Développement de microcarriers pharmacologiquement actifs transportant des cellules souches pour le traitement de maladies neurodégénératives

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« La recherche doit être gratuite, désintéressée et nécessaire. »

Georges Dumézil

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TABLE DES MATIERES

Abréviations :

6-OHDA	6-Hydroxydopamine
bFGF	Basic fibroblast growth factor
BDNF	Brain-derived neurotrophic factor
CSM	Cellules stromales mésenchymateuses
CSN	Cellules souches neurales
DARPP32	dopamine- and cAMP-regulated neuronal phosphoprotein
EGF	epidermal growth factor
GABA	Acide γ-aminobutyrique
GAD67	Glutamic acid decarboxylase 67
GAT1	GABA tranporter 1
GDNF	Glial cell line derived neurotrophic factor
GFP	Green fluorescent protein
IRM	imagerie à résonnance magnétique
LM	Laminine
MIAMI	Marrow-isolated adult multilineage inducible
MPA	Microcarriers pharmacologiquement actif
MPTP	1 - méthyle 4 - phényl 1,2,3,6-tétrahydro pyridine
NEM	Neurones épineux moyens
NT3	Neurotrophine-3
NGF	Nerve growth factor
PLGA	Acide poly(lactique-co-glycolique)
RPE	Retinal pigment epithelium
SNC	Système nerveux central
SNc	Subtancia nigra pars compacta
SNr	Subtancia nigra pars reticulata
TH	Tyrosine hydroxylase
VEGFA	vascular endothelial growth factor

INTRODUCTION GENERALE

Les maladies neurodégénératives, comprenant la maladie de Parkinson et la maladie de Huntington, forment un ensemble de pathologies affectant le système nerveux central (SNC). Elles induisent une dégénérescence irréversible des voies neuronales, conduisant à une importante diminution de la qualité de vie du patient et souvent à son décès.

La maladie de Parkinson est la deuxième maladie neurodégénérative la plus répandue dans le monde après la maladie d'Alzheimer. Plus de 2% de la population de plus de 65 ans en est atteinte et 7 à 10 millions de personnes à travers le monde en souffrent (Lees et al., 2009). Elle est caractérisée notamment par la mort des neurones dopaminergiques localisés dans la substance noire (SN) *pars compacta* (SNc) qui se projettent dans le striatum (noyau caudé et putamen chez l'homme) se traduisant par une baisse de la dopamine libérée (Lees et al., 2009). Cette diminution de la libération de dopamine dans le striatum induit l'apparition de symptômes moteurs caractéristiques : tremblements, rigidité musculaire, bradykinésie et une perte des reflexes de posture. D'autres zones du cerveau dégénèrent par la suite complexifiant les symptômes. L'étiologie de cette maladie est encore mal connue. Une agrégation notamment de l'α-synucléine au sein des neurones formant ainsi des corps de Lewy semble être à l'origine de cette mort neuronale (Volles and Lansbury, 2003, Gazewood et al., 2013). Plusieurs pistes sont étudiées pour comprendre l'origine de cette mort neuronale. De nombreux facteurs environnementaux tels que l'exposition aux pesticides ou aux métaux lourds semblent favoriser le développement de la pathologie et dans environ 5% des cas, des mutations génétiques sont impliquées.

La maladie de Huntington est une maladie génétique autosomale dominante avec une prévalence de 6 à 7 cas pour 100000 personnes à travers le monde. Elle

est causée par une mutation du gène codant pour la huntingtine localisé sur le chromosome 4. Le gène sain code pour une répétition du trinucléotide CAG codant pour la glutamine. Chez les patients souffrant de cette pathologie, cette répétition est amplifiée. Lorsque la protéine atteint un nombre de glutamine supérieur à 39, la pathologie se développe autour de 40 ans dans la plupart des cas. Il a été constaté qu'une répétition supérieure à 55 trinucléotides était associée à un développement et une évolution plus rapide de la maladie (Ho et al., 2001, Ha and Fung, 2012). Cette mutation induit, dans un premier temps, la mort des neurones épineux moyens (NEM) sécrétant du GABA (acide γ-aminobutyrique), localisés dans le striatum et se projetant vers le segment externe du globus pallidus ainsi que vers la SNc et SN reticulata (SNr). Par la suite la mort neuronale s'étend à des nombreuses autres régions du cerveau comme la substance blanche ou le cortex (Ha and Fung, 2012). Cette dégénérescence neuronale induit l'apparition de mouvements involontaires (choréiques et dystoniques) ainsi que des désordres psychiatriques tels que la dépression (Walker, 2007).

Malheureusement à ce jour il n'existe pas de traitement pour ces maladies pouvant stopper ou inverser la dégénérescence neuronale. Les seuls traitements existants visent à diminuer la sévérité des symptômes, permettant ainsi d'augmenter la qualité de vie de patients. En effet pour la maladie de Parkinson, le traitement le plus répandu est l'administration d'agents pharmaceutiques remplaçant la dopamine ou stimulant sa sécrétion (L-DOPA). Ils sont associés ou non à un inhibiteur de la décarboxylation de la dopamine (carbidopa ou benserazide) et à un inhibiteur de la recapture de la dopamine afin d'augmenter sa durée d'action (Figure 1A), qui peuvent induire l'apparition d'effet secondaire à long terme (Hauser, 2009, Ecker et al., 2009). Concernant la maladie de Huntington, les principaux traitements sont des

antichoréiques et des neuroleptiques qui permettent de réduire la sévérité des symptômes. A l'inverse de la maladie de Parkinson, une administration d'agents favorisant la sécrétion de GABA ou de son précurseur : le glutamate (Figure1B), ne suffit pas à stopper efficacement les symptômes. Toutefois leur administration peut provoquer l'apparition d'effets secondaires indésirables tels que des bradykinésies ou des épisodes de dépression (Adam and Jankovic, 2008). Il est donc nécessaire de développer et caractériser de nouvelles approches thérapeutiques pour ce type de maladie.

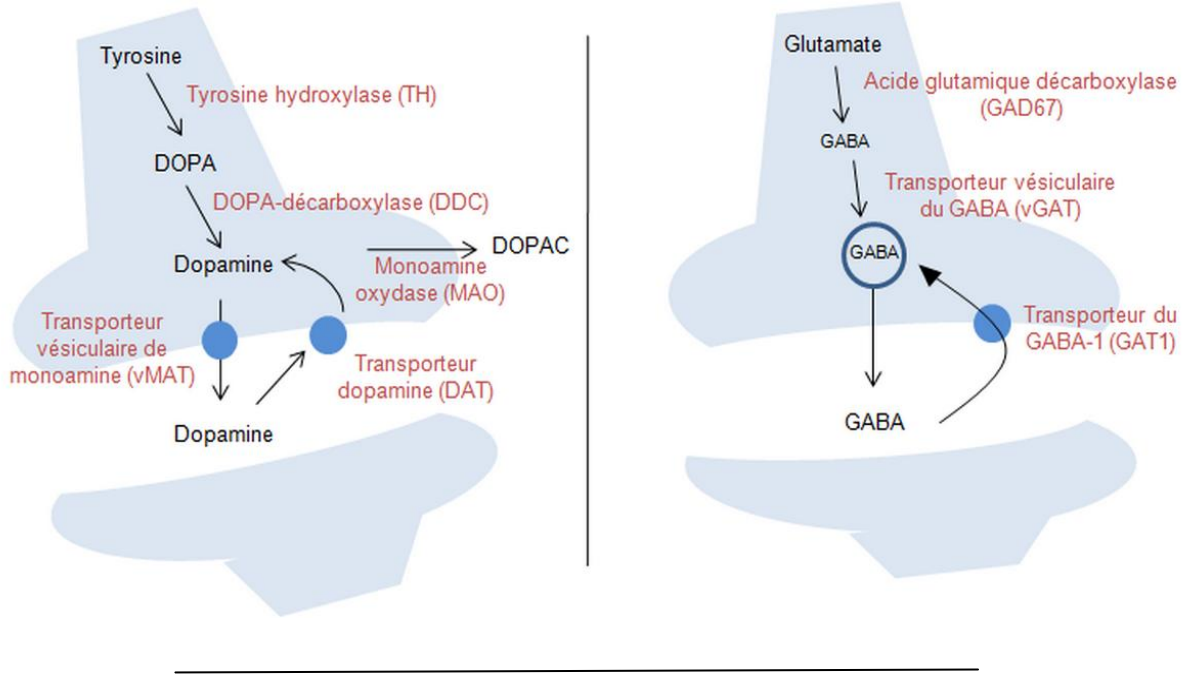

Figure 1 : *A) Schéma d'une synapse dopaminergique, la voie de synthèse et de la dégradation de la dopamine. La tyrosine hydroxylase (TH) permet de transformer la tyrosine en DOPA, le précurseurs de la dopamine. Il est également utilisé comme marqueur des cellules dopaminergiques. B) Schéma d'une synapse GABAergique et de la voie de synthèse du GABA. Le glutamate est transformé en GABA par l'action de l'acide glutamique décarboxylase (GAD67). Le GABA est ensuite recapturé dans la fente synaptique par le transporteur du GABA (GAT1). Ces deux protéines servent de marqueurs des cellules GABAergiques.*

La thérapie cellulaire consiste à greffer un tissu ou des cellules dans un organe afin de traiter une maladie ou de ralentir son évolution. Parmi tous les traitements basés sur la thérapie cellulaire, la greffe de moelle osseuse pour lutter contre les leucémies et les lymphomes (Karanes et al., 2008) et les greffes de peau dans les cas de brûlures (Kamel et al., 2013) sont les plus connus. Les organes composants le SNC, en particulier le cerveau, possèdent une faible capacité de réparation ou de régénération. De plus, aucun traitement curatif n'a à ce jour été développé contre les maladies neurodégénératives citées précédemment. La thérapie cellulaire apparait alors comme une alternative intéressante.

La thérapie cellulaire pour la maladie de Parkinson consiste à recréer localement la voie nigrostriée par l'implantation de tissus ou de cellules capables de produire de la dopamine ou son précurseur, la L-Dopa au sein du striatum, ou à protéger/réparer les neurones dopaminergiques du patient et ainsi à normaliser la quantité de dopamine dans le striatum (Figure 2). Pour la maladie de Huntington, une greffe de cellules capables de sécréter du GABA ne suffit pas, il est nécessaire de réparer les voies GABAergiques (Figure 3). Les premiers essais cliniques pour la maladie de Parkinson ont été réalisés avec des cellules chromaffines de la médullo-surrénale grâce à leur capacité à sécréter de la dopamine (Freed et al., 1990). Cette transplantation a permis d'obtenir des bénéfices fonctionnels, mais la survie des cellules greffées étant trop faible, la quantité de dopamine sécrétée était insuffisante (Ambriz-Tututi et al., 2012). Les cellules issues du glomus carotidien, capables de sécréter de la dopamine et du GDNF (glial cell derived neurotrophic factor), (Minguez-Castellanos et al., 2007, Lopez-Barneo et al., 2009) ainsi que les cellules de l'épithélium pigmenté de la rétine capable de sécréter de la L-DOPA ont été utilisées dans un essai clinique de la maladie de Parkinson. Toutefois des problèmes

éthiques et l'absence de bénéfices fonctionnels significatifs ont limité l'utilisation de ces cellules (Stover et al., 2005, Gross et al., 2011).

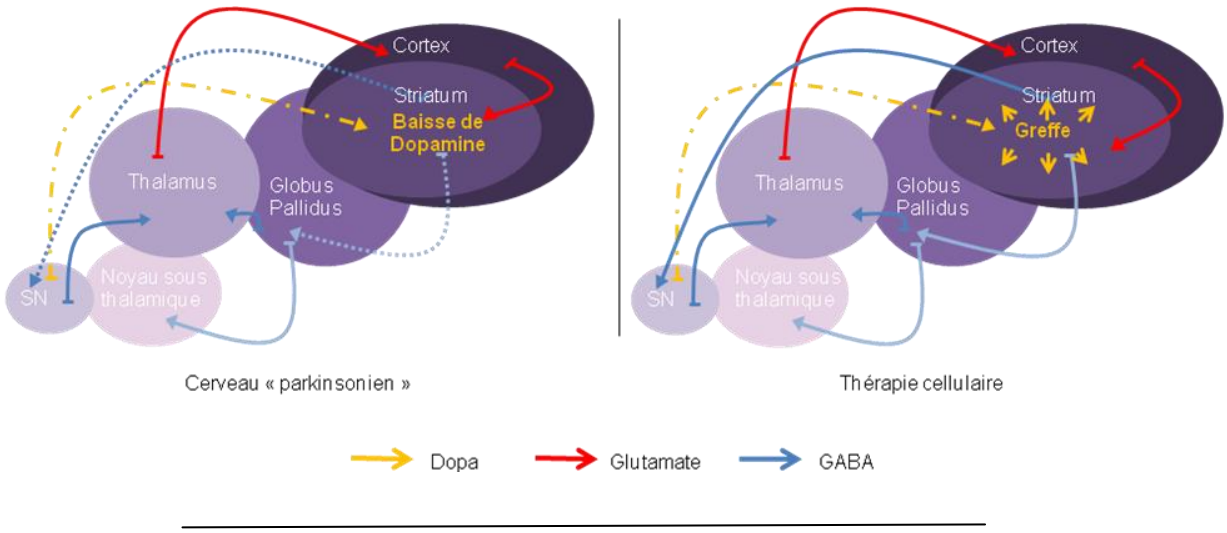

Figure 2 : *La maladie de Parkinson induit une dégénérescence de la voie dopaminergique nigrostriée (à droite). Une greffe de cellules capable de sécréter de la dopamine ou son précurseur, permet de restimuler les voies, GABAergiques ou cholinergiques, dépendantes de la dopamine (à gauche).*

L'implantation de tissus fœtaux, soit du mésencéphale ventral pour la maladie de Parkinson (Barker et al., 2013) soit d'éminences ganglionnaires pour la maladie de Huntington (Bachoud-Levi et al., 2000, Bachoud-Levi et al., 2006, Gallina et al., 2008, Gallina et al., 2010) a été étudiée à plusieurs occasions. Malgré une amélioration de l'état des patients, le développement de cette thérapie a été limité à cause de problèmes éthiques, notamment dus aux difficultés de survie des tissus 4 à 5 fœtus étant nécessaires pour traiter un unique patient. La thérapie cellulaire dans le SNC souffre principalement de deux limites qui sont la survie et l'intégration des cellules dans le tissu hôte et ce, avec la plupart des types cellulaires testés. En effet, seulement 10 à 20% des cellules initialement greffées survivent 24h après la greffe,

ce qui limite le potentiel de ces cellules à réduire les symptômes liés à la pathologie (Brundin et al., 2000, Lindvall, 2013, Lindvall and Bjorklund, 2011). Récemment de nouvelles études cliniques sont effectuées et vont permettre de réévaluer cette approche qui s'avère être une des plus intéressantes en terme d'efficacité, en particulier sur des stades précoces de la maladie (Barker et al., 2013).

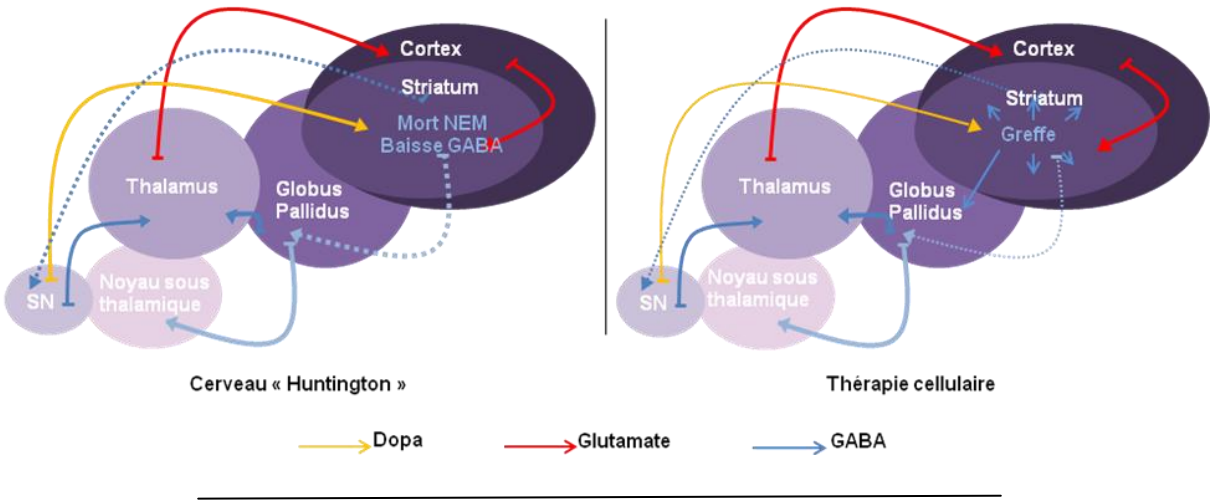

Figure 3 : *La maladie de Huntington induit une mort des neurones épineux moyens du striatum se projetant vers le globus pallidus ou la SNr (à droite). Une greffe de cellules capables de sécréter de l'acide gamma aminobutyrique (GABA) et de se reconnecter aux différentes voies neuronales permet de diminuer les symptômes de la pathologie (à gauche).*

Les cellules souches possèdent une capacité d'auto-renouvellement et de différenciation permettant d'obtenir un phénotype cellulaire désiré. Leur utilisation en thérapie cellulaire permet donc de palier au problème de disponibilité. Plusieurs études ont montré le potentiel thérapeutique des cellules souches neurales fœtales (CSN) dans les traitements de la maladie de Parkinson et de Huntington (Lescaudron et al., 2012). L'avantage de ces cellules est qu'elles permettent d'obtenir des neurones, des astrocytes ou des oligodendrocytes (Willerth, 2011). Mais leur

prélèvement soulève toujours des questions d'ordre éthique. Les cellules souches adultes ne présentent pas ce problème puisqu'elles peuvent être isolées chez l'adulte et dans certains cas permettent de les utiliser en autogreffe. Dans le cas des maladies neurodégénératives, deux types de cellules souches adultes se distinguent des autres. Chez l'adulte, les CSN sont issues de la zone sous-ventriculaire et de la zone sous granulaire (Bellenchi et al., 2013). Ces cellules peuvent se différencier naturellement en cellules nerveuses mais leur prélèvement reste délicat en raison de leur localisation dans le cerveau. Toutefois leur prélèvement, leur mise en culture puis réimplantation chez un patient parkinsonien a démontré des effets bénéfiques (Levesque et al., 2009). Les cellules stromales mésenchymateuses (CSM) de la moelle osseuse peuvent être prélevées aisément au niveau des os du bassin ou du sternum et permettent donc de pratiquer des autogreffes (Ding et al., 2011). Ces cellules se différencient naturellement en cellules de phénotype mésodermique : les ostéoblastes, chondrocytes, adipocytes, cellules stromales de la moelle et cellules musculaires entre autres. Il a également été démontré que ces cellules pouvaient se différencier vers un phénotype ectodermique et en particulier neural sous l'influence de différents facteurs (Tatard et al., 2004). Elles sont capables de sécréter d'importantes quantités de facteurs immunomodulateurs et de réparation tissulaire, ce qui les rend particulièrement attrayantes pour la thérapie cellulaire de maladies neurodégénératives. Le potentiel neuroprotecteur et neuroréparateur des CSM autologues a été démontré lors d'études cliniques sur l'ischémie cérébrale (Suarez-Monteagudo et al., 2009, Lee et al., 2010, Honmou et al., 2011) et la maladie de Parkinson (Venkataramana et al., 2010, Brazzini et al., 2010). A notre connaissance, il n'existe pas encore de résultats d'essais cliniques pour la maladie de Huntington mais des tests prometteurs sont en cours (Lescaudron et al., 2003).

La principale limite à l'utilisation des CSM est leur hétérogénéité (Li et al., 2008). Elles sont en effet composées de cellules souches et de précurseurs à différents stades de différentiation. De ce fait les résultats expérimentaux obtenus peuvent varier. Les cellules MIAMI (Marrow-isolated adult multilineage inducible cells) sont une sous-population homogène de CSM présentant un phénotype et un profil d'expression génique unique (D'Ippolito et al., 2006). Il a été démontré que ces cellules, cultivées sur de la fibronectine, sont capables de se différencier en cellules « neurones-like » suite à un traitement avec différents facteurs tels que la neurotrophine-3 (NT-3). Après traitements, les cellules présentent une croissance des neurites et expriment des facteurs neuronaux ainsi que certaines caractéristiques électrophysiologiques similaires à celles observées dans les neurones matures (Tatard et al., 2007a). Il a été démontré, par la suite, qu'un prétraitement de ces cellules avec de l'EGF (epithelial growth factor) et du bFGF (basic fibroblast growth factor) induit une diminution de l'expression des marqueurs de l'état souche tels que Oct4A, Notch1 et Hes5 et une augmentation des marqueurs de précurseurs neuraux tels que Nestin, Pax6 et de différentiation neuronale tel que Ngn2. De plus une augmentation de l'expression des récepteurs aux NT-3 est observée. Ce traitement permet donc d'augmenter la différenciation neuronale mais aussi d'augmenter la sensibilité des cellules au pouvoir différenciant du NT-3 (Delcroix et al., 2010a). Parallèlement des études ont démontré la capacité de la laminine, une protéine de la matrice extra-cellulaire, à induire une différenciation des CSM vers un phénotype neuronal (Mruthyunjaya et al., 2010).

L'ingénierie tissulaire est une approche novatrice qui consiste à associer les cellules avec des supports, des matériaux ou biomatériaux présentant différentes propriétés physicochimiques afin d'améliorer leurs effets biologiques et optimiser

ainsi la thérapie cellulaire. L'utilisation de ces supports peut agir sur le comportement des cellules et modifier ainsi leur morphologie, survie, prolifération et dans le cas des cellules souches, leur différenciation. L'association de supports avec des cellules souches adultes pour la thérapie de maladies neurodégénératives implique toutefois quelques limites. La taille de ces supports doit rester faible afin de faciliter l'implantation dans le cerveau, ils doivent être totalement biodégradables et non toxiques pour le tissu hôte et ils ne doivent pas activer de réponses immunitaires (Menei et al., 2005). Différentes sortes de support sont utilisés : les supports particulaires, qui ont été principalement utilisées pour augmenter la survie de cellules greffées. On recense des particules de collagène (Cherksey et al., 1996), de gélatine (Flores et al., 2007) ou encore polymériques (Vilos and Velasquez, 2012), tous principalement utilisés dans des études de la maladie de Parkinson. Il a notamment été démontré que l'utilisation de microparticules polymériques non biodégradable a permis d'augmenter la survie de cellules chromaffines (Borlongan et al., 1998) ou de précurseurs dopaminergiques du mésencéphale ventral (Saporta et al., 1997) lors d'une greffe dans un modèle de rats parkinsoniens ainsi qu'une amélioration de la motricité des animaux. L'association de microcarriers avec des cellules de l'épithélium pigmentaire de la rétine a notamment donné lieu à une étude clinique de la maladie de Parkinson (Stover et al., 2005). Toutefois, un essai clinique effectué en double aveugle n'a pas permis de démontrer les bénéfices de cette approche (Gross et al., 2011). Des supports sous forme de gels, qui peuvent être polymériques, lipidiques ou aqueux ; ont également été développés. L'utilisation de gel de gélatine-siloxane associée à des CSM a montré des avantages intéressants dans le cas d'ischémie cérébrale (Lu et al., 2007). De manière générale, ces supports permettent

de différentes façons d'améliorer le potentiel thérapeutique de CSN et de CSM (Williams and Lavik, 2009, Delcroix et al., 2010b).

Les microcarriers pharmacologiquement actifs (MPA) sont des microparticules développées dans notre laboratoire. Elles sont composées d'un copolymère, l'acide polylactique-co-glucolique (PLGA), sont biodégradables et biocompatibles avec le SNC (Tatard et al., 2005). Elles offrent aux cellules adhérées à leur surface un support en 3 dimensions et elles peuvent être enrobées avec des protéines de la matrice extra-cellulaire, telle que la laminine, afin de faciliter l'adhérence des cellules mais aussi d'en augmenter leur survie et leur différentiation. Enfin, il est possible d'encapsuler au sein de ces microparticules, un facteur de croissance qui sera libéré de façon contrôlée et prolongée durant la dégradation des microparticules, c'est-à-dire environ 2 mois (Figure 4) (Tatard et al., 2004, Tatard et al., 2007b, Tatard et al., 2005, Bouffi et al., 2010, Delcroix et al., 2011). Il pourra agir en association avec les protéines de la matrice extra-cellulaire afin d'optimiser la survie, la différentiation et l'intégration des cellules dans le tissu hôte.

L'effet bénéfique de l'utilisation de MPA associé à la greffe cellulaire a été démontré sur des cellules issues de phéochromocytomes de la glande médullo-surrénale du rat (PC12) (Tatard et al., 2004). Notamment, les effets de MPA libérant du NGF (nerve growth factor) sur des cellules PC12, ont été évalués dans un modèle de rats parkinsonien. Une augmentation de la différenciation des cellules greffées, associée à une baisse de prolifération et mort cellulaire, a été détectée lorsque les cellules étaient adhérées aux MPA. De plus des tests comportementaux ont permis de détecter une amélioration du comportement moteur ainsi qu'une diminution importante des stigmates liés à la pathologie (Tatard et al., 2004).

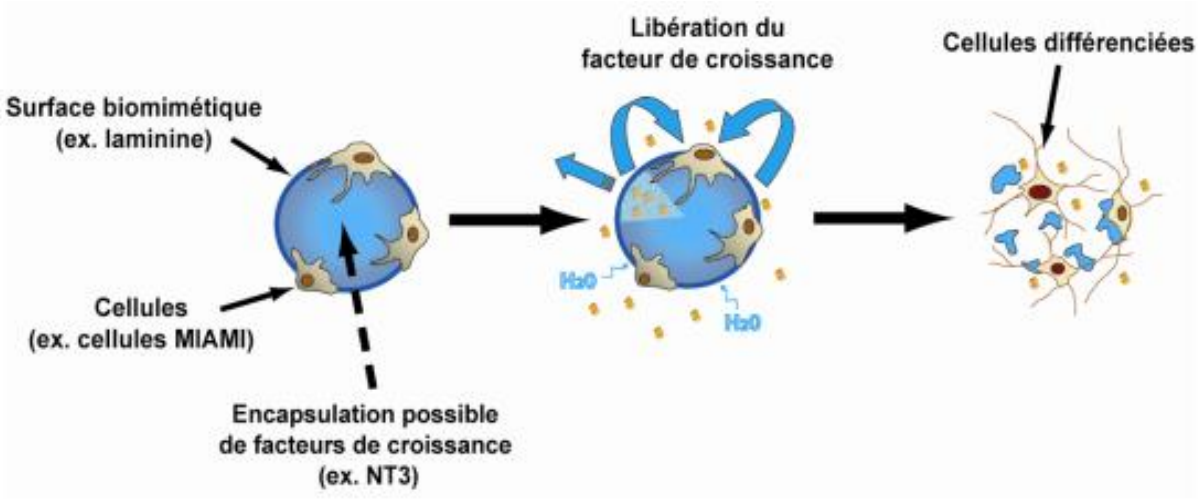

Figure 4 : *Des microsphères de PLGA, sont enrobées d'une surface biomimétique, constituée d'une protéine de la matrice extracellulaire afin de permettre l'adhésion de cellules et d'en modifier le comportement tel que la survie ou la prolifération. Un facteur de croissance peut être encapsulé au sein du PLGA. Il sera libéré de façon contrôlée et prolongée lors de la dégradation des microsphères pour favoriser la survie et la différenciation des cellules transportées. Enfin, après dégradation totale des microsphères, les cellules peuvent intégrer facilement le tissu hôte.*

Par la suite, cette stratégie d'ingénierie tissulaire a été testée sur des précurseurs dopaminergiques du mésencéphale ventral associés à des MPA libérant du GDNF. Après injection dans des rats parkinsoniens, il a été démontré une amélioration motrice des rats plus rapide et plus stable, ainsi qu'une augmentation de la survie et de l'intégration des cellules dans le tissu hôte et ce avec un nombre limité de cellules greffées (Tatard et al., 2007b). Les MPA libérant du GDNF ont même été utilisés seules, en tant qu'outil de libération contrôlée et prolongée du GDNF. Il a été démontré que le GDNF possède un pouvoir neuroprotecteur et peut induire une régénération des neurones dopaminergiques survivants, induisant ainsi une diminution des symptômes chez le rat (Jollivet et al., 2004). Cette approche d'ingénierie tissulaire pour la maladie de Parkinson a également été testée avec les

cellules MIAMI associées à des MPA recouverts de laminine et libérant du NT-3. La combinaison de ces différents facteurs a permis d'augmenter la survie cellulaire ainsi que la différentiation en cellules « neurones-like » capables d'exprimer la tyrosine hydroxylase (TH), l'enzyme responsable de la synthèse de dopamine (Garbayo et al., 2011b). De plus il a été démontré dans cette étude que le traitement des cellules MIAMI avec de l'EGF et du bFGF permet d'induire une neuroprotection encore plus importante (Delcroix et al., 2011).

Le potentiel neuroréparateur ou neuroprotecteur des cellules MIAMI semble provenir en grande partie de leur capacité à sécréter de grande quantité de facteurs neurotrophiques associés à leur pouvoir de différenciation en cellules neurones-like (Roche et al., 2013). Toutefois, l'utilisation de modèles *in vivo*, basée sur l'injection de neurotoxines dans des rongeurs est coûteuse, très longue à mettre en place et ne permet pas d'élucider facilement les mécanismes liés à cette réparation/protection neuronale. En effet, la transplantation de cellules peut avoir des effets difficiles à observer dans des modèles *in vivo* tels que la réponse du tissu hôte, les interactions des cellules du tissu hôte avec les cellules greffées ou encore la sécrétion de facteurs de croissance (Lindvall et al., 2004). Pour cela des modèles plus simples, plus rapides et moins onéreux doivent être mis en place afin de mieux comprendre et appréhender ces mécanismes d'action.

La culture organotypique permet de conserver une coupe d'organe en culture durant plusieurs semaines et confère un modèle simple et rapide pour étudier les mécanismes cellulaires et moléculaires du tissu cultivé (Gahwiler et al., 1997). La culture organotypique fut développée à la fin des années 50 sur des organes d'embryons de poulet tels que le cœur, les poumons ou les intestins (Loffredo Sampaolo, 1956, Loffredo Sampaolo and Sampaolo, 1956, Meerovitch, 1961). Puis

dans les années 60 la culture de coupes organotypiques de cerveau a été mise au point (Bousquet and Meunier, 1962). Afin d'optimiser la survie de ces coupes organotypiques, différentes méthodes de culture ont été développées. Le premier protocole notable est la technique, dite de «roller-tube» développée par Gahwiler en 1981 (Gahwiler, 1981). Dans ce protocole, des coupes sont placées de façon individuelle au fond d'un tube qui contient une petite quantité de milieu de culture. Une rotation lente de ces tubes permet d'obtenir une alternance entre la nutrition et l'aération de la coupe, ce qui permet d'obtenir une survie à long terme. Plus récemment un second protocole développé par Stoppini en 1991 propose une culture sur une membrane avec interface air/milieu (Stoppini et al., 1991). Le tissu est placé sur une membrane perméable sous laquelle est déposée une faible quantité de milieu de culture. Ainsi la coupe aspire les nutriments dont elle a besoin à travers la membrane d'un coté tandis que de l'oxygène peut être collecté de l'autre coté. Grâce à cette technique, les coupes organotypiques peuvent être utilisées pour étudier l'effet de l'injection d'un agent thérapeutique puisqu'elles ne sont jamais recouvertes de milieu. De plus, l'utilisation de coupes organotypiques a été rendue plus facile d'accès. Des coupes de nombreux organes ont pu être développées, tels la rétine (Lye et al., 2007), les intestins (Metzger et al., 2007) ou les tumeurs (Jung et al., 2002, Froeling et al., 2010) qui peuvent être issus de d'organismes variés, comme le lapin (Savas et al., 2001, Lye et al., 2007), le cochon (Dall and Zimmer, 2006) ou l'homme (Jung et al., 2002).

Depuis, des modèles de pathologies neurodégénératives ont été développés à partir de coupes organotypiques notamment pour la maladie d'Alzheimer (Shahani et al., 2006), de Parkinson (Matute, 2011, Stahl et al., 2009), de Huntington (Storgaard et al., 2000, Murphy and Messer, 2004, Reinhart et al., 2011) ou encore d'ischémie

cérébrale (Bahr, 1995, Finley et al., 2004, Jung et al., 2012, Xu et al., 2003). Ces coupes permettent à la fois d'étudier les propriétés anatomiques et physiologiques des neurones, les connexions et les voies neuronales. De plus, il est possible d'injecter un agent thérapeutique ou de greffer des cellules au sein de ces coupes et d'étudier ainsi plus facilement les interactions entre les cellules greffées et les cellules du tissu, la survie, la différentiation des cellules greffées ou encore les facteurs qu'elles sont capables de sécréter (Gahwiler et al., 1997, Sundstrom et al., 2005, Herlenius et al., 2012, Jaderstad et al., 2010).

Objectifs du travail de thèse :

De nombreux progrès ont été réalisés dans le domaine de la thérapie cellulaire, et en particulier en ingénierie tissulaire durant cette dernière décade. Toutefois de nombreuses questions restent en suspens, entre autres : le type de cellules le plus approprié, la quantité de cellules à greffer, les mécanismes liés à la mort cellulaire, la différenciation ou encore l'intégration des cellules greffées dans le tissu hôte. Il a été démontré à plusieurs reprises que les CSM, ainsi que les cellules MIAMI, possèdent, en plus de leur pouvoir de différenciation en cellules « neurones-like », un pouvoir neuroprotecteur/neuroréparateur de part leur capacité à sécréter des facteurs de croissance. Ce pouvoir a notamment été observé dans un modèle *in vivo* de la maladie de Parkinson. Toutefois, les mécanismes précis liés à la récupération fonctionnelle observée dans les modèles *in vivo* n'ont pas été clairement établis.

L'utilisation des coupes organotypiques dans le but de mimer des maladies neurodégénératives et d'en connaitre, de façon plus approfondie, les éventulles cibles thérapeutiques et donc d'optimiser la thérapie cellulaire. Par conséquent une revue bibliographique ayant pour but de montrer les avantages et inconvénients de l'utilisation des coupes organotypiques en tant qu'outils pour optimiser la thérapie cellulaire du cerveau, fait l'objet du **chapitre 1** de cette thèse.

L'utilisation des coupes organotypiques en tant qu'outils pour optimiser la thérapie cellulaire du cerveau (revue bibliographique).

Afin de comprendre les mécanismes cellulaires et moléculaires liés à la récupération fonctionnelle observée chez un modèle *in vivo* de rats hémi-

parkinsoniens (Delcroix et al., 2011), nous avons décidé de développer un nouveau modèle *ex vivo* de la maladie de Parkinson. Le but était alors de développer un modèle novateur, dans lequel une dégénérescence de la voie nigrostriée est obtenue sans ajout de neurotoxines, ni lésion manuelle. Le développement et la caractérisation complète de ce modèle font l'objet du **chapitre 2 :**

Modéliser la dégénérescence de la voie nigrostriée dans une culture organotypique, un nouveau modèle de la maladie de Parkinson.

Dans ce nouveau modèle, le nombre de gestes à effectuer est limité, diminuant ainsi le risque de biais dans le protocole, ce qui permet d'obtenir un modèle reproductible, rapide, peu coûteux et n'impliquant pas de techniques compliquées. Grâce à ce modèle, nous avons décidé d'effectuer une analyse comparative des effets des greffes de deux types de cellules souches, les cellules MIAMI et les CSN. De plus ces cellules seront greffées seules ou complexées à des MPA enrobés de laminine et délivrant du NT-3. Cette approche d'ingénierie tissulaire ayant déjà montré des effets bénéfiques, nous désirons comprendre les mécanismes impliqués dans la récupération fonctionnelle observée chez le rat, ainsi que les mécanismes permettant aux MPA d'améliorer le comportement des cellules greffées dans le tissu hôte. Ainsi la question directrice de ce travail de thèse développée dans le **chapitre 3** était :

Comment les MPA enrobés de laminine et délivrant du NT-3 associés aux cellules MIAMI et aux CSN permettent d'obtenir une réparation/protection de la voie nigrostriée dans un modèle *ex vivo* de la maladie de Parkinson ?

Forts de ces résultats nous avons transposé cette approche d'ingénierie tissulaire à une autre pathologie, la maladie de Huntington. Dans un premier temps nous avons donc cherché à développer un modèle *ex vivo* de cette pathologie, sans utilisation de neurotoxines pour obtenir la mort des neurones épineux moyens GABAergiques du striatum. Puis nous avons évalué la capacité de différenciation des cellules MIAMI et des CSN en NEM GABAergiques ainsi que leur potentiel neuroprotecteur sur les neurones du striatum hôte lorsque ces cellules sont greffées seules ou adhérées à des LM-MPA. Le développement et la caractérisation de ce nouveau modèle et l'évaluation de l'intérêt des cellules pour la maladie de Huntington sont abordés dans le **chapitre 4 :**

Développement d'un modèle *ex vivo* de la maladie de Huntington pour étudier le potentiel thérapeutique des cellules souches neurales et mésenchymateuses.

Dans ce nouveau modèle, nous nous sommes focalisés sur les aspects cellulaires liés à la pathologie, c'est-à-dire la mort des NEM GABAergiques du striatum. Une seule étape est nécessaire pour obtenir ce modèle, et donc un seul paramètre à contrôler, ce qui permet d'obtenir un modèle reproductible, rapide, peu coûteux et n'impliquant pas de techniques compliquées. Afin de développer une approche thérapeutique, différents tests ont d'abord été effectués *in vitro* afin de vérifier la capacité des MIAMI et des CSN à se différencier en neurones épineux moyens GABAergiques. Par la suite, nous avons effectué des greffes de ces cellules

complexées à des LM-MPA dans le modèle *ex vivo* de la pathologie et étudié le comportement des cellules dans le tissu hôte.

CHAPITRE 1 - Revue bibliographique

L’utilisation des coupes organotypiques en tant qu’outils pour optimiser la thérapie cellulaire du cerveau

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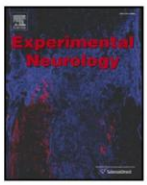

Review

Organotypic cultures as tools for optimizing central nervous system cell therapies

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ABSTRACT

Stem cell therapy is a promising treatment for neurological disorders such as cerebral ischemia, Parkinson's disease and Huntington's disease. In recent years, many clinical trials with various cell types have been performed often showing mixed results. Major problems with cell therapies are the limited cell availability and engraftment and the reduced integration of grafted cells into the host tissue. Stem cell-based therapies can provide a limitless source of cells but survival and differentiation remain a drawback. An improved understanding of the behaviour of stem cells and their interaction with the host tissue, upon implantation, is needed to maximize the therapeutic potential of stem cells in neurological disorders. Organotypic cultures made from brain slices from specific brain regions that can be kept in culture for several weeks after injecting molecules or cells represent a remarkable tool to address these issues. This model allows the researcher to monitor/assess the behaviour and responses of both the endogenous as well as the implanted cells and their interaction with the microenvironment leading to cell engraftment. Moreover, organotypic cultures could be useful to partially model the pathological state of a disease in the brain and to study graft–host interactions prior to testing such grafts for pre-clinical applications. Finally, they can be used to test the therapeutic potential of stem cells when combined with scaffolds, or other therapeutic enhancers, among other aspects, needed to develop novel successful therapeutic strategies or improve on existing ones.

Contents

Abbreviations: CI, cerebral ischemia; PD, Parkinson's disease; HD, Huntington's disease; SN, substancia nigra; DA, dopamine; GABA, gamma-aminobutyric acid; MSN, medium spiny neuron; CNS, central nervous system; NSC, neural stem cells; ES, embryonic stem; MSC, mesenchymal stromal cells; iPS cells, induced-pluripotent stem; TH, tyrosine hydroxylase; CA1, Cornu Ammonis layer 1; OGD, oxygen-glucose-deprivation; MPP+, 1-methyl-4-phenylpyridium; 3-NPA, 3-nitropropionic acid; NMDA, N-methyl-D-aspartic acid; NT-3, neurotrophin-3; NGF, nerve growth factor; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; DTG, 1, 3, di-o-tolylguanidine; HUCBC, human umbilical cord blood stem cells; 6-OHDA, 6-hydroxydopamine; QA, quinolinic acid; KA, kainic acid; polyQ, polyglutamine; PAMs, pharmacologically active microcarriers.

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Introduction

Neurological disorders such as cerebral ischemia (CI), Parkinson's disease (PD), or Huntington's disease (HD) have in common the loss of neurons in the brain. CI is a condition in which blood flow is curtailed to the brain resulting in neuronal death by oxygen and nutrient deprivation (Barrett and Meschia, 2010). The neurological signs and symptoms of PD are, in large part, a result of selective loss of neurons in the nigro-striatal dopaminergic pathway (for review Lees et al., 2009). In HD gamma-aminobutyric acid (GABA) ergic medium spiny neuron (MSN) death occurs at onset of disease manifestation (for review Walker, 2007). Unfortunately, PD and HD therapies only provide amelioration of symptoms but do not delay or halt neurodegeneration. In CI if the acute phase is not treated on time, the post-injury neuronal death is the cause of many disabilities. As of today, only thrombolytic therapy has shown any efficacy against CI. It is therefore essential to evaluate alternative therapeutic strategies such as cell therapy.

Cell therapy for the central nervous system (CNS) consists in cell injection into a lesioned brain tissue to restore a loss of function (Dunnett and Rosser, 2011). However, apart from poor cell engraftment issues that still need to be addressed cell availability and ethical concerns have limited the development and current clinical application of this therapy (Delcroix et al., 2010b; Liu and Huang, 2007). Stem cells that can differentiate into mature neural/neuronal cells can be used as alternative source of cells, as their self-renewal capacity allows the establishment of a cell bank avoiding availability and ethical difficulties. Neurons and glial cells can be generated from neural stem cells (NSC), embryonic stem (ES) cells, bone marrow-derived multipotent stromal cells also called mesenchymal stromal cells (MSC), and also lately, from induced-pluripotent stem (iPS) cells. Each type of stem cell has advantages and caveats that must be taken into consideration together with the type of application envisaged.

Brain organotypic slices, which can be maintained in culture for several weeks, confer a rapid and simple method to evaluate cellular interactions and mechanisms. Moreover, brain slices can be used to develop *ex vivo* models of neurological disorders and, in this way, they represent a link between *in vitro* studies and animal models. Cells can be grafted in organotypic slices allowing the researcher to understand how implanted cells interact with resident cellular matrix and injured residential cells and to predict how stem cells may behave *in vivo*. Thus, they represent a powerful tool to study cell therapy and neuroprotection.

In this review, after a brief overview of the clinical trials performed using cell therapy to treat cerebral disorders and their current limitations, we discuss how organotypic slices could address some of the key unanswered questions regarding cell therapy. We report the different *ex vivo* models of CI, PD and HD and review the studies carried out using these *ex vivo* models of neurological disorders to evaluate stem cell therapies. Finally, tissue engineering strategies for PD and other neurological disorders tested in organotypic slices are discussed.

Cell therapy clinical trials for neurological disorders

Here we will focus on discussing the clinical evaluation of cell-based therapies in which cells are implanted *via* stereotactic surgery for CI, PD and HD.

The first clinical trials in PD consisted in the striatal stereotactic implantation of adult cells, which may synthesize dopamine (DA) or its precursor, and are thus able to replace the lost DA in the striatum. Over the years, adult cells such as chromaffin cells (for review see Freed et al., 1990), human retinal pigment epithelium cells (for review see Gross et al., 2011; Stover et al., 2005) or carotid body cells (for review see Lopez-Barneo et al., 2009; Minguez-Castellanos et al., 2007) have been evaluated for PD therapy as they lack ethical issues and some of these cell types allow autografts to be performed. Those cell transplantation studies led to a certain improvement of motor functions but cell survival remained too weak limiting their efficacy. Most clinical trials of cell therapy for either PD or for HD consisted of striatal stereotactic implantation of minced foetal tissue or cell suspensions from the ventral mesencephalon or the ganglionic eminence, respectively. They were performed in order to restore lost DA within the striatum for PD patients and lost MSN for HD patients. These trials showed promising results (for review see Barker et al., 2013). However, in PD patients (Mendez et al., 2005), graft induced dyskinesias may occur and Lewy bodies were found in some long survival dopaminergic neurons (for review see Lindvall, 2013; Tomaskovic-Crook and Crook, 2011). But overall, the main limitations concern the poor availability of foetal tissue, the ethical issues associated with their use and the limited survival of the transplanted cells.

