of the monocytes/macrophages phenotype shift as a result of MSC presence (Vasandan et al., 2016; Zheng et al., 2018). Vesicles generated from the MSC membrane have been found to modulate the immune response by selectively targeting pro-inflammatory monocytes (Gonçalves et al., 2017). In addition, MSCs co-cultured with macrophages *in vitro* has been shown to change their phenotype and promote a shift from a M1 pro-inflammatory phenotype to a M2 anti-inflammatory phenotype (Vasandan et al., 2016).

MSCs can exert their effects even dead or at distance from the presumed site of action

The fate of MSCs when they encounter a highly inflammatory microenvironment is uncertain. In our study, MSCs were injected during the acute phase of inflammatory response post-SE. *In vivo*, the survival of these stem cells in such noxious environment has been reported to be short. Nevertheless, this limited lifespan would not hinder their immunomodulatory therapeutic action, that can be exerted once monocytes have phagocytosed debris from apoptotic MSCs (de Witte et al., 2018). Studies have revealed that apoptotic or dead MSCs can support protection in inflammatory microenvironments (Galleu et al., 2017, 2017; Weiss and Dahlke, 2019; Weiss et al., 2019; de Witte et al., 2016). In recent years, the therapeutic action of dying or apoptotic MSCs has received more attention following the results of numerous clinical trials in which MSCs were not detected in the targeted tissues but have demonstrated therapeutic effects (Weiss et al., 2019). A considerable amount of studies is in line with the hypothesis that M2 polarization of monocyte-macrophages may be achieved through efferocytosis of dying/apoptotic or non-necrotic MSCs (Weiss et al., 2019; de Witte et al., 2018).

The broad and not fully understood action of MSCs over the inflammatory response raises questions about the panel of inflammatory genes that we evaluated. These genes constitute a small proportion of the inflammatory genes involved in inflammatory processes,

and it is known that many other inflammatory molecules, other chemokines and growth factors have been identified as targets of the immunomodulatory action of mesenchymal stem cells in *in vivo* and *in vitro* studies (Lin et al., 2019).

In the event that MSCs survive, it has been reported that MSC may also exist as MSC1, which is rather a pro-inflammatory phenotype, and as MSC2, which promotes anti-inflammatory actions (Waterman et al., 2010). As it has been suggested that M1/M2 polarization of macrophages can be beneficial or deleterious depending on the time-window it occurs, MSC polarization towards MSC1 or MSC2 could also be helpful. Therefore, MSCs may have a MSC1 phenotype during the first hours following severe brain injuries, allegedly associated with the release of MIP1 α which contributes to the activation of M1 macrophages that would act as scavengers of cellular debris (Yan et al., 2014). When environmental signals drive the switch to the MSC2 phenotype, MSC would then release anti-inflammatory factors such as IL10, triggering the switch of monocyte-macrophages into the M2 type to promote repair processes. If ongoing studies aimed at determining whether MSC survive in the long term, either within the brain or at the periphery, provide positive results, further studies will be designed to investigate whether MSC change phenotype in the acute phase, during epileptogenesis and at epilepsy onset.

Regarding the fate of monocyte infiltrates at 5 days after SE, we observed that the rats that received MSCs had a greater number of monocyte-macrophages with a microglial phenotype (Iba1+) compared to rats subjected to SE that received saline instead of MSCs. This result suggests that MSCs may have promoted the transdifferentiation of infiltrating monocytes into a “microglial” monocyte-macrophage phenotype. Considering the scientific literature on the effect of MSCs on monocyte-macrophages polarization and the beneficial effects observed on cognition in rats subjected to SE and transplanted with MSCs, we propose that MSCs may have triggered in later stages of epileptogenesis a switch of M1 towards M2 phenotype in monocyte-macrophages. Ongoing studies are conducted to answer this question.

MSCs potentiate astrogliosis

MSC-based therapies for neurological disease aimed at neuroprotection and functional preservation frequently examine astrocyte reactivity as well as the state of the glial scar. Glial cells play an important role in brain homeostasis, and dysfunctions of astrocytes leading to

alterations of 1. water balance and buffering system for K^+ , e.g. through aquaporin-4 and inward rectifying K^+ channel downregulation, respectively, 2. glutamate uptake and conversion into glutamine, a substrate for the production of GABA in inhibitory GABAergic neurons, 3. extracellular concentration of adenosine, e.g. through increased expression of adenosine kinase, 4. the release of gliotransmitters such as glutamate, D-serine, ATP, among others, and 5. the production and release of inflammatory molecules, contribute to enhanced neuronal hyperexcitability and then seizures ((Devinsky et al., 2013; Dong and Benveniste, 2001). Here, we report five days after SE that rats that received intranasal administration of MSCs 6 hours post-SE had a greater surface area occupied by GFAP-positive cells. This higher astrocyte density generated by MSCs at 5 days post-SE raises the question of the role this reactivity may have. As for microglia and monocyte/macrophages, it is noteworthy that astrocytes exist in at least two distinct reactive states: A1 neuroinflammatory reactive astrocytes and A2 neuroprotective reactive astrocytes (Liddelow and Barres, 2017). Thus, astrocytes can also undergo phenotypic polarization processes (A1 towards A2) that would enhance their beneficial role by promoting extracellular glutamate clearance, and production of neurotrophic factors, for instance, thus supporting faster tissue repair and/or reducing the occurrence of severe damage (Amantea et al., 2015; Dabrowska et al., 2019; Wang et al., 2018). Prior research has shown that MSCs are able to modulate immune response through stimulation of astrogenesis. Injection of MSC into a spinal cord injury (SCI) model resulted in an increase in GFAP expression / astrogliosis 7 days later, facilitating the establishment of a neuroprotective and neurogenesis-promoting microenvironment (Kim et al., 2015). By contrast, other studies have shown that MSC transplantation induces a decrease in astrogliosis during the acute and chronic phase after injury demonstrating that the effect of MSCs clearly depends on the environment in which they are located.

At the hippocampal level, we also observed by RT-qPCR a significant increase in the level of GFAP transcripts 24 hours after SE in rats that received intranasal MSCs. Since neural stem cells (NSCs) express the GFAP marker at their earliest stage, one cannot exclude that this increase in GFAP mRNA level may relate to the enhancement of the self-renewal processes of neural progenitors. Further experimentations aimed at identifying markers for more advanced stages of neurogenesis (e.g. nestin and doublecortin) are needed to determine whether, in the long-term, MSCs promote the differentiation and survival of newborn neurons. Overall, our findings suggest that astrogliosis at 5 days post-SE may pave the way of a regenerative

niche for the surrounding cells, this hypothesis being supported by the functional results obtained on synaptic plasticity.

MSCs prevent the alteration of synaptic plasticity

Memory disorders are a frequent complaint in many epileptic patients. In preclinical studies, LTP is the most widely investigated physiological model of memory formation (Bliss and Collingridge, 1993; Cooke and Bliss, 2006). This form of synaptic plasticity has been shown to be severely impaired in experimental models of epilepsy, a consequence attributable to changes in the expression of N-methyl-D-aspartate (NMDA) receptors or their subunit composition (Postnikova et al., 2017).

Our results with single or repeated administration of MSCs showed a complete restoration of LTP, that returned to control level with 1 injection of MSC, or was greater than controls with 4 injections. In previous studies, improved cognitive outcome caused by MSCs has been attributed to the modulation of inflammatory processes, the differentiation of MSCs into brain cells, especially inhibitory neurons, the stimulation of neurogenesis and the enhancement of endogenous repair processes (Donega et al., 2013, 2014; Munoz et al., 2005; Paul and Anisimov, 2013; Shiota et al., 2018). More precisely, it has been reported that MSCs may promote neurogenesis of endogenous stem cells located in the subgranular area of the hippocampus, thus contributing to the restorative effort (Costa-Ferro et al., 2012; Kan et al., 2011). The question raised by these findings relates to the underlying mechanisms behind the restoration of long-term potentiation processes. Whole-cell patch clamp recordings from CA1 pyramidal cells showed that the biophysical properties altered by the induction of SE have been restored or prevented by MSCs (Dai et al., 2017). The underlying mechanisms may involve post-synaptic changes in the expression of NMDA receptors, or modification of transcription factors such as NFkB which is implicated in LTP processes (Gu et al., 2015; Voulgari-Kokota et al., 2012). The protective effect of MSCs on synaptic plasticity may be mediated at the cellular level by supporting the switch of microglial cells and/or monocyte macrophages to a more anti-inflammatory phenotype. In addition, other molecules of the MSCs secretome may have been acting as protectants of synaptic function by preventing abnormal sprouting of mossy fibers. Another explanation of the effect of MSCs can be related to GABAergic interneurons. Examination of resected hippocampus of epileptic patients has revealed a significant loss of these interneurons (Spreafico et al., 1998), an in an animal model

of TLE, the engraftment of GABAergic interneurons led to the reduction of seizure activity (Hunt et al., 2013). Intravenous infusion of MSCs rescued the loss of GAD67+ GABAergic interneurons induced by SE (Fukumura et al., 2018; Long et al., 2013). Further research is needed in our transplantation model to determine whether MSCs also have had an effect on interneuron subpopulations.

Neuroinflammatory processes have been suggested as contributing factors to these alterations. Neuronal excitability in epilepsy is highly related to the cytokine expression that has been shown to quickly alter the function of classical neurotransmitters by modulating their receptor assembly and phosphorylation at neuronal membranes (Ravizza et al., 2011; Vezzani and Viviani, 2015; Viviani et al., 2007). It has been shown that LTP could be altered by the presence of an inflammatory milieu in the hippocampus (Beattie et al., 2002; Cunningham et al., 1996). As discussed above, potential MSC-mediated phenotypic modulations of astrocytes, microglial cells and monocyte-macrophages may have operated in the surroundings of the neuronal populations by creating a protective environment, reducing the molecular damage underlying cognitive processes.

MSCs secretome may have played a distant protective role

Our findings at the cellular and molecular level in neuroinflammation and at the electrophysiological level suggest that the presence of MSCs directly in the injured area is not a requirement to observe beneficial effects. The paracrine mode of action of MSCs enables them to operate far from the suffering site by secreting soluble factors such as growth factors, angiogenic factors, anti-inflammatory molecules (da Silva Meirelles et al., 2009). This paracrine hypothesis is in line with the exhortation of Caplan in 2017 to change the name of Mesenchymal Stem Cells into Medicinal Signaling Cells because of their limited ability of differentiation *in vivo* (a feature generally inherent to stem cells), their therapeutic potential being rather related to their paracrine capacity (Caplan, 2017). Therefore, a growing number of studies are now focusing on the direct effect of injecting MSC secretome into models of brain aggression (Baez-Jurado et al., 2019; Cunningham et al., 2018; Harrell et al., 2019; Konala et al., 2016; Paul and Anisimov, 2013). Hence, culture medium of MSCs containing these biological factors could successfully be used in regenerative medicine (Sagaradze et al., 2019). The therapeutic potential of MSC-derived extracellular vesicles, also called exosomes, is also gaining considerable attention on the basis that vesicles carry trophic factors such as anti-

inflammatory molecules and growth factors (Baek et al., 2019; Dabrowska et al., 2019; Keshtkar et al., 2018; Long et al., 2017; Reiner et al., 2017). Nowadays, it is essential to accurately characterize the immunomodulatory and regenerative properties of these vesicles in order to consider in patients MSC-derived extracellular vesicles-based therapies.

Conclusion and future directions

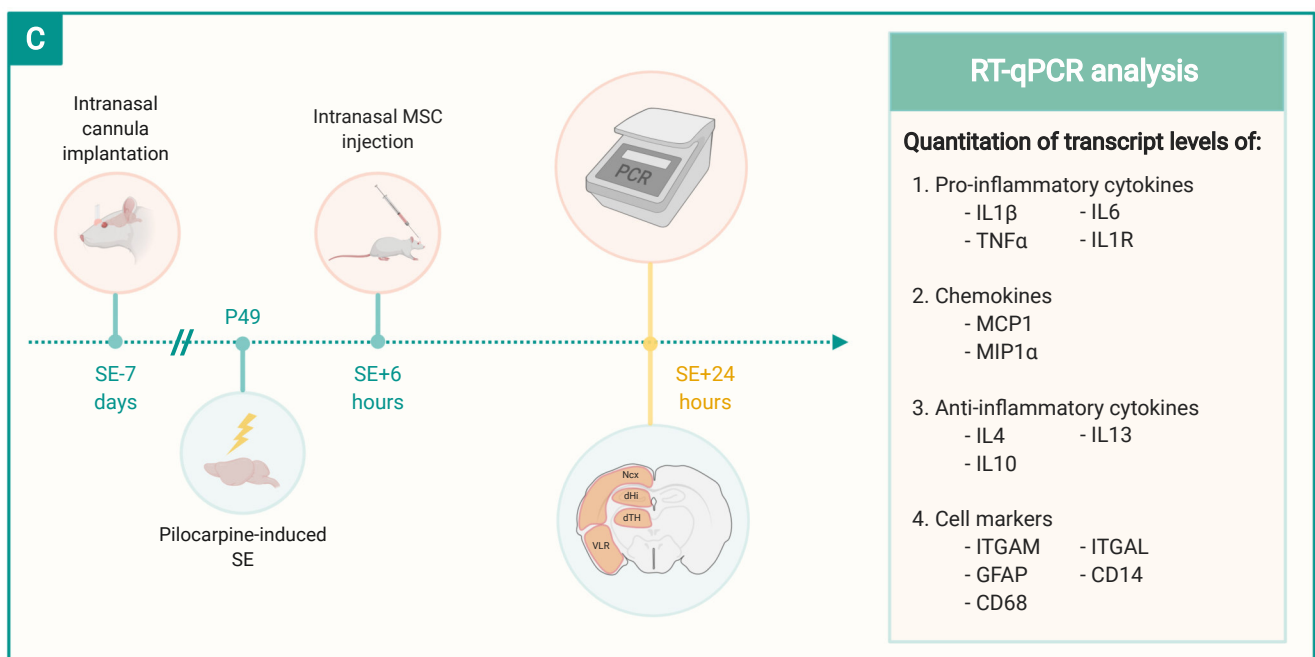
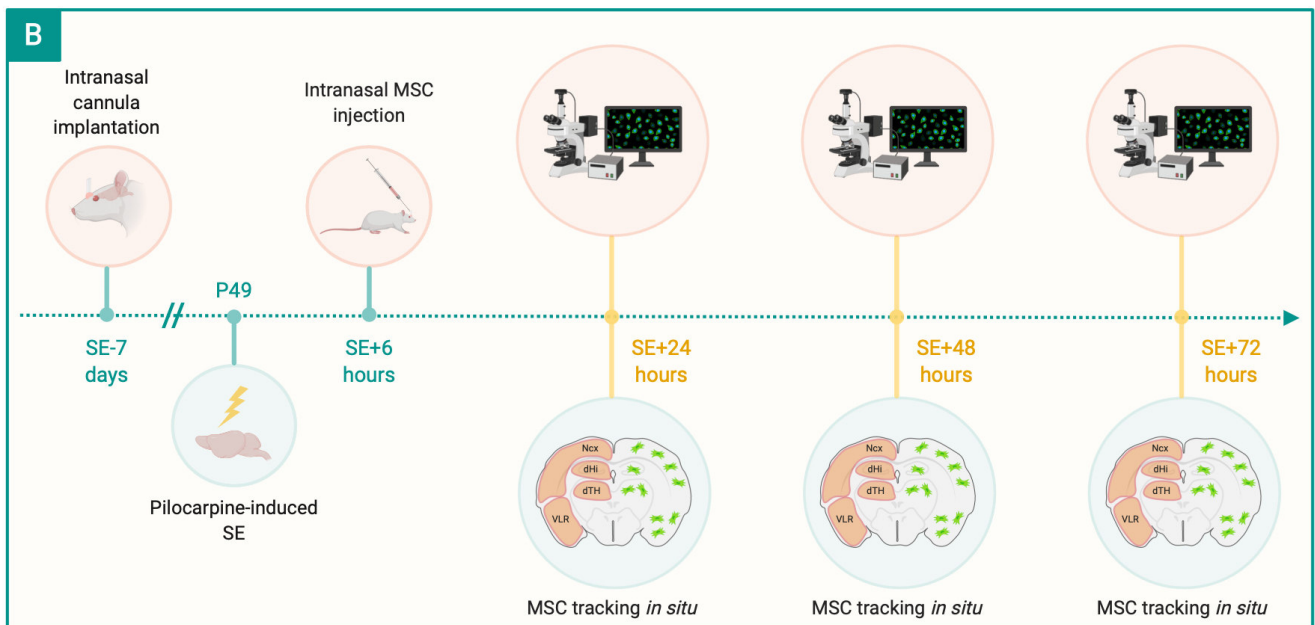
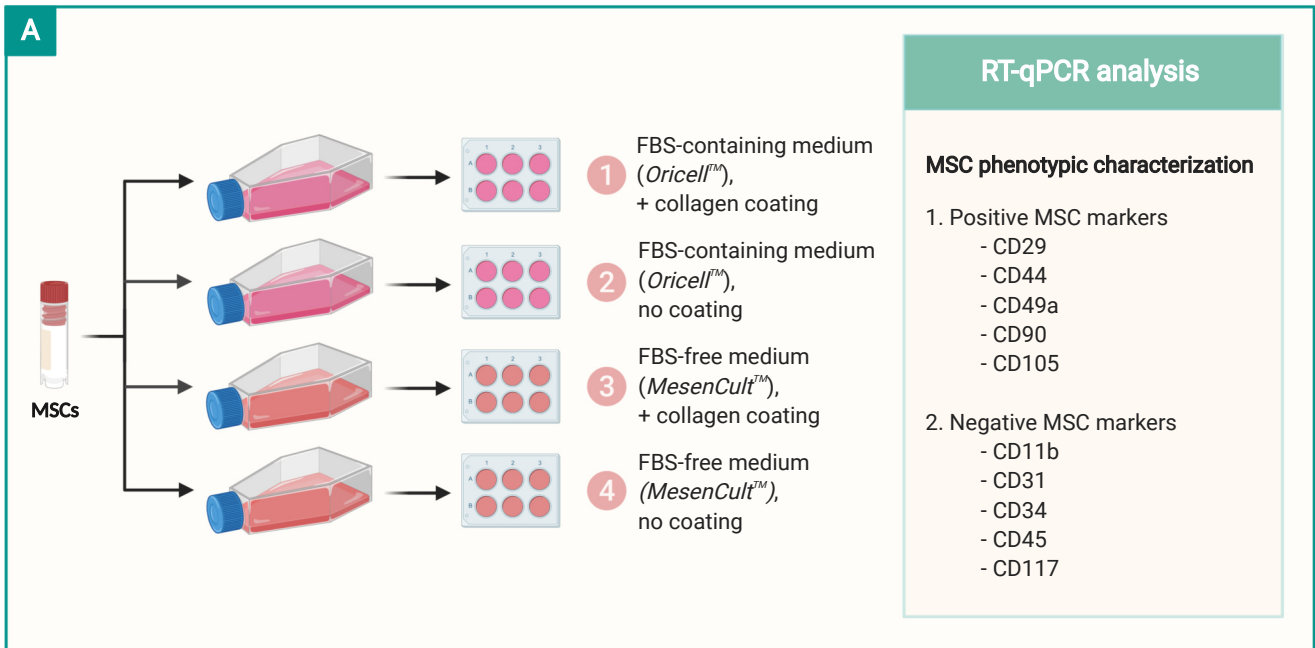
The engraftment of MSCs at the suffering site does not seem to be a prerequisite for their therapeutic action. Instead, their effects on epilepsy models, as well as in other neurological diseases, would rather involve a more general modification of the immune system through the secretion of trophic factors and inflammatory regulators by living, apoptotic or dead MSCs. Ongoing studies will allow us to complete the data on the fate of inflammation during the chronic phase of epilepsy after the intranasal injection of MSCs 6 hours posts-SE, both at the molecular level by quantifying the same set of inflammatory transcripts as those studied during the acute phase, and at the cellular level by evaluating the fate of the glial scar and monocytes-macrophages. It can be assumed that the presence of stem cells may promote the infiltration of monocytes over a longer period of time or may increase their survival into the brain parenchyma. Another worthwhile aspect to evaluate is the polarization of these monocytes-macrophages and of resident microglial cells and astrocytes. In addition, the assessment of hippocampal neuronal density after stem cell administration is also currently in progress and will provide insight into whether MSCs can prevent neuronal degeneration after pilocarpine-induced SE and/or promote the neurogenesis and survival of new neurons. Furthermore, the underlying mechanisms by which the MSCs modulate the molecular and cellular changes and act on long-term potentiation processes remain unclear. Many questions have yet to be answered, particularly regarding the fate of MSCs in the tissue, as well as their potential to counteract the development of epilepsy. Further investigations will determine in rats subjected to SE and transplanted with MSCs during the acute phase of epileptogenesis whether the protective effects observed on LTP are also associated with alleviation of behavioral and cognitive alterations. The Morris Water Maze test, used to assess the hippocampus-dependent spatial memory ability, will allow us to evaluate the potential effects of MSCs on cognitive deficits, while the Water Exploration Test and the O-maze, will provide a measure of anxiety disorders. The continuous EEG and video monitoring of spontaneous recurrent seizures will enable us to determine

whether treatment with MSCs as provided in our study can prevent the onset of seizures or reduce seizure frequency and severity. By then, these findings will provide us with the opportunity to conclude on the use of MSCs as an antiepileptogenic treatment that counteract SE-induced pathogenic mechanisms.

