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Interest of type 4 serotonergic receptor ligands for the treatment of cognitive deficits and associated hippocampal plasticity disorders

Candice Roux

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Candice Roux. Interest of type 4 serotonergic receptor ligands for the treatment of cognitive deficits and associated hippocampal plasticity disorders. Human health and pathology. Normandie Université, 2023. English. NNT : 2023NORMC406 . tel-04307315

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Normandie Université

THÈSE

Pour obtenir le diplôme de doctorat

Spécialité SCIENCES DE LA VIE ET DE LA SANTE

Préparée au sein de l'Université de Caen Normandie

Interest of type 4 serotonergic receptor ligands for the treatment of cognitive deficits and associated hippocampal plasticity disorders

Présentée et soutenue par
CANDICE ROUX

Thèse soutenue le 25/05/2023
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Santé, Environnement



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Scientific valorizations

Publications

Review

- **Roux CM**, Leger M, Freret T. **Memory Disorders Related to Hippocampal Function: The Interest of 5-HT4Rs Targeting.** *International Journal of Molecular Sciences.* 2021 Nov; 22(21):12082. <https://doi.org/10.3390/ijms222112082>.

Original articles

Published

- Lecouflet P, **Roux CM**, Potier B, Leger M, Brunet E, Billard JM, Schumann-Bard P, Freret T. **Interplay between 5-HT4 Receptors and GABAergic System within CA1 Hippocampal Synaptic Plasticity.** *Cereb Cortex.* 2021 Jan 1;31(1):694-701. doi: 10.1093/cercor/bhaa253. PMID: 32935845.
- **Roux CM**, Lecouflet P, Billard J.-M, Esneault E, Leger M, Schumann-Bard P, Freret T. **Genetic Background Influence on Hippocampal Synaptic Plasticity: Frequency-Dependent Variations between an Inbred and an Outbred Mice Strain.** *Int. J. Mol. Sci.* 2023, 24, 4304. <https://doi.org/10.3390/ijms24054304>.

In submission

- Heraudeau M, **Roux CM**, Lahogue C, Largillière S, Allouche S, Lelong-Boulouard V, Freret T. **Micropipette-guided Drug Administration (MDA) as a non-invasive chronic oral administration method in rats.** *Submitted* in *Journal of Neuroscience Methods*.

In preparation

- **Roux CM**, Zuba D, Esneault E, Leger M, Freret T. **Beneficial effects of 5-HT4Rs activation in mice: a transversal approach, from memory to its hippocampal correlates.** *In preparation.*

Oral communications

- 6^{ème} édition des journées scientifiques francophones du Club Alzheimer de Montpellier (15-16 November 2022) *online* 🏆
Roux CM, Leger M, Freret T “Interest of 5-HT4Rs targeting in the treatment of cognitive disorders and associated hippocampal dysfunction”
- **Ma thèse en 180s** (March 2021), Caen 🏆
« Pas de stress, y’a le RS » !
- **Juvenes Pro Medicina** (16 May 2020) *online*
Roux CM, Lecouflet P, Potier B, Leger M, Brunet E, Billard JM, Schumann-Bard P, Freret T « On the trail of understanding mechanisms underpinning beneficial effects of 5-HT4Rs activation on memory: a matter of hippocampal synaptic plasticity».

Posters

- **FENS 2022** (9-13 July 2022), Paris, France
Candice M ROUX, Sophie CORVAISIER, Marianne LEGER, Thomas FRERET. « Beneficial effects of 5-HT4Rs activation on memory are intimately linked to changes in hippocampal function ».
Lucas GEPHINE, **Candice M ROUX**, Thomas FRERET, Michel BOULOUARD, Marianne LEGER. “Vulnerability of Spatial Pattern Separation in a transgenic mouse model of Alzheimer’s Disease”.
- **1^{er} colloque interdisciplinaire du Groupe De Recherche Mémoire** (12-15 october 2021), Vers, France
Lucas GEPHINE, **Candice M ROUX**, Thomas FRERET, Michel BOULOUARD, Marianne LEGER « Etude de la fonction de séparation de patterns spatiale dans un modèle murin transgénique de la maladie d’Alzheimer »
- **NeuroFrance 2021** (19-21 mai 2021) *online*
Candice M ROUX, Pierre LECOUFLET, Jean-Marie BILLARD, Sophie CORVAISIER, Pascale SCHUMANN-BARD, Marianne LEGER, Thomas FRERET. « Effects of 5-HT4R activation on CA1 hippocampal synaptic plasticity: an add-on to the GABAergic hypothesis ».
- **Society for Neuroscience** (19-23 october 2019), Chicago, United-States of America
Michel BOULOUARD, Pierre LECOUFLET, **Candice M ROUX** ; Elie BRUNET, Marianne LEGER, Brigitte POTIER, Jean-Marie BILLARD, Pascale SCHUMANN-BARD, Thomas FRERET. « Interplay between 5-HT₄ receptors and GABAergic system within CA1 hippocampal synaptic plasticity ».

Awards and distinctions

- **6^{ème} édition des journées scientifiques francophones du Club Alzheimer de Montpellier (CALM)**
 - Award for the best presentation by “Fondation Vaincre Alzheimer”
- **Normandy final: Ma thèse en 180s**
 - Jury’s award
 - Audience’s award

Acknowledgments

*From the very first days I knew that acknowledgments would not be the easiest part of the manuscript and guess what – for once, I felt it right. Expressing thankfulness is always touchy, writing a thesis manuscript is hard, doing burpees is suicide. But thanking people that really matter - whether they closely participated to this adventure or from far far away 😊 - whether they have always been there, or whether they were just passing by – seems even beyond. The biggest frustration of this thesis is neither about unexpected results, sometime disappointing, nor experiments not going the way I thought nor having hard times (no offense to Sciences, I've just learned that it is by continuously trying that we finally success; so, the more you fail, the more you have chance to succeed! **J.Rouxel**). My biggest frustration today, is that there is no word to express the strength of the feelings I had, I have and I will have forever.*

- **To the committee members of this thesis,**

First, I would like to thank the committee members for accepting to be part of it and the time spent at examining the present work: **Pr Denis David**, **Pr Bruno Guiard** who made me the honor of being reviewers and **Dr Slavica Krantic**, **Dr David Virley** who made me the honor of being examiners.

Besides, my PhD was supported by the Convention Industrielle de Formation par la Recherche (CIFRE) fellowship from the Association Nationale de la Recherche et de la Technologie (ANRT) that I'd like to thank for making this thesis feasible.

- **To the direction of my two hosting laboratories,**

Je voudrais continuer en remerciant chaleureusement le **Dr Guillaume Froget**, PDG de PORSOLT, pour m'avoir permis de réaliser mes stages de master dans son entreprise et enfin de m'avoir permis de réaliser ma thèse entre les murs de PORSOLT.

Je tiens également à remercier **Thomas Freret** tout d'abord en sa qualité de directeur du laboratoire COMETE, pour avoir accepté de m'accueillir dans son unité de recherche sans me connaître (et sans même avoir lu mon CV 😊) – tu savais que je suis allée en Australie ?) - et ensuite, en tant que directeur de thèse (un peu de patience voir ci-dessous!).

- **To my thesis directors and supervisors,**

Je ne pourrais pas aller plus loin sans mentionner mes directeurs de thèse préférés (de toute façon, je n'en ai pas d'autres 😊), **Marianne, Thomas et Elise**. En plus d'être des directeurs de thèse, vous avez été de véritables sources d'inspiration et de véritables mentors.

Marianne et Thomas, je vous suis profondément reconnaissante pour les conditions de travail dans lesquelles vous m'avez permis d'évoluer - et je ne fais pas seulement référence à nos "mealtings" (coucou au Carline btw), ni aux bières des jours de la semaine qui se finissent en "I". Merci pour votre confiance et votre passion contagieuse. J'ai beaucoup appris à vos côtés et j'espère qu'un jour, je serais celle qui laissera ma porte grande ouverte tant pour les discussions scientifiques que pour les grosses marades (et parfois, rarement, très rarement ? mes pigneries). Malgré tous les obstacles et désillusions qu'une telle aventure peut comporter, vous avez rendu ces trois (+ un p'tit peu) années vraiment agréables pour mon petit singe. 🐒 Marianne, promis si on retourne courrir ensemble, je parlerai un p'tit peu moins...on y va quand ?!

Elise, (coucou Hibou 🦉) merci mille fois d'avoir passé ce coup de fil qui m'a amené ici et de m'avoir donné toutes les clés pour m'épanouir. Tu as toujours été un vrai soutien et travailler avec toi a été un reel plaisir. Merci pour

ta patience et ta douceur qui m'ont souvent facilité la tâche et rendu des situations plus faciles. J'ai bien essayé d'être un mini-toi mais j'ai des cannes de serin bien trop longues... 😊.

- **To COMETE's team, colleagues and friends, and both,**

Bien entendu, je tiens à remercier tous les membres de l'équipe de **COMETE**. Bien que ces années aient bénéficié de l'interaction avec de nombreuses personnes, j'aimerais adresser des remerciements particuliers à des collègues, des amis, et parfois les deux à la fois qui ont remplis mes tiroirs à souvenirs pour toujours...

Elpipi (Bro, le pire (co)pilote, mais le meilleur confident, Akpé pour tes mots toujours magiques), **Eva** (KiffKiff, la gardienne de ma santé mentale, merci à tes 28 personnalités différentes, je les aime toutes. Désolée d'avoir fait la gueule devant le vibratome), **So'** (la personne la plus apaisante & à la fois fonçeuse que j'ai jamais rencontré et la reine du BBQ, merci pour ton optimisme à toute épreuve). Nous avons eu tellement de bons moments que je pourrais écrire des centaines de lignes. Mais les gars, j'ai juste besoin que vous sachiez que vous avez rendu ces années incroyablement fantastiques.

Caro (la maman, merci pour les kit kat chunky), **Marie** (la pharmacologue aux conseils...particuliers 😊), **Mathilde** (ma voisine - ou la poissarde - je préfère préciser, pas parce que c'est ma voisine !), **Lucas** (mon voisin de bureau @Casper, à l'humour cinglant), **Imane** (la boxeuse girly insoupçonnée, et aussi un peu poissarde quand même) ; **Emna** (la Marseillaise qui croit que Bordeaux c'est le Nord !), merci de supporter mes bizarreries tous les jours, et de me soutenir même quand je suis - (parfois) - ronchon. J'ai hâte de partager le bureau encore un peu avec vous, nul doute quant au fait que nous avons des moments incroyables à venir ! À ceux/celles qui viennent de commencer, je vous souhaite de vivre votre thèse comme j'ai eu la chance de vivre la mienne (et n'oubliez jamais que les moments difficiles la rendent encore plus spéciale - ce qui est beaucoup plus facile à dire lorsqu'elle est presque terminée 😊...).

Jean-Marie, mon gourou de l'électrophy, merci d'avoir partagé ton savoir sans limites et d'avoir apporté des moments de légèreté quand je me battais avec des électrodes d'enregistrement trop fragiles à mon goût ou ma cuve d'enregistrement (" bah voilà quand tu veux "). J'ai aussi appris de toi que les plus belles ne sont pas celles qui ont le plus de choses à dire (On parle bien des tranches hein ?).

Germaine@**Véro**, Jean-Eud@**Michel** (quel beau prénom), pour des milliers de raisons, professionnelles et personnelles, merci 🙏. Je vous dois aussi quelques abdos pour ces moments de rires partagés (tout ça même sans avoir vu Michel sur le cerisier). Vous êtes tous les deux une véritable boule d'amour, de gentillesse et de générosité et je suis plus qu'heureuse de vous avoir dans ma vie. Grâce à vous (4 : Coucou **Elen&Tom**), je peux maintenant briller dans les conversations en parlant de Coman et de Zlatan (ah il est plus en équipe de France lui ?), et je comprends enfin les règles de ce sport fait pour les chochottes, c'est quoi déjà...ah oui, le foot ! Allez BREST. Bisous Bisous to you 2 + 2 😊 (tutuyutu, tuy tuy tuy tuy tuyuyutu...!).

Merci également à **Pascale** de m'avoir permis d'améliorer mes lifts (ah bon, c'est juste la surface qui rebonfit beaucoup ? rho...). Plus sérieusement, enseigner dans ton UFR était une véritable révélation pour moi, merci de m'avoir fait confiance. **Valentine**, merci pour ton partage de l'amour des aninmaux et de ta culture scientifique. Une pensée aux membres de **Necc'Ethos** qui furent partie des murs pour avoir généré cette atmosphère si agréable.

Un merci tout particulier à l'équipe technique : **Gégé**, **Stacy** et **Sophie** pour leurs précieux conseils et leur aide, notamment pour le "tutuch" et le génotypage. Mention spéciale pour avoir joué à la cygogne et à la babysitter avec bébés souris ! **Sophie**, merci tout particulier pour avoir toujours été à l'écoute et pour avoir partagé toutes ces anecdotes qui me feront toujours autant rire dans 10 ans (« -2µL je te dis ! ») – épique ! et **vive la Bretagne** ! **Eve**, merci pour ton sourire qui réchauffe les coeurs et ta gentillesse ainsi que pour toutes les tâches administratives que tu nous épargnes (et pour le café 😊). Merci à mon super ingénieur **Daniel** pour avoir

concrétisé mes pensées (un peu) floues et m'avoir fait gagner un temps précieux avec des scripts qui m'ont toujours semblé être du chinois...

Merci **aux stagiaires** qui m'ont aidé, j'espère avoir contribué à vous donner goût pour la recherche. Je vous jure, on passe quelques heures en dehors du labo quand même...

- **To Porsolt's team: "Oyez Oyez",**

Oyez Oyez bonnes gens. Depuis que je suis partie du nid, vous n'avez sûrement plus à râler parce que j'ai pris votre blouse ou mal rangé mes chaussures. Vous n'avez plus à m'écouter raconter ce que certains oseraient qualifier de conneries, ou encore me voir bouder parce que ça ne « marche pas », et me surprendre le samedi à papouiller mes souris (non en vrai, on travaillait !). J'espère que ça vous manque quand même un peu !

J'ai Porsolt dans la peau – littéralement 🐭 - mais aussi parce que c'est là que j'ai commencé à "grandir" (même si je faisais déjà bien mon 1m73). C'est également ici que j'ai rencontré des collègues et des amis incroyables, et parfois les deux. Je me souviendrai toujours de ce sentiment lors de ma première visite avant le premier stage. Je révisais mes fiches sur la LTP et l'hippocampe dans ma voiture, cachée devant le portail avant d'être accueillie par une tite blonde & un ti chauv', qui sont aujourd'hui deux des amis les plus proches que j'aie jamais eus, mes anges gardiens. Alors que je ne savais même pas ce qu'était la LTP et que je croyais encore que l'hippocampe c'était que dans la mer, vous m'avez fait confiance et m'avez donné ma chance, et "aujourd'hui" je soutiens une thèse sur ce sujet.

J'ai été accueillie à bras ouvert par un grand nombre de personnes au grand coeur, que je ne me ferais pas le défi de mentionner individuellement tellement j'ai peur d'en oublier....

Je me contenterai donc de remercier :

- La team "vivo" qui m'a accueillie en premier dans ses couloirs, alors que je déambulais à toute berzingue pour transférer mes coupes d'hippocampe dans le bain-marie à l'étage du dessous – et même sans ça en fait. Je n'aurais pas réalisé mon rêve de travailler avec des rollers aux pieds pour aller plus vite, mais j'ai appris tellement de choses grâce à vous. Merci de m'avoir transmis vos valeurs de solidarité, de rigueur et de persévérance.
- La team "vitro" que j'ai pu intégrer lors d'un de mes nombreux "retours" à Porsolt. Les couloirs étaient certes plus petits mais c'est là-bas que j'ai fait pousser mes premiers neurones (enfin ceux des rats) et aussi galéré à les faire coller sur les MEA. Merci de m'avoir ouvert les portes de ce monde qui m'était inconnu et de m'avoir réchauffé après être resté toute la journée sous la clim, en allant boire quelques verres.
- La team des "DE", qui m'a chaleureusement fait une place alors que je ne savais même pas ce qu'était une TC. Merci de m'avoir accompagné, soutenu, et d'avoir partagé sans limites vos connaissances et petites astuces. Un merci particulier à ceux qui m'ont partagé leur aquarium, et pour m'y avoir fait sentir comme un poisson dans l'eau, ou presque !
- La team "administrative", pour leur patience devant mon manque d'assuidité à remplir les feuilles d'heures et pour avoir rattrapé mes p'tites boulettes de p'tit boulet 🍩 (incluant les soins prodigués suite à des "petits" incidents... « Allô môman bobo »).

- **To (almost) lab's outsiders' friends,**

Comment ne pas mentionner **Dave, Anne&Kyom, Tata&Toto**, les enfants (**Mali, Abel, Lucie, Chloé**).

Merci de vous être occupés de moi comme d'un de vos p'tits pioupious pendant toutes ces années, de m'avoir consolé et fait passer des soirées et week-ends qui font oublier tous les problèmes (j'espère que mes parents vous ont payé très cher pour me supporter tout ce temps). **Dave**, merci d'avoir essayé de faire de moi une star du bad', malheureusement je crois que je suis restée une bad star tout court (coucou à la team du bad' de St Pierre la Cour, rassurez-vous j'ai toujours mon syndrome de la Tourette quand je joue). **Anne**, merci pour ces grands moments de complicité et ton amitié. **Kyom**, alors, ce Salvert post-thèse avec tout plein de rides, on se le

fait quand q'ça ? – sinon je peux aussi venir cuisiner chez vous, je fais ça prop', un p'tit mugcake au micro-ondes et zé parti...- Être la tiote d'un ami comme toi est un privilège qui me laisse presque sans mots. **Tata**, je comprends mieux pourquoi on t'appelle comme ça. J'ai parfois été un peu "tortellini" mais tu m'as toujours rendu "coquillette". Au fait, en parlant de pâtes, promis je ne me nourris d'autres choses maintenant, j'ai retenu la leçon, je ne veux pas perdre mes dents. Miss you Hibou. **Toto**, j'ai découvert que derrière cet Homme qui rôle sur un paddle gonflable, se cachait quelqu'un d'attentionné (et bricoleur !); merci pour ta bienveillance et ton amitié. **Les enfants**, merci pour votre douceur et d'avoir accepté que je pique votre nourriture 😊.

Un p'tit coucou à **Jordanie Trotro2021**, merci de m'avoir redonné un second souffle. Je n'aurais jamais imaginé faire des paquitos avec des bédouins au milieu du désert, après avoir éteint la lune pendant que Hamza le chasseur de méchants aux pantalons bleus nous berçait au son de sa brosse à dents électrique. Bisous les **crushers fous** mangeurs de boulets après que les lions se soient envolés devant la potale: PAQUITO!

Kalana, my friend. From the day we met, you became at your turn the lighter of someone's lantern. I feel lucky that it was mine. A lot of smiles from chutiduwa. 🍷

- **Last, but not least, to my family,**

Mam's, Pap's, vous aviez sans nul doute la place la plus ingrate dans cette aventure, mais pourtant, j'ai toujours pu compter sur votre soutien sans failles (même quand je tombe en panne à 400 km et qu'ils enlèvent les panneaux de l'autoroute exprès pour me faire louper la sortie). Je vous en ai fait voir des vertes et des pas mûrs comme ont dit...mais vous avez toujours donné le meilleur pour moi, quitte à accepter le manque de nouvelles et les pétales de plombs alors que vous n'aviez rien fait 😊. C'est grâce à vous que je suis ici et il n'y a pas de mots assez forts pour vous en remercier. Un jour, quelqu'un m'a fait écouter ces paroles et mon plus grand souhait serait que vous les suiviez. *"On vient de marier le dernier, tous nos enfants sont désormais heureux sans nous. Ce soir il me vient une idée, si l'on pensait un peu à nous, un peu à nous. On a toujours bien travaillé, on a souvent eu peur de n'pas y arriver, maintenant qu'on est tous les deux, si l'on pensait à être heureux, à être heureux. Tu m'as donné de beaux enfants, tu as le droit de te reposer maintenant, alors il me vient une idée, comme eux j'aimerais voyager, Hmm, voyager ».*

Mon coin 🐦, merci pour tes salades sa mère et tous ces fous rires (Jogging' du lux'!). Je n'aurais jamais cru que vivre avec toi me manquerait tant, surtout à l'époque où tu me poursuivais comme une fury avec ton doudou partout dans la maison et quand tu t'adonnais à placer soigneusement des agrafes devant ma porte de chambre pour que je me les plante dans les pieds. J'espère que maintenant tu m'aimes autant que je t'aime (t'enflames pas non plus, j'taime un peu) et que tu me crois enfin quand je te dis que je n'ai pas été adoptée (c'était toi en fait). Mais au moins, maintenant, je peux laisser les placards ouverts sans entendre "Candiiiice" 😊.

Tante Quechua, merci d'avoir fait de ton mieux pour me trouver un autre travail pendant toutes ces années. J'espère que tu n'es pas trop déçue que je ne possède pas de lavomatique ou d'entreprise de plomberie.

Une douce pensée pour mes mamies, **mamie-Laure**, comme l'or, **mamie Elen** l'artiste & mes deux **papys Michel**.

Mon tuktuk' 🐾, merci de m'avoir apporté toute la douceur et l'amour qu'un chat peut donner (les chats c'est quand même des branleurs.) et d'avoir réchauffé mes genoux lorsque je travaillais à la maison.

Mon doudou, ramène-nous des glaçons ! Ce n'est peut-être qu'une petite attention, mais je tiens à te le dire... : PS ILY.

& Enfin, merci à toutes les "Doris" 🐱



The best part of Science is knowing, for a moment something that nobody else in the world knows".

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Abbreviations

ACH: Acetylcholine	i.c.v.: intracerebroventricular
ACH-E: Acetylcholine Esterase	I/E: Inhibitory/Excitatory balance
aCSF: artificial CerebroSpinal Fluid	IF: Immunofluorescence
AD: Alzheimer's disease	IN: Interneurons
AMPA-(Rs): α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (receptors)	I/O: Input/Output
ArchT: Archaelhodopsin	i.p.: Intraperitoneal
BDNF: Brain-Derived Neurotrophic Factor	ITI: Inter-Trial Interval
CA (1,2,3): Cornus Ammonis 1,2,3	IQ: Interquartile
cAMP: cyclic Adenosine Monophosphate	KI: Knock-In
CANTAB: Cambridge Neuropsychological Test Automated Battery	KO: Knock-Out
CAMKII: Calcium-calmodulin-dependent Protein kinase II	LEC: Lateral Entorhinal Cortex
CNS: Central Nervous System	LD: Location Discrimination
CFC: Cross-Frequency-Coupling	LTM: Long term Memory
CREB: cAMP Response Element Binding protein	LTP: Long Term Potentiation
DG: Dentate Gyrus	LTD: Long Term Depression
DP: Depotentialion	MAPK: Microtubule Associated Kinase
DRN: Dorsal Raphe Nucleus	MDD: Major Depressive Disorder
EC: Entorhinal Cortex	MEA: Multi Electrode Array
EEG: Electroencephalogram	MEC: Medial Entorhinal Cortex
EGFP: Enhanced Green Fluorescent Protein	MF: Mossy Fibers
eIPSCs: evoked Inhibitory Post-Synaptic Currents	MFR: Mild Food Restriction
EM: Episodic Memory	mGLURs: metabotropic Glutamate Receptors
EPM: Elevated Plus Maze	mPFC: medial Prefrontal Cortex
ERK: Extracellular Regulating Protein	MRN: Medial Raphe Nucleus
fEPSPs: field Excitatory Post Synaptic Potentials	MTDL: Multi target directed ligands
GABA-(Rs): Gamma Amino-Butyric Acid (receptors)	MTL: Medial Temporal Lobe
GAD2: Glutamate Decarboxylase 2	NMDA-(Rs): <i>N</i> -methyl-D-aspartate (receptors)
GLUT: Glutamate	NOR: Novel Object Recognition
GPCR: G-protein coupled receptor	OF: Open-Field
GI: Gastro-intestinal	PBS: Phosphate Buffered Saline
HFS: High Frequency Stimulation	PD: Parkinson's disease
	PDL: Pulse Diode Laser
	PKA: Protein Kinase A
	PP: Perforant Path
	PPF: Paired-Pulse Facilitation

PRh: Perirhinal cortex
PS: Pattern Separation
PV: Parvalbumin
qEEG: quantitative Electroencephalography
RM: Recognition Memory
SC: Schaeffer Collaterals
SE: Spontaneous Exploration
SCHIZ: Schizophrenia
SCOP: Scopolamine
SEM: Standard Error Mean
s.c.: subcutaneous
SER: Serotonin
TBS: Theta Burst Stimulation
TOM: Temporal Order Memory
WM: Working Memory
5-HT: 5-Hydroxytryptamine
5-HT₁Rs, 5-HT₄Rs, 5-HT₆Rs: Type 1,4,6, serotonin receptors

Foreword

Tracing back to 1957 with the groundbreaking discovery of Scoville and Milner that hippocampal ablation led to anterograde amnesia, the hippocampus is widely accepted as the center of episodic memory (EM) processes. Notably through functional activity-dependent plasticity, the hippocampus is capable of encoding, storing, and retrieving episodes of everyday life. Consequently, alterations of hippocampal functioning (from the molecular to the cellular level) can lead to EM disorders. Consistent with this view, common psychiatric disorders such as schizophrenia (SCHIZ) and major depressive disorder (MDD) as well as neurodegenerative diseases such as Alzheimer's (AD) and Parkinson's (PD) which are characterized by EM disorders, also exhibit several hippocampal alterations.

Currently available therapies against memory impairments had still not proved sufficient medical efficacy. Indeed, the drugs developed exert only symptomatic effects and fail to counteract the evolution of brain alterations.

From 1990's onwards, type 4 serotonin receptors (5-HT₄Rs) started to become a therapeutic target of choice against memory disorders. Indeed *(1) Anatomically, 5-HT₄Rs are highly distributed in brain areas that are relevant for EM, such as the hippocampus, and their densities have been found to be reduced in cognitively impaired patients (2) Functionally, their blockade or depletion impairs EM while their activation improves memory in both healthy and animal models of cognitive disturbance as well as in healthy humans (3) Mechanistically, they appear to exert beneficial effects on the main pathological drivers of brain diseases.*

Hence, 5-HT₄Rs constitute a promising approach as a target for disease-modifying drugs that could prevent or limit the progression of several brain diseases. However, to gain in consideration in the field of memory disorders related to hippocampal (dys)function, there is a crucial need to better understand the mechanisms underlying the beneficial effects of their activation on memory.

The following introduction will provide a framework that will help to understand the interest of bridging 5-HT₄Rs to EM disorders by spanning key concepts of EM and its neurobiological substrates.

INTRODUCTION

Learning and memory

Episodic memory

In essence, memory is the capacity that allows us to connect day life events and learn from experiences. It is now well-recognized that multiple forms of memory co-exist. EM is the memory for past personal events, and usually includes the context in which the event took place (what, when, where?), as well as associated emotions, hence being at the core of the construction of our identity.

Traditionally, EM belongs to long-term declarative (or explicit or conscious) memory (Tulving, 1995). This classification of memory has been updated in 2003 in the *Memory NEO-Structural Inter-Systemic* model (MNESIS) model (Eustache & Desgranges, 2008) (**Figure 1**).

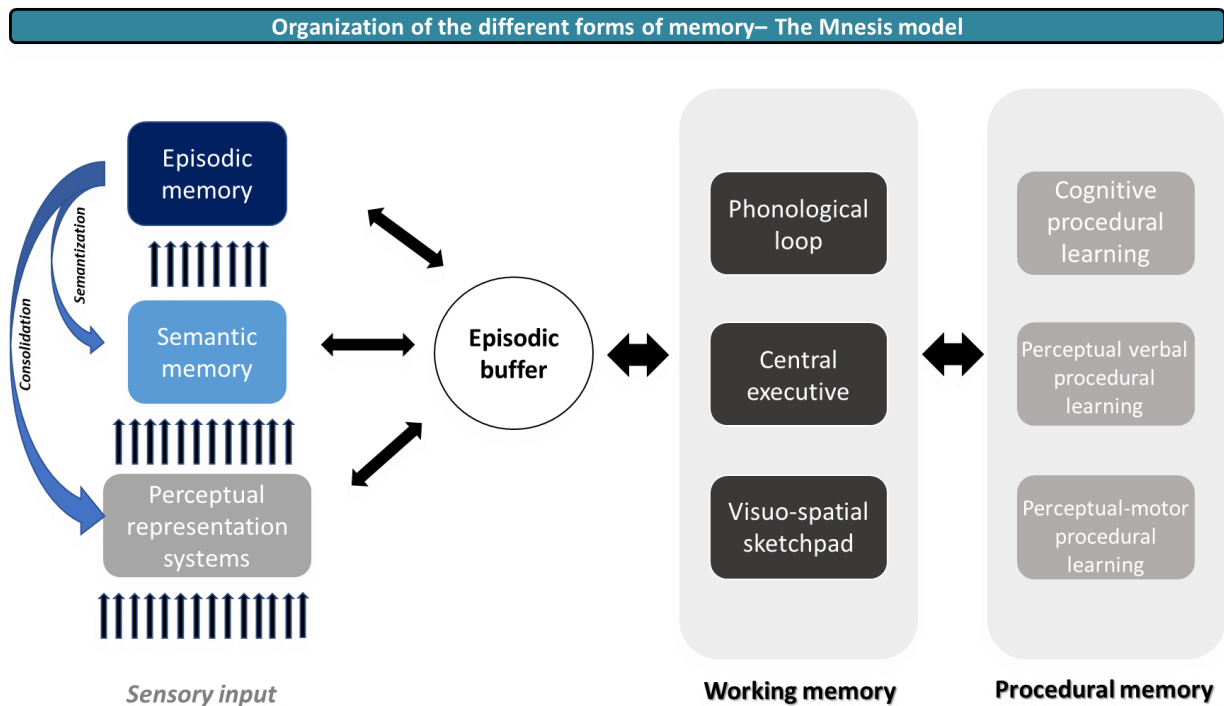


Figure 1: The Mnesis model.

(Adapted from Eustache and Desgranges, 2003).

This model reconciles original concepts described by pioneers (Atkinson & Shiffrin, 1968; Squire, 2004; Tulving, 1985, 1995) while adding a dynamic relationship between the different memory systems newly defined. According to the MNESIS model, EM is still a subset of long-term memory (LTM), which sits at the top of hierarchy from semantic (generic and context-free knowledge) memory and perceptual memory (shape and structure of words and items). Across time, EM can transfer to semantic memory through a process called semantization. LTM reciprocally interacts with another memory system for active temporary storage named working memory (WM). Due to the very limited capacity of WM and the fact that it can involve reactivation of long-term memories, an episodic buffer allows reciprocal transfer between LTM and WM.

Unfortunately, EM is particularly vulnerable to the effects of ageing and is found to be early disrupted in brain diseases such as AD (Tromp et al., 2015).

The accuracy of episodic memories relies on the quality of the encoding, consolidation and retrieval of information and requires the involvement of medial temporal lobe (MTL), and more particularly the hippocampus (**Figure 2**) (Tromp et al., 2015). During encoding, the inputs converge to the hippocampus where individual events bind together and become cohesive memories. Thereafter, consolidation phase is initiated allowing the persistence and maintenance of memories as well as their reorganization into LTM. Then, mental representations can be reactivated through retrieval (or recall) of the conscious experience of the event. MTL regions are interconnected with cortical regions (e.g., the retrosplenial and posterior cingulate cortex, the angular gyrus, and the medial and lateral prefrontal cortices (PFC)) to connect with past events.

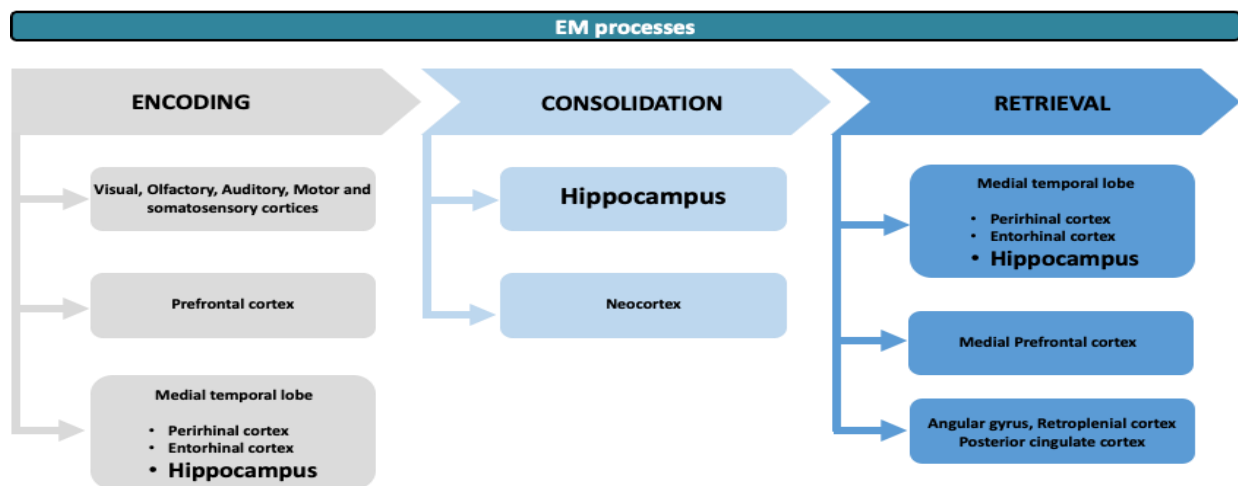


Figure 2: The three processes of episodic memory and their neural substrates.

(Adapted from Tromp et al., 2015)

Episodic-like memory (ELM) in rodents

Notwithstanding with the efforts made to model human EM in rodents, defining an experimental task that allows the assessment of personal experience still represents a challenge. Most of the current tasks in humans are based on verbal cues (California verbal learning test, RAVLT) or visual complex figures (Benton's Visual Retention Test, Rey's Complex Figure test), but all entail semantic and/or executive functions. Although these tasks cannot be directly translated to animals, the contents of episodic-like memory (ELM) can be modeled by the operationalization of the *souvenir* of the 'what', 'where' and 'when' components- also termed the 3W's - of a particular event. Different paradigms have thus been developed in rodents and are all based on spontaneous exploration (SE) of items. Among these tests is the novel object recognition test (NOR) (Ennaceur & Delacour, 1988) developed to assess the "what?" component of EM. Based on rodent's innate preference for novelty, this task measures the ability of an animal to discriminate between a novel object and an object previously encountered. Its popularity mainly lies in its potential of translatability from human studies. Indeed, it derives

from the “visual-paired comparison paradigm” widely used both in humans and non-human primates, in which the participant/subject has to discriminate between a familiar stimulus, paired with a new one (Rose et al., 2013). Another aspect of EM is the memory for the temporal order (“when?”) of object presentation/event experiencing (temporal order memory; TOM). The SE- based TOM task probes the animal’s ability to discriminate the recency of two familiar item/event presentation as rodents readily tend to explore old familiar than recent familiar objects indicating based on their relative recency (Barker & Warburton, 2011). This task derives from touch-screen based task used in humans (Hsieh et al., 2011).