Stem cells, which can be isolated from many sources, represent a potential candidate for cell therapy as they self-renew and present a large differentiation potential (for review see Benraiss and Goldman, 2011; Lindvall, 2013). The feasibility of using adult NSCs for PD cell therapy has been demonstrated but their poor availability and an improvement that returns back to baseline after 5 years post-operation suggests that efficacy is limited (Lévesque et al., 2009). Stereotactic implantation of autologous MSCs, which can differentiate into neuronal-like cells and secrete tissue repair and immunoregulatory factors (Tatard et al., 2004), has been evaluated in PD patients (Venkataramana et al., 2010) and in chronic stroke patients for their ability to repair the damaged neuronal tissue (Suarez-Monteagudo et al., 2009). It was concluded that MSCs could safely be grafted into the striatum of patients with good tolerance and no complications. Furthermore, in both cases, a decrease of symptoms associated with the disease was observed.

New clinical trials using adult stem cells are currently on going for the treatment of chronic stroke (NCT01714167 on Clinicaltrials.gov) or for PD (NCT01446614, NCT01453803 on Clinicaltrials.gov). To our knowledge, there are no clinical trials with stem cells for HD.

Cell therapy unanswered questions

As described above, cells from a variety of sources have shown various degrees of efficacy in clinical trials. However, cell therapy also presents some drawbacks that limit its use, such as poor cell survival and *in situ* differentiation, certain undesirable side effects and limited availability of foetal tissue. We now discuss the major issues and some future directions of the research associated with cell therapy for neurological disorders.

First of all, findings in a number of experimental models showed that neuronal precursor cell survival within the host tissue after transplantation was too weak (10–20%) and that cell death occurred within the first 3 weeks (Delcroix et al., 2010b; Liu and Huang, 2007; Olanow et al., 2003). *In situ* differentiation of stem cells was insufficient (at best

3–20%) (Delcroix et al., 2010b; Garbayo et al., 2011a). Moreover, cell survival does not depend only on the type of graft or immunosuppressive treatment but also on the site and method of injection of the cells (Modo et al., 2002). For example, dopaminergic cell suspension grafts implanted into the lesioned striatum presented 20% higher survival rate of tyrosine hydroxylase (TH)-positive neurons (dopaminergic neurons) in comparison to implantation in the intact striatum. Moreover, when a glass capillary was used for implantation, the survival of the single cell suspension was improved by four-fold against the use of a metal cannula (Nikkhah et al., 2009).

Furthermore, many unanswered questions still remain concerning cell therapy for neurological disorders that should be correctly addressed. For instance, which type of cell is more appropriate for a given neurological disorder knowing that they present different properties that fulfil different needs? Therefore, the dose of cells required for successful grafting should be investigated. With this aim in view, the mechanisms of action by which the stem cells survive, differentiate and engraft, should be particularly explored. The adequate cell number and processing must be well established for each disease (Delcroix et al., 2010b). A better selection of patients and surgical procedure improvement should result in a better outcome for this therapy (Bachoud-Levi et al., 2006; Kondziolka et al., 2000; Lévesque, 2009). The safety of the therapy and the way to manage the potential uncontrolled cell proliferation or tumourigenicity inherent to pluripotent stem cells (ES and iPS cells) must be evaluated. Do stem cells differentiate to undesired cell phenotypes? Do stem cells migrate to non-target sites? Are there functional consequences of these potential scenarios? All of these questions need to be addressed in the appropriate pathological context knowing that cell behaviour will differ depending on the immediate microenvironment. Therefore, cell therapy requires further investigations to increase therapeutic options in the future.

The development and optimization of better experimental models, such as organotypic slices, that allow for better control of the extracellular environment, that allow studying grafted precursors or stem cell responses, cell to cell interactions and the role of the microenvironment on the grafted cell behaviour will be essential to address these issues and further develop cell therapy.

Organotypic slice models as tools for efficient screening of cell behaviour

In vivo models are indispensable to prove safety, functional outcome and efficacy of a therapy, but in general they require high technical and financial resources, are laborious and time-consuming. Cell transplantation can have parallel effects that are too difficult to observe *in vivo* as host plastic responses, interference with host neuronal activity and growth factor secretion (Lindvall et al., 2004) and it is impossible to simultaneously test several conditions in the same animal. *In vitro* models are very quick and simple to develop. Using well-known chemical compounds they can mimic neuronal cell death and neuroprotective therapies can be tested. But those studies can only be performed on a simple cell culture or co-culture which cannot display the complexity of a tissue or an organ and its microenvironment (Zurich and Monnet-Tschudi, 2009).

Since their introduction, organotypic cultures of brain slices have become a useful tool to study physiological and pharmacological properties of tissues. More recently, approaches to lesion the area implied in a pathology were developed to mimic neurodegenerative disorders and in this way, to obtain *ex vivo* models. Brain slice models offer advantages over other *in vitro* models because they reproduce the *in vivo* environment while maintaining key *in vitro* features. Indeed, these brain slices are relatively inexpensive, easy to prepare and study. There is no need for lengthy animal surgery, or laborious monitoring of multiple physiological parameters, while minimizing ethical issues with the use of animals for experimentation. Furthermore, they maintain the cytoarchitecture and the microenvironment of the original tissue. This 3D environment has tremendous value for evaluating the efficacy of cell therapy, by providing external mechanical inputs, interactions between structures and cell adhesion parameters, which all profoundly affect intracellular signalling. In addition, they enable an easier way to study local cell implantation sites, grafted cell migration and also their interaction with the host environment, mainly glial cells and neurons (Abouelfetouh et al., 2004; Charriere et al., 2010; Jaderstad et al., 2010a, 2010b, 2011; Meng et al., 2011; Sarnowska et al., 2009a; Tanvig et al., 2009; Tonnesen et al., 2011). They are advantageous as they allow patch-clamp studies of neuronal electrophysiology (Finley et al., 2004; Jaderstad et al., 2010b; Kearns et al., 2006; Tonnesen et al., 2011) or studies on the influence of calcium or magnesium on the cells and their correlation with survival or apoptosis markers (Bickler and Fahlman, 2004; Turner et al., 2007).

This model allows for the control of the extracellular media whereby adding biological or pharmacological factors to study their effects represents an advantage over *in vivo* models (Fig. 1A). Therefore, the effect of growth factors or other chemicals on cell proliferation, cell differentiation or cell survival has been examined using various techniques (del Rio et al., 1991; Nakagami et al., 1997; Pozzo Miller et al., 1994; Su et al., 2011). Moreover, it permits to evaluate the activity of endogenous cells or stem cells and to learn from the environment, for example by the use of "reporter" stem cells, whose behaviour will highlight factors of the microenvironment (Park et al., 2006). Since the microglia remains functional, endogenous immunological interactions can also be assessed (Czapiga and Colton, 1999; Heppner et al., 1998). Furthermore, depending on the region of interest, many slices can be obtained, permitting the use of this *ex vivo* model for the screening of different therapeutic approaches. Moreover, various techniques may be employed to address basic cellular and molecular mechanisms (Cho et al., 2007) (Table 1). Sorensen et al., have also used organotypic slices from neonates rats as transient storage of dopaminergic donor cells before intracerebral grafting. In this way, pre-grafting examination can be performed as well as pooling and mixing of donor cells which are more mature when they are grafted (Sorensen et al., 1994).

Nevertheless, brain slices remain fragile, they can be easily distorted, and often flatten during the culture. Furthermore, they are more easily generated from young animals, which are more resistant and have stronger neuronal plasticity than adult tissue, which is the age at which the majority of neurological diseases take place. Functional outcome of a therapeutic strategy cannot be evaluated with this technique and generally slice cultures can be only maintained for a few weeks, so long-term assessments are not possible. Organotypic brain slices can only be used to test treatments that will be administrated in the brain by stereotaxy and the impact of the periphery cannot be taken into account. Moreover, the blood brain barrier is not conserved (Table 1).

In summary, even though organotypic cultures have certain limitations, they represent an easy, practical, and efficient way to screen different therapeutic strategies and to address many cellular and molecular mechanisms (Cho et al., 2007; Garbayo et al., 2011a; Sundstrom et al., 2005) prior or in parallel to pre-clinical models.

Organotypic slice preparation methods

Organotypic culturing was initiated at the end of 1950 with organotypic cultures of chick embryo organs as heart, lung, syrinx (Loffredo Sampaolo, 1956; Meerovitch, 1961) and then in the 60s with fragments of the adult rat hypophysis (Bousquet and Meunier, 1962). Several methods have been developed to maintain brain slices of around 150–400 μm in thickness alive in a long-term culture (Fig. 2). The most successful procedure was the "roller tube" technique described in 1981 by Gahwiler et al. (Gahwiler, 1981). In roller-tube cultures, individual slices are placed in flat-sided plastic culture tubes that contain a small amount of culture medium. During the slow rotation the tissue is covered by medium during half of a cycle, resulting in a continuous alternation of feeding and aeration,

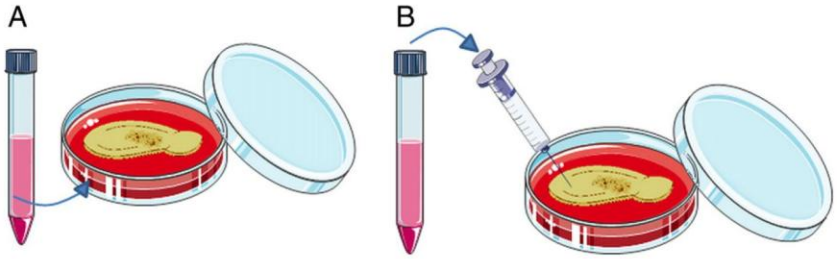

Fig. 1. Two major methods to study drugs or cell graft in brain organotypic slices. A) Agent can be added in the growth medium and cells will colonize the slice. B) Agent can be directly transplanted into the slice using a needle.

which is optimal for slice survival. More recently, in 1991, Stoppini et al. described the membrane interface culture method (Stoppini et al., 1991) that offers easier culture conditions. The tissue is placed directly on semi-porous membranes, and medium is added to the bottom of the culture dish. Slices can collect nutrients contained in the medium on one side and remain optimally aerated from the upper side. This technique is advantageous for studying the effect of a therapeutic agent added onto the organotypic slice, because these slices are never covered with embedding material or media. In general, slices cultured with the roller tube technique get thinner than cultures grown with the interface method due to tissue loss, but they can stay alive a longer time. Depending on the question, one may choose the more efficient technique for slice culturing according to the experiments to be performed. These techniques were further adapted to allow the development of organotypic cultures from different organs and from many hosts such as rats and mice but also rabbits (Savas et al., 2001) pigs (Dall and Zimmer, 2006) and human brain tissue (Jung et al., 2002).

Most organotypic brain slice cultures have been derived from neonatal (P0–P10) animals, but recently also adolescent or adult rats have been used and even human post-mortem brain tissue slices have been kept alive for a few weeks in culture (Finley et al., 2004; Noraberg et al., 2005; Su et al., 2011). The brain area the most cultured *in vitro* is the hippocampus, now usually prepared using Stoppini's method, with 6–7 day old rats. Over the years slice culture systems have been successfully established from a variety of brain regions including cortex, ventral midbrain, hypothalamus or cerebellum (Gahwiler et al., 1997). Furthermore, co-cultures of different brain slices have been developed, which allow for the assessment of inter-neural responses across brain regions. Nowadays, organotypic slices of many organs have been prepared as for example: liver (Mori et al., 1999), guts (Metzger et al., 2007), retina (Lye et al., 2007), tumour (Froeling et al., 2010) or bladder (Varley and Southgate, 2011) among others.

Stem cell transplantation studies using organotypic slices

Organotypic slices allow the study of endogenous cell activities. In addition, grafts of cells can also be performed in the slices and, in this way, graft–host interaction and grafted cell behaviour can be analyzed. We will here mainly report stem cell transplantation studies performed with organotypic cultures.

Migration of 5-bromodeoxyuridine-positive grafted cells was tracked and comparison of the migratory capacity of different stem cells showed that human NSCs and rat neurospheres from the subventricular zone seemed to migrate within the slice while MSCs remained at the implantation site (Tanvig et al., 2009). Recently, nanoparticles transporting CoPt, a magnetic product observable by MRI, were used to efficiently tag grafted cells in an organotypic model of spinal cord (Meng et al., 2011). Stem cell engraftment and interaction with the host tissue can also be monitored. It was thus reported that,

Table 1
Advantages and caveats of cell therapy pre-clinical studies performed in organotypic slices.

Cell transplantation studies	*Ex vivo*		
	References	Advantages	Caveats
1/Screening of therapeutic approaches	Sundstrom et al. (2005), Cho et al. (2007), Sarnowska et al. (2009b)	Six conditions can be tested with one rat in a few weeks. Interactions with host tissue easily explored	Functional outcome of a therapeutic strategy cannot be evaluated with this technique
2/Grafted cells/host interactions	Tanvig et al. (2009), Meng et al. (2011)	Easy follow-up of tagged cells	Cannot be conserved more than 1 month in culture
	Abouelfetouh et al. (2004), Sarnowska et al. (2009b), Charriere et al. (2010), Jeong et al. (2011)	Host cells remain functional, including microglia	Slices can be flattened or distorted Vascularization and blood brain barrier is not conserved
	Jaderstad et al. (2010b, 2011)	Cell functional integration within the host tissue is easily observed	Isolated organ/no interaction with periphery
	Kearns et al. (2006), Jaderstad et al. (2010a), Tonnesen et al. (2011)	Patch clamp studies on grafted cells may be performed	Slices from young pups/host plasticity differs from adult
3/Neurorepair mechanisms	Zhong et al. (2003), Park et al. (2006), Hall et al. (2009), Horn et al. (2009), Garbayo et al. (2011a, 2011b)	*Ex vivo* models of pathologies developed Host cells remain functional, including microglia	Functional outcome of a therapeutic strategy cannot be evaluated with this technique Slices difficult to prepare from adults/they do not reflect most of the pathological conditions
	Jaderstad et al. (2010a)	Allows patch clamp studies	
4/Neuroprotective factors secretion	Sarnowska et al. (2009a), Garbayo et al. (2011b)	Culture medium can be collected and analyzed	Isolated organ/no interaction with periphery
5/Adjuvant addition of biomolecules/chemical compounds	Abouelfetouh et al. (2004), Kearns et al. (2006), Tonnesen et al. (2011)	Can be added onto the culture medium of slice Can be injected directly into the slice	Blood brain barrier is not conserved

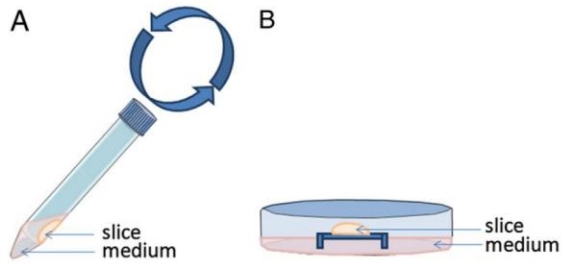

Fig. 2. The two major techniques used to culture brain organotypic slices (Gahwiler et al., 1997). A) Roller tube and B) semiporous membrane. See text for explanation.

human MSCs in contact with human organotypic spinal cord slices differentiate into neuronal cells (Jeong et al., 2011). A small percentage of MSCs are also able to differentiate into neuronal-like cells when deposited directly onto hippocampus slice. Three days post-grafting an increase of the length of the axon-like processes was observed as well as the number of neuronal-positive cells, which increased when retinoic acid was added into the culture medium (Abouelfetouh et al., 2004). In the same way, grafted MSCs could migrate into hippocampal tissue and differentiated into neuronal cells under the microenvironment pressure, but the differentiation rate remained very low (Charriere et al., 2010). As expected, NSC grafts within the striatum integrated morphologically forming part of the 3D cytoarchitecture. Moreover, they increased the overall survival of the organotypic cultures, reduced host cell necrosis and apoptosis while suppressing gliosis (Jaderstad et al., 2010b). Furthermore, connexin expression and early gap junctional intercellular communications were observed between grafted NSCs and host cells, which in this way protected neurons from death (Jaderstad et al., 2010a). It was shown that connexin43 expression and gap junction coupling was essential for neuroprotection (Jaderstad et al., 2011). Interestingly, connexin43 expression of NSC, decreased after transplantation into the host tissue, particularly with NSCs overexpressing neurotrophin-3 (NT-3), while connexin26 expression increased, following the pattern of neuronal differentiation (Jaderstad et al., 2010a, 2011). In a similar manner, bilateral interactions between cord blood-derived human or murine NSCs and the host cells were described. It was found that transplanted neurospheres behave differently depending on the implantation site, may integrate within the host tissue and activate host neuroblasts while inhibiting astrocytosis (Sarnowska et al., 2009b).

Many fundamental studies have been carried out with organotypic brain slices to evaluate endogenous cell or grafted cell behaviour. However, in order to better comprehend the mechanisms underlying proper graft integration in a pathological situation, these studies must be performed in organotypic *ex vivo* models, which mimic the neurological disorder. These models have been mainly used to study neurorepair and neuroprotection mechanisms in response to different drugs and growth factors, but a few studies have addressed grafted stem cell behaviour.

Evaluation of stem cell-based therapies in *ex vivo* models of neurological disorders

As stated above, we here consider neurological disorders for which organotypic cultures have raised particular interest to evaluate stem cell-based therapies. For each neurological disorder, we first describe the *ex vivo* model and then, we focus on the preclinical studies done in stem cell transplantation using organotypic cultures.

Cerebral ischemia

Ischemic brain injury occurs due to a cerebrovascular accident leading to a loss of blood supply to part of the brain. The most common type of stroke is characterized by the blockade of arterial vascularization at one site and, as a consequence, a lack of oxygen and glucose that induces cell death and tissue necrosis. Depending on the anatomical site of ischemic insult, CI can lead to hemiplegia, aphasia or motor dysfunction. CI, is the third cause of death in the US and in Europe with around 200 cases per 100,000 inhabitants per year (Barrett and Meschia, 2010) and almost 6 million victims every year according to the World Health Organization (Lloyd-Jones et al., 2010). Nevertheless, there has been a decrease of stroke mortality of 60% during the last thirty years due to early patient management. Ischemic stroke treatment begins with medication control aimed at avoiding the aggravation of cerebral injury, and a thrombolytic intravenous administration to dissolve the blood clot; if needed a surgical procedure to unblock vessels may be performed (Tarlov et al., 2012). These patients need to be treated within the first 3–6 h after stroke to avoid neuronal death; therefore cell therapy can be an attractive delayed treatment to repair damaged tissue, or sustain neuroprotection beyond this treatment window.

Cerebral ischemia *ex vivo* models

Several *ex vivo* approaches are currently used for CI modelling. Among them, hippocampal slices exposed to oxygen and glucose deprivation (OGD) are the most commonly used. This method models global cerebral ischemia, which causes a lack of oxygen and glucose to the brain, leading to the initial degeneration of the neuronal populations of the Cornu Ammonis layer 1 (CA1) in the hippocampus (Cho et al., 2007; Garbayo et al., 2011a; Noraberg et al., 2005; Raval et al., 2003; Xu et al., 2002). Hippocampal slice cultures are in general made using Stoppini's method. After careful dissection of the hippocampus, 300–400 μm transverse slices can be obtained (Fig. 3A). In those slices, the three main regions of the hippocampus, CA1, CA2 and CA3 are readily observed. Usually, two or three hippocampal slices are placed on a semi-porous membrane and cultured during a maximum of 3–4 weeks with an important conservation of neuronal circuits. The CI model is obtained by culturing the hippocampal slices in medium without glucose in an anaerobic chamber for 40 min (Raval et al., 2003; Xu et al., 2002). After that, slices are returned to normoxic conditions and to complete culture media. This OGD treatment induced CA1 hippocampus neuron death, which can reach 80% in only 24 h. After OGD, endogenous cell response (Bickler and Fahlman, 2004; Ziemka-Nalecz et al., 2013) and neuroprotective effects of different compounds, added into the culture media or directly onto the slice can be assessed (for review see Cho et al., 2007; Noraberg et al., 2005). Another way to produce selective cell death in the CA1 or in the CA3 region is by exposure of the hippocampal slices to Complex I inhibitors, such as rotenone or 1-methyl-4-phenylpyridium (MPP+), to Complex II inhibitors, such as 3-nitropropionic acid (3-NPA), or to excitotoxines, *i.e.* N-Methyl-D-aspartic acid (NMDA), or to kainic acid (KA) (Noraberg et al., 2005; Xu et al., 2003). These last models based on excitotoxicity only mimic a consequence of energy failure associated with CI. Thus, although they do not completely model CI pathology they correlate well with *in vivo* observations (Noraberg et al., 2005). On the other hand, OGD closely mimic CI pathology. However, variations in experimental setup can lead to different results and must be taken into account (Table 2).

Preclinical studies of stem cell therapies using cerebral ischemia organotypic cultures

MSC administration serves as a potential treatment for CI (Li et al., 2008). Thus, hippocampal organotypic slices have been used to test MSC effects on CI. It was first shown that a co-culture of OGD slices and MSCs lead to a significant reduction of dead cells in the CA1 area. The protective effect was probably exerted by diffusible factors such as nerve growth factor (NGF) brain derived neurotrophic factor (BDNF) or basic fibroblast growth factor (bFGF) (Zhong et al., 2003).

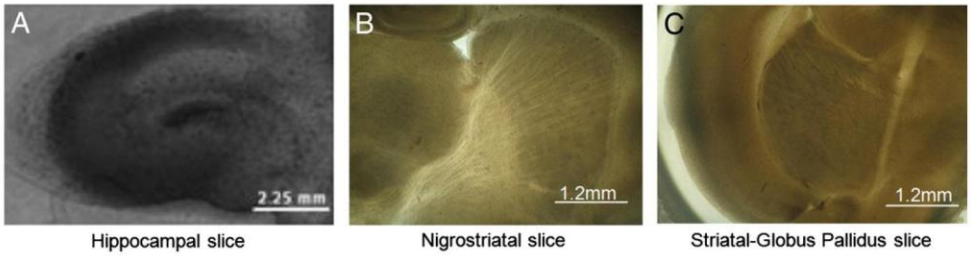

Fig. 3. Bright field images of organotypic cultures of A) hippocampal slices modeling cerebral ischemia, B) nigro-striatal slices modeling Parkinson's disease and C) striatal-globus pallidus slices modeling Huntington's disease.

More recently, a comparison of MSC and primary cortical cell effects on CI was performed using the OGD model. One day after OGD, the cells were transplanted on the surface of slices. An important decrease of CA1 region cell death was observed, and a concomitant neural differentiation for both types of grafted cells was detected after 7 days (Sarnowska et al., 2009b). Thereafter, OGD slices and cells were co-cultured without direct cell-tissue contact. An important decrease of cell death was also observed with both types of cells, suggesting that they were able to exert neuroprotection by secretion of particularly insulin-like growth factor, bFGF and NGF (Sarnowska et al., 2009a). A homogenous subpopulation of MSC, marrow-isolated adult multilineage inducible (MIAMI) cells, with a broad differentiation potential (D'Ippolito et al., 2006) have shown a reduction of hippocampus OGD induced cell death by, among others, neuroprotective factor secretion (Garbayo et al., 2011b). In another study, human umbilical cord blood stem cells (HUCBC) were compared to sigma receptor agonist 1, 3, di-o-tolylguanidine (DTG), a more classical functional *in vivo* treatment, in an OGD hippocampal slice. Unlike DTG, HUCBC mediated neuroprotection and limited inflammation by decreasing microglial derived nitric oxide levels detected in normoxic conditions, in the absence of a peripheral immune system (Hall et al., 2009). Nevertheless, in another paradigm an aggravation of the OGD induced lesion in hippocampal slices cultured with MSC conditioned medium was observed, probably involving GABA, NMDA or α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor agonists (Horn et al., 2009).

Neurodegenerative disorders: *ex vivo* models

Parkinson's disease

PD afflicts 2% of the population older than 65 years, with 7 to 10 million people affected worldwide. The neurological signs and symptoms of PD are, in large part, a result of selective loss of neurons in the nigrostriatal dopaminergic system. The causes of this neurodegeneration are not fully understood, but it seems that the dopaminergic neuronal death is induced, in part, by the aggregation of α-synuclein-containing Lewy bodies (Gazewood et al., 2013; Volles and Lansbury, 2003). The lack of DA causes the classical motor symptoms of bradykinesia, rigidity, resting tremors and loss of postural reflexes. PD is currently treated primarily with various DA replacement pharmacological agents either as monotherapy or in combination with a dopamine decarboxylation inhibitor such as carbidopa or benserazide. This therapy is very effective for the first few years of the illness, but undesirable side effects may appear later (Ecker et al., 2009; Hauser, 2009). Interestingly, neuronal degeneration in this pathology is quite localized at first, so cell therapy has been explored as an alternative approach. In a later stage, cell death related to PD first extends to basal forebrain, transentorhinal cortex, CA2 region of the hippocampus and then to the entire neocortex (Coelho and Ferreira, 2012; Halliday and McCann, 2010).

Parkinson's disease ex vivo models

The cortico-striatal and mesencephalon-striatal (nigro-striatal) pathways have been extensively studied for PD modelling (Fig. 3B). Organotypic co-cultures of cortex-striatum were first developed and characterized to study the cortico-striatal pathway (Plenz and Aertsen, 1996a; Plenz and Aertsen, 1996b). Further, organotypic rat ventral mesencephalon was co-cultured with striatal, cerebellum or hippocampus slices and an extensive innervation of the striatum by TH-positive fibres from the ventral mesencephalon was observed when co-cultured together (Ostergaard et al., 1990). As PD cell death appears first within the substancia nigra (SN), other groups made organotypic slices of this area to focus on dopaminergic cell bodies (Jaeger et al., 1989). Using this model, Testa et al. studied the molecular mechanism of rotenone (Testa et al., 2005), a pesticide well known to induce nigro-striatal pathway degeneration (Alam and Schmidt, 2002).

To cover the whole nigro-striatal pathway, and so merge many areas involved in PD, a team combined those two techniques to make organotypic cortex-striatum-mesencephalon cultures using the "roller tube" method from Garwhiler (Plenz and Kitai, 1996). For the preparation of these triple slice cultures, cortex, striatum and mesencephalon containing *SN* coronal sections from post-natal rat brain were gathered and mechanically stabilized on a coverslip by embedding them together with a membrane to reproduce the nigro-striatal pathway. Following attachment to a substrate, the dopaminergic regions of the SN extended processes into the striatum. They proved that cortex–striatum–mesencephalon culture maintained morphological and electrophysiological characteristics of the *in vivo* preparation (Plenz and Kitai, 1996). But this protocol necessitates performing 3 different slices and merging them onto a single coverslip, so a more skilled operator is required. This triple slice culture model has been used recently to make a comparison of three well-known dopaminergic specific neurotoxins, usually used for *in vivo* experiments: MPP^+ (Betarbet et al., 2002), rotenone (Perier et al., 2003), and 6-hydroxydopamine (6-OHDA) (Perese et al., 1989). In this article and in many others, the neurotoxins were directly included in the culture medium (Fig. 1A). In this way, the neurodegeneration is total and homogeneous between the different structures (Kress and Reynolds, 2005). To better mimic the *in vivo* model, in which the injection of neurotoxins is done stereotactically (Fig. 1B), a group developed organotypic cultures of the ventral mesencephalon with unilateral application of 6-OHDA in either the striatum or SN using a microelectrode to do the injections. In the case of cortical slices, this technique permits to introduce a simple method for generation of an internal control, by treating only one side of the brain, just as *in vivo* models (Stahl et al., 2009). In this *ex vivo* model, neurodegeneration is identically obtained as in animal models, so observed degeneration is similar allowing to a certain extent direct comparisons between these two models.

A new organotypic slice technique was developed by vibroslicing rat brain sagittally in order to keep the whole functional architecture and

Table 2
Development of neurological disorders *ex vivo* models.

	Ex vivo models	Observations/remarks	References
Cerebral ischemia	OGD treatment on hippocampal slices	Easy access and precise control of extracellular environment, which makes it suitable to study detailed molecular mechanisms associated with CI	Bickler and Fahlman (2004), Noraberg et al. (2005), Garbayo et al. (2011b), Ziemka-Nalecz et al. (2013)
	Complex I inhibitors as rotenone or MPP+, Complex II inhibitors as 3-NPA or excitotoxine as NMDA, Kainic acid	Correlate well with *in vivo* observations but only mimic a consequence of energy failure associated with CI	Xu et al. (2003), Noraberg et al. (2005)
Parkinson's disease	Addition of MPP^+, Rotenone or 6-OHDA on triple slice culture medium	A specific dopaminergic degeneration is obtained	Kress and Reynolds (2005)
	Unilateral application of 6-OHDA in either the striatum or SN	PD is identically obtained in animal models and allows generating an internal control	Stahl et al. (2009)
	Cortex–*corpus callosum*–striatum–SN slices	Mechanical denervation of the nigro-striatal pathway by cutting the medial forebrain bundle Allow studying all areas/pathways involved in this pathology	Cavaliere et al. (2010)
	Mouse sagittal slices exposed to 6-OHDA	Long-term viability with intact cytoarchitecture throughout cortical and subcortical regions	Kearns et al. (2006)
Huntington's disease	Kainic acid, quinolinic acid or 3-nitropropionic acid addition	Easy and rapid to develop and neurodegeneration similar to the one observed in HD but do not take account the genetic component of the pathology	Matyja (1986), Whetsell and Schwarcz (1989), Beal et al. (1991), Storgaard et al. (2000), Colle et al. (2012)
	Hippocampal slices established from the R6/2 transgenic mice	Provide a model to assess inhibitors of huntingtin aggregation in a system close to *in vivo* models	Jana et al. (2000), Smith et al. (2001), Smith and Bates (2004), Hay et al. (2004)
	Transfection with a mutant huntingtin gene	Mutant gene can be the same one used for R6/2 mice model Human mutant huntingtin can be used Merges all features of human HD cellular pathology	Murphy and Messer (2001), Reinhart et al. (2011)

pathways of the brain (Ullrich et al., 2011). This organotypic slice allows performing investigations on those pathways and also interactions with glial cells and capillaries, but no dopaminergic neurodegeneration of the nigro-striatal pathway is observed. In the same way, sagittal vibrosections of mouse brain were performed. Sagittal slices maintained neuronal pathway integrity during three weeks in culture and were immersed into 6-OHDA to induce a dopaminergic pathway degeneration (Kearns et al., 2006). Based on those studies, our team has begun the development of an *ex vivo* PD model in which a mechanical degradation of the nigro-striatal pathway is obtained. Sagittal slices are performed with an angle of 14° which induce the degeneration of the medial forebrain bundle which dispenses neurotoxin use (Daviaud et al., 2011).

Another organotypic model of nigro-striatal degeneration was developed by another group to study PD hallmarks. Two-or three-day old rat brains were separated in two hemispheres and the cerebellum was removed. The resulting brain blocks were cut dorso/ventrally with a vibratome using a 45° inclination. Slices containing cortex–*corpus callosum*–striatum–SN were selected. The PD model was obtained by a mechanical denervation of the nigro-striatal pathway, by cutting the medial forebrain bundle near the SN, thus leading to retrograde and anterograde neuronal degeneration in the SN and in the striatum, respectively. They compared mechanical lesion degeneration with 6-OHDA treatment of the whole slice. They showed that denervation induced a loss of about 60% of TH expression in the striatum and the SN in 3 days when this loss is about 30% in the same slices treated with 6-OHDA. The mechanical lesion model is an interesting tool to study mechanisms of neurodegeneration, as well as neuroregeneration without use of neurotoxins but it also damaged glutamatergic pathway and the GABAergic projections, while the 6-OHDA injection model is more specific for dopaminergic pathway (Cavaliere et al., 2010). In this way, scientists can either choose a dopaminergic specific model, which is closer to the *in vivo* model and mimics early PD, or a dopaminergic/glutamatergic/GABAergic degeneration, which is closer to an advanced PD model. Those two last models seem to be the more complete, simple and appropriate to study PD. Indeed, they allow studying all areas involved in this pathology and conserve many pathways that interact with this major neurodegeneration pathway. Furthermore they are based on unique slice culture, which is simpler to develop than co-cultures (Table 2).

One of the main advantages of these *ex vivo* PD models is that they provide a therapeutic screening platform without the need of time-consuming whole animal studies. However, the major drawback is that functional effects cannot be analyzed.

Preclinical studies of stem cell therapies using Parkinson's disease organotypic cultures

A few studies have used striatal organotypic cultures to monitor the interaction of grafted NSCs and the host striatal cells, but those slices did not present a nigro-striatal dopaminergic degeneration, which alter the micro-environment and may modify the cell/host interaction. A recent study, transplanted Wnt5a-overexpressing-NSCs, expanded as neurospheres, into PD organotypic cultures from 6-OHDA-treated-mouse striatum (Tonnesen et al., 2011). This study showed that grafted cells integrated the DA-denervated striatum and created bidirectional interactions with the host cells including host striatal presynaptic regulation of host cortical afferents to the grafted DA neurons and polysynaptic graft-to-host connections. Patch clamp studies were performed on grafted cells. An important depolarization of GABA receptor-mediated chloride currents in the DA neurons was observed, which is characteristic of endogenous dopaminergic neurons, but may also indicate partial immaturity. To confirm the importance of those results, similar experiments were performed in 6-OHDA-lesioned animals. *In vivo* grafted cells had similar morphological and electrophysiological properties than *ex vivo* grafted cells showing their maturity and integration in the organotypic slice.

Mouse embryonic stem cell derived neural precursor cells were implanted into a mouse sagittal slice previously treated with 6-OHDA. Viable cells were observed up to three weeks in culture and showed a dopaminergic differentiation when many factors as bFGF, sonic hedgehog or pleiotrophin were added in the culture media. Furthermore, patch clamp studies allowed recording action potentials, as well as postsynaptic currents, into grafted cells, localized by their capacity to express GFP, demonstrating functional integration and maturation within the host tissue (Kearns et al., 2006).

Huntington's disease

HD is an autosomal dominant genetic neurodegenerative disorder with a prevalence of 6 to 7 for 100,000 around the world (Walker, 2007). This pathology results in abnormal involuntary movements (chorea and dystonia), cognitive decline, behavioural changes and a range of psychiatric disorders, especially depression. These symptoms,

which gradually develop, are caused on an earlier stage by degeneration of the striatal neurons, especially of GABAergic striatal MSNs which project to the external segment of the globus pallidus, the SN *pars compacta* and *pars reticulata*. Then, the gradual neuronal loss extends throughout the brain (Ho et al., 2001). The huntingtin gene is localized on chromosome 4 and the normal allele on this site contains a trinucleotide repeat sequence (CAG) encoding polyglutamine, but this CAG repeat is amplified in patients suffering from HD. When the repetition reaches 39 or more, the pathology will develop generally at around 30–40 years. Although CAG repeats greater than 55 are associated with early age of onset (juvenile HD) and more rapid progression, there is no correlation between age of onset and number of repeats ranging from 40 to 50 (for review see Ho et al., 2001; Ha and Fung, 2012). No cure exists for this disease and antichoreic drugs or neuroleptics only reduce the severity of the symptoms. Moreover, their administration can cause side effects such as bradykinesia or depression, and are ineffective in stopping or reversing cell death (Adam and Jankovic, 2008). With HD, the neurodegeneration is mainly localized in the striatum at onset of the pathology, so cell therapy represents an attractive alternative treatment. Unfortunately, for advanced cases, HD is less optimal for cell therapy because the entire brain becomes involved (Wijeyekoon and Barker, 2011).

Huntington's disease ex vivo models

The first HD organotypic model developed dates back to 1986 with the study of the effects of KA administration on glial cells, both in the animal model of HD (which uses this drug to damage GABAergic neurons) and in striatal organotypic cultures. Indeed, glial cells have a major role in brain homeostasis and should be thoroughly investigated in neurodegenerative disorder models. This study proved that KA might act directly on astroglia cells by a transformation of the protoplasmic type of astroglia into the fibrous type; changes that were independent of neuronal damage (Matyja, 1986). The effect of quinolinic acid (QA) has also been tested on organotypic cultures of rat caudate nucleus or on combination cultures of frontal cortex and caudate nucleus. Light microscopy analysis proved that QA induced excitotoxic damage on the rat brain which models HD (Whetsell and Schwarcz, 1989). QA induced a loss of markers of spiny neurons (GABA and substance P), an increase in markers of aspiny neurons (somatostatin and neuropeptide Y) and a diminution of choline acetyltransferase activity, similar to changes observed in HD (Beal et al., 1991). Striatal degeneration of HD can also be caused *in vivo* by 3-NPA, a mitochondrial inhibitor. To better understand the neurotoxicity and possible indirect excitotoxicity mechanisms, 3-NPA was added into organotypic striatal and cortico-striatal slice culture medium. In this way, the authors proved that neuronal activity of the glutamatergic cortico-striatal pathway contributed to striatal pathology and that *in vivo* characteristics of 3-NPA toxicity can be reproduced in organotypic slices (Storgaard et al., 2000). Recently, QA, 3-NPA and the combination of these two toxins were tested to model HD in 400 μm striatal slices. The effects of NMDA receptor antagonist MK-801, of succinate and of reactive oxygen species were investigated. It was concluded that each model induces these processes in a differential manner: QA by NMDA receptor activation and 3-NPA by succinate dehydrogenase inhibition. Those effects were blocked by probucol, an antioxidant compound with scavenger properties (Colle et al., 2012). There is an important variety of those models, based on the different neurotoxins used and addition into the culture media or either directly injected onto the slices, leading to a heterogeneity of results obtained, which must be taken in consideration. Furthermore with that type of model, only the cellular aspect of HD can be studied, they can't take into account the genetic component of the pathology. Conversely they are easy and rapid to develop and do not involve high economic impact as normal rodents are used.

A model of organotypic hippocampal slices established from the R6/2 transgenic mice, which presents a CAG repetition, was developed showing the same formation of PolyQ aggregates in all of the hippocampus slice neurons, appearing first in the CA1 followed by the CA2 and later in the CA3 after 5 weeks. Creatine administration can block this aggregation *in vivo*, and this was confirmed in this organotypic model (Smith et al., 2001). Using this same model it was shown that a maintained overexpression of a chaperone protein HSP70, which is co-localized with nuclear aggregates in HD patients, could delay aggregate formation (Hay et al., 2004; Jana et al., 2000). In another study organotypic hippocampus slice of R6/2 mice is clearly established as a screening model of anti-aggregation components (Smith and Bates, 2004).

Another way to mimic HD is the transfection of HD-polyQ-GFP plasmids into cells within the slice. Three different non-viral transfection methods were tested on mouse cerebellum organotypic slices (Murphy and Messer, 2001). Mouse cortico-striatal slices could then be transfected with a mutant huntingtin gene and when exposed to malonate produced HD-like lesions and provided a new model of HD, conserving the correlation between CAG repetitions and aggregation length. A co-transfection of anti-HD single-chain Fv intrabodies can lead to a reduction of the physiological consequences and a diminution of cells expressing large aggregates after three days of culture (Murphy and Messer, 2004). More recently a new HD model was developed based on the acute transfection of rat cortico-striatal brain slices with DNA constructs derived from the human pathological huntingtin gene. This transfection of mutant huntingtin induced GABAergic MSNs death within 3 days. Furthermore, the transfected mutant gene is the same used for R6/2 mice model. Thereby, the model developed is very close to *in vivo* models, and merged all features of HD cellular pathology in a very specific manner. A screening of a chemical library of 74 neuroprotective and anti-inflammatory drugs implicated in HD was performed and Chemokine receptor, IκβB kinase complex and c-Jun N-terminal kinase particularly showed neuroprotective effects (Reinhart et al., 2011).

Those more recent models of HD are very powerful; they properly mimic the pathology including the genetic aspect of HD and as a consequence lead to the MSN death. Two majors techniques can lead to this model, in the first one organotypic slices are made directly on transgenic mice, and in the second organotypic slices are prepared from normal rodents and slices are transfected. The first protocol appears to be easier to develop because R6/2 mice can be purchased, so model acquirement does not demand specific knowledge but may be more expensive in a long term. Inversely, slice transfection involves high technology equipments and a skilled operator but normal rodents can be used, and a better control of the transfected cDNA construct obtained. Despite those very powerful HD *ex vivo* models, no therapy using stem cells has been studied using HD organotypic cultures.

Studies of stem cells with scaffolds using organotypic cultures

The development of new tools able to enhance the potential of cell therapy is needed. Tissue engineering is the combination of cells with materials, scaffolds, and biochemical or physico-chemical factors to improve their biological effects. In addition, cells are responsive to the architecture and to mechanical properties of the scaffolds, which can modulate morphology, proliferation and, in the case of stem cells, differentiation. Micro- or nano-capsules, micro- or nano-particles and gels, which can all be polymeric, aqueous, lipidic, are very different tools used to improve potential treatments (Subramanian et al., 2009; Williams and Lavik, 2009). In our laboratory we have developed a cell and drug carrier, named the pharmacologically active microcarriers (PAMs). They are polymeric biodegradable microspheres that offer a 3 dimensional support for cells. Moreover they can present a biomimetic surface of extracellular matrix protein to enhance survival and/or differentiation of grafted cells and lastly they can release a growth factor in a controlled manner during PAM degradation. Those combined parameters increase cell survival, differentiation and integration in the

host tissue (Bouffi et al., 2010; Delcroix et al., 2011; Tatard et al., 2004, 2005, 2007b).

Tissue engineering is an ongoing area of research that may also use organotypic slices to demonstrate its therapeutic potential (Fig. 4). With this aim and to mimic *in vivo* conditions, a co-culture of cortex organotypic slices and matrigel was developed. Matrigel is a gelatinous protein mixture, similar in composition to basement membrane with extracellular matrix and proteins, and allows 3D cell culture. The co-culture of matrigel with cortex organotypic slices allowed demonstrating axon elongation from the cortex to the matrigel and the ability of cells implanted into the gel to promote axonal growth. This *ex vivo* model provided a good means to screen transplanted cells for axon growth and cell–cell communication potential before *in vivo* experiments (Ishihara et al., 2011). To enhance CNS cell transplantation, gelatin–laminin-cryogels and dextran–laminin-cryogels transporting HUCBC were prepared. Cryogels are a cryogelation of a mixture of protein from the extracellular matrix with either gelatin or dextran. They can carry transplanted cells while offering them physical and stress protections and can promote cell differentiation. Organotypic hippocampal slice cultures were prepared as a 3D screening tool, in which aggregates of scaffold-conveying cells were transplanted into the CA1 region. It was proved that scaffold implantation did not induce inflammation, microglial activation and prevented astrocytic scar formation. Furthermore, laminin scaffolds attracted infiltration of neuroblasts making them an interesting tool for brain circuit regeneration (Jurga et al., 2011).

Neuroprotective properties of MIAMI cells adhered onto PAMs were demonstrated by our group using an *ex vivo* model of CI (Garbayo et al., 2011b). MIAMI cells can differentiate into neuronal-like cells (Tatard et al., 2007a) and after epidermal growth factor (EGF) and bFGF pretreatment their response to neuronal commitment is increased (Delcroix et al., 2010a). As many other cells, when combined to scaffolds their regenerative potential may be increased. One hour after OGD of hippocampal slice cultures MIAMI cells, or EGF–bFGF pre-treated MIAMI cells adhered onto PAMs, injected into the CA1 cell body layer of hippocampal slices significantly protected the CA1 region against ischemic death. Cell therapeutic value was significantly increased when delivering the cells complexed with fibronectin-coated microcarriers, by increasing stem cell survival, neuronal differentiation and paracrine secretion of different factors. Those results were then confirmed using an *in vivo* CI model (Garbayo et al., 2011b). MIAMI cells adhered to PAMs with a laminine biomimetic surface and delivering NT-3, improved survival and differentiation of stem cells and induced a strong functional recovery in an animal model of PD (Delcroix et al., 2011). Based on those results we have developed a new *ex vivo* PD model (Daviaud et al., 2011) to study the molecular mechanisms of this functional recovery using two different stem cells enabling to compare their mechanisms of action (Daviaud et al., 2011).

Conclusions

As described here, organotypic cultures of many organs were developed showing the great potential and interest of this model. Obviously, organotypic slices cannot replace *in vivo* models, which remain the only way to evaluate the functional outcome of a therapeutic strategy. However, organotypic brain slices can model different histopathological aspects of neurological conditions and can be used to address some of the difficulties limiting stem cell therapy approaches. Organotypic culture technique is fast, efficient and practical, allows reduction of animal number and preserves the 3D neuronal network. Many regions of the brain can be cultured alone or co-cultured with other regions. They can serve as screening platforms, to study the effects of drugs, cell grafts alone or combined with biomaterials injected onto the slices.