To conclude, early intervention with MSCs appears to be attractive for treating SE and restraining the development of chronic epilepsy symptoms, whereas delayed administration of MSCs during the chronic phase might be useful for easing spontaneous seizures and cognitive dysfunctions (Agadi and Shetty, 2015). Although transplantation into damaged brain areas represents a potential approach that may be useful, research aimed at facilitating delivery of MSCs using minimally invasive approaches must be encouraged to ease clinical use. Considering that epilepsy is a long-term chronic disease, its management by cell therapy may require more than one MSC injection. Our research showed here that intranasal MSCs treatment constitutes an alternative route of administration allowing to consider punctual treatments with minimal burden on patients. Preclinical studies and methodological considerations regarding the culture conditions of MSCs are still necessary to achieve clinical application of these cells intranasally, although these promising results represent a therapeutic hope for patients at risk to develop epilepsy after a severe brain insult.

ACKNOWLEDGMENTS

We thank Rachel Shadi Alary for her assistance in confocal microscopy and image analysis.



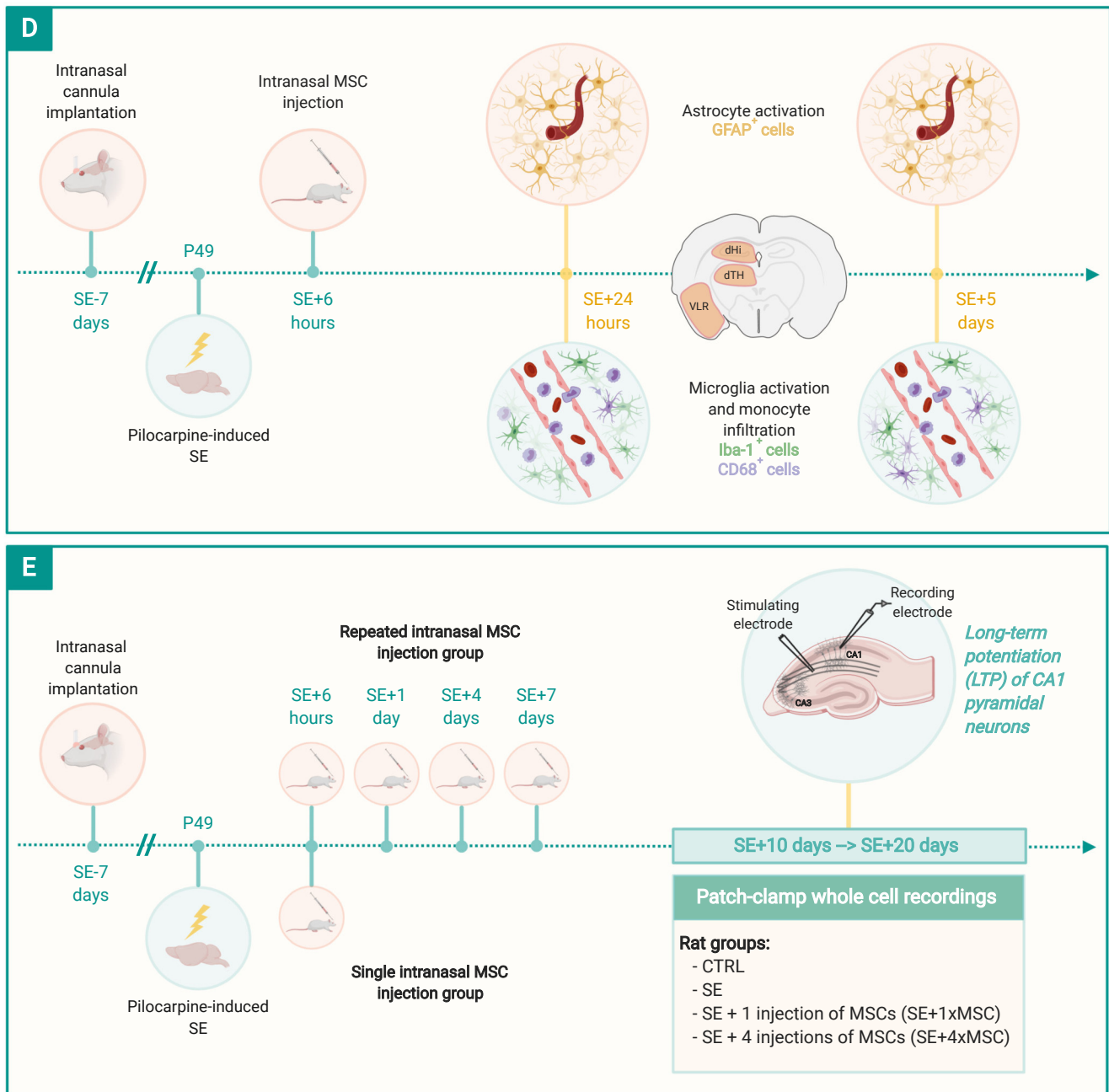


Figure 1. Study design. (A) MSCs were cultured in a medium containing FBS (OriCell® medium) or FBS-free (MesenCult® medium). The culture surfaces were coated or not with collagen I. RT-qPCR analysis were used to establish the MSC phenotype by measuring mRNA level of positive and negative markers of MSCs, as defined by the International Society for Cellular Therapy (Dominici, 2006). (B) To track GFP-expressing MSCs in the brain after intranasal injection 6h post-SE, fixed rat brains were collected at 24h, 48h and 72h post-SE. Brain sections were labelled with an anti-GFP antibody coupled with a fluorescent A633-conjugated secondary antibody to amplify the signal. Sections were then observed using confocal microscopy. (C) Transcript levels of pro- and anti-inflammatory cytokines, chemokines and cell markers were quantified in the dorsal hippocampus (dHi), the ventral limbic region (VLR), the dorsal thalamus (dTH) and the neocortex in rats subjected or not to pilocarpine-induced SE (SE) and treated or not with one intranasal MSC injection 6h post-SE. (D) Astrogliosis, microgliosis and monocytes/macrophages infiltration was evaluated by immunolabelling of GFAP, Iba-1 and CD68, respectively, in rats subjected to SE and treated or not with one intranasal MSC injection 6h post-SE and sacrificed 24h and 5 days after SE. (E) Long-term potentiation of CA1 pyramidal neurons in hippocampal slices from control rats (CTRL), and rats subjected to SE, that received one (SE+1xMSC) or four (SE+4xMSC) intranasal administration of MSCs. Abbreviations: AMG, amygdala; dHi, dorsal hippocampus; dTH, dorsal thalamus; IAC, insular agranular cortex; PC, piriform cortex; P49, post-natal day 49.

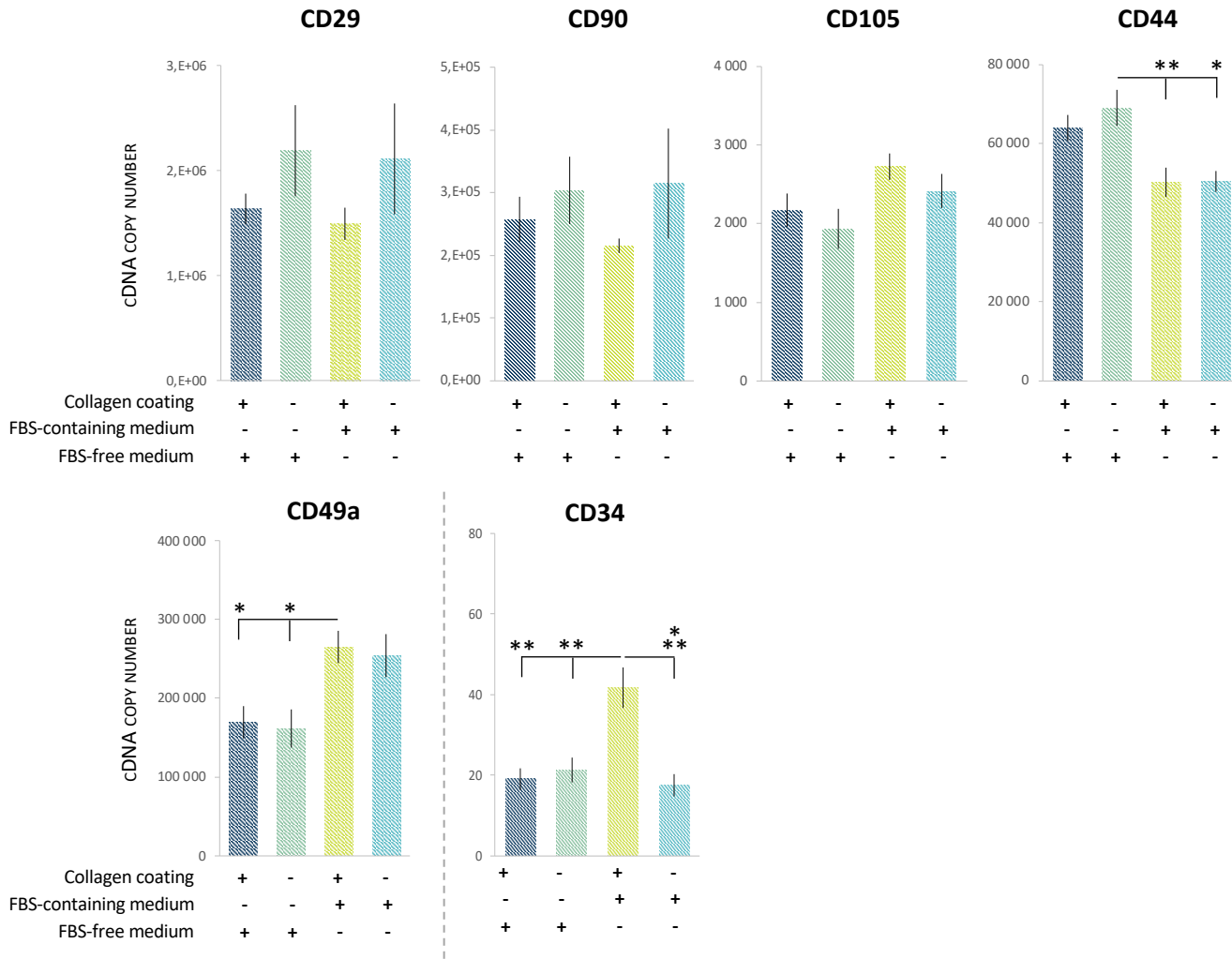


Figure 2. MSC phenotype is not affected by the absence of fetal bovine serum in the culture medium or by the coating of culture surfaces. Transcript values of positive markers of MSCs (CD29, CD44, CD49a, CD90 and CD105), as well as negative markers (ITGAM, CD31, CD34, CD45 and CD117) as defined by the ISCT (Dominici, 2006), were quantified by RT-qPCR in MSCs cultured in a medium that contains FBS (OriCell® medium) or in a FBS-free medium (MesenCult® medium), on a surface culture (6-well plates) coated or not with collagen I. Data for CD11b, CD31, CD45 and CD117 are not presented because none of the gene was detected by RT-qPCR. cDNA copy number are expressed by the mean \pm SEM. Tukey's post-hoc analysis following one-way ANOVA: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

Figure 3. Effect of intranasal MSC treatment on transcript levels of inflammatory markers in the hippocampus 24 hours after pilocarpine-induced SE. Transcript levels of pro-inflammatory cytokines (IL1 β , TNF α , IL6), chemokines (MCP1, MIP1 α), interleukin 1 receptor (IL1R) (A), anti-inflammatory cytokines (IL4, IL10, IL13) (B) and glial cell markers (ITGAM, GFAP) (C) were quantified by RT-qPCR in the hippocampus in control rats (CTRL, n=5) and in rats subjected to SE and treated (n=6) or not (n=7) by intranasal administration of MSCs 6h post-SE. Pro-inflammatory, anti-inflammatory and inflammation cell index were calculated as described in the Method section. Values are expressed in percent of CTRL (mean \pm SEM). Hashtags indicate statistical significance between CTRL and SE rats (CTRL vs SE ; CTRL vs SE+MSC). Asterisks indicate statistical significance between SE rats treated or not with MSCs (SE vs SE+MSC). Tukey's post-hoc analysis following one-way ANOVA: # or *: $p < 0.05$, ## or **: $p < 0.01$, ### or ***: $p < 0.001$, n.s: not significant.

HIPPOCAMPUS

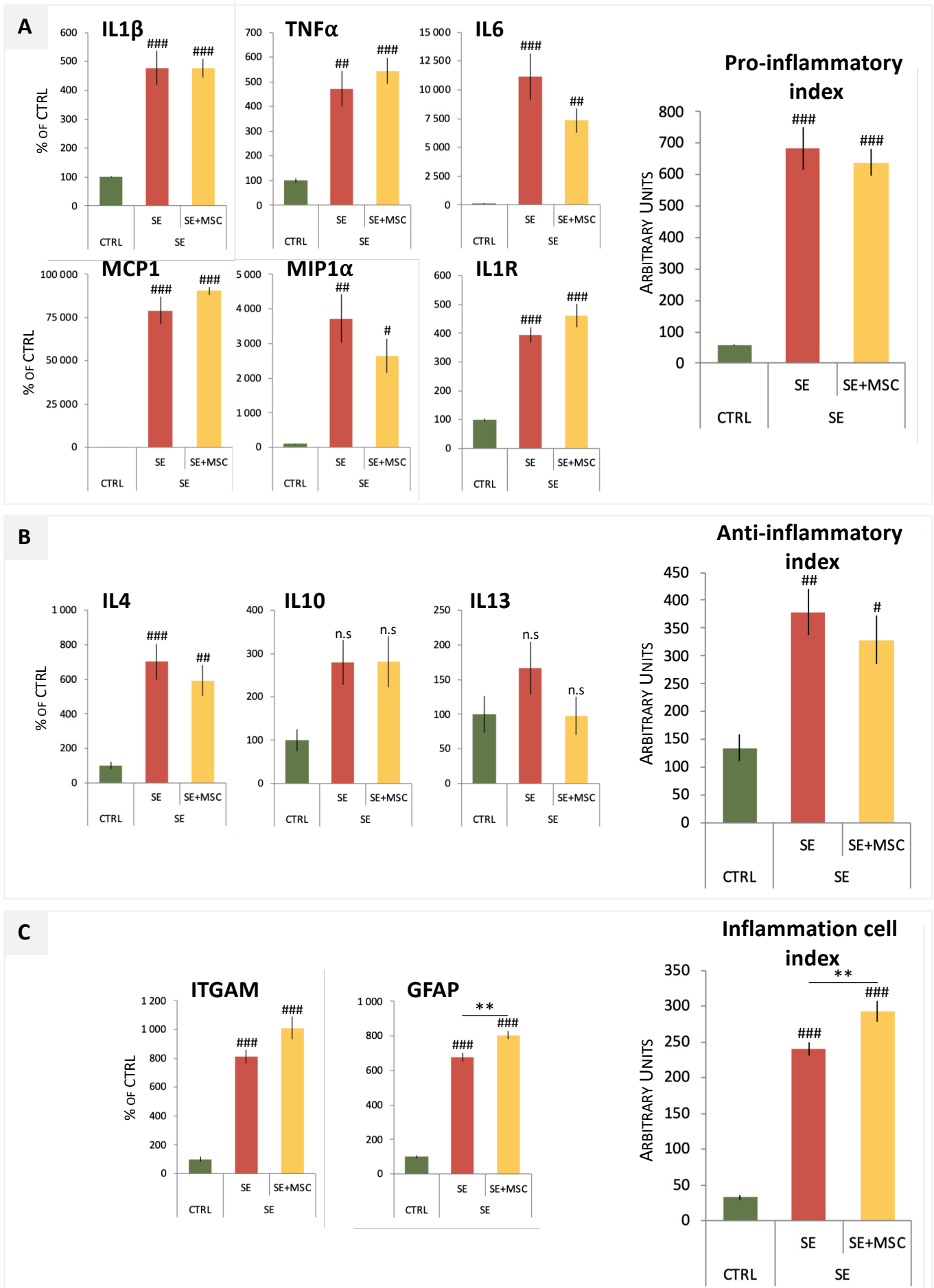
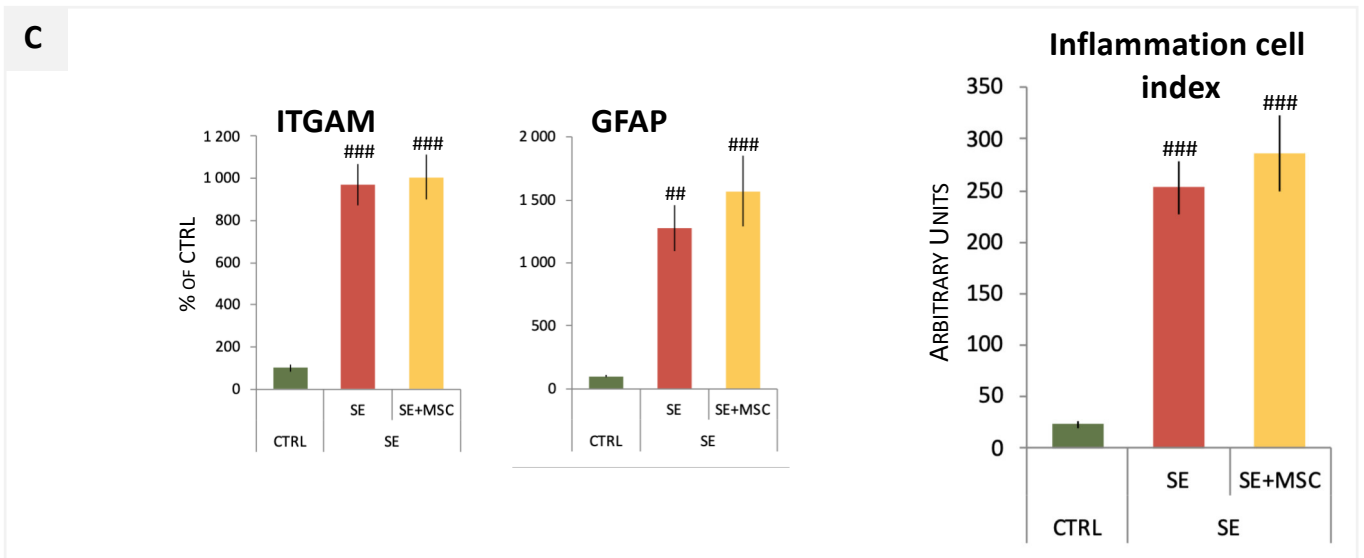
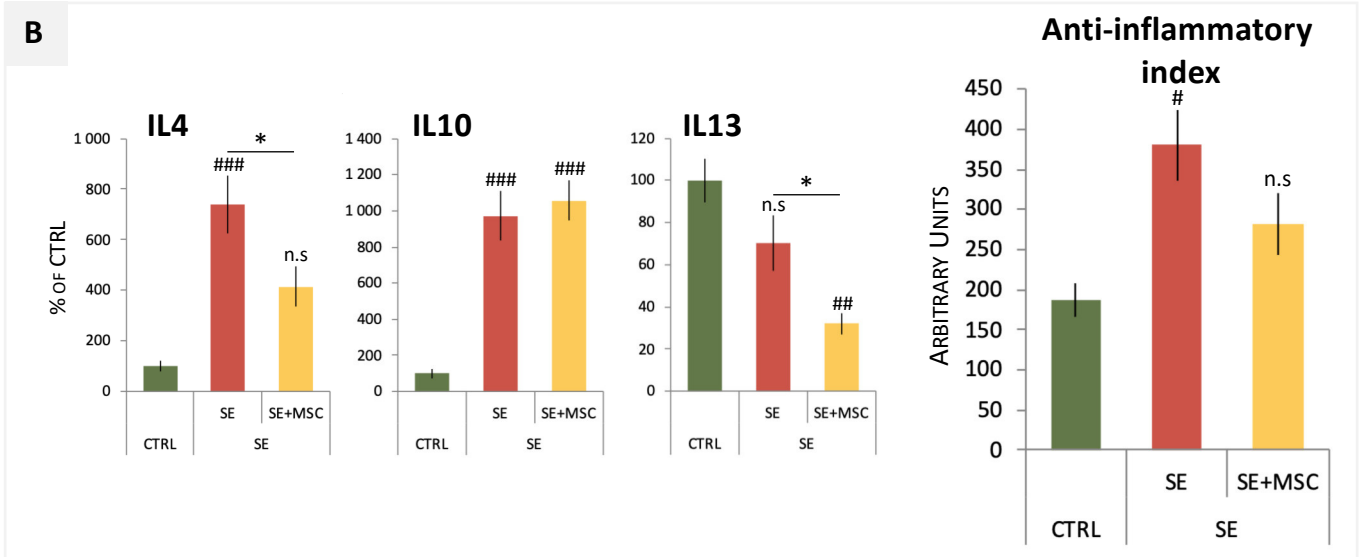
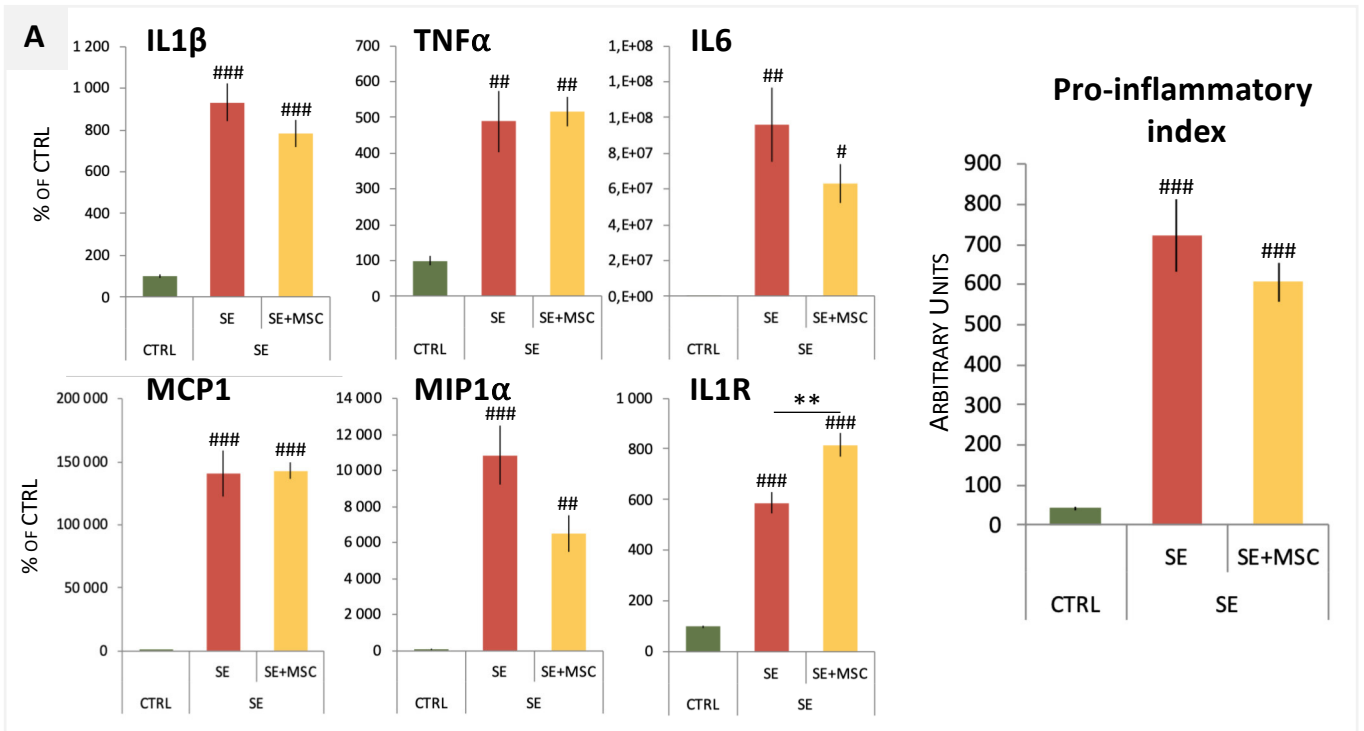