Recognition memory (RM) for both novelty and recency thus requires judgments of the previous occurrence of stimuli and the accuracy of the encoding of the information appears crucial. This function is thought to be supported by pattern separation (PS), which can be defined as a type of memory processing whereby highly overlapping sensory inputs are transformed into separate and distinct representations. PS can also be measured with SE-based paradigms whereby the distance between two similar objects varies across delays. More recently, a location discrimination (LD) task on touch-screen chambers – in which animals have to discriminate between two similar items separated with varying distances – has emerged (Oomen et al., 2013). Touch-screen assays are an extension of traditional operant systems with increased possibilities in terms of stimuli, hence facilitating reverse translation as it is widely used in humans. In fact, most of the tasks implemented derive from the Cambridge Automated Neuropsychological Test Associated Battery (CANTAB) which has been validated over the years as having diagnostic validity for assessing patients with disorders such as SCHIZ (Barnett et al., 2010), PD (Olde Dubbelink et al., 2013) and Autism Spectrum Disorder (Ozonoff et al., 2004).

Hippocampal long-term potentiation (LTP) and learning and memory

One of the most significant challenges in neuroscience has been to identify the cellular and molecular processes underlying learning and memory formation. Over the past century, a number of changes that accompany certain forms of acquisition and recall have been identified, and more particularly the forms that require the activation of afferent pathways to the hippocampus. Of these changes, activity-dependent hippocampal long-term potentiation (LTP) has received most attention. The most studied form of LTP is dependent on the glutamatergic *N*-Methyl-*D*-aspartate receptors (NMDA-Rs) (Lynch, 2004) (**Figure 3**).

NMDA-Rs dependent long-term potentiation (LTP)

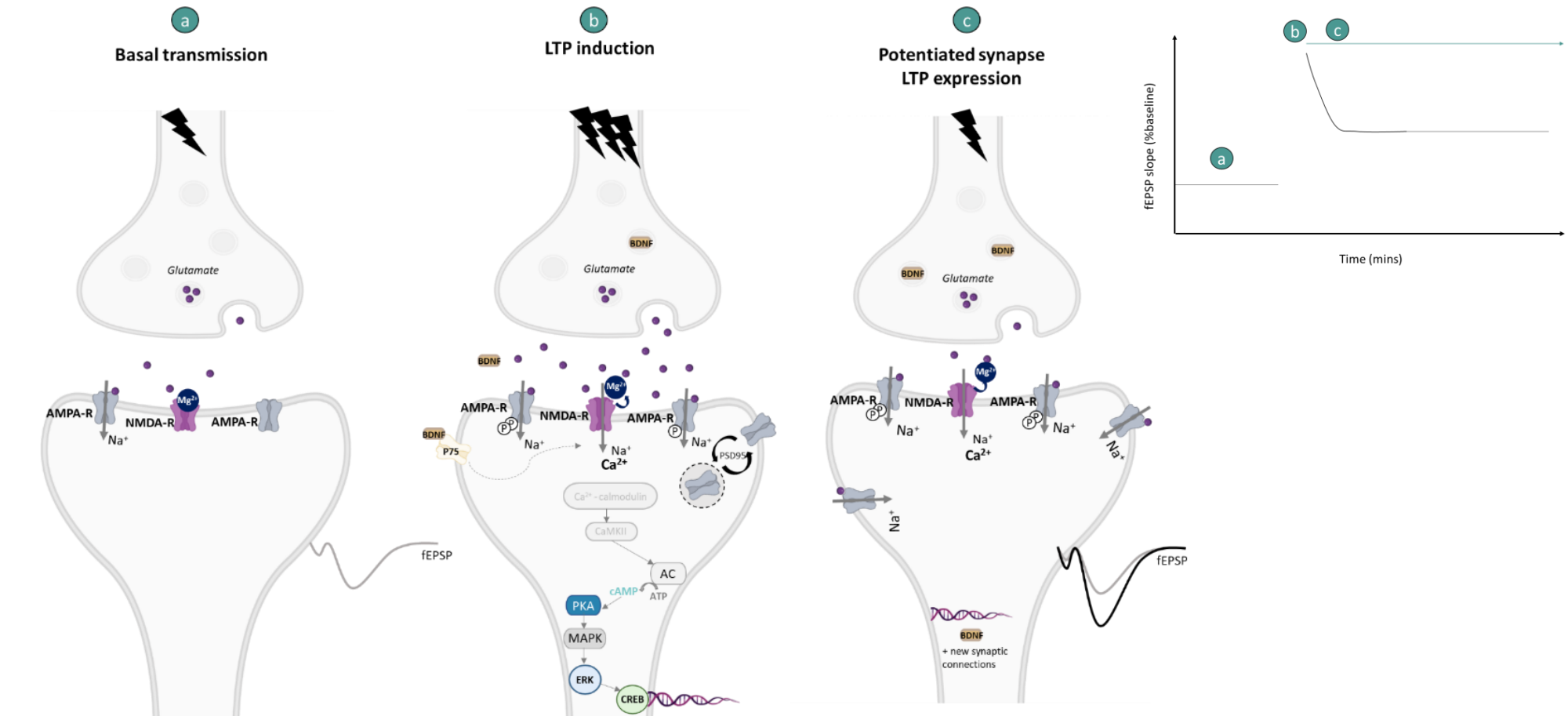


Figure 3: Graphical representation of the cellular and molecular mechanisms underlying LTP induction and maintenance.

Abbreviations: **AC**: adenylate cyclase; **AMPA-R**: α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors; **ATP**: adenosine tri-phosphate; **BDNF**: brain-derived neurotrophic factor, **Ca²⁺**: calcium ion; **CaMKII**: Calcium-calmodulin dependent protein kinase II; **cAMP**: cycli adenosine monophosphate; **CREB**: cAMP Response Element Binding protein; **PSD95**: Post synaptic densities protein 95; **fEPSP**: field excitatory post-synaptic potential; **MAPK**: mitogen activated protein kinase; **Mg²⁺**: magnesium ion; **NMDA-R**: *N*-methyl-D-aspartate receptors; **Na⁺**: sodium ion.

(Adapted from Bliss & Cooke, 2011).

NMDA-Rs act as coincidence detectors of (1) the presence of its ligand (glutamate) and co-agonist (D-serine and glycine) and (2) sufficient post-synaptic depolarization initiated by alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA-Rs) activity. Being particularly permeable to Ca^{2+} and Na^+ , NMDA-Rs activation leads to an important calcium influx onto the post-synaptic spine. In turns, it triggers intracellular cascades leading to a first phase of fast re-arrangements that last from 30-60 min called « Early-LTP or e-LTP » that is followed by a second phase of protein synthesis named « Late-LTP or l-LTP » that can last for hours and days (Baltaci et al., 2019).

The early phase of LTP is initiated by the rapid autophosphorylation of a calcium-calmodulin dependent protein kinase II (CaMKII) subsequent to calcium entry. It results in transient AMPA-Rs phosphorylation enhancing their conductance and exocytosis of new AMPA-Rs receptors. These new receptors are then inserted at the postsynaptic site of the stimulated synapse *via* interaction with transmembrane AMPA-Rs regulatory proteins (TARPs) and the post-synaptic density protein (PSD95). Also, silent synapses - that are inactive at rest due to the presence of only NMDA-Rs – become active and are newly addressed with AMPA-Rs, hence contributing to the strengthening of the post-synaptic response.

The late phase of LTP consists in sculpting synaptic connections (elongation, sprouting of dendritic spines) that are critical to the maintenance of LTP and conditions the persistence of memory trace. Such phenomenon requires *de novo* protein synthesis (such as the brain derived neurotrophic factor, BDNF) as well as the activation of transcription factors and synaptic structural changes. These processes are dependent on other calcium dependent proteins which activation is stimulated by an increase in intracellular cyclic adenosine monophosphate (cAMP) concentrations. For instance, cAMP activates protein kinase A (PKA) which leads ultimately to phosphorylation of the cAMP response element binding (CREB) and allows it to function as gene expression modifying transcription factor. CREB can also be stimulated by PKA through the phosphorylation of microtubule associated protein kinase (MAPK) such as the extracellular regulated kinase (ERK). Exocytosis of endogenous BDNF following strong stimulation is also able to activate MAPK through BDNF-TrkB signaling.

Importantly, it has been shown that LTP can be induced and be expressed by activating different intracellular pathways including calpain-dependent activity – a downstream target of ERK, according to the pattern of electrical activity (Zhu et al., 2015). Indeed, stimulation paradigms used to experimentally induce LTP are known as theta-burst stimulation (TBS) and high frequency stimulation (HFS) and have been shown to trigger different cellular pathways (Zhu et al., 2015). The frequency of these stimulation patterns is inspired from naturally occurring brain oscillations known as theta - θ - (4-12Hz) and gamma- γ -(30-100Hz) respectively. Changes in these frequency bands have been observed during spatial and contextual learning and were shown to naturally trigger LTP *in vivo* (Bragin et al., 1995; O'Keefe & Recce, 1993; Tort et al., 2008).

Although LTP mechanisms mainly involve glutamatergic signaling, many neurotransmission systems regulate hippocampal synaptic plasticity and downstream mnemonic functions, including the serotonergic one (**see the [Review article](#)**).

The serotonergic system and the hippocampus

Serotonin (5-hydroxytryptamine, 5-HT) is a small indolamine common to the animal (from submarine ascidies to humans) and plant kingdoms. In humans and other mammalian species, 5-HT plays a modulatory role in almost every physiological function. These include eating, reward, thermoregulation, cardiovascular regulation, locomotion, pain, reproduction, sleep-wake cycle, memory, cognition, aggressiveness, responses to stressors, emotion, and mood (Berger et al., 2009). Hence, dysfunctions of the serotonergic system are often associated with a number of human pathologies that include central nervous system (CNS) diseases such as SCHIZ, PD and AD (Ohno, 2019).

The role played by 5-HT system in memory lies on anatomical evidence that 5-HT receptors are widely distributed in crucial regions involved in these functions. Indeed, arising from two relatively small nuclei of the midbrain, namely the dorsal and medial raphe nuclei (MRN, DRN), serotonergic neurons project axons throughout the brain, and notably to the hippocampal formation, the striatum and frontal cortex (**Figure 4**) (Lesch & Waider, 2012).

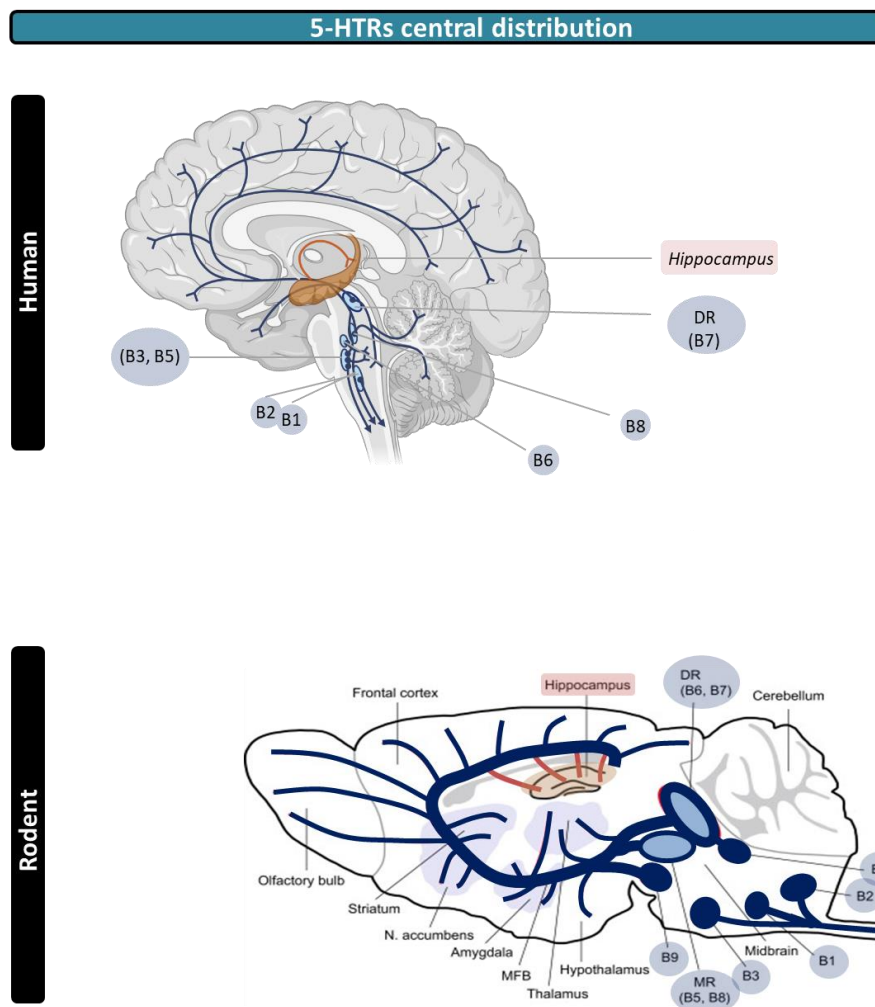


Figure 4: Patterns of central 5-HTRs distribution in the human brain (top) and rodent's brain (bottom).

(Adapted from Bockaert et al., 2004)

Further, experimental manipulations on 5-HT system revealed its modulatory role on memory processes. For instance, depletion of the 5-HT precursor (L-tryptophan) notably led to memory deficits. Since then, it is clear from both animal and humans studies that changes in 5-HT receptor expression are associated with marked changes in memory performances (for review see Coray & Quednow, 2022).

Based on structural, transductional and operational features, 5-HTRs have been grouped into 7 families (5-HT₁ - 5-HT₇) and 14 sub-types. Except for the 5-HT₃R subtype, which is ionotropic, they all belong to the metabotropic receptor's family (**Table 1**). Each of 5-HTR subtype has a specific regional distribution in the brain as well as sometimes opposite cellular effects according to the G protein they are coupled to (**Table 1**).

Table 1: List of 5-HTRs receptors, their central localization, coupling and cellular effects.

Family	Central distribution	Mechanism	Cellular effect
5-HT ₁	Pituitary gland, rostral raphe nuclei, hippocampus, prefrontal cortex, basal ganglia, amygdala, globus pallidus, putamen, caudate nucleus	Gi/o Adenylate cyclase (AC)	Inhibitory
5-HT ₂	Cerebral cortex, basal ganglia, amygdala, choroid plexus, hypothalamus, hippocampus, caudate nucleus, putamen, globus pallidus, substantia nigra	Gq Phospholipase C	Excitatory
5-HT ₃	Area postrema, tractus solitarius, limbic system, hippocampus, cerebral cortex	Ligand-gated ion channel	Excitatory
5-HT ₄	Prefrontal cortex, caudate nucleus, putamen, globus pallidus, hippocampus, substantia nigra	Gs Adenylate cyclase (AC)	Excitatory
5-HT ₅	Cerebral cortex, amygdala, cerebellum, hypothalamus, hippocampus	Gi/o Adenylate cyclase (AC)	Inhibitory
5-HT ₆	Dentate gyrus, hippocampus, olfactory tubercule, nucleus accumbens, amygdala, cerebellum	Gs Adenylate cyclase (AC)	Excitatory
5-HT ₇	Thalamus	Gs Adenylate cyclase (AC)	Excitatory

Among these 5-HTR subtypes, 5-HT₁Rs, 5-HT₄Rs, 5-HT₆Rs and 5-HT₇Rs subtypes have been extensively studied the field of learning and memory. Particularly, **5-HT₄Rs** activation was repeatedly shown to exert beneficial effects on memory performances in both humans and animals.

Type 4 serotonin receptors (5-HT₄Rs)

Generalities

5-HT₄Rs were identified in cultured mouse colliculi cells and guinea pig brain using a functional cAMP stimulation assay in the late 90's (1985). They are 7 transmembrane receptors that belong to the superfamily of G-coupled protein receptors (GPCR) (**Figure 5**), see the [Review article](#) for details). The intracellular C-terminal region which is the binding site of G-protein, is viewed as a key regulator of 5-HT₄Rs constitutive activity. The shorter is the C-terminal, the higher is the constitutive activity. Of most interest, the intracellular signaling cascade of 5-HT₄Rs shares common effectors with the classical signaling pathway involved in LTP (Lynch, 2004).

5-HT₄Rs transduction cascade

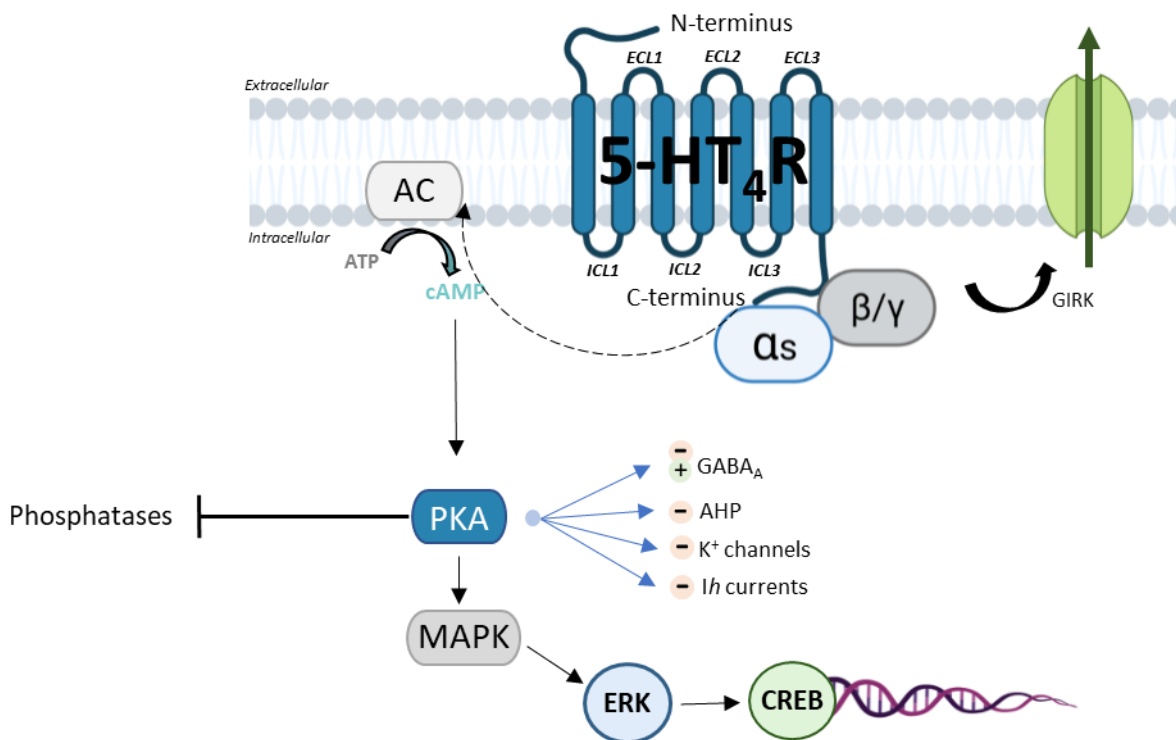


Figure 5: Schematic two-dimensional representation of 5-HT₄Rs. 5-HT₄Rs are composed of 3 intracellular (ICL) and 3 extracellular loops (ECL) and of an extracellular N-terminal domain for ligand binding as well as a C terminal intracellular which is coupled to the G protein.

(Adapted from Padayatti et al., 2013 and Bockaert, 2008)

Distribution

5-HT₄Rs are widely distributed both at the periphery and in the CNS. A number of studies based on autoradiography ³H]GR 113808 radioligand assay, positron emission tomography (PET)-based high resolution imaging and magnetic resonance imaging (MRI) contributed to draw a similar pattern of 5-HT₄Rs central distribution across species (**Figure 6**).

The highest densities of 5-HT₄Rs are found in olfactory bulb, islands of Calleja, basal ganglia and nucleus accumbens, hippocampus, and substantia nigra (Beliveau et al., 2017; Bockaert et al., 2004; Bockaert & Dumuis, 1998; Bonaventure et al., 2000). Within the nigro-striatal pathway 5-HT₄Rs are localized in cell bodies and terminals of GABAergic (GABA: γ -amino butyric acid) interneurons whereas in the cortex, the hippocampus and the amygdala, 5-HT₄Rs are likely localized on both cholinergic neurons and glutamatergic neurons (Bockaert et al., 2004; Huang & Kandel, 2007). Within the hippocampal formation, 5-HT₄Rs were found to be distributed within the pyramidal cell layer of all CA1, CA2, and CA3 subfields, as well as within the granular layer of the dentate gyrus and in the subiculum (Bonaventure et al., 2000).

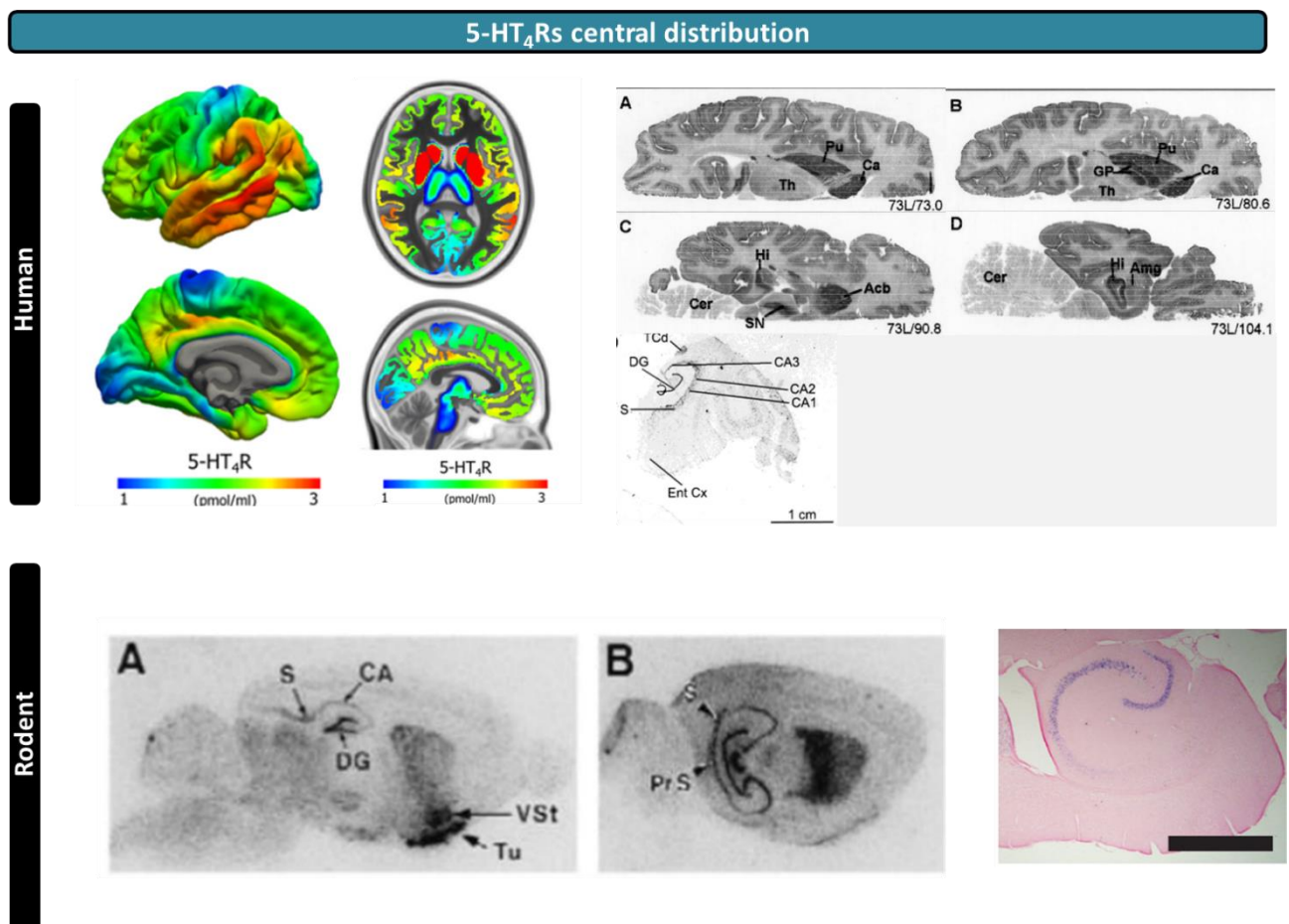


Figure 6: Central distribution of 5-HT₄Rs in human's and rodent's brain.

5-HT₄Rs ligands and memory

The identification of the involvement of 5-HT₄Rs in a number of important physiological functions rapidly raised their interest as therapeutic target notably in gastro-intestinal (GI) disorders and learning and memory, and prompted the development of 5-HT₄Rs agonists summarized in **Table 2**:

Table 2: List of main 5-HT₄Rs and their main characteristic

Agonist	pKi (binding)	pEC ₅₀	Efficacy	Status (indication)
5-HT	7.1-7.5	7.4-8	Full	
5-MeOT	6.2	7 -7.7	Full	
Tegaserod (HTF919)	8	8.8	Partial	Zelnorm®(GI)
Cisapride	7.1	7.25	Full/Partial	Prepulsid®(GI)
SC53116	7.5-8.1	7.64	Full	
SB205149		8	Full/Partial	
PRX-03140	7.7	6.9	Partial	Phase IIb (AD)
Prucalopride	8-8.6	7.5-7.8	Partial	Resolor®(GI)
Mosapride	6.8-7	7.1	Partial	
Y-34959	8.5	8.5	Full	
ML10302	7.9-9	7.7 -8.6	Partial	
SL65.0155	8.78	6.7-8.3	Partial	
RS67333	8.7	8.4	Partial	
RS67506	8.8	8.6	Partial	
BIMU-1	6.4-7	6.5 -8.4	Full/Partial	
BIMU-8	7-7.5	7.1 -8.1	Full	
PF-04995274	9.48	-	Partial	Phase I
SUVN-D4010	7.6	-	Partial	Phase II (AD)

Abbreviations: **pKi**: negative logarithm of constant affinity; **pEC₅₀**: negative logarithm of the concentration producing 50% of the maximal effect on cAMP production; **AD**: Alzheimer's disease; **GI**: gastrointestinal.

(Updated from Bockaert et al., 2004)

RS67333 has been one of the most studied agonists on behavioral memory in preclinical studies (**Table 3**).

As regards to clinical studies; this is only recently that a timely opportunity came through with the licensing of the partial 5-HT₄Rs agonist, prucalopride (Resolor®). It is the unique 5-HT₄Rs agonist that demonstrated beneficial effects on memory in healthy humans (de Cates et al., 2021, 2022; Murphy et al., 2020).

Table 3: Summary of the beneficial effects of 5-HT₄Rs activation**Preclinical studies**

Behavioral task	Model	Agonist	Treatment period	Dose (mg/kg)	Behavioral outcome	References
Social olfactory recognition test	Aged animals	SL65.0155	Acute	0.001 & 0.03	↑ performances from 0.01 mg/kg	(Moser et al., 2002)
Olfactory associative learning	5-HT ₄ Rs blockade induced deficits	RS67333	Acute	1	Reversal of deficits	(Marchetti et al., 2000)
	Healthy animals	RS67333 BIMU 1	5 injections Acute	1 1, 5, 10	↑ performances ↑ performances from 1 mg/kg	(Marchetti et al., 2004) (Letty et al., 1997; Marchetti-Gauthier et al., 1997)
Olfactory tubing maze	Healthy animals	RS67333	2-4 months (twice a week)	1	No effect	(Baranger et al., 2017)
	Transgenic mouse model of AD (5xFAD)	RS67333	2-4 months (twice a week)	1	↑ performances after 4-months treatment (but not after 2-months treatment)	
Linear maze	Aged animals	SL65.0155	Acute (i.p/p.o)	0.01 & 0.1	Reversal of deficits	(Moser et al., 2002)
Radial arm maze	SCOP induced deficits	SUVN-D4010	Acute (p.o)	1, 3, 10	Reversal of deficits	(Nirogi et al., 2021)
MWM	Healthy animals	RS67333	5 injections	1	↑ learning rate	(Lelong et al., 2001)
	SCOP induced deficits	SL65.0155	Acute (10 Days)	0.1 & 0.3	Reversal of learning deficits	(Moser et al., 2002)
		SSSP002392		0.3, 1.5, 7.5	Reversal of learning deficits	<i>Lo et al, 2014</i>
	Healthy animals	BIMU8	5 injections	30	↑ reference memory	(Teixeira et al., 2018)
Scopolamine induced deficits	PRX-03140	Acute (i.p)	0.03, 0.1, 0.3	No effect	(Shen et al., 2011)	
Barnes maze	CORT-induced deficits	RS67333	4 injections	1.5	Reversal of learning and reference memory deficits	(Darcet et al., 2016)
Object recognition	CORT-induced deficits	RS67333	4 injections	1.5	Reversal of deficits	(Darcet et al., 2016)
		RS67333	Acute (i.p)	1	↑ performances at 1 mg/kg when administered before acquisition	(Darcet et al., 2016; Freret et al., 2017a; Hotte et al., 2012; Lamirault & Simon, 2001; Levallet et al., 2009; Moser et al., 2002; Quiedeville et al., 2015)
	Healthy animals	SL65.0155	Acute	0.001 & 0.1	↑ performances at both doses	(Moser et al., 2002)
		RS67333	Acute (i.p)	0.3, 1.0, and 3.0	↑ performances at 0.3 and 1 mg/kg	(Nirogi et al., 2021)
		SUVN-D4010	Acute (p.o)	1, 3, 10	↑ performances at 1,3 mg/kg	
Place recognition	Healthy animals	RS67333	Acute (i.p)	0.0001, 0.01, 1	↑ performances at 1 mg/kg when administered before acquisition	(Lamirault & Simon, 2001)
Passive avoidance	SCOP, antagonist, dicyclomine induced deficits	BIMU 1/8	Acute (i.c.v. or i.p)	BIMU 1 (10 mg kg ⁻² or 5 µg/mouse BIMU 8 (30 mg kg ⁻¹ or 30 µg/mouse)	Reversal of deficits at highest doses	(Galeotti et al., 1998)

Introduction

	SCOP induced deficits	SC53116	Acute (i.c.v.)	10 µg/rat	Reverse deficits (when administered before training)	(Matsumoto et al., 2001)
		SSP002392	Acute	0.3, 1.5, 7.5	Dose-dependant reversal of induced deficits	(Lo et al., 2014)
	MK-801 induced deficits	RS67333	Acute	(6.25, 62.5 and 625 ng/mouse)	Impairment of consolidation	(Nasehi et al., 2015)
	SCOP induced deficits	RS67333	Acute	0.5, 1, 2	Reversal of induced deficits	(Freret et al., 2017a)
	Chemical lesion induced deficit	SL65.0155	Chronic (8,14 days) or acute i.p	1	Reversal of induced deficits	(Micale et al., 2006)
	Healthy animals	RS67333	Acute	0.5, 1, 2	No effect	(Freret et al., 2017a)
Auto-shaping	Chemical lesion induced deficit	BIMU 1/8	Acute	10-30	↑ performance when administered before pre-training only	(Meneses & Hong, 1997)
Spontaneous alternation	Healthy animals	VRX03011	Acute	0.1, 1, 5, 10	Dose-dependent ↑ of performance on delayed spontaneous alternation (no effect in standard protocol)	(Mohler et al., 2007)
	SCOP induced deficits	RS67333	Acute (i.p)	0.25, 0.5, 1	Reversal of induced deficits	(Freret et al., 2017a)
	Healthy animals	RS67333	Acute (i.p)	0.25, 0.5, 1	No effect	

Clinical studies

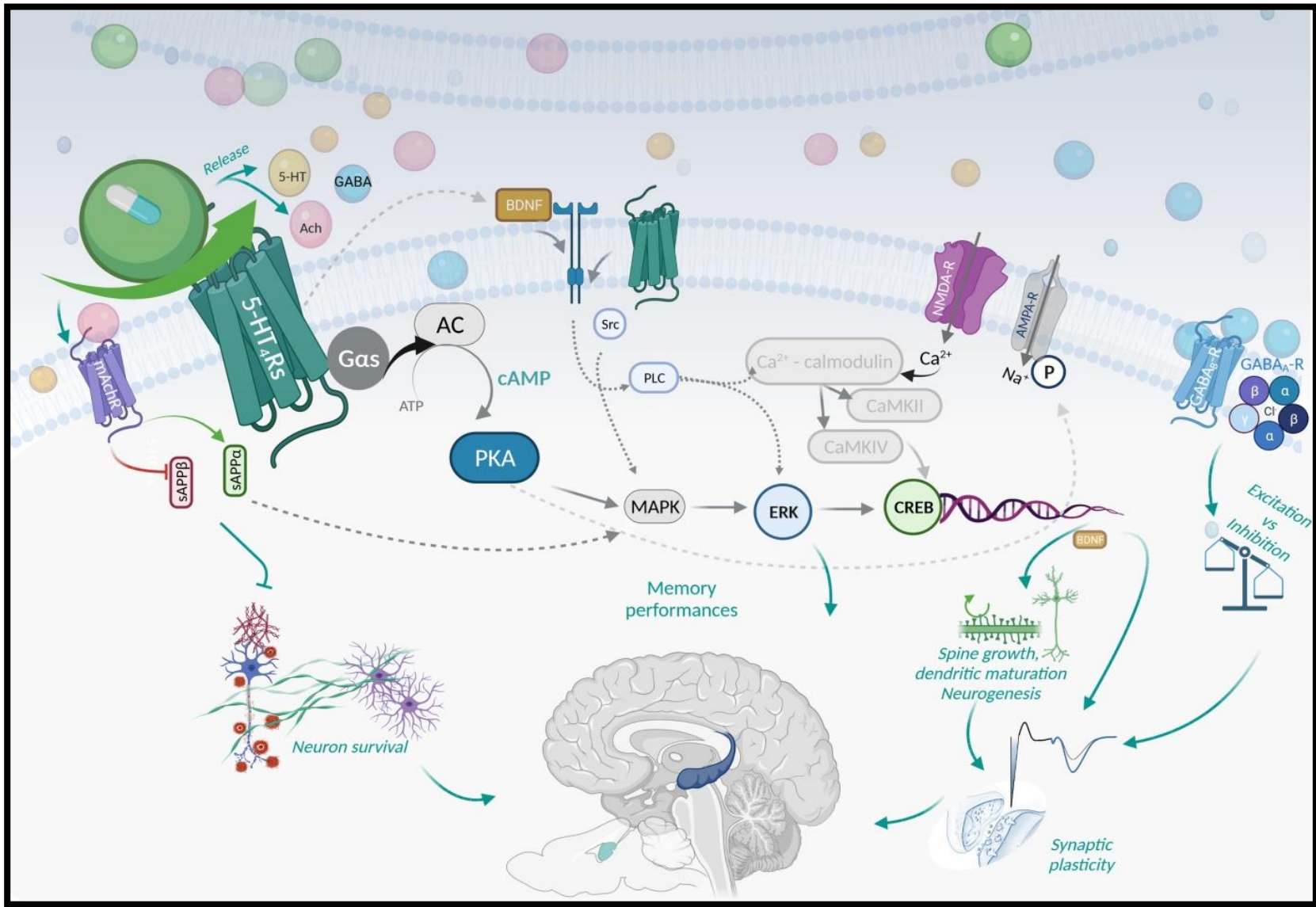
Rey auditory verbal learning task	Healthy volunteers	Prucalopride	Acute	1 mg	↑ performances	
N-back		Prucalopride	Acute	1 mg	No effect	
Contextual cueing		Prucalopride	Acute	1 mg	No effect	(Murphy et al., 2020)
Probabilistic instrumental learning task		Prucalopride	Acute	1 mg	↑ performances	
Memory encoding task		Prucalopride	6 days	1 mg	↑ performances	(de Cates et al., 2021)
Imaging face task		Prucalopride	6-days	1 mg	↑ accuracy	(de Cates et al., 2022)

Abbreviations: **AD**: Alzheimer's disease, **CORT**: corticosterone, **i.c.v.**: intracerebroventricular, **i.p.**: intraperitoneal, **p.o.**: per os; **SCOP**: scopolamine, ↑ improvement, ↓ impairment.