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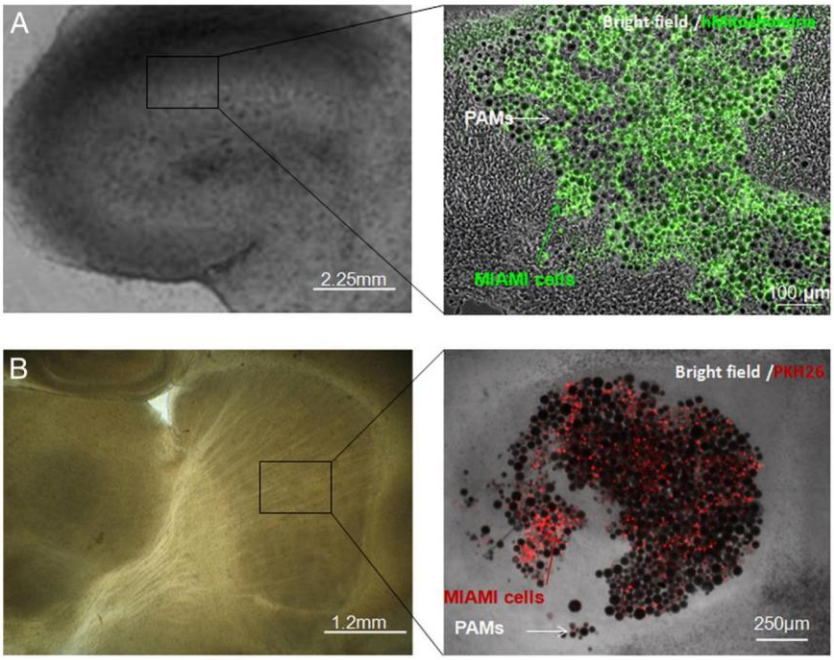

Fig. 4. Brain organotypic slices can be used for screening of stem cell therapy and tissue engeeniring applications. Pharmacologycally active microcarriers conveying A) immunostained stem cells injected into hippocampal organotypic cultures and B) PKH-26 labelled stem cells injected into nigrostrial organotypic cultures.

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CHAPITRE 2

Modéliser la dégénérescence de la voie nigrostriée dans une culture organotypique, un nouveau modèle de la maladie de Parkinson.

INTRODUCTION

Modéliser la dégénérescence de la voie nigrostriée dans une culture organotypique, un nouveau modèle de la maladie de Parkinson

L'objectif de cette thèse est d'étudier une nouvelle stratégie de médecine régénérative pour la maladie de Parkinson, et plus précisément, les mécanismes cellulaires et moléculaires impliqués dans la protection/réparation de la voie dopaminergique nigrostriée. Pour ce faire il était nécessaire de se demander quels modèles existent pour cette pathologie et lesquels permettraient d'effectuer cette étude le plus facilement possible et de manière complète.

Il existe de nombreux modèles *in vivo* de la maladie de Parkinson. Les plus utilisés sont obtenus par injection de neurotoxines (Tieu, 2011) (6-Hydroxydopamine (6-OHDA) ou 1 - méthyle 4 - phényl 1,2,3,6-tétrahydro pyridine (MPTP)) dans l'organisme qui induisent une dégénérescence spécifique des neurones dopaminergiques de la voie nigrostriée. Ces modèles représentent le seul moyen d'évaluer les bénéfices fonctionnels associés à l'administration d'un traitement. Toutefois, ces modèles sont couteux, très longs à obtenir et à étudier, et ne permettent pas d'étudier facilement les mécanismes cellulaires et moléculaires associés à une approche thérapeutique.

Comme vu dans le chapitre précédent, la culture organotypique permet de maintenir l'intégrité structurelle et le microenvironnement d'un tissu durant plusieurs semaines avec des méthodes de culture simples (Stahl et al., 2009, Herlenius et al., 2012, Daviaud et al., 2013). Des coupes organotypiques de cerveaux ont rapidement été développées. Elles permettent de maintenir l'architecture en trois dimensions de

l'organe, l'intégrité des voies neuronales, et le microenvironnement. Dès lors, l'utilisation des coupes organotypiques s'est démocratisée. Elles ont été utilisées pour mimer des pathologies telles que l'ischémie cérébrale, la maladie de Huntington (Diekmann et al., 1994, Storgaard et al., 2000, Smith and Bates, 2004, Murphy and Messer, 2004), la maladie d'Alzheimer (Shahani et al., 2006) ou encore la maladie de Parkinson (Kearns et al., 2006, Cavaliere et al., 2010, Plenz and Kitai, 1996).

Deux modèles *ex vivo* de la maladie de Parkinson ont déjà été mis en place. Dans le premier, des coupes sagittales de cerveaux subissent un bain de 6-OHDA afin de mimer la maladie de Parkinson (Kearns et al., 2006). Le second modèle développé utilise des coupes axiales observant un angle de 40° par rapport à la base du cerveau, permettant d'obtenir une coupe suivant le prolongement de la voie nigrostriée. Dans ce cas deux protocoles ont ensuite été testés : un bain de 6-OHDA ou une section manuelle de la voie nigrostriée permettant de mimer la pathologie (Cavaliere et al., 2010).

Nous avons alors décidé de créer un nouveau modèle *ex vivo* de la maladie de Parkinson avec pour objectif de rendre ce modèle aussi simple à faire que possible, c'est-à-dire sans ajout de neurotoxines ni manipulation supplémentaire pour mimer la pathologie. Il était également nécessaire que les techniques d'analyses classiques (immunomarquages, PCR quantitative…) puissent être utilisées sur ce modèle innovant. Il permettrait alors d'effectuer des études rapides de screening d'agents ou d'approches thérapeutiques pour la maladie de Parkinson. Pour cela, plusieurs angles de coupes du cerveau ont été testés dans le but d'obtenir une lésion de la voie nigrostriée tout en étant capable d'observer toutes les aires cérébrales impliquées dans la maladie de Parkinson, à savoir la substance noire, le faisceau médian du télencéphale et le striatum. Ces coupes devaient également pouvoir être

conservées durant un temps suffisamment long en culture afin de pouvoir y développer les stigmates de la maladie de Parkinson tout en laissant une fenêtre thérapeutique suffisamment longue pour pouvoir observer les effets d'une approche thérapeutique. En effet, le but final, est d'effectuer des greffes de cellules souches combinées ou non à des MPA dans ces coupes et d'y étudier le comportement et le devenir de ces cellules ainsi que du tissu hôte.

MODELING NIGROSTRIATAL DEGENERATION IN ORGANOTYPIC CULTURES, A NEW EX VIVO MODEL OF PARKINSON'S DISEASE

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MODELING NIGROSTRIATAL DEGENERATION IN ORGANOTYPIC CULTURES, A NEW EX VIVO MODEL OF PARKINSON'S DISEASE

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Abstract—Parkinson's disease (PD) is the second most frequent neurodegenerative disorder afflicting 2% of the population older than 65 years worldwide. Recently, brain organotypic slices have been used to model neurodegenerative disorders, including PD. They conserve brain three-dimensional architecture, synaptic connectivity and its microenvironment. This model has allowed researchers a simple and rapid method to observe cellular interactions and mechanisms. In the present study, we developed an organotypic PD model from rat brains that includes all the areas involved in the nigrostriatal pathway in a single slice preparation, without using neurotoxins to induce the dopaminergic lesion. The mechanical transection of the nigrostriatal pathway obtained during slice preparation induced PD-like histopathology. Progressive nigrostriatal degeneration was monitored combining innovative approaches, such as diffusion tensor magnetic resonance imaging (DT-RMI) to follow fiber degeneration and mass spectrometry to quantify striatal dopamine content, together with bright-field and fluorescence microscopy imaging. A substantia nigra dopaminergic cell number decrease was observed by immunohistochemistry against rat tyrosine hydroxylase (TH) reaching 80% after 2 days in culture associated with a 30% decrease of striatal TH-positive fiber density, a 15% loss of striatal dopamine content quantified by mass spectrometry and a 70% reduction of nigrostriatal fiber fractional anisotropy quantified by DT-RMI. In addition, a significant decline of medium spiny neuron density was observed from days 7 to 16. These sagittal organotypic slices could be used to study the early stage of PD, namely dopaminergic degeneration, and the late stage of the pathology with dopaminergic and GABAergic neuron loss. This novel model might improve the understanding of PD and may represent a promising tool to refine the evaluation of new therapeutic approaches.

Key words: Parkinson's disease, nigrostriatal organotypic slices, MRI, diffusion tensor imaging, dopamine, mass spectrometry.

INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disorder affecting 2% of the population older than 65 years, with 7–10 million people concerned worldwide (Lees et al., 2009; Gazewood et al., 2013). Clinically, the cardinal symptoms are tremor, rigidity of muscles, bradykinesia and loss of postural reflexes (Parkinson, 1817). The major symptoms are due, in part, to the lack of dopamine released in the striatum as a result of degeneration of dopaminergic neurons in the substantia nigra (SN). Dopaminergic neuronal death seems to be induced by α-synuclein aggregation, forming Lewy bodies which are toxic (Spillantini et al., 1997; Volles and Lansbury, 2003). At present, there is no cure for PD and treatments are merely symptomatic. Current therapy based on a dopamine replacement strategy consists mainly on the oral administration of the dopamine precursor L-3,4-dihydroxyphenylalanine (L-DOPA), but in long-term administration some secondary effects may appear (Ecker et al., 2009; Hauser, 2009). Novel drug and cell therapy approaches require extensive evaluation before routinely being used in humans (Poewe et al., 2012; Lindvall, 2013).

A wide variety of *in vitro* (Alberio et al., 2012) and *in vivo* models (Jackson-Lewis et al., 2012) have been developed to elucidate the pathogenesis, cell death mechanisms and to evaluate therapeutic strategies for PD. These PD models require a reproducible and well-characterized degeneration of the nigrostriatal dopaminergic system which is the main, but not the only pathway involved in PD. *In vivo* models can be broadly divided into genetic (Dawson et al., 2010) and neurotoxic models (Tieu,

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Abbreviations: 6-OHDA, 6-hydroxydopamine; BSA, bovine serum albumin; DARPP32, dopamine- and cAMP-regulated neuronal phosphoprotein; DT-RMI, diffusion tensor magnetic resonance imaging; FA, fractional anisotropy; H_2O_2, hydrogen peroxide; Hank's, Hank's Balanced Salt Solution; IgG, immunoglobulin G; LC–MS/MS, liquid chromatography–mass spectrometry; MEM, Minimum Essential Medium Eagle; MFB, medial forebrain bundle; MRI, magnetic resonance imaging; MSN, medium spiny neuron; NeuN, neuronal nuclei; NGS, normal goat serum; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline containing 1% Triton; PD, Parkinson's disease; RARE, rapid acquisition with relaxation enhancement; ROS, reactive oxygen species; SN, substantia nigra; TH, tyrosine hydroxylase.

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2011). However, *in vivo* studies require high technical and financial resources and they do not allow to simultaneously test several conditions in the same animal. Furthermore, it results in an important difficulty to address different engraftment mechanisms when testing cell therapy approaches. In contrast, *in vitro* experiments with dopaminergic neurons are easy to develop and use but they do not allow the study of cell interactions with the microenvironment or with the host tissue.

Recently, organotypic brain slice cultures have been used for PD modeling. Since their introduction, organotypic brain cultures have become a useful tool to study physiological properties of tissues, to monitor both acute and chronic effects of drugs and to study neurological disorders as in global cerebral ischemia or PD (Daviaud et al., 2013). Striatal–mesencephalon and cortex–striatum–mesencephalon co-cultures were developed to study tyrosine hydroxylase (TH)-positive neurons, being TH the rate-limiting enzyme in dopamine synthesis (Ostergaard et al., 1990). Those co-cultures maintained brain morphological and electrophysiological characteristics but did not mimic PD hallmarks (Plenz and Kitai, 1996). In order to better simulate *in vivo* models in which nigrostriatal degeneration is caused by dopaminergic neurotoxin injection, organotypic slices were next treated or injected with rotenone (Stahl et al., 2009) or 6-hydroxydopamine (6-OHDA) (Cavaliere et al., 2010). However, as in animal models, 6-OHDA led to neuronal death mediated by oxidative stress inducing selective dopaminergic neuron degeneration but did not produce extra-nigral pathology or Lewy body-like inclusions (Schober, 2004; Cavaliere and Matute, 2011). More recently, whole rat brain sagittal organotypic slices were developed which maintained dopaminergic and cholinergic neurons, as well as a complex capillary network and long nerve fibers (Ullrich et al., 2011). In the same way, sagittal sections of mouse brain were performed. These slices maintained the integrity of neuronal pathways during 3 weeks in culture and were immersed in 6-OHDA to induce a dopaminergic pathway degeneration (Kearns et al., 2006).

In the present study, we developed a sagittal organotypic culture model that includes all the areas involved in the nigrostriatal pathway in a unique slice without using neurotoxins to induce the dopaminergic lesion. The goal was to induce progressive nigrostriatal degeneration in a single step by mechanical transection of the medial forebrain bundle (MFB) while preparing the slices, in order to obtain a simple reproducible PD ex vivo model. We next studied slice survival while cultured with different media to maximize slice viability. The dopaminergic nigrostriatal pathway whose fibers run within the MFB was characterized by immunostaining against TH. Furthermore, progressive nigrostriatal degeneration was monitored combining two innovative approaches, magnetic resonance imaging (MRI) and mass spectrometry, rarely used for this type of study in organotypic slices. Fractional anisotropy (FA) measurements by diffusion tensor magnetic resonance imaging (DT-RMI) were used to follow and characterize MFB fiber degeneration in the organotypic slices. Indeed, DT-RMI provides quantitative information on water diffusion which can be used to evaluate the biological tissue properties. Furthermore, a liquid chromatography–mass spectrometry (LC–MS/MS) method to quantify striatal dopamine content directly in organotypic tissue was developed in this study. This new ex vivo PD model may represent a promising tool to refine the evaluation of new therapeutic approaches as cell therapy or tissue engineering, among others.

EXPERIMENTAL PROCEDURES

Preparation of nigrostriatal organotypic slices

Animal care and use were in strict accordance with the regulations of the French Ministry of Agriculture and all animal procedures were approved by animal experimentation ethics committee of Pays de la Loire. Every effort was made to minimize animal suffering and the number of animals used.

Timed pregnant Sprague–Dawley rats were purchased from Janvier (Saint Berthevin, France) or from SCAHU (Service commun d'animalerie hospitalo-universitaire, Angers University, France). Postnatal 6–8-day-old pups were used to prepare organotypic slices according to the Stoppini method (Stoppini et al., 1991) with modifications. Pups were anesthetized by an intraperitoneal injection of 80 mg/kg of ketamine (Clorketam 1000, Vetoquirol, Lure, France) and 10 mg/kg of xylazine (Rompum 2%, Bayar Health Care, Kiel, Germany). Animals were sacrificed, brains were rapidly dissected and cerebral hemispheres were separated and glued onto the chuck of a water-cooled vibratome (Motorized Advance Vibroslice MA752, Campdem Instruments, Loughborough, UK) to be sagittally cut. Under aseptic conditions, 400-µm slices were cut in different configurations in order to obtain a progressive degeneration of the nigrostriatal pathway. Finally, sagittal sections were cut alongside of the midline, with a 14-degree angle of the razor blade, and placed in sterile ice-cold Gray's Salt Balanced Solution (Sigma–Aldrich, St. Louis, MO, USA) supplemented with 6.5 mg/L of glucose and antibiotics (100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.25 µg/ml amphotericin B) (Sigma–Aldrich, St. Louis, MO, USA) for 1 h. Typically, about 10 slices can be obtained per hemisphere. The first 2–3 slices and the two last brain slices did not contain the three main areas involved in the pathology and were discarded. Three to four slices per hemisphere were next transferred to 30-mm diameter semiporous membrane inserts (Millicell-CM, Millipore, Bedford, MA, USA) within a 6-well plate and put in culture at 37 °C, 5% CO_2. A total of about 30 rat pups and about 120 organotypic slices were necessary to perform the whole characterization. For each condition, a minimum of three slices taken from three different rat pups were used.

Nigrostriatal organotypic slice culture

Slices were cultured at the air–liquid interface and maintained using two different protocols. In the first protocol the slices were cultured during 16 days in a

single medium: 50% Minimum Essential Medium Eagle (MEM) (Sigma–Aldrich, St. Louis, MO, USA), 25% Hank's Balanced Salt Solution (Hank's) (Sigma–Aldrich, St. Louis, MO, USA), 25% of decomplemented horse serum (Gibco, Life Technologies, Paisley, UK), 6.5 mg/ml of glucose, 1 mM of L-glutamine (Sigma–Aldrich, St. Louis, MO, USA) and antibiotics. In the second protocol tested, the slices were conserved in two different media. From days 0 to 3, a serum-containing medium identical to first protocol culture media condition was used: 50% MEM, 25% Hank's, 25% of decomplemented horse serum, 6.5 mg/ml of glucose, 1 mM of L-glutamine and antibiotics. From days 3 to 16, a serum-free medium was used: Neurobasal medium (Gibco, Life Technologies, Paisley, UK) supplemented with 6.5 mg/L of glucose, 1 mM of L-glutamine, 1x B27 supplements (Gibco, Life Technologies, Paisley, UK) and antibiotics. The media was changed the first day after culture and then every 2 days during the entire culture period for both culture media protocols.

Histological studies

At different times, ranging from 0 to 16 days, organotypic slices were washed with phosphate-buffered saline (PBS) (Lonza, Verviers, Belgium), fixed with 4% paraformaldehyde (PFA) (Sigma–Aldrich, St. Louis, MO, USA) in PBS pH 7.4 for 2 h and washed three times with PBS. Finally, slices were removed from membrane inserts and stored at 4 °C in PBS until use.

Immunofluorescence studies. Immunofluorescence was performed using antibodies against neuronal nuclei (NeuN) (clone A60, Merck Millipore, Billerica, MA, USA) and dopamine- and cAMP-regulated neuronal phosphoprotein (DARPP32) (BD Transduction Laboratories, Erembodegem, Belgium). Isotypic controls were performed for each antibody. Slices were incubated free-floating in PBS containing 1% Triton (PBST) (Sigma–Aldrich, St. Louis, MO, USA). After pre-blocking for 4 h with 4% bovine serum albumin (BSA, fraction V) (PAA Laboratories, Piscataway, NJ, USA), 10% normal goat serum (NGS) (Sigma–Aldrich, St. Louis, MO, USA) in PBST, slices were incubated for 48 h at 4 °C with mouse anti-NeuN (20 μg/ml in 4% BSA PBST) or with mouse anti-DARPP32 (0.25 μg/ml in 4% BSA PBST). After three washes with PBS, the sections were incubated for 2 h with the horse affinity-purified biotinylated anti-mouse immunoglobulin G (IgG) secondary antibody (7.5 μg/ml, Vector Laboratories, Les Ulis, France) at room temperature. Then, slices were washed and incubated for 2 h with Streptavidin Fluoroprobes R488 or R547H (Interchim, Montluçon, France) diluted 1:200 in PBS. Finally, the sections were rinsed and mounted using fluorescent mounting medium (Dako, Carpinteria, CA, USA).

TH immunohistochemistry. To observe dopaminergic degeneration a mouse anti-rat TH antibody (clone 6D7, Covance, Emeryville, CA, USA) was used and isotypic controls were performed. After pre-blocking for 4 h with 4% BSA, 10% NGS in PBST, slices were incubated for 48 h at 4 °C with mouse anti-rat TH (10 μg/ml in PBST). After three washes with PBS, slices were incubated for 2 h with the goat affinity-purified biotinylated ant-rabbit IgG secondary antibody (7.5 μg/ml in PBST) (Vector Laboratories, Les Ulis, France). Quenching of peroxidase was made with 0.3% hydrogen peroxide (H_2O_2) (Sigma–Aldrich, St. Louis, MO, USA) in PBST, at RT for 20 min. After three washes, sections were then incubated with Vectastain ABC reagent (Vector Laboratories, Les Ulis, France) in PBS was made for 2 h. Finally, sections were washed and revealed with 0.03% H_2O_2, 0.4 mg/ml diaminobenzidine (Sigma, St. Louis, MO, USA) in PBS supplemented with 2.5% nickel chloride and dehydrated before mounting.

Quantification of TH-positive fibers and cells

TH-positive fiber density in the striatum at different time-points ranging from 0 to 16 days was quantified using the Metamorph® software. The TH fiber density was estimated by subtracting the striatum TH staining intensity minus the cortex TH staining intensity, which represents the background. TH staining was expressed in% considering that TH staining at day 0 (intact slice) represents 100%. Results were presented as mean differences ± standard deviation and were calculated from two batches of three consecutive pictures in the center of the striatum and from six consecutive slices in the cortex above the striatum. Those pictures were taken from three different slices for each time-point.

The number of TH-positive neurons in the SN at different time-points ranging from days 0 to 16 was determined. Images were taken from three to four different slices for each time-point, in order to cover the whole SN. The number of positive-cells was manually counted assisted by Metamorph® analysis tool software. Results were presented as mean differences ± standard deviation.

Quantification of NeuN-positive neurons

The survival of total neurons in certain organotypic slice regions was estimated by immunofluorescence against NeuN. NeuN-positive neuron density was calculated, from days 0 to 16 using the Metamorph® software, At each time-point, six pictures taken from three different slices showing cortex, striatum and SN were used. NeuN staining was expressed in % considering that NeuN staining at day 0 (intact slice) represents 100%. Results were presented as mean differences ± average deviation.

Analysis of striatal dopamine content by mass spectrometry

Microdissected striata were dissociated by adding 200 μl of 1× reporter lysis buffer (Promega, Madison, WI, USA) followed by 14 s of ultrasonication. Then, 600 μl of pure acetonitrile were added to precipitate proteins. After a centrifugation (10,000*g*, 15 min, 4 °C), samples were dropped onto an Ostro 96-well plate (Waters S.A., Saint-Quentin, France) following manufacturer's

instructions to purify dopamine. Chromatography was performed using a Waters Alliance 2695 system (Waters S.A., Saint-Quentin-en-Yvelines, France) with a Uptishpere® C18 50DB 150 × 2.0 mm, 5 µm column (Interchim, Montluçon, France). The column was heated up to 40 °C, the mobile phase was composed of water/formic acid 0.1% – methanol/formic acid 0.1% (95/5) from 0 to 8 min and then water/formic acid 0.1% – methanol/formic acid 0.1% (5/95) from 8 to 10 min with a return to normal from 10 to 11 min. The flow-rate was 0.3 mL/min. High-performance liquid chromatography (HPLC) effluent between 1 and 4 min was directed into a Quattro Micro triple quadruple mass spectrometer (Waters S.A., Saint-Quentin, France). Ionization was achieved using electrospray in positive ion mode. The mass spectrometer operated in multiple reactions monitoring (MRM) mode. The (M H) + *m/z* transitions for each compound were 137 and 91. A typical retention time of dopamine was found to be 1.50 min. Quantification was achieved with QuantLynx (Waters S.A., Saint-Quentin, France) by comparison of the observed peak area ratios of dopamine samples to a calibration curve obtained under the same experimental conditions.

Visualization of the MFB by magnetic resonance imaging

Imaging was performed on a Biospec Avance III MR scanner (Bruker Biospin, Wissembourg, France) using a 20-cm bore 7 T magnet equipped with a BGA12S gradient/shim system capable of 675 mT/m maximum gradient strength and a ^{1}H cryoprobe.

For the *in vivo* experiments, the pups were sedated with a mixture of isoflurane/O_2 (~0.1%–0.1 l/min) and wrapped in a cotton blanket positioned over a warm water blanket. After a rapid scout set of images and a first and second order shim over the brain, a two-segments 2D-Echo planar imaging sequence (repetition time TR/Effective echo time TEeff 3500/30 ms) with 46 diffusion encoding directions, γ = 3 ms, γ = 17 ms, leading to a b = 1000 s/mm^2 was performed. The geometrical parameters were fixed at FOV = 14 mm × 14 mm. Three contiguous slices of 0.4 mm. The matrix size was defined at 128 × 96 leading to an in-plane resolution of 109 µm × 146 µm. As the number of experiment (Nexp) was fixed at 2, the total acquisition time was 22 min.

Organotypic slices were imaged at different times ranging from 0 to 16 days Orthogonal scout proton images were obtained using a rapid acquisition with relaxation enhancement (RARE) sequence (TR = 3.200 ms; (TE) = 60 ms; RARE factor = 4; FOV = 1.5 × 1 cm; matrix 256 × 128; Two contiguous slices of 0.3 mm, Nex = 4). A localized first and second order shim was performed on the slice prior to diffusion imaging. A four-segment 2D-Echo planar imaging sequence (TR/TEeff 3500/30 ms) with 46 diffusion encoding directions, =3 ms, =17 ms, leading to a b = 1000 s/mm^2 was performed. The geometrical parameters were fixed at FOV = 15 mm × 10 mm for a slice thickness of 300 µm. The matrix size was defined at 128 × 96 leading to an in-plane resolution of 117 µm × 104 µm. As the number of repetitions was fixed at 4, the total acquisition time was 43 min.

Statistical analysis

Data are presented as the mean value of three independent experiments ± standard deviation (SD), unless otherwise stated. Significant differences between samples were determined using an analysis of variance (ANOVA) test, followed by a Scheffe post hoc test which indicated if conditions were significantly different. *P*-value was set to 0.05, unless otherwise stated.

RESULTS

Morphology of nigrostriatal organotypic slices

After testing different angles of slice, 400-µm sagittal organotypic slices were cut alongside the brain midline (Fig. 1A). Morphological analysis of organotypic brain slices cultured from neonatal tissue just after preparation showed that many brain areas could be clearly identified using bright-field microscopy like the hippocampus, cortex, caudate nucleus-putamen, globus pallidus, ventral pallidum, SN and cerebellum among others (Fig. 1B). The nigrostriatal pathway, which is involved in PD, is composed of dopaminergic fibers that have their cell bodies in the SN, which send axons through the MFB, to the striatum where dopamine is liberated. Striatum observation under bright-field microscope allowed to identify those dopaminergic fibers (Fig. 1C) by TH immunohistochemistry, just as two-photon fluorescence microscopy of TH-immunostained nigrostriatal slices at day 0 allowed to easily visualize the dopaminergic cell soma in the SN and the nigrostriatal pathway from the SN to the striatum (Fig. 1D).

Culture media choice and viability of nigrostriatal organotypic slices

Culturing conditions remarkably affected organotypic culture viability. We found that, when culturing the slices with culture media condition 1 that contains 25% of serum, the slices underwent progressive and rapid flattening after 5–7 days of culture. This deleterious effect of serum has already been described (Kim et al., 2013). However, when culturing the slices with culture media condition 2 where serum-containing media is switched to serum-free media 3 days after the initiation of the cultures, organotypic slice survival was enhanced and a better preservation of the original brain morphology was observed. Using these conditions, organotypic slices can be kept viable for periods longer than 2 weeks in culture. Thus, culture media condition 2 was selected for further experiments.

Using NeuN immunofluorescence, the survival of neurons within specific brain regions of the organotypic slices using media condition 2 was further evaluated (Fig. 2A). Between days 1 and 6, a decrease of less than 10% ± 5% of NeuN-positive cells in the cortex was observed (Fig. 2B). In contrast, a significant 60% ± 10% and 80% ± 1% decrease of NeuN staining

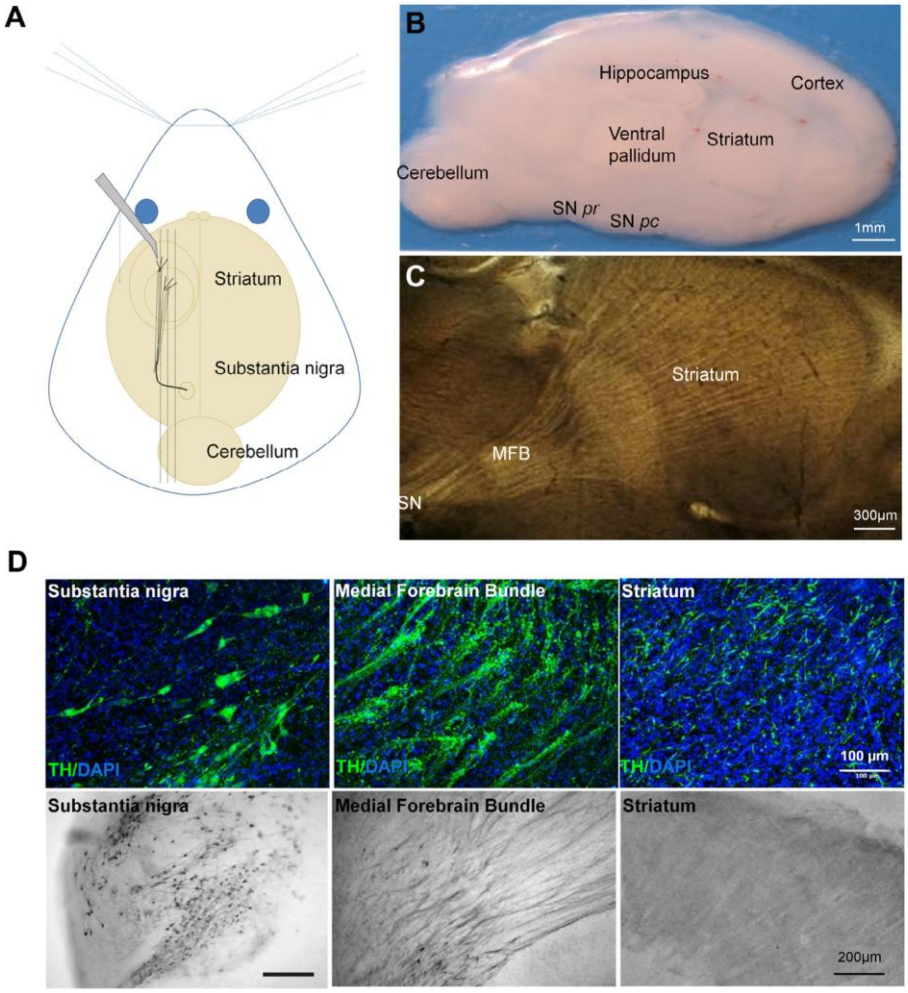

Fig. 1. Morphology of nigrostriatal organotypic slices shortly before preparation. (A) Schematic view of organotypic culture preparation. (B) Morphological integrity of nigrostriatal organotypic slice. (C) Bright-field observation of the dopaminergic medial forebrain bundle (MFB) fibers emerging from the SN to the striatum. (D) Representative images showing fluorescence and bright-field images of TH-immunostained nigrostriatal pathway.

occurred in the striatum and in the SN, respectively, between days 1 and 16 (Fig. 2B). *P*-value was set to 0.05 and experiments were performed with at least three brain slices from three different rat pups/condition.

Organotypic slice cultures display progressive degeneration of the nigrostriatal pathway

A thinning of the striatum within 3 days accompanied by a scarcity of fibers was observed by bright-field microscopy, predicting a reduction in dopaminergic terminals, which seemed to be total at day 16 (Fig. 3A). This dopaminergic deafferentation in the MFB was confirmed by immunochemistry against rat TH. A continuous loss of TH-positive fibers localized in this area was observed. At day 16, there were no TH-positive fibers left (Fig. 3A). A quantification of the striatal fiber diminution was made using Metamorph analysis tool. A 30% ± 20% decrease of TH fibers was detected at day 2 compared to day 0, reaching a decrease of 40% ± 20% at day 3. TH staining reached a 90% ± 15% decrease at day 4 and disappeared until day 16 ± 10% (Fig. 3B). *P*-value was set to 0.05 and experiments were performed with at least three brain slices from three different rat pups/condition.

Moving upstream in the nigrostriatal circuit, the number of TH-immunoreactive neurons in the SN was evaluated at days 0, 2 and 16. An important decrease of dopaminergic neurons in this area was already observed after 24 h, reaching 80% ± 20% after 48 h and being maximal after 16 days, with a 90% ± 15% loss (Fig. 3C). *P*-value was set to 0.01 and $n = 5$. The severe loss of DA nerve terminals in the striatum was accompanied with an important loss in the neurons of the SN.

Organotypic slice cultures showed loss of striatal dopamine content

Dopamine content in the striatum of the slices was measured by LC–MS/MS at different times after

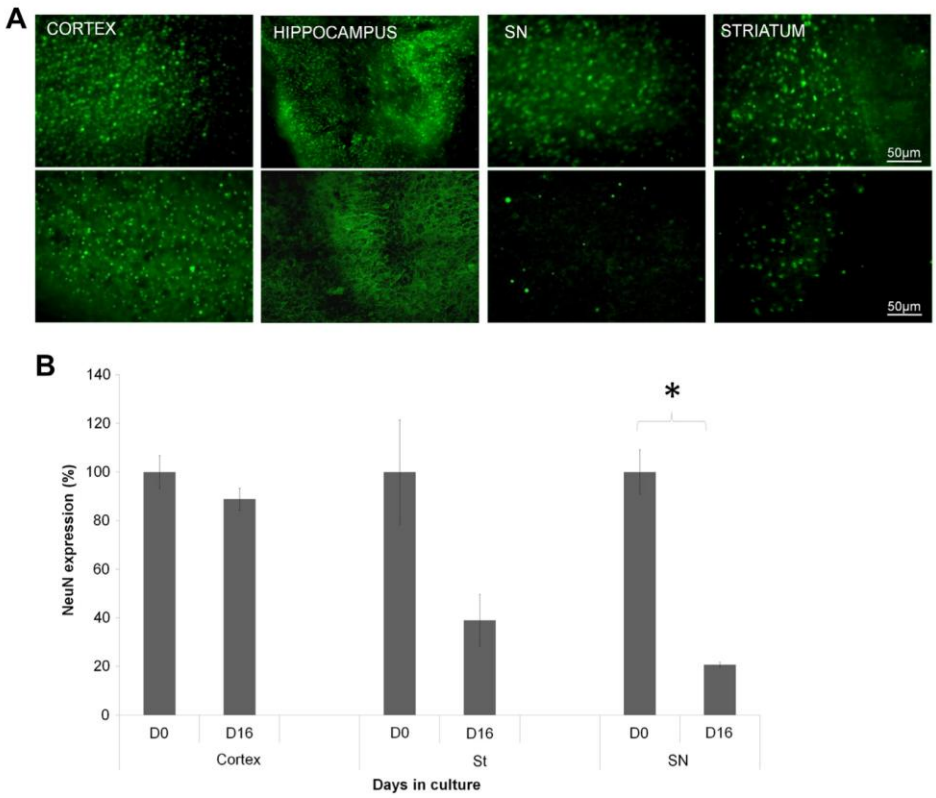

Fig. 2. Viability of nigrostriatal organotypic slices. (A) Immunofluorescence against neuronal nuclei (NeuN) showing four brain regions: cortex, hippocampus, striatum and substantia nigra (SN) at day 1 at the top and at day 16 at the bottom. (B) Quantification of the density of NeuN-positive cells in cortex, striatum and SN shows no important loss of staining in the cortex after 16 days in culture while NeuN expression decreased by 60% and 80% in the striatum and the SN respectively. *Significantly different results with $n = 3$ and P-value $= 0.05$.

organotypic preparation. As can be seen in Fig. 3D, dopamine content in the striatum diminished in the time-period examined. After 3 days of culture a reduction of 30% ± 15% of dopamine content was detected with a dopamine concentration decreasing from 65 ± 7 pg/ml at days 0 to 45 ± 5 pg/ml at day 3. Then, similar striatal dopamine content levels were found between days 3 and 16 (Fig. 3D). P-value was set to 0.05 and experiments were performed with at least three brain slices from three different rat pups/condition.

Delayed DARPP32-positive medium spiny neuron degeneration

An analysis by immunofluorescence of DARPP32-positive medium spiny neurons, the most numerous in the striatum, was performed at days 3, 6, 7, 11 and 16. Results showed that these neurons seemed to be only slightly affected by mechanical denervation before day 7. After 7 days in culture, a loss of about 50% of the striatal medium spiny neurons (MSNs) was detected; this loss may reach about 90% of the MSNs after 11 days in culture and seems to be total after 16 days. The loss of GABAergic MSCs observed from days 7 to 16 might not be due to the degeneration of the slice over the culture period since NeuN immunostaining showed that organotypic slices can be kept viable for periods longer than 2 weeks in culture (Fig. 4).

Organotypic slice cultures presented progressive loss of the MFB

Fiber MRI tractography was used to demonstrate the orientation and integrity of the MFB fibers *in vivo*. Cellular barriers encountered in highly directionally oriented tissues, such as white matter fibers, result in anisotropic water diffusion (smaller diffusion perpendicular than parallel to the fiber axis). The degree of directionality of diffusion can be quantified by the measure of the FA parameter. FA value ranges from 0 to 1 when anisotropy increases (Shepherd et al., 2003, 2006b). MFB visualization using tractography was possible in living rats (Fig. 5A). Then, MRI tractography of ex vivo slices from days 0 to 16 was performed, and despite the thinness of the tissue, MFB fibers could be observed. A progressive reduction of the FA from days 0 to 7 was detected (Fig. 5B). It reached 40% ± 10% at 24 h, 70% ± 15% at 48 h and 80% ± 1% at day 7. Afterward, the MFB could not be distinguished from the surrounding tissue and measurement could not be

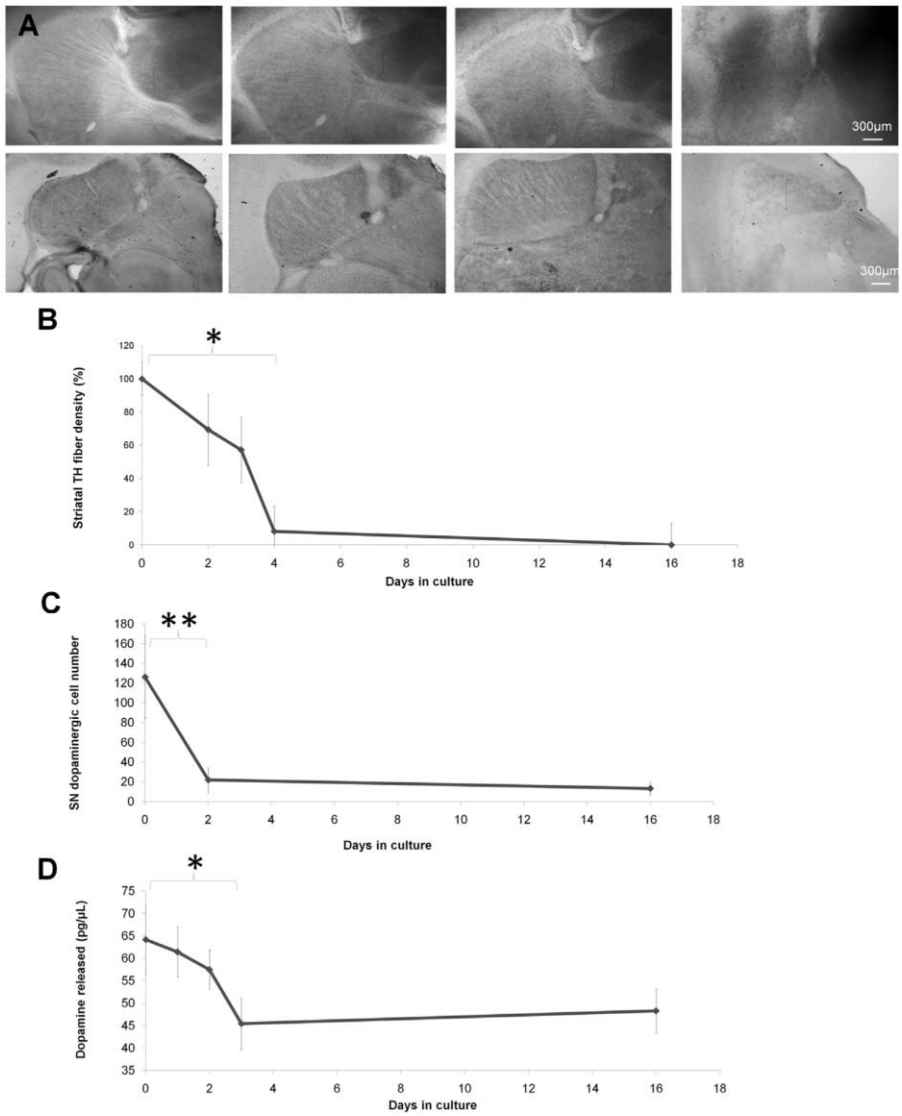

Fig. 3. Organotypic slice cultures display progressive anterograde degeneration of the nigrostriatal pathway. (A) Bright-field microscope observation (top) and TH immunostaining (bottom) allowed to observe dopaminergic fibers of the nigrostriatal pathway at days 0, 2, 3 and 16 (from left to right). A slimming of dopaminergic fibers of the MFB and the striatum is observed shown by the red arrows. (B) Striatal TH-positive fiber quantification. TH density in the striatum was of 70% at day 2, 60% at day 3 and 10% at day 4 compared to day 0 ($n = 3$). (C) Dopaminergic neuron quantification in the SN. The number of dopaminergic neurons remaining in the SN reached 20% at day 2 and 15% at day 14 ($n = 5$; P-value = 0.01). (D) Quantification of dopamine content in the striatum by LC–MS/MS. This quantification was based on the addition of the two masses of dopamine: M91 and M137. A 30% loss of striatal dopamine content was observed ($n = 3$). *Significantly different results with P-value = 0.05 unless otherwise stated.

unambiguously performed (Fig. 5C). P-value was set to 0.01 and experiments were performed with at least three brain slices from three different rat pups/condition.

Furthermore, TH immunochemistry showed a rapid degeneration of dopaminergic fibers in the MFB as well. Just after slice preparation, MFB fibers were intact. Three days later, an important degeneration occurred and varicosities were evident. At day 14, no more dopaminergic fibers were detected in the MFB by TH immunohistochemistry (Fig. 5B).

DISCUSSION

Organotypic cultures have great potential for disease modeling providing an ideal platform between *in vitro* and *in vivo* studies. They are easy to prepare and culture and interestingly, when sectioned appropriately, they retain the architecture and microenvironment of the original organ. Hence, organotypic brain slices have been used as models for neurological disorders such as stroke, epilepsy, cerebral ischemia or PD, among others (Daviaud et al., 2013). The present work sought to

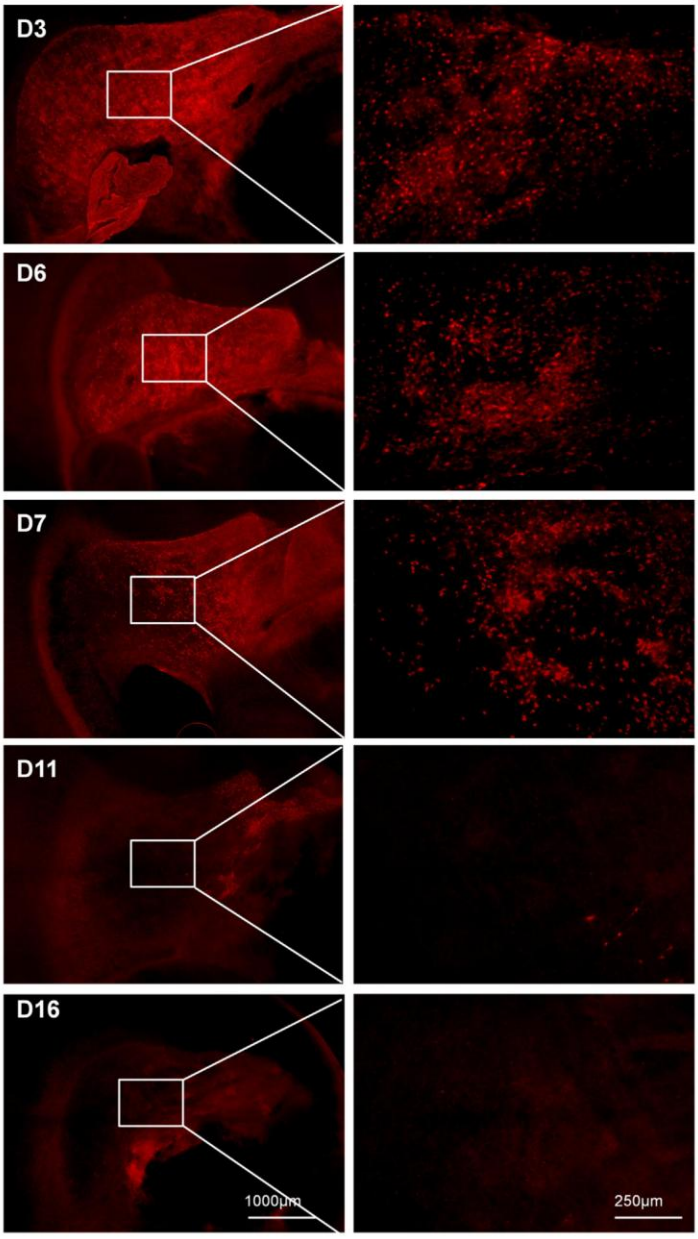

Fig. 4. Immunofluorescence against DARPP32, which highlights GABAergic medium spiny neuron (MSN) presence. Before day 6, no major differences were found, while a slight loss of DARPP32-positive neurons was detected at day 7. This decrease reached a significant level at day 11 and was total at day 16. Right column represents a 10-fold magnification of the area of the striatum in the insert.

develop and characterize a new ex vivo model able to mimic early and late stages of PD based on the mechanical transection of the nigrostriatal pathway obtained during slice preparation. This is the first report that characterizes progressive nigrostriatal degeneration in organotypic slices using MRI and the evaluation of dopamine content using quantitative mass spectrometry together with immunohistochemistry and immunofluorescence.