Figure 4. Effect of intranasal MSCs treatment on expression of inflammatory markers in the ventral limbic region 24 hours after pilocarpine-induced SE. As for Figure 3. Hashtags indicate statistical significance between CTRL and SE rats (CTRL vs SE ; CTRL vs SE+MSC). Asterisks indicate statistical significance between SE rats treated or not with MSCs (SE vs SE+MSC). Tukey's post-hoc analysis following one-way ANOVA: # or *: $p < 0.05$, ## or **: $p < 0.01$, ### or ***: $p < 0.001$, n.s: not significant.

VENTRAL LIMBIC REGION



DORSAL THALAMUS

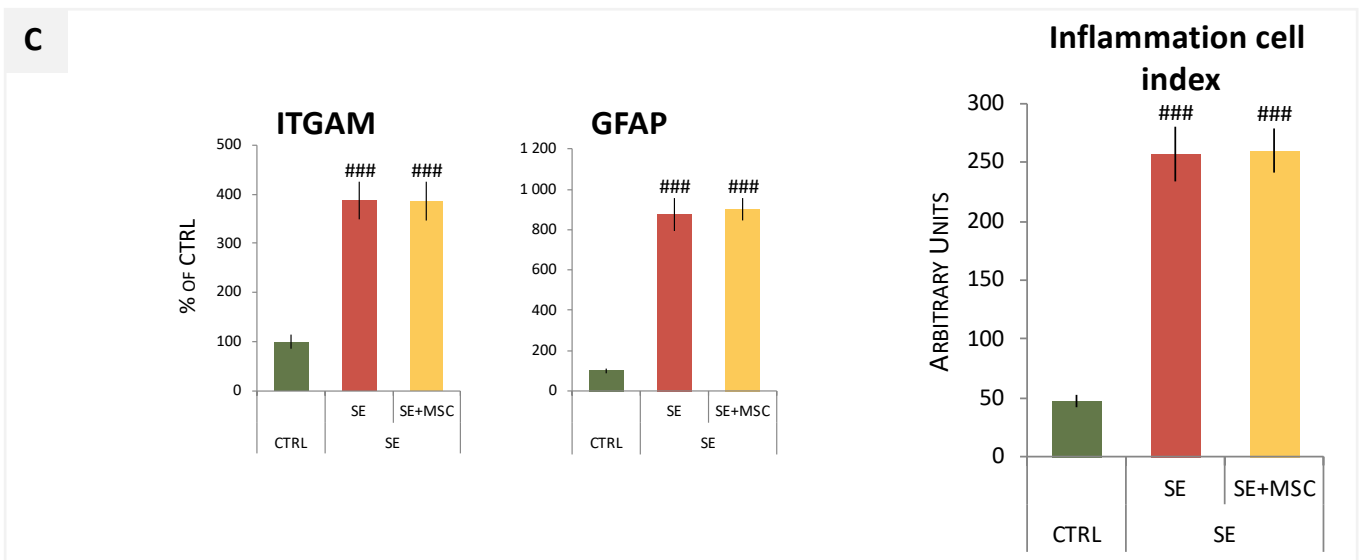
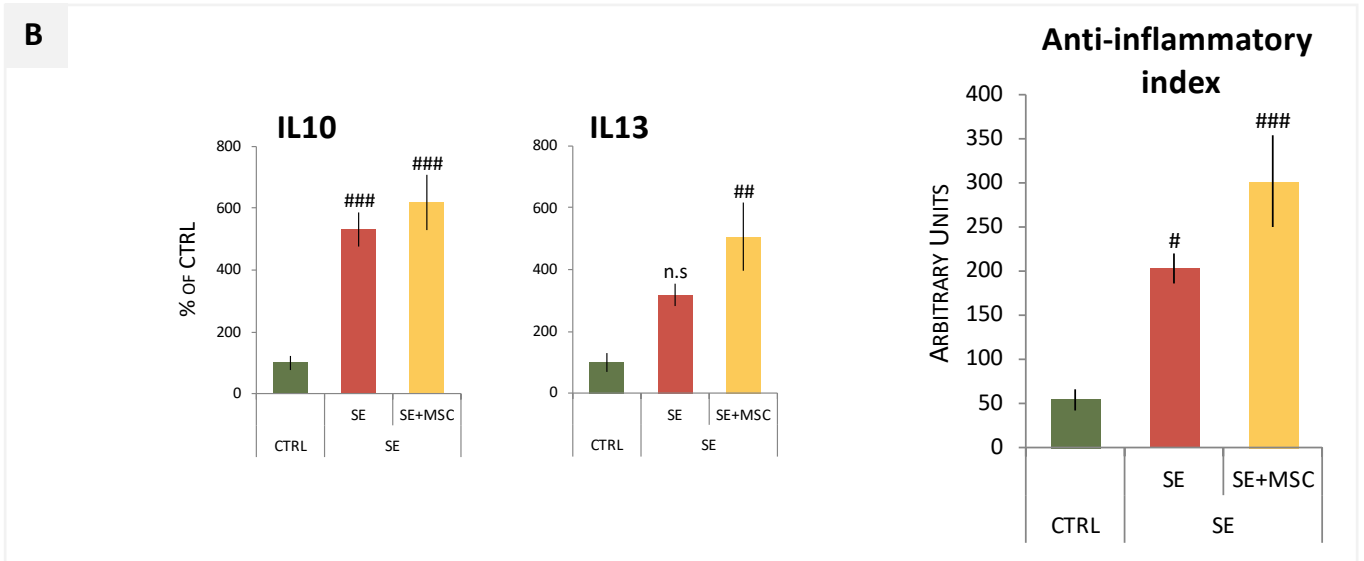
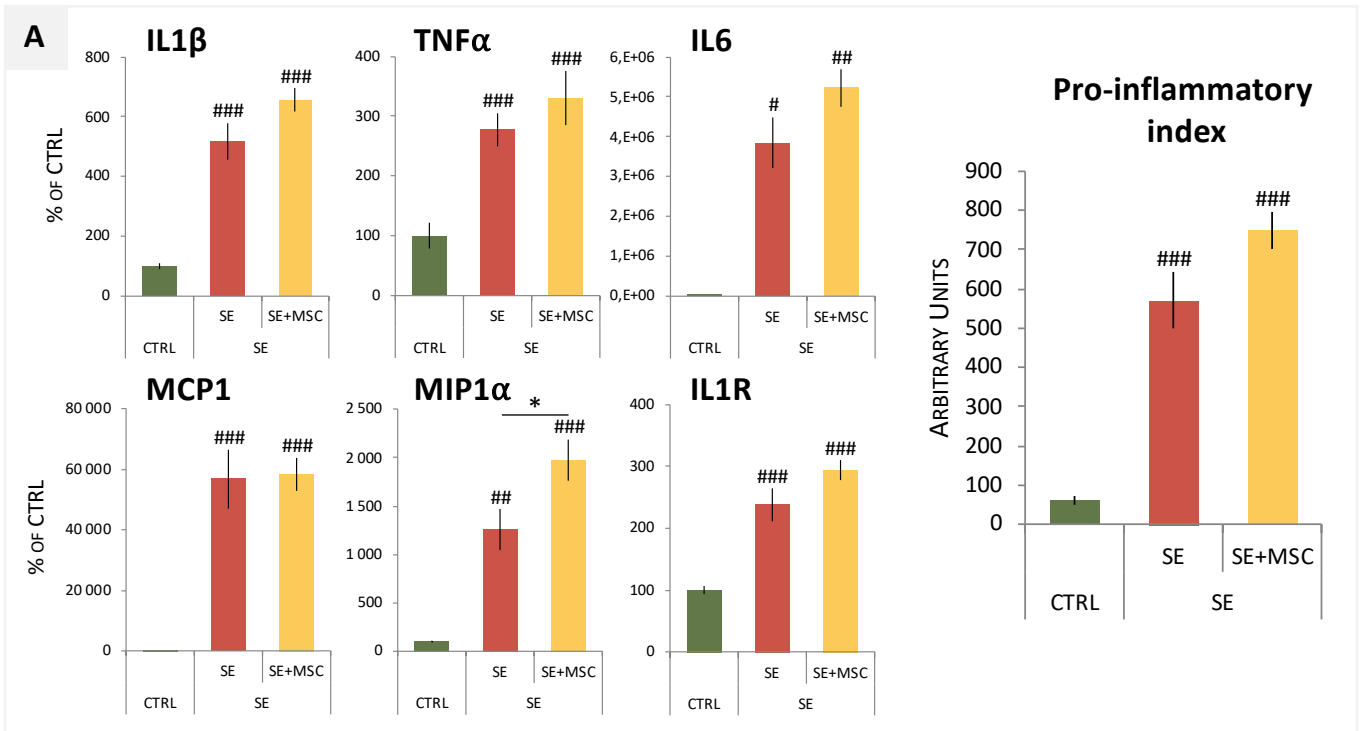
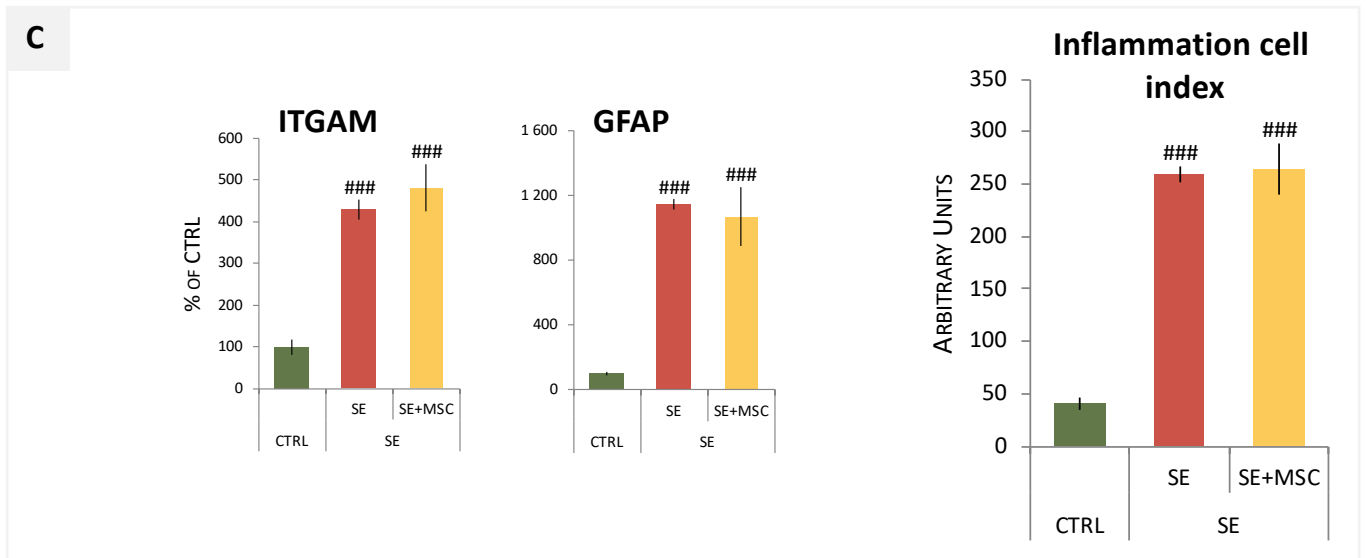
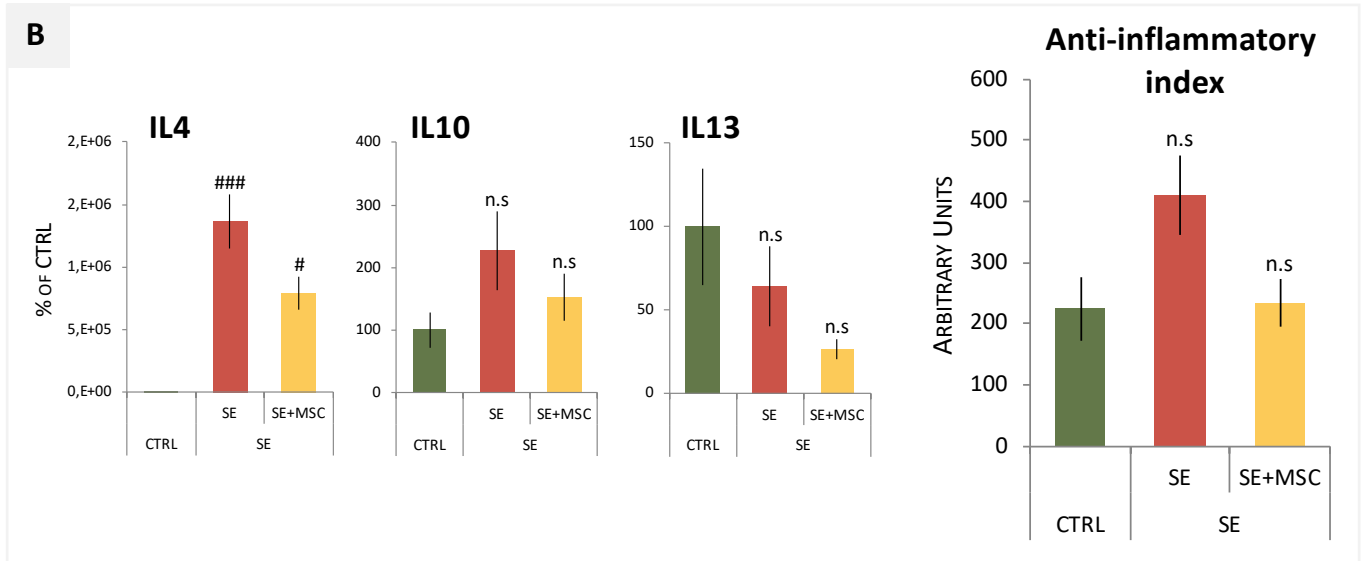
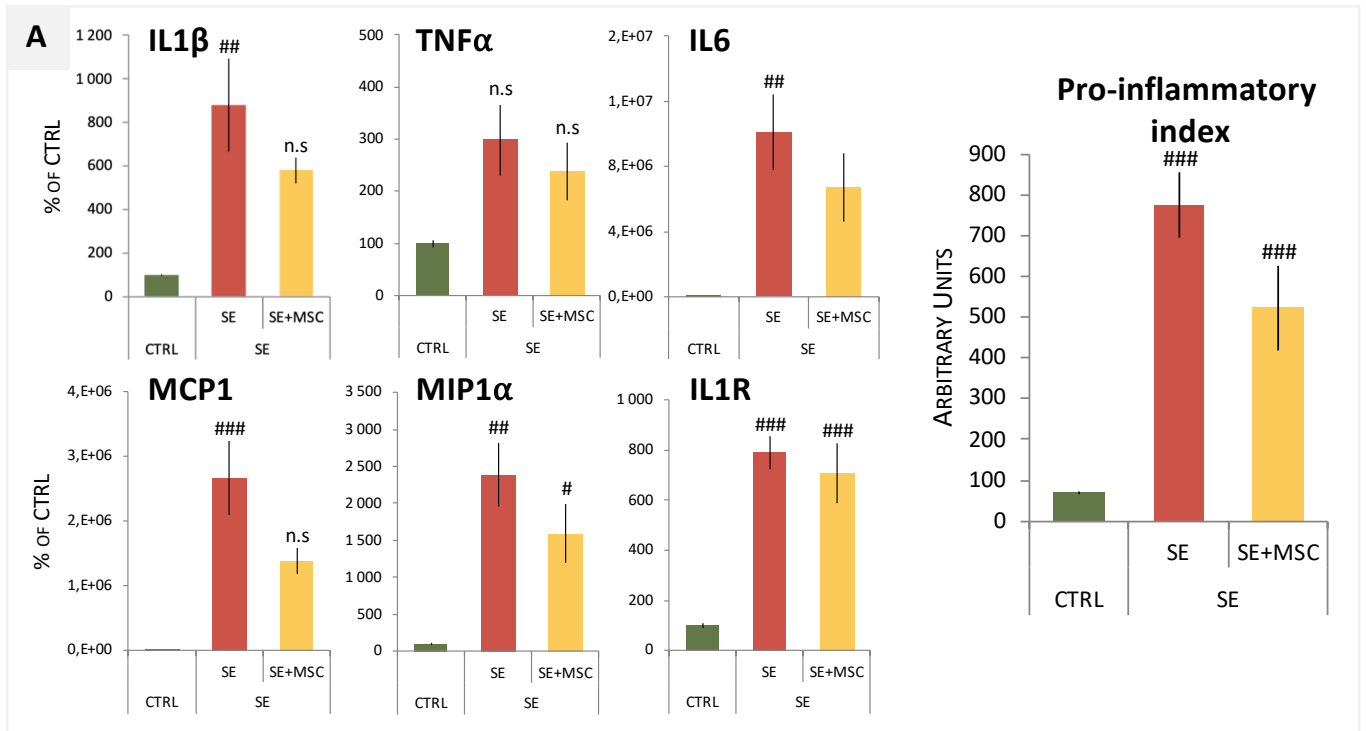


Figure 5. Effect of intranasal MSCs treatment on expression of inflammatory markers in the dorsal thalamus 24 hours after pilocarpine-induced SE. As for Figure 3. Hashtags indicate statistical significance between CTRL and SE rats (CTRL vs SE ; CTRL vs SE+MSC). Asterisks indicate statistical significance between SE rats treated or not with MSCs (SE vs SE+MSC). Tukey's post-hoc analysis following one-way ANOVA: # or *: $p < 0.05$, ## or **: $p < 0.01$, ### or ***: $p < 0.001$, n.s: not significant.