(Updated from Manahan-Vaughan 2017)

As depicted by the table above, the beneficial effects of 5-HT₄Rs activation on memory have mainly been studied in either healthy (or basal) conditions or in animal models of AD (genetically or pharmacologically-induced). Whilst mechanisms at work still remain to be elucidated, existing studies tend to suggest that 5-HT₄Rs activation could positively affect the hippocampal function resulting in cognitive enhancement and/or improvement. Within this framework, beneficial effects of 5-HT₄Rs could be extend beyond the field of AD to other CNS disorders having in common hippocampal alterations driving (at least in part) cognitive impairments.

The following **Review article** aims at further supporting the interest of 5-HT₄Rs activation in a broader range of memory disorders related to alterations of the hippocampal function. We identified and gathered from preclinical and clinical studies, the main hippocampal alterations that are common to CNS disorders associated with cognitive impairments such as AD, PD, SCHIZ and MDD. We then transposed the known beneficial effects of 5-HT₄Rs activation on the identified common pathological drivers. This helped us to draw an outline of the benefits that 5-HT₄Rs could represent in the research against memory impairments observed in the aforementioned diseases.





Review

Memory Disorders Related to Hippocampal Function: The Interest of 5-HT₄Rs Targeting

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Abstract: The hippocampus has long been considered as a key structure for memory processes. Multilevel alterations of hippocampal function have been identified as a common denominator of memory impairments in a number of psychiatric and neurodegenerative diseases. For many years, the glutamatergic and cholinergic systems have been the main targets of therapeutic treatments against these symptoms. However, the high rate of drug development failures has left memory impairments on the sideline of current therapeutic strategies. This underscores the urgent need to focus on new therapeutic targets for memory disorders, such as type 4 serotonin receptors (5-HT₄Rs). Ever since the discovery of their expression in the hippocampus, 5-HT₄Rs have gained growing interest for potential use in the treatment of learning and memory impairments. To date, much of the researched information gathered by scientists from both animal models and humans converge on pro-mnesic and anti-amnesic properties of 5-HT₄Rs activation, although the mechanisms at work require more work to be fully understood. This review addresses a fundamental, yet poorly understood set of evidence of the potential of 5-HT₄Rs to re-establish or limit hippocampal alterations related to neurological diseases. Most importantly, the potential of 5-HT₄Rs is translated by refining hypotheses regarding the benefits of their activation in memory disorders at the hippocampal level.

Keywords: 5-HT₄Rs; serotonin; hippocampus; memory disorders; therapeutic target; synaptic plasticity; cognition



Citation: Roux, C.M.; Leger, M.; Freret, T. Memory Disorders Related to Hippocampal Function: The Interest of 5-HT₄Rs Targeting. *Int. J. Mol. Sci.* **2021**, *22*, 12082. <https://doi.org/10.3390/ijms222112082>

Academic Editor:
Philippe De Deurwaerdere

Received: 9 September 2021

Accepted: 3 November 2021

Published: 8 November 2021

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1. Introduction

Memory impairments are a core symptom of a number of neurodegenerative diseases, such as Alzheimer's disease (AD) [1] and Parkinson's disease (PD) [2], but are also common to several psychiatric pathologies such as major depressive disorder (MDD) [3] and schizophrenia (SCZ) [4]. Whether or not this is the core symptom of these pathologies, alterations of memory function always have a severely disabling effect on a patient's everyday life. Indeed, memory function is a fundamental process which allows human beings to adapt from previous experiences and to progressively construct their unique identity [5].

Unfortunately, memory impairments remain therapeutically poorly apprehended. Over the past 30 years, only four drugs were approved to treat cognitive disorders. Initially developed in the context of AD—as the most prominent neurodegenerative disorder—the application domain of these drugs was thereafter extended to a larger number of pathologies. Among these drugs, three are acetylcholine esterase (ACh-E) inhibitors and the last is an N-methyl-D-aspartate receptor (NDMA-Rs) antagonist [6]. Regardless of their mechanism of action, they all show a limited efficacy and tolerance profile, leading to insufficient medical benefit. This contrasts with the large number of new therapeutic drug candidates tested in the field of preclinical studies, some demonstrating promising results. In 2008, over 172 drug development failures were registered in the field of AD [6]. Further, the only drug approved since 2003 was approved only very recently, with a use restricted to the United States [7].

Initially on the market for the treatment of gastrointestinal pathologies, type 4 serotonin receptors (5-HT₄Rs) ligands progressively earned a place in the sun as a promising therapeutic target for memory disorders [8,9]. Two years after their discovery in 1988 [10], 5-HT₄Rs started becoming the focus of intensive research for central nervous system (CNS) disorders, with over 100 patents of synthesized 5-HT₄Rs ligands registered by 2014 [9]. Even today, the modulation of 5-HT₄Rs remains a strategy of interest in the struggle against cognitive dysfunctions associated with psychiatric and/or neurological diseases [9].

This review will first discuss the current knowledge on memory function by focusing on the hippocampus and its alterations during physiological and pathological aging. Then, through a comprehensive discussion of the role of 5-HT₄Rs in hippocampal memory processes, the relevance of its pharmacological modulation as a future therapeutic strategy in memory disorders will be argued in a broad extent.

2. Episodic Memory Function and the Hippocampal Formation

From the second half of the 20th century, case studies of patients with amnesia, as well as the development of a large number of animal models with memory disorders, enabled major breakthroughs in the understanding of the brain memory system—or how the brain stores different kinds of information. The idea of the existence of different forms of memory stems from this wealth of clinical work and fundamental studies. Often viewed as the most sophisticated, episodic memory is characterized by the capacity to re-experience a past personal event, situation or experience in the context in which it originally occurred [11]. A characteristic feature of episodic memory resides in the ability to bind together various interrelated stimuli and their spatial, temporal and conceptual relationships, to build up coherent memory representations [12]. Unfortunately, episodic memory shows the largest degree of decline in age-related cognitive impairments such as in AD [1] or even in several psychiatric contexts, such as MDD [3]. This review will mainly focus on episodic memory impairments and on the key related brain structure, namely the hippocampus.

2.1. The Hippocampal Formation

Lying deep in the medial temporal lobe (MTL), the hippocampus sits at the top of a hierarchy of cortical systems in which later stages integrate information from previous ones. This allows it to build complex representations and to influence earlier stages of operations through back projections—the proper definition of episodic memory [11]. Such consideration fuels the broad consensus that the hippocampus and surrounding MTL structures play a critical role in the encoding and subsequent retrieval of new long-term episodic memories.

A turning point in cognitive neurosciences came from patient case studies with hippocampal damage. One of the most famous examples comes from the post-surgery follow-up of patient H.M. (Henry Molaison) that enabled the role played by the hippocampus in episodic memory to be highlighted [13]. Following these clinical observations, several animal models with lesions of distinct brain structures, notably the hippocampus, were developed. First in rodents [14,15] and then in a non-human primate species [16], all models highlighted the hippocampus as having a core role in memory function. Since then, the sheer number of studies performed in experimental models of amnesia has demonstrated the role of the hippocampus in episodic memory [17–19] and demonstrated its anatomic-functional specialization. Thus, the ventral (or anterior) and the dorsal (or posterior) part of the hippocampus (in rodents and primate, respectively) differ markedly in their afferences/efferences and consequently in their dedicated role [20]. The ventral hippocampus (VH) has robust efferent connections to the rostral hypothalamus and amygdala and is mostly involved in the emotional components of memory processes [21]. Hence the ventral part of the hippocampus attracts much of the work on memory impairment related to psychiatric disorders, such as anxiety-induced depression and post-traumatic stress disorder. Conversely, the dorsal hippocampus (DH) is mainly involved in spatial memory processing [22], with outputs primarily projecting to the dorsal lateral septum and the

mammillary body [20]. Further, the discovery of place cells (within the CA1) [23]—which activate specifically when a person is in a precise location (spatial information)—reinforced the theory of an anatomic-functional segregation within the hippocampus.

2.2. The Hippocampal Formation Circuitry

Composed of three cyto-architectonically distinct regions, i.e., the dentate gyrus (DG), the subiculum and the cornus ammonis (CA) with its three subfields (CA1, CA2 and CA3), the hippocampal formation forms a trisynaptic loop. The entorhinal cortex (EC) is the major source of both input and output of information within the hippocampus [24].

Before being projected into the hippocampal formation through the EC, information may arise either from the parahippocampal gyrus or the perirhinal cortex, respectively encoding spatial and object representations. Mostly concentrated within the superficial layers (II-III) of the EC, this flow of information can reach the pyramidal neurons of the CA1 area by two distinct pathways. Indeed, the apical shafts of the CA1 area can be reached either directly (1/6 synapses) thus constituting the perforant path (PP), or using a tri-synaptic pathway, i.e., first passing by the DG, then the CA3 (through mossy fiber projections, MF), to finally reach CA1 through the Schaffer collateral pathway (SC). Finally, CA1 pyramidal neurons send their axons to the subiculum which flows information out to the EC, within its deep layers (V-VI) [24] (Figure 1A).

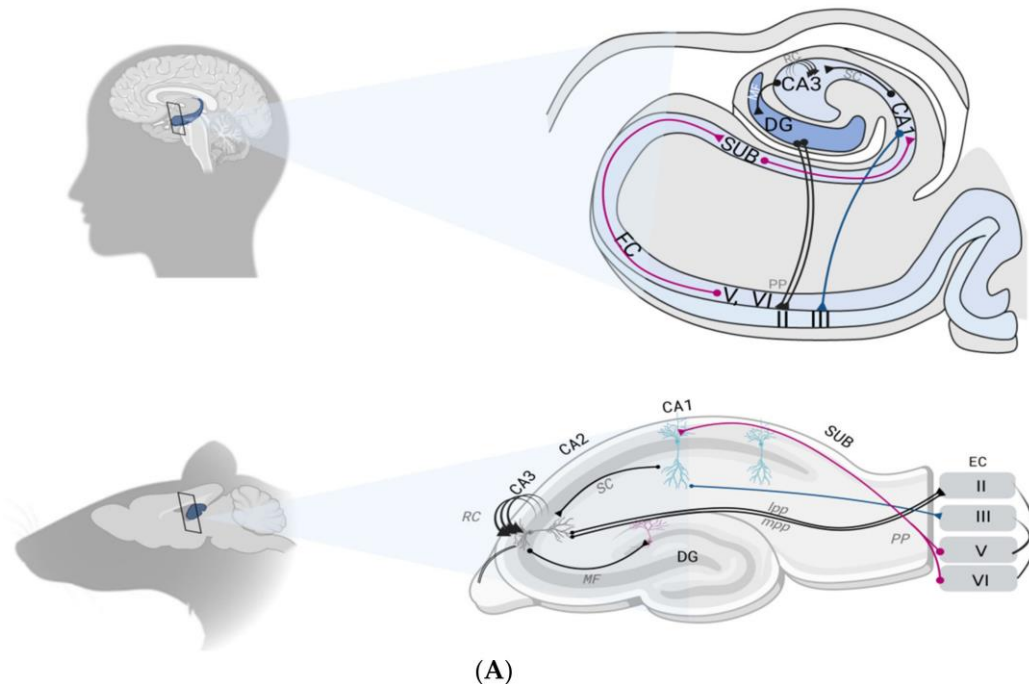


Figure 1. Cont.

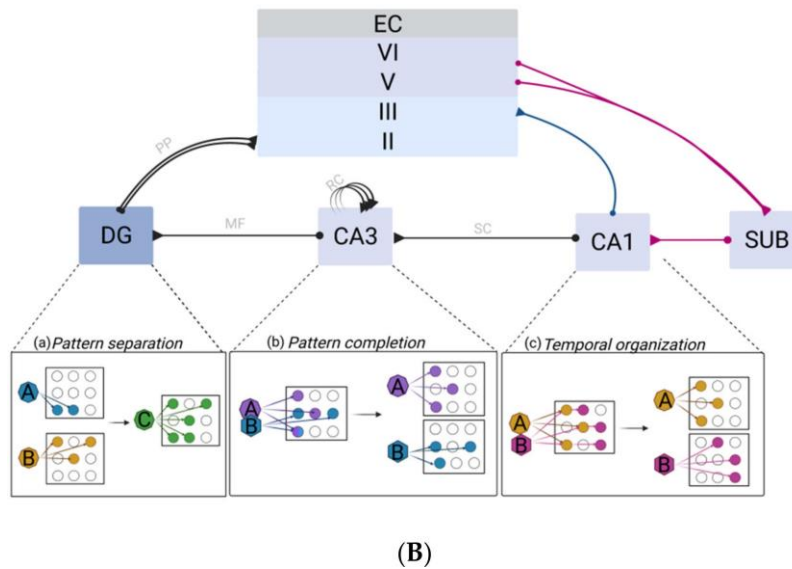


Figure 1. (A) Schematic representation of the location of hippocampal formation in both humans and rodents (left). Circuitry organization of the hippocampal formation in both species is depicted (right). Main inputs to the hippocampus are provided by superficial layers of the EC. Inputs converge to the CA1 through both the tri-synaptic pathway (DG, CA3 and CA1) and monosynaptic pathway, directly to the CA1 through the layer II of the EC. Recurrent collaterals (RC) of the CA3 contact other CA3 neurons and form the auto-associative network. The CA1 connection with the subiculum provides the main hippocampal outflow back to the deep layers of the EC (adapted from Small et al. 2011). (B). Representation of the functional specialization of each hippocampal subfield. The DG–CA3 axis is assigned to pattern separation (a), a function allowing it to disambiguate sensory inputs from similar experiences. Two similar inputs (A) and (B) are thus represented as two non-overlapping inputs. The pattern-separated signals from the DG are then projected onto the CA3 via the mossy fibers (MF) pathway. The CA3 is specialized in pattern completion (b), a process by which a partial or degraded subset (A) and (B) of the initial input can re-activate the retrieval of the whole context through a generalization process (C). The CA1 performs temporal organization of sequentially activated place cells (c). During spatial navigation, temporally close events (A→B) activate place cells in sequences that are then played out separately on a compressed time scale as a specific theta sequence (A/B). Abbreviations: CA1, CA3: cornus ammonis 1,3; DG: dentate gyrus; EC: entorhinal cortex; lpp, lateral perforant path; MF: mossy fibers; mpp, medial perforant path; PP, perforant path; SC, schaffer collateral pathway; RC: recurrent collaterals; SUB: subiculum.

2.2.1. The Dentate Gyrus–CA3 Pathway: Pattern Separation and Completion

The DG is renowned for its crucial role in the non-overlapping encoding of episodes presenting a high degree of similarity to limit interferences through a process known as pattern separation (PS) [24]. Compared with other hippocampal subfields, DG is more prone to representing highly similar scenes in a distinct fashion (Figure 1B). Therefore, damage to the DG leads to PS capacity impairment, both in humans and in animal models [25,26]. Hence, the DG acts as a competitive learning network precluding redundancy, where only the most relevant input patterns are selected among the continuous flow of information from diverse origins arising from the EC. Sparse recoding of EC inputs is achieved by keeping a low proportion (1 to 2%) of active DG excitatory granule cells (GC). This first selection stage is enabled by three main characteristics of the DG: the DG exhibits the highest densities of GABAergic interneurons compared with other subfields, thus providing strong inhibition to GC; GC have a low firing rate; and the GC receive outnumbered projections from the EC (from ~110,000 EC fibers, GC receive ~1.2 million inputs in each rat hippocampal hemisphere) [24].

The pattern-separated signals from DG are then projected onto the CA3 via the MF pathway, which constitutes the second selection stage. Indeed, MF synapses exhibit sparse but powerful connection to the CA3. Each CA3 cell receives ~50 MF inputs [24]. Such a method of projection favors a randomizing effect, since a set of neurons will be active

for a unique event, leading thus to very different representations in the CA3 even for two highly similar events. This diluted connectivity substantially contributes to the final orthogonalization of the information which is essential to PS [24].

PS is fundamental, and any impairment of this capacity ultimately hampers the holistic retrieval of multidimensional episodes: the pattern completion. The aim of pattern completion is to enable the recall of a whole memory from partial cues (Figure 1B). This function mainly relies on the hippocampal CA3 network [27]. In fact, the unique presence of recurrent axon collaterals (EC) in CA3 neurons accounts for the highest number of synapses observed in CA3 pyramidal cells' dendrites. The CA3 area is viewed as a single operating network (auto-associator), which allows arbitrary associations between inputs [24,27]. Subsequently, an event is represented by a set of concurrent neuronal firing, by which each feature can be re-activated by RC during recall [24,28].

Another particularity conferred by the RC to the CA3 region is its ability to act as an auto-attractor [24,27]. An auto-attractor maintains the steady firing of a set of neurons, which were first selectively activated during a specific task, particularly a spatial task. This function is fundamental since the probability of overlapping two events in a same space location is high. Further, the high plasticity of the CA3 area within a very short timing window is likely to allow rapid, "on the fly" encoding of information, thus facilitating associations between any spatial location [24,28].

2.2.2. Multifaceted Roles of the CA1

Considered as the primary output of the hippocampus, the CA1 area performs accurate representation of a whole context, resulting from integrative computation between its two inputs [29]. This is supported by the generally assumed fact that direct input from EC is not sufficient to trigger an action potential in the CA1 by itself, but requires concurrent CA3 input. In line with a set of evidence regarding selective damage to the CA1, the discovery of sequence cells broadens the spectrum of functions that are assigned to this region with a role in the temporal aspects of memory [30] (Figure 1B). Accordingly, the double set of CA1 afferent would then allow CA1 cells to compare incoming information—corresponding to the currently occurring event—from the lateral EC, with information arising from the CA3 which represents familiar information [24,29]. In addition, back projections from the CA1 to the neocortex support the role of the CA1 in the memory consolidation process. Indeed, plasticity of CA3–CA1 synapses allows the whole episode encoded in CA3 to be represented in CA1 in longer term types of memory, than CA3. The CA1 can ensure an efficient recall by acting as a recorder of the recall activity of CA3 from a partial cue [29].

2.3. Synaptic Plasticity as a Correlate of Hippocampal Memory

The unraveling of mechanisms by which the hippocampus encodes and stores information has a long history. Efforts made over the past decades of research on this topic have progressively lead to the now widely accepted synaptic plasticity and memory hypothesis: *"activity-dependent synaptic plasticity is induced at appropriate synapses during memory formation, and is both necessary and sufficient for the encoding and trace storage of the type of memory mediated by the brain area in which that plasticity is observed"* [31]. Indeed, a defining characteristic of the hippocampus is this incredible ability to undergo activity-dependent functional and morphological remodeling via plasticity mechanisms. Over a century ago, Ramón y Cajal raised the idea that the dynamics of neural circuits (i.e., the changes in the efficacy of synapse transmission) would serve memory function. He was the first to propose the cellular theory of memory storage as an anatomical change in synaptic functional connections. This foreshadowed the Hebbian theory *"cells that fire together wire together"* that led to the assumption that associative memories are formed by synaptic plasticity, driven by temporal contiguity of pre- and post-synaptic activity [32]. This appealing cellular basis for learning and memory was further supported by the discovery of long lasting potentiation of synaptic strength, now known as long term potentiation (LTP). The characteristics of LTP

(cooperativity, associativity, input specificity, as well as its durability) serve as non-trivial explanations for the great capacity, rapid acquisition and stability of memory [33].

Bliss and Lomo were the first to demonstrate the existence of LTP in the hippocampus following brief trains of high-frequency stimulation (HFS-100 Hz) [34]. Following this pioneering work, thousands of papers have been published on *ex vivo* hippocampal LTP, using different sets of stimulating protocols [35], such as theta-burst stimulation (TBS-5 Hz). As a matter of fact, the potentiation effects have a deep relationship with rhythmic bursts of activity that mimic naturally occurring brain oscillations [36]. Respectively described as gamma- γ - (30–100 Hz) rhythms for HFS and theta- θ - (4–12 Hz) rhythms for TBS, these oscillatory frequencies are observed during spatial and contextual learning [23,37,38]. Importantly, phase-amplitude coupling between theta and gamma oscillations has been reported across species, including mice, rats, and humans. Additionally, this phase-amplitude coupling is known to play a critical role in hippocampus-dependent memory processes [39].

Further, performance in hippocampal-dependent memory tasks has been associated with changes in LTP [40,41]. Inhibitors of hippocampal LTP were found to block both learning and retention when assessed in spatial memory tasks [40,41]. Additionally, several biochemical changes that occur after induction of LTP also arise during memory acquisition [42]. Since then, LTP has become a prototypical experimental model for the assessment of basic mechanisms involved in learning and episodic-like memory formation [35].

The induction of hippocampal LTP—in almost all of its subfields—is dependent on NMDA-Rs (with the exception of the MF-CA3 which can also display a form of LTP independent of NMDA-Rs) [42]. Therefore, the critical event leading to induction of LTP is the influx of calcium ions into the postsynaptic spine upon NMDA-Rs activation. Subsequent to calcium entry is the increase in calmodulin kinase II (CaMKII) activity that contributes to enhanced AMPA conductance and new addressing to the membrane. In addition, two other major pathways that involve different protein kinases, cyclic adenosine-monophosphate (cAMP)-dependent protein kinase (PKA) and extracellular regulated kinase (ERK), have also been identified as triggered by NMDA-Rs activation [42]. Downstream extracellular signals, including brain-derived neurotrophic factor (BDNF) have further been proposed to support the long lasting changes in synaptic function [42,43].

Nevertheless, hippocampal synaptic plasticity does not resume to LTP. Depotentialization (DP)—the reversal of LTP—and long term depression (LTD), which denotes the weakening of synapses, were also described in the hippocampus [43]. Both are necessary to specific forms of memory—also termed flexibility—that requires extinction of the obsolete memory traces, such as in the novelty recognition task [44]. These two synaptic plasticity processes are induced by low-frequency stimulation (LFS-1 Hz), which ranges around the hippocampal delta frequency band (0.5–4 Hz). Otherwise, both synaptic plasticity processes seem to rely upon similar mechanisms to LTP at a molecular level [45].

2.4. Neurotransmission Systems in the Hippocampus

Cellular events supporting learning and memory are the result of complex interactions between various neurotransmission systems. Most knowledge regarding these processes stems from the observation of the dysfunction of these systems in pathological conditions or from experiences of pharmacological manipulation [42,46]. The neurotransmitters and neuromodulators systems involved in hippocampal memory function are incontestably numerous. Therefore, this discussion is limited to those having a key pivotal role and/or having demonstrated a strong relationship with hippocampal serotonergic function [47], and more specifically with 5-HT₄Rs, which are the core of this review.

2.4.1. The Glutamatergic System

The excitatory amino acid glutamate is the most abundant amino acid transmitter in CNS and is largely involved in learning and memory. The hippocampus is comprised of 90% of glutamatergic cells and hence is enriched in glutamate receptors, mainly AMPA receptors (AMPA) (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor)

and NMDA receptors (NMDARs) [48]. The major role played by these two ionotropic receptors, as well as by metabotropic receptors (mGluRs) [42], in synaptic plasticity (LTP process) and thus in memory formation, is widely accepted.

In rodents, pharmacological disruption of glutamatergic-mediated neurotransmission is accompanied by memory deficits in hippocampal dependent tasks (such as Morris water maze (MWM), passive avoidance, and radial maze tasks). In contrast, activation of glutamatergic transmission was related to improved memory performance [46]. Furthermore, the hippocampal atrophy in cognitively impaired patients, as well as the observed compensatory NMDARs' over-activation (leading to excitotoxicity) also contributed to ascribing the glutamatergic system at the core of cognitive processes. Based on these observations, NMDARs antagonist-based therapies were proposed as an interesting strategy in AD (Memantine, MEM) [49] and MDD (Ketamine) [50]. However, despite its promising beneficial effects in preclinical studies, MEM showed poor clinical efficacy [49]. Of most interest, 5-HT₄Rs have been shown to be expressed on glutamatergic neurons and consequently could be a target to modulate the glutamatergic system [51]. Hence, this constitutes an interesting avenue of research for the treatment of memory disorders.

2.4.2. The GABAergic System

Memory function homeostasis relies on an intricate balance between excitatory and inhibitory transmission. γ -Aminobutyric acid (GABA) represents the major inhibitory neurotransmitter of the CNS. Although hippocampal GABAergic interneurons account for only 10 to 15% of the total neuron population, their large anatomical and functional diversity across all subfields of the hippocampus allows a powerful regulation of cellular and network activity [52]. Indeed, hippocampal GABAergic inputs arise from the medial septum (MS) and specifically innervate hippocampal GABAergic interneurons. Back projections to the MS form a reciprocal loop, which is considered to play a critical role in the generation of hippocampal rhythmic activity. Therefore, GABAergic interneurons strictly regulate both spatial and temporal extents of hippocampal activity, under a synchronized activity at theta frequency of neuronal populations. Moreover, both *in vivo* and *in vitro* studies have also underlined a role of GABAergic interneurons in driving gamma oscillations [52]. Finally, the bursts of population of pyramidal cells that occur during slow-wave sleep in sharp-wave ripples appeared related to an increase in DG interneurons firing [52].

Whether memory improvements are supported by blockade or activation of GABA-receptors (GABARs) is often controversial, GABAergic neurotransmission clearly appears to be involved in memory function. The main source of these discrepancies may be due to the type of GABARs which are targeted. GABA_ARs are ionotropic receptors, permeable to chloride and mediate fast tonic inhibition on post-synaptic sites. Their activation is associated with altered memory performance [53]. Conversely, metabotropic GABA_BRs that are preferentially located on post-synaptic terminals, mediate slow phasic inhibition [52]. Their blockade may have beneficial effects on memory [53] and can modulate LTP [54].

Interestingly, application of the 5-HT₄Rs agonist BIMU-8 was found to stimulate GABA release in guinea pig hippocampal slices [55,56] (Table 1, Figure 2). Additionally, through different conditioning protocols of LTP induction, the authors of this current study recently demonstrated an interplay between 5-HT₄Rs activation and GABAergic neurotransmission within the hippocampal CA1 area [57]. This reinforces the interest of 5-HT₄Rs as a modulatory target to treat memory disorders.

Table 1. Summary of pathological drivers of hippocampal atrophy contributing to memory impairment in AD pathology and beneficial effects of 5-HT₄Rs ligands. Abbreviations: Dose/Con.: dose/concentration; mg/kg.d: mg/kg per day; ↑ denotes an increase; ↓ denotes a decrease; Aβ: beta-amyloid peptide; Ach: acetylcholine; AD: Alzheimer's disease; APP: amyloid precursor protein; APP cleaving enzyme 1; Bace-1: beta-site; cAMP: cyclic adenosine monophosphate; CREB: cAMP response element-binding protein; EC: entorhinal cortex; GABA: gamma-aminobutyric acid; icv: intracerebroventricular; IL-1β: interleukin 1 beta; IPSPs: inhibitory postsynaptic potentials; MCP-1: monocyte chemoattractant protein-1; MMP-9: matrix metalloproteinase 9; NA: not applicable; pCREB: phosphorylated cAMP response element-binding protein; PD: Parkinson disease; sAPPα: soluble alpha-amyloid precursor protein; 5-HIAA: 5-hydroxyindoleacetic acid; 5-HT: serotonin; 5-HTR: serotonin receptor.

Alteration	Contributing Factor	5-HT ₄ Rs Agonist	Dose/Con.	Treatment Duration	Preclinical Model	Target Brain Disease	Outcome of 5-HT ₄ Rs Activation	References
Hippocampal volume loss	Aβ-mediated cell death (Dysfunction in APP metabolism)	VRX-0311	0.1 nM–10 μM	30 min	CHO cells stably expressing the human 5-HT _{4(e)} receptor and APP695	AD	Concentration-dependent ↑ sAPPα	Mohler et al. 2007
		Prucalopride	1 μM	2 h	HEK-293 expressing SEAP-tagged APP and 5-HT ₄ Rs	AD	↑ sAPPα secretion (50%) through stimulation of α-secretase	Cochet et al. 2013
		Prucalopride	1 μM	30 min	CHO cell line expressing sAPPα and 5-HT ₄ Rs	AD	↑ sAPPα secretion	Lezoualc'h and Robert, 2003 Robert et al. 2001
		SSP-002392	5 mg/kg	26–37 days	APP/PS1 mice (4–5 and 12 months)	AD	↓ soluble and insoluble hippocampal Aβ40 and Aβ42 ↓ total number of Aβ deposits in mice aged 4–5 months ↓ Bace-1, Adam17 (50%) and Nicastrin ↑ astrogliosis and microgliosis (Aβ degradation)	Tesseur et al. 2013
			10 nM		SH-SY5Y human neuroblastoma cell line	NA	↑ sAPPα release	
		RS67333	2 mg/kg		APP/PS1 mice (7–8 months)	AD	No change in Aβ ↑ sAPPα production (102%, 265%, 343% of control at 8 h, 24 h, and 48 h respectively)	Hashimoto et al. 2012
		RS67333	3 μM	1 h, 2 h, 4 h, 8 h, 24 h and 48 h	H4/AβPP/5-HT ₄ cells	AD		
		RS67333	3 mg/kg	10 days	Female Tg2576 transgenic mice (10–12 months)	AD	↓ in Aβ load (30%)	
		RS67333	-	30 min	COS-7 cells transiently expressing 5-HT ₄ Rs and SEAP-APP	AD	↑ sAPPα release	Giannoni et al. 2013

Alteration	Contributing Factor	5-HT ₄ Rs Agonist	Dose/Con.	Treatment Duration	Preclinical Model	Target Brain Disease	Outcome of 5-HT ₄ Rs Activation	References
Network plasticity impairments	Neuro-inflammation	RS67333	1 mg/kg.d (twice a week)	3 months	5xFAD female mice	AD	↑ hippocampal sAPP α (1.5 fold) ↓ in A β load (hippocampus: 48%, EC: 55%) ↓ A β 42 levels in the insoluble and soluble fractions (33% and 53 % respectively) ↓ in A β load in EC (31–33%) Dose-dependent ↓ of A β levels	Baranger et al. 2017
		RS67333	1 mg/kg.d (twice a week)	3 months	5xFAD female mice	AD	90–95% depletion of both A β 40 and A β 42 at 30 μ M Protection from A β -mediated cell death (increase in neuronal survival) ↓ astroglial reactivity (61%)	Cho and Hu, 2007
		RS67333	1 mg/kg.d	2 weeks	5xFAD male mice (4 months)	NA	↓ pro-inflammatory mediators IL-1 β (25%) and MCP-1 30%) after 4 months' treatment	Baranger et al. 2017
	Synaptic loss and connectivity alterations	RS67333	1 mg/kg.d (twice a week)	3 months	5xFAD male mice	AD	↓ astrogliosis (49%) ↓ microgliosis (57%) Potentiates learning-induced spine growth (+6% relative to controls)	Giannoni et al. 2013
		SL65.0155	0.01 mg/kg	4 days	Adult C57BL/6j mice	NA	Boosts phosphorylation of cofilin (regulator of neuronal morphology and spinogenesis) Prompts dendritic spine maturation (increasing the number of active axo-spinous excitatory synapses in dendritic branches of principal neurons)	Restivo et al. 2008
	↓ plasticity-related proteins	BIMU-8	10 μ M	10 min	N1E-155 Neuroblastoma cells	NA	Boosts numbers of excitatory synapses ↑ cAMP levels (with stronger effect of Velusetrag)	Schill et al. 2020
		Prucalopride	1.5- 3 mg/kg	Single dose	(MPTP)-induced PD model mice	PD	↑ pCREB positive cells in DG	Ishii et al. 2019
		Velusetrag	3 mg/kg	3 mg/kg	Adult male Sprague-Dawley rats	NA	↑ pCREB/CREB ratio	Pascual-Brazo et al. 2011
		SSP-002392	0.0001 -1 mmol/L	0.01–1 mmol/L	SH-SY5Y human neuroblastoma cells	NA	↑ cAMP production (with stronger effect of SSP-002392) Ach-dependent increase in electrically-evoked GABA release at low concentration (0.2–0.4 μ M)	Tesseur et al. 2013
		BIMU-8	0.2–2 μ M	45 min	Guinea pig hippocampal slices	NA	Ach-dependent inhibition of electrically-evoked GABA release at higher concentration (0.7–2 μ M)	Bianchi et al. 2002
	Inhibitory vs. excitatory imbalance	Zacopride	10 μ M	5 min	Guinea pig hippocampal slices	NA	↑ IPSPs	Bijak and Misgeld, 1997
		VRX-03011	1–5 mg/kg	Single dose	Adult male Long Evans rats	NA	↑ Ach outflow under mnemonic demand	Mohler et al. 2007

Table 1. Cont.