Previous PD organotypic models used principally SN (Jaeger et al., 1989), ventral mesencephalon–striatum (Ostergaard et al., 1990), entorhinal–hippocampal (Diekmann et al., 1994), cortex–striatum (Plenz and Aertsen, 1996a,b) or complex triple slice cultures (Plenz and Kitai, 1996) that did not allow an easy study of all brain regions involved in this pathology. In the present work, brain sagittal slices were used since they contain

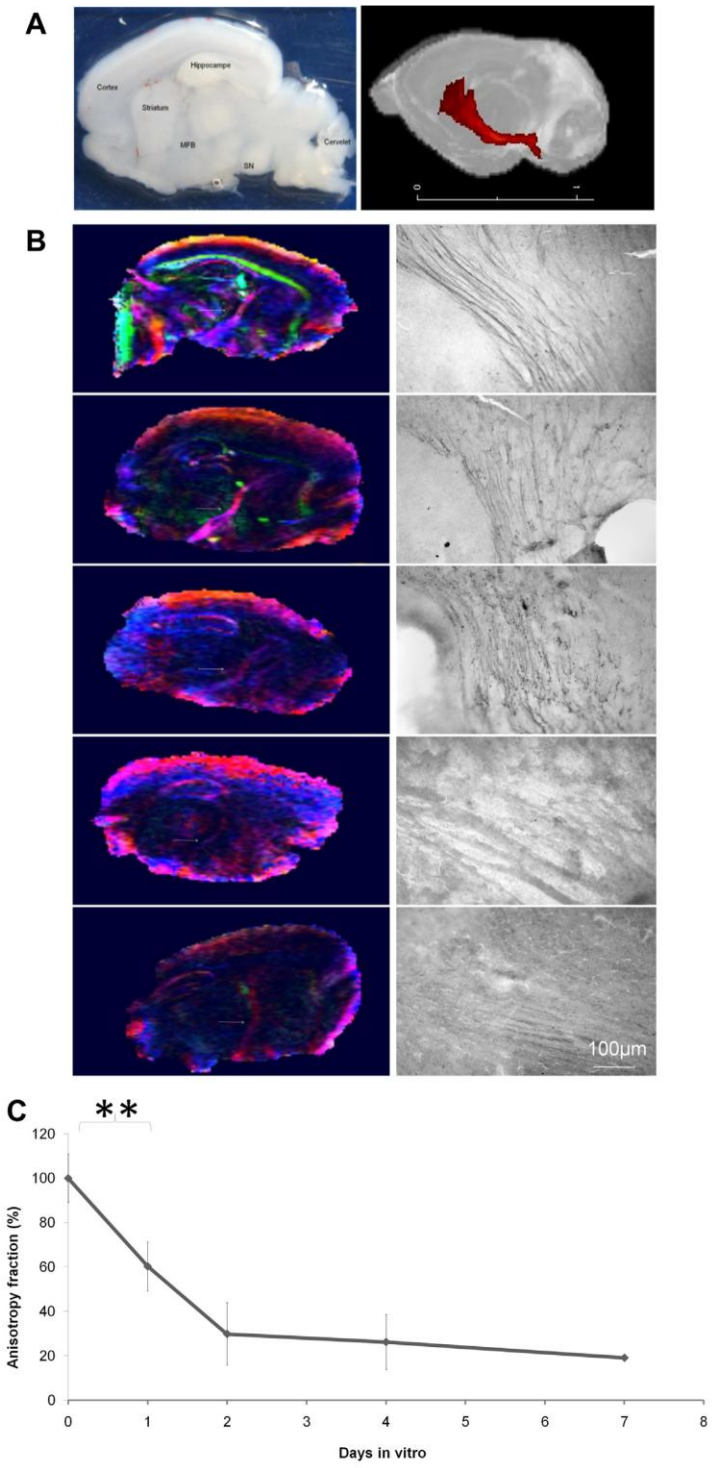

Fig. 5. Organotypic slice cultures presented a progressive loss of dopaminergic fibers in the medial forebrain bundle. (A) Observation of the medial forebrain bundle in living rat by MRI. (B) Observation of the medial forebrain bundle in organotypic slices from days 0 to 7 (from top to bottom) by MRI (indicated by the arrows) and immunochemistry against rat TH. (C) Anisotropy fraction quantification of the MFB fibers. A loss of anisotropy of 40% was observed after 24 h, 70% after 3 days and reached 80% after 7 days.*Significantly different results with $n = 3$ and P-value = 0.01.

in a unique slice many anatomical structures as corpus callosum, cortex, hippocampus, striatum, globus pallidus, SN and cerebellum among others. In addition, many of the original neuronal connections between brain structures are better preserved in brain sagittal slices (Ullrich et al., 2011). Furthermore, the subgranular zone of the dentate gyrus and the subventricular zone, two neural stem cell niches, are conserved which allows to study neurogenesis (Garzon-Muvdi and Quinones-Hinojosa, 2009). Sagittal sections remain the most complete type of organotypic slices since they allow the observation of cholinergic, GABAergic and dopaminergic neurons as well as of vascular brain capillaries or cortico-striatal and cortico-hippocampal nerve fibers (Kearns et al., 2006; Ullrich et al., 2011).

Organotypic slices are generally cultured using either, the roller tube culture method described by Gahwiler (1981) with a slice survival rate of up to 6–8 weeks, or the membrane interface air-medium method developed by Stoppini et al. (1991), which offers a survival of about 2–3 weeks. The membrane interface technique that maintains brain slices on a porous membrane filter at the interface between medium and a humidified atmosphere was used in the present study, since this method provides an easier access to the slice culture preparation. Regarding the culture of the slices, the selected culture media protocol used a serum-free media from days 3 to 16. This allows having a well controlled media and studying solely the effect of added drugs or cell grafts on the behavior of brain cells. Ullrich et al. (2011) observed that, in their organotypic whole brain model, nerve growth factor or glial cell line-derived neurotrophic factor addition in the serum-containing media was necessary to support cholinergic or dopaminergic neuron survival, respectively. Moreover, with this culture media protocol whole slice survival seemed to be good enough with no significant flattening or distortion. In contrast, Ullrich et al. (2011) observed that, in their organotypic whole brain model, nerve growth factor or glial cell line-derived neurotrophic factor addition in the serum-containing media was necessary to support cholinergic or dopaminergic neuron survival, respectively. However, a culture media containing 25% of heat-inactivated horse serum was used in that study. It has been reported that serum-containing media may induce a strong degeneration of neurons in slice culture (Kim et al., 2013). In other studies (Kearns et al., 2006; Cavaliere et al., 2010) in which organotypic slices were cultured in a serum-free media with B27 supplements similar to our culture conditions, a long-term viability of dopaminergic neurons in organotypic culture was observed. Furthermore, we did not notice an important cell death, by NeuN staining, in the cortex or hippocampus, two regions that are not affected by the sectioning. This proved that a good neuronal survival was obtained, at least until 16 days, allowing the analysis of the pharmacological effects of injected drugs or cells on the organotypic slices during this time-period.

Previous studies of the nigrostriatal pathway used cultures and co-cultures with no loss of dopaminergic fibers that only allow assessing developmental, plastic or regenerative properties of DA neurons (Jaeger et al., 1989; Ostergaard et al., 1990; Diekmann et al., 1994; Plenz and Kitai, 1996; Plenz and Aertsen, 1996a; Ullrich et al., 2011). The single organotypic slices developed by Cavaliere et al. (2010) or Kearns et al. (2006) containing the nigrostriatal pathway could be conserved in culture about 12–14 days and 3 weeks, respectively, without SN dopaminergic neuron loss. This difference of survival could be explained by the fact that Kearns used mouse brain slices which are smaller and easier to keep in culture while Cavaliere used rat brain slices. The PD model developed by Kearns, uses 300–400 μm slices of the brain hemispheres cut in the parasagittal plane using a vibratome. To obtain the dopaminergic neuronal loss those slices were incubated in a 20 mM 6-OHDA bath for 10 min (Kearns et al., 2006). In the model developed by Cavaliere, axial slices of the hemispheres are performed with an angle of 45° compared to the base of the brain, therefore along the axis of the MFB without sectioning the fibers. Consequently slices can be conserved in culture with no dopaminergic degeneration. To obtain the dopaminergic neuronal loss, two protocols were tested. In the first one, slices were incubated in a 100 μM 6-OHDA bath for 60 min and in the second protocol, a transection in the middle of the MFB was performed (Cavaliere et al., 2010). After the 6-OHDA treatment, degeneration observed by Kearns et al. (2006) reached 46% in the SN and 60% in the striatum, after 2 weeks in culture while Cavaliere et al. (2010) observed a 30% degeneration both in the SN and in the striatum within 24 h. The hand-operated denervation tested by Cavaliere induced a larger degeneration as 60% decrease in TH-positive neurons was observed both in the SN and in the striatum after 3–5 days *in vitro*. In the present model, MFB dopaminergic fiber death is induced during slice preparation. Indeed, the slice is performed along the nigrostriatal pathway, perpendicular to the one made by Cavaliere, so that the MFB fibers are sectioned at their base inducing the degeneration (Fig. 1).

The characterization of the progressive nigrostriatal degeneration was achieved by combining different techniques. MRI studies showed a 70% MFB fiber FA decrease in 2 days, suggesting fiber degeneration. This method has been used to address brain tissue structure on sliced and fixed samples (Shepherd et al., 2006a; Flint et al., 2010) and to demonstrate early pathological changes in parkinsonian patients (Yoshikawa et al., 2004), but to the best of our knowledge, this is the first report describing nigrostriatal pathway degeneration in an organotypic slice PD model by DT-RMI. TH immunostaining quantification revealed a rapid loss of 80% of the dopaminergic cell bodies in the SN and of 30% of striatal fiber density at 2 days. A 15% loss of striatal dopamine content quantified by mass spectrometry after 2 days was also observed, as a result of the terminal loss in the striatum. Those results suggest that in our model, the dopaminergic degeneration occurs first in the SN area, which then affects the MFB and striatal fibers. It is difficult to

correlate this observation with PD pathology as the primary cellular locus of dysfunction is a controversial issue (Kordower et al., 2013). This anterograde fiber degeneration confirms the report of Beirowski et al. (2005) showing this directionality in fiber degeneration after sectioning. The relatively quick dopaminergic nigrostriatal pathway degeneration allows obtaining a therapeutic window of around 14 days to study novel therapeutic approaches.

The lesion seems to be quite localized at first to the nigrostriatal pathway, but from days 7 to 16 an important loss of striatal GABAergic MSN occurred. The late GABAergic pathway degeneration observed after 8 days in culture could be due to the damage induced after the sagittal slice preparation as previously reported by Cavaliere et al. (2010). This strong striatal MSN death explains the important decrease of NeuN expression in the striatum as GABAergic MSN represents about 90% of the striatal neurons. Thus, this new ex vivo PD model could be used to model early and late stages of the pathology. Actually, from days 0 to 8, a degeneration of the nigrostriatal pathway occurred associated with a dopamine loss in the striatum which corresponds to an early stage of the disease. Then, from days 8 to 14, a degeneration of GABAergic neurons appears which matches the later PD phase.

In spite of the high complexity of PD, experimental models that mimic as close as possible the symptoms and the temporal progression of the disease are needed. Obviously, the model presented in this study differs from a chronic neurodegenerative disorder. Indeed in the human pathology, neurodegeneration is very progressive, occurs over several years and declares mainly in people over 60 years. In our model, the neurodegeneration is observed in 2 weeks, in 7-days-old rat pups which differs from the human pathology. However, despite their limitations, this model offers a good possibility to choose the degree of degeneration and therefore the stage of the disease. Furthermore a new therapeutic approach can be tested in only a few weeks allowing a rapid screening. Recently also adolescent and adult rats have been used and even human post-mortem tissue slices have been kept alive for a maximum time of 1 week (Finley et al., 2004; Su et al., 2011; Fernandez-Bueno et al., 2012).

Moreover, in PD, dopaminergic neuronal death may be induced by the formation of Lewy bodies mainly composed of α-synuclein aggregation and other aggregated proteins (Volles and Lansbury, 2003; Spillantini et al., 1997). Unfortunately, Lewy body formation was not analyzed in our model. However, it has also been shown that neuronal death in the SN may occur in part by an important overproduction of reactive oxygen species (ROS) which may lead to Lewy bodies formation and toxicity (Blandini, 2013). Thus, since a 2 μM concentration of ROS was detected in intact hippocampal organotypic slices (Ganesana et al., 2012), ROS formation may be re-created in our model during sagittal slice preparation as well. Indeed, the slice preparation induces an important lesion and a little inflammation of the slice can be observed during the first day in culture. It could be interesting to study this possibility in future studies.

As dopaminergic degeneration has been completely characterized using several techniques, this allows choosing the degree of dopaminergic denervation before starting the screening of new treatments. Indeed, this model would be useful to test both neuroprotective and neuroregenerative strategies. In addition, the model might be a useful tool to understand the fundamental molecular mechanism underlying degeneration. Within this line, axonal regeneration (Ishihara et al., 2011) and glial response (Ziemka-Nalecz et al., 2013) after axotomy could be studied in the model presented here. Moreover, using MRI that produces detailed images of a living brain tissue over time without using radioactive ligands (Petridou et al., 2006), fiber tracts can be visualized in organotypic slices and pathway integrity assessed by calculation of FA and the study of neuronal metabolism using magnetic resonance spectroscopy (Delli Pizzi et al., 2013). This could be used to evaluate the neuroregenerative potential of axonal growth therapeutics among others (Ullrich et al., 2011). To this end, efficacy changes over time can be evaluated using the same treated organotypic slice, thus reducing costs. As demonstrated in this study, LC–MS/MS can also be used to quantify a neurotransmitter. In addition the association of mass spectrometry and microdissection allows performing this neurotransmitter analysis in a chosen area and not only in the culture media (Krabbe et al., 2009).

In summary, this model represents a promising tool to quickly and efficiently test new and better treatments for PD, including cell therapy or tissue engineering. The development and optimization of simple ex vivo models with precise control of the extracellular environment and a complete characterization of the neurodegeneration, allows studying not only the therapeutic effect of the treatment under evaluation but also stem cell responses and the role of the microenvironment. A better comprehension of all the parameters involved in the therapeutic effect is thus obtained. Using this model, a comparative study between two stem cell types associated with microparticles for PD treatment is currently being performed (Daviaud et al., 2011, unpublished results).

CONCLUSION

We have prepared and characterized a relevant ex vivo model of PD using innovative and conventional techniques. This new model allows to study dopaminergic, cholinergic or GABAergic neurons as well as blood vessels or glial cells and their interactions. It is easy to obtain, and the dopaminergic degeneration is reproducible and does not involve complicated surgical process, and does not need neurotoxin injection. Furthermore, this model can be use to study the early stage of the pathology, namely dopaminergic

neurodegeneration, or later stage of the disease with dopaminergic and GABAergic neuron loss.

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DISCUSSION

L'utilisation de coupes organotypiques a permis le développement d'un nouveau modèle de la maladie de Parkinson. Ce nouveau modèle se veut simple d'utilisation. En effet, la section de la voie nigrostriée se fait durant la préparation des coupes sagittales de cerveau. Ainsi aucune manipulation supplémentaire n'est nécessaire, telle que l'injection de neurotoxines ou la dénervation manuelle, ce qui diminue le risque d'obtenir des biais techniques durant la préparation du modèle, le rendant plus accessible et reproductible. De plus ce type de coupe sagittale permet l'étude de nombreuses aires cérébrales telles que le cortex, l'hippocampe, le striatum, le faisceau médian du télencéphale, la substance noire ou encore le thalamus. Ces coupes permettent donc d'étudier de nombreuses voies : dopaminergiques, GABAergiques, sérotoninergiques ou encore les capillaires sanguins (Ullrich et al., 2011).

Une caractérisation poussée du modèle a été effectuée avec des techniques classiques telles que les immunomarquages et avec des techniques novatrices telles que l'imagerie à résonnance magnétique (IRM) et la spectrométrie de masse. Nous avons pu démontrer que les coupes développées possèdent une survie maximale de 16 jours. De plus, la mort des neurones dopaminergiques dans la substance noire est observée en 2 jours. Il s'en suit alors une dégénérescence antérograde des fibres du faisceau médian du télencéphale détectée par IRM. Enfin une déafférentation totale des fibres dopaminergiques localisées dans le striatum est observée par immunomarquage, corrélée à une baisse de la dopamine dosée par spectrométrie de masse. Après 7 jours en culture, une dégénérescence importante des neurones épineux moyens GABAergiques du striatum a été constatée et devient totale après

11 jours en culture. De ce fait le modèle développé permet d'étudier la maladie de Parkinson à un stade précoce, c'est-à-dire avec seulement une dégénérescence des voies dopaminergiques ou à un stade plus tardif dans lequel une dégradation des voies GABAergiques s'ajoute à celle des voies dopaminergiques.

Le but de notre étude est de mieux comprendre les mécanismes cellulaires et moléculaires liés à une amélioration comportementale des rats parkinsoniens suite à une greffe de cellules souches combinées ou non à des MPA (Delcroix et al., 2010a). Il est donc nécessaire de travailler dans un modèle de lésion partielle afin de pouvoir étudier le phénomène de neuroprotection. Un modèle de lésion partielle de la voie nigrostriée est obtenu en 2 jours ce qui laisse une fenêtre thérapeutique de 14 jours. Ainsi lors de greffes de cellules souches couplées ou non aux MPA dans ce modèle, des études de survie et de différenciation des cellules greffées ainsi que des études du comportement du tissu hôte liées notamment à l'évaluation de la sécrétion de facteurs neurotrophiques, pourront être effectuées, durant ces 14 jours, de manière beaucoup plus rapide, simple et moins coûteuse que par l'utilisation de modèle *in vivo*.

CHAPITRE 3

Comment les MPA enrobés de laminine et délivrant du NT-3 associés aux cellules MIAMI et aux CSN permettent d'obtenir une réparation/protection de la voie nigrostriée dans un modèle ex vivo de la maladie de Parkinson ?

INTRODUCTION

Comment les MPA enrobés de laminine et délivrant du NT-3 associés aux cellules MIAMI et aux CSN permettent d'obtenir une réparation/protection de la voie nigrostriée dans un modèle *ex vivo* de la maladie de Parkinson ?

Les microcarriers pharmacologiquement actifs (MPA) sont utilisés en ingénierie tissulaire afin d'améliorer la survie, la différenciation et l'intégration de cellules transplantées dans un tissu hôte. En effet, les MPA apportent aux cellules qu'elles convoient un support biomimétiques en trois dimensions associé à la libération prolongée et contrôlée d'un facteur de croissance (Tatard et al., 2005, Garbayo et al., 2011a).

La preuve de concept a été démontrée dans un modèle de rats parkinsoniens. L'utilisation de MPA recouvertes de fibronectine libérant du NGF (nerve growth factor) associés aux cellules PC12 (Tatard et al., 2004) et l'utilisation de MPA libérant du GDNF associés à des précurseurs dopaminergiques du mésencéphale ventral (Tatard et al., 2007b) ont tous les deux permis d'obtenir une augmentation de la survie des cellules greffées et une différenciation vers un phénotype dopaminergique. De plus une amélioration du comportement moteur des rats a également été observée.

Les CSN sont les cellules souches propres du système nerveux central. Elles représentent une référence de différenciation neuronale. Pour palier à des problèmes d'ordre éthique lors de l'utilisation de CSN prélevés chez le fœtus humain, nous avons décidé d'utiliser une lignée de CSN humaine exprimant la GFP (green

flurorecent protein). Cette lignée est immortalisée par l'expression de vMyc dont le promoteur est sous contrôle de l'EGF et du bFGF. De ce fait une simple suppression de ces facteurs de croissance induit une différenciation neuronale des cellules (Villa et al., 2000). Les cellules MIAMI sont une sous population homogène de cellules souches mésenchymateuses présentant un profil d'expression génique unique (Roche et al., 2013). Leur survie et leur différenciation en cellule de type neuronal sont dépendantes de la présence du NT-3 (Tatard et al., 2007a). Par la suite, il a été démontré que la laminine permettait d'induire une survie et une différenciation importante des cellules MIAMI (Delcroix et al., 2011).

Il a alors été démontré que l'association des MPA enrobés de laminine et libérant du NT-3 (LM-MPA-NT3) permet d'augmenter la survie des cellules MIAMI et leur différenciation vers des cellules exprimant la TH après greffe dans des rats hémi-parkinsoniens. Une stimulation de la neuroprotection et de la croissance de fibres dopaminergiques du système nigro-strié associée à l'amélioration du comportement des rats parkinsoniens fut observée suite à la greffe de ces complexes. Il a également été démontré que le NT3 permet d'augmenter la survie et la différenciation des CSN (Lu et al., 2011, Yang et al., 2010). De plus ce type cellulaire a déjà été utilisé pour des essais cliniques de la maladie de Parkinson, avec un résultat intéressant (Levesque et al., 2009). Toutefois, les mécanismes associés à l'amélioration comportementale liée à ces nouvelles approches thérapeutiques nécessitent d'être étudiés de manière plus approfondie. En effet, afin d'apporter une nouvelle approche thérapeutique en essais pré-cliniques puis cliniques, il est non seulement nécessaire de prouver les bénéfices apportés par cette approche comparée aux autres déjà en place mais aussi de pouvoir expliquer et gérer chaque paramètre lié à ces bénéfices.

Ce chapitre décrit l'étude des mécanismes cellulaires et moléculaires liés à l'amélioration du comportement des rats. Pour cela, le modèle *ex vivo* de la maladie de Parkinson développé dans le chapitre précédent est utilisé. L'effet de la stratégie thérapeutique, consistant à l'injection de LM-MPA-NT3 associés à des cellules MIAMI, sur la protection de la voie dopaminergique nigrostriée, a dans un premier temps du être confirmé. Des cellules MIAMI et des CSN humaines ont été greffées seules ou adhérées à des MPA enrobés de laminine et délivrant du NT3 dans le striatum de coupes organotypiques (modèle *ex vivo* de la maladie de Parkinson). Le devenir de chaque type cellulaire a été étudié, ainsi que les réponses du tissu hôte suite à ces greffes. Enfin nous avons analysé les facteurs neuroprotecteurs exprimés par les deux types de cellules avec et sans les MPA afin de comprendre leurs mécanismes d'action. De plus nous avons à chaque fois comparé le devenir et le comportement des cellules MIAMI à celui des CSN, qui sont les cellules souches naturelles du SNC, et représentent donc ici une référence en terme de différenciation neuronale.

Neuroprotective mechanisms of human stem cells complexed with pharmacologically active microcarriers in an *ex vivo* model of Parkinson's disease

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Abstract:

Parkinson's disease (PD) is an incurable neurodegenerative disorder. Cell therapy has great potential but also some limits to locally repair nigrostriatal pathway. We have previously demonstrated recovery after treatment with human marrow-isolated adult multilineage inducible cells (MIAMI) cells when adhered on neurotrophin-3 (NT3) releasing pharmacologically active microcarriers (PAMs) in rat model of PD. In this study, we extend this analysis by elucidating survival, differentiation and neuroprotective mechanisms of MIAMI cells compared to human neural stem cells (NSC) both adhered to NT3 releasing PAMs in an *ex vivo* model of PD. Stem cells differentiated into dopaminergic neuron-like cells *in situ*, and PAMs improved stem cell survival and differentiation when releasing NT3. qPCR analysis showed that the neuroprotective effect of these cells could be mediated by VEGF, HGF or GDNF for MIAMI cells and STC1 for NSCs. Secreted human VEGF and human STC1 were quantified in the culture medium of organotypic slices. VEGF induced angiogenesis/protection of blood vessels that may improve graft survival besides a direct VEGF protection potential of dopaminergic neurons. These results show a prospective interest of human NSC-PAMs and MAMI-PAMs in tissue engineering for PD.

Keywords:

Parkinson’s disease, neural stem cells, Marrow-isolated adult multilineage inducible cells (MIAMI), organotypic slice culture, Neurotrophin-3, Laminin

Abbreviations:

basic fibroblast growth factor (bFGF), brain derived neurotrophic factor (BDNF), complementary DNA (cDNA), Disease Rating Scale (UPDRS), diaminobenzidine (DAB), Dorsal Root Ganglia (DRG), Dulbecco's Modified Eagle Medium (DMEM), dopamine (DA), epidermal growth factor (EGF), extracellular matrix (ECM), glial cell line derived neurotrophic factor (GDNF), green fluorescent protein (GFP), hepatocyte growth factor (HGF), human mitochondria (hMito), human stanniocalcin-1 (hSCT1), human vascular endothelial growth factor (hVEGFA), Laminin (LM), Marrow-isolated adult multilineage inducible cells (MIAMI), mesenchymal stromal cells (MSC), messenger RNA (mRNA), nerve growth factor (NGF), Neural stem cells (NSC), Neurotrophine-3 (NT3), Parkinson’s disease (PD), pharmacologically active microcarriers (PAMs), Phosphate-Buffered Saline (PBS), Poly-D-lysine (PDL), poly(lactic-co-glycolic acid) (PLGA), retro transcription quantitative polymerase chain reaction (RT-qPCR), tyrosine hydroxylase (TH), Unified Parkinson’s Disease Rating Scale (UPDRS)

1. Introduction

Parkinson's disease (PD) is a chronic and progressive neurodegenerative disorder. The neurological symptoms mainly result from the degeneration of the nigro-striatal dopaminergic neurons that lead to a severe dopamine (DA) deficiency in the striatum required for motor control. Currently there are no curative treatments for this neurodegenerative disorder and existing symptomatic treatments may cause severe motor side effects in the long term (1-2). Interestingly, neuronal degeneration in PD is relatively localized at first, before expansion to other brain areas, so a localized striatal DA release by a cell replacement therapy is an alternative therapeutic strategy. Cell transplantation studies led to a certain improvement of motor functions, but overall, poor cell availability and survival of the transplanted cells limit its application (3-4). Among the different cell sources, neural stem cells (NSC) and mesenchymal stromal cells (MSC) are good candidates for cell therapy studies because of their potential to differentiate into neuron-like cells (5). In addition, they have already been tested in clinical trials for PD demonstrating the feasibility, efficacy and safety of this alternative treatment (6). Stereotactic injection of autologous adult NSCs produced an increase in DA uptake within the putamen and an improvement of the Unified Parkinson's Disease Rating Scale (UPDRS) of more than 80%. However, symptoms reappeared after 60 months (7). Stereotaxic implantation of autologous bone-marrow-derived MSC (8) or intra-arterial injection (9) also induced a 30% and a 50% improvement in the UPDRS, respectively, allowing the reduction of the medication. However, despite these encouraging results, stem cell-based therapies for PD are still facing poor survival and engraftment of cells that limit their effectiveness and use. All this evidence supports the need to implement strategies

that will enhance stem cell survival, differentiation and engraftment, leading to functional recovery.

In this context, pharmacologically active microcarriers (PAMs) provide a powerful tool to improve cell engraftment. PAMs are biodegradable and non cytotoxic polymeric microspheres, made of poly lactic-co-glycolic acid (PLGA) coated with extracellular matrix (ECM) proteins that provide a 3-dimensional (3D) support for the cells. Furthermore, PAMs can release a growth factor in a controlled and prolonged manner during microsphere degradation. These combined properties increase cell survival, differentiation and integration in the host tissue (4, 10). The proof of concept of the capacity of PAMs associated with marrow-isolated adult multilineage inducible (MIAMI) cells for neural tissue repair has recently been obtained in PD and in cerebral ischemia rat models (11-12). MIAMI cells are a homogeneous human MSC subpopulation with a unique gene expression profile, which can overcome the problem of heterogeneity and inconsistent effects usually observed with MSC (13). It was demonstrated that survival and neuronal differentiation of MIAMI cells was dependant of the presence of neurotrophine-3 (NT3) in the culture media. (14). In addition, the pre-treatment of MIAMI cells with epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) and a laminin (LM) substrate enhanced this differentiation and allowed a better response to neuronal commitment (15). In a rat model of PD, striatal implantation of MIAMI cells pre-committed towards the dopaminergic phenotype adhered onto LM-coated PAMs releasing NT3 (LM-PAM-NT3) improved stem cell survival and dopaminergic differentiation. This led to the protection/repair of the nigro-striatal pathway and to functional recovery of the PD rats (11). Furthermore, implantation of EGF and bFGF pre-treated MIAMI cells also induced functional recovery in PD rats probably, due to the release of glial cell line

derived neurotrophic factor (GDNF) (15). Further studies are needed to fully understand the stem cell mode of action in this PD context and the contribution of PAMs to neural repair, in order to maximize the therapeutic potential of this approach. However, the use of *in vivo* models for investigating the effects of therapeutic approaches is time consuming and involves high technical and financial resources.

Organotypic cultures made from brain slices, which can be kept in culture for several weeks after injecting molecules or cells, represent a remarkable tool to address cell therapy issues. Grafts of NSCs into organotypic slices have been performed showing a functional integration of stem cells within the host tissue (16-17). Furthermore, organotypic slices can model the pathological state of PD. Thus, it is possible to test the potential of stem cells in a PD context (for review see (18)). To this end, we previously developed an *ex vivo* model of PD in which dopaminergic fibers degeneration is obtained without neurotoxin injection. In that model, a 30% striatal dopaminergic deafferentation is obtained within 2 days and became total after 4 days (19).

Using this *ex vivo* model of PD, we sought to investigate and compare the therapeutic effects of MIAMI cells and NSCs when administered alone or combined with LM-PAMs releasing or not NT3. To our knowledge, this is the first report of a comparative study of the effect of two different adult stem cells in the same model. Furthermore, the use of organotypic slices to test tissue-engineering strategies has been rarely used. We hypothesized that NSCs will mainly replace the lost dopaminergic cells while MIAMI cells will mainly protect the nigrostriatal pathway by secreting pro-angiogenic and neuroprotective factors. To evaluate their mechanisms of action and their therapeutic potential stem cell- PAM complexes were grafted in

PD organotypic slices and stem cell survival and proliferation, when administered alone or combined with PAMs, were studied. The neuronal and dopaminergic differentiation capacity and the protection/repair potential of stem cell-PAM complexes were also assessed. Finally, stem cell survival and neuroprotective mechanisms were clarified by studying their secretory phenotype and the impact on the organotypic slice microenvironment.

2. Materials and methods

2.1 Cells culture

2.1.1 Culture of MIAMI cells

MIAMI cells were expanded *in vitro* from passage 4-5 on fibronectin (Sigma Aldrich, St Louis, USA) coated flasks at 125 cells/cm² in low oxygen tension in Dulbecco's Modified Eagle Medium-low glucose (DMEM, Gibco, Life Technologies, Paisley, UK), supplemented with 3% KTE serum, 30µg/ml ascorbic acid and a mixture of lipids (working concentration of 510nM lipoic, 70nM linolenic and 150nM linoleic acid, all from Sigma). Then a 10 days treatment with an addition of 20ng/mL of EGF, bFGF (both from R&D systems, Lille, France) and 5µg/ml of Heparin (Sigma Aldrich, St-Louis, USA) is conducted to enhance neuronal specification. Cells were fed every 3 days by changing half medium, and split every 5 days

2.1.2 Culture of NSC cells

To overcome the ethic limitations associated with the isolation and the use of NSCs, a lineage of NSCs expressing green fluorescent protein (GFP), namely hNSC1, kindly provided by Alberto Martinez Serrano from Madrid University (20-21) was used. This lineage was perpetuated by the expression of v-myc, whose promoter

was under control of epigenetic (EGF, bFGF) mitogenic stimuli. hNSC1 cells were expanded *in vitro* from passage 10 to 15 on Poly-D-Lysine (PDL, Sigma Aldrich, St-Louis, USA)) coated flasks. Cells grows in a DMEM/F12 (1:1) (Glutamax, Gibco, Life Technologies, Paisley, UK) medium supplemented with 6,5mg/L of glucose (Sigma Aldrich, St-Louis, USA), Hepes (Hepes Buffer 1M, Sigma Aldrich, St-Louis, USA), 0,5% of Albumax (Gibco, Carlsbad USA), 1% of N2 supplements (Gibco, Life Technologies, Paisley, UK), 20ng/ml of EGF and bFGF both from R&D Systems (R&D systems, Lille, France), 1% of non essentials amino acids (NEAA, Biowhitaker Lonza, Belgium) and antibiotics/antimyotics (100 U/mL penicillin, 0.1 mg/mL streptomycin, 0.25 µg/mL amphotericin B, Sigma Aldrich, St-Louis, USA) under normoxic conditions. Cells were fed every 3 days by changing half medium, and split every 5 days, keeping 20% of old medium.

To induce differentiation of those cells, EGF and bFGF were withdrawn from the culture medium, in this way v-myc was down regulated, decreasing their immortality capacity (20). We evaluated their differentiation capacity by immunostaining against nestin, glial fibrillary acidic protein, β3-tubuline and medium neurofilament (data not shown).

2.2 Formulation of NT3 microspheres

NT3-loaded microspheres were prepared using an s/o/w emulsion solvent extraction-evaporation method previously described here (11, 22). A poly(lactic-co-glycolic acid) (PLGA) copolymer with a lactic:glycolic ratio of 37.5:25 (MW: 25,000 Da) was used (Phusis, Saint Ismier, France). The protein loading was 1 µg of NT3 (Peprotech, Rocky Hill, USA) with 5 µg of human serum albumin (HSA, Sigma Aldrich, St-Louis, USA) per mg of microspheres. First, NT3 and HSA were

precipitated separately using a process previously described (11). Briefly, 1,077 g of cold glycofurol (Tetraglycol, Sigma Aldrich, St-Louis, USA) was added to 10 µl of a NaCl solution (0.3 M) containing 50 µg of NT3 and 1 mg poloxamer 188 (Lutrol F68, BASF, Ludwigshafen, Germany). After 30 min on ice, the protein particles were harvested by 30 min centrifugation at 10,000 g. HSA solid particles were produced in a similar manner, adding 1.077 g of cold glycofurol on 25µl of a NaCl solution (0.3 M) containing 250 µg HSA and 5 mg poloxamer. After supernatant removal, the NT3 and HSA solid particles were mixed with 670 µL of organic phase made of 50 mg PLGA dissolved in a 3:1 methylene chloride:acetone solution. This organic phase was emulsified in a poly(vinylalcohol) (Mowiol$^{4\text{-}88}$,Kuraray Specialties Europe, Frankfurt, Germany) aqueous solution (90 ml, 4% w/v) maintained at 1°^{C} and mechanically stirred at 550 rpm for 1 min (Heidolph, RZR 2041, Merck Eurolab, Paris, France). After addition of 100 ml of deionized water and stirring for 10 min, the resulting o/w emulsion was added to 500 mL deionized water and stirred for a further 20 min to extract the organic solvent. Finally, the microspheres were filtered on a 0.45 nm filter (SVLP type, Millipore SA, Guyancourt, France). Empty/blank microspheres were prepared in the same way without NT3.

2.3 LM-PAM formulation

To obtain LM-PAMs, PLGA microspheres were coated with a combination of LM (Sigma Aldrich, St-Louis, USA) and PDL (Sigma Aldrich, St-Louis, USA) molecules to favor cell attachment as previously published (11). Briefly, 5mg of microspheres were resuspended in phosphate buffer saline (PBS, Lonza, Verviers, Belgium). Coating solution was prepared in PBS, at a final concentration of 40 µg/mL-60 µg/mL of PDL/LM. This solution was mixed to the microsphere suspension

and placed under rotation at 15 rpm at 37°C during 1h30. After coating, LM-PAMs were washed 3 times in distilled sterile water, lyophilized and finally kept at -20°C for long-term storage. Thus, we will distinguish LM-PAMs releasing NT3 (LM-PAM-NT3) and PAMs without NT3 (LM-PAMs). When no precision is given, the term PAMs refers to both LM-PAM-NT3 and LM-PAMs-not releasing NT3.

2.4 Characterization of LM-PAM-NT3

The average volume diameter and the size distribution of microspheres were evaluated using a Multisizer Coulter Counter (Beckman Coulter, NYON, Swiss).

Encapsulation yield was determined as previously reported (11). Briefly, after dissolution of microspheres in acetone, centrifugation and evaporation, protein quantification was performed with a NanoOrange® kit (Invitrogen, Cergy Pontoise, France) following the manufacturer's guidelines.

To assess NT3 release kinetics, 5mg of LM-PAM-NT3 were dispersed on 500µl PBS (Lonza, Verviers, Belgium) supplemented with 0.1% BSA (Fraction V, PAA Lab, Austria) and antibiotics (100 U/mL penicillin, 0.1 mg/mL streptomycin, 0.25 µg/mL amphotericin B, Sigma Aldrich, St-Louis, USA). Every day from day 1 to day 5 and then every 3 days, sample was centrifugated at 295g for 10 min. Supernatants were collected and conserved at -20°C prior to bioassay. Then LM-PAM-NT3 were resuspended in 500µL PBS-BSA-antibiotics.

The bioactivity of the NT3 released from the LM-PAM-NT3 was evaluated *in vitro* from n=3 experiments by determining the neuron-like differentiation of dorsal root ganglia (DRG) cells after NT3 treatment. Sprague Dawley rat pups aged between 2 and 5 days were sacrificed by CO2 asphyxia. The spine was quickly picked up and DRG were extracted and mechanically triturated into dissection

medium composed of Hank's Balanced Salt Solution (HBSS, Lonza, Verviers, Belgium), 1% B27 (Gibco, Life Technologies, Paisley, UK), 0,5mM Glutamax (Gibco, Life Technologies, Paisley, UK) antibiotics (Sigma, St Louis, USA) and collagenase (2mg/ml, Gibco, Life Technologies, Paisley, UK). The DRG were, settled and the supernatant containing cell suspension was kept. This step was performed twice. Then after a centrifugation, cells cultured onto PDL (5µg/cm², Sigma Aldrich, St-Louis, USA) coated inserts, into a 24 wells plate at a rate of 10.000 cells/cm². A standard range was performed by an addition of recombinant human NT3 (0, 5, 15 and 30ng/ml) diluted in PBS BSA 0.1% onto DRG cells. To test the activity of NT3 released by LM-PAM-NT3, released media or LM-PAM-NT3 were directly added to the culture media for 5 days. Then, cells were fixed with cold PFA 4% during 15min and then washed with PBS. Immunofluorescence against β3-tubuline was performed to observe NT3 induced neuron-like differentiation. Only β3-tubuline positive cells which exhibit a large neurite outgrowth, were counted using Metamorph® software and compared with the standards to confirm NT3 effectiveness and evaluate the quantities released. Animal care and uses were in strict accordance with the regulations of the French Ministry of Agriculture and of the animal experimentation ethic committee, protocol number CEEA.2010.13, of Pays de la Loire.

2.5 Formation of PAM/stem cell complexes

MIAMI cells were detached and pelleted at 295g for 10 min. Pellets were resuspended in culture medium supplemented with 3% FBS (Lonza, Verviers, Belgium) and NSCs pellets in culture medium supplemented with 1% of FBS. 0.50mg lyophilized microspheres were resuspended in coated eppendorf tubes (Sigmacote, Sigma, St Louis, USA) containing DMEM-F12 (Gibco, Life Technologies, Paisley,

UK), for 15 min. PAMs suspension was mixed with of 0.5mL of cell suspension ($2.5x10^5$ cells/0.50 mg PAMs). The mixture was then gently flushed and plated in 1.9 cm2 Costar ultra low adherence plate (#3473, Corning, Avon, France). Plates were incubated at $37°^C$ during 4h for MIAMI cells and 5h for NSCs, to allow cell attachment on PAM surface. PAMs/cell aggregates were pelleted by centrifugation at 200g for 2 min. Cell adhesion to PAM surface was assessed by microscopic observation and cells adhered to PAMs were quantified using the Cyquant cell proliferation assay (CyQuant Cell proliferation Assay kit, Invitrogen). Complexes were further studied using light and fluorescence microscopy and scanning electron microscopy. Samples were prepared for scanning electron microscopy analysis as previously described (10). For fluorescence microscopy, NSCs were observed by their own capacity to express GFP and microspheres were stained by PKH26 following manufacturer protocol (Sigma Aldrich, St-Louis, USA)

2.6 Preparation and culture of Parkinson's disease organotypic slices

Animal care and use were in strict accordance with the regulations of the French Ministry of Agriculture and all animal procedures were approved by animal experimentation ethic committee of "Pays de la Loire", protocol number CEEA.2010.13. Every effort was made to minimize animal suffering and the number of animal used.

Organotypic PD cultures were prepared as previously described (19). Six to eight days Sprague Dawley rats pups were sacrificed after anesthetic and were brains removed. Cerebral hemispheres were separated and glued on the vibratome plate on their central side. 400µm sagittal slices were obtained using a vibratome (motorized vibroslice, Campben instruments, Loughborough, England) along the

nigrostriatal pathway with an angle of the razor blade of 14°. Nigrostriatal slices were then dived into Grey's Salt Balanced Solution (Sigma Aldrich) supplemented with 6,5mg/L of glucose (Sigma Aldrich, St-Louis, USA) and antibiotics (Sigma Aldrich, St-Louis, USA). Three to four slices per hemisphere were next transferred to 30 mm diameter semiporous membrane inserts (Millicell-CM, Millipore, Bedford, MA, USA) within a 6-well plate and put in culture at 37ºC, 5% CO2. From day 0 to day 3, a serum containing medium was used: 50% MEM (Minimum Essential Medium Eagle, Sigma Aldrich), 25% Hank's (Hank's Balanced Salt Solution, Sigma Aldrich), 25% of horse serum (decomplemented horse serum, Gibco), 6.5mg/ml of glucose, 1 mM of L-glutamine (Sigma Aldrich, St-Louis, USA) and antibiotics (Sigma Aldrich, St-Louis, USA). From day 3 to day 16, a serum free medium was used: neurobasal medium (Gibco) supplemented with 6.5mg/L of glucose, 1mM of -glutamine (Sigma Aldrich, St Louis, USA), B27 supplements (50x, B27 supplements, Gibco) and antibiotics (Sigma Aldrich, St-Louis, USA) this media was changed every two days.

2.7 Injection of stem cell/ PAM complexes into organotypic slices

Two days after organotypic slice preparation (Figure 1), treatments were injected into the striatum using a 22-gauge needle (Hamilton, Bonaduz, Switzerland) connected to a micromanipulator. Eight experimental groups were tested: (1) non-treated organotypic slices, (2) culture media, (3) NSC cells, (4) MIAMI cells, (5) NSC LM-PAM, (6) MIAMI LM-PAM, (7) NSC LM-PAM-NT3 and (8) MIAMI LM-PAM-NT3. Each experimental groups were tested in n=3 unless otherwise stated. Total injection volume consisted of 2µl of culture media containing approximately 50.000 cells alone or adhered to 0.1 mg of PAMs. Injections were done at 0.5µl/minute infusion rate.

The needle was left in place for 5 min to avoid the cells being expelled from the organotypic slices.

2.8 Histological study

One and fourteen days after injection (Figure 1), organotypic slices were fixed by addition of 5ml of ice cold 4% PFA (Sigma, St Louis, USA) in PBS (Lonza, Verviers, Belgium) pH 7.4 during 2 hours. Then slices are washed in PBS three times. Non specific sites are blocked with PBS, Triton 1% (PBS-T, Triton X-100, Sigma, St Louis, USA), BSA 4% (Fraction V, PAA Lab, Austria), NGS 10% (Sigma, St Louis , USA) during 4 hours at RT under agitation (expect for CD31 study, in this case, 0.05% Triton is employed).