Figure 6. Effect of intranasal MSCs treatment on expression of inflammatory markers in the neocortex 24 hours after pilocarpine-induced SE. As for Figure 3. Hashtags indicate statistical significance between CTRL and SE rats (CTRL vs SE ; CTRL vs SE+MSC). Asterisks indicate statistical significance between SE rats treated or not with MSCs (SE vs SE+MSC). Tukey's post-hoc analysis following one-way ANOVA: # or *: $p < 0.05$, ## or **: $p < 0.01$, ### or ***: $p < 0.001$, n.s: not significant.

NEOCORTEX



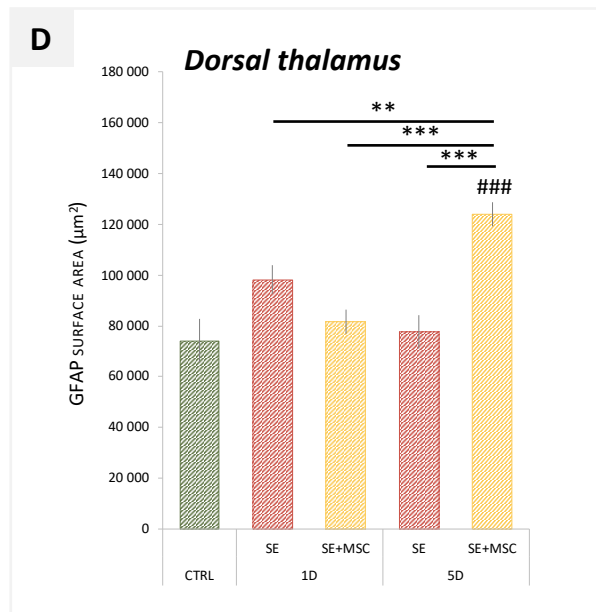
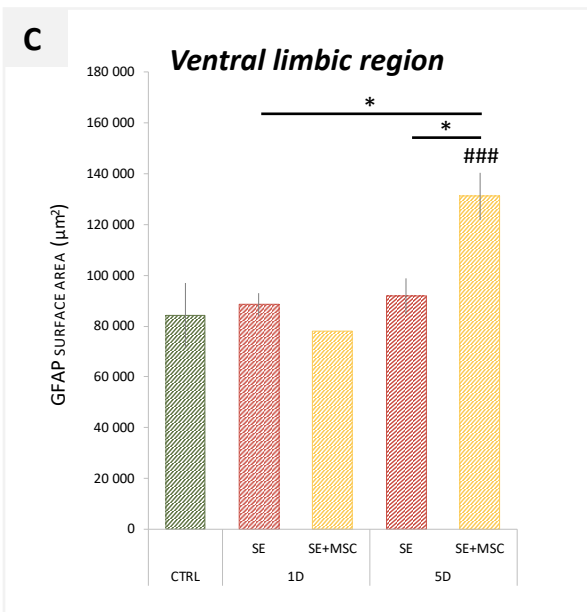
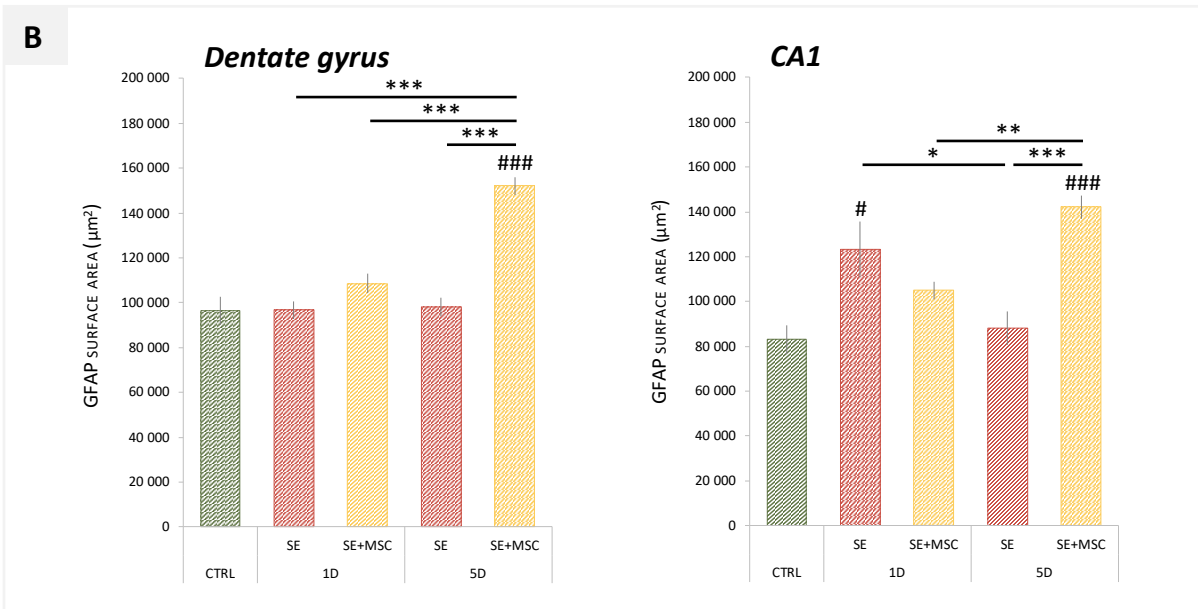
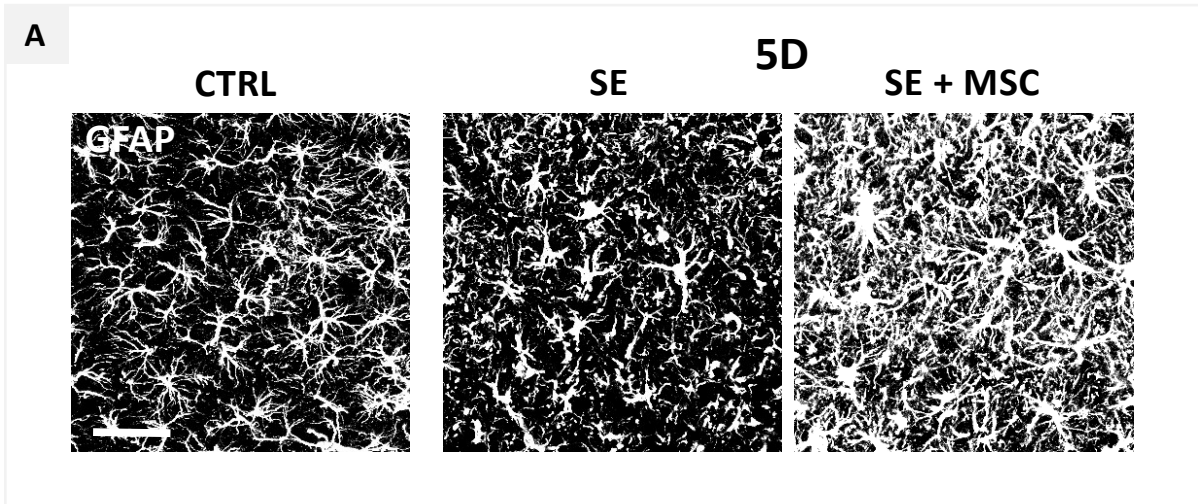


Figure 7. MSC treatment enhance astrogliosis 5 days after pilocarpine induced-SE. Immunofluorescence detection was performed in the ventral limbic region **(A)** using two specific antibodies directed against GFAP. Representative images selected at Bregma -4.16 mm from control rats (CTRL) and rats sacrificed 5 days (5D) after SE with intranasal administration of MSCs 6 hours post-SE or not. Confocal microscope images were acquired at 20X magnification. **(B-D)** Quantitation of the surface area occupied by GFAP signal in the dentate gyrus and in CA1 pyramidal cell layer of the hippocampus **(B)**, in the ventral limbic region **(C)** and the dorsal thalamus **(D)**, in sections selected at Bregma -4.16 mm from rats sacrificed 1 day (1D) or 5 days (5D) after SE with intranasal administration of MSCs 6 hours post-SE (1D-SE+MSC, n=7; 5D-SE+MSC, n=9) or not (1D-SE, n=8; 5D-SE, n=6), and in control rats (CTRL, n=3). Values are expressed by the mean \pm SEM. Hashtags indicate statistical significance between CTRL and SE rats (CTRL vs SE ; CTRL vs SE+MSC). Asterisks indicate statistical significance between SE rats treated or not with MSCs (SE vs SE+MSC). Tukey's post-hoc analysis following two-way ANOVA: # or *: $p < 0.05$, ## or **: $p < 0.01$, ### or ***: $p < 0.001$. Total surface per quantified area: 408 193,21 μm^2 . Scale bar: 50 μm .

HIPPOCAMPUS – *Dentate Gyrus*

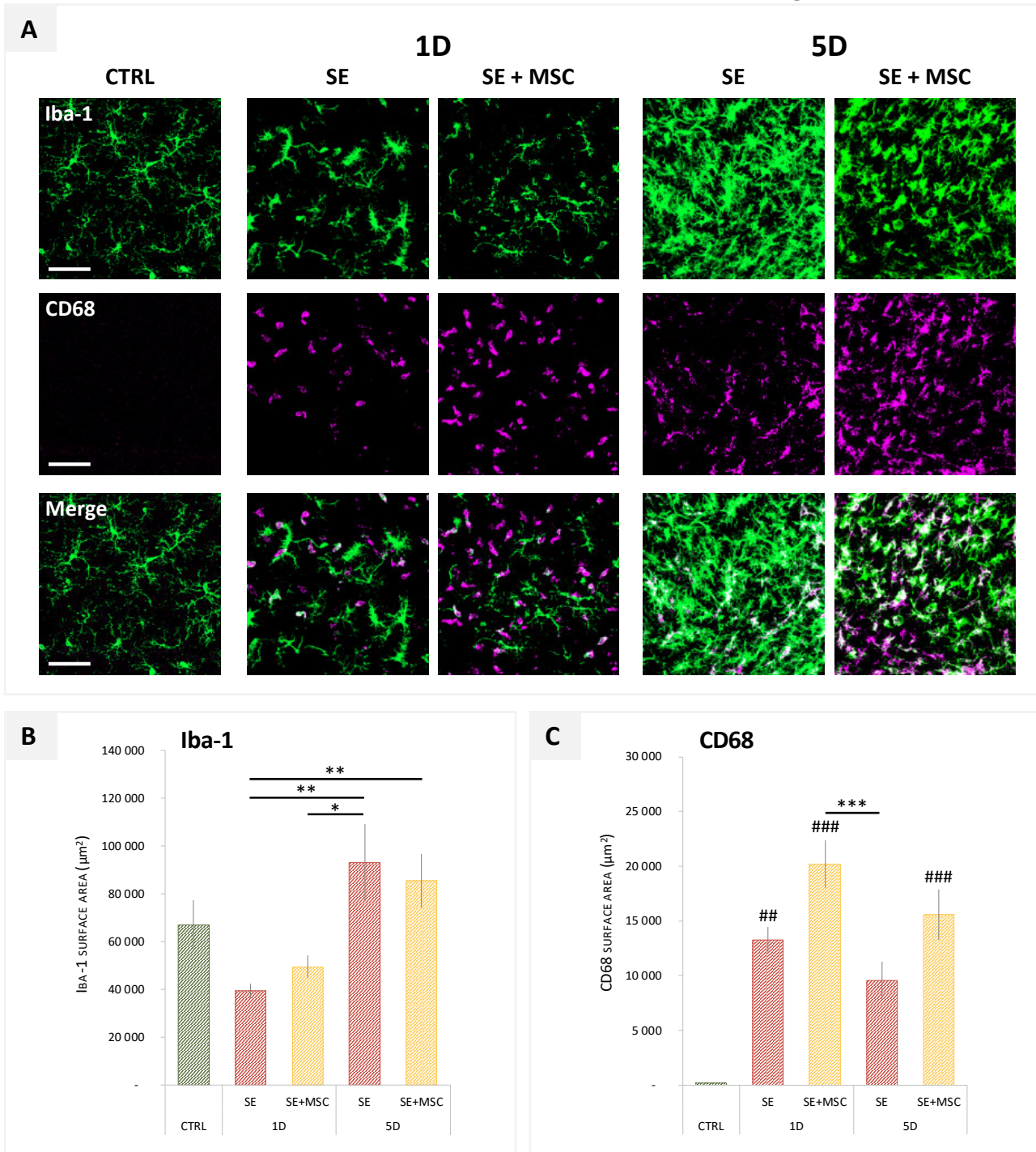


Figure 8. Effects of MSC on microgliosis and monocytes/macrophages infiltration in the dentate gyrus during epileptogenesis in rats subjected to pilocarpine-induced SE. (A) Immunofluorescence detection of Iba-1 and CD68 in the dentate gyrus. Representative images selected at Bregma -4.16 mm from control rats (CTRL) and rats sacrificed 1 day (1D) or 5 days (5D) after SE with intranasal administration of MSCs 6 hours post-SE or not. Confocal microscope images were acquired at 20X magnification. **(B-C)** Quantitation of the surface area occupied by Iba-1 **(B)** and CD68 **(C)** signal in the dentate gyrus in sections selected at Bregma -4.16 mm from rats sacrificed 1 day (1D) or 5 days (5D) after SE with intranasal administration of MSCs 6 hours post-SE (1D-SE+MSC, n=7; 5D-SE+MSC, n=9) or not (1D-SE, n=8; 5D-SE, n=6), and in control rats (CTRL, n=3). Values are expressed by mean \pm SEM. Hashtags indicate statistical significance between CTRL and SE rats (CTRL vs SE ; CTRL vs SE+MSC). Asterisks indicate statistical significance between SE rats treated or not with MSCs (SE vs SE+MSC). Tukey's post-hoc analysis following two-way ANOVA: # or *: $p < 0.05$, ## or **: $p < 0.01$, ### or ***: $p < 0.001$. Total surface area quantified : 408 193,21 μm^2 . Scale bar: 50 μm .

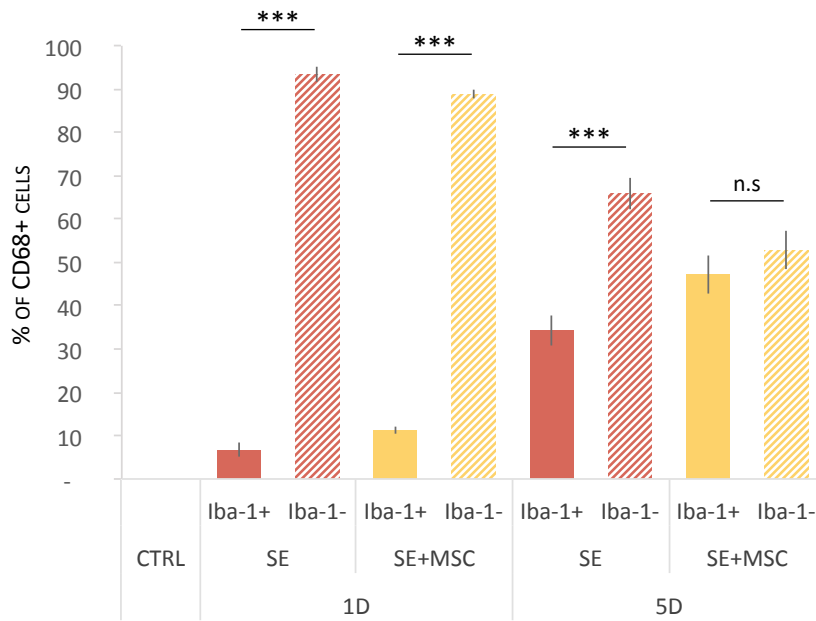


Figure 9. Quantification of dentate gyrus CD68+ monocytes/macrophages that express or not the Iba-1 microglial marker. CD68-positive/Iba-1-negative and CD68-positive/Iba-1-positive cells have been quantified in the dentate gyrus of the hippocampus in rats sacrificed 1 day (1D) or 5 days (5D) after SE with intranasal administration of MSCs 6 hours post-SE (1D-SE+MSC, n=7; 5D-SE+MSC, n=9) or not (1D-SE, n=8; 5D-SE, n=6), compared to controls (CTRL, n=3). Values are expressed by mean \pm SEM. Sidak post-hoc analysis following one-way ANOVA: ***: $p < 0.001$, n.s: not significant.