Alteration	Contributing Factor	5-HT ₄ Rs Agonist	Dose/Con.	Treatment Duration	Preclinical Model	Target Brain Disease	Outcome of 5-HT ₄ Rs Activation	References
		<i>Renzapride</i>	1 mg/kg (systemic) 100 μM (icv)	Single dose	Adult female Wistar rats	NA	Concentration-dependent ↑ hippocampal 5-HT levels (200%)	Ge and Barnes 1996
		<i>RS67333</i>	1.5 mg/kg.d	3 days	Adult male Sprague-Dawley rats	NA	↑ 5-HT levels (73%) ↓ 5-HIAA levels (27%) (no effect of 5-HT ₄ Rs agonist in acute conditions)	Licht et al. 2010
		<i>BIMU-8</i>	0.2–4 μM	5 min	Guinea pig hippocampal slices	NA	↑ Ach outflow after electrical stimulation (but not at rest)	Siniscalchi et al. 1999

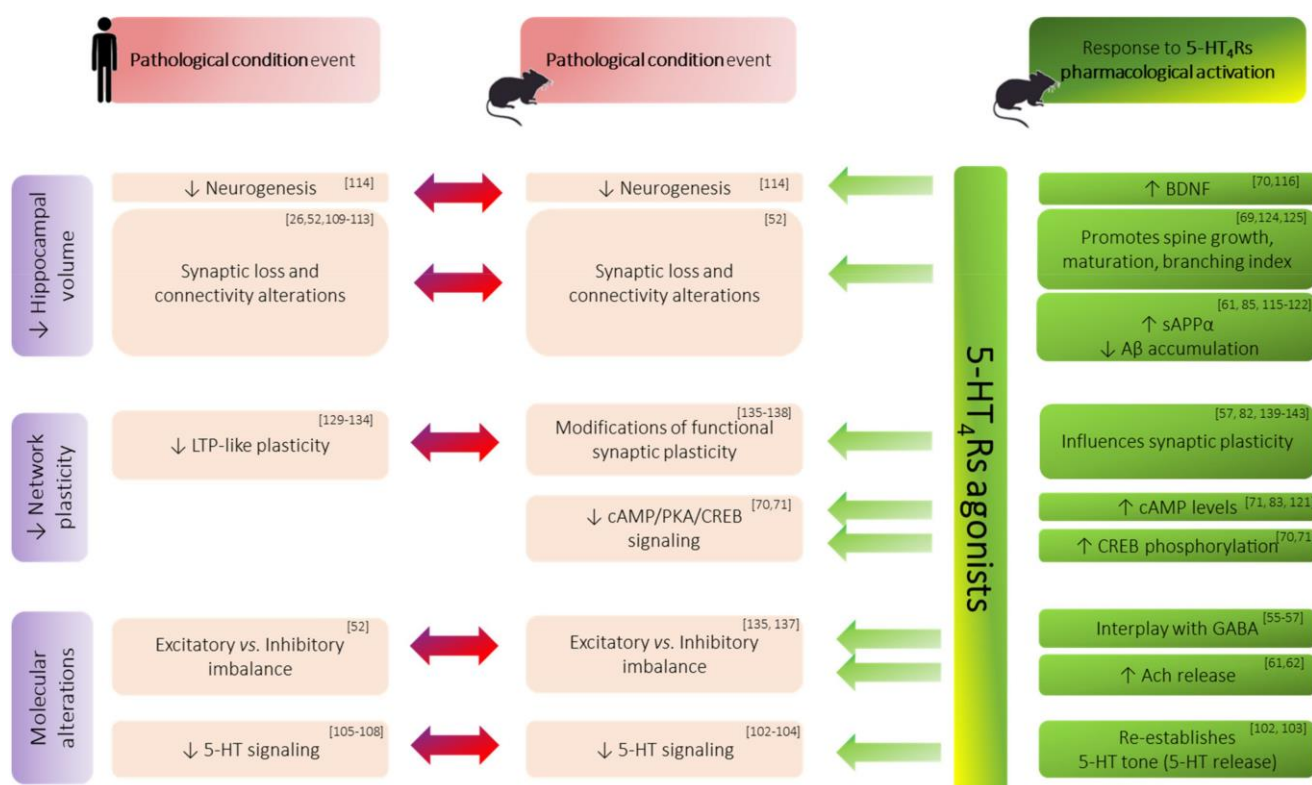


Figure 2. Summary of major hippocampal alterations (purple boxes) associated with memory impairments in both human and animal models of amnesic condition (red boxes). The beneficial effects of 5-HT₄Rs pharmacological activation are represented at each level of alteration (green boxes). ↑ denotes an increase; ↓ denotes a decrease. Abbreviations: Aβ: beta-amyloid peptide; Ach: acetylcholine; BDNF: brain derived neurotrophic factor; cAMP: cyclic adenosine monophosphate; CREB: cAMP response element-binding protein; GABA: gamma-aminobutyric acid; LTP: long-term potentiation; PKA: protein kinase A; sAPPα: soluble alpha-amyloid precursor protein; 5-HT: serotonin; 5-HTR: serotonin receptor.

2.4.3. The Cholinergic System

The hippocampus receives regulatory cholinergic inputs from the septal nuclei via the pre-commissural branch of the fornix. Cholinergic inputs are known to play an important role in hippocampal-dependent memory, either through nicotinic (ionotropic, mainly α7 sub-type) or metabotropic M1-M5 receptors [58].

Numerous humans and animal studies have linked acetylcholine (Ach) neurotransmission to learning and memory. Indeed, the increased release of hippocampal Ach during a memory task (notably spatial) was demonstrated to be positively correlated to improve-

ments of learning performance [59]. Additionally, the administration of muscarinic receptor antagonists (atropine, scopolamine) induced cognitive impairments. Scopolamine is even considered as a gold standard in preclinical research to identify potential anti-amnesic properties of drug candidates. Therefore, high expectations have been placed on this neurotransmission system in the search for new drugs to treat memory disorders. Hence, three of the four drugs in the market to date (galantamine, rivastigmine, donepezil) aim to increase Ach levels by inhibiting the enzyme responsible for its degradation (AChE) [6].

Of most interest, Ach inputs mostly contribute to pacing intra-hippocampal theta rhythm [58]. This activity rhythm is critical to memory since it favors cellular excitability through the suppression of various potassium currents. Additionally, the cholinergic system is particularly influential in its interaction with the neuro-modulatory serotonergic system [60]. In line with the scope of this review, it is worth mentioning that 5-HT₄Rs activation increases hippocampal outflow of Ach [61,62] (Table 1, Figure 2). First observed *ex vivo* in guinea pig hippocampal slices, this boosting effect of 5-HT₄Rs agonists on Ach release was also identified *in vivo* and was interestingly found to be specific to the memory process [61].

2.4.4. The Serotonergic System

Among monoaminergic systems, the serotonergic system is the most projected system in the brain. Mainly originating from dorsal and median raphe nuclei (DRN and MRN), serotonergic neurons send projections to the hippocampus [63]. All seven 5-HT receptors (5-HTRs) subfamilies are expressed in the hippocampus, each having a unique distribution pattern, although pattern overlapping is also observed [64,65].

The main supporting evidence for the involvement of 5-HT in memory function comes from observations of memory impairment after 5-HT depletion in human and animal model studies [66]. Numerous studies demonstrated the modulatory function exerted by the serotonergic system in memory function either in animal models or in human, both in physiological and in pathological aging condition [67].

As stated earlier, the identification of 5-HT₄Rs on hippocampal glutamatergic neurons [51] strongly supports an interplay between the serotonergic and the glutamatergic system that could undoubtedly benefit memory function. Hence, the serotonergic system appears to be central to memory function in that it has intimate interactions with both the two major neurotransmission systems and other neuromodulator systems [47].

3. Relevance of 5-HT₄Rs Modulation in Memory Disorders

5-HT₄Rs belong to excitatory G α s (stimulatory alpha subunit) protein-coupled receptors (GPCR). Their activation exerts a stimulatory effect through the activation of adenylate cyclase (ADC) as a primary mode of signal transduction on cAMP concentration. This second messenger interacts with various other proteins including PKA, which is known to modulate the activation of gene expression modifying transcription factors, such as the cAMP response element-binding protein (CREB) [68]. Additionally, an intriguing aspect of metabotropic 5-HTRs is their ability to elicit non-canonical pathways that can be G-protein independent. With regard to 5-HT₄Rs, their activation can initiate phosphorylation of their associated non-receptor tyrosine kinase Src, which activates mitogen-activated protein kinases (MAPK) including the extracellular signal-regulated kinases (ERK) [68,69]. Quite interestingly, these molecular actors also appear to be involved in LTP. Moreover, cAMP/signaling and BDNF expression were found to be disrupted in a number of animal models of neurological disorders [70,71] and found to be enhanced after 5-HT₄Rs activation [70,71] (Figure 2). Altogether, this raises the interest of 5-HT₄Rs-targeting in plasticity-related memory enhancement.

3.1. Insights from Animal Behavior Investigations

The idea that 5-HT₄Rs agonists are promising drug candidates for memory impairments—especially those related to hippocampal dysfunction—was firstly supported by behavioral stud-

ies on different animal models [72,73]. On one hand, cognitive impairments were often reported following antagonism (either pharmacologic agent or optogenetic construct) of 5-HT₄Rs [74]. Surprisingly, the genetic ablation of 5-HT₄Rs did not alter learning and memory capacities in mice. However, the deleterious effect of scopolamine (a cholinergic antagonist) on long term memory was enhanced in 5-HT₄Rs KO mice [75]. On the other hand, a very large number of preclinical studies reported consensual data supporting the beneficial effects of 5-HT₄Rs activation on memory performance. Overall, administration of 5-HT₄Rs agonists increased the learning rate in a hippocampus-dependent spatial task, such as the MWM [76] and the object recognition test [77–79]. 5-HT₄Rs agonists also restored memory impairments in animals treated with cholinergic antagonists [80–82], in aged animal [77,83] and in transgenic models of neurological diseases [84,85]. Additionally, it was recently reported that intra-hippocampal injection of a 5-HT₄Rs agonist reduced sleep deprivation-induced memory impairments [86]. These behavioral effects of 5-HT₄Rs modulation were extensively reviewed [73,87]. Likewise, chronic 5-HT₄Rs activation was found to counterbalance learning and memory deficits induced by stress-induced depression [3].

Additionally, 5-HT₄Rs have also been considered as an associative target of choice. Indeed, given the multidimensional and complex aspect of the pathogenesis of memory disorders, a new approach has emerged that consists of the simultaneous modulation of more than one target. After having proved the efficacy of 5-HT₄Rs stimulating activity in co-administration protocols with different AchE inhibitors [78,88], the first multi-target drug ligand (MTDL) associating both activities has been designed. Named as Donecopride, this drug candidate was mainly developed for application in the field of AD [89]. Indeed, these promising results argue for the development of other MTDLs combining 5-HT₄Rs agonistic activity with a different secondary target (other than AchE inhibitor) to be used for different medical application [90].

These observations constitute the first line of evidence for an interest in 5-HT₄Rs activation in disorders related to hippocampal dysfunction. However, a limitation of preclinical research has certainly been the lack of investigation of 5-HT₄Rs' functional and/or expression alteration in animal models that display memory deficits [91]. In order to clarify if 5-HT₄Rs changes are causative or involved in the etiology of diseases, their expression pattern needs to be assessed on a cellular level in preclinical models.

3.2. Distribution of 5-HT₄Rs in CNS and Memory Disorders

The distribution of 5-HT₄Rs within the brain is mainly restricted to the limbic system, thus intimately tied to memory function. The highest 5-HT₄Rs mRNA levels and densities are found in caudate, putamen, accumbens, and in the hippocampal formation [92–94]. Within the hippocampal formation, the highest expression is found in the granule cell layer of the DG, followed by the pyramidal cell layer of the CA. Further, 5-HT₄Rs exhibit a layered distribution within CA subfields, with the highest densities identified in the stratum oriens and stratum radiatum. This suggests a localization of receptors at both basal and apical dendritic fields of pyramidal cells. Radio-ligand assays also show strong labelling in the stratum lucidum of the CA3 area, probably reflecting the presence of 5-HT₄Rs on MF [95,96].

Ligand binding studies also help to reinforce the idea that 5-HT₄Rs play a pivotal role in memory function. In fact, the hippocampal density of 5-HT₄Rs was found to be inversely correlated with episodic memory test performance in healthy subjects [97]. Further, it has also been observed that a striking feature of aging is the dramatic decrease in 5-HT₄Rs density that occurs [93,98]. Likewise, the loss of 5-HT₄Rs expression was also observed in different cohorts of patients suffering from memory deficits [91,99] and was correlated with the stage of the disease. For instance, a post-mortem brain analysis in AD patients reported a 70% decrease in hippocampal 5-HT₄Rs [100], a change that was positively correlated to amyloid beta peptide load [98]. Additionally, reduced 5-HT₄Rs binding was observed in the hippocampus in an animal model of depression [101] (Figure 2).

Moreover, it has been proposed that improvement of memory performance in patients who suffer from memory disorders is supported by up-regulation of 5-HT₄Rs, which in turns stimulates hippocampal 5-HT release as shown in rodents [102,103] (Table 1, Figure 2). Indeed, there is now a large body of preclinical data showing a dynamic positive correlation between central 5-HT levels and 5-HT₄Rs densities. For instance, 5-HT₄Rs KO mice have diminished tissue levels of 5-HT (and its main metabolite, 5-HIAA) [104]. Hence, 5-HT₄Rs activation could enhance 5-HT global tone through the positive feedback loop projecting from the prefrontal cortex to the DRN and thus, to the hippocampus [91]. If so, this could account for the variation of 5-HT₄Rs expression observed in AD. Indeed, an upregulation of 5-HT₄Rs expression occurs at the pre-clinical stage of the disease and continues along with dementia progressing (up to mild stage), as if a compensatory strategy was put in place (in response to decrease in interstitial 5-HT levels), until exhaustion [98]. Indeed, the loss of serotonergic cells in AD patients can reach above 70% in the DRN and MRN [105] and can even be reduced to undetectable levels [106,107]. This ultimately contributes to a decrease in hippocampal 5-HT neurotransmission, which has been identified as a correlate of cognitive impairment [108] (Figure 2). Altogether, the changes in 5-HT₄Rs density may reflect the abnormal range of 5HT levels required for memory functioning. Hence, the clinical stage of the disease during which 5-HT₄Rs may be used appears critical.

3.3. Morphological/Structural Alterations of Hippocampal Formation in Memory Disorders

Although a host of brain changes are likely to be responsible for cognitive decline, structural and functional hippocampal alterations were identified as one major correlate. Therefore, magnetic resonance imaging (MRI) scan has become one of the most common markers associated with cognitive scales performed in aging studies or in clinical practice to measure brain disease burden [109]. Whilst hippocampal atrophy is an important imaging correlate of memory impairments observed in numerous brain disorders, its pattern of alteration may vary according to the disease and the stage of the disorder.

For instance, within hippocampal formation, the EC appears to be most resistant to the effects of normal aging, as changes are mainly restricted to the DG and CA3. In contrast, the EC is most vulnerable to AD while the DG and CA3 remain relatively preserved. With regard to the CA1 area and the subiculum, they are mainly affected in SCZ and MDD respectively. Unlike AD, no prominent cell loss has been identified in aging, SCZ and MDD, suggesting rather, functional alterations such as connectivity dysfunction [109]. Consistently, an MRI-based study using diffusion tensor imaging to detect dendritic integrity revealed age-related alterations of DG and CA3 dendrites in aged patients [26] (Figure 2). Nevertheless, the measure of hippocampal volume was found to be sensitive enough to aging and to neurodegenerative and psychiatric disorders. For instance, after the age of 70, total hippocampal volume is believed to decrease at a rate of ~1.5% a year [110]. Additionally, hippocampal volume loss has been shown to reach 10 to 15% in mild cognitive impaired (MCI) patients [110]. Patients suffering from schizophrenia, PD or depression also exhibit hippocampal volume reduction of 4–6% relative to healthy subjects [111–113].

Of most interest, several lines of evidence now support that 5-HT₄Rs agonists could limit such hippocampal deterioration at different levels, notably in AD context (see Table 1 for extensive details).

First, the above reported hippocampal volume loss—either due to aging or pathological condition—can be compensated, at least partly, through neurogenesis boost, which is altered in various neurological and psychiatric diseases [114]. However, it has been shown that sub-chronic treatment with 5-HT₄Rs agonists induced an increase in BDNF expression in the CA1 (72%) as well as in the DG (52%), this latter demonstrating a neuro-proliferative activity [70]. Further, increased levels of other neurotrophic factors have also been reported after 5-HT₄Rs agonist treatment, such as the soluble (non-amyloidogenic) form of the amyloid precursor protein alpha (sAPP α) (Table 1, Figure 2). The functions of sAPP α include—but are not limited to—proliferation, neuroprotection, synaptic plasticity, memory formation, neurogenesis and neuritogenesis in cell culture and animal models. Quite inter-

estingly, sAPP α production was found to be promoted following acute [115] and chronic 5-HT $_4$ Rs activation in various conditions that include cell lines overexpressing 5-HT $_4$ Rs (50% increase) [61,116–119] as well as neuroblastoma cell line [61], and cultured neurons from a mouse model of AD [85,120–122]. A similar effect was observed in vivo both in healthy mice (2-fold increase) [115] and in AD mice models (1.5-fold increase) [84,85]. In the context of AD, the effects of 5-HT $_4$ Rs activation on sAPP α production would confer an additional benefit through a reduction in amyloid load (31–55% in a mouse model of AD [84,85]) by limiting the amyloidogenic pathway. Indeed, accumulation of neurotoxic A β in key hippocampal regions appears to be the primary cause of neuronal death leading to hippocampal atrophy [123] (Table 1, Figure 2).

Second, additional data supporting the putative role of 5-HT $_4$ Rs in preserving hippocampal integrity come from studies focusing on dendritic spines hosting excitatory synapses. The latter are dynamic structures, whose formation, shape, volume and collapse depend on neural activity. Therefore, they influence (but also can in return be influenced) the learning processes and memory performance [18]. In mice, pharmacological activation of 5-HT $_4$ Rs was shown to selectively potentiate the learning-induced dendritic spines' growth (+6%) within the hippocampal CA1 (Table 1, Figure 2). This was not found in other brain structures that are not as much implicated in memory processing (i.e., primary visual cortex) [69]. Moreover, in a recent study using high resolution time lapse FRET imaging on neuronal dendrites, 5-HT $_4$ Rs activation was found to prompt maturation of synaptic connections via the 5-HT $_4$ R/G13/RhoA signaling cascade [124]. By activating PKA and BDNF/TrkB signaling pathways, 5-HT $_4$ Rs activation also promoted total dendritic length, number of primary dendrites and branching index in vitro [125]. Since spines represent potential sites of postsynaptic excitatory input, boosting their growth and maturation may translate into an increase in the number of excitatory synapses.

Finally, it is worth mentioning that reactive astrocytes are found both in human AD patients and AD mice models. Post-mortem morphological brain studies demonstrate close interaction between astrocytes and A β deposition in AD patients. In fact, reactive astrocytes are thought to be involved in A β production by upregulating β -secretase activity and APP in the diseased brain [126]. In this way, any strategy that would participate in a reduction in astrogliosis may substantially contribute to a reduction in A β load and subsequent neuronal loss. IL-1 β and MCP-1 are two key pro-inflammatory mediators involved in glial reactivity whose levels have been found to be reduced by 30% to 45% following chronic 5-HT $_4$ Rs activation in an early onset mouse model of AD [84] (Table 1). Consequently, astrogliosis and microgliosis were reduced by 50–60% and 57% respectively in the EC, an area of the hippocampal formation that is particularly susceptible to degeneration in AD, as previously discussed [84,85]. Of note, astrogliosis reduction was even more pronounced with a longer duration of 5-HT $_4$ Rs agonist treatment [85]. Hence, 5-HT $_4$ Rs modulation could modify AD pathogenesis by targeting inflammatory pathways in glial cells.

The demonstration of such beneficial effects of 5-HT $_4$ Rs ligands holds promise for the development of disease-modifying drugs, which represents a yet unmet medical need. Of course, upstream correction of the pathological drivers of the disease is crucial to significantly improving the downstream symptoms and to prevent progressive cognitive deterioration. To date, preclinical studies that showed beneficial effects of 5-HT $_4$ Rs on hippocampal function have been mainly performed in either non-pathological conditions or in experimental models of the disease (cell lines or animal models). However, it seems important to stress that the pathology of AD shares a number of hippocampal alterations with ageing, SCZ, MDD and PD as discussed above. This ultimately raises the hope for potential translation of such beneficial effects of 5-HT $_4$ Rs in a large number of brain diseases.

3.4. Functional Synaptic Plasticity Impairments

Considered as the cellular support of memory, LTP has received much attention in the search for a better understanding of the mechanisms involved in memory disorders. Veritably, impairment of hippocampal synaptic function is often considered as an early de-

tectable feature of aging and/or pathological stage, well before the first memory symptom appearance or before the observation of hippocampal atrophy.

Downregulation of plasticity-related proteins such as cAMP and CREB have, for instance, been observed in the hippocampus of both animal models of AD, and AD patients [127] (Table 1). In this regard, there is accumulating evidence for a beneficial action of 5-HT₄Rs agonists on cAMP/CREB signaling. Consistently, increases in both cAMP and CREB levels as well as the phosphorylated form (active form) of CREB (pCREB) were found both in healthy rats [70] and the neuroblastoma cell line [121] following 5-HT₄Rs activation.

Interestingly, such effects have been investigated for the first time in a mouse model of PD. In this study, the cAMP and pCREB levels were found to be increased in the DG following an acute treatment with 5-HT₄Rs agonist and correlated to facilitation of memory performance [71].

Additionally, recent technical developments using repetitive Transcranial Magnetic Stimulation (rTMS) and ElectroEncephaloGraphy (EEG), has enabled the non-invasive investigation of LTP in human cortical tissue [43,128]. Thus, a decrease in LTP-like plasticity was observed in various conditions, including aging [129], MDD [130,131], SCZ [132,133] and AD [134] (Figure 2). Although based on a cortical readout, the conclusions drawn are overall consistent with those coming from the deep electrophysiological recordings, conducted in different animal models. Indeed, despite a few discrepancies (mainly related to differences in protocols used, such as animal species, strain, sex, electric conditioning stimulation), most preclinical studies reported an impaired hippocampal synaptic plasticity [135–138]. Those alterations could be specific to the condition investigated (aging, model of neurodegenerative disease such as AD), and also of the hippocampal subfields targeted (DG, SC-CA1 pathway).

However, the effects of 5-HT₄Rs modulation on synaptic plasticity have been little studied, with only eight studies performed between 2001 and present, and results varying according to the hippocampal subfield investigated (Table 2).

Table 2. Compilation of electrophysiological investigations of synaptic plasticity in rodents after pharmacological 5-HT₄Rs activation. ↑ denotes an increase; ↓ denotes a decrease; = denotes no change. Abbreviations: CA1, CA3: cornus ammonis 1,3; DG: dentate gyrus; DP: depotentiation; HFS: high frequency stimulation; LTD: long term depression; LTP: long term potentiation; LFS: low frequency stimulation; SUB: subiculum; TBS: theta burst stimulation.

Method	Hippocampal Area	Plasticity	Conditioning Stimulus	5-HT ₄ Rs Agonist	Effects of 5-HT ₄ Rs Activation on Plasticity	Reference
In vivo	DG	LTP	HFS (200 Hz)	RS67333	↓	Kulla and Manahan-Vaughan
		LTP	HFS (200 Hz)	5-Methoxytryptamine	=	
		LTP	HFS (10 × 400 Hz)	RS67333	Transient ↑ and curtailed	Marchetti et al. 2004
		LTP	HFS (200 Hz)	RS67333	Curtailed	Twarkowski et al. 2016
	CA3	DP	LFS (5 Hz)	RS67333	Blocked	
		LTD	LFS (1 Hz)	RS67633	↓	
		LTP	HFS (4 × 100 Hz)	RS67333	↓	Twarkowski et al. 2016
		LTD	LFS (1 Hz)	RS67333	↓	Matsumoto et al. 2001
Ex vivo	CA1	LTP	HFS (5 × 400 Hz)	SC53116	↑	
		LTP	HFS (4 × 100 Hz)	RS67333	=	Kemp and Manahan-Vaughan 2005
	CA1	LTD	LFS (1 Hz)	RS67333	↓	
		LTP	HFS (1 × 100 Hz)	RS67333	=	Lecouflet et al. 2020
	SUB	LTP	TBS (4 × 5 Hz)	RS67333	↓	
		LTP	HFS (4 × 100 Hz)	RS67333	=	
		LTD	LFS (1 Hz)	RS67333	↑	Wawra et al. 2014

The sole study which investigated the subiculum plasticity did not show any change of in vivo LTP following 5-HT₄Rs activation [139]. Conversely, regarding DG and CA3 plasticity, all three in vivo studies reported an impaired time-course of LTP, which returned to baseline levels after 5-HT₄Rs activation [140–142]. Finally, regarding hippocampal CA1 subfield, conflicting results were reported. Indeed, the first research group reported an enhanced in vivo LTP after intracerebroventricular (icv) injection of 5-HT₄Rs agonist [82]. Additionally, this enhancement of LTP magnitude was blocked either by 5-HT₄Rs antagonist or scopolamine. Conversely, the second research group did not observe any change of in vivo LTP. However, the icv administration of 5-HT₄Rs agonist fully blocked learning- induced depotentiation of LTP [143], therefore suggesting a role for 5-HT₄Rs in behavioral meta-plasticity. Interestingly, an electrophysiological experiment was recently conducted *ex vivo* on a hippocampal slice to investigate the effects of 5-HT₄Rs activation on synaptic plasticity [57]. Opposite results were again observed *ex vivo*, but here they were linked to the frequency stimulation used to induce LTP. Thus, LTP was found to be specifically impaired after θ -burst, but not γ -burst. Within the hippocampus, the interaction between γ and θ rhythmic activities is critical for memory formation and the two experimental protocols impact network activity differently. Indeed, contrary to γ -burst, θ -burst efficacy of induction mainly relies on fine regulation of GABAergic neurotransmission, through notably a disinhibition process mediated by GABA auto-receptors [36,144]. While strengthening the theory of a tight interplay between 5-HT₄Rs and the GABAergic system [55–57] these results argue in favor of targeting the 5-HT₄Rs to treat memory disorders. Indeed, altered GABA neurotransmission—and the corollary imbalance between excitatory and inhibitory neurotransmission—has been repeatedly reported in many memory–neural circuit disorders [52] (Figure 2).

Finally, it should be noted that 5-HT₄Rs agonists prevent persistent LTD in all subfields of hippocampal formation (CA3, DG and CA1) [80,143] with the exception of one study which reported an increase [139] (Table 2). In the same way, 5-HT₄Rs agonists prevent DP in the DG [142] (Table 2).

Overall, the aforementioned set of data seems difficult to reconcile with the conventional view that increased LTP amplitude correlates with improved memory. However, literature data conversely suggest that stimulation of 5-HT₄Rs may reset plasticity to a baseline level, rather than potentiate or reduce the synaptic strength. In the same way, it has been proposed that LTP decays may reflect a reset in hippocampal circuits back to a certain level, so that new information can be more effectively processed later [145]. This is based on the principle of homeostatic plasticity whereby network excitability is comprised of uncompensated LTP and LTD. Indeed, either insufficient or excessive synaptic plasticity prevents learning and memory formation [146]. The key feature of this popular model lies in synaptic gain adjustment: prolonged increase in activity downscales synapses to maintain an overall average firing rate, and vice versa [147].

In other words, the above discussed data support the fact that 5-HT₄Rs, through their modulatory effects on synaptic plasticity processes, will enable the hippocampus to ensure its filtering role of information during acquisition and more variable changes in the downstream areas. This perspective seems consistent with clinical data that suggest that an increased signal-to-noise ratio within the hippocampus improves the encoding accuracy, a function which is thought to be mainly supported by the DG where 5-HT₄Rs are most abundantly expressed [148]. Overall, this is based on the core idea that the hippocampus yields a limited storage space, where relevant information is temporarily stored. The storage process is then triggered whenever the environment configuration is significant [24,148]. Hence, at a network level, it is reasonable to think that it might be beneficial to reduce excitability in regions that are primarily involved in information filtering, such as DG-CA3, to make any new event more salient. Further, a higher activation level in the DG/CA3 hippocampal region was reported both in MCI patients and in animal models of age-related memory loss. Disruption of this hyperactivity by pharmacological manipulations was associated with an improvement in cognitive function [149]. Interestingly, most electro-

physiological studies consent on increased excitability of hippocampal pyramidal cells following 5-HT₄Rs activation. Indeed, activation of ADC also leads to potassium channel inhibition and subsequent reduction in neurons after hyperpolarization [73]. Hence, 5-HT₄Rs agonists were found to enhance population spike amplitude in CA1 hippocampal slices, both in healthy animals [150,151] and in a mouse model of AD [151]. Recent work also suggests a regulatory role of 5-HT₄Rs in GC cells' excitability [152]. At the cellular level, 5-HT₄Rs activation results in tonic depolarizing currents [124]. Taken altogether, 5-HT₄Rs activation beneficial effects would appear to be two-fold. First, it would preserve the excitatory/inhibitory (E/I) balance, through either direct or indirect modulation of GABAergic neurotransmission. Second, 5-HT₄Rs would transiently exert a boosting effect on synaptic efficiency, through a selective increase in neurons' ability to fire action potentials whenever the incoming input is strong enough to alleviate inhibition.

Moreover, according to the gating hypothesis which suggests that levels of activity are transferred thorough the hippocampus, a high degree of CA3 activation will provide strong inhibitory inputs to CA1. In contrast, small fluctuations in CA3 activity will not provide sufficient excitation to bring enough CA1 neurons above activation threshold [24]. This raises the important need of evaluating the effects of 5-HT₄Rs activation on synaptic plasticity within the different hippocampal subfields simultaneously.

3.5. 5-HT₄Rs in Clinical Trials

The patent applications relating to 5-HT₄Rs modulators were very recently the subject of a literature review [9]. Among the most promising 5-HT₄Rs agonists was SL65.0155 (also called Caperserod) developed by Sanofi-Aventis. Despite its encouraging results in the preclinical field [83,153], to the best of the authors' knowledge, this compound did not reach clinical trials (as it is not referenced in [ClinicalTrials.gov](https://clinicaltrials.gov) data base).

To date, four 5-HT₄Rs partial agonists have been tested in clinical trials to treat memory disorders. As such, VRX-03011 (also called PRX-03140) from Epix Pharmaceuticals (NCT00672945) [154] reached the milestone safety and proof of concept phase (phase 2b) of clinical trials for the treatment of AD. However, since then, no additional information appears on the clinical trials website and no original papers have been published, suggesting that research on this compound has been discontinued. In 2011, after demonstrating encouraging results in pre-clinical investigations, PF-04995274 (Pfizer, NCT03516604) reached phase 1 of clinical trials against cognitive impairment in AD, but has shown limited blood brain barrier permeation [9]. Quite recently, SUVN-D4010, a novel, potent, highly selective 5-HT₄Rs partial agonist intended for the treatment of cognitive disorders, was found to be safe and well tolerated in healthy human subjects, even in elderly population (Suven Life Sciences, NCT02575482 and NCT03031574). Lastly, the results published last year regarding prucalopride are also of high interest. Indeed, while already approved by the FDA in 2018 to treat chronic idiopathic constipation, prucalopride was investigated in a battery of cognitive tests related to hippocampal functions. In healthy human subjects, prucalopride showed beneficial effects on learning and memory performance (NCT03572790) [148] and is currently under investigation for its role in depression. Evidence for improved memory performance after 5-HT₄Rs activation in humans was extended by a very recent fMRI study. Following prucalopride intake, hippocampal activity during memory recall was significantly increased compared with volunteers receiving a placebo [155].

Overall, arguments to consider 5-HT₄Rs as a target of choice for the treatment of memory impairments mainly stem from preclinical evidence. In fact, only a few experiments were performed on humans. Therefore, beyond the encouraging results of preclinical studies, it is wise to be cautious when editing conclusions because the touchy step of "translation from bench to bedside" often holds many disappointments. The human and rodents' hippocampus display quite common structural anatomy and play a similar function in memory process. However, memory function (and so its integrity) relies on several distributed regions in the whole brain that conversely may display striking difference across species (notably cortical regions). For instance, in the model of hippocampal dis-

engagement of long-term memory [156] the hippocampus would be crucial for recent memory retrieval, while cortical areas would play a key role for remote memory retrieval.

4. Concluding Remarks and Future Perspectives

By spanning key aspects of hippocampal alterations that pave the way to decline in memory function, this review draws an original outline of the interest of 5-HT₄Rs targeting for memory impairment.

The use of 5-HT₄Rs ligands in the treatment of memory deficits is still an ongoing challenge but has long been—and still unfortunately is—restricted to AD and MDD. However, as highlighted in this review, a number of functional and morphological changes within the hippocampus are a common denominator of a broader range of both normal ageing and neurological diseases (such as PD, MDD, SCZ). A large amount of data from both animal models and humans have now reached a consensus on the fact that 5-HT₄Rs activation can attenuate some of these hippocampal alterations. This ultimately raises the exciting potential of restoring—or at least limiting—memory decline in these pathologies. Nevertheless, a deeper understanding of the mechanisms at work is still needed and would help further development. In this view, studies that investigate 5-HT₄Rs effects on hippocampal function in a more integrated view should provide substantial insights. This constitutes an interesting framework for the authors' current research that recently revealed a modulatory effect on HFS-induced LTP, measured *ex vivo* after *in vivo* administration of a 5-HT₄Rs agonist (unpublished data, [157]). These changes were accompanied with variations in the levels of the hippocampal neurotransmitter highly involved in memory function and associated synaptic plasticity.

Author Contributions: Conceptualization, writing—original draft preparation, C.M.R.; writing—review and editing, M.L. and T.F.; visualization, M.L. and T.F.; supervision, M.L. and T.F. Illustrations have been designed using BioRender. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments : This work was supported by the Convention Industrielle de Formation par la Recherche (CIFRE) fellowship from the Association Nationale de la Recherche et de la Technologie (ANRT). We gratefully thank Johnny Vo for reading the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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OBJECTIVES

Objectives

Early decline in EM is a core symptom of a number of neurodegenerative diseases such as AD and PD as well as psychiatric pathologies such as SCHIZ or MDD which strongly and negatively impact the quality of life. The hippocampus is critical for episodic memory and is highly vulnerable to alterations that characterize this wide range of brain diseases. This makes the targeting of the hippocampal function of most interest against associated memory disorders. Despite all the efforts that have been made, there remains an unmet need for the discovery of effective therapies against memory impairments. Over the last 30 years, studies tackling memory deficits are increasingly considering 5-HT₄Rs as a major therapeutic target. Indeed, the benefits of their activation on memory is now widely acknowledged, in both healthy humans and animals as well as in pathological animal models. However, mechanisms at work still remain elusive. While a number of studies have provided some cues on cellular and molecular mechanisms, there is still a lack of conciliation between all these hypotheses and a lack of direct link with behavior. This leaves with the feeling that all raised hypotheses would represent several pieces of the same big puzzle yet unassembled. An in-depth comprehension of the underlying mechanisms of action would allow to encourage their targeting as part of current developing therapeutic strategies against memory disorders such as Multi-Target Directed Ligands (MTDL).