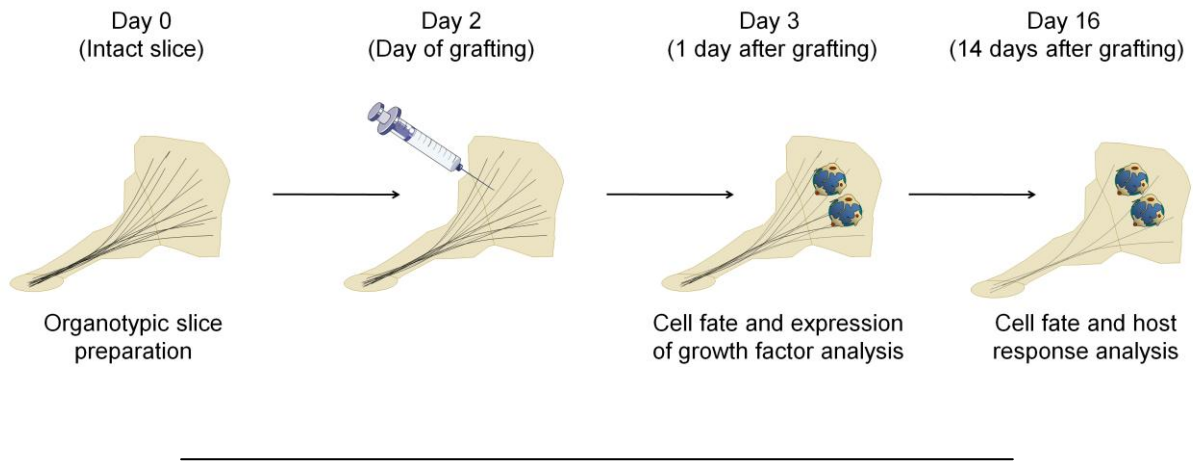

Figure 1: *Experimental protocol. At day 0, nigrostriatal pathway remains intact but the degeneration is initiated. Two days after slice preparation, cells-PAMs complexes were transplanted in the striatum. At day 3 (1 day after grafting), a 30% loss of striatal TH positive fibers density was detected compared to day 0 Grafted cells fate and growth factor expression were analyzed. At day 16, (14 days after grafting), dopaminergic degeneration is complete. Grafted cells fate and host tissue responses were studied.*

2.8.1 Tyrosine hydroxylase immunochemistry

To observe dopaminergic neurons a mouse anti rat tyrosine hydroxylase (TH) antibody (10µg/ml in PBST, clone 6D7, Covance, Emeryville, CA, USA) was used to study the nigrostriatal pathway of the PD slice, and a polyclonal rabbit anti-human TH (1:100, AS-DOUB-LX immunization program, Liège, Belgium) antibodies was used to asses dopaminergic differentiation of grafted cells. Slices were incubated 48h with the primary antibody diluted in PBS-T, BSA 4% at 4°C. After washes, slices were incubated with the biotinylated anti-mouse or anti-rabbit secondary antibody (7.5µg/ml, Vector Laboratories, Burlingame, USA). Then slices were washed and quenching of peroxidase was made with 0.3% H2O2 (Sigma, St Louis, USA) in PBS-T, at RT for 20 min. After PBS washes, incubation with Vectastain ABC reagent (Vector Laboratories, Burlingame, USA) in PBS was performed at RT for 2h. Sections were washed and revealed with 0.03% H2O2, 0.4 mg/mL diaminobenzidine (DAB, Sigma, St Louis, USA) in PBS, 2.5% nickel chloride (Sigma, St Louis, USA) and dehydrated before mounting. Isotypic controls and/or omission of the primary antibody were performed to assess the specificity of the immunostainings.

TH-positive rat fiber quantification in the striatum at different time-points ranging from three to sixteen days was performed using the Metamorph® software. The TH fiber density was estimated by subtracting the striatum TH intensity from the TH intensity of the cortex, which represents the background. Results were presented as mean differences +/- average deviation and were calculated from 6 pictures taken from 4 different rats for each group.

2.8.2 Anti-mitochondria, β3-tubulin, Ki67 and CD31 immunofluorescence

A mouse anti rat CD31 antibody (10µg/ml, clone TLD-3A12, Abcam, Paris, France) was used to see brain slices vascularization. MIAMI cells were detected using a mouse anti-human mitochondria antibody (10ng/ml, clone MTCO2, Abcam, Paris, France). NSC cells were observed using their own potential to express GFP. A mouse anti human β3-tubulin (2ng/ml, clone SDL.3D10, Sigma, St Louis, USA) or rabbit monoclonal anti β3-tubulin (1:400, EP1331Y, Abcam, Cambridge, UK), a mouse anti-human CD31 (300ng/ml, clone WM59, BD Pharmingen, Franklin Lakes, USA) and a monoclonal mouse anti-human Ki67 (350ng/ml, MIB-1, Dako, Glostrup, Denmark) antibodies were used to characterize grafted cells differentiation. Slices were incubated 48h with the primary antibody diluted in PBS-T, BSA 4% at 4°C. After washes, slices were incubated with the corresponding biotinylated mouse or biotinylated rabbit secondary antibody (7.5 µg/ml, Vector Laboratories, Burlingame, USA). Then slices were washed and incubated with Streptavidin Fluoroprobes R488 or R547H (Interchim, Montluçon, France) diluted 1:200 in PBS for 2 hours before mounting with a fluorescent mounting medium. Isotypic controls and/or omission of the primary antibody were performed to assess the specificity of the immunostainings.

The intensity and density of positive stained cells in the striatum was quantified using the Metamorph® software for all markers but CD31. Pictures of CD31 positives blood vessels were taken with 10x magnifications for each slice in which blood vessels appeared in green. The number of positive blood vessels was counted under visualization using Metamorph® software Results were presented as mean differences +/- average deviation and were calculated from 3 to 4 different slices.

2.9 Dopamine quantification by mass spectrometry

Quantification of DA in the striatum was performed at day 0 and day 16 after slice culture as previously described in (23). Briefly, microdissected striatums were dissociated by adding 200 µl of 1x reporter lysis buffer (Promega, Madison ,USA) followed by 14 sec of ultrasonication. Then, 600 µl of pure acetonitrile were added to precipitate proteins. After a centrifugation (10000g 15min 4°C), samples were dropped onto an Ostro^{TM} 96-well plate (Waters S.A, Saint-Quentin, France) following manufacturer´s instructions to purify DA. Chromatography was performed using a Waters Alliance 2695 system (Waters SA, Saint-Quentin-en-Yvelines, France) with a Uptishpere$^{®}$ C18 50DB 150x2,0mm, 5 µm column (Interchim, Montluçon, France). Ionization was achieved using electrospray in positive ion mode. The mass spectrometer operated in multiple reactions monitoring (MRM) mode. The (M H) + m/z transitions for each compound were 137 and 91. A typical retention time of DA was found to be 1.50 min. Quantification was achieved with QuantLynx (Waters S.A, Saint-Quentin, France).

2.10 Laser microdissection

24 hours after implantation (Figure 1), grafted cells alone or complexed with PAMs were isolated from the striatum using laser microdissection, to study messenger RNA (mRNA) expression by retro transcription quantitative polymerase chain reaction (RT-qPCR). Organotypic slices were dehydrated by 4 successive 4min ethanol baths (70°, 90°, 100° and 100°). Immediately after slices dehydration, microdissection was carried out at RT with the LMD6000 microdissection system and software from Leica (Leica Microsystems, Wetzlar, Germany), using a UV laser with a wavelength of 355 nm. Two dehydrated organotypic slices were mounted on a

polyethylene-naphthalate membrane-coated Petri dish (Leica Microsystems, Wetzlar, Germany). The microdissection was achieved with settings: Power 128; Speed 1; Specimen balance 0 and 6.3x objective. Areas of approximately 3.10^6 µm², corresponding to the whole striatum were selected and collected in the cap of a 0.5 mL microfuge tube containing 70 µL of RNA Later (Life Technologies, Carlsbad, CA, USA). Striatum from two organotypic slices were pooled, centrifuged and kept at -80°until RNA extraction. LMD was performed for no longer than 30min per Petri dish.

2.11 Reverse transcription and real time quantitative PCR.

Design of primers specific for human genes and PCR were performed as described elsewhere (11, 15) (see table 1). Cells were lysed in a 1% β–mercaptoethanol containing buffer and RNA extracted following treatment by DNAse to remove any traces of genomic DNA (Total RNA isolation Nucleospin RNA II, Macherey Nagel, Hoerdt, France). First strand complementary DNA (cDNA) synthesis was performed with a Ready-To-Go You-Prime First Strand Beads kit in combination with random hexamers (Amersham Biosciences, Orsay, France) using 1 mg RNA according to the manufacturer's guidelines. Following first strand cDNA synthesis, cDNAs were purified (Qiaquick PCR purification kit, Qiagen, Courtaboeuf, France), eluted in 50 µL RNAse free water (Gibco, Life Technologies, Paisley, UK). Five microliters of cDNA (1:20) were mixed with iQ SYBR Green Supermix (Biorad, Hercules, CA, USA) and primer mix (0.2 mM) in a final volume of 15 µL. Amplification was carried on a Chromo4 thermocycler (Biorad, Hercules, CA, USA) with a first denaturation step at 95°^{C} for 3 min and 40 cycles of 95°^{C} for 10s, 55°^{C} for 15 s and 72°^{C} for 15s. Several housekeeping genes, glyceraldehyde-3-phosphate dehydrogenase (Gapdh, NM_002046), hypoxanthine phosphoribosyltransferase 1

(Hprt1,NM_000194), beta actin (Actb, NM_001101), 30S ribosomal protein S18 (Rps18, NM_001093779) and heat shock 90 kD protein 1 beta (Hspcb, NM_007355) were tested for normalization. The relative transcript quantity (Q) was determined by the delta cT method and relative quantities (Q) were normalized using the multiple normalization method described here. (24).

2.12 ELISA assays

Every two days, from day 0 to day 16, slice culture media were removed and stored at -80°C until use. ELISA assays were performed to detect human stanniocalcin-1 HGF (hSCT-1, DuoSet ELISA, R&D systems) and human vascular endothelial growth factor (hVEGFA, specific to 121, 165a and 165b forms, DuoSet ELISA, R&D systems) following the manufacturer instructions. Every sample was tested in triplicate. Standard points were diluted in Neurobasal media. Optical density was read at 450nm and 580nm with plate reader (Labststem, Multiskan Ascent, Vienna, Virginia, USA.). Wavelengths read at 450nm were subtracted from 580nm to correct optical imperfections.

2.13 Statistical analysis

Data are presented as the mean value of three independent experiments +/- standard deviation (SD), unless otherwise stated. Significant differences between samples were determined using an ANOVA test, followed by a Scheffe post-hoc test which indicated if conditions were significantly different. P-value was set to 0.05, unless otherwise stated.

Table 1 : gene Primers used for RtqPCR

Gene	Full name	Accession Number	Sequence
BDNF	Brain-derived neurotrophic factor	**000011.9**	Qiagen, ref #QT00235368
GDNF	Glial cell line-derived neurotrophic factor	011675.2	Qiagen, ref #QT00001589
HGF	Hepatocyte growth factor	001010934	F = 5'-TCTGGTTCCCCTTCAATAGC-3' R = 5'-GTGTTCGTGTGGTATCATGG-3'
HSPB1	Homo sapiens heat shock 27kDa protein 1	001540	F = 5'-GACCAAGGATGGCGTGG-3' R = 5'-CTAGCTTGGGCATGGGG-3'
NT3	Neurotrophin 3	001102654.1	Qiagen, ref #QT00204218
NGF	Nerve growth factor	002506	Qiagen, ref #QT00043330
VEGFa	Vascular endothelial growth factor a	001204384	F = 5'-CAGCGCAGCTACTGCCATCCA-3' R = 5'-CAGTGGGCACACACTCCAGGC-3'
STC1a	Stanniocalcin-1	003155	F = 5'-AGGCAAGGCTGACTTCTCTG-3' R = 5'-AACTACTTGTCGCATTGGGG-3'
hTSG6	Tumor necrosis factor, alpha-induced protein 6	007115	F = 5'-TCACATTTCAGCCACTGCTC-3' R = 5'-TGATCATATCGTCAGTTGTAGTGAA-3'
NTRK3	neurotrophic tyrosine kinase, receptor, type 3 , Variant A	AF058389.1	F = 3'GAACCTCTACTGCATCAACG-5' R = 3'ACTATCCAGTCCACATCAGG-5'
TKd-TRK3	Neurotrophic tyrosine kinase, receptor, type 3 transcript variant 3 (isofrm C)	AF058390.1	F = 3'AGCCGGACACGTGGGTCTTTT-5' R = 3'CTTGGAATGTCCGGGAAGGCTTA-5'

3. Results

3.1 Stem cell NT3 receptor expression

mRNA expression levels on NSCs and MIAMI cells of NTRK3 and Tkd-NTRK3, the main NT3 receptors, were evaluated by RT-qPCR. NTRK3 which represents all variants of NT3 receptors and Tkd-NTRK3 were expressed both in NSC cells and MIAMI cells suggesting that both stem cells may respond to this neurotrophin and confirming previous reports (25). Furthermore, NSCs highly expressed both NT3 receptors (10-fold more than MIAMI cells) (Figure 2A).

3.2 Characterization of PAMs: LM-PAMs release bioactive NT3 and form PAM/stem cell complexes

The mean particle size of PAMs measured using a Multisizer Coulter Counter was 60.92 µm +/- 20.04µm. Observation of the microspheres and PAMs by brightfield microscopy and with scanning electron microscope was performed to ensure the quality of the formulation. Microspheres were perfectly spherical with a smooth surface, and no pores on their surface (Figure 2B). The yield of the microencapsulation of NT3 was around 100% determined by Nanoroange® total protein assay.

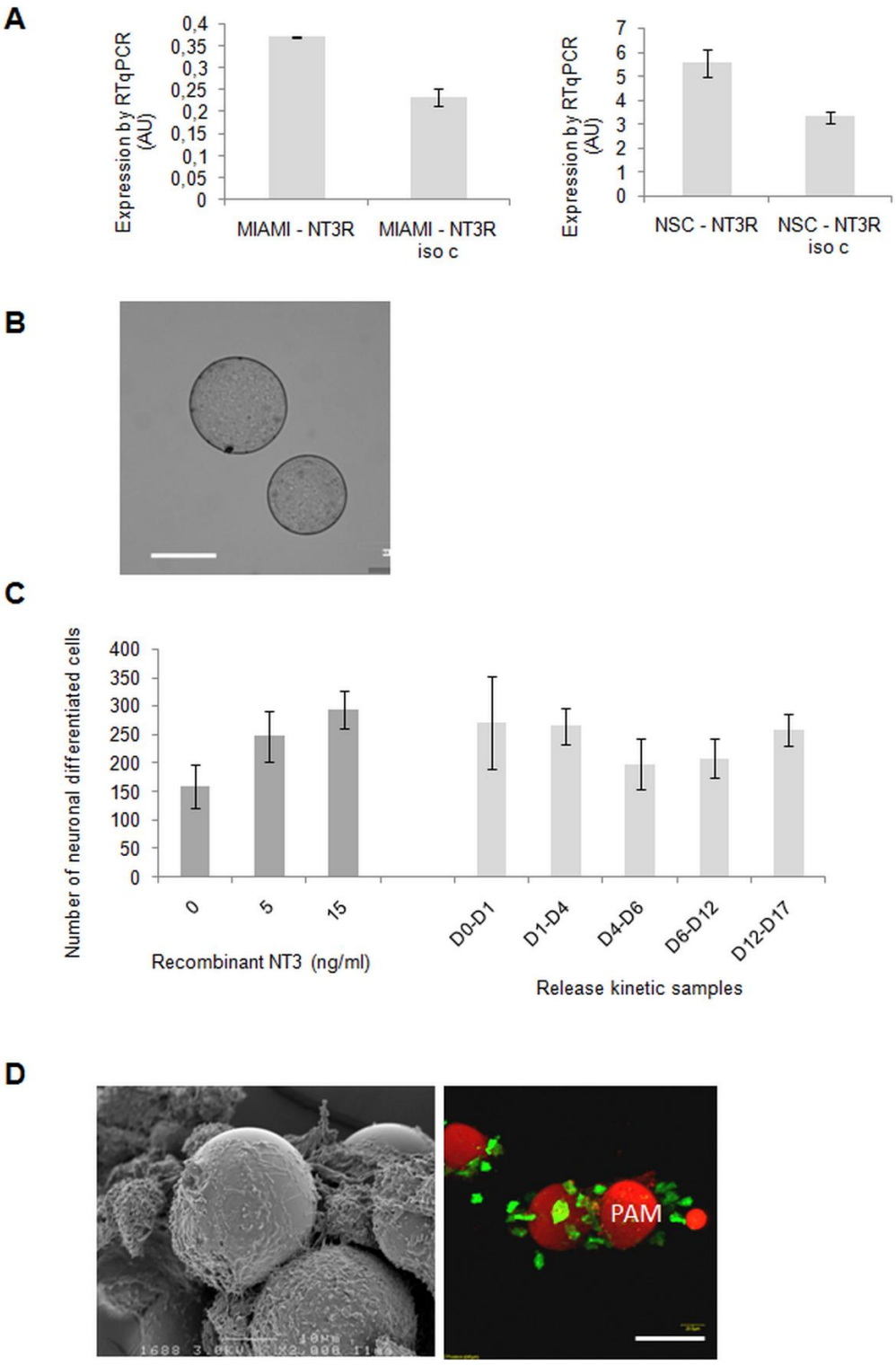

Figure 2: *Characterization of stem cells and PAMs alone and associated. A) RTqPCR against NT3 receptors for MIAMI cells and NSCs. Both types of cells express NT3R, particularly NSCs. B) Observation of microparticles by Brightfield microscopy. Microparticles were perfectly spherical with a smooth surface and a mean diameter of 60µm.C) Number of neuronal differentiated dorsal root ganglion cells. A minimum of 5-10ng bioactive NT-3 was released from LM-PAM-NT3 at every time-point. D) Observation of PAMs by scanning electronic microscope when complexed with MIAMI cells and by immunostaining when complexed with GFP positive NSCs. Scale bar was 60µm.*

NT3 released by PAMs was characterized by a bioassay on rat DRG. This bioassay allowed assessing the amount of bioactive NT3 released over 16 days. Released NT3 induced DRG cell differentiation into β3-tubulin expressing cells just as well as recombinant human NT3 directly added into the culture medium. We observed that 10 to 15ng of bioactive NT3/0.5mg LM-PAM-NT3 were released from day 0 to day 1 and from day 1 to day 3. From day 3 to day 6 and day 6 to day 12 a slight decrease of NT3 release was detected which reached about 5ng. Finally, NT3 release increased from day 12 to 17 with 5-10ng/0.5mg LM-PAM-NT3 (Figure 2C). Furthermore, NT3 contained within the microspheres was bioactive 8 months after microsphere preparation when conserved lyophilized at -20°C (data not shown).

PAMs with a PDL/LM surface showed a zeta potential of 34.5 +- 2.6 mV, which is satisfactory for cell adhesion. Both stem cells adhered well to PDL/LM PAMs as observed by fluorescence microscopy and MEB (Figure 2D). The percentage of cells adhered onto PAM's surface at the end of the cell attachment protocol was about 94%. It was also noticed that cells remained mainly adhered onto the surface of PAMs even after the transplantation process.

3.4 Effects of grafted stem cell/PAM complexes on the organotypic nigrostriatal pathway.

3.4.1 Nigrostriatal pathway repair/protection

Immunohistochemistry against rat TH was used to evaluate the degree of the nigrostriatal lesion and therefore the efficacy of the different treatments. At day 3 after slice culture a 40% decrease of TH fiber density was observed compared to day 0. Striatal TH fiber density continued to decrease reaching 100% at day 16 in the non-treated slices as recently reported (19). Rat TH fiber density in the striatum was

quantified one day after grafting and was found to be identical for all the treated groups (data not shown). It was slightly higher after media injection, and more than 2.5-fold higher for grafts of NSCs alone or complexed to PAMs, compared to non-treated slices. Injection of MIAMI cells alone did not have an important effect in TH striatal fiber innervation as no differences were detected with media injection control. TH-positive fiber innervation increased 3-fold when slices were injected with MIAMI LM-PAMs as well as with LM-PAM-NT3 alone. The greatest degree of TH fiber density within the grafted striatum was found in the MIAMI LM-PAM-NT3 treated group, where a significantly 3.8-fold increase was observed (Figure 3A). This may suggest a secretion of tissue repair factors by MIAMI cells particularly when adhered onto LM-PAM-NT3

One day after organotypic slice culture TH-positive fibers within the MFB were still observed, however after 16 days in culture no TH staining could be detected, highlighting the degeneration of the dopaminergic neurons. Fourteen days after transplantation of NSCs or MIAMI cells, both adhered onto LM-PAM-NT3, the degeneration of the dopaminergic fibers within the MFB was less important. Indeed, dopaminergic fibers with some varicosities could still be observed (Figure 3B).

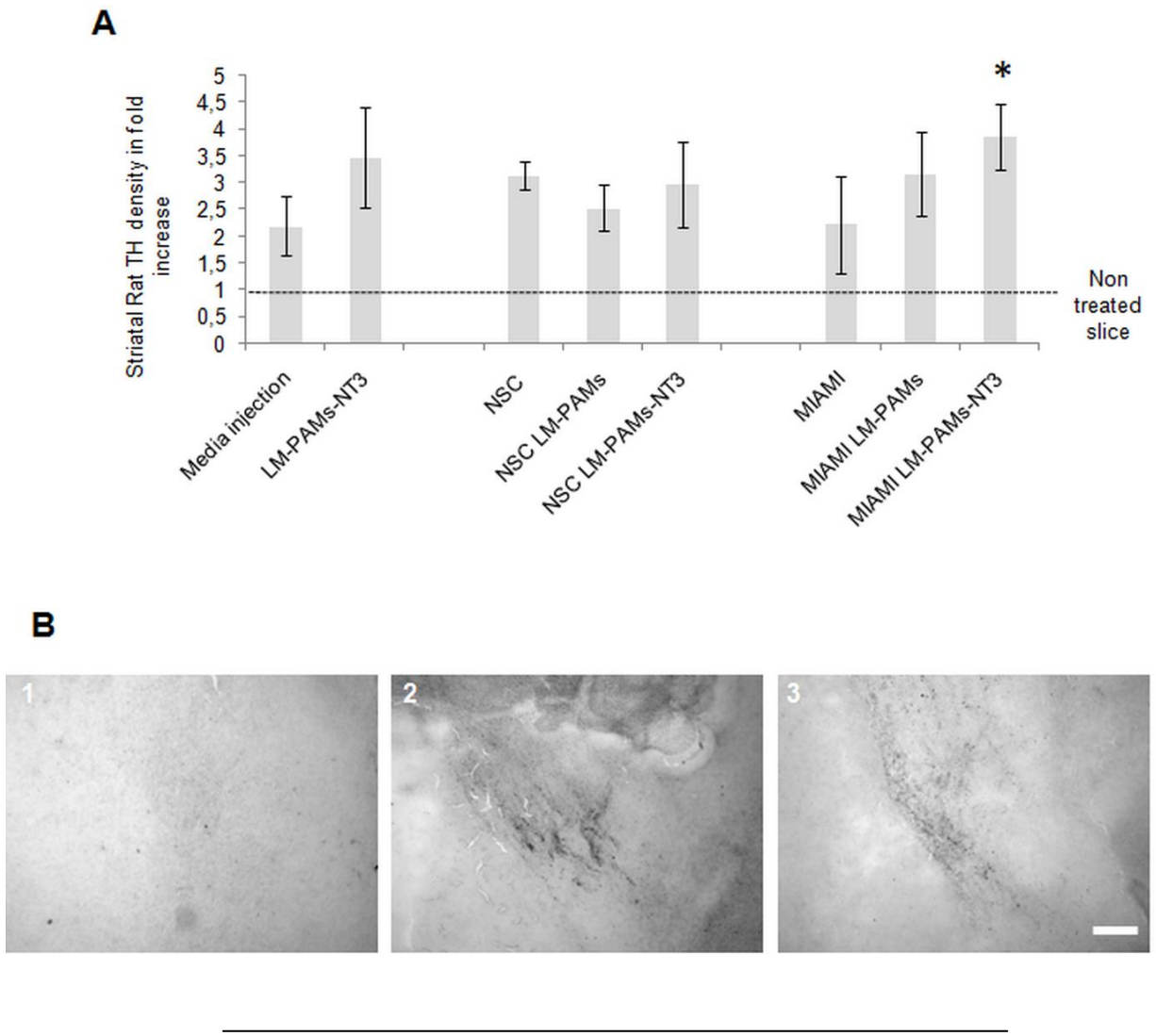

Figure 3: *Protection/repair of the nigro-striatal pathway at day 16. A) Quantification of rat TH density in the striatum; in fold increase against non-treated slices. The only significant rise of rat TH density was obtained with MIAMI/LM-PAM-NT3 (n=3 and p-value= 0.05). B) TH immunochemistry of MFB dopaminergic fibers at day 16. A total disappearance of the fibers was observed for non-treated slices (1) while injection of MIAMI /LM-PAM-NT3 complexes (2) or NSC/LM-PAM-NT3 (3) complexes seemed to protect those fibers.*

3.4.2 Striatal dopamine levels

Striatal DA level was quantified by LC MS/MS to assess if grafted cells could restore striatal DA content. In non-treated PD organotypic slices, a statistically significant 30% ± 0.7% reduction in striatal DA content compared to intact slices was detected 3 days after slice culture and remained at similar levels thereafter (19). Injection of MIAMI cells alone or complexed to LM-PAM-NT3 induced a recovery of DA levels with 96% ± 3% and 81% ± 15% of DA content, respectively. Nonetheless, striatal DA content of PD organotypic slices grafted with NSC (adhered or not to PAMs) significantly reached normal levels 16 days after graft with 103% ± 6% (P-value was set to 0.05 and n=2).

3.5 Fate of stem cell/PAM complexes after injection in PD organotypic cultures

Human specific mitochondria (hmito) for MIAMI cells and GFP expression for NSCs were used to evaluate the fate, survival and integration of stem cells injected alone or in combination with PAMs in the PD organotypic slices, one and fourteen days after implantation. Grafted cells in the striatum exhibited a rounded morphology suggesting that they maintained the morphology of stem cells during the first days after implantation (Figure 4A and 4D). Fourteen days after grafting, NSCs showed important neurite outgrowth with a neuronal-like morphology (Figure 4A), and MIAMI cells exhibited a bipolar neuron-like morphology (Figure 4D). No important differences in GFP-expressing NSC density can be observed when cells are grafted alone or in combination with PAMs 14 days after graft (Figure 4A), while more hmito-expressing cells can be observed when MIAMI cells were grafted adhered onto PAMs (Figure 4D). Expression of Ki67, a cell cycle-related nuclear protein detected in all phases of the active cell cycle, was evaluated to verify that NSCs did not

proliferate after grafting. Many of the GFP-expressing NSCs were double-labeled for Ki67, indicating that a large proportion of the cells continued to proliferate one day after grafting (Figure 4B). A strong decrease of Ki67-expressing NSCs was observed 14 days after graft (Figure 4B) and no evident cell proliferation was detected for MIAMI cells at both time-points.

Those observations were quantified and, for NSCs, a slight increase of GFP expressing cell density was detected one day after graft when cells were adhered on PAMs. Fourteen days after grafting, no differences were found between those two conditions. Furthermore, for cells grafted alone, as for NCSs adhered on PAMs, a strong decrease of cell number was observed after 14 days in culture. Interestingly, the same results were obtained for Ki67 expression (Figure 4C). Indeed, a slight increase in cell proliferation was observed 24h after transplantation when the cells were adhered onto PAMs, however, the expression of Ki67 by NSCs decreased 14 days after grafting (2-3-fold less than at day 1). There were no significant differences in cell proliferation when NSCs were adhered on LM-PAMs or on LM-PAM-NT3. These results suggest that most of the cells were induced to differentiate over time.

For MIAMI cells a 2-fold significant increase of cell number was detected one day after grafting when complexed to PAMs. Fourteen days after transplantation this increase in cell number was even more important (3.3-fold), suggesting that PAMs stimulate cell survival. The pro-survival effect of PAMs occurred during the first 24h and it slowed down cell death thereafter (Figure 4E).

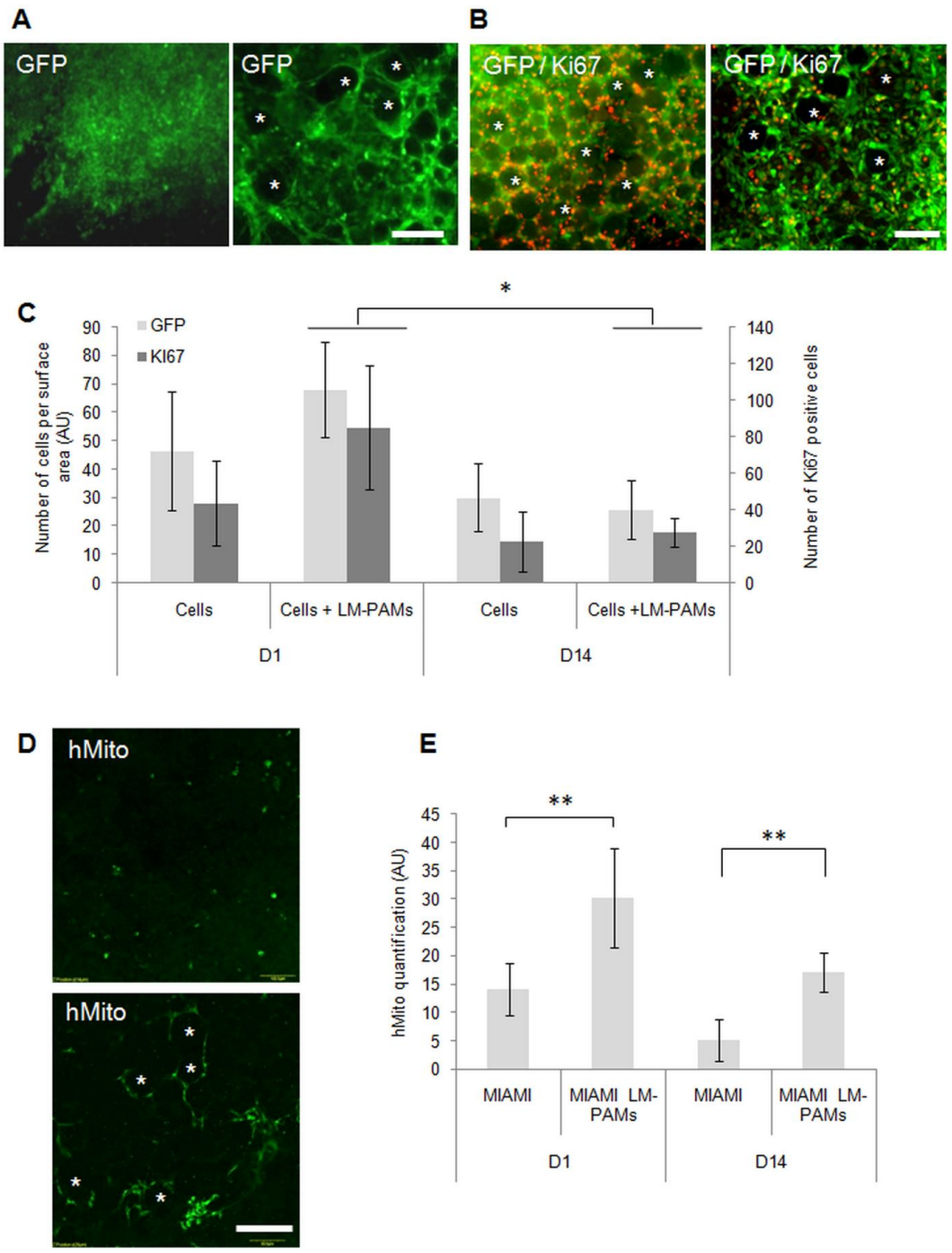

Figure 4:*Stem cell survival and proliferation A) GFP expressing NSCs observed by fluorescence microscope 14 days after grafting alone (left) or complexed with PAMs (right). No important differences in GFP expression were noticed. B) Immunofluorescence against human Ki67 highlighting the proliferation of grafted hNSC1 cells 1 day (left) and 14 days (right) after grafting when complexed to PAMs. An important decrease of Ki67 expression was observed over time. C) GFP and Ki67 expression quantification. A significant decrease of GFP and Ki67 expression was noticed 14 days after grafting when NSCs were complexed to PAMs. * Significantly different results with α=0.05. D) Immunofluorescence against HMito14 days after grafting MIAMI cells alone (above) or in combination with PAMs (below). An increase of HMito-positive-cells was noticed when the cells were adhered onto PAMs. E) HMito positive-cells quantification. After 1 and 14 days in situ, MIAMI cells number increased 2 or 3 times when adhered onto to PAMs.** significantly different results with α=0.01. The asterisks in the picture highlight the PAMs. (n=3) Scale bar is 100µm*

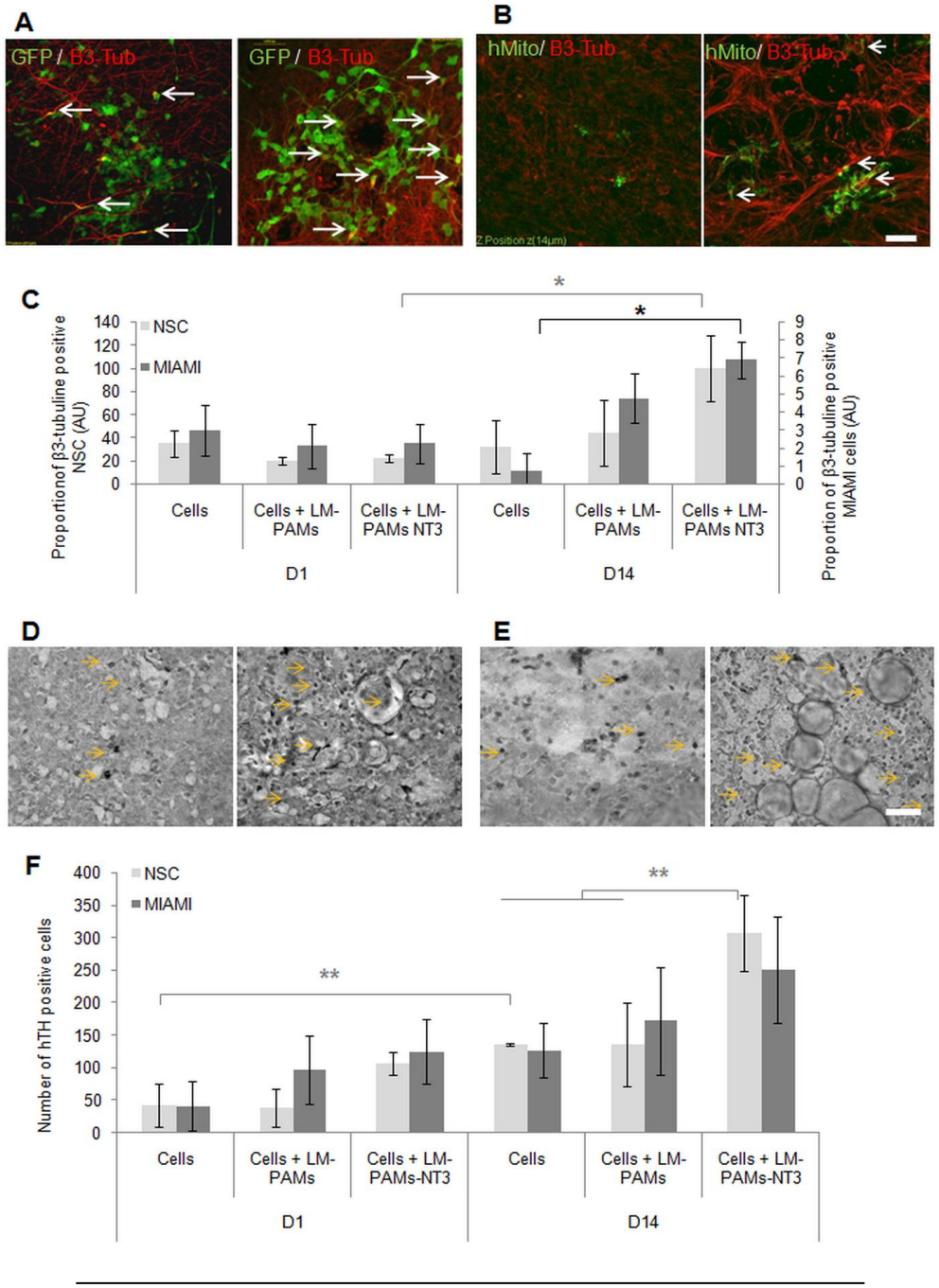

Figure 5: *Differentiation potential of grafted cells. A) β3-tubulin expression of NSCs and B) MIAMI cells, alone (left) and adhered on LM-PAMs-NT3 (right), observed by immunofluorescence 14 days after grafting. White arrows indicate β3-tubulin positive cells. Scale bar is 50µm. C) β3-tubulin expression quantification of grafted cells 1 day and 14 days after grafting when adhered or not on PAMs. hNSC1 as MIAMI cells showed a significant expression of β3-tubulin after 14 days in situ when complexed with LM-PAM-NT3 (n=3 and * significantly different results with α=0.05).D) Human TH expression of NSCs. E) and MIAMI cells, alone (left) and adhered on LM-PAM-NT3 (right), observed by immunohistochemistry 14 days after grafting. Yellow arrows indicate hTH positive cells. Scale bar is 50µm. F) Quantification of TH expression 1 and 14 days after grafting cells alone and combined to PAMs. TH expression is very weak 24h after graft for NSC and MIAMI cells with a slight increase of this expression when cells were adhered on LM-PAM-NT3. 14 days after grafting TH expression increased and was significantly different for NSC/LM-PAM-NT3 compared to the 2 other conditions at this time-point. An increase of TH expression was also observed for MIAMI cells when adhered on LM-PAM-NT3 (n=3 and ** significantly different results with α=0.01).*

3.6 Differentiation of grafted cells to a dopaminergic phenotype

Expression of β3-tubulin was used to determine neuronal differentiation of grafted cells 1 and 14 days after injection with or without PAMs in the PD organotypic slices. For both types of cells, β3-tubulin expression remained low 1 day after grafting for all conditions tested, but the expression increased for MIAMI cells and NSCs at day 14 when adhered onto LM-PAM-NT3 (Figure 5A and 5B). Quantification of the ratio of cells expressing β3-tubulin among the cells present at day 14 revealed no changes in β3-tubulin expression when cells were grafted alone, while a 6.7-fold increase of β3-tubulin expression was detected for MIAMI cells grafted with LM-PAMs. Nevertheless, a higher ratio of NSCs expressed β3-tubulin (10-fold more), in all conditions tested, compared to MIAMI cells. Furthermore, a significant increase was observed when cells were adhered onto LM-PAM-NT3, with a 3-fold and a 9-fold more important ratio of β3-tubulin expression of NSCs and MIAMI cells, respectively compared to cells grafted alone (Figure 5C).

Immunohistochemistry against human-specific TH (hTH), the rate-limiting enzyme in DA synthesis, illustrates the differentiation of grafted cells toward a neuronal dopaminergic-like phenotype. A small number of cells expressed TH at day one, but more importantly an increase in the number of TH-expressing cells was observed 14 days after grafting in both MIAMI cells and NSCs, when adhered onto LM-PAM-NT3 compared to cells transplanted alone (Figure 5D and 5E). After counting the TH-positive cells, an overall trend toward a dopaminergic phenotype of NSCs and MIAMI cells was observed at day one, particularly when the cells were complexed to LM-PAM-NT3. Fourteen days after grafting, a 3-fold more important number of hTH-positive NSCs was observed. Furthermore, this increase was 2.3 times higher when NSCs were adhered on LM-PAM-NT3. For MIAMI cells there were

a higher number of TH-positive cells when they were complexed to LM-PAM-NT3, but this increase was not significantly different from the number of TH-positive cells counted when the cells were grafted alone (Figure 5F).

3.6 Repair and protection mechanisms

3.6.1 *In vitro and ex vivo mRNA expression of tissue repair factors*

The expression of mRNA *in vitro* allowed detecting which factors may be secreted by cells alone, and by cells 4h and for 24h after adhesion to PAMs. NSCs mainly expressed hVEGFA and brain derived neurotrophic factor (BDNF) while nerve growth factor (NGF) was expressed at a lower level. MIAMI cells expressed very high levels of hVEGFA (about 20-fold more than NSCs), BDNF and low levels of GDNF and NGF confirming previous results (11). Interestingly, growth factor expression by NSCs was similar whether they were associated or not to PAMs, while for MIAMI cells, hVEGFA expression almost doubled at 24h when adhered on LM-PAMs (Figure 6A).

Twenty four hours after implantation in PD organotypic slices, grafted complexes were isolated using laser microdissection, and mRNA expression was quantified using RTqPCR to analyze if cellular patterns of growth factor expression could change due to the host microenvironment (Figure 6B). As observed *in vitro* (Figure6A), NSCs expressed mainly hVEGFA and also a minor quantity of STC-1. A slight increase of hVEGFA expression was observed when NSCs were adhered onto PAMs. MIAMI cells expressed hVEGFA, almost 4-fold more than NSCs, an important quantity of hepatocyte growth factor (HGF), which is not expressed by NSCs, and low levels of STC-1, nerve growth factor and GDNF (Figure 6B).