HIPPOCAMPUS – CA1

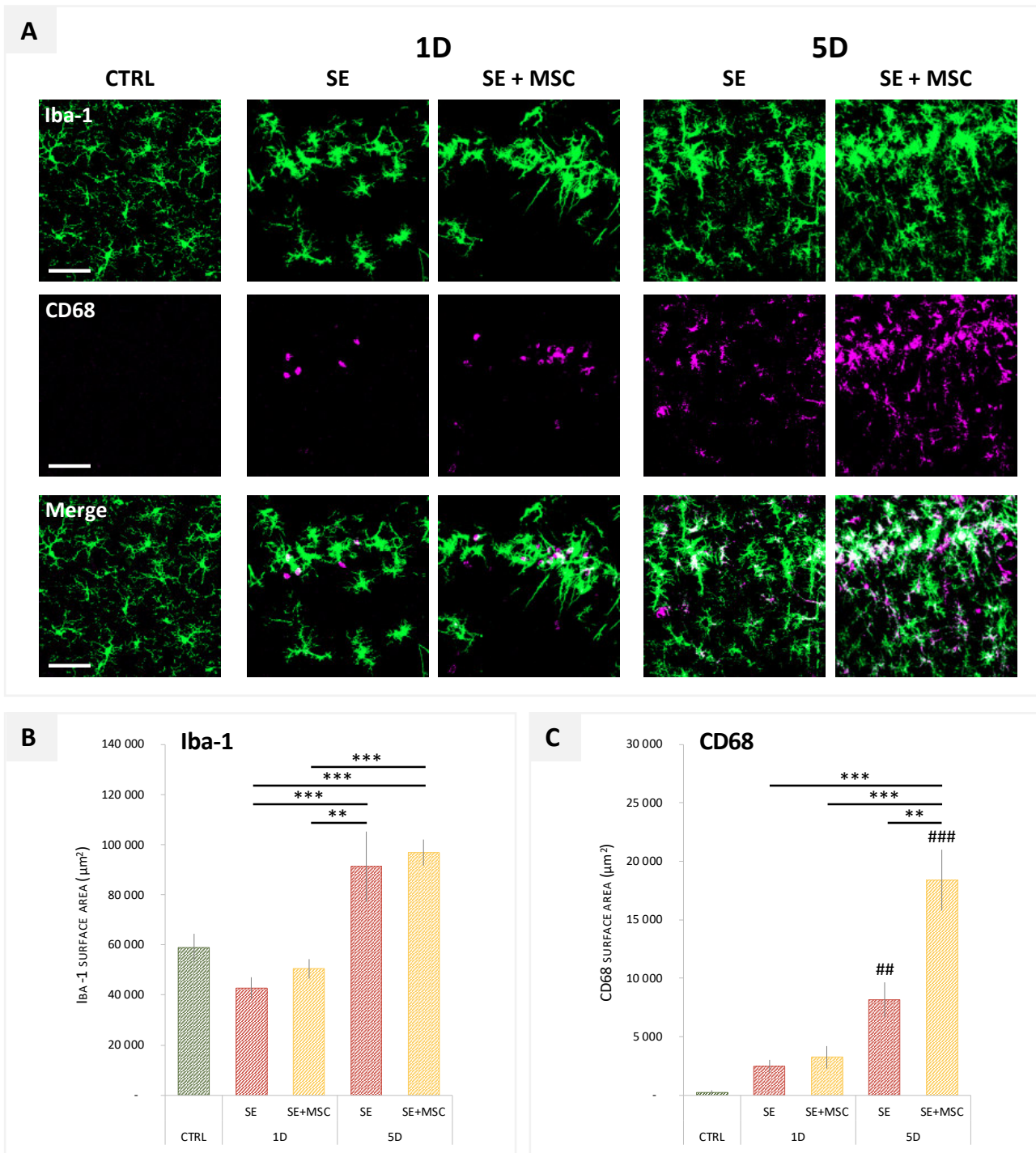


Figure 10. Effects of MSCs on microgliosis and monocytes/macrophages infiltration in CA1 pyramidal cell layer during epileptogenesis in rats subjected to pilocarpine-induced SE. (A) As for Figure 8. Values are expressed by mean \pm SEM. Hashtags indicate statistical significance between CTRL and SE rats (CTRL vs SE ; CTRL vs SE+MSC). Asterisks indicate statistical significance between SE rats treated or not with MSCs (SE vs SE+MSC).

VENTRAL LIMBIC REGION

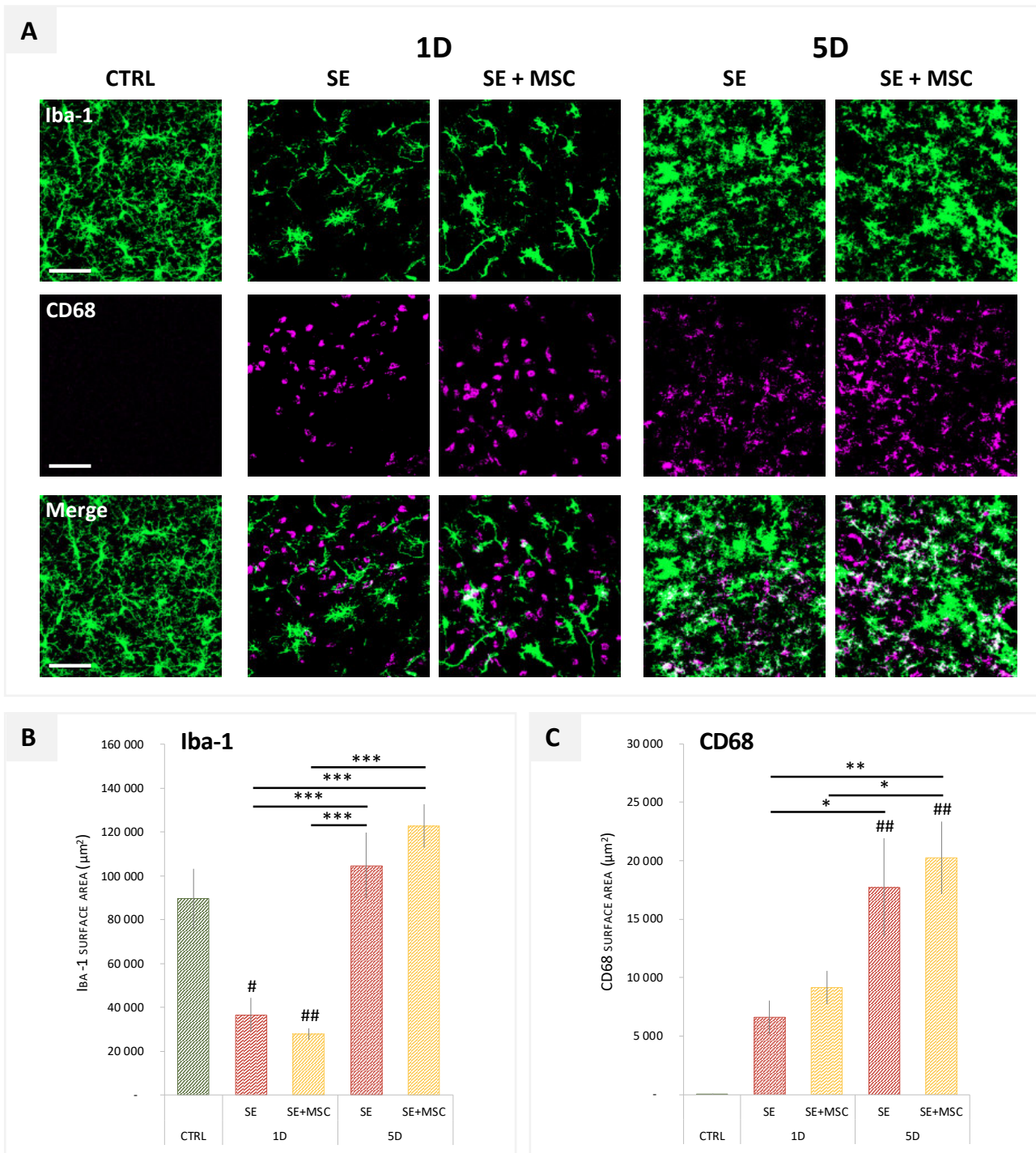


Figure 11. Effects of MSC on microgliosis and monocytes/macrophages infiltration in the surviving area of the ventral limbic region during epileptogenesis in rats subjected to pilocarpine-induced SE. (A) As for Figure 8. Values are expressed by mean \pm SEM. Hashtags indicate statistical significance between CTRL and SE rats (CTRL vs SE ; CTRL vs SE+MSC). Asterisks indicate statistical significance between SE rats treated or not with MSCs (SE vs SE+MSC).

DORSAL THALAMUS

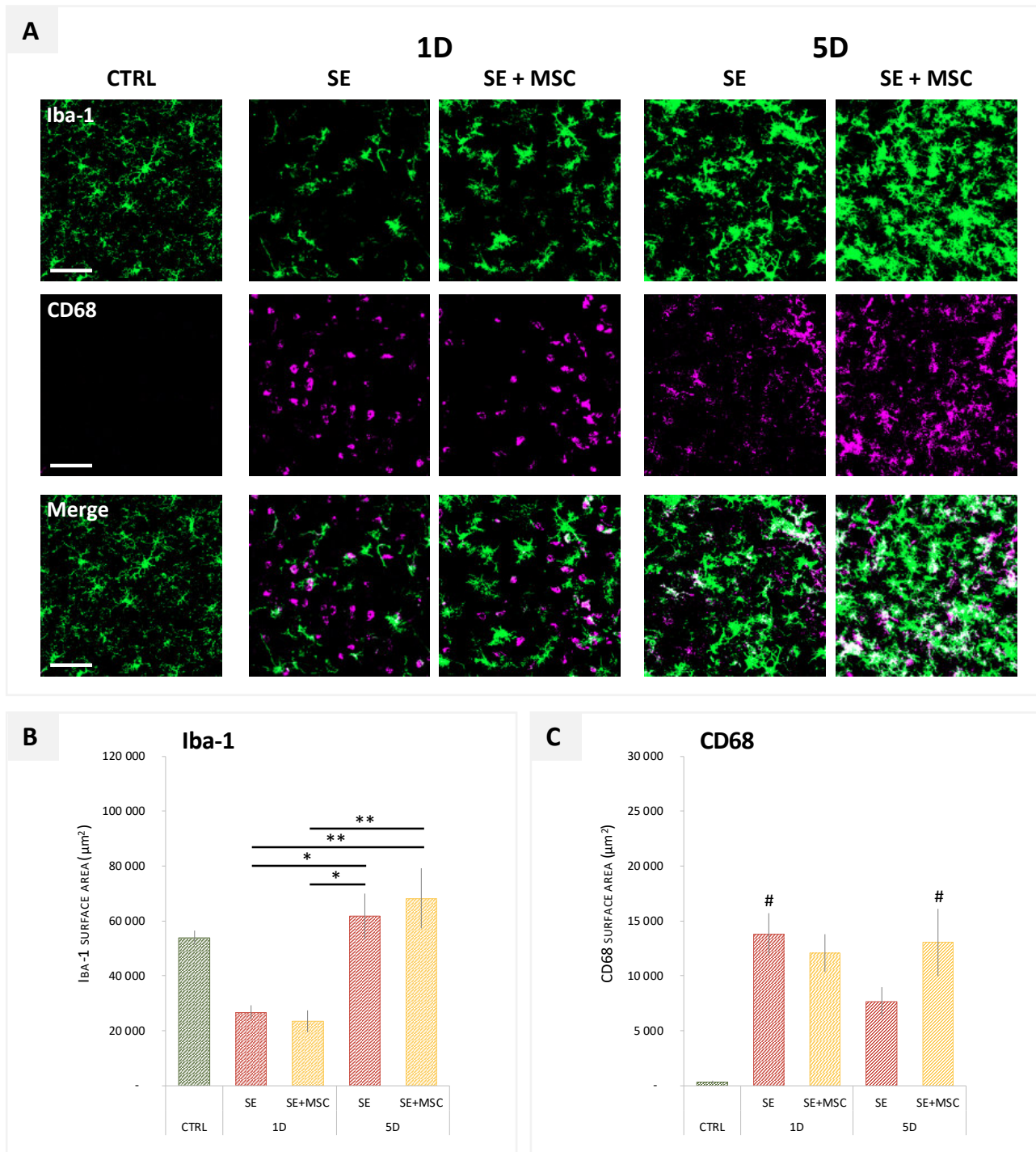
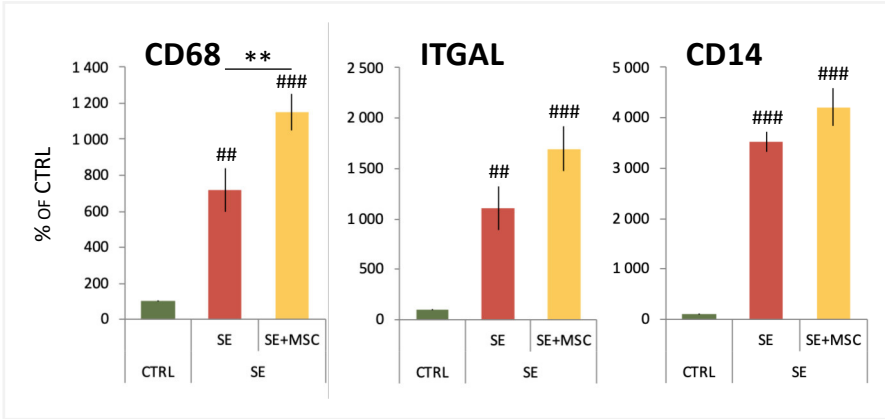
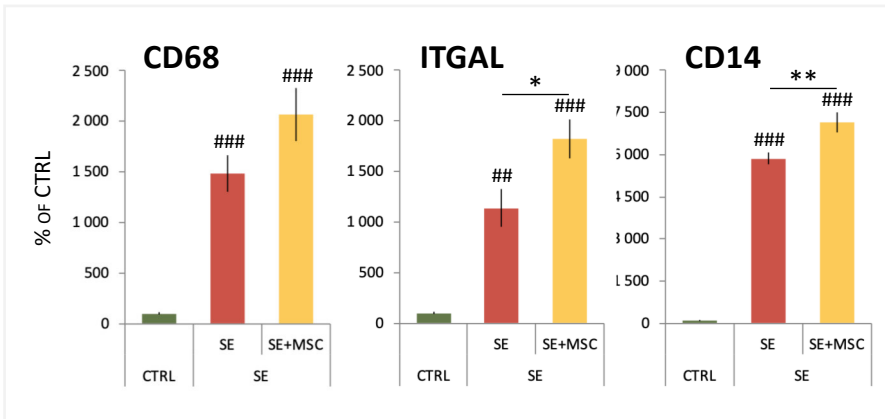


Figure 12. Effects of MSC on microgliosis and monocytes/macrophages infiltration in the surviving area of the dorsal thalamus during epileptogenesis in rats subjected to pilocarpine-induced SE. (A) As for Figure 8. Values are expressed by mean \pm SEM. Hashtags indicate statistical significance between CTRL and SE rats (CTRL vs SE ; CTRL vs SE+MSC). Asterisks indicate statistical significance between SE rats treated or not with MSCs (SE vs SE+MSC).

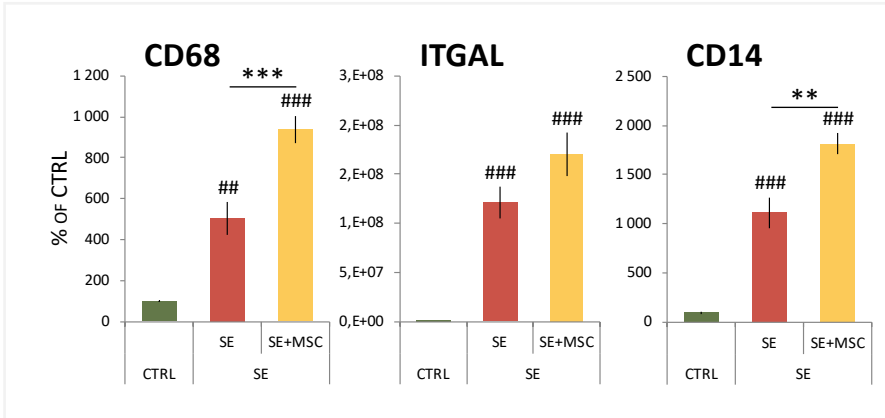
HIPPOCAMPUS



VLR



DORSAL THALAMUS



NEOCORTEX

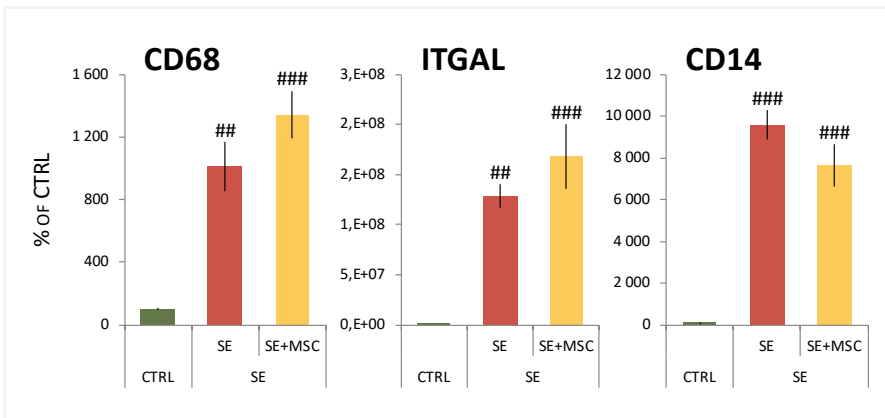


Figure 13. Effect of intranasal MSC injection on expression of monocyte/macrophage markers 24 hours after pilocarpine-induced SE. As for Figure 3. Hashtags indicate statistical significance between CTRL and SE rats (CTRL vs SE ; CTRL vs SE+MSC). Asterisks indicate statistical significance between SE rats treated or not with MSCs (SE vs SE+MSC). Tukey's post-hoc analysis following one-way ANOVA: # or *: $p < 0.05$, ## or **: $p < 0.01$, ### or ***: $p < 0.001$, n.s: not significant.

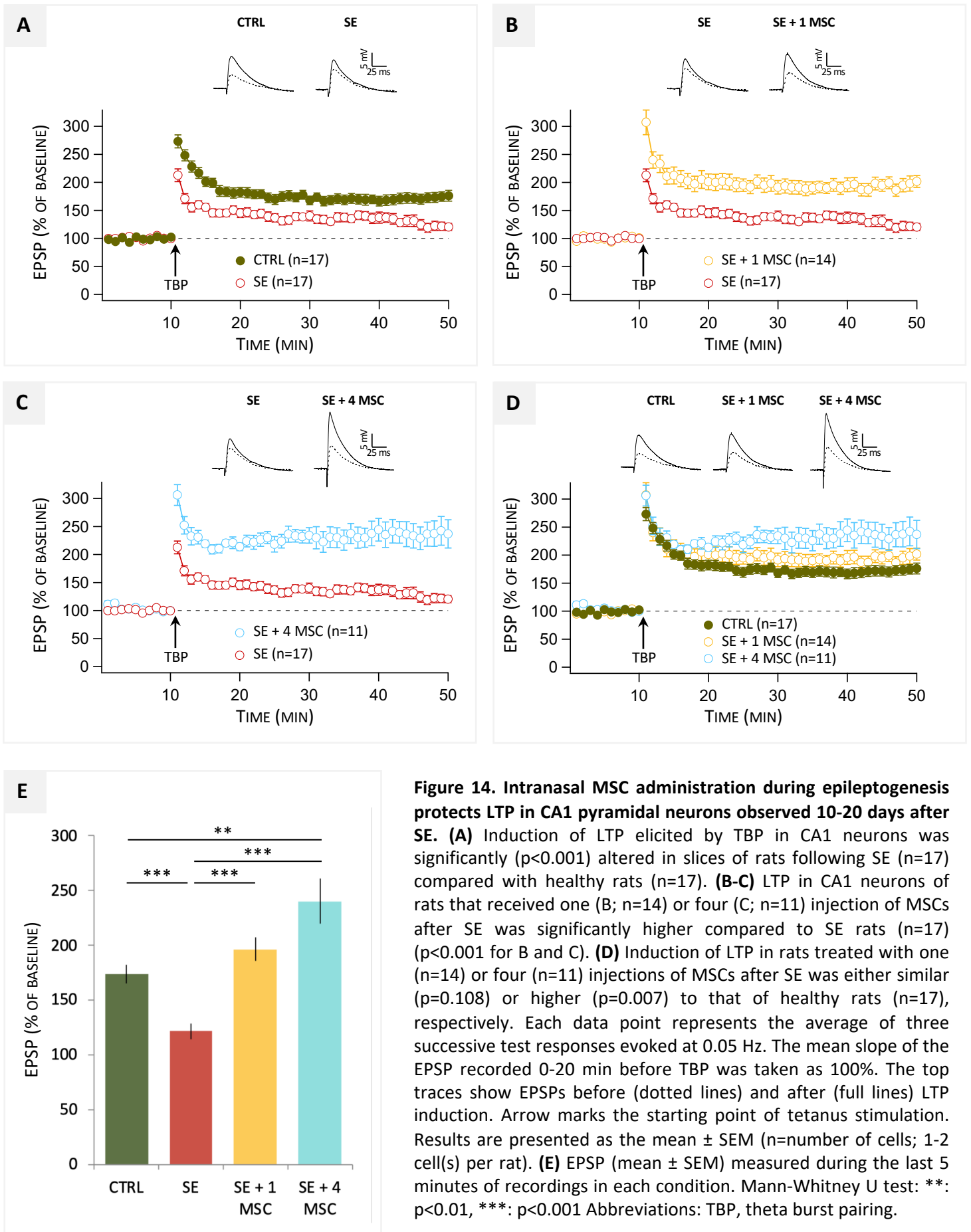


Figure 14. Intranasal MSC administration during epileptogenesis protects LTP in CA1 pyramidal neurons observed 10-20 days after SE.