Hence, the aim of this thesis was to investigate the mechanisms underpinning the beneficial effects of 5-HT₄Rs activation on hippocampal-dependent memory.

- **The first objective was to evaluate the effects of 5-HT₄Rs pharmacological activation on three hippocampal-dependent features of episodic memory.**

To this end, the effects of acute systemic administration of the 5-HT₄Rs agonist RS67333 were investigated in a LD task, a NOR test and a TOM test which are validated probes to respectively assess the “where”, “what”, and the “when” components of episodic-like memory in rodents. To gain in translatability to the clinics, the LD task was performed on touchscreen operant chambers following preliminary methodological development of the task at COMETE’s laboratory.

- **The second objective was to investigate the neurobiological correlates of the behavioral effects of 5-HT₄Rs activation at the hippocampal level.**

In fact, 5-HT₄Rs have been shown to exert modulatory effects on a number of cellular and molecular aspects related to behavioral memory performances, and notably hippocampal synaptic plasticity.

Since *in vivo* synaptic plasticity is governed by hippocampal oscillations that co-exist in different frequency bands, the effects of systemic administration of RS67333 on brain waves patterns were investigated by quantitative electroencephalography (qEEG).

Besides, for the first time, CA1 hippocampal synaptic plasticity was measured *ex vivo* following either direct bath application or acute systemic treatment with the 5-HT₄Rs at a dose which is acknowledged to have beneficial

effects on memory. *Ex vivo* electrophysiological recording technique on isolated hippocampal slices was chosen because it allows a precise control of the environment and limit the interactions with interconnected brains areas while preserving the integrity of the canonical tri-synaptic fibers pathway (Xiong et al., 2017). Up to date, the sole *ex vivo* study involving 5-HT₄Rs pharmacological stimulation was performed in the subiculum and reported unchanged LTP (Wawra et al., 2014).

Meanwhile, the molecular aspect was tackled through the quantification of hippocampal neurotransmitters following acute systemic treatment with RS67333 in the same conditions.

The use of such transversal approach allowed us to dissect the hippocampal function at different levels to understand to what extend and how, it could be involved in the beneficial effects of 5-HT₄Rs activation on memory.

Finally, within a methodological development framework, we aimed at developing telemetric EEG in mice as well as a set-up for extracellular recordings at Porsolt's laboratory to increase their capabilities in the domains of cognition and epilepsy for early stage drug development.

MATERIALS AND METHODS

Materials and methods

Animals

All the experiments were approved by the regional ethics committee (Comité d'Ethique Normandie en Matière d'Expérimentation Animale, CENOMEXA; agreement numbers: 21467 and 29543), in compliance with the European directive 2010/63/EU on the protection of animals used for scientific purposes.

Experiments were conducted on different mice strains (**Table 4**). Mice were housed in groups of 5-8 in standard polycarbonate cages (43x26x19 cm) with *ad libitum* access to food and water (except when specifically mentioned). They were maintained on 12:12 light/dark cycle (lights off at 7:00a.m) in controlled environment for temperature ($21 \pm 2^\circ\text{C}$) and humidity ($55\% \pm 10\%$). Mice were allowed to adapt to this environment for a minimum of 1 week prior experiments.

Table 4: Summary of mice strains used.

Experimental models :		
Strain	Breeder	Stock No.
NMRI (National Medical Research Institute)	Janvier Labs, Le Genest St Isle, France	N/A
C57BL/6Rj	Janvier Labs, Le Genest St Isle, France	N/A
B6J.Cg-Gad2 ^{tm2(cre)Zjh} /MwarJ (GAD2-IRES-Cre KI)	The Jackson Laboratory, USA	#028867
B6.Cg-Gt(ROSA)26Sor ^{tm40.1(CAG-aop3/EGFP)Hze} /J (Ai40D KI)	The Jackson Laboratory, USA	#021188

Abbreviations: **N/A** : Non Applicable, **GAD**: Glutamate decarboxylase; **KI**: Knock-in.



Figure 7: NMRI mouse

NMRI male mice aged of 3-5 months old were used for electrophysiology experiments of **Articles 1 and 2**. This outbred strain of mice is among the most widely outbred strain used in biomedical research (Jensen et al., 2016).



Figure 8: C57BL/6Rj mouse

C57BL/6Rj male mice aged of 3-6 months old were used for experiments of **Articles 2 and 3**. C57BL/6 is among the most widely inbred mice strain used in biomedical research (Jensen et al., 2016). In addition to being well characterized on a behavioral standpoint this strain yields a stable genetic background that offers a wide range of possibilities for the generation of transgenic mice.



Figure 9: *GAD2-CRE Ai40D transgenic mouse*

Optogenetic experiments were performed on a double transgenic mouse line (named as **GAD2-CRE-Ai40D**) obtained from two simple transgenic mice lines described in **Table 4**. These mice expressed a photo-sensitive protein **ArchT** in **GABAergic interneurons**.

They were used at the age of 10-13 months old without gender distinction. These mice were developed at the University Center of Biological Resources (CURB, Caen, France) by Cre/lox recombination (**Figure 10**). To this end, two transgenic mouse lines (Ai40D and GAD2-IRES-Cre) built on C57BL/6J genetic background were bred to obtain the first generation (F0) (**Table 4, Appendix A**). Matings were carried using continuous polygamous (harem) breeding system with one male bred to 2-5 females. After weaning (3-4 weeks after born) mice were housed in groups of 2-4 in standard polycarbonate cages (36 x20 x14 cm). From F2 generation (**Appendix A**), selection of mice carrying the genes of interests for further breeding and final testing was performed by genotyping (hair sampling). The protocol used follows that described by The Jackson Laboratory: Protocol 9943 for Separated PCR Assay (**for detailed method see Appendix B**).

Note: genetic abnormalities have been observed in F1 generation mice (absence of udder) leading to difficulties in maintaining F2 generation. We therefore resorted to pups' adoption with SWISS female whenever possible. Moreover, males from F2 generation had poor fertility rate. These technical issues severely impacted the total number of animals in the generation of interest.

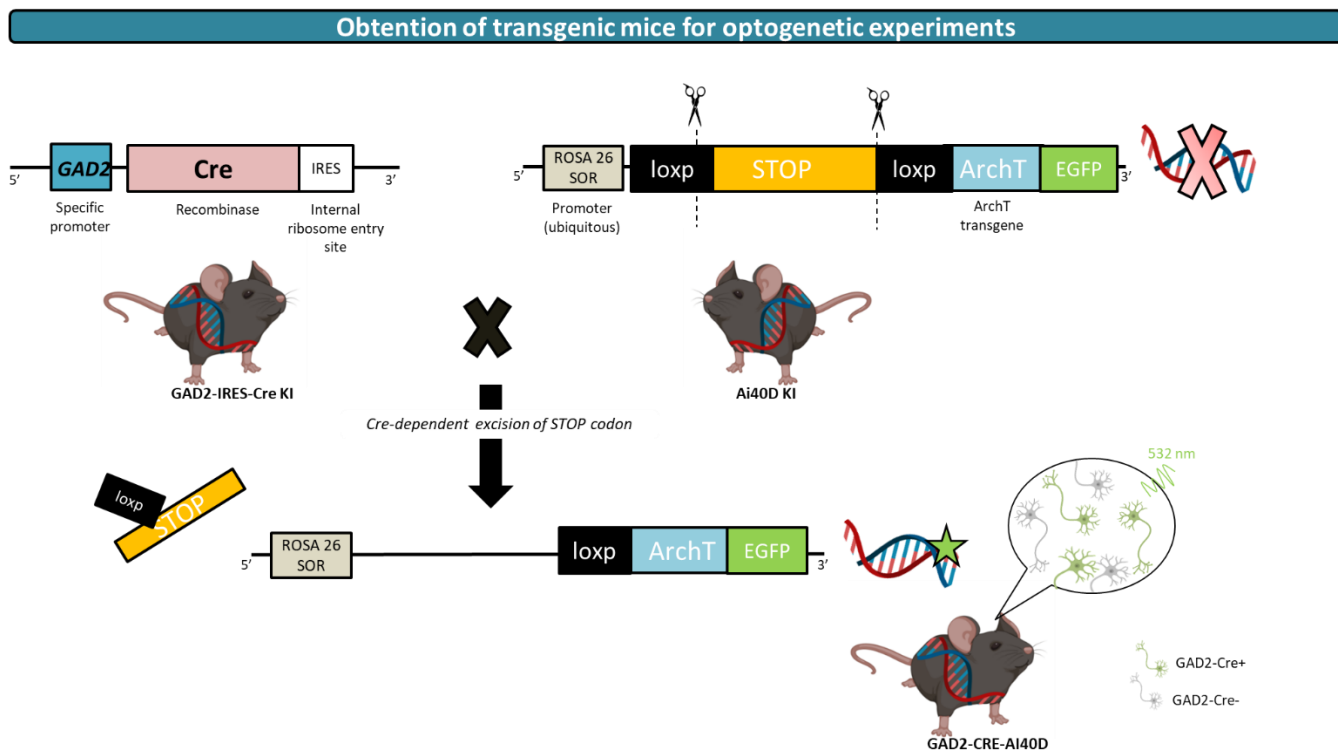


Figure 10: Principle of Cre-lox recombination for the the obtention of GAD2-CRE-Ai40D transgenic mice.

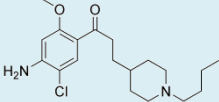
GAD2-IRES-Cre knock-in (KI) mice have Cre recombinase enzyme expression directed to glutamate decarboxylase 2 (GAD2) positive neurons. GAD2 encodes GAD65, which is present preferentially in presynaptic terminals for activity-dependent synthesis of vesicular GABA (γ -aminobutyric acid) (Pan 2012). This makes GAD2 a suitable pan GABA driver for Cre-dependent targeting of GABAergic interneurons (Taniguchi et al., 2011). On the other hand, Ai40D KI mice have the gene encoding for the photosensitive protein ArchT which expression is restricted to the presence of Cre recombinase enzyme.

Drugs

All experiments aimed at deciphering mechanisms underlying the pro-cognitive effects of **RS67333**. RS67333 is a **5-HT₄R partial agonist** among the most **affine (pKi=8.7)** and the most **selective**. Indeed, except for sigma receptors (σ R) for which it has an affinity at the micromolar range (pKi σ_1 R=8.9 and pKi σ_2 R=8.0), it displays low affinity for other receptors such as serotonergic, dopaminergic and muscarinic ones (Bockaert et al., 2004; R. Eglén, 1995). In addition, RS67333 displays **good brain penetration with logPe= -4.73 ± 0.02** (Pe represents the effective permeability coefficient determined by parallel artificial membrane permeability assay (PAMPA)).

Additional drugs and specific information are summarized in **Table 5**:

Table 5: Summary of the drugs used

Drugs						
Substance	Pharmacological activity	Supplier	CAS No.	Concentration (μM)	Perfusion duration (mins)	Reference
RS67333 HCl or (1-(4-Amino-5-chloro-2-methoxyphenyl)-3-(1-butyl-4-piperidinyl)-1-propanone)	5-HT ₄ Rs agonist 	Tocris Bioscience, Bristol, UK	168986-60-5	1 mg/kg (in vivo use) 1, 10 μM (ex vivo use)	i.p. – 30 mins (<i>in vivo</i> use) 15min minimum (<i>ex vivo</i> use)	(R. Eglen, 1995; Fontana et al., 1997).
RS39604 HCl 1-[4-Amino-5-chloro-2-(3,5-dimethoxyphenyl)methoxy]-3-[1-[2-methylsulphonylamino]ethyl]piperidin-4-yl]propan-1-one hydrochloride	5-HT ₄ Rs antagonist	Tocris Bioscience, Bristol, UK	167710-87-4	1 μM	15min minimum	(R. Eglen, 1995)
APV or 2-amino-5-phosphonovaleric acid	NMDA-R antagonist	Sigma Aldrich	79055-68-8	50 μM	15min minimum	(Latif-Hernandez et al., 2016)
NBQX disodium salt 2,3-dioxo-6-nitro-7-sulfamoyl-benzof[<i>f</i>]quinoxaline	AMPA-R antagonist	Sigma Aldrich	479347-86-9	10 μM	15min minimum	(Goldstein & Litwin, 1993)
Bicuculline methiodide	GABA _A -R antagonist	Sigma Aldrich	40709-69-1	10 μM	15min minimum	(Chapman et al., 1998)
CGP55845 HCl (2S)-3-[[[(1S)-1-(3,4-Dichlorophenyl)ethyl]amino]-2-hydroxypropyl](phenylmethyl)phosphinic acid hydrochloride	GABA _B -R antagonist	Sigma Aldrich	149184-22-5	1 μM	15min minimum	(Davies et al., 1993)
CGP 36216 HCl (3-Aminopropyl)(ethylphosphonic)phosphonic acid hydrchloride	GABA _B -R Antagonist (pre-synaptic)	Tocris Bioscience, Bristol, UK	1781834-71-6	100 μM	15min minimum	(Ong et al., 2001)
CGP35348 (3-Aminopropyl)(diethoxymethyl)phosphonic acid	GABA _B -R Antagonist (post-synaptic)	Tocris Bioscience, Bristol, UK	123690-79-9	200 μM	15min minimum	(Stäubli et al., 1999)

All pharmacological substances were dissolved in distilled water except RS39604 and CGP55845 which were dissolved in DMSO at a final concentration of 0.2% and 0.1% respectively.

Abbreviations: **AMPA-R**: α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; **Conc**: concentration; **GABA_{A/B}-R**, gamma-amino-butyric acid (A/B) receptor; **HCl**: Hydrochloride; **i.p.** : intraperitoneal; **NMDA-R**: N-methyl-D-aspartate receptor; **μM** : micromolar.

Electrophysiology

Extracellular recordings on acute hippocampal slices

Experimental set-up

The specific equipment used to perform extracellular recordings on mice hippocampal slices are listed in **Table 6**:

Table 6: List of the equipment required to perform extracellular recordings in rodent hippocampal slices.

Electrophysiology setup for extracellular recordings		
<i>Reagents or resources</i>	<i>Supplier</i>	<i>Reference/Model</i>
Binocular microscope	Olympus®	Sz30
Micromanipulator (stimulation electrode)	WPI®	MM301R
Tungsten stimulating electrode	Phymep®	UESMGSEKKNM
Isolated pulse stimulator	Digitimer Ltd®	DS2A
Micromanipulator (recording electrode)	WPI®	DC3001L
Borosilicate capillaries	Phymep®	GC-150F-10
Recording electrode headstage	NPIelectronics®	Ext02-B-MA
Electrode holder	NPIelectronics®	EH-01/PPH-OP-BNC
Pipette puller	Narishige®	PB7
Flow pump	Masterflex	77122-14
Extracellular amplifier/filter 1	NPIelectronics®	Ext02-B
Humbug	Questiscientific®	-
Amplifier/Filter 2	Tektronics®	AM502
Data acquisition card	National Instrument®	BNC-2110
Software	Anderson & Collingridge, University of Bristol, UK	WinLTP® version 2.30

The experimental set up for extracellular recordings comprises different electrical equipment that are interconnected (**Figure 11**). Central to the set-up is a **data acquisition card**, connected to the central unit of the computer. The data acquisition card is also connected to the **isolated stimulation unit** which allows the control of electrical stimulation delivery by the **software**. Neurons network depolarizations are collected by the **recording electrode** and processed through its headstage to a first **extracellular amplifier** which filters (band-pass: 0.1Hz- 500Hz) and amplifies (x10) the signal. The signal is again filtered through a **humbug** which removes 50Hz noise generated from surrounding industrial electrical stream. The output of the humbug connects with a **second amplifier** (x100) and **filter** (10-1000Hz). The signal is finally processed back through the data acquisition card which allows its visualization on the software.

Electrophysiology set-up for extracellular recordings

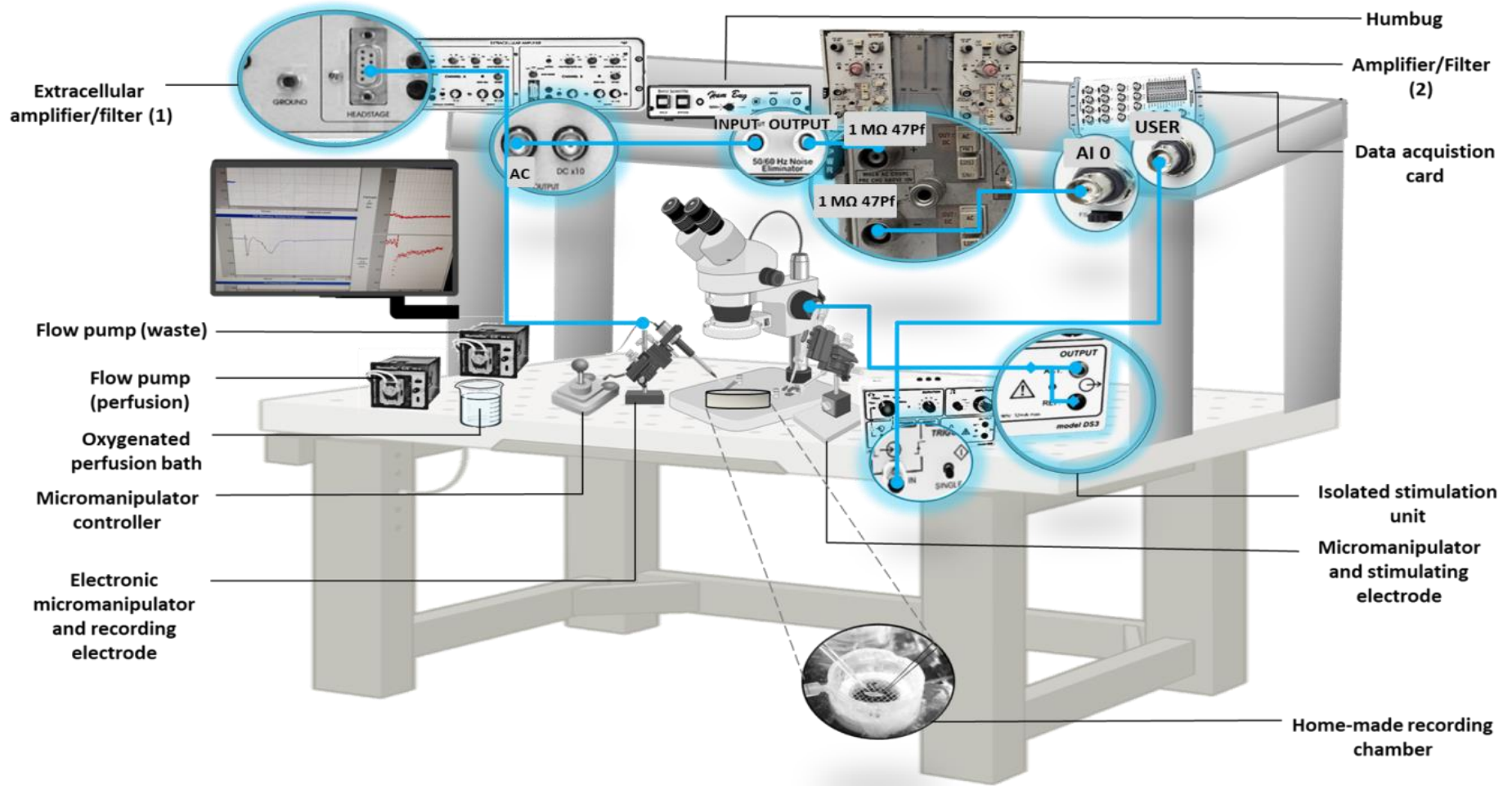


Figure 11: Schematic representation of the experimental set-up used for extracellular recordings.

Slice preparation and recordings

Transverse hippocampal slices preparation (400 μ m thickness) (Figure 12A) and extracellular recordings were performed according to the method described elsewhere (Bortolotto et al., 2011; Lecouflet et al., 2021).

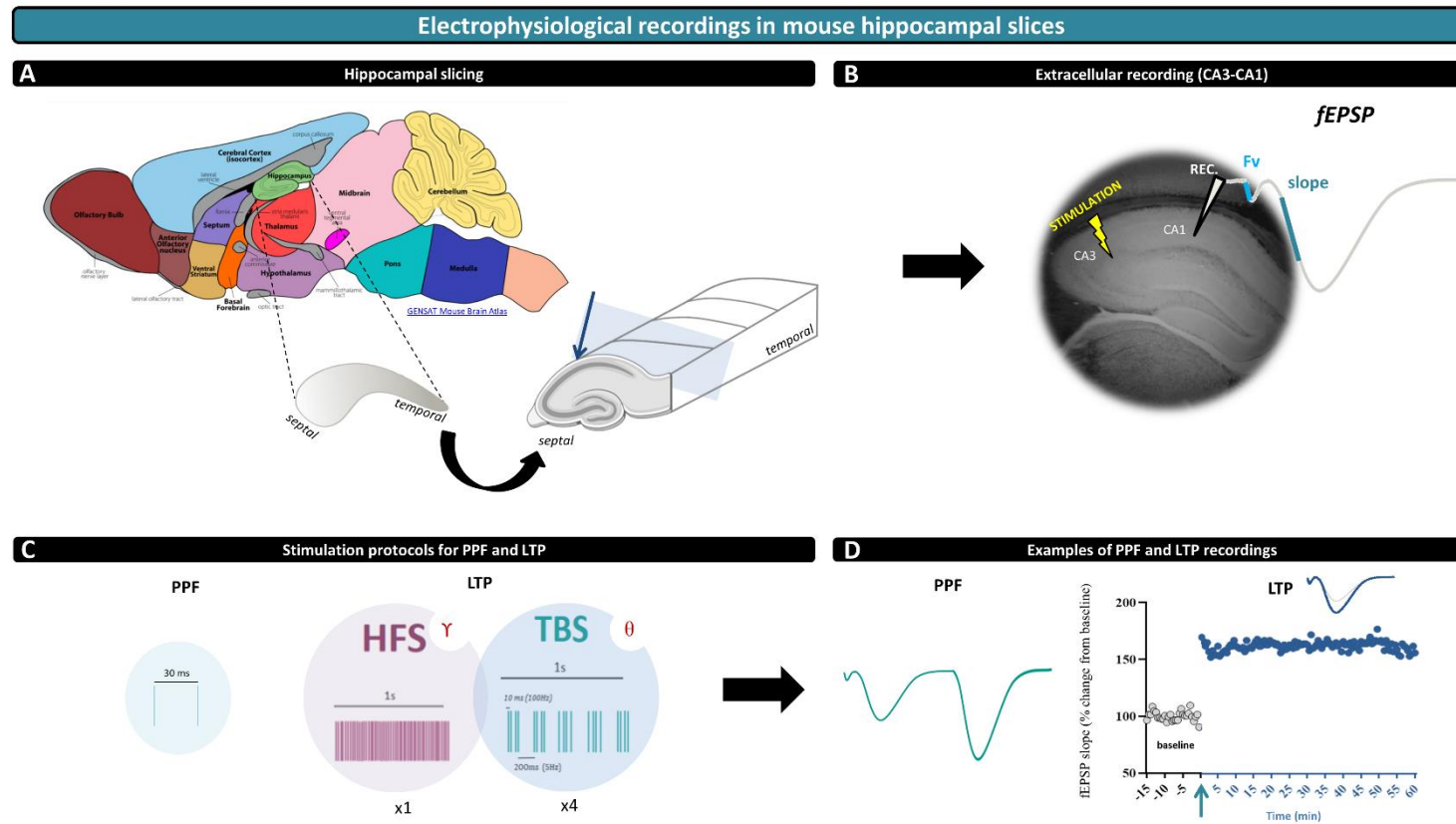


Figure 12: Graphical overview of the method used for *ex vivo* electrophysiological recordings on mouse hippocampal slices.

(A) Schematic view of mouse hippocampus and its orientation for slicing (adapted from GENSAT mouse brain atlas). (B) Photography of a mouse hippocampal slice with schematic representation of electrical stimulation and recording electrodes placement. fEPSP is depicted with Fv and slope (C) Protocols used for paired-pulse facilitation (PPF) and LTP induction by HFS or TBS. (D) Example of a PPF representative trace and LTP recording including baseline (white circles), LTP induction (blue arrow) and post-tetanic response (blue circles).

Abbreviations: fEPSP: field excitatory post-synaptic potential; Fv: fiber volley; HFS: High Frequency Stimulation; PPF: Paired-Pulse Facilitation; REC: recording; TBS: Theta-Burst Stimulation

Extracellular recordings coupled to optogenetic

Optogenetic technique is used to induce reversible cell loss of function at millisecond timescale precision. This is allowed by the expression of light-sensitive proteins named as opsins which are divided in two main categories (**Figure 13**). **Microbial opsins (Type I)** include depolarizing (Channelrhodopsins) and **hyperpolarizing** (Halorhodopsins and **Archaerhodopsins**) types. They are widely spread in archea, bacteria, algae and fungi where they are essential for light-sensing and photosynthetic activity. They are distinguished from their mammalian (Type II) homologues by their direct ion transport function instead of activating intracellular cascades (Han, 2012).

Ai40D mice conditionally express the green-yellow light-activated outward proton pump Archaerhodopsin (ArchT3.0 or ArchT) from algae *Halorubrum sodomense* (strain TP009) with enhanced photocurrents and which is widely used for neuronal silencing (Han, 2012).

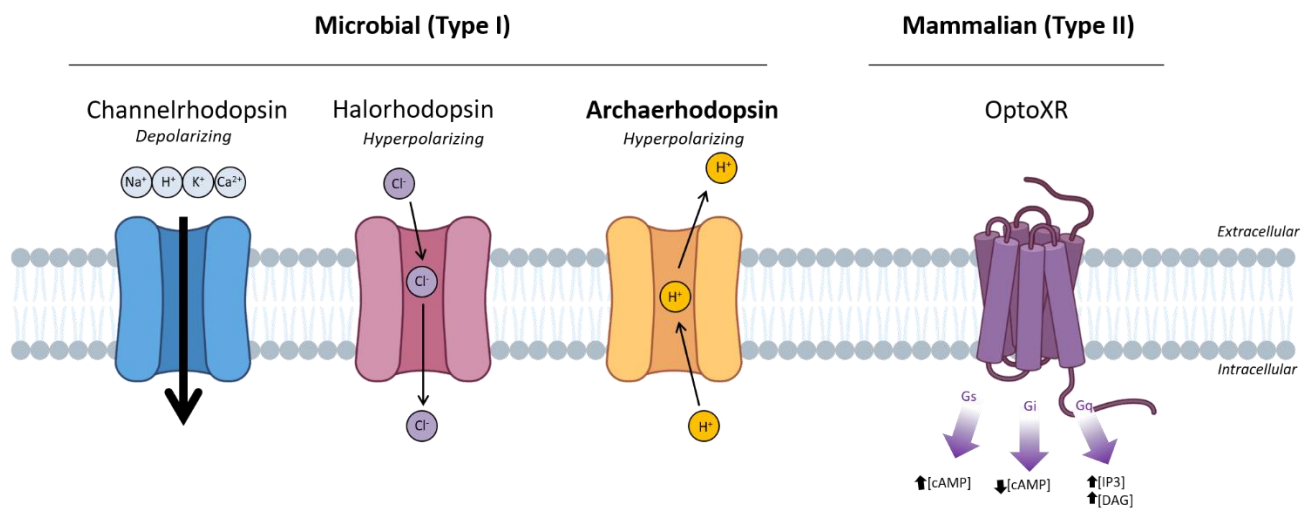


Figure 13: Different types of opsins

Abbreviations: **cAMP**: cyclic adenosine monophosphate; **DAG**: diacylglycerol; **IP3**: inositol triphosphate.

(Adapted from Han et al., 2012)

ArchT transgene is coupled to the enhanced fluorescent green protein (**EGFP**) reporter which allows visual confirmation of ArchT expression by **immunofluorescence (IF)** (**Appendix C**).

Optogenetic experiments coupled to electrophysiological recordings were performed using the set-up and experimental conditions previously described (**Figures 11 and 12**). Specific additional equipment used for optogenetic is listed in **Table 7**:

Table 7: List of specific equipment for optogenetic experiments.

Specific equipment for ex vivo optogenetic stimulation

<i>Reagents or resources</i>	<i>Supplier</i>	<i>Reference/Model</i>
PDL 532™ laser	Cobolt®	0532-06-91-0100-100
Coupler Schäfter + Kirchhoff	Cobolt®	80042
Optical fiber (200µM 0.22NA)	Avantes	FC-UVIR200-2
Software	Cobolt®	Cobolt Monitor (version)
Eye protection	UNIVET®	5X7L.00.00.653

Abbreviations: **DPL**: pulse diode laser, **NA**: numerical aperture.

Optogenetic control of **GABAergic neurons** was performed using a green laser (**532nm**) coupled to a 200µm diameter optical fiber through a collimator. The laser was manually turned **ON** at **1mW** at the tip of the optical fiber during the whole duration of the TBS (**Figure 14**). Manual control of laser settings was performed using the dedicated software (**Table 7**)

Extracellular recordings coupled to optogenetic control of interneurons

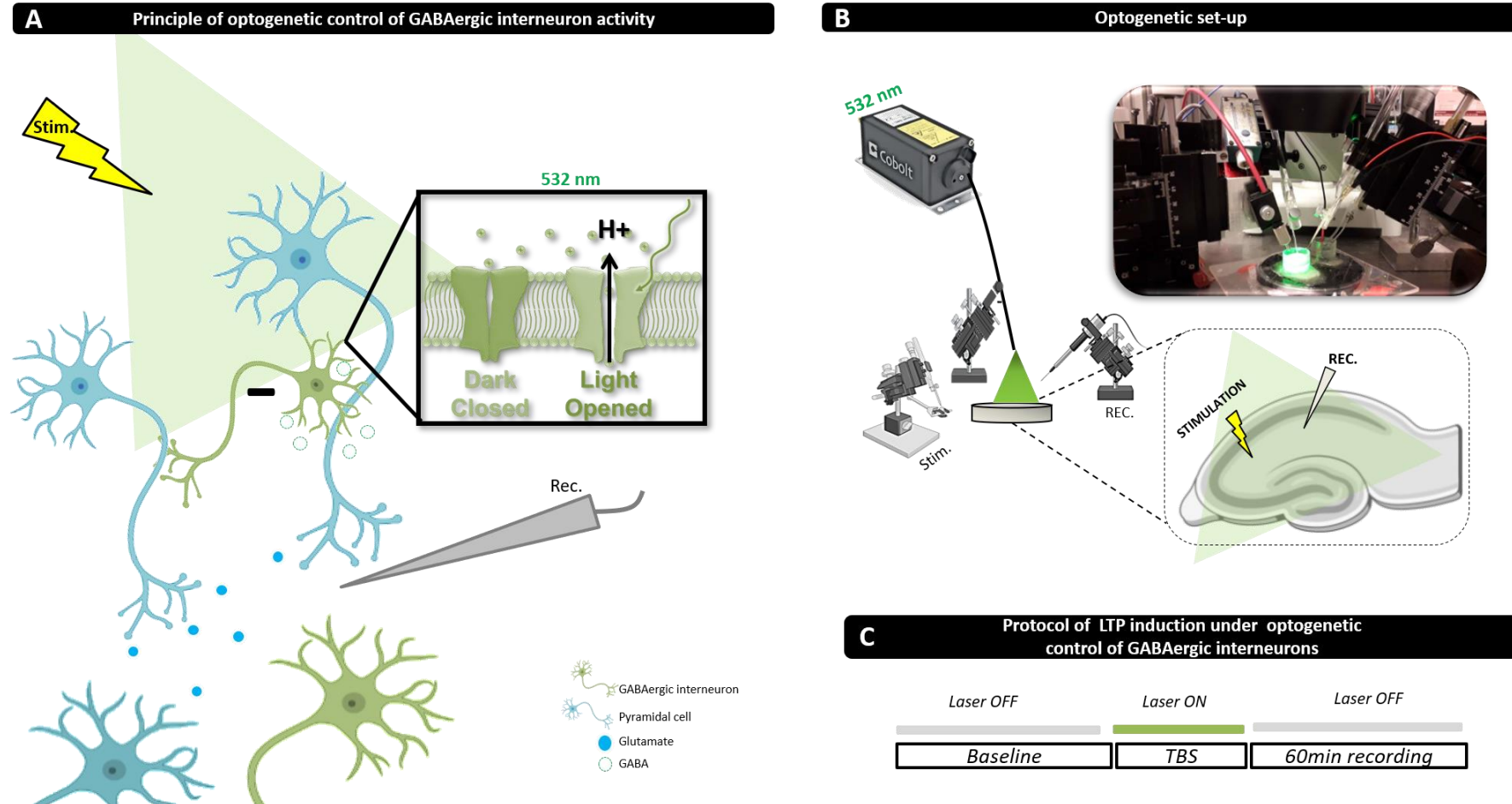


Figure 14: Graphical overview of *ex vivo* optogenetics experiments on mouse hippocampal slices.

(A) Principle of light-activated optogenetic control of GABAergic interneurons coupled to extracellular recordings. (B). Experimental set-up adapted for simultaneous electrophysiological recording and optogenetic control. (C) Experimental design used for simultaneous optogenetic experiment coupled to LTP measurements. Abbreviations: *Rec*: recording; *Stim*: Stimulation; *TBS*: Theta-Burst Stimulation.

Patch clamp recordings

Evoked GABAergic inhibitory post-synaptic currents (eIPSPs) were measured by whole-cell patch clamp (**Figure 15**). Experiments were performed at « *Biophotonics and neuronal networks* » laboratory (UMR-S 9188, Université de Paris Sud), following the protocol described by Potier and collaborators (Potier et al., 2006) and detailed in **Article 1**.