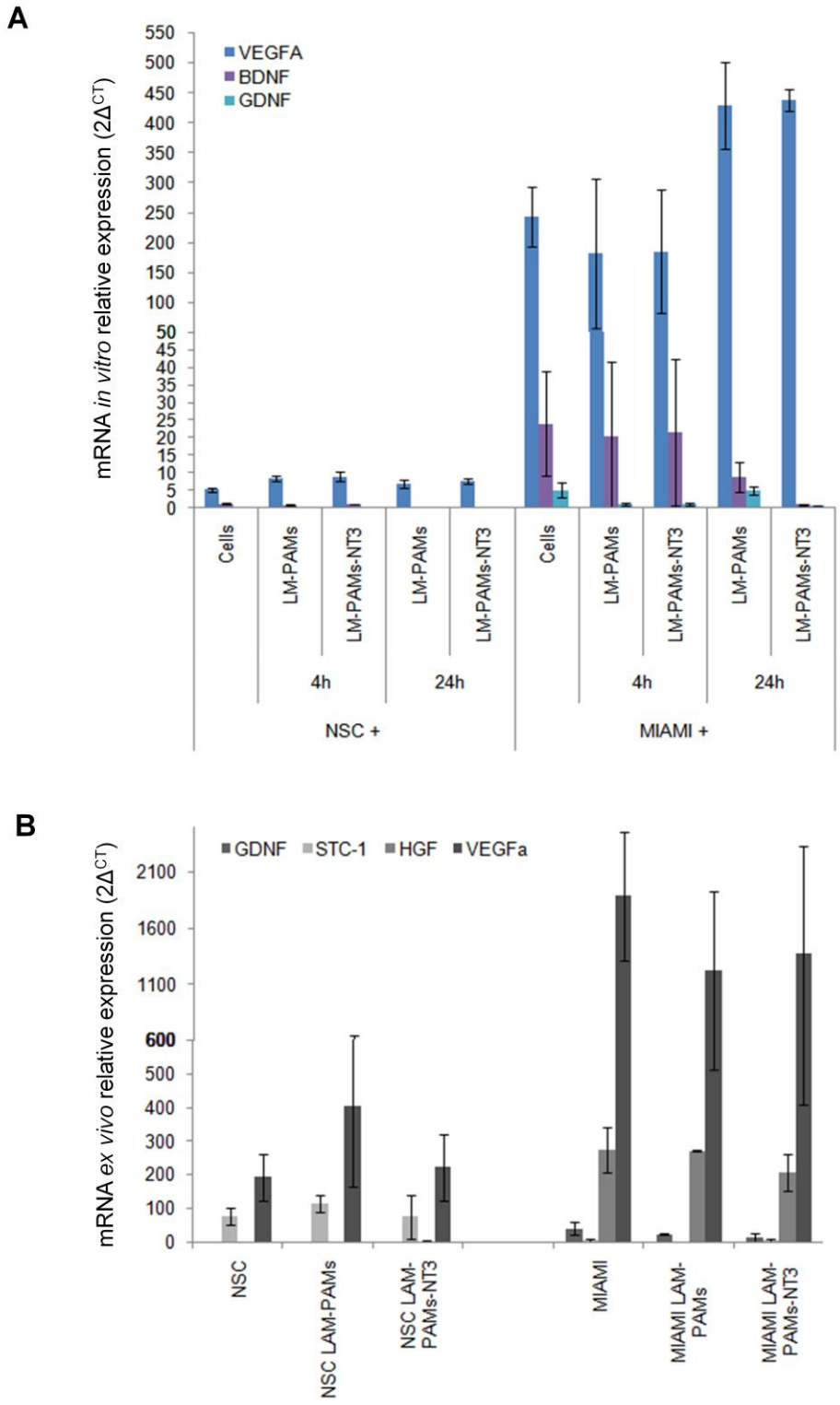

Figure 6: *Expression profile of stem cells A) In vitro RTqPCR of cells alone, or 4h and 24h after adhesion on PAMs. For both cells VEGFA mRNA is the most expressed followed by BDNF mRNA, with an expression almost 50x higher of VEGFA for MIAMI cells. B) Ex vivo RTqPCR of cells grafted alone or adhered on PAMs after isolation by laser microdissection. The most expressed mRNA is VEGFA followed by STC1 for NSCs.HGF and GDNF are also expressed by MIAMI cells. Expression of VEGFA mRNA is still 4-fold higher in MIAMI cells than NSCs.*

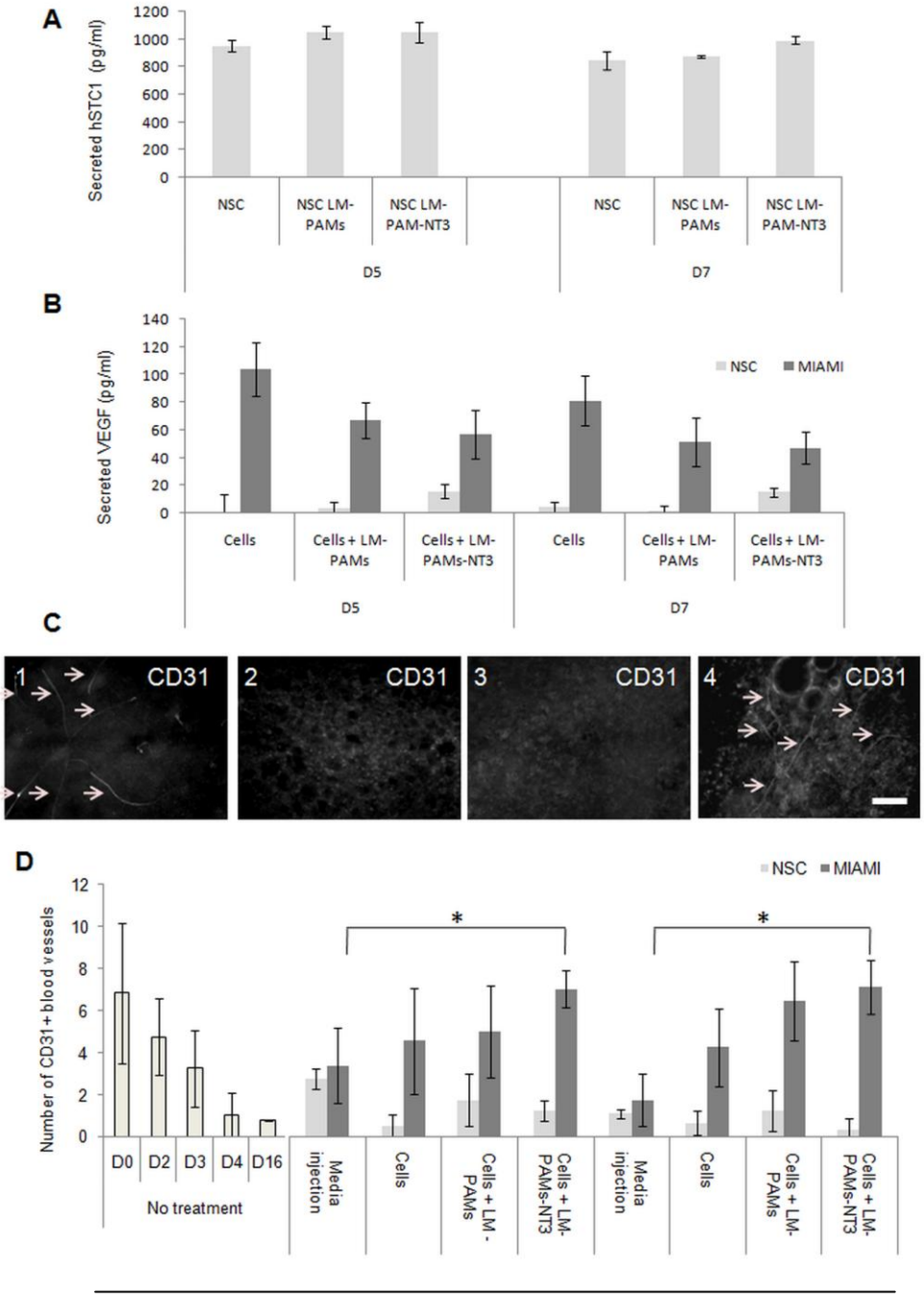

Figure 7: *Grafted cells protein expression and effects A) ELISA quantification of secreted STC1 in culture medium of stem cell grafted organotypic slices. STC1 secretion decreased along time, and is slightly higher when cells adhered to PAMs B) ELISA quantification of secreted VEGFA in culture medium stem cell grafted organotypic slices. For MIAMI cells, VEGFA secretion, decreased overtime and with PAMs adherence. For NSC cells, VEGFA secretion remained very low. C) Immunofluorescence against CD31-positive cells in 1) intact slice, 2) non treated slice at day 16, 3) slice at day 16 with NSC/LM-PAM-NT3 graft and 4) slice at day 16 with MIAMI/LM-PAM-NT3 graft. Blood vessel number increased after MIAMI/LM-PAM-NT3 implantation. White arrows show the blood vessels. Scale bar is 50µM. D) Quantification of blood vessels in the slice. In 4 days, a total degradation of blood vessels was detected. No amelioration was observed after grafting NSCs even when complexed with PAMs, while a significant protection of blood vessels was observed after MIAMI cells implantation, particularly when adhered on LM-PAMs-NT3, (n=3 and p-value= 0,01).* significantly different results with α=0.05*

3.6.3 Analysis of secreted STC-1 and hVEGFA proteins in slice cultures.

Interestingly, STC-1 was secreted by NSCs from 1 day to 14 days after grafting, with around 2350 ± 170 pg/mL at day 3 (after slice culture) and reached 310 ± 70pg/mL at day 16. A little increase was observed when cells were adhered on LM-PAM-NT3 (Figure 7A). As MIAMI cells expressed very low levels of STC1 mRNA, no ELISA assays were performed on those cells for this protein.

Around 115 ± 20pg/mL of hVEGFA secreted by MIAMI cells was detected 1 day after grafting in the slice culture media, which decreased continually to day 16, with around 53 ± 7pg/ml hVEGFA. Moreover, secretion decreased when cells were adhered on LM-PAMs and LM-PAM-NT3. Conversely, for NSCs, hVEGFA secretion was very low (almost 6-fold lower than MIAMI cells), but increased slightly when adhered onto LM-PAM-NT3 (Figure 7B).

3.6.4 CD31 expression and blood vessels counting in organotypic slices

The paracrine stem cell-mediated effect of hVEGFA around the grafted area on PD organotypic slices was assessed by immunofluorescence against CD31, a marker of the endothelial cells of blood vessels (Figure 7C). CD31-positive blood vessels in the nigrostriatal slices were quantified from day 0 to day 16 of culture. At day 0, the number of blood vessels was around 7 ± 3 and decreased to almost zero ± 1 from day 4 to day 16 suggesting the disappearance of blood vessels in the organotypic slices when no treatment is administered. Media injection did not induce significant blood vessel formation neither did LM-PAM-NT3 grafted alone (not shown) at day 3 or at day 16. In contrast, grafted MIAMI cells induced CD31 expression, suggesting blood vessel maintenance. Moreover, the number of vessels increased when cells were adhered on PAMs becoming statistically significant when cells were

adhered onto LM-PAM-NT3 at day 3 and day 16. Therefore, the treatment with MIAMI cells LM-PAM-NT3 promoted the normalization of blood vessel number in the PD organotypic slices. This effect however, was not observed with NSC cells alone or complexed to PAMs. Blood vessel number remained between 2 and 0 in all cases (Figure 7C).

4. Discussion

The conditions for achieving more consistent efficacy in cell replacement studies for PD are being evaluated in recent clinical trials. However, combinatorial cell and drug delivery strategies to enhance cell survival and differentiation still need to be developed and implemented to improve cell or stem cell engraftment. Organotypic models of neurodegenerative disorders can be useful to better understand the mechanisms by which stem cell therapy combined or not to drug delivery may exert beneficial effects, as they may be kept in culture for a few weeks and maintain the cytoarchitecture of the original tissue (18)

In this study grafts were performed two days after slice preparation (Figure 1). Indeed, after two days a degeneration of 80% of the TH-positive neurons in the substantia nigra associated with a 30% dopaminergic denervation in the striatum was recently observed (26). This permits the development of an early stage of PD, which allows observing a protection/repair of the nigrostriatal pathway during a therapeutic window of 14 days (26-27). In this study, we first confirmed our previous observations *in vivo* showing a significant protection/repair of the striatal dopaminergic fibers after a graft of MIAMI/LM-PAM-NT3 (11). In the present study, the protection was mainly assessed in the striatum, but a protection of the fibers localized in the MFB also seems to be induced. However, only a slight neuroprotection, which was not

significantly different from the injection of LM-PAM-NT3 only, was observed after a NSC/LM-PAM-NT3 graft. NT3 has already been reported as an anti-apoptosis factor on 6-OHDA treated PC12 cells (28) and as a neuroprotective factor in adult PD rats (29). It is important to highlight that grafted cell differentiation and neuroprotection/repair assessed by grafted cells combined to PAMs was observed only 14 days after grafting, which is a short time window. Indeed, neuroprotection studies using this type of strategy were evaluated after 6 weeks (30) or 8 weeks (11) in hemi-parkinsonian rats.

In this study we further confirmed, using bioassays performed with either the LM-PAM-NT3 directly in contact with the DRG cells or with released media collected throughout time from LM-PAM-NT3; that released NT3 was still in a bioactive form and was stable for many months. The release profile obtained from LM-PAM-NT3 shows an incomplete release of the encapsulated protein, as previously reported with PLGA-based carriers (11, 30-31). However, this bioassay showed that NT3 possessed a strong neuronal differentiating capacity starting from 5ng/mL on DRG cells. With the release kinetics observed here, more than enough NT3 was released in only 24 hours to induce differentiation. Moreover, cell differentiation was still induced by released NT3 up to 17 days. This allowed an important therapeutic window for MIAMI cells and NSCs to differentiate toward a neuronal phenotype in response to NT3 as they present NT3 receptors. Indeed, it has been shown that MIAMI cells could differentiate to neuron-like cells under the effect of NT3 (11, 32). The same observations were made for NSCs *in vitro* (33) and *ex vivo* (34).

The grafted cells acquired a neuronal-like morphology at day 16 suggesting that they integrated into the host tissue, however no induction of neuronal differentiation was observed over time as no change in β3-tubulin expression was

detected. Nevertheless, the level of expression of β3-tubulin by NSCs was 10-fold more important at all time points, compared to MIAMI cells. Indeed, it was observed that a few NSCs in culture already express β3-tubulin. Moreover, it has been reported that this NSC cell line expresses β3-tubulin with a rate of about 9% after a simple depletion of proliferative factors *in vitro* (20). An increase in neuronal-like differentiation of MIAMI cells was observed after adhesion to LM-PAMs highlighting a differentiation potential of LM as already reported *in vitro* in a previous study (11). The neuronal differentiation was further enhanced by the release of NT3 confirming our previous observations *in vitro*, showing that NT-3 was necessary for the cells to acquire a neuronal-like morphology (14). These results demonstrate that LM-PAM-NT-3 induce a neuronal-like differentiation of MIAMI cells by the combined action of laminine and NT-3. For NSCs an enhanced expression of β3-tubulin was only observed whey they were combined to LM-PAM-NT3 at 16 days, we can therefore assume that mainly NT3 induced their neuronal differentiation (33-34).

Furthermore, the microenvironment of the lesioned dopaminergic denervated striatum seemed to induce a dopaminergic differentiation of grafted cells. Indeed, a significant increase in TH expression was noticed for NSCs grafted alone after 14 days while no TH expression was detected i*n vitro*. No effect of LM-PAMs was noticed on TH expression, highlighting that LM was able to stimulate neuronal but not dopaminergic differentiation for MIAMI cells. A significant increase of TH expression for NSCs and MIAMI cells was quantified when cells were grafted with LM-PAM-NT3, particularly for NSCs. These results further confirm and quantify previous observations in a PD paradigm and allow a better understanding of this dopaminergic differentiation mechanisms induced by LM-PAM-NT3 (11, 30). We also focused on DA striatal content after stem cells grafts. A slight increase of DA content in the

striatum after MIAMI/LM-PAM-NT3 graft was observed, so we can assume that this DA is mainly due to a repair/protection of the host dopaminergic pathway. For NSCs a significant increase of DA content, which reached normal levels after grafting of NSC LM-PAM-NT3, was observed. This result suggests that the quantified DA may be released by differentiated NSCs and by the remaining dopaminergic cells. This effect may be enhanced by the more important number of NSCs compared to MIAMI cells. All these data suggest that NSC grafts combined to LM-PAM-NT3 mainly act as a dopaminergic neuron replacement therapy in this PD paradigm.

MIAMI cells and particularly MIAMI/LM-PAM-NT3 complexes may exert neuroprotective effects on the nigro-striatal pathway through their potential to secrete tissue repair factors (11-12). In the present study, we observed *in vitro* that MIAMI cells expressed BDNF and GDNF as well as high levels of hVEGFA, which was also expressed by NSCs, although at lower levels. BDNF and GDNF have both been reported as neuroprotective factors whose effects were, in part, mediated by autophagy regulation (35) and oxidative stress diminution (36), respectively. A greater neuroprotective effect was usually obtained with GDNF in pre-clinical trials (37-38), which was then evaluated in clinical trials in PD patients (39). Besides NT3 delivered by the PAMs, hVEGFA was highly expressed *ex vivo* and was also secreted by MIAMI cells but not by NSCs, which mainly secreted hSTC1 in the PD organotypic cultures. hVEGFA has already been tested as neurorescue molecule in PD. It was showed that a graft of hVEGFA-expressing cell line in 6-OHDA treated rat induces a strong behavioral recovery and a protection of rat TH-positive cells in the striatum and in the SNc (40-41). Another possibility is that hVEGFA secreted by MIAMI cells and NT3 released by PAMs have a synergetic effect as already reported (42).

As hVEGFA is a well known pro-angiogenic factor, a quantification of blood vessels in the brain slices was performed. No maintenance or increase of blood vessel number was observed over time in the organotypic cultures after grafting NSCs, which did not secrete hVEGFA, although its mRNA was detected. The hVEGFA primer used recognizes all the 14 isoforms of hVEGFA, while the ELISA antibody only recognizes VEGF165, 165b and 121. In this eventuality, we could assume that isoforms, which bind to the ECM (165) may be secreted by NSCs while diffusible isoforms (121) by MIAMI cells (44). Furthermore, NSCs differentiate in an important manner in TH expressing cells able to secrete high quantity of DA, which can block hVEFGA expression (43). After grafting MIAMI cells, which secrete hVEGF, a significant increase CD31 expressing-cells was observed 1 day and 14 days after transplantation, particularly when complexed to LM-PAM-NT3. However, it was observed that hVEGFA had a tendency to decrease in time and when adhered onto PAMs. Apart from the fact that it bound to its receptor, so that it was not found in the media, this observation can also be explained by the fact that during those 14 days, MIAMI cells differentiated into neuron-like cells expressing TH (43). Nevertheless, blood vessels were observed 14 days after grafting particularly with cell-PAMs complexes, suggesting that another angiogenic growth factor was secreted. An important expression of HGF by MIAMI cells, which has also been identified as a member of angiogenic growth factors was detected (45). This factor could have an effect on the increase of CD31-expressing cells observed in this study. Furthermore, an important expression of heat shock protein beta-1 (HSPB1) was detected for MIAMI cells as for NSCs, which increases hVEGFA gene transcription, leading to VEGFA-mediated angiogenesis (46). But as HSPB1 expression was

almost similar for both types of cells, this expression cannot explain the differences detected in CD31 expression between NSCs and MIAMI cells.

hVEGFA may also increase grafted cells survival by creating new blood vessels around the graft enhancing oxygen and nutrients supply (47). Furthermore, it has been proven that hVEGFA can directly and indirectly act to enhance dopaminergic neurons and MSCs survival (41, 48-49). Indeed, it was previously reported that PAMs releasing VEGFA in a prolonged manner induced a strong increase of Bcl2 activity in MSCs and therefore a decrease of apoptosis (48). This result suggests that in our study, hVEGFA secreted by MIAMI cells may act as an anti-apoptotic factor in the graft microenvironment, explaining cell survival. Moreover, after adhesion to PAMs, a significant increase of survival was observed for MIAMI cells confirming previous reports in PD and cerebral ischemia paradigms (11-12). Indeed, survival increase induced by PAMs may be due to the 3 dimensional scaffolds (31) and to the released which NT3 is known to play a role in this process (14). Survival enhancement of cells when adhered on a three dimensional scaffold was mainly described, especially with retinal pigmental epithelium cells (50), which led to a clinical trial in a PD paradigm (51). The increased number of cells observed 1 day after grafting when they are complexed to PAMs may explain the neuroprotective effects of these complexes as more neuroprotective factors may be secreted at this early time-point. In contrast, no significant increase of NSC survival was detected due to the 3D scaffold. However, NSCs survival remains higher than MIAMI cells for every time point. The high secretion of STC1 by NSCs may act by autocrine effect to induce a protection of cells against mitochondrial membrane defects induced by reactive oxygen species as reported with neural crest-derived cells.(52).

5. Conclusion

The biological mechanisms by which MIAMI cells and NSC complexed or not on LM-PAM-NT3 induce neuroprotection were in part elucidated using an ex vivo model of PD. A small fraction of MIAMI cells differentiate into dopaminergic neuron-like cells, but induce a significant recovery in only 14 days with a slight increase of DA content. The repair/protection potential may act by HGF, GDNF and mostly hVEGFA secretion. On the other hand, NSC can differentiate to dopaminergic neurons able to secrete DA and a slight neurorepair/protection potential was associated to these cells, which can secrete STC1. NSCs may therefore act as a replacement therapy for PD treatment, while MIAMI cells mainly protect host remaining dopaminergic cells. We can suggest that the graft of both types of cells with LM-PAM-NT3 could be a powerful and original approach to treat PD.

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DISCUSSION

Grâce à cette étude nous avons pu démontrer que les coupes organotypiques en plus de représenter un support intéressant et efficace pour y développer et étudier des maladies neurodégénératives, représentent également un outil puissant pour effectuer une étude mécanistique liée à la greffe de cellules souches dans un paradigme de maladie neurodégénérative.

Dans un premier temps, nous avons confirmé le pouvoir neuroprotecteur d'une greffe de cellules MIAMI-LM-MPA-NT3 sur la voie dopaminergique nigrostriée. En effet une augmentation significative de la densité des fibres dopaminergiques fut détectée dans le striatum mais fut aussi observée dans le faisceau médian du télencéphale. Nous avons donc cherché les mécanismes liés à cette neuroprotection. Dans un premier temps, il a été mis en évidence que la laminine permet d'augmenter significativement la survie des cellules MIAMI en particulier durant les premières 24h, alors qu'elle ne semble pas avoir d'effet sur les CSN. La différenciation des deux types de cellules souches en cellules « neurones-like » exprimant la β3-tubuline et la tyrosine hydroxylase, l'enzyme permettant la synthèse de dopamine, s'effectue faiblement sans MPA, juste grâce au microenvironnement du striatum lésé. Une légère augmentation de cette différenciation est observée quand les cellules sont adhérées aux MPA blanches et encore plus lorsqu'elles sont adhérées aux LM-MPA-NT3. De plus ces cellules, en particulier les CSN, sont capables de sécréter de la dopamine et ainsi de pouvoir diminuer les symptômes liés à la pathologie. Par la suite, nous avons pu démontrer que les cellules MIAMI étaient capables de sécréter d'importantes quantités de VEGFA (vascular endothelial growth factor A), contrairement aux CSN. L'importance de la sécrétion du VEGFa dans la

neuroprotection induite par les cellules MIAMI avec ou sans MPA a par la suite été analysé. En effet il a déjà été démontré que ce facteur possède un puissant pouvoir angiogénique. Nous avons pu démontrer que cette sécrétion de VEGFa permettait de bloquer la dégradation des vaisseaux sanguins observée dans les coupes organotypiques. Suite à la greffe de CSN, aucune protection/formation de vaisseaux sanguins n'a pu être détectée.

En plus de ce pouvoir angiogénique, le VEGFA possède aussi un pouvoir de protection des neurones dopaminergiques (Pitzer et al., 2003, Yasuhara et al., 2004). Une équipe a également démontré qu'une sécrétion constante de VEGFA dans le striatum de rats hémi-parkinsoniens induit une importante amélioration motrice chez les rats associée à une protection des neurones dopaminergiques dans la substance noire ainsi que dans le striatum, ce qui vient confirmer nos travaux (Yasuhara et al., 2005). Les cellules MIAMI pourront donc agir au sein du SNC en se différenciant en neurones dopaminergiques capables de sécréter de la dopamine mais surtout en sécrétant des facteurs neuroprotecteurs tels que le VEGFA. En comparaison les CSN vont principalement se différencier en neurones dopaminergiques en grand nombre capables de sécréter une quantité encore plus importante de dopamine. Par contre leur potentiel de neuroprotection reste assez faible malgré une sécrétion très importante de STC1 connue pour être un puissant anti-apoptotique (Durukan Tolvanen et al., 2013, Yeung et al., 2012). Ces deux types cellulaires ont donc des comportements différents après implantation dans le tissu hôte mais pouvant être complémentaires. Une approche novatrice et intéressante serait donc de greffer ces deux types de cellules en même temps dans un modèle *ex vivo* puis, *in vivo* de la maladie de Parkinson.

CHAPITRE 4

Développement d'un modèle ex vivo de la maladie de Huntington pour étudier le potentiel thérapeutique des cellules souches neurales et mésenchymateuses.

INTRODUCTION

Développement d'un modèle *ex vivo* de la maladie de Huntington pour étudier le potentiel thérapeutique des cellules souches neurales et mésenchymateuses.

L'étude de cette nouvelle approche d'ingénierie tissulaire pour la maladie de Parkinson ayant donné des résultats satisfaisants, nous avons décidé de développer une approche similaire concernant la maladie de Huntington.

Dans un premier temps, nous avons cherché à développer un nouveau modèle *ex vivo* de la maladie de Huntington qui se focalise sur l'aspect cellulaire de la pathologie mais ne nécessitant pas d'injection de neurotoxines. Les modèles *ex vivo* de la maladie de Huntington existants consistent en l'injection dans une coupe striatale d'une neurotoxine telle que l'acide kaïnique, l'acide quinolinique ou encore l'acide 3-nitropropionique (Matyja, 1986, Whetsell and Schwarcz, 1989, Storgaard et al., 2000). Une grande diversité de toxines et de protocoles existent créant ainsi des différences au niveau des résultats. D'autres modèles plus complexes existent, prenant en compte l'aspect génétique de la pathologie. Ils peuvent être obtenus en préparant des coupes organotypiques à partir de souris transgéniques R6/2 ou bien en utilisant des coupes organotypiques de rongeurs normaux et en y transfectant le gène muté de la huntingtine (Smith and Bates, 2004, Smith et al., 2001, Murphy and Messer, 2001, Murphy and Messer, 2004, Reinhart et al., 2011). Mais ces derniers modèles sont plus longs et plus coûteux à mettre en place.

Ce nouveau modèle *ex vivo* sera ensuite utilisé pour effectuer une étude du potentiel thérapeutique de l'implantation de cellules MIAMI et de CSN associées ou non à des MPA pour la maladie de Huntington. De précédents travaux ont permis de démontrer que les cellules souches mésenchymateuses et les CSN sont capables de

se différencier en cellules exprimant l'acide gamma aminobutyrique (GABA) (El-Akabawy et al., 2011, Bosch et al., 2004) et qu'elles peuvent induire une protection/réparation des voies GABAergiques lésées dans la maladie de Huntington conduisant à une amélioration comportementale chez les rongeurs (Amin et al., 2008, Lee et al., 2005, Lescaudron et al., 2003). Il a également été démontré que le BDNF était un facteur clé dans la protection/réparation des neurones GABAergiques et qu'il pouvait donc avoir un effet thérapeutique pour la maladie de Huntington (Samadi et al., 2013, Reiner et al., 2012, Giampa et al., 2013). De plus, le BDNF semble pouvoir stimuler la différenciation des précurseurs neuraux en neurones épineux moyens (NEM) GABAergiques (Ivkovic et al., 1997, Aubry et al., 2008, Ma et al., 2012).

Ce chapitre décrit donc la mise au point d'un modèle innovant de la maladie de Huntington. Le but était d'obtenir une coupe organotypique dans laquelle les principales zones impliquées dans la pathologie, à savoir le striatum et le globus pallidus, pouvaient être étudiées. Lors de la préparation de ces coupes organotypiques, une lésion des voies GABAergiques devait être obtenue sans ajout de neurotoxines afin d'obtenir un modèle, simple, reproductible et mono-paramétrique de la maladie de Huntington. Par la suite le potentiel des cellules MIAMI et des CSN à se différencier en NEM exprimant le GABA a été étudié. L'effet du BDNF dans ce contexte a également été exploré. Enfin les cellules souches associées ou non à des MPA enrobés de laminine ont été greffées dans le modèle *ex vivo* de la maladie de Huntington et une étude du devenir cellulaire, de la différenciation *in situ* et du potentiel de neuroprotection des complexes cellules LM-PAM a été réalisée.

Pharmacologically active microcarriers complexed with human stem cells differentiation potential in an innovative *ex vivo* model of Huntington's disease

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Abstract:

Huntington's disease (HD) is an autosomal dominant genetic neurodegenerative disorder for which no cure exists. Cell therapy has great potential but also some limits to locally repair striatal GABAergic medium spiny neurons. In this study, we evaluated the therapeutic potential of the transplantation of human marrow-isolated adult multilineage inducible cells (MIAMI) cells compared to neural stem cells when adhered on laminin coated pharmacologically active microcarriers (PAMs) for the treatment of HD. In the present study, we developed an organotypic HD model from rat brains that includes the main areas involved in the pathology in a single slice preparation, without using neurotoxins to induce the GABAergic lesion. The mechanical transection of projection medium spiny neurons obtained during slice preparation induced HD-like histopathology. This innovative model was characterized and showed a slice survival of 18 days with a loss of DARPP32 and GAD67 expression of 50% and 20% respectively in 3 days We then showed that a GABAergic MSNs differentiation commitment could be obtained with MIAMI cells and NSCs by addition of valproic acid and BDNF in the culture media. Stem cells differentiated into GABAergic neuron-like cells in situ, a slight MSNs differentiation of 15% was obtained with MIAMI cells while about 80% of the total numbers of NSCs differentiate in DARPP32 expressing cells. No significant improvement was detected when cells were implanted with PAMs. Moreover, a slight protection of host GAD67 and DARPP32 positive neurons was detected after the implantation of both types of stem cells. These results show a prospective interest of human NSC-PAMs and MAMI-PAMs in tissue engineering for HD.

Keywords:

Huntington's disease, neural stem cells, Marrow-isolated adult multilineage inducible cells (MIAMI), organotypic slice culture, pharmacology active microcarriers, Laminin

Abbreviations:

Huntington's disease (HD), medium spiny neurons (MSNs), Marrow-isolated adult multilineage inducible (MIAMI), human mesenchymal stromal cells (hMSC), Neural stem cells (NSCs), gamma-Aminobutyric acid (GABA), brain derived neurotrophic factor (BDNF), pharmacologically active microcarriers (PAMs), neurotrophine-3 (NT3), kaininc acid (KA), quinolinic acid (QA), 3-nitropropionic acid (3-NPA), messenger RNA (mRNA), Poly-D-Lysine (PDL), poly(lactic-co-glycolic acid) (PLGA), laminin (LM), phosphate buffer saline (PBS), Sonic hedgehog (SHH), normal goat serum (NGS), human mitochondria (hMito), green fluorescent protein (GFP), dopamine- and cAMP-regulated neuronal phosphoprotein (DARPP32), glutamic acid decarboxylase-67 (GAD67), GABA transporter-1 (GAT1), paraformaldehide (PFA), neuronal nuclei (NeuN), diaminobenzidine (DAB), complementary DNA (cDNA), Neurofilament medium (NFM), Bovine serm albumin (BSA), valproic acid (Valp Ac)

1. Introduction

Huntington's disease (HD) is an autosomal dominant genetic neurodegenerative disorder with a prevalence of 6 to 7 for 100.000 around the world (Walker, 2007). This pathology results in abnormal involuntary movements (chorea and dystonia), cognitive decline, behavioral changes and a range of psychiatric disorders. These symptoms, which gradually develop, are in part caused by degeneration of the gamma-Aminobutyric acid (GABA) expressing (GABAergic) striatal medium spiny neurons (MSNs) which project to the external segment of the globus pallidus, and to the SN *pars reticulata* (Ho et al., 2001). The huntingtin gene is localized on chromosome 4 and the normal allele contains a trinucleotide repeat sequence (CAG) encoding polyglutamine, but this CAG repeat is amplified in patients suffering from HD. When polyglutamine repetition reaches 39 reaches or more, the pathology will develop generally at around 30-40 years (for review see Ha and Fung, 2012). No cure exists for this disease and antichoreic drugs or neuroleptics only reduce the severity of the symptoms. Moreover, their administration can cause important side effects (Adam and Jankovic, 2008). With HD, the neurodegeneration is mainly localized in the striatum at onset of the pathology, so cell therapy may represents an attractive alternative treatment (Wijeyekoon and Barker, 2011).

Most clinical trials of cell therapy for HD consisted of striatal stereotactic implantation of cell suspensions from ganglionic eminence. They were performed in order to restore lost striatal GABAergic MSNs. These clinical trials showed promising results, but overall, the poor availability of fetal tissue, the ethical issues associated with their use and the limited survival of the transplanted cells restrained the development of this therapy (Gallina et al., 2008, Gallina et al., 2010). The use of stem cells that have great proliferative and differentiation capacity can overcome

those limitations but to our knowledge no clinical trials with stem cells for HD were already performed.

Marrow-isolated adult multilineage inducible (MIAMI) cells are a unique human mesenchymal stromal cells (hMSC) subpopulation exhibiting a homogeneous morphology and a unique gene expression profile (Roche et al., 2013, D'Ippolito et al., 2006). Furthermore they have a high neuron-like differentiation capacity and can secrete neuroprotective factors (Tatard et al., 2007, Garbayo et al., 2011, Rahnemai-Azar et al., 2011). Neural stem cells (NSCs) are the natural stem cells of the central nervous system, and represent a reference for brain cell therapy. It has already been reported that MSCs and NSCs were able to differentiate into GABA expressing cells (El-Akabawy et al., 2011, Bosch et al., 2004) and that, particularly MSCs, could protect/repair the striatal MSN and induce functional recovery in HD models (Amin et al., 2008, Lee et al., 2005, Lescaudron et al., 2003). It has been reported that brain derived neurotrophic factor (BDNF) was a key factor for protection and repair of GABAergic MSNs, and that it could have an important therapeutic effect on HD (Samadi et al., 2013, Reiner et al., 2012, Giampa et al., 2013). Moreover, BDNF was mainly used to induce neuronal precursor cells differentiation in a GABAergic MSNs phenotype (Ivkovic et al., 1997, Aubry et al., 2008, Ma et al., 2012). However, despite encouraging results, cell-based therapies for HD are still facing poor survival and engraftment of cells that restrain their use. All this evidence supports the need to develop strategies to optimize stem cell survival, engraftment, differentiation and contribution to functional recovery.

In this context, pharmacologically active microcarriers (PAMs) provide a powerful tool for cell integration. PAMs are biodegradable and biocompatible polymeric microparticles made of poly (lactic-co-glycolic acid) and provide a 3-

dimensionnal support coated with extracellular matrix proteins. Furthermore, PAMs can encapsulate a growth factor, which will be released in a controlled manner during microparticles degradation. Those characteristics increase cell survival, differentiation and integration in the host tissue (Tatard et al., 2004, Tatard et al., 2005). We previously showed that neurotrophine-3 (NT3) releasing PAMs were a powerful tool to increase MIAMI cell therapy and induced motor improvement in a PD paradigm (Delcroix et al., 2011). In order to maximize the therapeutic potential of this approach, a complete understanding of the stem cell mode of action in this HD paradigm and the contribution of PAMs to neural repair is required. A wide variety of *in vivo* models were developed to explore cell death in HD as well as to evaluate therapeutic approaches for HD (Gardian, 2006, Kim et al., 2011, Mangiarini et al., 1996). However, the use of *in vivo* models for exploring the molecular and cellular mechanisms of the therapeutic approaches is time consuming and involves high technical and financial resources.

Organotypic cultures made from brain slices that can be kept in culture for several weeks after injecting molecules or cells represent a remarkable tool to address cell therapy issues. Recently, organotypic brain slice cultures have been used for HD modeling. Thus, it is possible to test the potential of stem cells in a HD context (Daviaud et al., 2013b). First HD organotypic models were obtained by kainic acid (KA), quinolinic acid (QA) or-nitropropionic acid (3-NPA) administration in striatal organotypic cultures (Matyja, 1986, Whetsell and Schwarcz, 1989, Storgaard et al., 2000). A model established from the R6/2 transgenic mice was developed and was clearly established as a screening model of anti-aggregation components (Smith et al., 2001, Smith and Bates, 2004). HD was also modeled by the transfection of HD-polyQ plasmids or with DNA constructs derived from the human pathological

huntingtin gene into cells within the slice (Murphy and Messer, 2001, Murphy and Messer, 2004, Reinhart et al., 2011).

To this end, we developed a coronal organotypic culture model that includes the main areas involved in HD in a unique slice that do not need neurotoxins to induce the GABAergic MSNs depopulation. The goal was to induce progressive striatal MSNs degeneration in a single step while preparing the slices, in order to obtain a simple reproducible HD *ex vivo* model. We next studied slice survival and we characterized the MSNs depopulation in the striatum and the GABAergic pathway degradation whose fibers run from the striatum to the globus pallidus. This new *ex vivo* model of HD was then used to study a new approach of tissue engineering for HD. A complete characterization of BDNF induced differentiation of MIAMI cells and NSCs was first performed *in vitro* and a complete protocol was implemented to have a GABAergic MSN differentiation. Thereafter, NSCs and MIAMI cells associated with laminin (LM) coated PAMs, were grafted in the lesioned striatum. A study of grafted cells survival, neuronal and GABAergic differentiation was performed.

2. Material and methods

2.1 Huntington's disease organotypic slice culture preparation

Animal care and use were in strict accordance with the regulations of the French ministry of agriculture and all animal procedures were approved by animal experimentation ethic committee of "Pays de la Loire". Every effort was made to minimize animal suffering and the number of animal used.

Timed pregnant Sprague-Dawley rats were purchased from Janvier (Saint Berthevin, France) or from SCAHU (Service commun d'animalerie hospitalo-

universitaire, University of Angers, France). Postnatal 6 to 8 day old pups were used to prepare organotypic slices according to Stoppini method (Stoppini et al., 1991) with modifications (Daviaud et al., 2013a). Pups were anesthetized by an intraperitoneal injection of 80 mg/kg of ketamine (Clorketam 1000, Vetoquirol, Lure France) and 10 mg/kg of xylazine (Rompum 2%, Bayar Health Care, Kiel Germany). Animals were sacrificed; brains were rapidly dissected and removed. Under aseptic conditions, 400µm slices were cut in different configurations in order to obtain a progressive degeneration of the GABAergic MSNs. Finally, cerebellum and olfactive bulbs/prefrontal cortex were cut off and brains were glued, on their dorsal side, onto the chuck of a water cooled vibratome (Motorized Advance Vibroslice MA752, Campdem instruments). Coronal sections were cut with a 14-degree angle of the razor blade, and placed in sterile ice-cold Grey's Salt Balanced Solution (Sigma Aldrich, St Louis, USA) supplemented with 6.5 mg/L of glucose and antibiotics (100 U/mL penicillin, 0.1 mg/mL streptomycin, 0.25 µg/mL amphotericin B) (Sigma Aldrich, St Louis, USA) for one hour. Typically, about 10 slices can be obtained per hemisphere. The first 2-3 and the 2 last brain slices did not contain the main areas involved in the pathology and were discarded. Three to four slices per hemisphere were next transferred to 30 mm diameter semiporous membrane inserts (Millicell-CM, Millipore, Bedford, MA, USA) within a 6-well plate and put in culture at 37ºC, 5% CO2. For each condition, a minimum of three slices taken from 3 different rat pups were used.

Slices were conserved in two different media. From days 0-3, a serum containing medium was used: 50% MEM (Minimum Essential Medium Eagle, Sigma Aldrich), 25% Hank's (Hank's Balanced Salt Solution, Sigma Aldrich), 25% of horse serum (decomplemented horse serum, Gibco), 6.5mg/ml of glucose, 1 mM of L-

glutamine (Sigma Aldrich, St-Louis, USA) and antibiotics (Sigma Aldrich, St-Louis, USA). From days 3-18, a serum free medium was used: Neurobasal medium (Gibco, Life Technologies, Paisley, UK) supplemented with 6.5 mg/L of glucose, 1mM of L-glutamine, 1x B27 supplements (Gibco, Life Technologies, Paisley, UK) and antibiotics. The media was changed the first day after culture and then every two days during the entire culture period.

2.2 Cells culture

2.2.1 Culture of MIAMI cells

MIAMI cells were expanded *in vitro* from passage 4-5 on fibronectin (Sigma Aldrich, St Louis, USA) coated flasks at 120 cells/cm² in low oxygen tension in DMEM-low glucose (Gibco, Life Technologies, Paisley, UK), supplemented with 3% fetal bovine serum, 30µg/ml ascorbic acid and a mixture of lipids (working concentration of 510nM lipoic, 70nM linolenic and 150nM linoleic acid, all from Sigma). Then a 10 days treatment with an addition of 20ng/mL of EGF, bFGF (both from R&D systems, Lille, France) and 5µg/ml of Heparin (Sigma Ladrich, St-Louis, USA) is conducted to enhance neuronal specification. Cells were fed every 3 days by changing half medium, and split every 5 days

2.2.2 Culture of NSC cells

A lineage of NSC expressing green fluorescent protein (GFP) (hNSC1) was kindly provided by Alberto Martinez Serrano from Madrid University [21-22]. hNSC1 cells were expanded *in vitro* from passage 10 to 15 on Poly-D-Lysine (PDL, Sigma Aldrich, St-Louis, USA)) coated flasks. Cells grows in a DMEM/F12 (1:1) (Glutamax, Gibco, Life Technologies, Paisley, UK) medium supplemented with 6.5mg/L of

glucose (Sigma Aldrich, St-Louis, USA), Hepes (Hepes Buffer 1M, Sigma Aldrich, St-Louis, USA), 0.5% of Albumax (Gibco, Carlsbad USA), 1% of N2 supplements (Gibco, Life Technologies, Paisley, UK), 20ng/ml of EGF and bFGF both from R&D Systems (R&D systems, Lille, France), 1% of non essentials amino acids (NEAA, Biowhitaker Lonza, Belgium) and antibiotics/antimyotics (100 U/mL penicillin, 0.1 mg/mL streptomycin, 0.25 µg/mL amphotericin B, Sigma Aldrich, St-Louis, USA) under normoxic conditions. Cells were fed every 3 days by changing half medium, and split every 5 days, keeping 20% of old medium.

2.3 GABAergic differentiation

2.3.1 *In vitro* cell differentiation assessment

NSCs and MIAMI cells were seeded at 3000 cells per cm² on glass cover coated with PDL (5µg/cm², Sigma Aldrich, St-Louis, USA) and LM respectively (2µg/cm², Sigma Aldrich, St-Louis, USA). Then, different conditions were tested to obtain the best GABAergic differentiation protocol (Table 1). Briefly, for MIAMI cells or NSCs, DMEM-low glucose (Gibco, Life Technologies, Paisley, UK) or DMEM/F12 (Glutamax, Gibco, Life Technologies, Paisley, UK) were respectively supplemented with B27/N2 (both from Gibco, Life Technologies, Paisley, UK), Sonic hedgehog (SHH, Peprotech, Rocky Hill, USA), valproic acid (Sigma Aldrich, St-Louis, USA) or BDNF (Peprotech, Rocky Hill, USA).

2.3.2 In vitro differentiation analysis

After treatment, cells were fixed by addition of 1ml of ice cold 4% paraformaldehide (PFA, Sigma, St Louis, USA) in DPBS (Lonza, Verviers, Belgium) pH 7.4 during 15min. Then cells were washed in DPBS three times. Non specific

sites are blocked with DPBS, Triton 0.1% (PBS-T, Triton X-100, Sigma, St Louis, USA), bovine serum albumin 4% (BSA, Fraction V, PAA Lab, Austria), normal goat serum 10% (NGS, Sigma, St Louis, USA) during 45 min at RT.

A mouse anti human β3-tubulin (2ng/ml, clone SDL.3D10, Sigma, St Louis, USA) or rabbit monoclonal anti β3-tubulin (1:400, EP1331Y, Abcam, Cambridge, UK), a mouse anti human neurofilament medium (NFM, 1:50, clone NN18, Sigma Aldrich, USA), a monoclonal rabbit anti human dopamine- and cAMP-regulated neuronal phosphoprotein (DARPP32, 1:400, clone EP721Y, Abcam, Paris, France), A mouse anti glutamic acid decarboxylase-67 antibody (GAD67, 5µg/ml, clone 1G10.2, Millipore SA, Guyancourt, France), a rabbit anti GABA transporter-1 (GAT1, 500ng/ml, Millipore SA, Guyancourt, France) and a monoclonal mouse anti-human Ki67 (350ng/ml, MIB-1, DakoGlostrup, Denmark) antibodies were used to characterize cell differentiation. Cells were incubated on night with the primary antibody diluted into PBS-T, BSA 4% at 4°C. After washes, slices were incubated with the biotinylated mouse or rabbit secondary antibody (7,5 µg/ml, Vector Laboratories, Burlingame, USA) for 1 hour at RT. Then slices were washed and incubated with Streptavidin Fluoroprobes R488 or R547H (Interchim, Montluçon, France) diluted 1:200 in PBS for 1 hour before mounting with a fluorescent mounting medium.

The intensity of positive stained cells was quantified using the Metamorph® software for all markers. Intensity of the staining was then divided by the total number of cell. Results were presented as mean differences +/- average deviation and were calculated from 3 different experiments.

MIAMI cells	B27/N2	SHH	Ac Valp	BDNF
Medium 1	-	-	-	-
Medium 2	-	-	-	30ng/ml
Medium 3	-	-	10µM, 3 days	30ng/ml
Medium 4	-	-	10µM, 5 days	30ng/ml
Medium 5	-	200ng/ml	10µM, 3 days	30ng/ml
Medium 6	1/100[e]	-	10µM, 3 days	30ng/ml

NSCs	B27/N2	Ac Valp	BDNF
Medium 1	-	-	-
Medium 2	-	-	30ng/ml
Medium 3	-	10µM, 3 days	30ng/ml
Medium 4	-	4µM, 3 days	30ng/ml
Medium 5	1/100[e]	10µM, 3 days	30ng/ml

Table 1: Summary of the GABAergic medium spiny neurons differentiation media tested on MIAMI cells (top) and NSCs (Bottom). For MIAMI cells based medium was made of DMEM-low glucose and antibiotics while for NSCs was medium was made of DMEM/F12 and antibiotics.

2.3 Preparation of laminin coated microparticles

Microparticles were prepared using an s/o/w emulsion solvent extraction-evaporation method previously described here [13, 23]. A PLGA copolymer with a lactic:glycolic ratio of 37.5:25 (MW: 25,000 Da) was used (Phusis, Saint Ismier, France). Organic phase was made of 50 mg PLGA dissolved in a 3:1 methylene chloride:acetone solution. This organic phase was emulsified in a poly(vinylalcohol) (Mowiol[4-88],Kuraray Specialities Europe, Frankfurt, Germany) aqueous solution (90 ml, 4% w/v) maintained at 1°C and mechanically stirred at 550 round per min (rpm) for 1 min (Heidolph, RZR 2041, Merck Eurolab, Paris, France). After addition of 33 ml

of deionized water and stirring for 10 min, the resulting o/w emulsion was added to 190 mL deionized water and stirred for a further 20 min to extract the organic solvent. Finally, the microparticles were filtered on a 5 µm filter (SVLP type, Millipore SA, Guyancourt, France).

2.4 PAMs preparation

To obtain PAMs, PLGA microspheres were coated with a combination of LM (Sigma Aldrich, St-Louis, USA) and PDL (Sigma Aldrich, St-Louis, USA) molecules to favor cell attachment as previously published (Delcroix et al., 2011). Briefly, 5mg of microspheres were resuspended in phosphate buffer saline (PBS, Lonza, Verviers, Belgium). Coating solution was prepared in PBS, at a final concentration of 6 µg/mL-9 µg/mL of PDL/LM. This solution was mixed to the microsphere suspension and placed under rotation at 15 rpm at 37°C during 1h30. After coating, PAMs were washed 3 times in distilled sterile water, lyophilized and kept at -20°C for long-term storage.

2.5 Stem cells/ LM-PAMS complexes injection into organotypic slices

Three days after organotypic slice preparation, treatments were injected into the striatum using a 22-gauge needle (Hamilton, Bonaduz, Switzerland) connected to a micromanipulator. Eight experimental groups were tested: (1) non-treated organotypic slices, (2) culture media, (3) NSC cells, (4) MIAMI cells, (5) NSC-LM-PAMs and (6) MIAMI-LM-PAMs. Each experimental groups were tested in n=3 unless otherwise stated. Total injection volume consisted of 1.5µl of culture media containing approximately 37.000 cells alone or adhered to 0.075 mg of LM-PAMS.

Injections were done at 0.5µl/minute infusion rate. The needle was left in place for 5 min to avoid the cells being expelled from the organotypic slices.