(A) Induction of LTP elicited by TBP in CA1 neurons was significantly ($p < 0.001$) altered in slices of rats following SE ($n = 17$) compared with healthy rats ($n = 17$). **(B-C)** LTP in CA1 neurons of rats that received one (B; $n = 14$) or four (C; $n = 11$) injection of MSCs after SE was significantly higher compared to SE rats ($n = 17$) ($p < 0.001$ for B and C). **(D)** Induction of LTP in rats treated with one ($n = 14$) or four ($n = 11$) injections of MSCs after SE was either similar ($p = 0.108$) or higher ($p = 0.007$) to that of healthy rats ($n = 17$), respectively. Each data point represents the average of three successive test responses evoked at 0.05 Hz. The mean slope of the EPSP recorded 0-20 min before TBP was taken as 100%. The top traces show EPSPs before (dotted lines) and after (full lines) LTP induction. Arrow marks the starting point of tetanus stimulation. Results are presented as the mean \pm SEM (n =number of cells; 1-2 cell(s) per rat). **(E)** EPSP (mean \pm SEM) measured during the last 5 minutes of recordings in each condition. Mann-Whitney U test: **: $p < 0.01$, ***: $p < 0.001$. Abbreviations: TBP, theta burst pairing.

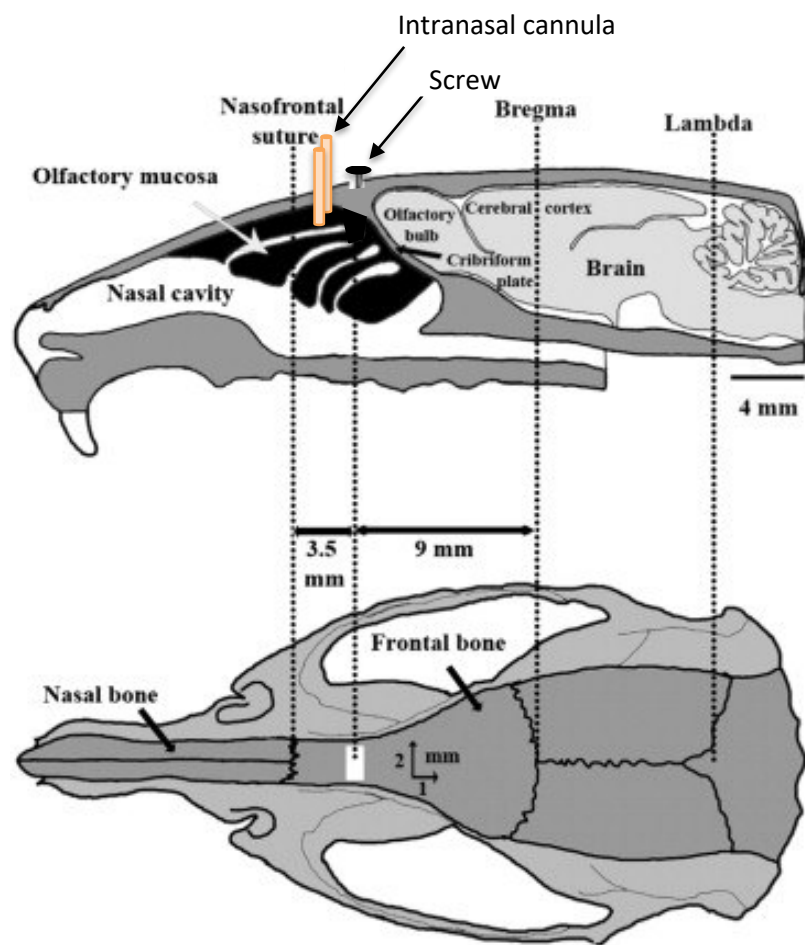


Figure S1. Localization of the implanted intranasal cannulas for MSC administration.

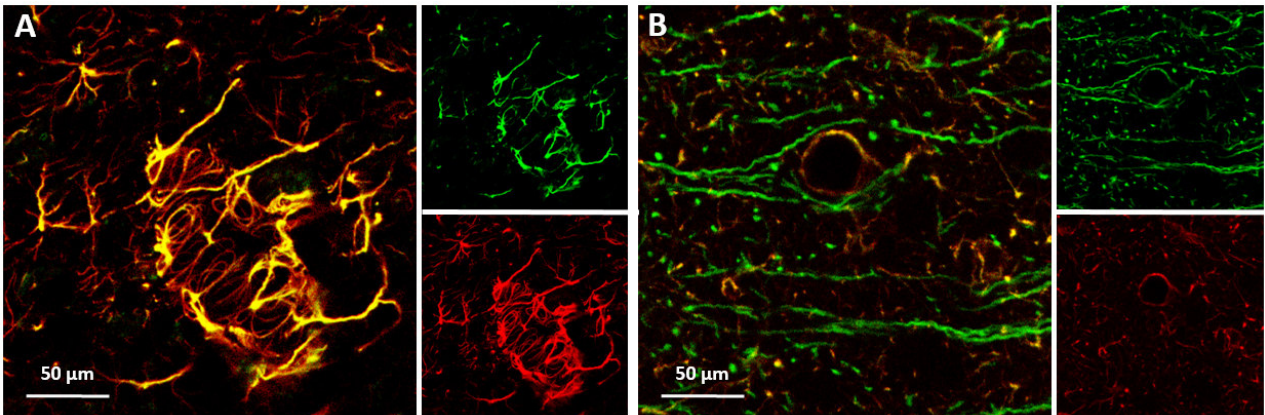


Figure S2. Non-overlapping of GFAP immunofluorescent labelling obtained with two different antibodies. Double immunohistochemical labelling of GFAP in the hippocampus (A) and in the piriform cortex (B) of a rat 7 weeks after pilocarpine-induced SE. The rabbit polyclonal anti-GFAP antibody (AB5804; Chemicon) is visualized in green while the mouse monoclonal anti-GFAP antibody (G3893; Sigma-Aldrich) is visualized in red. Colocalization is displayed in yellow when red and green are superimposed. These observations suggest that GFAP epitopes recognized by the two antibodies are not accessible (or present) in the same manner within the same structure or between two different structures. Scale bar: 50 μm .

Table S1. Primer sequences – *Rattus Norvegicus*

cDNA	Primer sequences – <i>Rattus norvegicus</i>	Product sizes (bp)	GenBank ID#
CD14	F-AAAGAACTGAAGCCTTTCTCG R-AGCAACAAGCCGAGCATAA	89	NM_021744.1
CD29	F-GAAGCTCACGTGCATGTTGT R-GCTACAATTGGGATGATGTCG	68	NM_017022.2
CD31	F-TCCTGAGGGTCAAGGTAATAGC R-CTCCAGACTGTACATCGTTACCC	78	NM_031591.1
CD34	F-CGGCTATTTCTGATGAACC R-CCTCCACCATTCTCCGTGTA	87	NM_001107202.2
CD44	F-TGATTCTTGCCGTCTGCAT R-CTGTTCCATTGCCACTGTTG	87	NM_012924.2
CD45	F-GGGGTTGTTCTGTGCTCTGT R-CCGTGCTTTGCGTAGAGACT	97	NM_001109890.1
CD49a	F-GGGCTACTGCTGCTAATGCT R-GGCCTTTGAAGAATCCAATC	62	NM_030994.2
CD68	F-CTTTCTCCAGCAATTCACCTG R-ACTGGCGCAAGAGAAGCA	99	NM_001031638.1
CD73	F-CATTGCCAGAAGGTGAGAGG R-TGTAGAGAAAGGTGTTGGTGTG	65	NM_021576.2
CD90	F-TGTTGGGAAAGGGTAGGA R-GCTCCTTGAGGCAGAGAAG	132	NM_012673.2
CD105	F-TATTCTCACACAGTGCCCC R-CCGATGCTGTGGTTGGTACT	95	NM_001010968.2
CD117	F-GCTCCTCCGAGTGTGTTTGA R-GCCCGTCATTATGGAAGGCT	98	NM_022264.1
GFAP	F-ACATCGAGATCGCCACCTAC R-GGATCTGGAGGTTGGAGAAA	90	NM_017009.2
IFN γ	F-TTTTGAGCTCTGCCTCAT R-AGCATCCATGCTACTTGAGTTAAA	107	NM_138880.2
IL1 β	F-TGTGATGAAAGACGGCACAC R-CTTCTTTGGGTATTGTTTGG	70	NM_031512.2
IL1R1	F-CACGGAATGAGACGATGGAAG R-ACGAAGCAGATGAACGGATAG	250	NM_013123.3
IL4	F-GTAGAGGTGTCAGCGGTCTG R-TTCAGTGTTGTGAGCGTGGA	70	NM_201270.1
IL6	F-CCCTTCAGGAACAGCTATGAA R-ACAACATCAGTCCAAGAAGG	74	NM_012589.1
IL10	F-AGTGGAGCAGGTGAAGAATGA R-TCATGGCCTTGTAGACACCTT	62	NM_012854.2
IL13	F-AGTCCTGGCTCTCGCTTG R-GATGTGGATCTCCGCACTG	63	NM_053828.1
ITGAL	F-GAAGGATACCAAGGGACCAA R-AGGGGATCTCCTTAGTGACTG	88	NM_001033998.2
ITGAM	F-ACTCTGATGCCTCCCTTGG R-TCCTGGACAGTTGTTCTCA	72	NM_012711.1
MCP1	F-CGGCTGGAGAACTACAAGAGA R-TCTCTTGAGCTTGGTGACAAATA	78	NM_031530.1
MIP1 α	F-TCCACGAAAATTCATTGCTG R-AGATCTGCCGTTTCTCTTG	92	NM_013025.2
TNF α	F-TGAACTTCGGGGTGATCG R-GGGCTTGTCACTCGAGTTTT	122	NM_012675.3

HIPPOCAMPUS

	CTRL		SE		SE + MSC	
	Mean	SEM	Mean	SEM	Mean	SEM
IL1β	1 552	21	7 414	917	7 402	519
TNFα	5	1	26	4	30	3
IL6	10	4	1 079	192	709	99
MCP1	171	18	135 413	13 794	154 894	3 843
MIP1a	59	4	2 187	417	1 554	285
IL1R	365	15	1 441	95	1 686	148
IL4	28	6	199	29	168	25
IL10	42	10	118	22	120	25
IL13	2 719	719	4 535	1 041	2 658	741
ITGAM	2	0	12	1	15	1
GFAP	12 156 087	1 120 258	82 221 001	3 092 075	97 828 738	2 964 365
CD68	2 108	53	15 121	2 513	24 217	2 139
CD14	832	80	29 340	1 611	35 059	3 066
ITGAL	102	6	1 128	223	1 734	229

Table S2. Number of cDNA copies (mean \pm SEM) after reverse transcription in the hippocampus of control rats (CTRL, n = 5), rats subjected to SE (SE, n = 7), and rats subjected to SE that received 6 hours later an intranasal administration of MSCs (SE+MSC, n = 6). Brains were removed 24h after SE and transcript values were measured by RT-qPCR.

VLR

	CTRL		SE		SE + MSC	
	Mean	SEM	Mean	SEM	Mean	SEM
IL1β	1 479	139	13 773	1 349	11 569	929
TNFα	6	1	29	5	30	2
IL6	0	0	5 314	1 160	3 497	605
MCP1	131	18	183 944	23 856	186 661	8 494
MIP1a	57	9	6 245	943	3 741	578
IL1R	338	17	1 984	149	2 751	154
IL4	35	7	258	40	145	28
IL10	32	8	310	43	337	35
IL13	10 097	1 059	7 095	1 313	3 228	504
ITGAM	1	0	12	1	12	1
GFAP	4 156 553	551 549	53 190 808	7 591 517	65 185 500	11 665 571
CD68	1 775	224	26 260	3 216	36 648	4 708
CD14	704	73	41 288	1 367	50 346	2 402
ITGAL	94	10	1 072	175	1 715	179

Table S3. Number of cDNA copies (mean \pm SEM) after reverse transcription in the ventral limbic region (VLR) of control rats (CTRL, n = 5), rats subjected to SE (SE, n = 7), and rats subjected to SE that received 6 hours later an intranasal administration of MSCs (SE+MSC, n = 6). Brains were removed 24h after SE and transcript values were measured by RT-qPCR.

DORSAL THALAMUS						
	CTRL		SE		SE + MSC	
	Mean	SEM	Mean	SEM	Mean	SEM
IL1 β	46	4	239	28	303	19
TNF α	8	2	22	2	26	4
IL6	0	0	200	33	273	25
MCP1	515	80	292 694	50 805	300 498	28 971
MIP1a	54	4	684	115	1 074	116
IL1R	303	19	722	80	893	48
IL4	0	0	0	0	0	0
IL10	49	10	259	27	300	44
IL13	104	33	331	37	528	116
ITGAM	9	1	33	3	33	3
GFAP	4 008 343	417 454	35 151 657	3 254 292	36 157 210	2 201 210
CD68	501	20	2 540	403	4 693	336
CD14	711	80	7 937	1 107	12 913	792
ITGAL	0	0	734	98	1 029	134

Table S4. Number of cDNA copies (mean \pm SEM) after reverse transcription in the dorsal thalamus of control rats (CTRL, n = 5), rats subjected to SE (SE, n = 7), and rats subjected to SE that received 6 hours later an intranasal administration of MSCs (SE+MSC, n = 6). Brains were removed 24h after SE and transcript values were measured by RT-qPCR.

NEOCORTEX						
	CTRL		SE		SE + MSC	
	Mean	SEM	Mean	SEM	Mean	SEM
IL1 β	46	1	407	99	269	28
TNF α	14	1	43	10	34	8
IL6	0	0	514	117	342	106
MCP1	425	46	11 350 694	2 407 741	5 868 279	853 036
MIP1a	80	7	1 911	340	1 274	318
IL1R	379	37	2 999	245	2 682	461
IL4	0	0	237	38	137	22
IL10	66	19	149	41	100	25
IL13	2 517	874	1 613	604	666	146
ITGAM	12	2	53	3	60	7
GFAP	6 619 878	759 509	75 755 104	2 196 461	70 792 578	11 998 830
CD68	485	29	4 910	770	6 502	738
CD14	387	57	37 047	2 668	29 566	3 884
ITGAL	0	0	761	72	996	190

Table S5. Number of cDNA copies (mean \pm SEM) after reverse transcription in the neocortex of control rats (CTRL, n = 5), rats subjected to SE (SE, n = 7), and rats subjected to SE that received 6 hours later an intranasal administration of MSCs (SE+MSC, n = 6). Brains were removed 24h after SE and transcript values were measured by RT-qPCR.

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BOX 9 | HIGHLIGHTS OF STUDY 4

- Intranasal MSCs administration does not significantly reduce the explosive neuroinflammation observed 24h after status epilepticus (SE) in the hippocampus, the ventral limbic region, the dorsal thalamus and the neocortex;
- MSCs increase the astrocyte proliferation in the hippocampus, the ventral limbic region and the dorsal thalamus 5 days after SE;
- MSCs promote the infiltration and transdifferentiation of monocyte-macrophages into microglial-like cells in the hippocampus after SE;
- Intranasal mesenchymal stem cells (MSCs) administration prevent synaptic function alterations in the hippocampus of rats subjected to pilocarpine-induced SE and allow to recover a long-term potentiation similar to that of controls.

CHAPTER 6

DISCUSSION & PERSPECTIVES

The work carried out in this thesis allowed us to address inflammation broadly in the epileptic brain, from epileptogenesis phase following brain injury (*status epilepticus*), to the chronic phase. In the first study, we have demonstrated by investigating the inflammation in resected hippocampus of epileptic patients that not all patients have a high level of inflammation. However, when present, the inflammation is of low grade within the hippocampus. We then demonstrated that short-term delay in processing of resected tissue leads to large decrease of targeted mRNAs, even those of housekeeping genes. This result precluded the use of post-mortem tissues as controls and prompted the use of animal models of epilepsy to evaluate the extent of inflammation in the epileptic hippocampus above control values. Data on rats modeled a large part of the variability observed in patients and allowed us to establish that, in contrast to the early phase of epileptogenesis during which inflammation is highly elevated, inflammation during the chronic phase may be indistinguishable from control animals or higher, and that epilepsy can be active regardless of the inflammatory status. The contribution of animal models also allowed us to show that not all brain insults, known as epileptogenic, associated with high neuroinflammatory response necessarily leads to epilepsy. In addition, by studying one of the prototypic markers of inflammation, interleukin-1 β (IL1 β), we have determined that the major cellular contributors to the production of this cytokine during the acute phase were microglial cells.

It is now increasingly recognized that peripheral leukocytes infiltrate brain tissue during epileptogenic cerebral aggression (Fabene et al., 2013; Navarro, 2007; Ravizza et al., 2005; Varvel et al., 2016; Vinet et al., 2016; Zattoni et al., 2011), certainly contributing to the pathophysiology of epilepsy, although their harmful role is currently being questioned (Kronenberg et al., 2018). The purpose of the second study was to determine how to monitor the fate of infiltrating monocytes. We have shown **in rats** that circulating monocytes infiltrating the brain parenchyma following SE expressed CD68 as a specific marker, not found on resident microglial cells, even in their activated state, contrasting with mouse models of epileptogenesis (Zattoni et al., 2011). Such specificity in *cd68* gene expression in rats enabled us to differentiate monocytes from the pool of microglial cells over time. We have provided evidence 1) that monocytes underwent transdifferentiation processes into (brain) tissue monocyte-macrophages (mo-MPs), bearing morphological features of activated microglial cells, as evidenced by ITGAM (CD11b) marker, some of them up to the expression of the

specific microglial marker Iba-1, and, 2) that this phenotype was maintained in the long term during the chronic phase of epilepsy during which transdifferentiated monocytes fully integrated the microglial network. Moreover, we have provided evidence that a large majority of monocytes expressed heparan sulphate chains (HSCs) on their surface at the time of extravasation. HSCs have been shown to be involved in infiltration processes. Here, we report that migration/integration of infiltrating cells into the parenchyma was associated with the degradation of cell surface HSCs, likely through the action of heparanase, constitutively expressed by infiltrating monocytes (Parish, 2006) and neurons (Navarro, 2007; Navarro et al., 2008).

In the light of our results obtained in Study 2, we considered developing a transgenic rat that would allow us to follow the fate of infiltrating monocytes, using a reporter gene encoding Green Fluorescent Protein (GFP). The specific expression of GFP in circulating monocytes will be triggered by tamoxifen-induced activation of a CreERT2 whose expression will be under control of the rat cd68 gene regulatory elements. However, due to constraints imposed by the companies we contract out the production of the transgenic rats, which mostly use Sprague-Dawley rats from Charles River Laboratories, we had to evaluate whether the inflammatory response in Sprague-Dawley (SD) rats from Charles River Laboratories (CRL) was similar to that previously characterized, in the first study of this thesis, in the hippocampus of SD from Harlan/Envigo (HAR/ENV) laboratories. In a first part of study 3, we refined the characterization of the inflammatory response following pilocarpine-induced SE in HAR/ENV SD rats, by examining, in addition to the hippocampus, 3 other brain structures more or less affected by neurodegenerative processes, i.e. the ventral limbic region (VLR, that includes the amygdala, the piriform cortex and the insular agranular cortex), the dorsal thalamus and the neocortex. We found that the expression of inflammatory molecules varied according to the brain area and to the model (weaning or juvenile) considered, and irrespective of the presence or absence of subsequent neurodegenerative processes. By studying these same inflammatory variables in the hippocampus of the CRL rat, in addition to neuronal degeneration, we observed that pro-inflammatory molecules were expressed to a lesser extent in CRL SD rats compared to HAR/ENV SD rats, while anti-inflammatory molecules were expressed in the opposite way. Furthermore, the data obtained in CRL SD rats reinforced our hypothesis that the extent of inflammation is not directly related to neurodegeneration since

the CRL rats suffered from a much more severe neuronal deficits than HAR/ENV SD rats, despite a less pronounced inflammatory response to SE.

Finally, in the fourth study, we tested a stem cell-based therapy approach to counteract the effects of pilocarpine-induced *status epilepticus* with the hypothesis that the mesenchymal stem cells that we used, known notably for their anti-inflammatory properties, could lower the inflammatory response occurring after a pro-epileptogenic insult and act as an anti-epileptogenic or a disease-modifying treatment. We showed that intranasal administration of mesenchymal stem cells 6 hours after SE had no significant effect on the induction of inflammatory molecules in the first 24 hours after SE, although their presence resulted in a modulation of the transdifferentiation of infiltrating monocytes into monocyte-macrophages, as well as an increase in astrocyte density. On a functional level, we investigated whether one or four intranasal injections of MSCs could counteract the onset or reduce the severity of cognitive impairment by studying long-term potentiation, a mechanism underlying the learning and memory processes. We observed that injection(s) of MSCs 6 hours post-SE restored the synaptic plasticity of hippocampal pyramidal neurons with a return to the same level as in control animals.