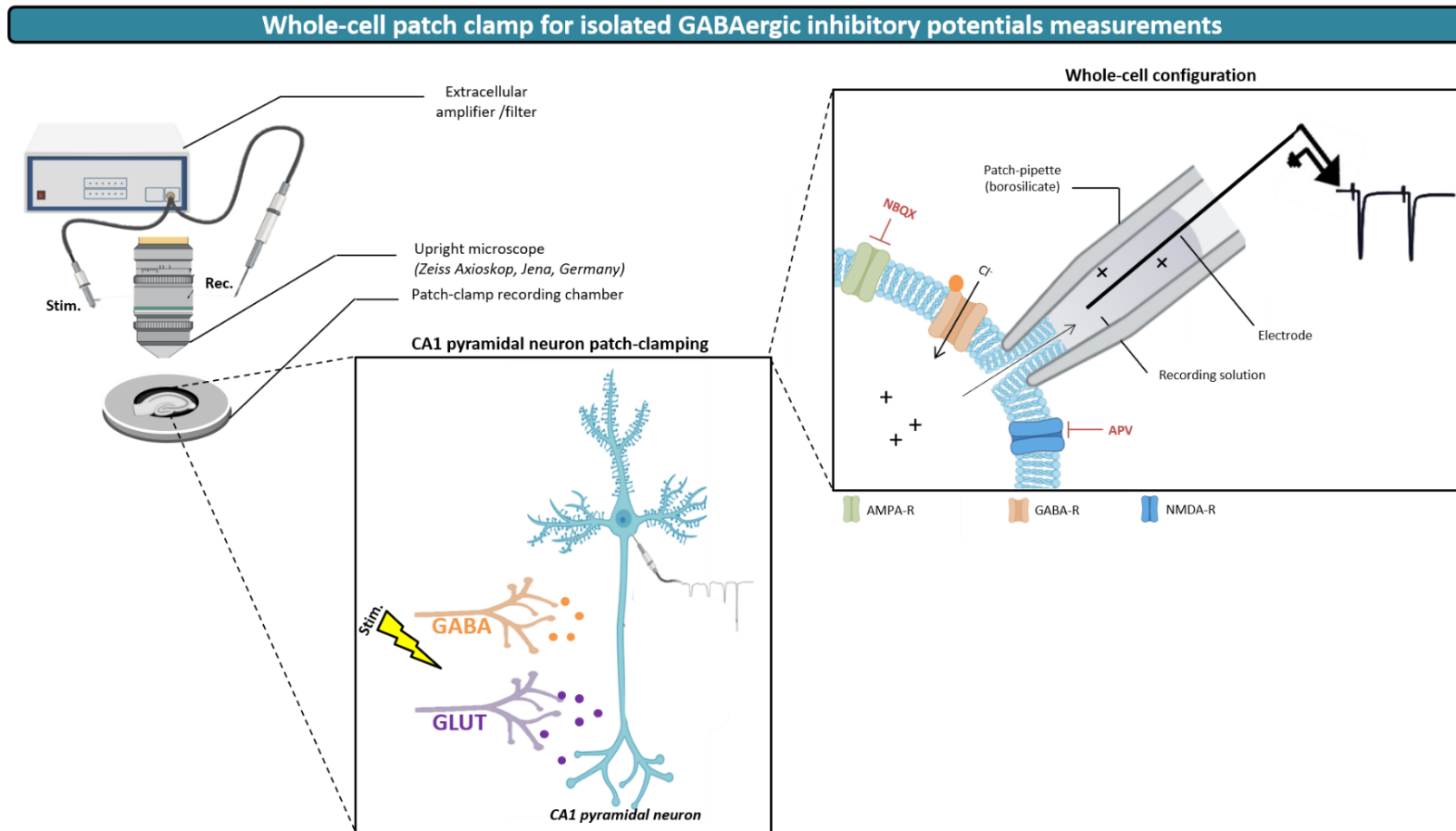


Figure 15: Graphical overview of patch-clamp recordings on mouse hippocampal slices.

Quantitative electroencephalography in conscious mice

The effects of RS67333 on hippocampal rhythms were investigated *in vivo* using electroencephalographic recordings. The method used in awake mouse is briefly described below (Dürmüller et al., 2000). An overview of the experimental design is provided in **Figure 16** and a list of materials and resources is provided in **Table 8**:

Table 8: List of specific equipments required for the recording of qEEG in the conscious mouse.

Specific equipment for EEG recordings		
<i>Materials or resources</i>	<i>Supplier</i>	<i>Reference/Model</i>
Head connector	Emka® Technologies	N/A
Telemetry transmitter	Emka® Technologies	RodentPack2
Battery for transmitter	Power One®	PR48
DIN 84 Stainless screws (cheese head slotted)	N/A	119364
Platinum iridium wire	Phymep®	778000
Dental cement	Dentalon®	Kulzer GmbH
Insulated copper wire	Phymep®	357-918
Wireless receiver	Emka® Technologies	RodentPack2
PoE switch	Netgear	Plus Switch
Data acquisition software	Emka® Technologies	IOX v2.10.0.40
Data analyzes software	Emka® Technologies	ecgAUTO v3.5.5.22

Abbreviations: **N/A**: Non-Applicable; **PoE**: Power over Ethernet.

EEG implant fabrication

Customized home-made implants were prepared for hippocampal EEG recordings as depicted in **Figure 16A**. Briefly, a reference electrode and a depth hippocampal electrode were welded to a head connector. The reference electrode consisted in a stainless screw welded to flexible copper wire and the depth electrode of a pair of insulated platinum iridium twisted together. On the extremity of the depth electrode, reaching the hippocampus wires were cut at 45°C so than there is one end longer than the other one (~1mm separation between the two wire ends), and insulation was removed using fire flame.

The surgical procedure is described in **Article 3** and depicted in **Figure 16B**.

Electroencephalographic recordings

EEG signals were transmitted to a wireless receiver which could record up to 8 mice (1 biopotential) simultaneously (**Figure 16C**). The receiver is connected to the data acquisition software through a Power over Internet (PoE) switch. Field potential were sampled at 500Hz and band-pass filtered between 1 and 100Hz. After completion of the study, electrode placement was verified by immunohistochemistry (Nissl staining) and animals showing incorrect electrode placement were excluded from analysis

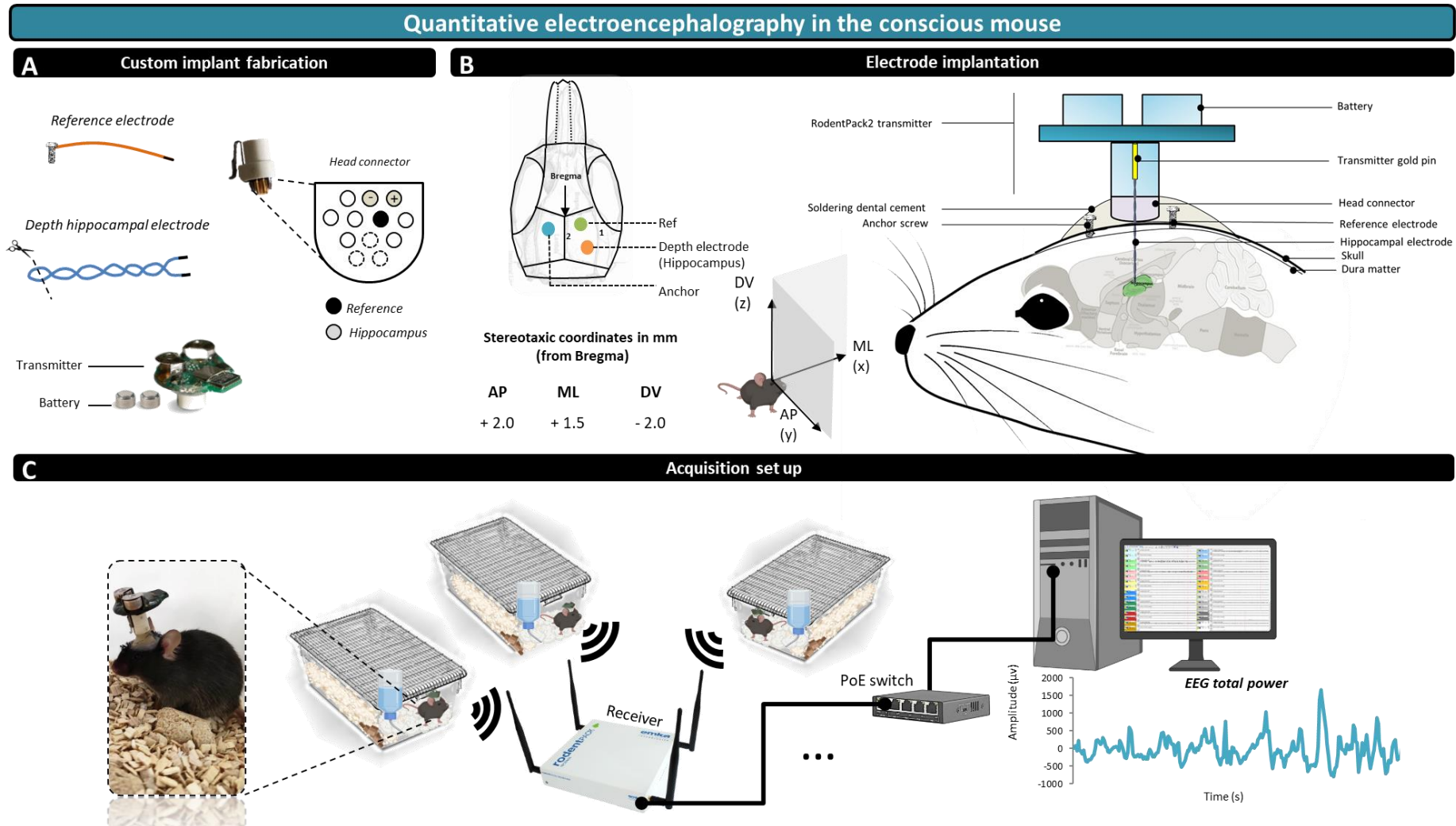


Figure 16: Graphical overview of qEEG recordings in the conscious mouse.

(A) Customized implant fabrication. (B) Surgical procedure for electrode implantation. (C). Schematic view of the set-up for home-cage simultaneous EEG recordings in mice with an example of raw data trace. Abbreviations: AP: antero-posterior; DV: dorso-ventral; ML: medio-lateral; PoE: Power over Ethernet; Ref: Reference

Behavioral tests

All behavioral tests were performed during the dark phase of the animals' cycle. Mice were allowed to acclimate to the testing room at least 30min before each daily session.

Location Discrimination task using automated touch-screen chambers

The LD task allows to assess spatial PS which measures the ability to discriminate between two similar stimuli. This task was performed in automated touch-screen chambers following the method previously described (McTighe et al., 2009; Oomen et al., 2013)

A description of the apparatus as well as the of the main outline of the experimental protocol are provided in **Article 3**. Visual overview is given in **Figure 17**.

Mice were separated into 2 groups as follows:

Group	First injection (i.p)	Second injection (s.c)
Control	NaCl 0.9%	NaCl 0.9%
RS67333 1 mg/kg	RS67333	NaCl 0.9%

Treatment randomization was performed using the number of intermediate training sessions to reach criterion. Pharmacological treatments were consecutively performed 30min prior each probe test session.

Pattern separation test using semi-automated touch-screen chamber

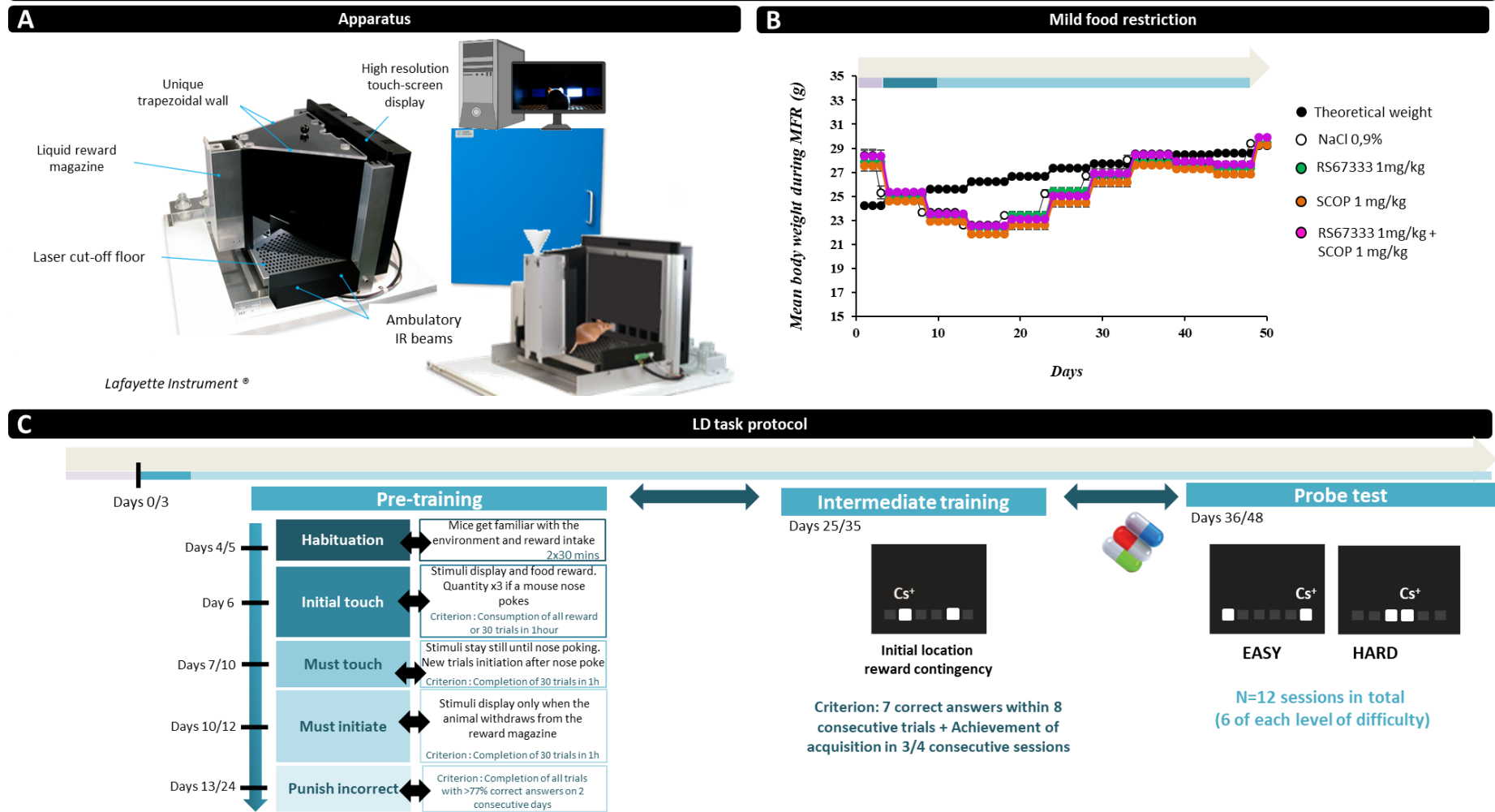


Figure 17: Graphical overview of Location Discrimination task on touch-screen operant chambers. **(A)** Description of operant touch screen chambers with an example of a mouse performing LD task (adapted from Horner et al. 2013) **(B)** Example of mild food restriction protocol and its effects on mice body weight curve before and during experiment. **(C)** Overview of the protocol for LD task adapted from Oomen et al., 2013. Abbreviations: Cs: Conditioning stimulus. SCOP: scopolamine

Spontaneous object recognition tasks

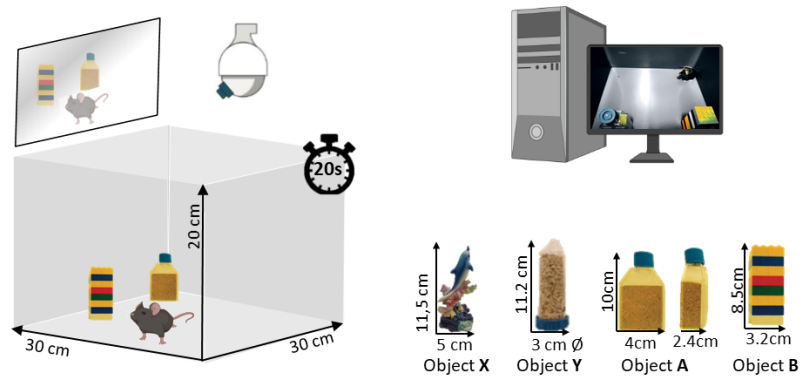
Spontaneous object exploration-based paradigms were used to assess recognition memory for novelty and temporal memory using the NOR test and the TOM test respectively. RM allows individuals to recognize experienced stimuli (Eichenbaum et al., 2007) while TOM is defined as the ability to order past items/events in time (Hannesson et al., 2004).

The essence of NOR resides in the assumption that if rodents have the choice between a familiar and a novel object, they will spend more time exploring the novel object which appears as more appealing considering that they remember the physical and sensorial characteristics of the previously encountered object. The exploration of a novel object is based on rodent's neophilia and triggers episodic-like memory formation (Ennaceur & Delacour, 1988). Rodents also prefer objects which they have not seen very recently over those they have investigated more recently. This is taken as a measure for relative recency which is the primary outcome measure of TOM (Mitchell, 1998).

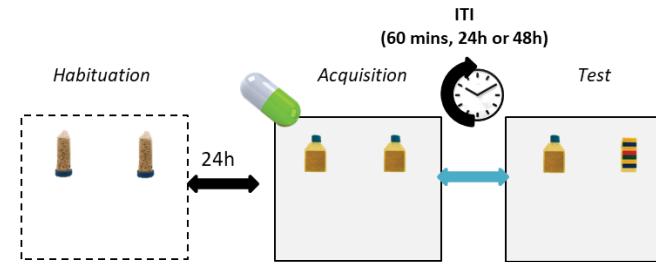
For both tests, a description of the apparatus as well as the of the outline of the experimental protocols are provided in [Article 3](#). Visual overview and details are depicted in **Figure 18**:

Behavioral task based on spontaneous object recognition task

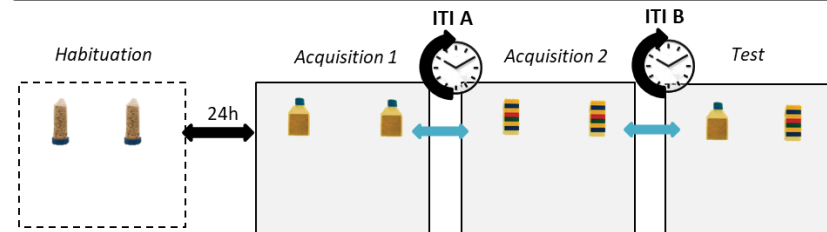
A Apparatus and experimental conditions



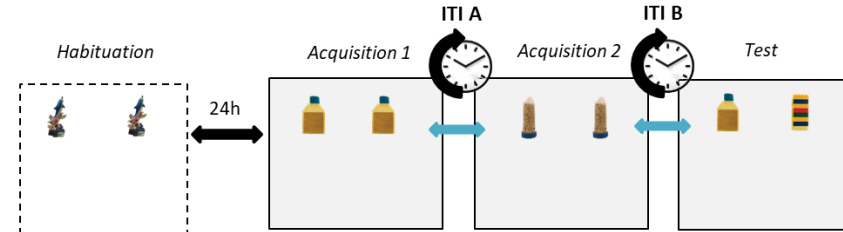
B Experimental design NOR



C Experimental design TOM



D Experimental design of NOR adapted from TOM



Design	ITI A (mins)	ITI B (mins)
(a)	5	5
(b)	5	60
(c)	60	5
(d)	60	24h

Figure 18: Graphical overview of behavioral tests based on spontaneous object exploration paradigms.

(A) Experimental conditions for behavioral tests based on spontaneous object exploration (B) Overview of the protocol and the different experimental designs used for the set-up and optimization of TOM test. (C) Overview of the protocol used for NOR test. (D) Protocol used for the adapted version of NOR test from TOM task. Abbreviations: **NOR**: Novel Object Recognition; **ITI**: Inter-Trial- Interval; **TOM**: Temporal Order Memory Task.

Implementation and optimization of the protocols

The protocol used for the **NOR** test follows that described by Leger et al (Leger et al., 2013a) (**Figure 18B**).

In order to emphasize pro-cognitive effects, we had to optimize the inter-trial intervals (ITIs) in a way that mice in basal conditions could not discriminate the novel object. Therefore, we performed preliminary tests with different inter-trial intervals (ITIs). We tested 1h, 24h and 48h-ITIs test and found that mice displayed poor discrimination performances only after a 48h delay between sampling and test session. Hence, a delay of 48h was used to assess the pro-cognitive potential of RS67333.

TOM was evaluated using an adaptation of protocols described elsewhere (Barker et al., 2019; Barker & Warburton, 2011; Dere et al., 2005) (**Figure 18C**). Among the different it is tested, poor discrimination performance, corresponding to a natural forgetting condition was observed when using 1h ITI A and 24h ITI B. Therefore, these ITI were chosen to assess the pro-cognitive potential of RS67333.

In order to confirm that the preference of mice for the familiar object in the TOM task was due to recency memory and not to forgetting of the previously encountered object, we adapted the protocol (*i.e.* 2 acquisition phases following which we introduce a novel object in the test phase (**Figure 18D**)). Intact performances were expected to be observed.

Biochemical assays

Quantification of the four hippocampal neurotransmitters glutamate (**GLUT**), **GABA**, acetylcholine (**ACH**), and serotonin (**SER**) was performed following acute systemic treatment (*i.p.* administration) of either RS67333 at 1mg/kg or NaCl 0.9% (**Figure 19A**). Thirty minutes (30min) after treatment, hippocampi were collected, weighted and stored into a freezer (-80°C). Biochemical analyses were processed by « Plateforme de Recherche et d'Innovation en Spectrométrie de Masse et Métabolomique » (PRISMM platform, Caen) following the method described elsewhere (Party et al., 2019).

For tissue homogenization, samples were crushed in tubes containing internal standard and formic acid 2% (**Figure 19B**). Samples were centrifuged and supernatant were injected in the **Ultra-High-Performance Liquid Chromatography coupled to tandem Mass Spectrometry (UHPLC-MS/MS)** system (**Figure 19C**). For compounds detection, the UHPLC system was interfaced with an electrospray triple quadrupole mass spectrometer. LabSolutions 5.86 SP1 software was used to process the data. Lower limits of quantification in the injected solutions were 0.002ng/g for GLUT, 0.025ng/g for GABA, 25.000ng/g for ACH and 3.000ng/g for SER calculated for 25mg sample (7µL injection volume).

Quantification of hippocampal neurotransmitter by U-HPLC/MS-MS

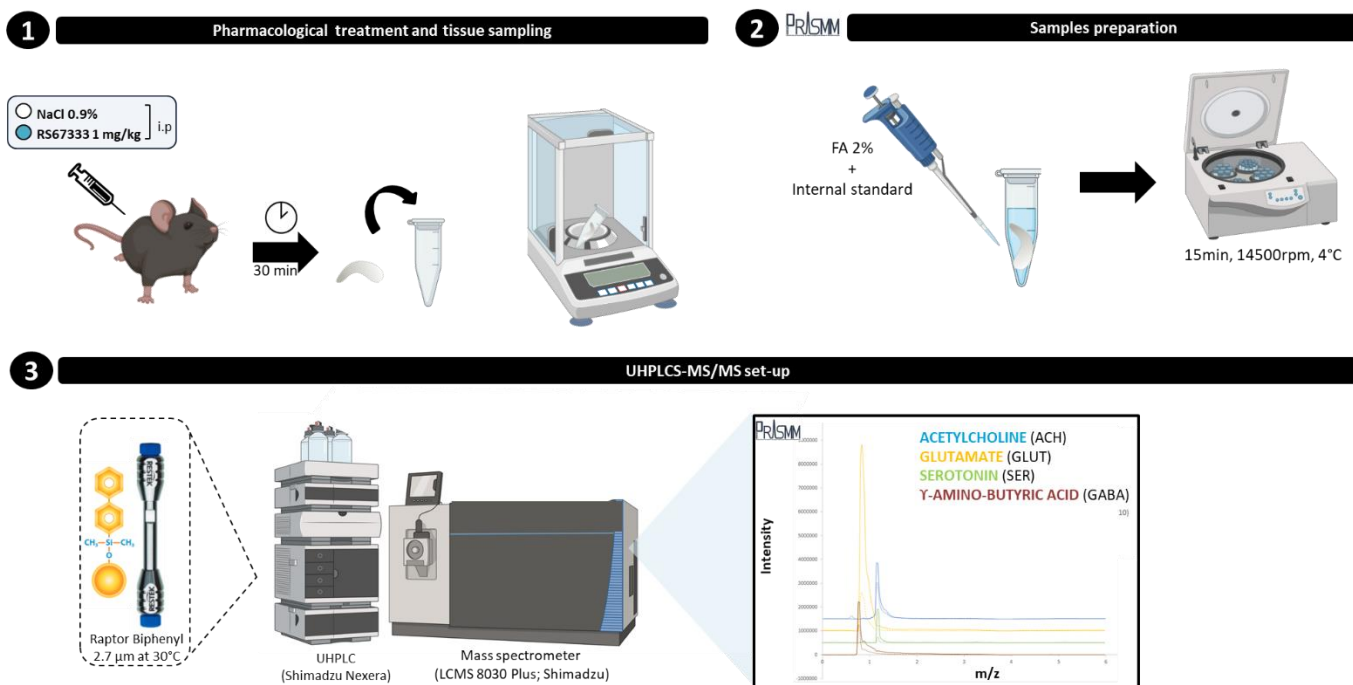


Figure 19: Graphical overview of hippocampal neurotransmitter quantification.

(A) pharmacological treatment and hippocampi sampling (B) samples preparation (C) Samples analysis using UHPLC-MS/MS method. Abbreviations: **UHPLC-MS/MS**: Ultra High-Performance Liquid Chromatography coupled to Tandem Mass Spectrometry.

Statistical analyzes

Data that follow a Gaussian distribution and have equal variance are represented as mean ± standard error mean (SEM) and were analyzed using parametric statistical tests. Data sets that do not meet one of these criteria are represented as median ± interquartile (IQ) and were analyzed using non-parametric statistical tests.

Statistical analyzes were performed using RStudio® software (version 1.2.5001) and GraphPad Prism® (version 8.2.0). Data were considered significant when p value was <0.05. All statistical tests used and the conditions in which they were used are summarized in the table below:

Table 9: Summary of the different statistical analyzes used.

		No. of groups		Post-hoc* (>2)	Comparison with theoretical value
		2	>2		
Statistical test	Parametric	Student t-test (paired and unpaired)	ANOVA	Tukey's	One sample t-test
		ANOVA for repeated measures		Šídák's	
	Non-parametric	Mann-Whitney	Kruskall-Wallis	Dunn's of Multiple Comparisons using Rank Sums.	Wilcoxon signed-rank test
		ANOVA-type for repeated measures (package nparLD)		Pairwise comparison	
	Normal distribution	Shapiro Wilk test			
	Homoscedasticity (equal variance)	Levene test			
	Outliers	Grubb's test			

* Post-hoc test can be performed only if significant differences are observed following ANOVA.

EXPERIMENTAL RESULTS

Article 1

Interplay between 5-HT₄ Receptors and GABAergic System within CA1 Hippocampal Synaptic Plasticity.

Cerebral Cortex, 2020,00: 1–8

Article 2

Genetic background influence on hippocampal synaptic plasticity: frequency-dependent variations between an inbred and an outbred mice strain.

IJMS, 2023, 24, 4304

Article 3

Beneficial effects of 5-HT₄Rs activation in mice: a transversal approach, from memory to its hippocampal correlates.

In preparation

ARTICLE 1

ARTICLE 1: Interplay between 5-HT₄ Receptors and GABAergic System within CA1 Hippocampal Synaptic Plasticity.

Authors : Pierre Lecouflet, **Candice M. Roux**, Brigitte Potier, Marianne Leger, Elie Brunet, Jean-Marie Billard, Pascale Schumann-Bard and Thomas Freret

Published in *Cerebral Cortex* (2020)

Contextual overview:

Given the well-known beneficial effects of 5-HT₄Rs activation on learning and memory as well as the pivotal role of hippocampal synaptic plasticity in such cognitive processes, it appears crucial to investigate the influence of 5-HT₄Rs activation on hippocampal synaptic plasticity.

The effects of 5-HT₄Rs agonist have already been shown to influence hippocampal synaptic plasticity under certain experimental conditions. Up to date, most of the studies have been performed *in vivo* and unfortunately reported inconsistent results. By contrast, investigation of *ex vivo* hippocampal synaptic plasticity following 5-HT₄Rs agonist perfusion had never been performed and may help to dissect the specific influence of 5-HT₄Rs activation on isolated hippocampal plasticity.

The aim of this article was to better understand the mechanisms by which 5-HT₄Rs activation induces cognitive improvement by focusing on hippocampal synaptic plasticity changes measured *ex vivo*.

Two main questions were addressed in this article:

- **Does 5-HT₄Rs activation influence CA1 hippocampal synaptic plasticity *ex vivo*?**

- **If so, what are the mechanisms underlying these effects?**

ORIGINAL ARTICLE

Interplay between 5-HT₄ Receptors and GABAergic System within CA1 Hippocampal Synaptic Plasticity

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Abstract

The type 4 serotonin receptor (5-HT₄R) is highly involved in cognitive processes such as learning and memory. Behavioral studies have shown a beneficial effect of its activation and conversely reported memory impairments by its blockade. However, how modulation of 5HT₄R enables modifications of hippocampal synaptic plasticity remains elusive. To shed light on the mechanisms at work, we investigated the effects of the 5-HT₄R agonist RS67333 on long-term potentiation (LTP) within the hippocampal CA1 area. Although high-frequency stimulation-induced LTP remained unaffected by RS67333, the magnitude of LTP induced by theta-burst stimulation was significantly decreased. This effect was blocked by the selective 5-HT₄R antagonist RS39604. Further, 5-HT₄R-induced decrease in LTP magnitude was fully abolished in the presence of bicuculline, a GABA_AR antagonist; hence, demonstrating involvement of GABA neurotransmission. In addition, we showed that the application of a GABA_BR antagonist, CGP55845, mimicked the effect of 5-HT₄R activation, whereas concurrent application of CGP55845 and RS67333 did not elicit an additive inhibition effect on LTP. To conclude, through investigation of theta burst induced functional plasticity, we demonstrated an interplay between 5-HT₄R activation and GABAergic neurotransmission within the hippocampal CA1 area.

Key words: electrophysiology, hippocampus, memory disorders, serotonin

Introduction

The serotonin type 4 receptor (5-HT₄R) has gained increasing interest in the field of new therapeutic strategies to treat memory disorders. Indeed, better cognitive performances were recently observed in healthy human subjects after a single intake of prucalopride (a 5-HT₄R agonist), a drug clinically authorized in some countries for the treatment of irritable bowel (Murphy 2019). Moreover, numerous preclinical studies have

shown beneficial effects of either acute or chronic pharmacological activation of 5-HT₄R on memory and learning functions. Conversely, blockade of these receptors leads to learning and memory impairments (Galeotti et al. 1998; Marchetti et al. 2000; Hagen and Manahan-Vaughan 2017). More interestingly in the field of Alzheimer's Disease (AD), its activation, both in vivo and in vitro (Robert et al. 2001; Cochet et al. 2013; Tesseur et al. 2013), enables the inhibition of amyloid protein precursor processing

(APP), favoring soluble APP α (sAPP α) production rather than amyloid beta peptide (A β). Hence, a decrease in amyloid load as well as in neuroinflammation markers has recently been described in a transgenic mouse model of AD after chronic treatment with RS67333, a 5-HT $_4$ R agonist (Baranger et al. 2017). In addition, this disease-modifying effect was associated with decreased memory impairments (Giannoni et al. 2013; Baranger et al. 2017). Finally, it is worth mentioning that in response to the early degeneration of the serotonergic system (Smith et al. 2017), 5-HT $_4$ R density is upregulated through early and mild stages of the disease (Madsen et al. 2011). Such phenomenon should strengthen the effect of a pharmacological intervention on this receptor.

In line with its cerebral expression and notably within the hippocampus (Marner et al. 2010), behavioral studies investigating the effect of 5-HT $_4$ R modulation have focused on hippocampus-dependent memory tasks. A recent study reported that optogenetic activation of serotonergic fibers in the CA1 area was associated with an increase in spatial memory performances (Teixeira et al. 2018). Quite interestingly, this activation also elicited synaptic potentiation, which was blocked by 5-HT $_4$ R antagonism.

Hippocampal synaptic plasticity, notably long-term potentiation/depression (LTP/LTD), is widely recognized as a cellular mechanism for memory storage (Morris et al. 1986; Kemp and Manahan-Vaughan 2007; Goh and Manahan-Vaughan 2013; Takeuchi et al. 2014). Observation of the different ranges of brain electrical activity frequencies has further helped to set up *in vivo* protocols of conditioning stimuli. Thus, high-frequency stimulation (HFS, 40–120 Hz also called γ -frequency) was historically the first discovered pattern to elicit LTP, whereas thereafter theta-burst stimulation (TBS) was described (TBS or θ -burst, 5–10 Hz frequency) (Larson and Lynch 1986; Bliss and Collingridge 1993). Within the hippocampus, the interaction between γ and θ rhythmic activities is critical for memory formation. However, owing that it mimics hippocampal electrical activity recorded while a rat performs a behavioral task, θ rhythm is thought to be more characteristic to memory functions (Buzsaki and Moser 2013; Larson and Munkacsy 2015). Besides, HFS and TBS protocols impact network activity differently since only the second requires specific GABAergic regulation through the GABA $_B$ receptors to induce LTP (Stelzer et al. 1994; Perez et al. 1999). With regard to 5-HT $_4$ R, literature argues for a complex regulatory role of this receptor on hippocampal synaptic plasticity. Indeed, the modulation of this receptor may (or may not) affect both LTP and LTD differently, according to the subzone considered (Hagena and Manahan-Vaughan 2017). Within the CA1 area, the two different studies conducted *in vivo* have led to conflicting results. One research group showed that the activation of 5-HT $_4$ R enhanced LTP (Matsumoto et al. 2001), whereas another reported no effect on LTP but an inhibited LTD (Kemp and Manahan-Vaughan 2005). However, in any case, neither LTP nor LTD in the CA1 area was affected by 5-HT $_4$ R blockade.

So far still misunderstood, neurobiological processes underlying 5-HT $_4$ R regulation of functional plasticity might imply the GABAergic system. Indeed, the activation of 5-HT $_4$ R has been shown to modulate GABA release from hippocampal slices (Bijak and Misgeld 1997; Bianchi et al. 2002) and to regulate GABA $_A$ receptors in the cortex (Cai et al. 2002). The present study therefore aims to better characterize the interplay between 5-HT $_4$ R and GABA $_A$ receptors involved in the modulatory effect of hippocampal Schaeffer's collateral-CA1 synaptic plasticity. To this end,

effects of 5-HT $_4$ R activation on two conditioning protocols for LTP induction were investigated.

Materials and Methods

Animals

Experiments were performed on adult (10–14 weeks old) Naval Medical Research Institute male mice (Janvier Labs, France), weighing 25–30 g. Mice were housed in groups of 8 within standard polycarbonate cages, with food and water *ad libitum* and maintained in a regulated environment (22 ± 1 °C) under 12 h reversed light/dark cycle (light on from 8 pm to 8 am). All experiments complied with the European Community guidelines and French law on animal experimentation.