2.7 Histological study

At different times, ranging from 0 to 18 days, organotypic slices were washed with phosphate buffer saline (PBS) (Lonza, Verviers, Belgium), fixed with 4% PFA (Sigma Aldrich, St Louis, USA) in PBS pH 7.4 for 2h and washed three times with PBS. Finally, non specific sites are blocked with PBS, Triton 1% (PBS-T, Triton X-100, Sigma, St Louis, USA), BSA 4% (Fraction V, PAA Lab, Austria), NGS 10% (Sigma, St Louis, USA) during 4 hours at RT under agitation.

2.7.1 Anti-mitochondria, β3-tubulin and DARPP32 immunofluorescence

Immunofluorescences were performed using antibodies against neuronal nuclei (NeuN) (1:100, clone A60, Merck Millipore, Billerica, USA) to analyze slice survival. A monoclonal mouse anti rat DARPP32 (250ng/ml, BD transduction laboratories, Franklin Lakes, USA) antibody was used to study the striatal MSNs of the slice. MIAMI cells were detected using a mouse anti-human mitochondria antibody (hMito, 10ng/ml, clone MTCO2, Abcam, Paris, France). NSC cells were observed using their own potential to express GFP. A mouse anti human β3-tubulin (2ng/ml, clone SDL.3D10, Sigma, St Louis, USA) or rabbit monoclonal anti β3-tubulin (1:400, EP1331Y, Abcam, Cambridge, UK) and a monoclonal rabbit anti human DARPP32 (1:400, clone EP721Y, Abcam, Paris, France) antibodies were used to characterize grafted cells differentiation. Slices were incubated 48h with the primary antibody diluted into PBS-T, BSA 4% at 4°C. After washes, slices were incubated with the biotinylated mouse or rabbit secondary antibody (7.5 µg/ml, Vector

Laboratories, Burlingame, USA). Then slices were washed and incubated with Streptavidin Fluoroprobes R488 or R547H (Interchim, Montluçon, France) diluted 1:200 in PBS for 2 hours before mounting with a fluorescent mounting medium.

The number of double positive stained cells in the striatum was quantified using Metamorph® software for all markers and then divided by the total number of cells. Results were presented as mean differences +/- average deviation and were calculated from 3 to 4 different slices.

2.7.2 GAD67 immunochemistry

A mouse anti GAD67 antibody (5µg/ml, clone 1G10.2, Millipore SA, Guyancourt, France) was used to observe striatal-globus pallidus GABAergic pathways and to study GABAergic differentiation of grafted cells. Slices were incubated 48h with the primary antibody diluted into PBS-T, BSA 4% at 4°C. After washes, slices were incubated with the biotinylated anti-mouse secondary antibody (7,5µg/ml, Vector Laboratories, Burlingame, USA). Then slices were washed and quenching of peroxidase is made with 0.3% H2O2 (Sigma, St Louis, USA) in PBS-T, at RT for 20 min, then after PBS washes, incubation with Vectastain ABC reagent (Vector Laboratories, Burlingame, USA) in PBS was made at RT for 2h. Sections were washed and revealed with 0.03% H2O2, 0.4 mg/mL diaminobenzidine (DAB, Sigma, St Louis, USA) in PBS, 2.5% nickel chloride (Sigma, St Louis, USA) and dehydrated before mounting.

GAD67-positive fiber quantification in the striatum at eighteen days was quantified using the Metamorph® software. Results were presented as mean differences +/- average deviation and were calculated from 6 pictures taken from 4 different rats for each group.

2.8 Reverse transcription and real time quantitative PCR.

The following experimental details were performed following the guidelines of the PACEM core facility (‘‘Plate-forme d'Analyse Cellulaire et Moléculaire”, Angers, France). Sense and antisense desalted primer pairs (Eurofins MWG Operon, Ebersberg, Germany) were mixed in RNAse free water at a final concentration of 5 µM (Table 2). Total RNA of cells were extracted and purified using RNeasyMicrokit (Qiagen, Courtaboeuf, France), and treated with DNase (10 U DNase I/µg total RNA). RNA concentrations were determined using a ND-2000 NanoDrop (Thermo Fisher Scientific, Wilmington, Delaware USA) and used for normalization of the input RNA in the Reverse transcription. RNA integrity was verified on Experion RNA StdSens chip (Bio-Rad). First strand complementary DNA (cDNA) synthesis was performed with a SuperScriptTM II Reverse Transcriptase (Invitrogen), in combination with random hexamers, according to the manufacturer’s instructions. Following first-strand cDNA synthesis, cDNAs were purified (Qiaquick PCR purification kit, Qiagen, Courtaboeuf, France) and eluted in 40 µL RNAse free water (Gibco). 3ng of cDNA was mixed with MaximaTM SYBR Green qPCR Master Mix (Fermentas) and primer mix (0.3 µM) in a final volume of 10µL. Amplification was carried out on a Chromo4 thermocycler (Biorad) or LightCycler 480 (Roche) with a first denaturation step at 95°C for 10 min and 40 cycles of 95°C for 15 s, 60°C for 30 s. After amplification, a melting curve of the products determined the specificity of the primers for the targeted genes. Several housekeeping genes, Glyceraldehyde-3-phosphate dehydrogenase (Gapdh), Beta-2-microglobulin (B2M), Beta actin (Actb), and Heat shock 90 kDa protein 1 beta (Hspcb) were tested for normalization. The GeNorm^{TM} freeware (http://medgen.ugent.be/-jvdesomp/genorm/) was used to determine that GAPDH, B2M, ACTB and HSPCB were the three most stable

housekeeping genes. The relative transcript quantity (Q) was determined by the delta Cqmethod $Q=E^{(Cq\ min\ in\ all\ the\ samples\ tested\ -\ Cq\ of\ the\ sample)}$, where E is related to the primer efficiency (E=2 if the primer efficiency=100%). Relative quantities (Q) were normalized using the multiple normalization method described in Vandesompele et al (Vandesompele et al., 2002). Q normalized=Q/(geometric mean of the three most stable housekeeping genes Q). The $2^{(-\Delta\Delta Ct)}$ method was retained, using a housekeeping gene and gene of interest (Livak and Schmittgen, 2001) tested on control sample and treated sample.

Gene	Full name	Accession Number	Sequence
TUJ1	Neuron-specific class III beta-tubulin	**006086**	F = 5'-CCAGTATGAGGGAGATCG-3' R = 5'-CACGTACTTGTGAGAAGAGG-3'
GAD67	Glutamic acid decarboxylase	**000817**	F = 5'-GGTGGCTCCAAAAATCAAAGC-3' R = 5'-CAATGTCAGACTGGGTAGCG-3'
DARPP32	dopamine- and cAMP-regulated neuronal phosphoprotein	**181505**	F = 5'-GAGAGCCTCAGGAGAGGG-3' R = 5'-CTCATTCAAATTGCTGATAGACTGC-3'

Table 2: human specific gene primers used for RtqPCR

2.9 Statistical analysis

Data are presented as the mean value of three independent experiments +/- standard deviation (SD), unless otherwise stated. Significant differences between samples were determined using an ANOVA test, followed by a Scheffe post-hoc test

which indicated if conditions were significantly different. P-value was set to 0.05, unless otherwise stated.

3. Results

3.1. Morphology of coronal organotypic slices.

Sagittal, coronal and axial slices were first compared 1 day and 7 days after slice preparation. The goal was to induce a mechanical degeneration of striatal-globus pallidus GABAergic pathway (Figure 1A). GABAergic MSNs from the striatum to the globus pallidus could be easily identified and characterized in organotypic slices with those three different angles of slice.

An anti-DARPP32 immunofluorescence was performed to assess which of those slice angles will induce the strongest GABAergic DARPP32 positive MSNs depopulation in a short window time. As observed in figure 1B, only a small number of cells degenerated in sagittal and axial slices after 7 days in culture. However a strong and rapid degeneration was obtained in coronal slices for which almost no MSNs could be observed after 7 days in culture (Figure 1B).

Morphological analysis of organotypic coronal brain slices cultured from neonatal tissue just after preparation showed that many brain areas could be clearly identified using bright-field microscopy like the cortex, striatum, globus pallidus, corpus callosum, ventral pallidum and the substantia innominata among others (Figure 1C). Furthermore GABAergic pathways between the striatum and the globus pallidus which is mainly involved in HD can be easily identified by GAD67 or DARPP32 immunostaining (Figure 1C).

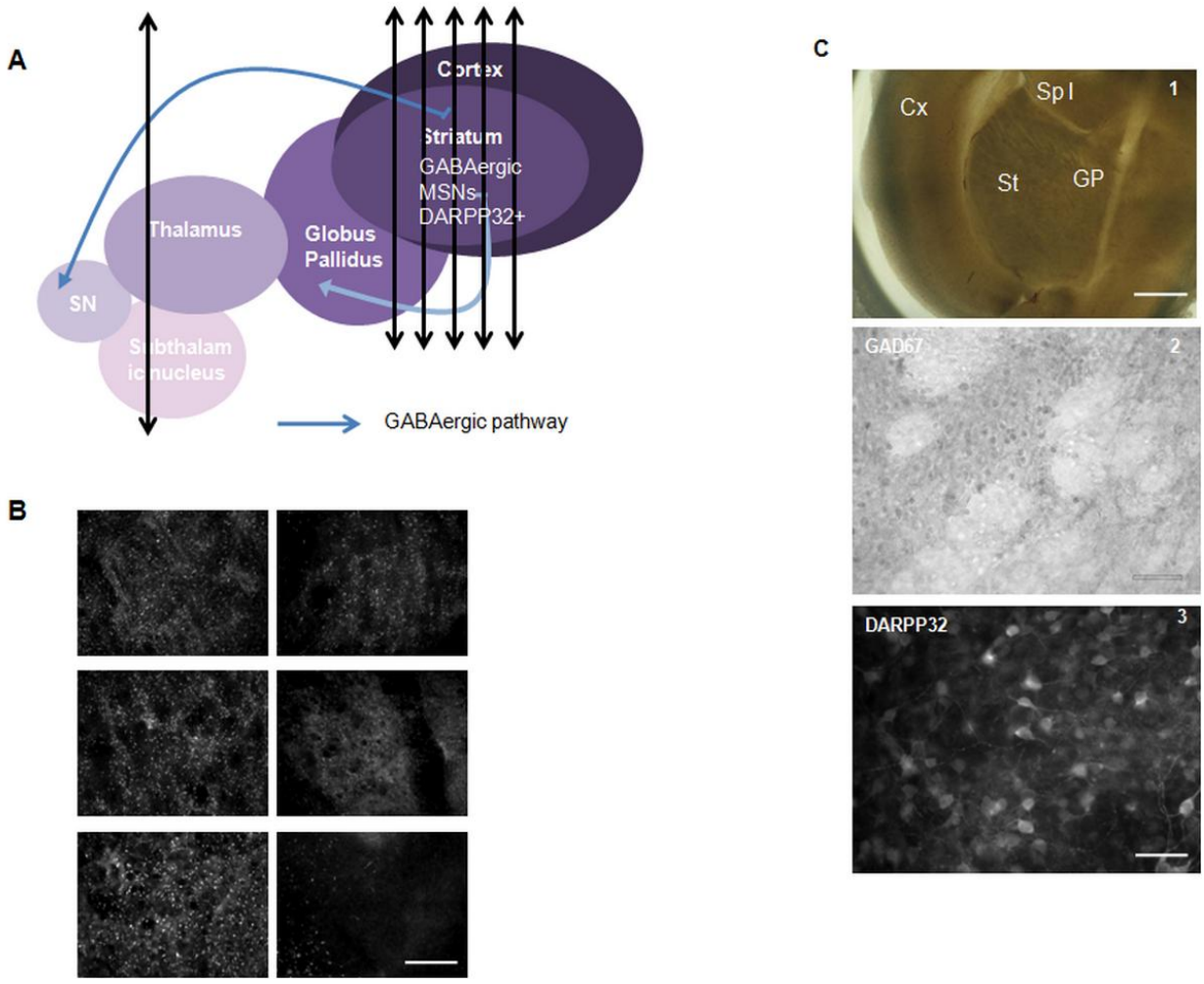

Figure 1: *A) Sagittal schematic overview of the main brain areas and of the two GABAergic pathways involved in Huntington's disease. B) Immunofluorescence against DARPP32, highlighting the medium spiny neurons in sagittal, axial and coronal slices (from up to down) at day 0 and after 7 days in culture. Scale bar represents 200µM. C) Brightfield observation of the coronal slice. Cortex (Cx), striatum (St), globus pallidus (GP) and lateral septum (Sp L) can be easily observed (1). Scale bar represents 1000µM. GAD67 (2) and DARPP32 (3) immunostainings demonstrated that GABAergic neurons and MSNs can be studied in these slices. Scale bar represents 50µM.*

3.2 Coronal organotypic slices viability

Appearance of the whole striatum-globus pallidus organotypic slice was observed by brightfield microscopy. Eighteen days after slice preparation, no important distortion or flattening of the slices was detected. However, an important thinning of the striatum within 6 days was observed by bright field microscopy, predicting a reduction in striatal neurons (Figure 2A). Using NeuN immunofluorescence, the survival of neurons within specific brain regions of the organotypic slices was assessed. A first evaluation allowed observing an important disappearance of striatal neurons in the striatum after 18 days in culture while no differences were detected in the cortex (Figure 2B). A quantification of NeuN positive cells was performed at day 0, 14 and 18. Between days 1-18, no important differences were quantified within the cortex while a slight decrease of 25% in NeuN expression was detected between day 14 and day 18 in the lateral septum, admitting that day 0 represents 100%. In the striatum a decrease of about 50% of the NeuN positive cells were detected at day 14 and reached 70% at day 18 (Figure 2C).

3.3. Organotypic slice cultures display progressive degeneration of the GABAergic pathway

An important thinning of the striatum within 6 days was observed by bright field microscopy, predicting a reduction in GABAergic MSNs (Figure 2A). This striatal cell death was confirmed by immunostaining against rat GAD67 and against DARPP32 to quantify MSNs GABAergic neurons, which are for vast a majority DARPP32-positive neurons (Ehrlich, 2012). The density of the GAD67-expressing neurons decreased dramatically from day 4 onwards and DARPP32-positive neurons from day 2 onwards, when compared to day 0 (Figure 3A). A quantification of those markers

showed a loss of the GABAergic marker density, GAD67, of 10% at day 2, 30% at day 4, 35% at day 6, when compared to day 0. It was complete by day 18 with only 1% staining left (Figure 3C). A15 % loss of DARPP32 staining was detected at day 1 and reached a significant level with 35% at day 2, 50% at day 3 and 75% at day 4 compared to day 0, and was complete by day 18 (Figure 3B).

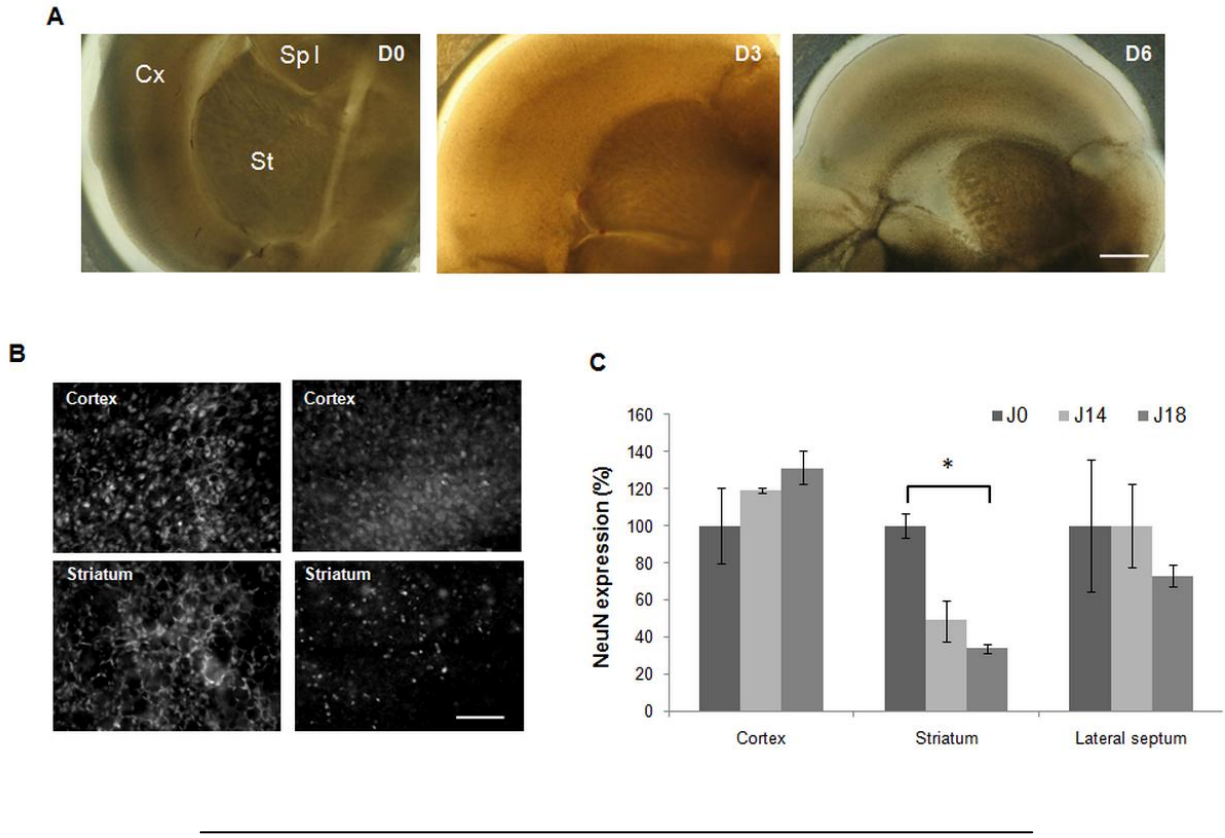

Figure 2: *A) Brightfield observation of the coronal slice at day 0, 3 and 6. No flattening or distortion of the slice could be observed. A sliming of the striatum, expecting a loss of striatal neurons was observed at day 6.Scale bar represents 1000µM. B) Immunofluorescence against neuronal nuclei (Neun) in the cortex (up) and in the striatum (below) at day 0 (left column) and after 18 days in culture (right column). A disappearance of NeuN staining was observed at day 18 in the striatum. Scale bar represents 100µM. C) Quantification of NeuN expressing cells in the cortex, the striatum and the lateral septum at day 0, 14 and 18. NeuN expression dramatically decreased in the striatum over time while no important differences were observed in the cortex or in the lateral septum. N=3 and * : significant result with p=0.05*

3.4. *In vitro* cell differentiation analysis

No main differences in β3-tubulin messenger RNA (mRNA) expression were observed between the three conditions tested (MIAMI cells in expansion MIAMI cells treated with EGF/bFGF and MIAMI cells after MSNs differentiation commitment). Only a slight decrease was noticed after inducing neuronal differentiation of MIAMI cells. DARPP32 was slightly expressed for all conditions and no significant differences were observed between the different conditions. A significant expression of GAD67 was detected after E/F treatment and induction of neuronal differentiation. Concerning NSCs, an increase of DARPP32 and GAD67 mRNA expression was noticed after neuronal differentiation induction. Moreover, NSCs express 4-fold more β3-tubulin, 3500-fold more DARPP32 and 750-fold more GAD67 than MIAMI cells for all conditions tested (Figure 4A).

For both type of cells, five different treatments were tested based on two protocol developed to obtained mature GABAergic MSNs from embryonic stem cells (Aubry et al., 2008, Ma et al., 2012). It was detected that an addition of 10µM of valproic acid during 3 days followed by 30ng/ml of BDNF during 7 days in the culture medium induced the commitment of stem cells toward GABAergic MSNs expressing cells (Figure 5A and Figure 6A). Indeed with this condition a slight increase in β3-tubulin and GAD67 expression was detected in MIAMI cells as well as a significant increase in DARPP32 expression compared to E/F MIAMI cells (Figure 5B). Concerning NSCs, a slight increase in β3-tubulin and GAT1 expression were detected as well as a significant increase in GAD67 expression compared to NSCs in expansion (Figure 6B).

It was also showed that a 5 days treatment of valproic acid instead of 3 days did not permit to enhance neuronal or GABAergic differentiation and moreover,

induced a more important number of degenerating cells.. The same conclusion was made with a supplemental 5 days treatment with SHH. However the addition of B27 and N2 supplements in the culture medium induces an increase in Ki67 expression, highlighting a more important cell proliferation (not shown).

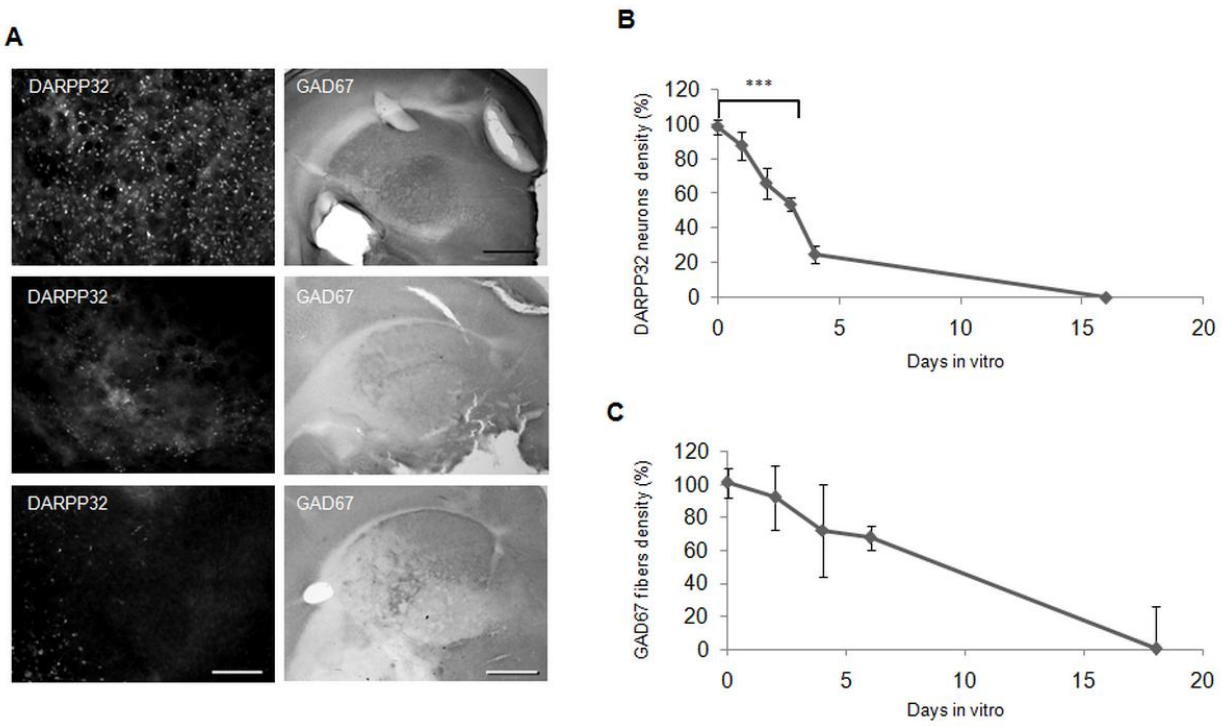

Figure 3: *A) Immunofluorescence against striatal DARPP32 positive neurons at day 0, 2 and 4 top to bottom (left column, scale bar is 200µM). Immunohistochemestry against striatal GAD67 positive neurons at day 0, 4 and six, top to bottom (left column, scale bar is 1000µM). B) Striatal DARPP32 neurons density decreased of 15% at day 1, 35% at day 2, 50% at day 3, 75% at day 4 and became total at day 16 holding the fact that day 0 represents 100%. C) Striatal GAD67 positive neurons quantification. A 10% decreased was detected at day 2 which reached 30% at day 4, 35% at day 6 and reached 100% at day 18, holding the fact that day 0 represent 100%. N=4 and *: significant result with p=0.05*

3.5 Histological study

3.5.1 Stem cells GABAergic differentiation potential

Immunofluorescence against β3-tubulin was used to determine neuronal-like commitment of stem cells 15 days after grafting in the HD organotypic slices. For both types of cells, β3-tubulin expression remained low 15 day after grafting for all conditions tested even when complexed with LM-PAMs (Figure 7A).

Only a few DARPP32-positive cells co-labeled by hMito were detected after grafting MIAMI cells even when associated to LM-PAMs. For NSCs, a strong expression of DARPP32 was detected in GFP+NSCs when grafted alone or complexed with PAMs (Figure 7B).

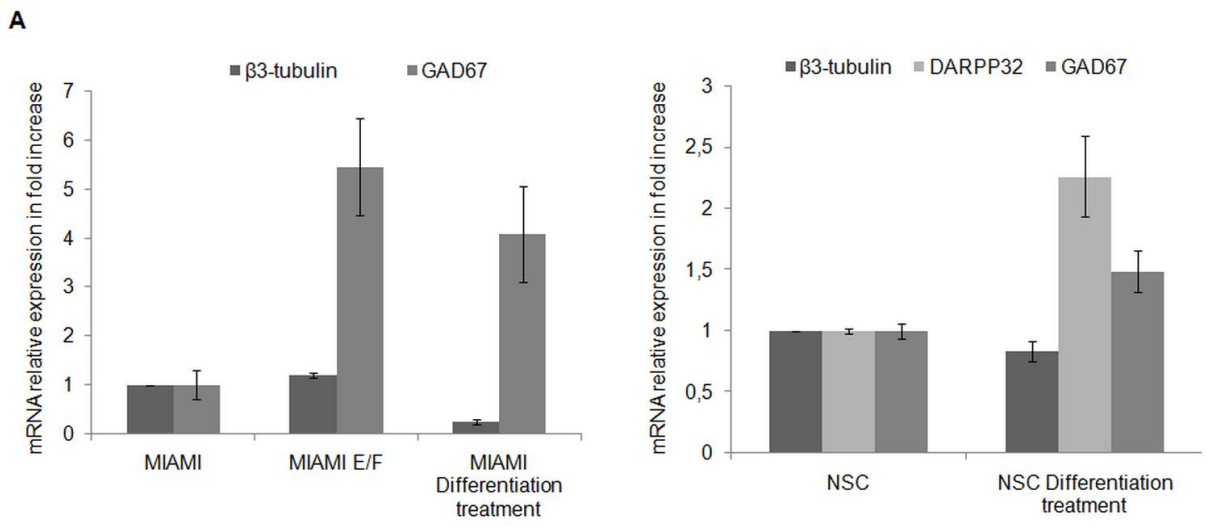

Figure 4: *A) In vitro mRNA expression of neuronal and GABAergic differentiation markers. For MIAMI cells no important differences in β3-tubuline expression was noticed between the different conditions. An expression of DARPP32 mRNA was detected after differentiation treatment (not shown), an important increase GAD67 expression was observed for E/F and valproic acid/BDNF treated MIAMI cells. Regarding NSCs, only a slight increase of GAD67 and DARPP32 was observed after treatment of cells with valproic acid/BDNF compared to cells in expansion.*

Quantification of the proportion of positive cells confirmed that β3-tubulin expression was very low with no differences detected after adhesion to PAMs. For MIAMI cells, DARPP32 expression remained low for cells adhered or not to PAMs. For NSCs, DARRP32 expression detected was close to 80% of the total number of cells, when cells were grafted alone and associated to PAMs (Figure 7C). Unfortunately, no GAD67 expression, which highlights GABAergic neurons maturation, was detected for MIAMI cells neither for NSCs.

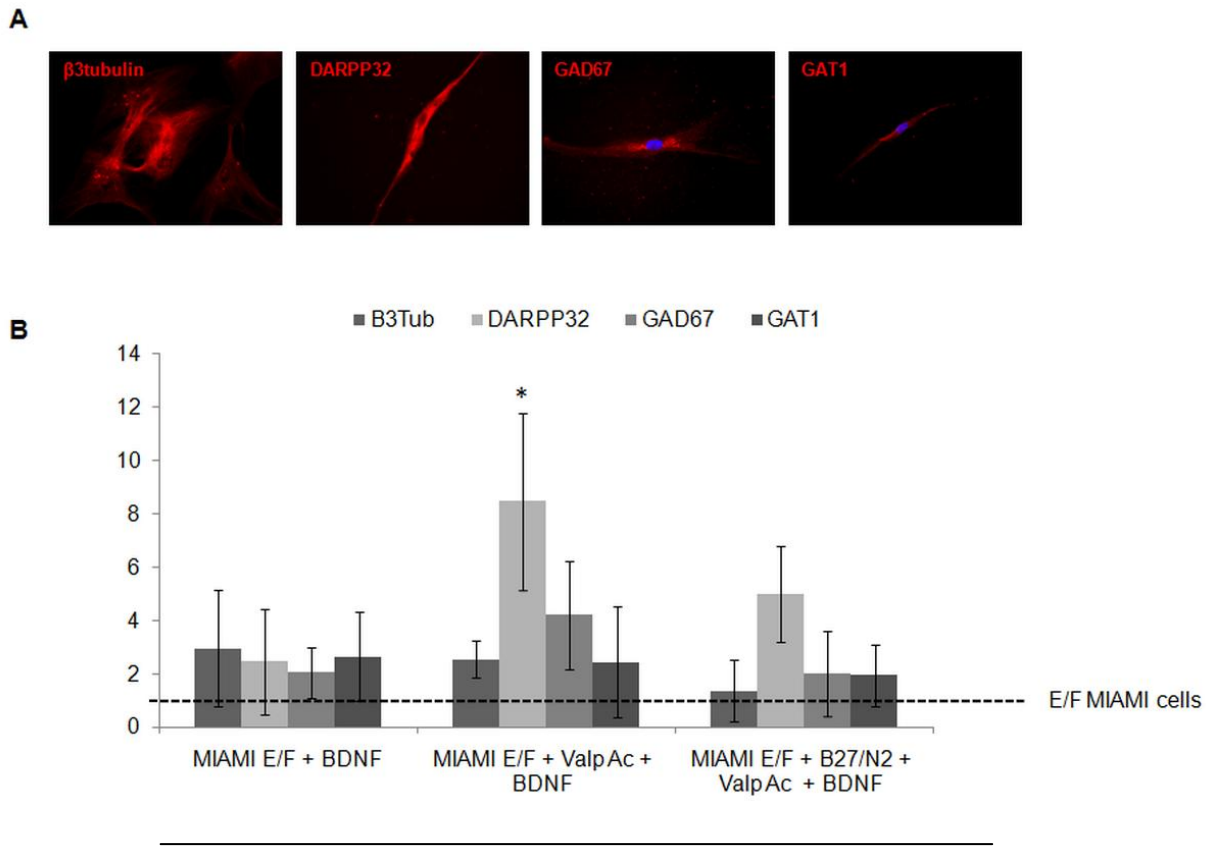

Figure 5: *A) In vitro immunofluorescence of MIAMI cells against β3-tubuline, NFM, DARPP32, GAD67, GAT1 and Ki67. B) MIAMI cells expression of different markers in fold increase against MIAMI E/F. The most important GABAergic MSNs differentiation of MIAMI cells was obtained after a 3 days treatment with valproic acid followed by a 7 days treatment with BDNF. In this case an increase of 2.5-fold of β3-tubuline expression, 1.6-fold of NFM, 8.4-fold of DARPP32, 4-fold GAD67 and a 2.4-fold GAT1 expression were detected. The proliferation marker Ki67 was no more expressed in this condition. Scale bar is 50µM and *: significant result with p=0.05*

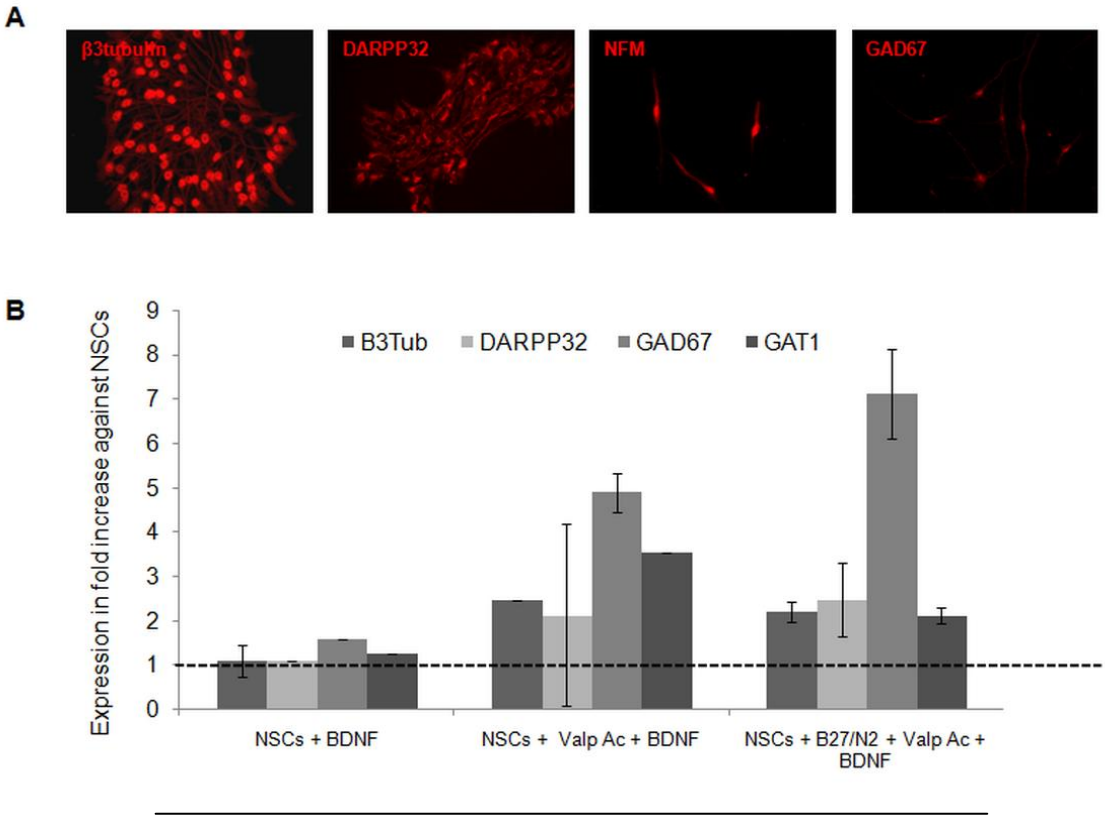

Figure 6: *A) In vitro immunofluorescence of NSCs against β3-tubuline, NFM, DARPP32, GAD67, GAT1 and Ki67. B) NSCs expression of different markers in fold increase against non treated NSCs. The most important GABAergic MSNs differentiation of MIAMI cells was obtained after a 3 days treatment with valproic acid followed by a 7 days treatment with BDNF. In this case an increase of 2.4-fold of β3-tubuline expression, 3-fold of NFM, 2-fold of DARPP32, 4.9-fold GAD67 and 3.5-fold GAT1 expressions were detected. No differences in Ki67 expression were detected. Scale bar is 50µM*

3.5.2 Neuroprotection/repair assessed by stem cells grafting

Immunostainings against rat GAD67 and DARPP32 were used to determine the density of GABAergic fibers and MSNs respectively, in the striatum. The effect of the different treatments on GABAergic fibers density was then assessed 15 days after grating. For non treated slices, no GAD67- or DARPP32-positive neurons were found in the striatum or the globus pallidus (1), while some positive neurons were found after MIAMI (2), MIAMI-LM-PAMs (3), NSC (4) and NSC-LM-PAMs grafting (5). However no real improvement of host cell protection could be observed when cells were grafted complexed to PAMs (Figure 8A and 8B).

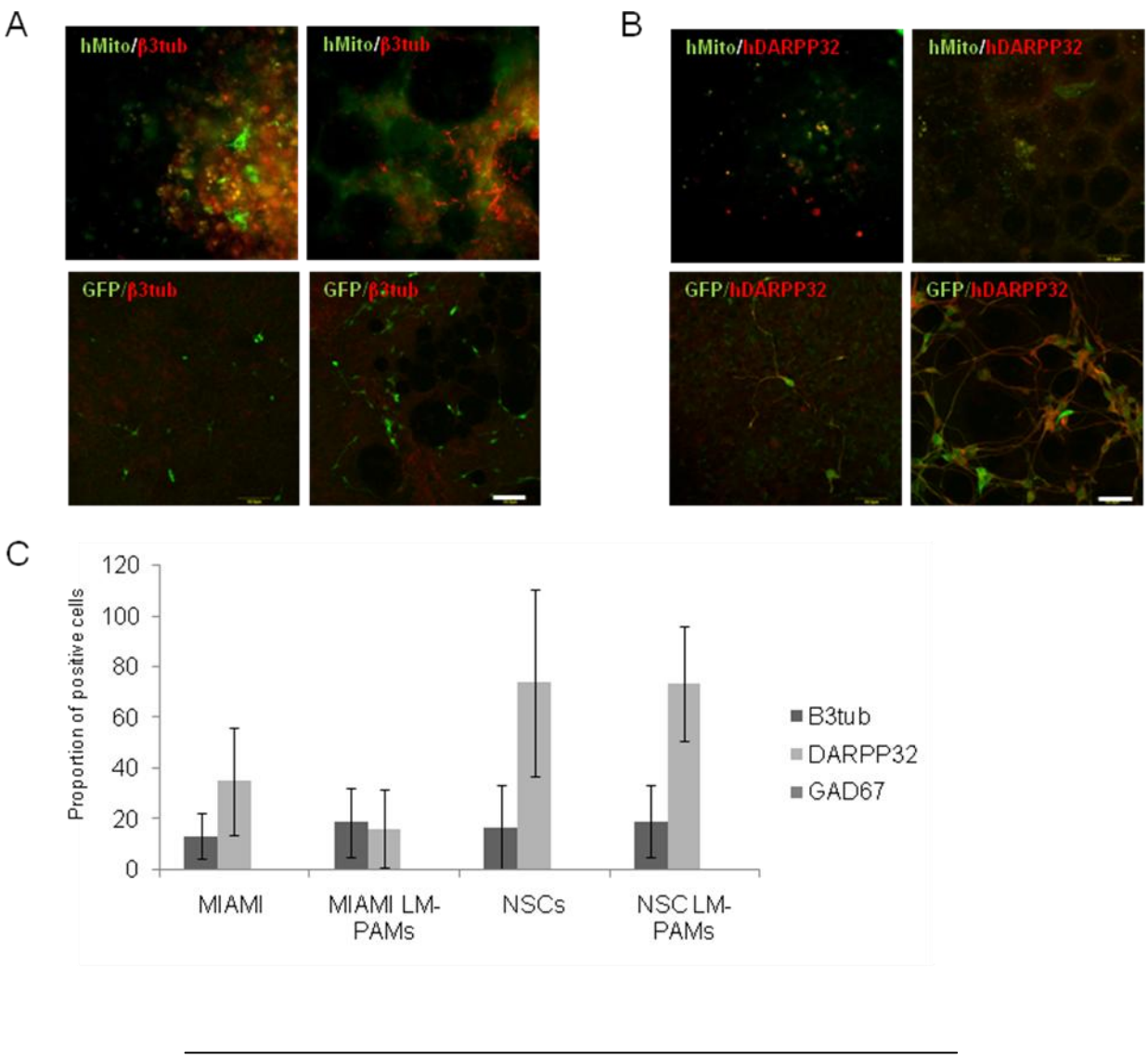

Figure 7: *Ex vivo GABAergic MSNs differentiation of grafted cells. A) Immunofluorescence against β3-tubuline and B) DARPP32 for MIAMI cells (top) and NSCs (bottom) 15 days after grafting of cells alone (left column) and associated to LM-PAMs (right column). C) Quantification of marker expression 15 days after grafting alone or complexed to LM-PAMs. No important differences were observed in β3-tubuline or DARPP32 expression when cells were complexes to LM-PAMs. The same observations were made for NSCs. Unfortunately no GAD67 expressing cells was identified. Scale bar is 50µM*

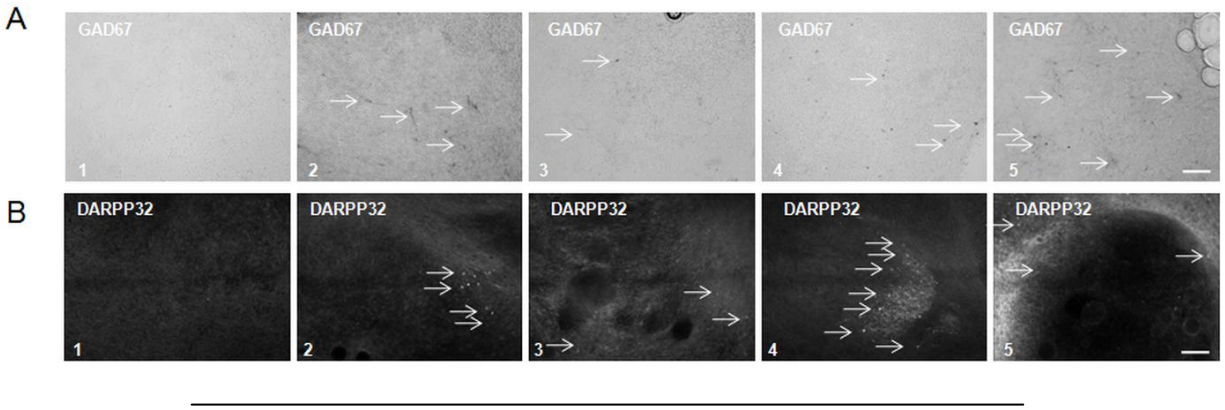

Figure 8: *Grafted cells neuroprotective potential. A) Immunohistochemestry against rat GAD67 positive cells and B) immunofluorescence against rat DARPP32 positive neurons at day 18 for non treated slice (1), MIAMI cells (2), MIAMI/LM-PAMs (3), NSCs (4) and NSCs/LM-PAMs (5). Scale bar is 50µM and the white arrows indicate positive cells.*

4. Discussion

Organotypic cultures represent an ideal bridge between *in vivo* and *in vitro* studies. They are easy to prepare and to keep in culture during many weeks, and they maintain the original circuits, pathway and microenvironment of the brain architecture. Organotypic slices have been widely used to model neurological pathologies (for review (Daviaud et al., 2013b). The present work sought to develop and characterize an innovative *ex vivo* model of HD. This model had to be very simple and rapid to apply, to allow quick studies of novel therapeutic approaches. This model was based on the mechanical transection of the GABAergic MSNs pathways from the striatum to the GP in a one step slice preparation. In this report, a complete characterization of the fate of MSNs and GABAergic pathway was assessed by immunostaining.

Stopinni's organotypic culture method was chosen in the present study, since it provides an easier access to the slice culture preparation (Stoppini et al., 1991).

Regarding the culture of the slices, the selected culture media protocol used a serum-free media from day 3 to 16. This allows having a well controlled media and studying solely the effect of added drugs or cell grafts on the behavior of brain cells. Advantages of this culture media on slice survival were already described in our previous report (Daviaud et al., 2013a). Moreover, it has been reported that serum containing media induces a decrease in neuronal sprouting (Kim et al., 2013) particularly for striatal GABAergic neurons (Dahl-Jorgensen et al., 1999). In the present study we did not notice any important cell death, by NeuN staining, in cortex or lateral septum during the 18 days of culture, while we observed a significant decrease of NeuN expression in the striatum in 14 days of culture. This proved that the striatal neurons were the only ones to be dramatically affected by the slice sectioning and that the organotypic slice survival is important enough until 18 days of culture, to study new therapeutic approaches.

Plenz & Kitai mentioned in 1998 mentioned that in triple slice organotypic culture, MSNs showed similar morphological and electrophysiological characteristics than MSNs described *in vivo* (Plenz and Kitai, 1998). The addition in the culture medium of neurotoxins as KA, QA, or 3-NPA induces a lesion of the striatal neurons, and therefore a model of HD (Matyja, 1986, Whetsell and Schwarcz, 1989, Storgaard et al., 2000). But for those models many protocols for each neurotoxin exist which created an important variability in the results obtained. More recent models also take into account the genetic aspect of the pathology. A model established from the R6/2 transgenic mice was developed and was clearly established as a screening model of anti-aggregation components (Smith et al., 2001, Smith and Bates, 2004). HD was also modeled by the transfection of HD-polyQ plasmids or with DNA constructs derived from the human pathological huntingtin gene into cells within the slice

(Murphy and Messer, 2001, Murphy and Messer, 2004, Reinhart et al., 2011). But those last models were time-consuming and expensive to develop.

The model developed here allowed to study all the areas involved in HD. The degeneration of GABAergic MSNs pathways was initiated during sectioning of the brain in a single step. The characterization of the progressive GABAergic MSNs degeneration was achieved by immunostainings. To our knowledge this is one of the first reports that characterize the striatal GAD67 and DARPP32 positive neurons degeneration overtime in this type of model. It revealed that a rapid loss of DARPP32 expression neurons occurred in the striatum followed by a decreased in GAD67 expression. A decrease of more than 50% and 30% of the DARPP32 and the GAD67 positive cells respectively was detected 3 days after slice preparation compared to day 0. This allows the development of a early model of the disease, in which a window of time of 15 days was obtained to study new therapeutic approaches.