Altogether, these promising results pave the way for further key questions and future prospects that have yet to be addressed.

I. Neuroinflammation in epilepsy: where are we headed now?

1. Neuroinflammation: a very heterogeneous variable in patients and animals

The inflammatory variables we analyzed in Study 1 were measured at one point in time, i.e., at the time of surgery for hippocampal resection in TLE patients. Considering the diversity of parameters that can change from one patient to another, such as the time and intensity of the last seizure, the frequency of seizures, or multiple treatment adjustments, the values attributed to these variables could have been different if they had been measured at another time. This is why it is important to emphasize that beyond the inter-individual variability, there may also be intra-individual variability.

Study 1 and Study 3 allowed us to highlight the great variability of inflammatory processes in epilepsy. The different results obtained in Study 3 in which inflammation was

assessed in the hippocampus of rats from Charles River Laboratories (CRL) and compared to that obtained in Study 1 with rats from Harlan/Envigo Laboratories (HAR/ENV) shows that the examination of inflammation and the findings of the different investigations can be subjected to considerable discrepancies. We did not expect to find so many differences at the behavioral, cellular and molecular levels when we decided to compare the inflammatory response following SE between SD rats from HAR/ENV and CRL. In fact, we initiated this study just to reassure ourselves that the risk was minimal by switching from HAR/ENV rats to CRL rats. Of course, beyond the fact that the conclusions are not what we expected, this study has allowed us to have a much broader reflection and to put into perspective the conclusions obtained in the literature on neuroinflammation. Indeed, these results only underline the fact that when one tries to model a human pathology, characterized by very different clinical pictures added to a great pathophysiological heterogeneity, it is not surprising that the different animal models used lead to variable results, very different from one model to another. And the underlying question is whether it is possible to imagine one day finding a common denominator for all the pathophysiological profiles, which would make it possible to foresee that each patient benefits from the same treatment. Or if, on the contrary, if such a common denominator did not exist, the only possibility would then be to personalize the therapeutic approach, based on a set of biomarkers that would make it possible to refine the clinical picture of each patient.

The involvement of inflammatory mediators in neuronal excitability, synaptic plasticity and neuronal survival has been suggested in numerous studies (Vezzani and Viviani, 2015; Vezzani et al., 2008; Yirmiya and Goshen, 2011). The direct consequences of neuroinflammation on the processes of neuronal degeneration should nevertheless be taken with caution. We have shown in CRL rats, where the inflammatory balance is in favor of anti-inflammation, that the neurodegeneration observed in the dentate gyrus of the hippocampus is more prominent than in HAR/ENV rats. Considering this, it is important again to take a step back from the molecules highlighted in our studies, and to remember that they represent only few of the many inflammatory mediators among others. Furthermore, other genetic and epigenetic factors may affect sensitivity to these mediators and suggest that the presence of cytokines in the brain during epileptogenesis or epilepsy cannot be seen as a go/no-go paradigm for neuronal death. As discussed in the review of Löscher (2017), other parameters

may influence the physiological responses observed in experimental designs in animals, such as genetic background, animal source, sanitary status, housing conditions at the supplier's animal husbandries and then in the laboratory, maternal care at the earliest age, shipping conditions to the laboratory, duration of acclimatization before experimentation, age at testing, sex, handling habituation or inter-experimenter variation (Löscher, 2017). Some will see this variability as a disadvantage since the results obtained in the different scientific studies may differ depending on the epilepsy model used, the species and lineage of the animals studied or the animal supplier, but it is rather a tremendous asset to actually model the diversity that exists in humans. In fact, the broad spectrum of action of a treatment can only be validated if it has similar effects on different experimental models and/or on animals from different suppliers. Conversely, if the treatment does not show similar effects across models, this does not mean that it is ineffective. Rather, it means that it will be targeted at a more specific population of patients, whose demand for personalized care is growing today, and will therefore be consistent with the personalized medicine treatments that are increasingly in demand today.

2. Taking a step back from our understanding of the neuroinflammatory picture

The work conducted in this thesis focused on the commonly studied prototypical markers of inflammation, namely the pro-inflammatory cytokines IL1 β , IL6, TNF α , the chemokines MCP1 and MIP1 α and the anti-inflammatory cytokines IL4, IL10 and IL13. The major contribution of our study lies in the establishment of inflammation indexes, which brings new insights into how to deal with the large amount of transcriptomic data obtained with RT-qPCR for instance. However, it is important to note that these markers are not the only one involved in neuroinflammation and that other inflammatory mediators such as molecules of the prostanoid family or reactive oxygen species are also involved in the overall neuroinflammatory picture (Vezzani et al., 2019).

Regarding the different cell types involved in inflammation, our studies have focused on microglial cells, infiltrating monocytes and astrocytes. However, these cells are not the only one involved in the production of inflammatory mediators. Indeed, other cell types of the cerebral parenchyma, notably neurons and endothelial cells, or immune cells from the

periphery, especially neutrophils or T-cells, will infiltrate the cerebral parenchyma in many cerebral pathologies, including epilepsy, and be involved in inflammatory processes (Rana and Musto, 2018; Ravizza et al., 2008; Zattoni et al., 2011). More research is needed nowadays to ascertain more accurately the extent to which each of these cell types will participate in the production of inflammatory molecules, and to take into account the dynamic aspect of this production over time. It is very likely that each of these cell types will have a different role depending on the time window considered, from the early times of epileptogenesis after a pro-epileptogenic brain injury, to the later times during the chronic phase of epilepsy, during ictal or interictal periods. Presently, one of the main technological limitations slowing down access to these scientific and valuable scientific insights is related to tissue dissociation protocols for cell isolation which are likely to alter cell phenotype (van den Brink et al., 2017), justifying our choice to obtain transcriptomics data from whole tissues of the different regions investigated.

Two-photon imaging could be a valuable tool for assessing the behavior of the different cell types in their natural environment and their interaction with surrounding cells. However, in our case, two main problems hinder the use of this technology: 1) transgenic animal models in rats are still in their infancy and, even if they tend to become more widespread in the future, they are still relatively high cost resources not easily affordable by all laboratories; 2) the realization of experimental models for two-photon monitoring requires surgical procedures to create a brain window which, on the one hand, can alter physiological processes and, on the other hand, can de facto create a cerebral inflammation that would be paradoxical considering the variables we are studying. Research models such as zebrafish can be used to circumvent these limitations and represent attractive tools for scientific investigation due to the transparency of the embryo, the ease of manipulation and maintenance, the high fertility, the rapid embryonic development and the low cost (Brenet et al., 2019; Kundap et al., 2017; Stewart et al., 2012).

3. From laboratory to clinical practice: relevance of these results for epileptic patients

To translate these results into clinical applications, the challenge today remains in the identification of new peripheral biomarkers to 1. pinpoint and target patients potentially more responsive to anti-inflammatory treatments or therapies acting on inflammation, and 2. to determine the optimal time windows to enhance the efficacy of such treatments with reduced dosages applied. Combination therapies represent a valuable tool for the management of such multi-factorial chronic disease in which inflammation is only one aspect of the overall pathophysiological processes. Numerous biomarkers are currently being studied to uncover novel diagnostic, prognostic and predictive factors for epilepsy. Among the biomarkers of interest that can be easily quantified peripherally are microRNAs, amino acids, complement proteins or reactive oxygen species (Kobylarek et al., 2019; Terrone et al., 2019; Vezzani et al., 2019). For technical reasons, our study 1 did not allow us to have access to blood tissue and thus to search for possible peripheral biomarkers that would have correlated with one or more of the inflammatory mediators found in the hippocampus of some patients. The existence of such correlations would be of interest in future studies to determine whether peripheral markers could predict the cerebral inflammatory status. Nevertheless, caution should be exercised with the potential conclusions given, since epileptic patients who undergo surgery and whose resected tissue can be collected for scientific research purposes represent only a very small percentage of the general epileptic population. Despite this, such results would represent a breakthrough to allow better targeting of patients eligible for inflammation-based therapy in order to either prevent the onset of epilepsy in patients at risk or to modulate the progression of the disease in drug-resistant epileptic patients.

Regarding the bidirectional link between inflammation and seizures, our study has in no way tested the possible roles of the observed changes in the expression of inflammatory molecules on the recurrence of seizures and provides no information on the eternal dilemma of whether inflammation is the cause or the consequence of seizures. Therefore, any discussion of the impact of inflammation on the functional alterations observed is therefore purely hypothetical. Experimental models of conditional blockade of the expression of specific inflammatory mediators could demonstrate the direct involvement of cytokines in the initiation and perpetuation of seizures *in vivo*. Such models are now possible with CRISPR-Cas9

technology. However, even if the alteration of gene expression can be controlled over time, the results following its interruption (KO models) or, on the contrary, its definitive activation (KI models), must be interpreted with caution. In fact, very many mediators of inflammation are involved in cerebral homeostasis, so that the sudden and definitive alteration (which is rarely the case) of one of them, even in adulthood, may lead to compensation by other signaling pathways that are difficult to identify or anticipate and lead to misinterpretations over the long term (Yirmiya and Goshen, 2011).

Finally, data on cognitive and neuropsychiatric co-morbidities were also not available for the patients included in our Study 1. Considering that inflammatory processes have been shown to play a key role in processes such as synaptic plasticity, neurotransmitter metabolism, dopaminergic transmission and neurogenesis, deregulation of inflammatory mediator expression in the brain is thought to be involved in comorbidities associated with epilepsy (Paudel et al., 2018; Vezzani et al., 2019). We have shown in our animal models that inflammatory levels can be elevated in structures of the limbic system, such as the amygdala, involved in mood and emotions. For this reason, it would have been of interest to determine whether a particular inflammatory profile found in patients, either in brain areas removed together with the hippocampus during surgery resection, or in blood samples, could have been associated with certain types of comorbidities.

II. Tracking (brain) monocyte-macrophages into the epileptic brain: one step closer to uncovering their role?

1. Specific markers of monocytes-macrophages

The purpose of the second study was to identify a marker to specifically track monocytes infiltrating the brain following pilocarpine-induced SE in rats, in order to differentiate this cell population from resident microglial cells and to determine whether these monocytes were only transiently present in the tissue or whether they persisted over time. We showed that CD68 was a marker that was expressed only on the surface of cells from the periphery, since its presence was not detected in control rats on one hand, and its expression was barely detected at 7h post-SE, always on cells located near blood vessels, on the other hand. Nonetheless, the specificity of this marker still requires further study. It is not

known today whether this marker is expressed by all monocytes or only by a particular subpopulation, and this is a question that remains to be answered.

In addition, we have also shown that monocytes found in the hippocampus possessed heparan sulfate chains (HSC) in the early stages of infiltration, and that as early as 2 days post-SE, these chains were no longer present on their cell surface. However, the detection of HSCs shows that some monocytes do not have these chains on their surface. The question raised here is whether the HSCs are no longer present because they have already been degraded by heparanase to promote monocyte migration into the brain parenchyma, or whether there are subpopulations of monocytes, some of which lack expression of these HSCs. Further studies using Fluorescence Activated Cell Sorting (FACS) will soon allow us to provide some answers to these questions concerning the more precise phenotype of circulating monocytes and monocytes isolated in the brain at different times after SE using tissue dissociation protocols.

As outlined in Studies 1 and 2, the main problem in histological studies in rats lies in the availability of reliable and validated antibodies to identify certain cell types. CD68 is certainly not the only marker allowing the identification of monocyte-macrophages. The marker CD11a (ITGAL) has recently been proposed as a specific marker for these infiltrating monocytes, allowing them to be differentiated from the pool of residential microglial cells (Shukla et al., 2019). Nevertheless, CD11a has been proposed in a mouse model and given the differences between the markers used in mice and those used in rats, it is now necessary to investigate whether CD11a is located on the same cells as CD68 in our models. We have shown in study 4 that mRNA level of CD11a is correlated with that of CD68 24 hours after SE. Based on these results, the next step will be 1. to determine all the time-course of CD11a expression in the brain after SE in our different experimental models (i.e. SE induction at P21 and P42 in Harlan/ENV rats, and SE induction at P42 in Charles River rats) and 2. to investigate by immunohistological labelling where this marker is detected. Flow cytometry approach will also enable us to assess the relative proportion of immune cells expressing CD68 and/or CD11a markers in peripheral blood.

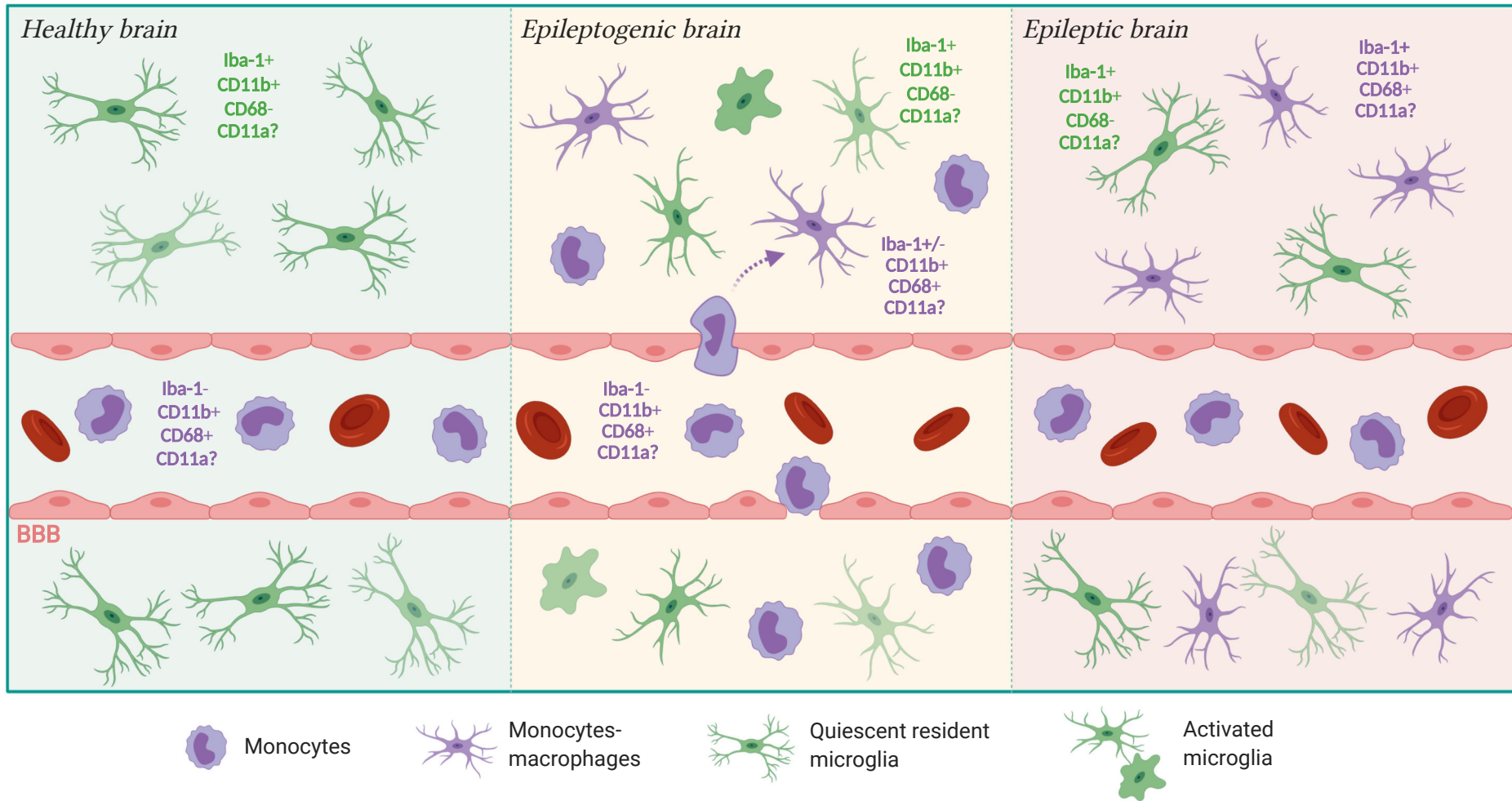
Figure 1 of this chapter schematically summarizes the morphological and phenotypic fate of monocyte-macrophages, from the phase preceding cerebral aggression (i.e. before SE), through what happens in the brain during epileptogenesis and then in the epileptic brain.

2. CD68: a new tool to explore the contribution of monocyte macrophages to neuroinflammation and their function in the pathophysiology of epilepsy?

Despite the considerations mentioned in the previous section, the identification of a marker such as CD68 opens up potential avenues for tracking these monocytes over time and determining their contribution to the inflammatory status, in addition to defining their functional role. Our hypothesis is that these monocyte-macrophages would play antagonistic functions at various stages of the pathology. We propose that during the acute phase following the brain injury, i.e. during epileptogenesis, infiltrating monocytes and their transdifferentiation into monocyte-macrophages will support the resident microglial cells to efficiently remove debris from the different cell populations injured by the cerebral insult. Then, during the late stages of epileptogenesis and during chronic epilepsy, we suggest that monocyte-macrophages may be at the origin of an important release of pro-inflammatory molecules, thus supporting a non-homeostatic environment that may promote the perpetuation of the physiopathological processes involved in the genesis of seizures and/or in the appearance of the molecular and cellular dysfunctions underlying the cognitive and psychological disorders associated with epileptic seizures. To investigate this hypothesis, we intend to use the CD68 marker to unambiguously distinguish resident microglial cells from monocyte-macrophages by creating a transgenic rat strain in which only circulating monocytes and CD68+ tissue monocyte-macrophages will express the EGFP transgene. This expression will be conditioned over time by the acute administration of tamoxifen. This will allow us to monitor the fate of monocyte-macrophages over time and to determine the extent to which these cells participate in the production of inflammatory mediators using an *in situ* hybridization approach.

Subsequently, we will address the question of the precise role of these monocytes in epileptic tissue by selectively depleting CD68+ monocytes, either prior to the induction of SE, during epileptogenesis, or during the chronic phase of epilepsy. This selective ablation will be achieved through the presence of the diphtheria toxin receptor transgene (DTR) which will be expressed only by the CD68+ cells. Subsequently, injection of the diphtheria toxin at the desired time will result in the selective death of the DTR-expressing cells. This model will first allow us to determine whether the absence of monocyte-macrophages at different stages of

epileptogenesis aggravates the development of the disease. In a second step, we will investigate whether their absence once epilepsy is developed modifies brain inflammation and reduces the severity of the disease, along with modifying the excitability and synaptic plasticity of the pyramidal neurons of the hippocampus as well as the associated cognitive and behavioral disorders. Deciphering the role of monocytes-macrophages into the diseased epileptic brain will be key to target these cells for potential therapeutic intervention.



Chapter 6 - Figure 1. Monocytes (in purple), normally present in the bloodstream (*healthy brain*), infiltrate the cerebral parenchyma following pilocarpine-induced SE and breakdown of the blood-brain barrier (BBB) (*epileptogenic brain*). These infiltrating monocytes gradually change morphology and transdifferentiate into monocyte macrophages with a morphology similar to activated microglial cells (in green). This phenotype persists in the brain throughout the chronic phases of epilepsy (*epileptic brain*). The CD68 marker allows the differentiation of these two cell populations since it is only found on the infiltrating monocytes.

III. Potential and challenges of MSCs for treating temporal lobe epilepsy

1. The quest for the immunomodulatory effect of MSCs

The immunomodulatory properties of MSCs are one of their advantages that promotes their use in many preclinical and clinical studies. In Study 4, in which MSCs were injected 6 hours after pilocarpine-induced SE, we observed no significant effect on molecular inflammation measured at 24 hours post-SE by RT-qPCR in four cortico-limbic or thalamic structures of interest. However, we observed cellular changes at 5 days post-SE in rats that received intranasal MSCs, including greater transdifferentiation of monocyte-macrophages and increased astrogliosis. Other experiments will investigate whether MSCs impact other pro- or anti-inflammatory molecules, growth factors or anti-apoptotic factors. These results will very soon be complemented by cellular and molecular studies of inflammation during the chronic phase of epilepsy. We showed in Study 1 that inflammation during the chronic phase of epilepsy was low grade in the model where SE was induced at juvenile age, as opposed to what we observed in animals in which epilepsy was induced at weaning in which the inflammatory level had returned to the control level. To investigate whether MSCs can alleviate inflammation in the chronic phase and lower the low-grade inflammation all the inflammatory mediators studied at 24 hours post-SE will be quantified at 7 weeks post-SE by RT-qPCR. We will also investigate during the chronic phase whether the MSCs have an effect on glial scar formation.