Pharmacology

All drugs used were perfused at least 15 min before any recording to ensure full diffusion in the tissues and full expression of their effects. Based on its pharmacological profile (Eglen et al. 1995; Hegde et al. 1995), the selective 5-HT $_4$ R agonist (RS67333) was used at 10 μ M. GABA $_A$ and GABA $_B$ receptor antagonists (bicuculline methiodide and CGP55845, respectively) were used at 10 and 1 μ M, respectively. Except for the RS compound obtained from Tocris biosciences®, all others pharmacological compounds were purchased from Sigma-Aldrich®.

Extra-Cellular Recordings

Mice were deeply anesthetized (isoflurane 5%) and decapitated. The brain was rapidly extracted from the skull and submerged for half a minute in ice-cold artificial cerebrospinal fluid (aCSF). The aCSF composition was as follows (in mM): NaCl 124, KCl 3.5, MgSO $_4$ 1.5, NaH $_2$ PO $_4$ 1.2, CaCl $_2$ 2.5, NaHCO $_3$ 26, D-glucose 12. The solution was bubbled with an O $_2$ /CO $_2$ carbogen gas mixture (95%/5%) to keep the pH around 7.4. Hippocampi were removed from each hemisphere and cut in 400 μ m thickness transverse slices with a tissue chopper (McIlwain®). Slices were then allowed to recover in a holding chamber containing aCSF at 28 °C for at least an hour to recover before recordings.

For electrophysiological recordings, slices were placed between two nylon meshes and completely submerged in a recording chamber perfused with a constant flow rate of 2 mL/min of bubbled aCSF at room temperature. All drugs were applied via direct bath perfusion. Extracellular synaptic responses in the CA1 area were elicited by stimulation of the Schaeffer collateral. Stimulation pulses (0.02 msec duration) triggered by a computer controlled by the WinLTP® software (Anderson and Collingridge 2001) were delivered by a stimulus isolation unit through a bipolar electrode. Responses were recorded with glass micropipettes filled with 2 M NaCl placed in the apical dendritic layer of the CA1 area.

For LTP recording, stimulation pulses were delivered every 10 sec and field excitatory postsynaptic potentials (fEPSP) were recorded. The mean slope of three successive fEPSPs was considered, as a data point and the electrical intensity of the pulses was set to obtain a baseline fEPSP slope of 0.1 mV/s. After 15 min of stable recordings for the baseline, a conditioning stimulus was applied to induce LTP. The conditioning stimulus was realized either through a HFS protocol (i.e., 100 Hz tetanus for 1 sec) or a TBS protocol (i.e., four repetitions of five bursts at 0.1 Hz—each burst constituted of four pulses at 100 Hz—separated by 200 ms at 5 Hz). Baseline recordings were resumed for 60 min after the

conditioning stimulus. Therefore, the last 15 min recordings, reflecting LTP magnitude, were used for statistical analysis. In addition, for the TBS protocol, the area under curve (AUC) of the two first burst responses was calculated. We then evaluated the potentiation of the second burst corresponding to AUC2/AUC1, which reflects the efficacy of the TBS.

Whole-Cell Patch-Clamp Recordings

Evoked inhibitory postsynaptic currents (IPSCs) were recorded at room temperature in CA1 pyramidal cells from acute hippocampal slices of four different animals perfused with aCSF. Borosilicate patch pipettes (5 M Ω) were filled with (in mM) CsCH₃O₃S 140, CsCl 6, MgCl₂ 2, HEPES 10, EGTA 1.1, QX-314 5, ATP 4, (pH 7.3; 290mosM) to optimize synaptic currents. Membrane currents were acquired and filtered at 2 Hz using an AxoPatch 1-D amplifier (Axon Instruments). Online acquisitions and analysis were performed using WinLTP software. Series resistance was compensated and regularly monitored throughout the experiment and recordings showing unstable (>20%) series resistance were rejected. GABA_R-dependent synaptic current (IPSC) was evoked at 0.07 Hz by electrical stimulation of the Schaffer collateral/commissural pathway using a bipolar electrode located in the "stratum radiatum", in the presence of NBQX and APV to block glutamatergic transmission. RS67333 was applied through the perfusion for 30 min and then washed out. Time-course of the effect of RS67333 on IPSC amplitude was monitored from the 10 min preceding application (baseline measurement) until the 10 min after the wash out.

Statistical Analyses

Data are expressed as mean \pm standard error of the mean (SEM). Analysis of variance (ANOVA) was performed via R[®] software, and a *P* value less than 0.05 was considered significant. When necessary, univariate *t*-test and post hoc multiple comparison test with a Bonferroni–Dunn correction factor were used.

Results

RS67333 (5-HT₄R agonist) did not Affect HFS-Induced LTP, but Inhibited TBS-Induced LTP

When using the HFS protocol, ANOVA with repeated measurements of mean fEPSP slopes during the last 15 min showed no group effect ($F_{(1,14)} = 0.397, P = 0.5388$), no time effect ($F_{(30,420)} = 1.101, P = 0.3293$), and no group \times time interaction ($F_{(30,420)} = 0.823, P = 0.7350$) (Fig. 1A). Thus, RS67333 did not change the stable and robust tetanus-induced LTP normally expressed in control conditions (respectively $137 \pm 8\%$ vs. $131 \pm 7\%$ of baseline, $n = 8$ slices for each condition).

Conversely, when using the TBS protocol, ANOVA revealed a group effect ($F_{(1,14)} = 14.907, P = 0.0017$), a time effect ($F_{(30,420)} = 1.488, P = 0.0494$), but no group \times time interaction ($F_{(30,420)} = 1.129, P = 0.2948$) (Fig. 1B). In the control condition, LTP magnitude was of $150 \pm 9\%$ of baseline value, whereas its value dropped down to $116 \pm 3\%$ in RS67333-treated slices ($n = 8$ slices for both conditions). Moreover, although stable over time in the control condition (one-way ANOVA, $F_{(30,210)} = 0.695, P = 0.8822$), a significant decrease in LTP magnitude was observed in RS67333-treated slices (one-way ANOVA, $F_{(30,210)} = 1.8828, P = 0.0078$).

Further, having a look at AUCs' ratio values for the two first bursts of TBS conditioning, one-way ANOVA revealed a group

effect, with RS67333 treated slices having an AUC ratio significantly lower than control group ($F_{(1,14)} = 4.654, P = 0.0488$, Fig. 1C). Also, only the control group displayed a ratio significantly higher than 100% (univariate *t*-test, $P = 0.0490$ and 0.8370 for control and RS67333 treated groups, respectively).

Blockade of GABA_A Receptors Suppressed the Inhibitory Effect of RS67333 (5-HT₄R agonist) on TBS-Induced LTP

We then assessed whether the inhibitory system is involved in the effect of RS67333 on TBS-induced LTP. Thus, RS67333-induced modulation of synaptic plasticity was measured in the presence of bicuculline (GABA_A receptors antagonist, 10 μ M), and compared with both control conditions (i.e., aCSF with or without bicuculline). ANOVA with repeated measurements showed neither group effect ($F_{(2,22)} = 0.080, P = 0.9231$), nor time effect ($F_{(30,660)} = 1.394, P = 0.0806$), and no group \times time interaction ($F_{(60,660)} = 0.811, P = 0.8438$) (Fig. 2A). Accordingly, TBS-induced LTP was similar with or without bicuculline ($148 \pm 10\%$ and $148 \pm 5\%$ of baseline, respectively) and not different from the LTP observed in RS67333 plus bicuculline-treated slices ($145 \pm 6\%$ of baseline). This last result suggests that the decreased magnitude of TBS-induced LTP observed with 5-HT₄R activation involves the contribution of the GABA_A receptors.

Activation of 5-HT₄R had no Effect on GABA_A Receptor Activities of CA1 Pyramidal Cells

ANOVA of the time course of evoked IPSCs revealed neither condition effect ($F_{(2,9)} = 0.631, P = 0.5539$), nor time effect ($F_{(39,351)} = 1.201, P = 0.1984$), or condition \times time interaction ($F_{(78,351)} = 0.806, P = 0.8747$). No effect of 5-HT₄R activation was noticed since the amplitude of evoked IPSCs remained constant after the addition of RS67333 in the recording medium, as well as during washout (Fig. 2B). These results therefore demonstrated that 5-HT₄R activation does not directly change inhibitory transmission in CA1 pyramidal neurons.

GABA_B Receptors Blockade Mimicked Effects of 5-HT₄R Activation on LTP

We then tested the effects of an antagonist of the second type of GABAergic receptors, GABA_B receptors. ANOVA with repeated measurements showed a group effect ($F_{(3,39)} = 8.573, P = 0.0002$), a time effect ($F_{(30,1170)} = 1.583, P = 0.0243$), and a group \times time interaction ($F_{(90,1170)} = 1.475, P = 0.0035$) (Fig. 3). Compared with the control slices group ($142 \pm 4\%$ of baseline, $n = 15$), the magnitude of TBS-induced LTP was significantly decreased in slices infused with either RS67333 or CGP55845 (selective GABA_B receptor antagonist) alone (respectively, $P = 0.0006$ and 0.0002 ; $116 \pm 3\%$ and $115 \pm 7\%$ of baseline, $n = 8$ and 9), or in combination ($P = 0.0009$; $119 \pm 6\%$ of baseline, $n = 10$). Furthermore, all three treated groups were not different from each other ($P > 0.5$). Thus, the concurrent 5-HT₄R activation and GABA_BR blockade did not produce a stronger impairment of TBS-induced LTP than modulation of each of these receptors separately.

Discussion

We demonstrated here for the first time that 5-HT₄R activation in the CA1 field of the hippocampus leads to highly contrasting

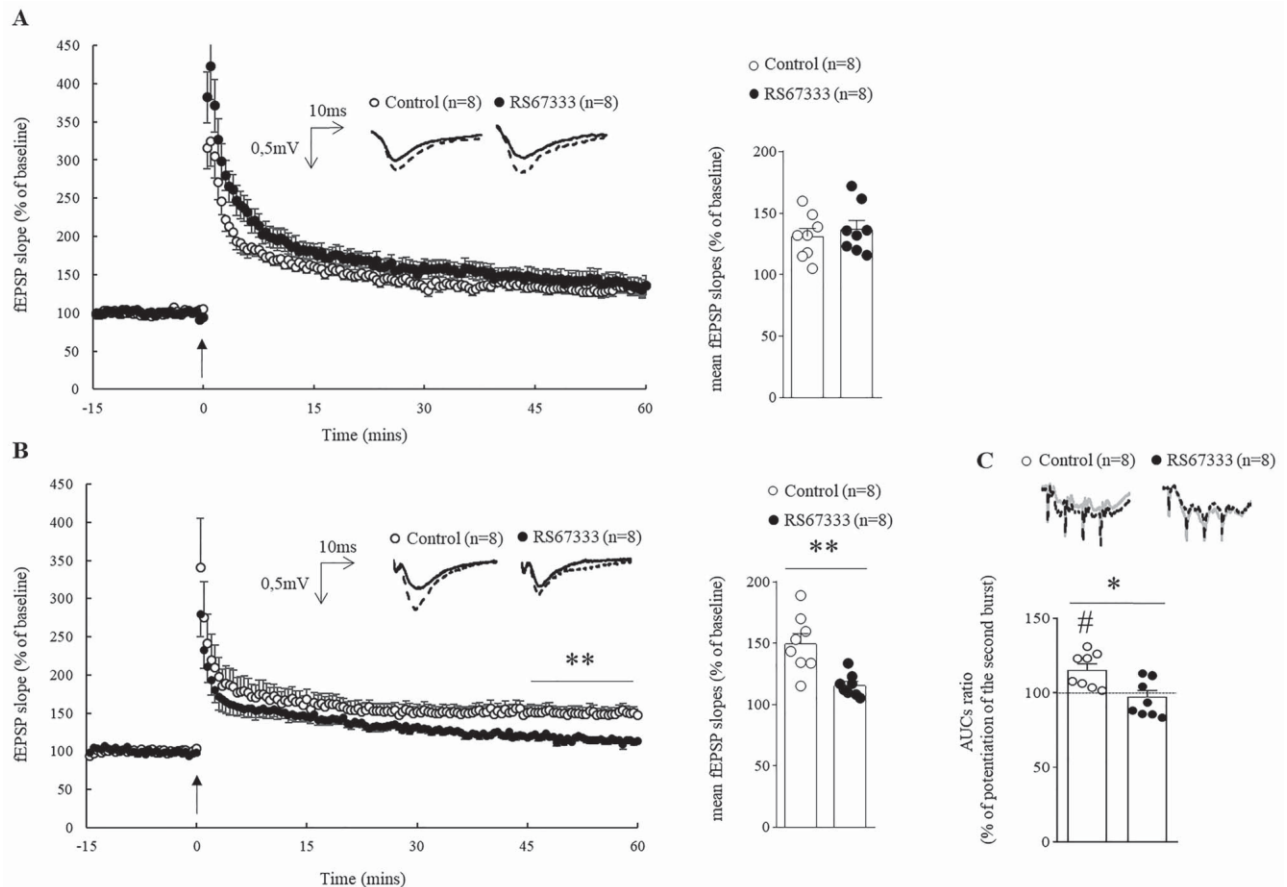


Figure 1. Effects of pharmacological activation of 5HT₄ R (RS67333, 10 μ M) on CA1 hippocampal slices during HFS- and TBS-induced LTP. Data are expressed as mean \pm SEM. A. and B. Time course of fEPSP, after HFS- and TBS-induced LTP, respectively. Arrow marks the time when conditioning stimulation was applied and mean of the last 15 min are displayed. C. AUCs ratio during TBS conditioning stimulus. Insets show representative traces of fEPSP before and after conditioning stimulation (* and ** for $P < 0.05$ and 0.01 vs. control; # for $P < 0.05$ vs. 100%).

effects on the expression of LTP, according to the conditioning protocol used. Indeed, through ex vivo experiments, we observed either an unaffected LTP or a conversely highly decreased potentiation (HFS vs. TBS). Furthermore, we showed that the key difference between the two conditioning stimulation protocols stands in the recruitment of GABA_BR, is central in the effects of 5-HT₄R.

The classic HFS conditioning protocol (100 Hz tetanus for 1 sec) is one of the most frequently used in the literature. Although using such protocol, we observed no effect of 5-HT₄R activation on the magnitude of CA1 hippocampal LTP. Interestingly, this result is consistent with an in vivo study conducted in the CA1 area of freely moving rats [Kemp and Manahan-Vaughan 2005]. In this study, Manahan-Vaughan's team used a similar (100 Hz tetanus for 1 sec) induction protocol but repeated it four times. However, as stated earlier, among the only two in vivo studies published so far, the other revealed contrasting results. Led by the group of Matsumoto, they showed an enhanced LTP after 5-HT₄R activation [Matsumoto et al. 2001]. One would have been tempted to rely on the methodological difference in the origin of the signal recorded to explain the discrepancy. Indeed, whereas we and Manahan-Vaughan group recorded fEPSPs slopes (dendritic response), the population spike (PS) amplitudes (somatic response) were recorded in the study of Matsumoto. However, previous works on effects of 5-HT₄R activation

collected in other hippocampal formation areas (i.e., dentate gyrus [Kulla and Manahan-Vaughan 2002; Twarkowski et al. 2016] and CA3 area [Twarkowski et al. 2016]) indicated that changes in fEPSP slopes are similar to those in PS amplitudes. Hence, it is more likely that the entirely different LTP induction protocol (five trains at 1 Hz, each composed of eight pulses at 400 Hz) used by the group of Matsumoto would account for such discrepancy.

More sensitive to variations of GABAergic neurotransmission than the HFS protocol, the TBS protocol is viewed as a more physiological pattern of stimulation [Larson and Munkacsy 2015]. Indeed, TBS mimics two particularities of hippocampal physiology: the complex spike discharges of pyramidal neurons [Ranck 1973] and the rhythmic modulation of their excitability during theta rhythm [Rudell et al. 1980]. Hippocampal theta rhythm was originally described as the arousal rhythm [Green and Arduini 1954]. Although at first discussed in line with motor behavior [Vanderwolf 1969], it is now rather associated with the updating of the cognitive spatial map (within hippocampal place cells) [O'Keefe and Nadell 1978], as well as with memory and learning processes [Hasselmo 2005; Buzsaki and Moser 2013]. Numerous studies have shown that TBS-induced LTP is more susceptible than HFS-induced LTP [Larson and Munkacsy 2015] to various experimental manipulations, many of which also lead to memory deficits.

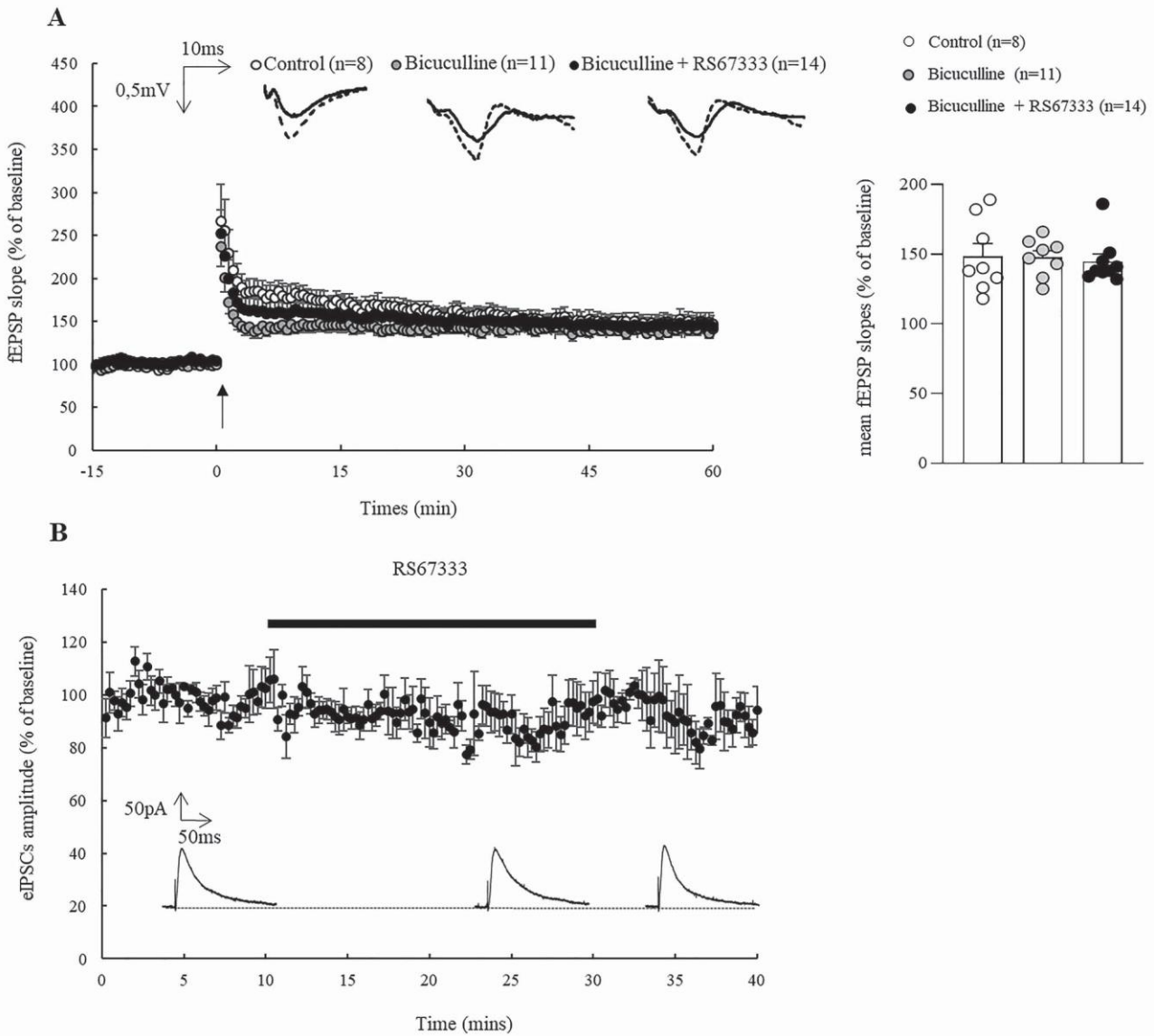


Figure 2. Effects of pharmacological activation of 5HT₄R (RS67333, 10 μ M) on evoked fEPSP slopes after TBS-induced LTP in GABA_AR blockage condition (A) and on evoked IPSCs in CA1 pyramidal cells (B). Data are expressed as mean \pm SEM. Bicuculline (10 μ M) was used as GABA_AR antagonist. For evoked IPSCs, holding potential was set at -30 mV (N = 4). Insets show representative traces of fEPSPs and IPSCs (* and ** for $P < 0.05$ and 0.01 vs. control).

Although using the TBS protocol, we reported for the first time that the activation of 5-HT₄R led to a significant decrease of LTP magnitude. Of note, one *ex vivo* experiment has investigated the effect of serotonin application on rat hippocampal slices (Corradetti et al. 1992). Realized before the discovery of 5-HT₄R, authors of this study reported no change of HFS-induced LTP (consistent with our previously discussed results) and a decrease of primed burst-induced LTP. Interestingly, the primed burst protocol used shares close properties with our TBS protocol, in such a way that a similar time interval was used between the priming pulse and the following burst (four pulses at 100 Hz). Hence, in line with our result, one might hypothesize that LTP impairments observed at that time with serotonin application rely on 5-HT₄R activation.

Next, we further explored the mechanisms at work that could account for differential effects of RS67333 (or of serotonin application) on LTP according to the stimulation protocol

used. A quarter of a century ago, the power of the afferent stimulation was argued to likely overcome the inhibitory effect of serotonin. This was suggested to account for the absence of effect of serotonin application during HFS protocol. Here, we first investigated whether a direct effect of 5-HT₄R activation on either α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) or NMDAR (N-methyl-D-aspartate receptor) activity was involved. On basal synaptic transmission (relying on AMPAR activity), we reported no effect of either 1 or 10 μ M of RS67333 (see Supplementary Fig. 1A). With regard to NMDAR, although a decrease of their activity would have explained impairments of LTP, RS67333 had no effect on NMDAR activation—whatever the dose considered (see Supplementary Fig. 1B). Furthermore, HFS-induced LTP also requires NMDAR and was unaffected by RS67333. Hence, RS67333 effect on TBS-induced LTP cannot be explained by modulation of NMDAR activity.

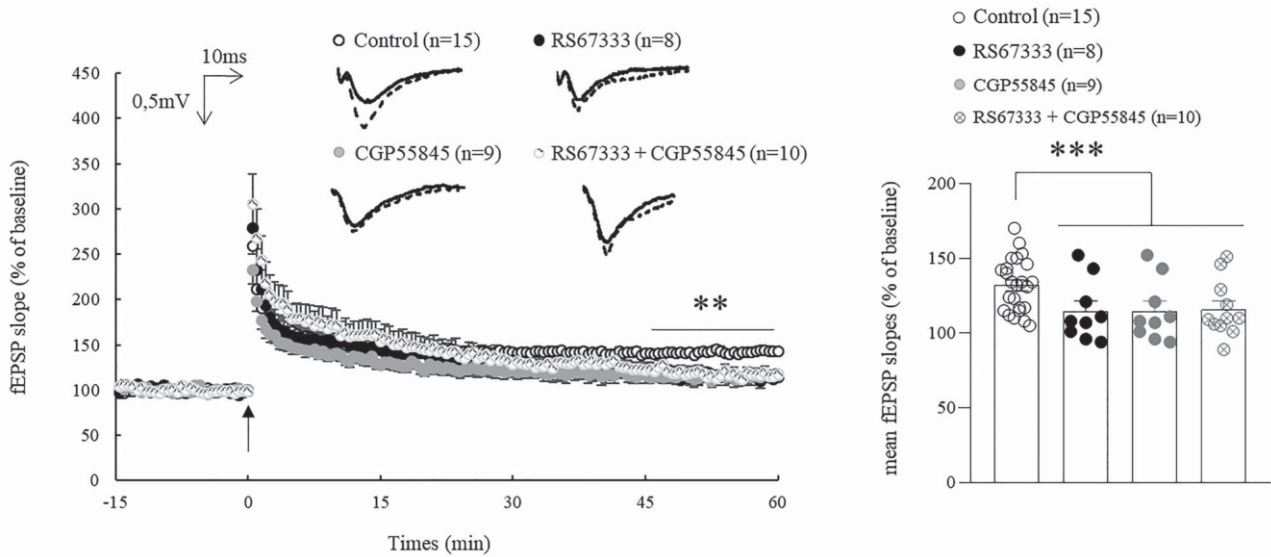


Figure 3. Effects of pharmacological activation of 5HT₄R (RS67333, 10 μ M) on evoked fEPSP slopes after TBS-induced LTP in GABA_BR blockage condition. Data are expressed as mean \pm SEM. CGP55845 (1 μ M) was used as GABA_BR antagonist. Insets show representative traces of fEPSPs (* and ** for $P < 0.05$ and 0.01 vs. control).

As stated earlier, several studies have advanced converging arguments for a role of GABAergic transmission in the modulatory role of 5-HT₄R on synaptic plasticity (Bijak and Misgeld 1997; Bianchi et al. 2002; Cai et al. 2002). Here, we reported that 5-HT₄R activation did not change the amplitude of evoked GABA_AR-dependent IPSCs at low frequency. This result is consistent with no effect of the 5-HT₄R agonist (BIMU-8) on evoked GABA release from hippocampal slices at similar concentration (Bianchi et al. 2002).

Conversely, effects of 5-HT₄R activation on TBS-induced LTP appear to rely on GABA-dependent mechanisms. Indeed, when RS67333 was applied, we did not observe burst potentiation across conditioning stimuli during the TBS protocol. Increase of action potential firing across burst repetition is the key property of the TBS protocol. It results from cumulative loss of synaptic inhibition (Larson and Munkacsy 2015), also called postburst hyperpolarization. This phenomenon is caused by GABA_AR activation and is more or less suppressed according to the burst interval used, with a maximum effect at 200 msec (Grover et al. 2009).

In our experiments, when a postsynaptic GABA_AR antagonist was applied (bicuculline), we did not observe any change of TBS-induced LTP. This result confirms the loss of inhibition resulting from the TBS protocol used. Nevertheless, when co-applied with RS67333, bicuculline fully blocked the 5-HT₄R activation effect on TBS-induced LTP. Thereafter, an effective GABAergic neurotransmission is mandatory for RS67333 to impair TBS-induced LTP.

With regard to GABA_BR, they are located both on pre- and postsynaptic elements. Here, when a nonselective antagonist was used (CGP55845), TBS-induced LTP displayed an impairment similar to this observed in condition of 5-HT₄R activation (RS67333). Besides, when both compounds were co-applied, no additive effect was observed. Conversely, a selective postsynaptic GABA_BR antagonist (CGP35845) prevented the RS67333

suppressive effect on TBS-induced LTP (see [Supplementary Fig. 3](#)).

Taken altogether, our results argue for an interplay of 5-HT₄R activation with GABAergic functioning. But, still remains open the question of the nature of this interaction and how it contributes to in vivo beneficial effect on memory performances. A direct effect through 5-HT₄R localized on GABAergic interneurons seems unlikely. Indeed, an in situ hybridization study has reported that expression of 5-HT₄R mRNA does not seem to co-localize with the Gad-65 mRNA (a marker of GABAergic interneurons) in the hippocampus (Penas-Cazorla and Vilaro 2015). An indirect pathway would then require the release of other neurotransmitters, such as acetylcholine, whose receptors are present on interneurons in the CA1 (Bianchi et al. 2002).

On a more integrated dimension, while considering LTP as a substrate of memory trace encoding, it would have been expected that RS67333 increases LTP magnitude. Indeed, the administration of 5-HT₄R agonists has demonstrated promnesic effects both in animals and humans. However, one has first to consider that excessive (as well as insufficient) synaptic plasticity will prevent learning and memory formation (Barnes et al. 1994). Thus, dampening (as well as increasing) LTP magnitude may—in some cases—support promnesic effect. Second, hippocampal synaptic plasticity is neither just about the CA1 subfield nor only the LTP process. For instance, some models of sequence memory processing suggest that DG-CA1 network is specialized in encoding new spatiotemporal sequences for long-term storage in CA3, whereas the CA1 network is thought to underlie the comparison of current events with past experiences stored in CA3 (Lee et al. 2004). Such anatomo-functional dissociation of learning-specific activity might support contrasting effects of 5-HT₄R activation on LTP within the different subfields of hippocampal formation. Finally, considering both LTP and LTD as the two main synaptic plasticity processes, their balance—rather than LTP alone—would inform

about expected memory performances (Hanson and Madison 2010). Interestingly, preliminary unpublished data demonstrated that 5-HT₄R activation leads to an enhanced LTD magnitude. Therefore, modulation of 5-HT₄R is of importance to maintain synaptic plasticity within a nonexcessive range.

Supplementary Material

Supplementary material can be found at *Cerebral Cortex* online.

Notes

Authors wish to warmly thank Nicole Turnbull for proofreading the manuscript, spelling corrections and editing English. *Conflict of Interest*: None declared.

Funding

Normandy University and Normandy Regional Health Agency (Pharmacy residency of Dr. Pierre Lecouflet).

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Summary

➤ Does 5-HT₄Rs activation influence CA1 hippocampal synaptic plasticity *ex vivo*?

This study demonstrated that **5-HT₄Rs activation influences CA1 hippocampal synaptic plasticity independently of the control of other brain areas.**

We showed a protocol-dependent impairment of *ex vivo* CA1 hippocampal LTP following 5-HT₄Rs pharmacological activation by direct bath perfusion of RS67333. While no change in LTP magnitude was reported following HFS protocol, RS67333 impaired LTP induced by TBS protocol, which is more sensitive to the GABAergic transmission modulation.

➤ What are the mechanisms underlying LTP reduction?

Given the higher sensitivity of the TBS protocol, compared to the HFS one, to the GABAergic transmission, we further investigated the putative role played by the GABA-R in the effects of 5-HT₄Rs activation on TBS induced LTP. We showed that the LTP reduction induced by 5-HT₄Rs activation was dependent on GABA_A-R albeit no change in GABA_A-R excitability was observed. In addition, RS67333 mimicked the effects of a GABA_B-R antagonist which decreases glutamate and GABA release.

Altogether, even if the exact nature of the interaction remains to be determined, we highlighted an **interplay between 5-HT₄Rs signaling and GABAergic neurotransmission** within CA1 hippocampal plasticity.

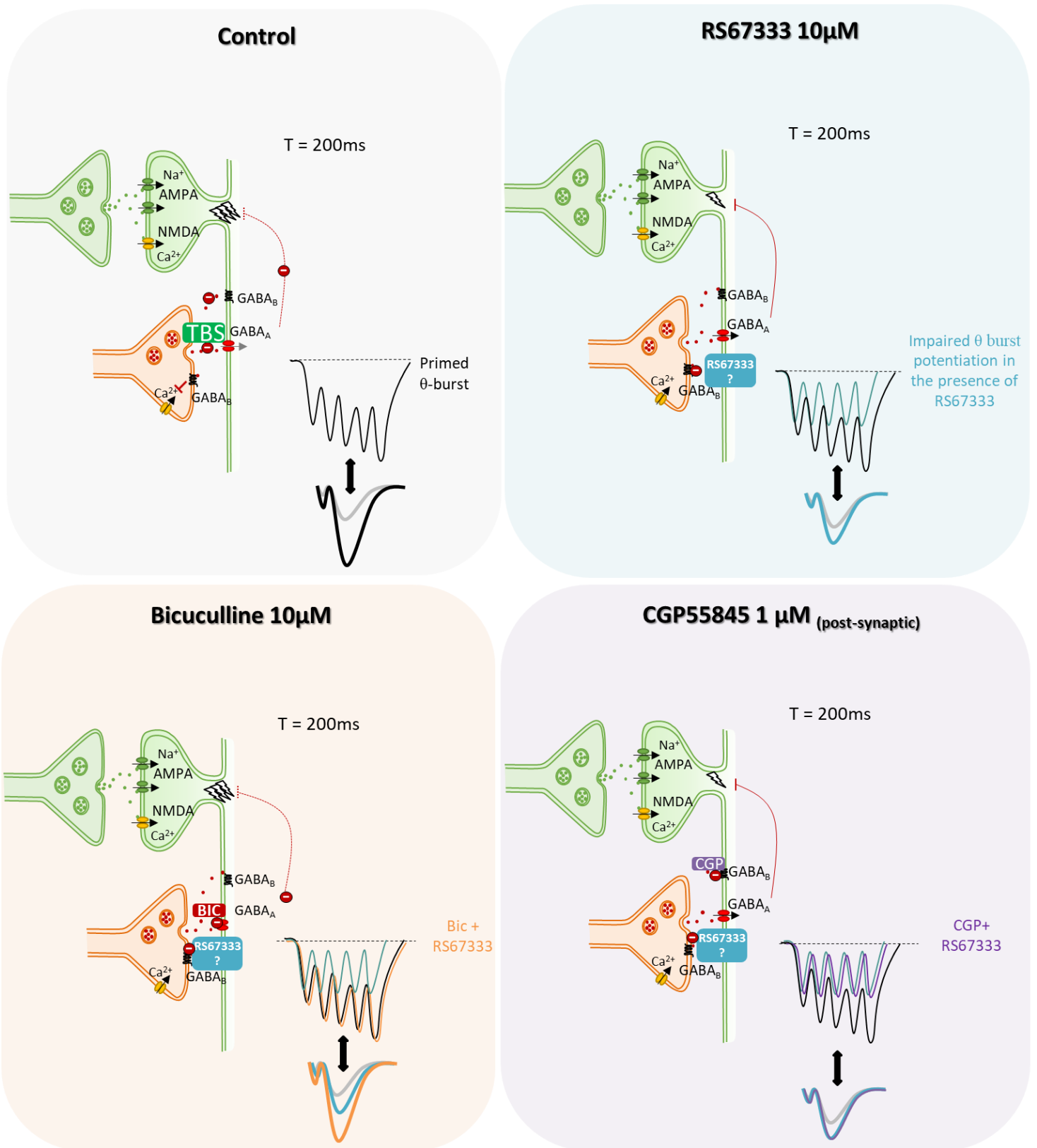


Figure 20: Putative mechanism of action of RS67333 on hippocampal synaptic plasticity

(Adapted from Larson & Munkácsy, 2015)

COMPLEMENTARY RESULTS

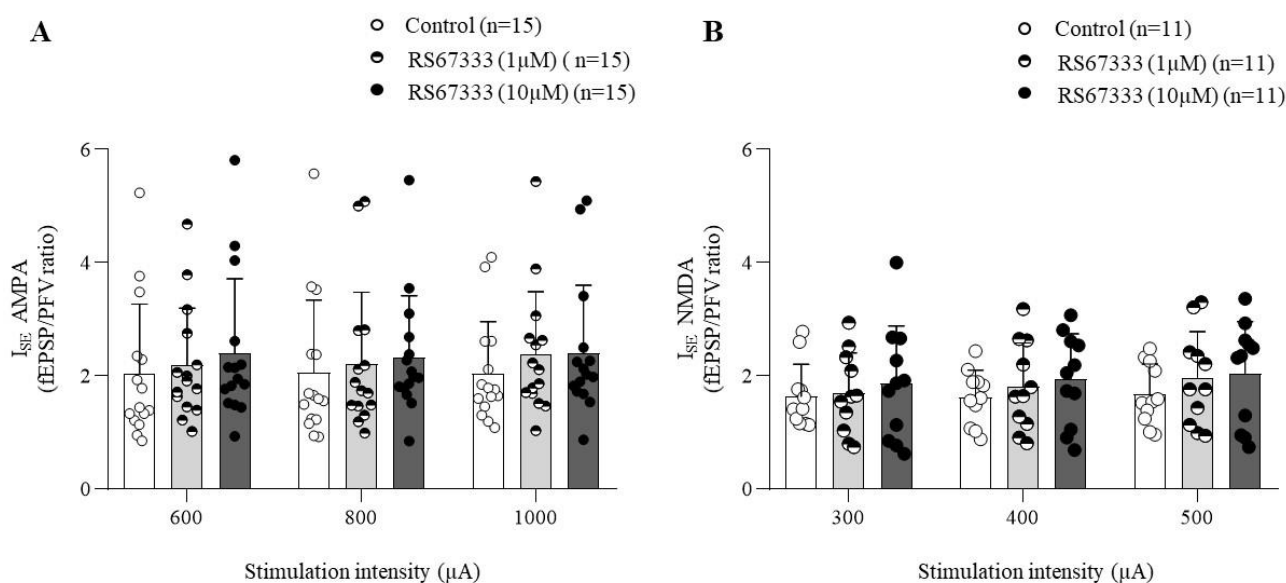
Supplementary methods:

For basal glutamatergic transmission investigation, the NMDA receptor antagonist APV was added to the bath (50 μ M). To investigate NMDAR activity, fEPSP were recorded in a low magnesium aCSF (0.1mM) supplemented with the non-NMDA receptor antagonist NBQX (10 μ M). fEPSP and fiber volley (FV) slopes were recorded at increasing stimulus intensity, varying from 600 to 1000 μ A and 300 to 500 μ A, respectively for basal glutamatergic transmission and NMDAR activity. Index of synaptic efficacy (I_{SE}) corresponds to fEPSP slope/FV slope ratio. To ascertain its specificity of action, RS67333 (10 μ M) was tested in 5-HT₄Rs KO mice (Jackson lab, n=13), as well as in wild type mice in a competitive condition with combination of RS39604 (1 μ M, a highly selective antagonist of 5-HT₄R, n=8) and finally at very low dose (1 μ M, n=12). To further characterize 5-HT₄R effects on LTP, we investigated contribution of post-synaptic GABA_B receptors using GCP35348 at 200 μ M (selective post-synaptic GABA_BR antagonist) in combination with RS67333 at 10 μ M (n=10).