The combination of stem cells and polymeric bioactive scaffold to enhance cell survival and differentiation need to be developed and studied to improve therapeutic approaches for HD. An evaluation of MIAMI cells and NSCs *in vitro* differentiation capacity was first performed. It was previously reported that the differentiation of neural precursors into striatal progenitors *in vitro* could be assessed by addition of BDNF, SHH or DKK1 in the culture media. A mature GABAergic MSNs was obtained after 60 days of treatment (Aubry et al., 2008, Ma et al., 2012). Our aim was to perform a transplantation of pre-differentiated stem cells in the *ex vivo* model of HD. Indeed we previously observed that a transplantation of mature differentiated cells implies an important cell death. Conversely it was showed that a graft of undifferentiated ES cells induced a proliferation of cells and an overgrowth of the

graft (Aubry et al., 2008). Five different treatments were tested and it was observed that a 3 day treatment with valproic acid followed by a 7 day treatment with BDNF induce an increase in DARPP32 and GAD67 expression in MIAMI cells as in NSCs.

We then focused on grafted cells behavior. Cells alone or complexed to LM-PAMs were grafted at day 3, when an early stage of HD was obtained. The grafted cells acquired a neuronal-like morphology at day 18 suggesting that they integrated into the host tissue. However, only 10-15% of stem cells were expressing β3-tubulin even when cells were adhered on LM-PAMs. Furthermore, about 15% of MIAMI cells and 80% of NSCs were expressing DARPP32 highlighting their potential to differentiate in MSNs, and no important differences were detected when cells were complexed to LM-PAMs. Unfortunately no GAD67 expression was detected in stem cells. ES cells differentiated into striatal progenitors were grafted in quinolinc acid treated rats, and exhibited a 15% of DARPP32 positive neurons after 13 weeks in situ (Aubry et al., 2008) while a lateral ganglionic eminence like progenitors grating revealed a 50% of DARPP32 expression (Ma et al., 2012). Those results indicated that the microenvironment of the lesioned striatum may secrete growth factors that induce MSN differentiation of grafted cells and that NSCs were particularly sensitive to those factors. To better characterize MIAMI cells and NSCs stage of differentiation, a complete mRNA or protein study, of Sox1, Pax6, Six3 or Nestin or, which are markers of stem cells and neural progenitor cells, could be performed

MIAMI cells are well known to induce neuroprotection by secreting neuroprotective growth factor (Garbayo et al., 2011, Roche et al., 2013, Daviaud et al., Submitted) as BDNF, GDNF or VEGFA. Interestingly it was previously reported that the addition of GDNF on organotypic slices culture media enhances a more

robust expression of DARPP32 in striatal neurons (Snyder-Keller et al., 2008). Furthermore, BDNF was a powerful factor to induce protection of striatal neurons and to induce an improvement in behavioral outcome in HD rats (Reiner et al., 2012, Samadi et al., 2013). Moreover, it has been shown that MSCs induce a functional recovery of in vivo models of HD (Lescaudron et al., 2003, Rossignol et al., 2011, Lin et al., 2011). In this study, grafted cells neuroprotective potential was analyzed by immunostainning against rat DARPP32 and GAD67. It was observed that MIAMI cells may exert a neuroprotection on striatal neurons. Indeed, some GAD67 and DARPP32 positive neurons were observed in the striatum after MIAMI cells implantation, adhered or not on LM-PAMs, while a complete disappearance of those neurons was detected in non-treated slice. A comparison was performed with hNSCs and the same conclusions were made. Indeed we previously showed that this NSCs lineage was able to secrete high quantity of STC1, which is known to be an anti-apoptotic and anti-oxidative damage factor (Daviaud et al., Submitted, Block et al., 2009).

The development of LM coated-PAMs releasing BDNF transporting MIAMI cells or NSCs could be an efficient strategy to treat HD. Indeed, besides the lesioned microenvironment, BDNF released by PAMs could increase GABAergic differentiation of MIAMI cells and NSCs and could also protect the host striatal neurons.

Conclusion

An innovative *ex vivo* model of HD was developed and characterized. This model focuses only on the cellular aspect of HD and not on the genetic one. It allows

screening of new drugs or therapeutic approaches to be performed with a therapeutic window of 15 days. We then confirmed that both type of cells could be committed to cells with a GABAergic MSNs phenotype in vitro with to a simple treatment of valproic acid and BDNF. We further showed that both types of cells were able to protect striatal neurons probably by growth factor secretion. The lesioned striatum microenvironment induced a slight MSN-like differentiation of the cells. We can suggest that the development of LM coated-PAMs releasing BDNF transporting MIAMI cells or NSCs could be an efficient strategy for HD.

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Disclosure of interests

There is no disclosure of interest in this publication.

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DISCUSSION

Grâce à l'utilisation de cultures organotypiques, un modèle innovant de la maladie de Huntington a été développé. Il s'agit d'un modèle simple et reproductible, ne nécessitant pas d'injection de neurotoxines, la dépopulation des NEM GABAergiques étant initialisée lors de la coupe. De plus cette coupe permet d'étudier de nombreuses zones cérébrales impliquées dans la pathologie telles que le cortex, le striatum ou le globus pallidus. La coupe et la dégénérescence obtenue ont été caractérisées par immunomarquages contre des marqueurs des NEM mais aussi des neurones GABAergiques. Nous avons alors pu démontrer que ces coupes coronales possèdent une survie maximale de 18 jours. Une mort des NEM a été détectée, elle atteint 35% en deux jours, 45% en 3 jours et environ 80% en 4 jours. La densité des fibres GABAergiques dans le striatum diminue d'environ 40% en 4 jours. De ce fait, une lésion partielle des neurones du striatum est observée en 3 jours environ. C'est à ce temps que nous avons décidé d'effectuer les greffes de complexes cellules souches-LM-MPA, laissant ainsi une fenêtre thérapeutique de 15 jours.

Nous avons d'abord voulu vérifier la capacité des cellules MIAMI et des CSN à se différencier en NEM GABAergiques. En effet, il est nécessaire que les cellules donnent des neurones « GABAergique-like » afin de former des synapses avec leur cible (Mazzocchi-Jones et al., 2011, Nakao and Itakura, 2000). Nous nous sommes basés sur différents protocoles de différenciation des précurseurs neuraux en NEM GABAergiques qui ont été mis au point précédemment (Ivkovic et al., 1997, Aubry et al., 2008, Ma et al., 2012). Cinq traitements différents ont été testés et pour les deux types de cellules souches. Il s'est avéré qu'un engagement vers une différenciation « neurone-like » exprimant DARPP32 (dopamine- and cAMP-regulated neuronal

phosphoprotein) et GAD67 (Glutamic acid decarboxylase) était obtenu après 3 jours de traitement à l'acide valproïque suivi de 7 jours de traitement au BDNF.

Après implantation dans le tissu hôte, une différenciation assez faible des cellules MIAMI en cellules « neurone-like » exprimant DARPP32 a été observée même quand elles furent implantées avec les LM-MPA. Concernant les CSN, elles semblent exprimer faiblement la β3-tubuline mais près de 80% des cellules semblent exprimer DARPP32, que les cellules soient greffées seules ou complexées aux LM-MPA. Malheureusement aucune cellule n'exprimant GAD67 n'a pu être détectée.

Dans le chapitre précédent nous avons mis en évidence une expression de différents facteurs de croissance par les cellules MIAMI, tels que le VEGFA, le BDNF et le GDNF alors que les CSN sécrètent majoritairement de la STC1. Or il a été démontré que le BDNF permet d'augmenter l'expression de DARPP32 dans des neurones du striatum (Snyder-Keller et al., 2008) et qu'il semble induire une neuroprotection importante des NEM GABAergiques dans des modèles *in vivo* de la maladie de Huntington (Reiner et al., 2012, Samadi et al., 2013). Nous avons donc cherché à mettre en évidence une neuroprotection des neurones GABAergiques du striatum hôte induite par les cellules MIAMI ou les CSN quand elles sont associées ou non aux LM-MPA. Nous avons pu observer que 18 jours après la greffe aucun neurone exprimant DARPP32 ou GAD67 ne pouvait être observé dans des coupes non traitées alors que de nombreux neurones étaient encore présents dans les coupes ayant subit une greffe de cellules MIAMI ou des CSN.

Ces deux types cellulaires semblent donc avoir un potentiel intéressant pour une approche thérapeutique de la maladie de Huntington par leur capacité à se différencier en NEM, et par leur potentiel de neuroprotection. Le BDNF étant un

facteur clé dans la maladie de Huntington, l'utilisation des MPA enrobés de laminine et délivrant du BDNF pourrait être une approche thérapeutique originale et intéressante pour stimuler la différenciation des cellules MIAMI et NSC mais aussi induire une neuroprotection plus importante des cellules du tissu hôte.

DISCUSSION GENERALE

DISCUSSION GENERALE

Face aux limites liées aux traitements pharmacologiques et aux autres approches thérapeutiques, la thérapie cellulaire est devenue un des traitements les plus prometteurs pour traiter les maladies neurodégénératives. Cependant, malgré de nombreux essais pré-cliniques et cliniques montrant des bénéfices fonctionnels, la thérapie cellulaire du système nerveux central n'est pas encore utilisable comme traitement de routine (Lescaudron et al., 2012, Yoo et al., 2013). En effet, en plus des nombreux problèmes éthiques associés à l'isolation de ces cellules et aux risques d'infections (Piroth et al., 2013), ces vingt dernières années les implantations de cellules ou tissus fœtaux ont démontré une grande diversité de résultats en essais cliniques, due à l'absence d'un protocole unique (Barker et al., 2013, Gallina et al., 2011).

Durant cette thèse nous avons décidé de nous focaliser sur deux populations de cellules souches adultes. Ces cellules, tout comme les cellules ES, sont capables de proliférer en culture et peuvent également se différencier vers un phénotype cellulaire donné sous l'effet de facteurs de croissance particuliers. Les cellules souches neurales (CSN) représentent des candidates intéressantes pour les maladies neurodégénératives puisque ce sont des cellules souches propres au système nerveux central. Elles peuvent être prélevées dans la zone sous ventriculaire, le gyrus denté, ou au niveau du bulbe olfactif. Le prélèvement de ces cellules chez un patient reste risqué mais permettent d'effectuer des autogreffes, palliant ainsi aux problèmes éthique et d'immunosuppression (Zhao et al., 2003). De plus, des essais cliniques ont démontré un gain fonctionnel intéressant des CSN dans la maladie de Parkinson (Levesque et al., 2009). Néanmoins, pour des raisons

éthiques nous ne pouvions utiliser des CSN primaires. Nous avons donc eu recours à une lignée de CSN, les cellules HNSC.100 développées par l'équipe du Dr Martinez-Serrano (Villa et al., 2000). Cette lignée est immortalisée par vMyc dont le promoteur est gardé sous contrôle de l'EGF et du bFGF. Dès que ces deux mitogènes ne sont plus présents dans le milieu de culture, les cellules n'expriment plus Vmyc et perdent leur immortalité (Villa et al., 2002). De plus, les études effectuées sur cette lignée ont permis de démontrer qu'elle est stable et garde son stade multipotent durant sa culture. Il a également été démontré que ces cellules s'intègrent parfaitement après implantation dans le cerveau de rats sains. Un arrêt de la prolifération cellulaire est rapidement détecté. Les cellules perdent rapidement le marqueur d'état souche nestine et se différencient majoritairement en astrocytes (Rubio et al., 2000).

Les cellules stromales mésenchymateuses (CSM) peuvent être facilement isolées à partir de nombreux tissus comme la moelle osseuse, le cordon ombilical ou la synovie. Elles sont donc immunocompatibles et leur utilisation ne soulève aucun problème éthique. De plus elles ont montré des capacités de différenciation vers un phénotype ectodermal (Barzilay et al., 2009) et neuronal (Ross and Verfaillie, 2008). Enfin, leur utilisation dans des études cliniques de maladies neurodégénératives a démontré d'importantes capacités de neuroprotection (Venkataramana et al., 2010).

Toutefois, l'utilisation des cellules souches adultes a montré de nombreuses limites. Une mort importante des cellules implantées est rapidement détectée, de plus, un taux assez faible de cellules implantées se différencient en cellules neuronales matures. L'ingénierie tissulaire est une approche novatrice qui consiste à associer les cellules avec des supports, des matériaux ou biomatériaux présentant

différentes propriétés physicochimiques afin d'améliorer leurs effets biologiques et ainsi optimiser la thérapie cellulaire. Toutefois cette approche prometteuse n'a que très peu été développée dans des études cliniques. L'utilisation de microcarriers de gélatine (Spheramine) associés à des cellules de l'épithélium pigmentaire de la rétine (hPRE) ont permis d'obtenir une amélioration comportementale importante lors d'essais pré-cliniques (Watts et al., 2003) mais malheureusement des études cliniques n'ont pas montré de réel bénéfice chez les patients atteints de la maladie de Parkinson (Gross et al., 2011). La compréhension des mécanismes mis en jeu dans les approches d'ingénierie tissulaire est donc essentielle pour améliorer les traitements pour les maladies neurodégénératives.

Cette thèse se consacre à l'étude du fonctionnement de nouvelles approches thérapeutiques pour les pathologies neurodégénératives, plus particulièrement la maladie de Parkinson et la maladie de Huntington. Cette thèse fait suite à de précédents travaux dans lesquels il a été démontré que l'implantation d'une sous population de CSM, les cellules MIAMI, dans des rats hémi-parkinsoniens induit une protection / réparation des voies dopaminergiques lésées et une amélioration comportementale chez le rat. Cette augmentation était d'autant plus importante quand ces cellules étaient complexées à des MPA enrobés de laminine et délivrant de la neurotrophine-3 (LM-MPA-NT3). De plus nous avons utilisé, dans cette étude, des cellules MIAMI traitées à l'EGF et au bFGF avant implantation. En effet un plus grand pouvoir neuroprotecteur a été détecté suite à ce traitement en dépit d'une survie cellulaire plus faible due à l'absence de MPA (Delcroix et al., 2010a, Delcroix et al., 2011). Le but de cette thèse est d'expliquer les mécanismes cellulaires et moléculaires induisant cette protection par une étude du comportement cellulaire (phénotype et intégration) et des facteurs de croissance sécrétés par les cellules

dans le tissu hôte dans un contexte de la maladie de Parkinson puis de tester une approche similaire dans le cas de la maladie de Huntington.

Cette caractérisation approfondie devait permettre d'augmenter nos connaissances sur le fonctionnement des cellules MIAMI et des MPA, et de pouvoir ainsi améliorer ces nouvelles approches thérapeutiques pour faciliter un éventuel transfert vers des études cliniques. Nous avons également décidé d'étudier la transplantation des CSN en comparaison aux cellules MIAMI afin de mieux pouvoir appréhender les modes de fonctionnement de ces deux différents types cellulaires qui ont tous les deux montré des améliorations motrices dans des essais cliniques de la maladie de Parkinson (Venkataramana et al., 2010, Levesque et al., 2009)

Pour cette étude nous avons décidé de développer de nouveaux modèles pour ces deux pathologies. En effet, les modèles *in vivo* existants sont la plupart du temps longs et coûteux à mettre en place. Les coupes organotypiques de cerveau permettent de conserver l'architecture et les circuits neuronaux intacts ainsi que le microenvironnement du tissu hôte durant 2 à 3 semaines en culture. Ils permettent ainsi d'étudier rapidement et facilement les effets d'une stratégie thérapeutique sur les cellules neurales. Différents modèles *ex vivo* de maladies neurodégénératives ont déjà été développés grâce à l'utilisation de coupes organotypiques, notamment de la maladie de Parkinson, la maladie de Huntington, d'Alzheimer ou d'ischémie cérébrale (Chapitre 1). Ces modèles étaient obtenus majoritairement par administration d'une neurotoxine, se rapprochant ainsi des modèles *in vivo* existants. Toutefois, un nombre important de ces neurotoxines existent, elles peuvent être administrées à différentes concentrations et différents modes d'administration ont été

mis en place. Tous ces paramètres induisent une difficulté à comparer et analyser les différentes approches thérapeutiques.

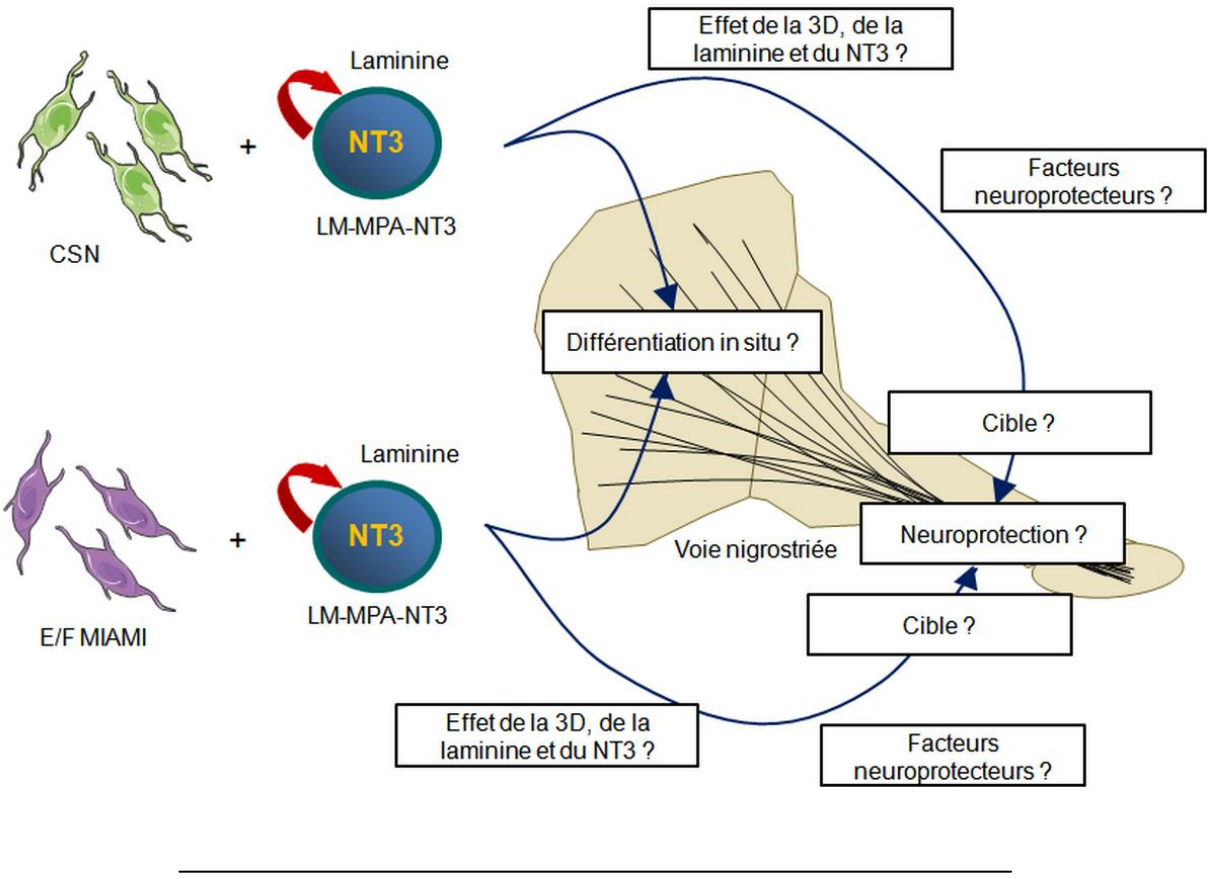

Figure 5 : *Schéma résumant le but de cette étude sur la maladie de Parkinson. Des greffes de cellules MIAMI et CSN associées à des LM-MPA-NT3 sont effectuées dans un modèle de la maladie de Parkinson. Le devenir et le comportement des cellules greffées et des cellules du tissu hôte seront analysés.*

Nous avons donc tenu à mettre au point des modèles simples et mono-paramétriques pour ces deux pathologies qui soient rapides à obtenir et peu coûteux. Nous avons pu démontrer que lors de la préparation des coupes organotypiques, le choix de l'angle de coupe est primordial. En effet nous avons cherché à obtenir un

angle de coupe qui permet de sectionner la voie dopaminergique nigrostriée pour la maladie de Parkinson et les voies GABAergiques pour la maladie de Huntington. Nous avons par la suite caractérisé ces modèles. Une survie de plus de deux semaines a été observée avec une dégénérescence progressive et reproductible des voies dopaminergiques et GABAergiques mais suffisamment rapide pour obtenir une fenêtre thérapeutique de 14 jours et 15 jours respectivement permettant d'étudier de nouvelles stratégies thérapeutiques. Nous avons également pu démontrer qu'en plus des études classiques telles que les immunomarquages ou les qPCR, il était possible d'utiliser des approches innovantes telles que la spectrométrie de masse, la microdissection laser ou encore l'IRM pour caractériser ces coupes.

Nous avons pu démontrer que ces modèles *ex vivo* de maladies neurodégénératives permettent d'effectuer des screenings de nouveaux traitements de manière rapide, simple et efficace. Toutefois, ces nouveaux modèles ne peuvent en aucun cas remplacer les modèles *in vivo* qui restent les seuls modèles permettant d'évaluer une amélioration comportementale liée à un traitement. De plus ces coupes ne permettent pas d'étudier un traitement à long terme puisqu'elles ne peuvent être gardées plus d'un mois en culture. Enfin, elles ne permettent d'évaluer que des approches thérapeutiques dans lesquelles le traitement est injecté directement dans le tissu ou dans le milieu de culture, aucun autre mode d'administration ne peut être testé.

Concernant la maladie de Parkinson, le modèle *ex vivo* a donc été utilisé pour analyser les mécanismes de neuroprotection de la voie nigrostriée, observés suite à l'implantation de cellules MIAMI dans les rats. Nous avons dans un premier temps confirmé que l'implantation de cellules MIAMI associées à des LM-MPA-NT3 induit

une protection significative des fibres dopaminergiques de la voie nigrostriée. Toutefois nous n'avons pas pu observer de protection induite par les cellules MIAMI E/F greffées sans MPA comme cela a été observé précédemment (Delcroix et al., 2011). Les CSN n'induisent qu'une protection assez faible équivalente à la greffe de PAM-NT3 seules. Par la suite nous avons démontré que le support de laminine en 3 dimensions fournit par les MPA augmente significativement seulement la survie des cellules MIAMI confirmant les résultats obtenus précédemment (Delcroix et al., 2011, Garbayo et al., 2011b). Une fois adhérées à des LM-MPA-NT3, les cellules MIAMI se différencient de façon significative en cellules « neurone-like » exprimant la TH capables de sécréter de la dopamine en quantité limitée. La neuroprotection induite par les CSM pré traitées avec du EGF/bFGF a été expliquée par une plus forte sécrétion de BDNF, GDNF et NGF dans une étude précédente (McCoy et al., 2008) alors que dans l'étude de Delcroix *et al.*, cette neuroprotection était plus attribuée à une expression importante de STC1 et de GDNF (Delcroix et al., 2011). Dans notre contexte *ex vivo*, nous avons pu confirmer une expression de BDNF et de GDNF par les cellules MIAMI mais restant relativement faible. Toutefois aucune expression de STC1 n'a pu être détectée avec ce type cellulaire. Cependant, nous avons pu démontrer que ces cellules sont capables de sécréter d'importantes quantités de VEGFA et de HGF qui sont connues pour avoir des propriétés pro-angiogéniques et neuroprotectrices (Yasuhara et al., 2004, Bussolino et al., 1992). Enfin nous avons observé une augmentation du nombre de vaisseaux sanguins situés autour du greffon dans le tissu hôte, corrélée à une sécrétion de VEGFA. Cette protection des vaisseaux sanguins n'a pas été observée avec les CSN qui ne sécrètent pas de VEGFA, montrant l'importance de ce facteur dans le phénomène de neuroprotection. Toutefois ces cellules sont capables de se différencier de manière importante en

neurones exprimant la TH lorsqu'elles sont associées à des LM-MPA-NT3. Elles sont également capables de sécréter une quantité importante de dopamine, dont le taux mesuré retourne alors à un niveau normal. Nous pouvons donc supposer, qu'une fois ces cellules greffées, elles retrouvent des caractéristiques de CSN non modifiées

Dans ce contexte, nous avons mis en évidence que les cellules MIAMI vont protéger la voie nigrostriée par la sécrétion de facteurs de croissance alors que les CSN vont plutôt substituer localement les neurones dopaminergiques dégénérés. Les LM-MPA-NT3 sont un outil remarquable permettant à la fois d'augmenter la survie des cellules souches et leur différenciation vers un phénotype dopaminergique et ainsi d'améliorer la thérapie cellulaire.

Toutefois, certains mécanismes n'ont pas été élucidés. Nous n'avons pas pu démontrer les mécanismes permettant aux LM-MPA d'augmenter la survie cellulaire *in situ.* Lors de la préparation des coupes organotypiques, il a été démontré qu'une libération de réactifs dérivés de l'oxygène peut induire la mort des cellules implantées. Nous pouvons supposer que l'apport d'un support biomimétique aux cellules permet de les protéger de l'apoptose. Il serait donc intéressant de quantifier le nombre de cellules en apoptose quand elles sont implantées seules et en association avec des LM-MPA. De plus une très forte quantité de Stanniocalcin-1 est sécrétée par les CSN. Ce facteur est connu comme étant un puissant facteur anti-apoptotique (Durukan Tolvanen et al., 2013, Bironaite et al., 2013) et pourrait donc à la fois agir sur la survie des cellules greffées mais aussi des cellules du tissu hôte.

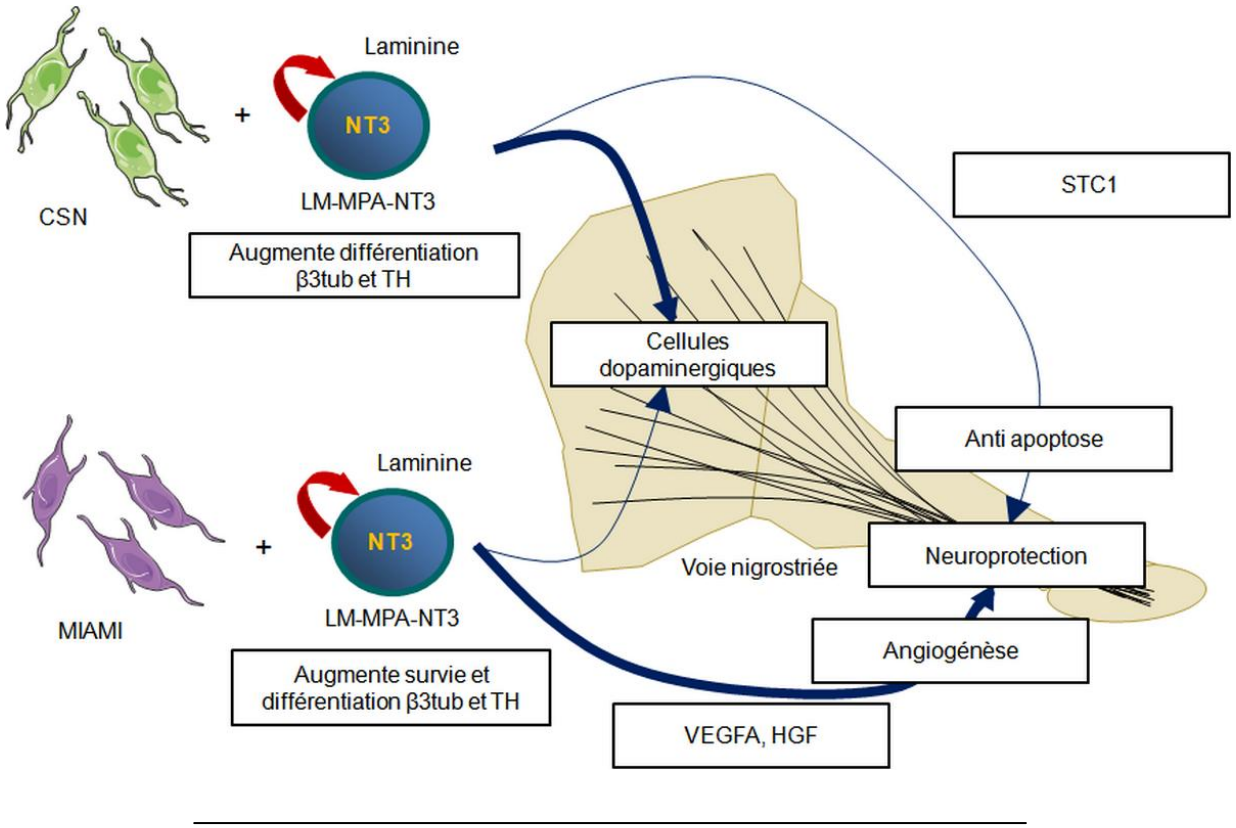

Figure 6 : *Schéma résumant l'action des MPA sur les cellules et les principaux modes de fonctionnement des complexes cellules/MPA sur le modèle ex vivo de la maladie de Parkinson.*

De plus il a été démontré dans des cellules de neuroblastomes, qu'une augmentation de l'expression de STC1 semblait induire une augmentation de l'expression de MAP-2c, un marqueur spécifique des axones. La STC1 pourrait donc être responsable de la différenciation des cellules greffées vers un phénotype neuronal et induire la formation d'axones (Wong et al., 2002) qu'il serait intéressant de mettre en évidence dans cette étude. Toutefois durant cette étude nous n'avons pas pu mettre en évidence de rapport entre cette sécrétion et la survie des CSN, ni la protection des fibres dopaminergiques de la voie nigrostriée. Il serait donc intéressant d'étudier de façon approfondie les effets de ce facteur sur les CSN et les cellules MIAMI.

Concernant le projet portant sur la maladie de Huntington, nous avons voulu évaluer le potentiel des cellules MIAMI et des CSN à se différencier en NEM GABAaergiques *in vitro* avant de les implanter dans le modèle *ex vivo.* Pour ce faire nous nous sommes basés principalement sur deux études dans lesquelles des cellules ES sont différenciées étape par étape en neurones striatales exprimant DARPP32 et GAD67 (Aubry et al., 2008, Ma et al., 2012). Nous avons évalué la différenciation des cellules MIAMI et des CSN suivant plusieurs protocoles, puis les complexes cellules LM-MPA ont été greffés dans les coupes organotypique de la maladie de Huntington afin d'étudier l'effet du microenvironnement du striatum lésé sur la différenciation des cellules avec ou sans PAMs, et d'étudier la capacité des cellules à protéger les NEM GABAergiques. En effet il a déjà été précédemment démontré que les cellules MIAMI étaient capables de se différencier en cellules « neurones-like » présentant une activité électrophysiologique similaire à celle des neurones matures ainsi qu'une croissance de « neurites-like », quand elles sont stimulées par des facteurs de croissances en particulier le NT-3 (Tatard et al., 2007a, Delcroix et al., 2010a). La voie de transduction permettant au NT-3 d'induire la différenciation en cellules de type neuronal a été étudiée. La fixation du NT-3 sur son récepteur stimule la voie de kinase MEK/ERK via l'action de Rac1b pour bloquer la prolifération et stimuler le développement de neurites (Curtis et al., 2012). Toutefois, aucune étude à ce jour n'a étudié le potentiel de ces cellules à se différencier vers un phénotype GABAergique.

Nous avons pu démontrer qu'une différenciation des cellules MIAMI et des CSN pouvait être induite par l'utilisation successive d'acide valproïque et de BDNF. Les complexes cellules/LM-MPA ont par la suite été greffés dans les coupes organotypiques. Après 15 jours de culture une différentiation relativement faible des

cellules MIAMI en NEM exprimant DARPP32 a pu être observée alors que cette différenciation atteint 80% chez les CSN. Toutefois aucune expression de GAD67 n'a pu être détectée. Aucune amélioration de cette différenciation n'a été détectée lorsque les cellules sont adhérées aux LM-MPA. Enfin, les greffes de cellules MIAMI et de CSN ont permis d'induire une protection des cellules du striatum de l'hôte. Nous pouvons donc supposer que ces cellules souches, en particulier les CSN, peuvent remplacer les NEM GABAergiques dégénérés dans un contexte de la maladie de Huntington. D'après nos résultats précédents nous pouvons supposer que les effets observés sur la protection des neurones du striatum hôte sont du à la sécrétion de facteurs de croissance tels que le VEGFA, BDNF et le GDNF pour les cellules MIAMI et via la STC1 pour les CSN.

Nous avons vu que le BDNF permet d'obtenir une différenciation partielle des cellules souches *in vitro*. De plus, il a été démontré que le BDNF permet d'augmenter l'expression de DARPP32 dans des neurones du striatum (Snyder-Keller et al., 2008) et qu'il semble induire une neuroprotection importante des NEM GABAergiques dans des modèles *vivo* de la maladie de Huntington (Reiner et al., 2012, Samadi et al., 2013). Ce facteur pourrait donc être un facteur clé dans le traitement de la maladie de Huntington. Il serait intéressant de pouvoir encapsuler ce facteur au sein des MPA afin d'améliorer cette stratégie. Le milieu d'injection des complexes cellules/LM-MPA-BDNF pourrait contenir de l'acide valproïque. Ainsi les effets du microenvironnement, de l'acide valproïque et du BDNF permettraient d'obtenir une différenciation *in situ* des cellules souches vers un phénotype de NEM GABAergiques mature.

Toutefois une thérapie cellulaire pour la maladie de Huntington semble plus difficile à mettre en place que pour la maladie de Parkinson. En effet, dans la maladie

de Parkinson les symptômes sont dus à une perte de libération de dopamine dans le striatum et il a été démontré une forte diminution des symptômes lorsque le taux de dopamine dans le striatum est normalisé. Au minimum, les cellules greffées peuvent donc être utilisées comme des pompes à dopamine. Or dans la maladie de Huntington, l'apparition des symptômes est liée à une dégénérescence des voies GABAergiques. Mais il a été prouvé que normaliser la quantité de GABA dans le striatum ou le globus pallidus ne suffisait pas pour atténuer les symptômes liés à la pathologie. Dans ce cas les cellules souches greffées doivent se différencier en NEM GABAergique et doivent se reconnecter au niveau cortical et au niveau du globus pallidus (Mazzocchi-Jones et al., 2011, Nakao and Itakura, 2000). Dans ce cas les CSN semblent avoir un avantage sur les cellules MIAMI puisque 80% des cellules implantées se différencient en NEM à projection exprimant DARPP32.

Les cellules souches embryonnaires (ES) sont également des candidates intéressantes pour la thérapie cellulaire substitutive de maladies neurodégénératives. Il a été démontré que ces cellules peuvent se multiplier de façon virtuellement illimitée (Suda et al., 1987), et qu'elles peuvent donner lieu à n'importe quel type cellulaire quand elles sont cultivées sous les bonnes conditions (Smith, 2001). Toutefois, de nombreux essais pré-cliniques ont permis de montrer que l'utilisation de ces cellules présente de nombreux désavantages. En effet, même si de nouvelles techniques ont été développées, la culture des cellules ES est régulièrement faite sur des couches de cellules nourricières d'origine animale ce qui limite leur passage en essais cliniques. Si ces cellules ne sont pas greffées à un stade suffisamment avancé en terme de différenciation, des formations de tumeurs, les tératomes, ont été détecté (Andrews, 2002). Enfin comme toute transplantation de tissu ou d'organe, l'implantation de ce type cellulaire nécessite une immunosuppression

constante (Bradley et al., 2002). Plus récemment un nouveau type de cellule a été développé pour palier aux problèmes éthiques, les cellules souches pluripotentes induites (iPS). Ce protocole décrit pour la première fois en 2007 (Takahashi and Yamanaka, 2006) permet de reprogrammer des cellules prélevées chez un patient, par exemple des fibroblastes, en cellules pluripotentes. Ces cellules présentent un potentiel de différenciation important, permettent des autogreffes et peuvent être obtenues en quantité virtuellement illimitée. Toutefois le protocole en place pour obtenir ce type cellulaire est encore coûteux et long à mettre en place. Il serait toutefois intéressant de pouvoir étudier la différenciation des cellules iPS, lorsqu'elles sont associées à des MPA libérant un facteur de croissance et de la comparer aux cellules MIAMI et aux CSN.

A ce jour les CSM restent donc intéressantes pour la thérapie cellulaire. Elles font partie des rares cellules à être capables d'agir de deux façons différentes. En effet dans le cas de la maladie de Parkinson, elles peuvent se substituer partiellement aux neurones dopaminergiques puisqu'elles sont capables de produire de la dopamine. Elles peuvent également agir comme cellules neuroprotectrices par la sécrétion de facteurs de croissance et de facteurs immunorégulateurs. Elles sont utilisées dans la recherche contre la maladie de Parkinson, de Huntington mais aussi pour l'ischémie cérébrale ou la réparation cardiaque ou cartilagineuse. Dans chaque cas l'utilisation des microcarriers pharmacologiquement actifs a démontré des avantages intéressants permettant d'optimiser l'approche thérapeutique.

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« Development of pharmacologically active microcarriers transporting human stem cells for the treatment of neurodegenerative disorders »
- Cell culture (Primary and lineage)
- Microsphere and pharmacologically active microcarriers formulation
- Development of novel *ex vivo* models of neurodegenerative disorders
- Preparation and culture of organotypic slices
- Grafts of cells-microparticles complexes in organotypic slices
- Cellular makers analysis by immunostaining, SDS pages, Western Blot and ELISA
- mRNA extraction, purification
- Design of primers
- Analysis of gene expression by RTqPCR
- Laser microdissection on organotypic slices

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2010 **Master internship,** Laboratoire de Physiologie Moléculaire des Semences. Angers
« Intearaction between the three proteins MtSAP1, MBF1 and HR, during stress response in Medicago Truncatula »
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Supervision of practical work in animal physiology, pharmacokinetic and animal experimentation bases

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Scientific communications

Oral Characterization of pharmacologically Active Microcarriers/stem cells complexes and study of stem cells differentiation in an *ex vivo* model of Parkinson's disease
Nicolas Daviaud, Elisa Garbayo, Laurence Sindji, Claudia N Montero Menei
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Pharmacologically Active Microcarriers Carrying Stem Cells For Tissue Engineering In An Organotypic Model of neurodegenerative disorders
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Journée scientifique de l'école doctorale Biologie-Santé - 07 Novembre 2011- Talmont Saint Hilaire

Développement de microcarriers pharmacologiquement actifs transportant des cellules souches humaines pour la thérapie de maladies neurodégénératives
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Pharmacologically Active Microcarriers carrying human stem cells : Regenerative potential in an ex vivo model of neurodegenerative disorders.
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Development And Characterization Of A New Ex Vivo Model Of Parkinson's Disease Using Organotypic Slices Without Neurotoxin Injection
N Daviaud, E Garbayo, L Sindji, P C Schiller, M Perez-Pinzon, C N. Montero-Menei
Tissue engineering and regenerative medicine international society (TERMIS), 17-20 June 2013

Pharmacologically Active Microcarriers Carrying Stem Cells For Tissue Engineering In An Organotypic Model Of Parkinson's Disease
N Daviaud, E Garbayo, L Sindji, P C Schiller, C N Montero-Menei
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Posters

Pharmacologically Active Microcarriers carrying human stem cells : Regenerative potential in an ex vivo model of neurodegenerative disorders.
Nicolas Daviaud, Elisa Garbayo, Laurence Sindji, Alberto Martinez-Serrano, Miguel Perez-Pinzon, Paul Schiller, Claudia N Montero-Menei
Journée Scientifique de l'IFR 132 - 8 Décembre 2011 – Angers

Visualisation du faisceau médian du télencéphale dopaminergique dans un modèle de coupe organotypique *ex vivo*
N. Daviaud[1], F. Franconi[2], L. Lemaire[1], C.N. Montero-Menei[1]*
Société française de résonnance magnétique en biologie et médecine – 21>23 Mars 2012 - Marseille

Pharmacologically active microcarriers transporting neural and mesenchymal stem cells for tissue engineering of neurodegenerative disorders
Nicolas Daviaud, Elisa Garbayo, Laurence Sindji, Miguel Perez-Pinzon, Paul C Schiller, Claudia N Montero-Menei- **World Stem Cells Summit** - December 2012 – MIAMI

Evaluation of a cell and drug carrier therapeutic approach in an ex vivo model of Parkinson's disease.
Nicolas Daviaud, Elisa Garbayo, Laurence Sindji, Miguel Perez-Pinzon, Paul C Schiller, Claudia N Montero-Menei. **International Society of Drug Delivery Sciences and Technology** – September 2013

Articles

Organotypic cultures as tools for optimization of central nervous system cell therapies.
Nicolas Daviaud, Elisa.Garbayo., Paul C Schiller, Miguel Perez-Pinzon, Claudia N. Montero-Menei,
Exp Neurol 248: 429-440.

Modeling nigrostriatal degeneration in organotypic cultures, a new ex vivo model of Parkinson's disease
Nicolas Daviaud, Elisa.Garbayo., Miguel Perez-Pinzon, Claudia N. Montero-Menei,
Neuroscience 256C: 10-22.

Behavioral characterization and VEGFa mediated neuroprotection of mesenchymal stem cells compared to neural stem cells in an ex vivo model of Parkinson's disease
Nicolas Daviaud, Elisa.Garbayo., Paul C Schiller, Claudia N. Montero-Menei,
Submitted to Biomaterials

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Thèse de Doctorat

Nicolas Daviaud

Développement de microcarriers pharmacologiquement actifs transportant des cellules souches pour le traitement de maladies neurodégénératives.

Pharmacologically active microcarriers conveying stem cells for neurodegenerative disorders treatment

Résumé

L'association de cellules souches adultes à des vecteurs polymériques bioactifs est une approche prometteuse pour traiter les pathologies neurodégénératives comme la maladie de Parkinson ou la maladie de Huntington. Il a été précédemment démontré que la transplantation d'une sous population de cellules stromales mésenchymateuses, les cellules MIAMI, associées à des microcarriers pharmacologiquement actifs (MPA) enrobés de laminine et délivrant de la neurotrophine-3 (LM-MPA-NT3) permettait d'obtenir une réparation/protection de la voie nigrostriée. L'objectif de ce projet est de comprendre les mécanismes cellulaires et moléculaires, par lesquels les cellules greffées, associées ou non à des MPA, induisent cette neuroprotection. Nous avons d'abord mis au point et caractérisé des modèles *ex vivo* de la maladie de Parkinson et de la maladie de Huntington dans lesquels la dégénérescence des neurones dopaminergiques et GABAergiques, respectivement, est obtenue sans injection de neurotoxines. Par la suite, nous avons évalué le comportement de cellules MIAMI greffées, en comparaison à des cellules souches neurales (CSN), associées ou non à des MPA. Puis nous avons étudié les mécanismes permettant d'obtenir une protection des voies neuronales lésées dans ces nouveaux modèles. Nous avons pu déterminer que les cellules MIAMI agissent majoritairement par sécrétion de facteurs de croissances tels que le VEGFA, le BDNF et le GDNF qui peuvent protéger les voies neuronales lésées alors que les CSN vont remplacer les neurones dopaminergiques lésés dans la maladie de Parkinson ou les neurones épineux moyens lésés dans la maladie de Huntington en plus de sécréter des facteurs comme la STC1.

Mots clés
Ingénierie tissulaire, cellules MIAMI, cellules souches neurales, maladie de Parkinson, maladie de Huntington, microcarriers pharmacologiquement actif

Abstract

Stem cells and polymeric bioactive scaffold complexes represent a promising therapeutic approach for neurodegenerative disorders as Parkinson's disease and Huntington's disease. It has been previously reported that the transplantation of a subpopulation of mesenchymal stromal cells (MIAMI cells) complexed to pharmacologically active microcarriers coated with laminin and releasing neurotrophine-3 (LM-PAM-NT3) induced a repair/protection of the nigrostriatal pathway. The objective of this project was to determine the cellular and molecular mechanisms induced by a graft of stem cells, associated or not to LM-PAM-NT3, which lead to this neuroprotection. To simplify this study, we first developed and characterized innovative *ex vivo* models of Parkinson's disease and Huntington's disease in which dopaminergic and GABAergic pathways degeneration respectively were obtained without neurotoxin injection. We then evaluated the behavior of grafted MIAMI cells compared to neural stem cells associated or not to LM-PAM-NT3. Thereafter, we evaluated the mechanisms inducing the protection/repair of the lesioned neuronal pathways in those novel models. We determined that MIAMI cells will marginally replace lost neurons and will mainly act by secreting factors such as VEGFA, BDNF or GDNF which can protect dopaminergic or GABAergic pathways. Neural stem cells will principally act by replacing lesioned dopaminergic neurons in Parkinson's disease or medium spiny neurons in Huntington's disease, besides the secretion of protective factors as STC1.

Key Words
Tissue engineering, MIAMI cells, neural stem cells, Parkinson's disease, Huntington's disease, pharmacologically active microcarriers