The effect of MSCs on inflammation was far below our initial assumptions. Indeed, results presented by Shetty and colleagues at the 12th annual meeting of the International Society for Stem Cell Research and summarized in a 2015 review by the same group (Agadi and Shetty, 2015) announced very promising results. Indeed, the intraperitoneal administration of human bone marrow-derived MSCs, 1h post-SE induced by kainic acid, was reported to reduce neuronal degeneration and inflammatory response. However, these results have not yet been published. Expecting to lower the inflammatory level 24h post-SE may have been over ambitious in our claims, especially since the MSCs were injected 6 hours after the onset of SE, while, as shown in Study 1, the peak of induction of the inflammatory mediators occurred in the brain 7 hours post-SE. We made the choice to inject them at 6 hours post-SE to be as close as possible to the peak of chemokine induction, hoping to promote cell

migration to the areas where these chemoattractive molecules are most produced; indeed, MSCs have chemokine receptors on their surface. We may have injected MSCs earlier, within a couple of hours after SE for example, with the idea to get a more significant effect on the expression of inflammatory mediators. Nevertheless, it is important to remember that the immunomodulatory and trophic properties of MSCs can be modulated by the environment they will encounter at the time of their transplantation. As for injecting MSCs prior to the induction of SE, when considering a translational approach to the clinic, it is difficult to imagine that MSCs can be used in healthy subjects as prophylactic treatment, which is why we ruled out this option.

The release of inflammatory mediators is not the only one to be considered when talking about neuroinflammation. Our research themes give great importance to the monitoring and determination of the role of monocytes infiltrating the epileptic brain, in particular their participation in the neuroinflammatory picture. Therefore, additional investigation aimed at better understanding the relationship between MSCs and infiltrating monocytes will be of interest in the laboratory. This may be possible with the creation of the transgenic rat mentioned in the previous section in which the infiltrating monocytes will be fluorescent and can be depleted at the desired time. The question of how the presence of MSCs acts on invading monocytes and how the presence or absence of invading monocytes will modulate the immunomodulatory effects of MSCs in brain tissue remains today a key question to answer.

2. MSCs: a new tool for cognitive remediation?

We showed that intranasal injection of MSCs had an effect on long-term potentiation (LTP), the cellular and molecular mechanism underlying the learning and memory processes. In animals receiving a single administration of stem cells 6h after SE, we observed that LTP was maintained at the level of controls, whereas it was significantly altered in SE animals. In animals receiving 4 injections of MSCs, at 6h, 1 day, 4 days and 7 days post-SE, LTP was not only protected; it outreached that of the control animals. These rather impressive functional results raise questions about the effects of MSCs on behavior. Further research will now investigate whether these effects can be transposed to a more integrated level by studying the effects of MSCs on the symptomatology of epilepsy, i.e. the severity and frequency of seizures, as well as associated comorbidities such as memory and anxiety disorders. We will

first investigate whether spatial learning abilities are improved in rats receiving stem cells by measuring their performance in Morris Water Maze, before evaluating the effect of stem cells on anxiety disorders with the Water Exploration Test (Fares et al., 2013) and the O-maze. Finally, we will measure on the same animals by video EEG if the administration of MSCs reduces the number and severity of seizures during the chronic phase. Regardless of the effects observed in these behavioral tests, it will be interesting to investigate in parallel at the cellular level whether MSCs can have an effect on the long-term survival of hippocampal neurons or on the formation and maturation of new neurons.

The results obtained on the cellular mechanisms underlying memory processes after intranasal delivery of MSCs following SE are promising. Nonetheless, they raise questions about the molecular and cellular bases of these actions. Over the past years, many studies have focused their attention not on the direct effect of stem cells as a tool for cell therapy, but on the secretome of these cells (Drago et al., 2013). The secretome is defined as the set of molecules and vesicles produced by MSCs and secreted in their environment. *In vitro*, an increasing number of studies have tested the effect of the conditioned medium in which mesenchymal stem cells have been cultured on diverse cell populations including immune cell populations such as monocytes, macrophages or brain cell populations such as microglia, astrocytes or neurons. In order to test whether the secretome of mesenchymal stem cells can have the same effect as the cells themselves, it would be interesting to test *ex vivo* and *in vivo* the effects of the MSCs secretome on LTP, firstly by incubating hippocampal slices in secretome bath directly, and secondly, by injecting the secretome *in vivo* through the intranasal route, as performed in this thesis.

Mesenchymal stem cells have been used as a therapeutic tool in a highly lesional model of SE induction in juvenile rats. In this model, neuronal degeneration and associated edematous tissue lesions are much more severe than in a model of SE induced in weaning rats. Another question raised by our findings is whether the injection of MSCs in a model where neuronal lesions are much less important, i.e. the model of SE induction at P21, may have similar, inferior, or better effects on cognition.

Another aspect to consider in light of our results on LTP concerns the time window in which the MSCs were injected. Our cell grafting protocol in which MSCs were injected during epileptogenesis phase aimed at counteracting the development of epilepsy or easing the

severity of the disease. At present, it is legitimate to wonder what effect the injection of stem cells might have during the chronic phase, when the brain is already epileptic and the cognitive and behavioral disorders are already installed. Only a few published studies have investigated whether stem cells (mesenchymal stem cells or bone marrow mononuclear cells) can have an effect when injected during the chronic phase of epilepsy. Although these studies have shown promising results such as a reduction in the number of seizures, improved learning and memory abilities, reduced neurodegeneration and improved electroencephalographic observations (Costa-Ferro et al., 2012; Huicong et al., 2013; Venturin et al., 2011), they now need to be replicated and confirmed.

3. Nose-to-brain delivery of stem cells: a realistic goal in human?

The innovative aspect of our study using stem cells in an attempt to modulate the pathophysiology of epilepsy lies in the route of administration we have adopted, the intranasal route. The question that remains unanswered today in our study concerns the fate of cells injected via this route, and, if they infiltrate the brain, their migration path. In order to answer these questions, future *in vivo* experiments will be carried out with MSCs that have previously been labelled with iron nanoparticles (USPIOs: ultrasmall superparamagnetic iron oxide nanoparticles). The MSCs will be injected according to the same protocol as in the study 4, i.e. 6 hours after SE, and we will search on histological sections for the localization of MSCs using Perls' Prussian blue staining. If labelled cells are found in region of interest, the next step will consist in seeking for the route of migration of USPIO-labelled MSCs by MRI. This will allow us to evaluate the time of presence of MSCs after intranasal injection as well as their preferred migration route.

The question raised by the use of the intranasal cell and drug delivery pathway in preclinical models is that of its possible translation to humans. The delivery of molecules through the intranasal route is already a technique gaining increasing interest (Dhuria et al., 2010; Djupesland et al., 2014; Erdő et al., 2018; Li et al., 2015; Lochhead and Thorne, 2012). Indeed, the practical aspect of this route of administration makes it attractive especially for treating chronic pathologies for which the heaviness of the treatments constitutes an additional burden altering the quality of life of the patients. However, while drug administration by this route is already used in clinical practice, intranasal cell administration is nascent, and many questions will need to be answered before routine use of this delivery

route can be made. Questions concerning the differences in nasal anatomy between rodents used in the laboratory and humans need to be further investigated. Rodents are forced to breathe through the nose and have a nasal structure that is optimized for olfaction, whereas the human nasal structure is more specialized for breathing and protection of the respiratory system (Djupesland et al., 2014; Erdő et al., 2018; Li et al., 2018). In addition, nasal turbinates, complex skeletal structures that support the nasal epithelium and increase the surface area of the nasal passages, are found in the nasal chamber of both rodents and humans. Nevertheless, nasal cones in rodents are more abundant, resulting in a relative larger epithelial surface area than in humans. This may result in an increase in the absorption of treatments in rodent studies compared to humans, so before adapting this type of treatment, prior dosing studies should be conducted in a species-specific manner. By contrast, the mucociliary clearance rate in humans is three to four times slower than in rats, which potentially allows more time for the absorption of therapeutics.

Concerning the transport and migration of cells or molecules administered intranasally, the routes that have been mentioned are those of the olfactory nerve pathway, the trigeminal nerve pathway, or the passage of cells/molecules through the blood vessels, the olfactory epithelium being richly vascularized (Djupesland et al., 2014; Erdő et al., 2018; Li et al., 2018). Of these different pathways, transport along the olfactory nerves can provide direct and targeted delivery to structures of the limbic system as the olfactory bulb comes into direct contact with limbic structures involved in the pathophysiology of numerous psychiatric and neurodegenerative disorders. All the differences between rodent and human nasal anatomy and the absorption of cells/drugs by the nasal epithelium have not yet been fully elucidated. Understanding and addressing these issues may be the key to unlocking the ultimate potential of intranasal administration for the treatments of brain disorders in humans since this delivery route offers the necessary flexibility and repeatability for treating chronic conditions.

4. Enhancing the therapeutic potential of MSCs

4.1. Combination therapy: MSCs and enriched environment

One of the research topics of interest to the laboratory is the study of the effect of environmental enrichment (EE) on SE-induced cognitive impairment (Fares et al., 2013). A

pilot study carried out by the team a few years ago on children suffering from epilepsy (Env'Epi project) showed that a multimodal enrichment of their environment by activities followed over a period of 6 months led to a considerable improvement of their quality of life associated with a decrease in their anxiety. The activities followed included art therapy, pottery, adapted physical activity and musical stimulation. In animals, the study of the effects of EE was performed using the Marlau™ cage, a cage designed to increase social interaction, voluntary exercise, entertaining activities and cognitive stimulation through the exploration of maze contained in the cage whose configuration is changed 3 times a week. The results of this study showed that when rats were subjected to SE at weaning before being housed in the Marlau™ cage, the emergence of cognitive impairment was prevented (Fares et al., 2013). Therefore, and in light of the promising results we obtained on LTP in our study 4, it has emerged the question whether a cumulative effect of EE and MSCs can be observed. The more accurate question is whether the combination of enriched environment can render MSCs more potent, and whether there are additive or synergistic effects. These questions are driven by other studies that have shown encouraging results when MSCs administration was combined with environmental enrichment (Cho et al., 2016; Kubota et al., 2018; Mu et al., 2019). Therefore, further studies will investigate in the model we used so far whether the housing of rats in Marlau™ cages combined with intranasal administration of MSCs following pilocarpine-induced SE can result in change in cellular and molecular processes as well as in a functional outcome that exceeds that of these therapeutic approaches applied on their own.

4.2. *In vitro* preconditioning

A growing number of research projects have been carried out in recent years to improve the immunomodulatory, repair and migration properties of MSCs. To this end, stem cells have been genetically modified in an effort to improve their capacity and/or to dissect which mechanisms were involved in the migration or therapeutic properties of MSCs. However, it is difficult to imagine that genetically modified stem cells will be routinely transplanted into humans. As an alternative to these genetic modifications of stem cells, preconditioning has been used.

The idea that MSCs can be licensed by pro-inflammatory stimuli to increase their immunosuppressive ability raises the possibility of regulating MSCs functions *in vivo* in

different pathologies and has been suggested to improve their clinical efficacy (Marigo and Dazzi, 2011). Licensing/preconditioning has also been shown to promote the migration and integration of MSCs into tissues of interest. Hence, a large number of *in vitro* studies have aimed to optimize cell culture conditions in order to enhance the cell expression of appropriate factors for their tissue homing as well as of immunomodulatory and trophic factors, or to promote cell survival (Crisostomo et al., 2008; Ferreira et al., 2018; Hu and Li, 2018; Luo et al., 2016; Najjar et al., 2018; Ponte et al., 2007; Redondo-Castro et al., 2017; Ullah et al., 2019).

Preliminary *in vitro* experiments have also led us to investigate the effect of hypoxia-like preconditioning or preconditioning with interleukin. Following our study comparing the effect of a serum-free medium and a serum-containing medium on the phenotype of MSCs, we tested the effect of preconditioning with deferoxamine (DFO), an iron chelating agent, used to mimic hypoxia. DFO was put in the culture medium of MSCs at different concentrations and various exposure times (10h, 24h, 48h or 72h) were tested. The cells were then harvested, and the expression of a specific set of genes was quantified by RT-qPCR, including genes for MSCs identification, genes involved in their homing, and genes of pro- or anti-inflammatory cytokines and chemokines. The preliminary results of this study are presented in figures 1A and 2A. We observed that a long exposure time to DFO, in addition to affecting the survival of MSCs, induces a modification of their own phenotype (**Appendix - Fig. 1A**). By contrast, the expression of inflammatory mediators such as TNF α , MCP1, IL10 or IL13 appears to be favored by longer incubation times under hypoxia-like conditions (**Appendix - Fig. 2A**). In a second study, we then sought to compare the effects of DFO exposure to treatment with interleukin-1 α and β and examined the effect of the DFO+ IL1 α / β combination. The results are presented in Figures 1B and 2B in the Appendix. Compared to controlled conditions, it appears that the combination of DFO and interleukins leads to a strong increase in the expression of the stromal-derived factor-1 (SDF-1), also called CXCL12, which is reportedly involved not only in the chemotaxis of lymphocytes and macrophages, but also in the regulation of neurotransmission, neurotoxicity and neuroglial interactions (Guyon, 2014; Li and Ransohoff, 2008) (**Appendix - Fig. 1B**). However, its CXCR4 receptor, which is believed to be involved in MSCs migration (Liu et al., 2011), was undetectable in both preconditioned and non-preconditioned MSCs. Further research will be needed to complete

these data and to investigate whether preconditioning of MSCs has a positive effect on their migration capacity towards inflammatory signals.

5. Translational application of MSCs: are we there yet?

The aim of our project was to test the effects of intranasal injection of stem cells just after an epileptogenic brain injury, viewed as a preventive anti-epileptogenic or disease-modifying treatment. We are aware that in a more translational perspective, the search for treatments that seek to cure epileptic patients prevails over the search for preventive treatments that could be taken during the epileptogenesis phase. Nevertheless, considering the numerous traumas or brain injuries that can lead to the development of epilepsy, it is equally crucial that attempts be directed at developing safe and reliable treatments to people at risk to develop epilepsy. As outlined in this section of the discussion, however, many challenges remain to be overcome before the use of stem cells as a first-line disease-modifying therapy becomes more widely known and accepted. While there are now a few clinical trials testing the effect of MSCs in epileptic patients by injecting the cells, often by intravenous injection combined with the intrathecal route (Hlebokazov et al., 2017; Milczarek et al., 2018; Slobina et al., 2019), to our knowledge, none has so far attempted to prevent epilepsy by injecting MSCs, and even less by the intranasal route. If such transposition is envisioned in humans, further work will be needed to determine the best time window(s), the dosage and frequency of MSCs administration, as well as evaluating their effects in combination with antiepileptic treatments.

Stem cells also represent a new tool for personalized medicine since injections can be made in an autologous manner, using patient's own cells and by adapting the MSC-based treatment according to the characteristics of the patient's pathophysiology. Mesenchymal stem cell-based drug delivery is also gaining a lot of credit, but still faces methodological challenges such as the concerns about the toxicity to nontarget peripheral tissues or the need to adopt standardized methodologies to favor comparisons across studies (Krueger et al., 2018).

It is now known that MSCs do not have the same properties depending on their tissue source, on the passage they are used and on growth culture conditions. In order to further expand their use, it will be important to clearly document these variations in MSCs

characteristics and to ensure consistency in culture protocols between different studies by respecting good cell culture practices, thereby reducing potential biases related to cell culture conditions. In addition, other culture conditions will have to be controlled. In our study, we have sought to avoid the use of fetal bovine serum which is recognized as a source of potential variability in cell culture (Gottipamula et al., 2013; Stein, 2007). However, the required steps prior to large-scale use in humans must also include the establishment of standard procedures and the development of chemically-defined cell culture media (McGillicuddy et al., 2018).

Other potential hindrance for the use of stem cells in humans today lie not only in the ethical issues of the source of the stem cells, but also in their rate of rejection after transplantation or their potential side effects and uncontrolled proliferation leading to the formation of teratomas (Musiał-Wysocka et al., 2019). Nevertheless, MSCs have substantial strengths that make them attractive candidates compared to other stem cell types and offset some of the above-mentioned limitations: 1. their availability in adults in bone marrow and adipose tissue makes it possible to bypass the ethical questions often raised about the use of embryonic or fetal stem cells; 2. their low immunogenicity leads to a low rate of rejection after transplantation. Nevertheless, MSCs have substantial strengths that make them attractive candidates compared to other stem cell types and offset the above-mentioned limitations; 3. they have already demonstrated their safety in clinical studies. Future studies and observations will be needed to investigate the long-term effects of MSCs therapies, including adverse effects. Although caution must be exercised today with regard to the use of these cells in humans and that the various limitations discussed here need to be resolved, the clinical use of MSCs remains attractive due to their potential therapeutic advantages, including their trophic and immunomodulatory characteristics.

To conclude, stem cell-based therapy aimed at preventing or treating epilepsy is still in its infancy, along with the use of stem cells to treat other neurological diseases, and there is still a long way ahead before they become a first-line treatment for chronic conditions. But although there are still many challenges to overcome in the use and understanding of mesenchymal stem cells as an effective therapeutic tool tailored to each disease and each patient, there is no doubt that the future years will see the development of the research environment needed to ensure that stem cells become the new, cutting-edge therapeutic technology of tomorrow.

“The time will come when diligent research over long periods will bring to light things which now lie hidden. A single lifetime, even though entirely devoted to the sky, would not be enough for the investigation of so vast subject... And so this knowledge will be unfolded only through long successive ages. There will come a time when our descendants will be amazed that we did not know things that are so plain to them... Many discoveries are reserved for ages still to come, when memory of us will have been effaced.”

~ Seneca

APPENDIX

APPENDIX



Appendix - Figure 1. Preconditioning of MSCs can impact their phenotype. (A) mRNA expression levels of positive MSC markers (CD73, CD90, CD105), chemokines receptors (CXCR1, CCR1) and chemokine SDF-1 in MSCs stimulated with the iron chelator deferoxamine (DFO) at 100 μ M (DFO 100), 200 μ M (DFO 200) or 400 μ M (DFO 400) for 10, 24, 38 or 72 hours and compared to respective unstimulated MSCs (CTRL). **(B)** Transcript levels of positive MSC markers (CD73, CD90, CD105), chemokines receptor CCR1 and chemokine SDF-1 in MSCs stimulated for 10 hours with either DFO at 200 μ M (DFO), with IL1 α / β at 1 ng/mL (IL1) or with a combination of DFO 200 μ M and IL1 α / β 1 ng/mL (DFO+IL1) and compared to unstimulated MSCs (CTRL). Data are expressed in number of cDNA copy.

APPENDIX



Appendix - Figure 2. Preconditioning of MSCs modify their expression of inflammatory mediators. Transcripts levels of inflammatory mediators measured in MSCs after preconditioning. **(A)** mRNA expression levels of pro-inflammatory cytokines (IL6, TNF α), chemokines (MCP1, MIP1 α) and anti-inflammatory cytokines (IL10, IL13) in MSCs stimulated with the iron chelator deferoxamine (DFO), a hypoxia mimetic agent, at 100 μ M (DFO 100), 200 μ M (DFO 200) or 400 μ M (DFO 400) for 10, 24, 38 or 72 hours and compared to respective unstimulated MSCs (CTRL). **(B)** Transcript levels of pro-inflammatory cytokines (IL1 β , IL6, TNF α), chemokines (MCP1, MIP1 α) and anti-inflammatory cytokines (IL10) in MSCs stimulated for 10 hours with either DFO at 200 μ M (DFO), with IL1 α / β at 1 ng/mL (IL1) or with a combination of DFO 200 μ M and IL1 α / β 1 ng/mL (DFO+IL1) and compared to unstimulated MSCs (CTRL). Data are expressed in number of cDNA copy.

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