Supplementary results:

RS67333 (5-HT₄R agonist) did neither affect basal glutamatergic transmission, nor NMDA activation

Glutamatergic signaling is at the heart of excitatory transmission system. Besides, LTP expression is closely related to activation of the NMDA subtype of glutamate receptors. Thus, we looked at the effects of RS67333 on both basal neurotransmission (AMPA-mediated, n=15 for all groups) or isolated NMDA-mediated current (n=11 for all groups). ANOVA with repeated measurement revealed neither group effect (respectively, $F_{(2,42)}=0.351$ and $F_{(2,30)}=0.543$, $p=0.7063$ and $p=0.5864$), nor stimulation intensity effect (respectively, $F_{(2,54)}=0.413$ and $F_{(2,60)}=1.686$, $p=0.6630$ and 0.1939), or group x stimulation intensity interaction (respectively, $F_{(4,84)}=0.29$ and $F_{(4,60)}=0.282$, $p=0.8800$ and 0.8886) (**suppl figures 1A and 1B**). Whatever the dose considered, RS67333 did not modify activity of AMPA- or NMDA-mediated currents

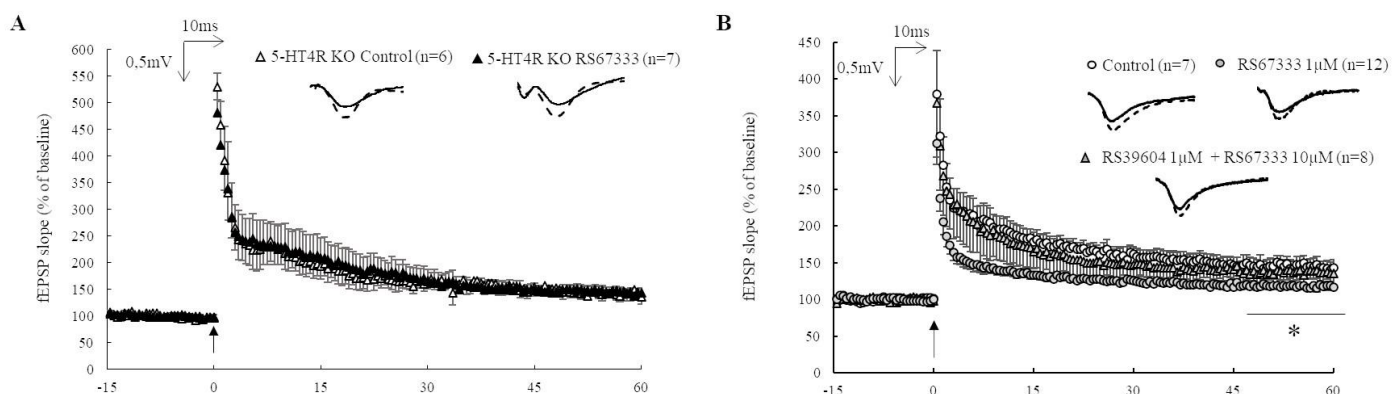


Supplementary figure 1: Effects of pharmacological activation of 5HT₄Rs (RS67333) on index of either AMPA or isolated NMDA receptors synaptic efficacy.

Effects of RS67333 on AMPAR-Rs-mediated transmission. (**B**) Effects of RS67333 on NMDA-Rs-mediated transmission. (I_{SE} defined as the fEPSP/PFV ratio) was displayed according to intensity stimulation.

Selectivity towards 5-HT₄Rs – specificity of action of RS67333 on TBS-induced LTP.

A. With 5-HT₄Rs KO mice (Jackson Laboratory, n=6 for control condition and 7 for RS67333). ANOVA with repeated measurements of mean fEPSP slopes during the last 15min revealed neither group effect ($F_{(1,11)}=0.001$, $p=0.9748$), nor time effect ($F_{(29,319)}=1.123$, $p=0.3070$), and no group x time interaction ($F_{(29,319)}=1.320$, $p=0.1299$). **B.** ANOVA with repeated measurements of mean fEPSP slopes during the last 15min revealed a group effect ($F_{(2,24)}=4.223$, $p=0.0268$), due to a statistical difference only between low dose RS67333 and control group ($p=0.0124$, $n=7$) (**supplementary figure 2**). Altogether, these results demonstrated the specificity of action of RS67333 on LTP, through 5-HT₄Rs.

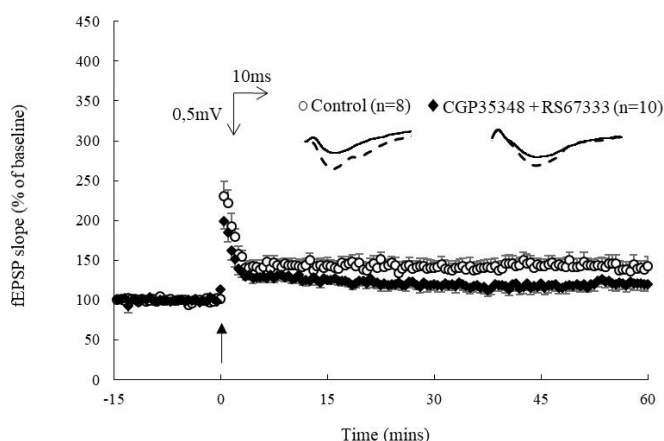


Supplementary figure 2: Specificity of action of RS67333 towards 5HT₄Rs

Data are expressed as mean±SEM. **(A)** Time course of fEPSP slope after TBS-induced LTP in 5-HT₄R KO mice with application of RS67333 (10µM). **(B)** Time course of EPSP with low dose of RS67333 (1µM) and with combined application of working dose of RS67333 (10µM) and RS39604 (selective 5HT₄R antagonist, 1µM). Insets show representative traces of fEPSPs (* $p<0.01$ versus control).

CGP35348 (selective post-synaptic GABA_BR) alleviated RS67333 impaired TBS-induced LTP.

ANOVA with repeated measurements revealed neither group effect ($F_{(-1.16)}= 4.146$ $p=0.0586$), nor time effect ($F_{(31.496)}=0,7558$; $p=0.08283$), and group x time interaction ($F_{(31.496)}=1.361$, $p=0.057$) (**suppl figure 3**). Thus, post-synaptic GABA_B receptor blockade prevents the suppressive effect of RS67333 on TBS-induced LTP.



Supplementary figure 3: Effects of pharmacological activation of 5HT₄Rs (RS67333, 10µM) on evoked fEPSP slopes after TBS-induced LTP in postsynaptic GABA_BR blockage condition.

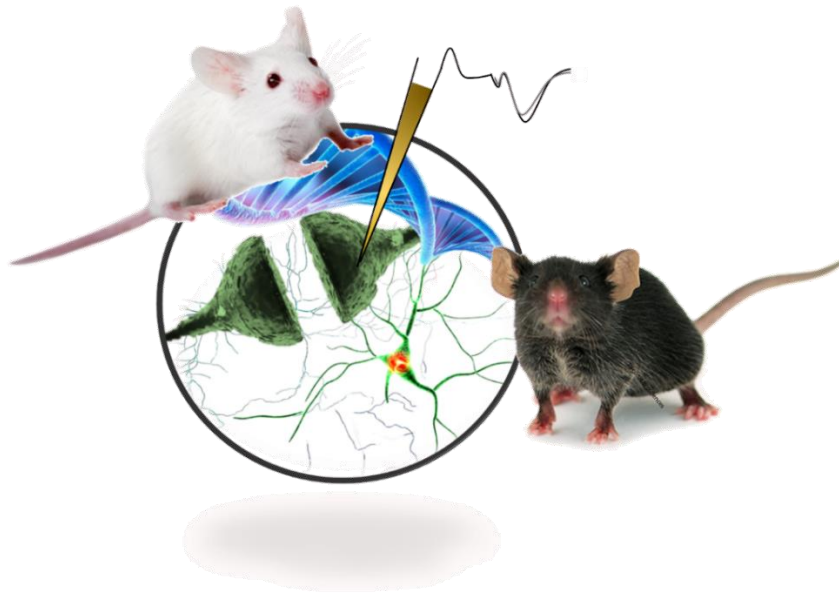
Data are expressed as mean±SEM. CGP35348 (200µM) was used as selective postsynaptic GABA_ARs antagonist. Insets show representative traces of fEPSPs

ARTICLE 2

ARTICLE 2: Genetic background influence on hippocampal synaptic plasticity: frequency-dependent variations between an inbred and an outbred mice strain.

Authors: Candice M Roux, Pierre Lecouflet, Jean-Marie Billard, Elise Esneault, Marianne Leger, Pascale Schumann-Bard and Thomas Freret.

Published in *IJMS* (2023)



Contextual overview:

NMRI mice belong to a Swiss-type outbred strain and have long been widely used in experimental biology including in Neurosciences. However, outbred strains are often snubbed due to their believed high level of heterogeneity in terms of experimental outcomes. Additionally, the growing interest for transgenic animal models contributed to put outbred strains on the sideline in preclinical research to the advantage of inbred mice strains – and notably C57BL/6 - which progressively became the most popular choice for scientists.

Over the last decade, important consideration has raised as regard to the choice of the sub-strain. Indeed, several studies reported significant differences in terms of LTP magnitude according to the genetic background. While strains differences have been investigated in a number of mice and rats inbred sub strains, such differences have never been apprehended between outbred *versus* inbred strains.

Finally, at COMETE laboratory, NMRI mice have been well-characterized from behavioral standpoint (Freret et al., 2017a; Leger et al., 2015; Lelong et al., 2003) as well as in experiments involving electrophysiological recordings (**see Article 2**). However, further experiments aiming at confirming the involvement of GABAergic neurotransmission in the effects of RS67333 using optogenetic technique led us to resort to the use of transgenic mice on a C57BL/6Rj background.

In this second study, we therefore addressed the following issue:

- **Is CA1 hippocampal synaptic plasticity similar between NMRI outbred mice stock and C57BL/6Rj inbred strain**



Article

Genetic Background Influence on Hippocampal Synaptic Plasticity: Frequency-Dependent Variations between an Inbred and an Outbred Mice Strain

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Abstract: For almost half a century, acute hippocampal slice preparations have been widely used to investigate anti-amnesic (or promnesic) properties of drug candidates on long-term potentiation (LTP)—a cellular substrate that supports some forms of learning and memory. The large variety of transgenic mice models now available makes the choice of the genetic background when designing experiments crucially important. Furthermore, different behavioral phenotypes were reported between inbred and outbred strains. Notably, some differences in memory performance were emphasized. Despite this, investigations, unfortunately, did not explore electrophysiological properties. In this study, two stimulation paradigms were used to compare LTP in the hippocampal CA1 area of both inbred (C57BL/6) and outbred (NMRI) mice. High-frequency stimulation (HFS) revealed no strain difference, whereas theta-burst stimulation (TBS) resulted in significantly reduced LTP magnitude in NMRI mice. Additionally, we demonstrated that this reduced LTP magnitude (exhibited by NMRI mice) was due to lower responsiveness to theta-frequency during conditioning stimuli. In this paper, we discuss the anatomo-functional correlates that may explain such hippocampal synaptic plasticity divergence, although straightforward evidence is still lacking. Overall, our results support the prime importance of considering the animal model related to the intended electrophysiological experiments and the scientific issues to be addressed.

Keywords: synaptic plasticity; genetic background; hippocampus; electrophysiology; memory



Citation: Roux, C.M.; Lecouflet, P.; Billard, J.-M.; Esneault, E.; Leger, M.; Schumann-Bard, P.; Freret, T. Genetic Background Influence on Hippocampal Synaptic Plasticity: Frequency-Dependent Variations between an Inbred and an Outbred Mice Strain. *Int. J. Mol. Sci.* **2023**, *24*, 4304. <https://doi.org/10.3390/ijms24054304>

Academic Editor: Hiroki Toyoda

Received: 31 December 2022

Revised: 8 February 2023

Accepted: 9 February 2023

Published: 21 February 2023



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1. Introduction

Hippocampal synaptic plasticity is widely known as a key cellular support for memory [1]. Almost half a century ago, long-term potentiation (LTP, i.e., an activity-dependent enhancement of synaptic transmission) was described in mammalian brains, first in vivo in the anesthetized rabbit [2], then shortly after ex vivo in guinea pig hippocampal slices [3]. Since these pioneer experiments, thousands of papers have been published on LTP, using both different experimental protocols and biological materials [4].

Among existing conditioning protocols, high-frequency stimulation (HFS) and theta-burst stimulation (TBS) are the two electrical stimulations that have been the most widely used to induce LTP within the CA1 area of the hippocampus [1,5]. Both mimic naturally occurring hippocampal electric oscillations. Respectively described as gamma- γ - (30–100 Hz) and theta- θ - (4–12 Hz) rhythms, these oscillatory frequencies are observed during spatial and contextual learning [6–8]. Therefore, considering the pivotal role of hippocampal LTP in memory, it became common to correlate changes in LTP strength with behavioral performance in hippocampal-dependent tasks [9]. The HFS protocol—as the first historically described pattern of LTP induction—still remains the most often used, but the

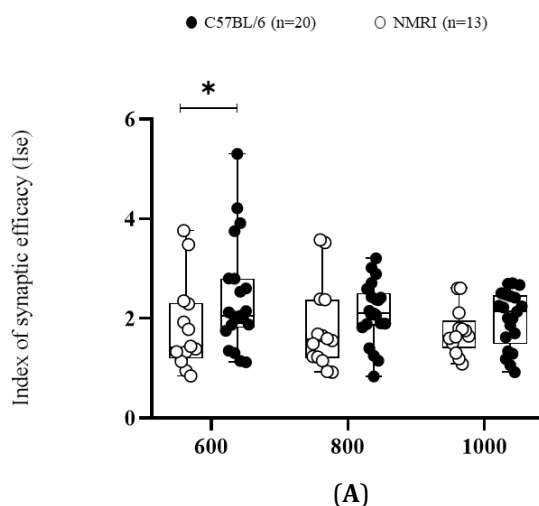
TBS pattern appears to be favored when investigating modulations of neurotransmission systems, particularly the GABAergic tone (GABA: γ -aminobutyric acid) [5].

Although the first LTP experiment was performed on a rabbit, most works carried out since in neuroscience research have been conducted in mice [10]. Their use has enabled major breakthroughs over the last decades, notably in the understanding of memory processes and for better management of neurological diseases with memory impairments [10]. To this end, hundreds of different mice strains, resulting from careful breeding for the selection of desirable phenotypes, have been designed. This large variety of mice strains now offers a wealth of choices of biological materials, but raises, at the same time, the question of biological variability between genotypes. The same holds true when considering the nature of neurobiological mechanisms in LTP induction and development.

Historically, pharmacological and behavioral research used outbred mice strains [11,12], defined as a closed population (for at least four generations) of genetically variable mice, thus displaying a high level of heterozygosity [13]. However, following the intensive and rapid development of genetic engineering, inbred mice (stemming from 20 consecutive generations of sibling mating) became by far the most popular choice for scientists [14]. Inbred strains yield a stable genetic background for the generation of a wealth of transgenic mice. As early as the 1920s, several lines of evidence started to demonstrate the profound influence of the genetic background on behavioral outcomes. Previously, synaptic plasticity correlates of memory were mainly investigated between inbred strains or sub-strains [9,15] and only one study aimed at comparing inbred and outbred strains of mice [16]. Hence, the research community ran a number of studies on both strain subtypes with little awareness of the importance of strain selection while interpreting the results. Among the most widely used inbred and outbred mice subtypes are C57BL/6 and NMRI mice, respectively. Few studies were aimed at comparing the memory capacities of these two strains, and the expression of synaptic plasticity had not yet been investigated. Hence, the objective of this study was to investigate and compare the functional synaptic plasticity at CA3/CA1 hippocampal synapses of inbred C57BL/6 strains and outbred NMRI stock.

2. Results

First of all, the efficacy of basal synaptic transmission was measured using the index of synaptic efficiency (ISE) corresponding to the field excitatory post-synaptic potential (fEPSP) slope/pre-synaptic fiber volley (Fv). This ratio significantly differed between strains ($F_{(1,\infty)} = 5.3249$, $* p < 0.05$). C57BL/6 exhibited a higher ISE compared to NMRI mice at the lowest stimulation intensity (600 mV) (Figure 1A).



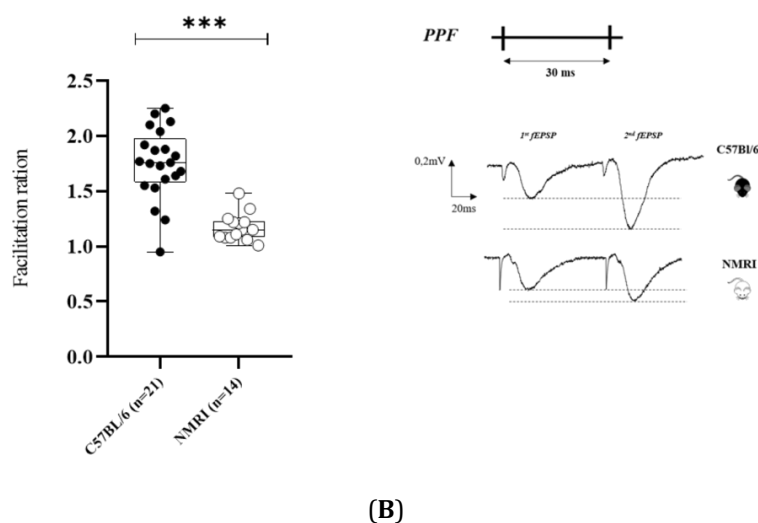


Figure 1. Basal synaptic transmission and paired-pulse facilitation are higher in C57BL/6 than in NMRI. **(A)** Basal synaptic transmission determined by I/O curves in slices from C57BL/6 mice ($n = 20$ slices/ $n = 14$ mice) and NMRI mice ($n = 13$). Data are expressed as median \pm interquartile. ANOVA for repeated measures ($* p < 0.05$). **(B)** Facilitation ratio in slices from C57BL/6 mice ($n = 21$ slices/ $n = 14$ mice) and NMRI mice ($n = 14$ slices/ $n = 11$ mice) (left). Stimulation pattern of PPF with corresponding representative traces of paired-pulse-induced fEPSP from NMRI and C57BL/6 mice (right). Each 2nd fEPSP slope was divided by the slope of the 1st fEPSP. Data are expressed as median \pm interquartile. Mann–Whitney U test ($*** p < 0.001$).

Pre-synaptic short-term plasticity reflected by paired-pulse facilitation (PPF) was also found to be decreased in NMRI compared to C57BL/6, as shown by the decreased facilitation index ($*** p < 0.001$) (Figure 1B).

As regards long-term plasticity recordings, it is worth mentioning that, independently of the mice strain, LTP was successfully induced within the CA1 field, as shown by the significant difference between the baseline and the last 15 min of recordings (Figure 2) using both the HFS protocol (C57BL/6: $143 \pm 4\%$; $### p < 0.001$, $n = 19$ and NMRI: $149 \pm 18\%$, $n = 13$; $### p < 0.001$ versus the theoretical value of 100%) and the TBS protocol (C57BL/6: $169 \pm 10\%$, $### p < 0.001$, $n = 19$ and NMRI: $127 \pm 4\%$, $n = 22$; $### p < 0.001$, vs. the theoretical value of 100%).

When making an inter-strain comparison, using the HFS protocol (Figure 2A), the LTP magnitude did not differ between strains ($143 \pm 4\%$ versus $149 \pm 18\%$ for C57BL/6 and NMRI, respectively). Nevertheless, the coefficient of variation (CV), which is an index of within-strain variability, was higher in NMRI mice than in C57BL/6 ($*** p < 0.0001$, cf. Table 1).

Conversely, the magnitude of TBS-induced LTP was higher in hippocampal slices of C57BL/6 mice than in NMRI ones ($169 \pm 10\%$ versus $127 \pm 4\%$). Statistical analysis of the last 15 min of fEPSP recordings revealed a strain effect ($F_{(1,\infty)} = 24.7450$, $*** p < 0.001$). No significant effect of time ($F_{(11,\infty)} = 1.4467$, $p = 0.1415$) or group \times time interaction ($F_{(11,\infty)} = 0.9134$, $p = 0.5283$) was detected (Figure 2B). The strain difference was confirmed when the medians of the last 15 min of recording were compared between groups (Mann–Whitney, $*** p < 0.001$). Furthermore, NMRI displayed a lower CV than C57BL/6 mice ($*** p < 0.001$, cf. Table 1).

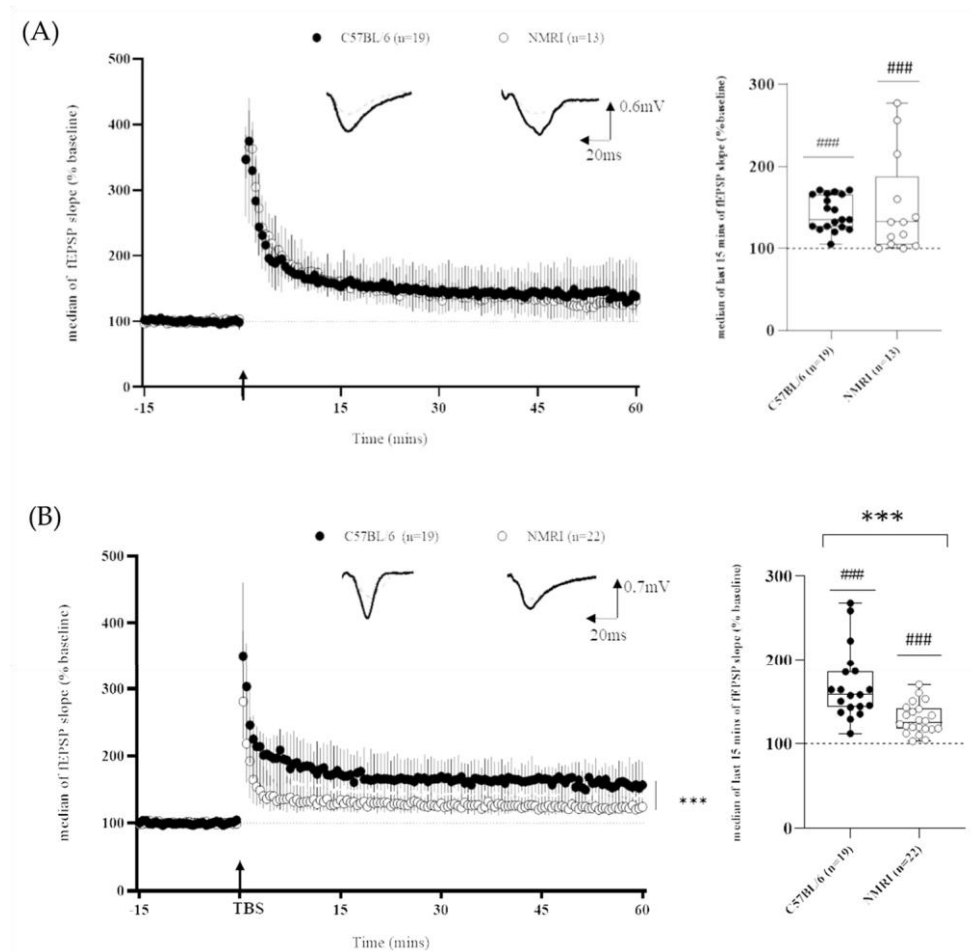


Figure 2. Differential LTP expression in outbred and inbred mice strains depends on stimulating frequency. **(A)** Time course of fEPSP slope after HFS-induced LTP in slices from C57BL/6 mice ($n = 19$ slices/ $n = 14$ mice) and NMRI mice ($n = 13$ slices/ $n = 10$ mice) (left) and corresponding last 15 min of fEPSP slope (right). **(B)** Time course of fEPSP slope after TBS-induced LTP in slices from C57BL/6 mice ($n = 19$ slices/ $n = 13$ mice) and NMRI mice ($n = 22$ slices/ $n = 18$ mice) (left) and corresponding last 15 min of fEPSP slope (right). Data are expressed as median \pm interquartile. Arrow marks the time when conditioning stimulation was applied. Insets show representative traces of fEPSP before (dashed line) and after (full line) conditioning stimulation (univariate test, ### $p < 0.001$: last 15 min of recording versus theoretical value of 100%; ANOVA for repeated measures *** $p < 0.001$ and Mann–Whitney U test *** $p < 0.001$ for inbred versus outbred comparison).

Interestingly, the facilitation (AUC of second burst relative to the first one) was clearly marked in C57BL/6 mice (### $p < 0.001$), while it was more discreet and non-significant in NMRI mice (Figure 3A).

Thus, consistent with their higher exhibited TBS-LTP magnitude, C57BL/6 mice also showed a higher degree of facilitation across bursts compared to NMRI mice (* $p < 0.05$; *** $p < 0.001$) (Figure 3B).

Table 1. Summary of phenotypic properties and main electrophysiological results in C57BL/6 and NMRI mice.

Zootechnical Parameters			
Strain	C57BL6/Rj	NMRI	
Genetics	Inbred	Outbred	
Coat	Black	White (albino)	
Litter size at birth	6.53	14.8	Janvier Labs 2011 data
Median life span (months)	27–31	17	Gower & Lamberty, 1993
Spatial behavioral performance (Morris water maze)			
Learning rate	C57BL6 vs. NMRI		
Escape latency	No difference C57BL6 < NMRI		Klapdor et al., 1996; Salari et al., 2018 Vicens et al., 1999; Vicens et al., 2002
Memory performance	C57BL6 vs. NMRI		
Time spent in target quadrant	No difference		Vicens et al., 2002; Salari et al., 2018; Klapdor et al., 1996; Vicens et al., 1999
Platform crossing	No difference		Salari et al., 2018; Klapdor et al., 1996
(Ex vivo) hippocampal synaptic plasticity			
HFS-LTP	C57BL6	NMRI	
Magnitude (%)	143 (+/-4)	149 (+/-18)	No difference
Coefficient of variation (%)	12%	41%	C57BL/6 < NMRI
TBS-LTP	C57BL6	NMRI	
Magnitude (%)	169 (+/-10)	127 (+/-4)	C57BL/6 > NMRI
Coefficient of variation (%)	26%	14%	C57BL/6 > NMRI

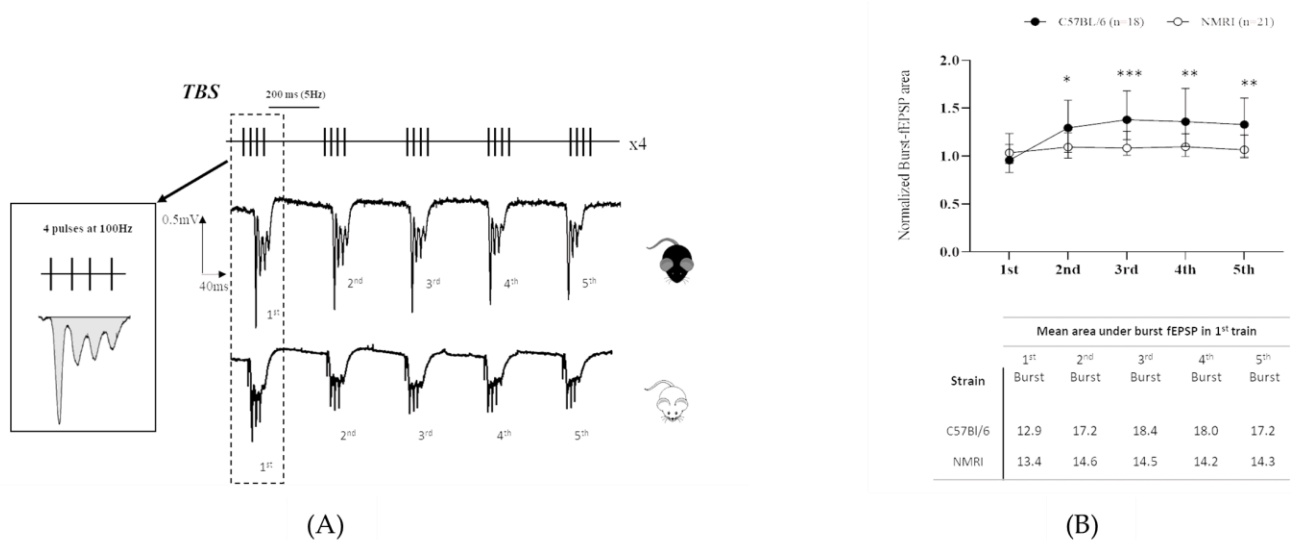


Figure 3. C57BL/6 mice exhibit higher degree of facilitation than NMRI outbred stock during TBS. **(A)** Typical TBS pattern of stimulation with corresponding sample trace of burst-induced fEPSP from NMRI and C57BL/6 mice. Area of burst-induced fEPSPs was measured as the total fEPSP areamarked in shadow. **(B)** Normalized burst-fEPSP areas from C57BL/6 mice ($n = 18$ slices/ $n = 12$ mice) and NMRI mice ($n = 21$ slices/ $n = 17$ mice). Each burst-fEPSP was normalized to the first one. Data are expressed as median \pm interquartile. Mann–Whitney U test for comparison of 2nd vs. 1st burst (### $p < 0.001$) and for comparison between C57BL/6 and NMRI groups: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3. Discussion

Hippocampal synaptic excitability/plasticity is often discussed independently of mice-strain considerations. Here, we showed for the first time the existence of different electrophysiological profiles from the dorsal hippocampus between inbred and outbred mice strains and therefore shed light on the need to pay attention to the genetic background.

Basal hippocampal synaptic transmission was found to be higher in C57BL/6 mice than in NMRI mice. This electrophysiological parameter reflects glutamate transmission (i.e., neurotransmitter release and subsequent AMPA receptor activity/recruitment and/or densities). Similarly, functional short-term plasticity (PPF) was higher in C57BL6 mice compared to outbred NMRI mice. PPF reflects presynaptic calcium signaling that influences the probability of neurotransmitter release (Pr). During the second stimulus, Pr depends directly on the remaining (after the first stimulus) presynaptic stock of free calcium. Therefore, the higher the stock, the higher the Pr will be, but the lower the PPF will be. To sum up, these first results demonstrated that C57BL/6 mice are more prone (than NMRI ones) to presynaptic discharge in response to electrical stimuli.

Finally, we unveiled long-term plasticity differences using two conditioning protocols (TBS and HFS). While both mice strains displayed comparable levels of LTP following the HFS protocol, C57BL6 mice displayed a higher level of TBS-induced LTP compared to NMRI mice.

At the post-synaptic site, the *N*-methyl-D-aspartate receptor subtype of glutamate receptors (NMDA-R) is critical to LTP induction, and previous experiments interestingly showed genetic differences in NMDA-R/glutamate receptor-channel complex expression [17]. However, this is unlikely to explain our results, as no difference was noted when using the HFS-induced LTP protocol (which also relies on NMDA-R). A deeper investigation of plasticity-related proteins or molecules that are preferentially involved in TBS would be of interest. An LTP deficit in inbred DBA/2 mice was, for instance, previously shown to correlate with a decrease in hippocampal protein kinase C compared with the C57BL/6 mice strain (Matsuyama et al., 1997). Similarly, given that pyramidal cell excitability is a determinant for LTP induction, it would be worth investigating underlying molecular mechanisms, such as type 5 metabotropic glutamate receptors (mGluR5) [18], potassium channels [19] or frequency-dependent cell responsiveness [20].

Of note, our results are somewhat consistent with previous data from the literature. For instance, LTP differences between C57BL/6 and other inbred sub-strains studied were strikingly pronounced when using the TBS protocol, whereas they were more subtle when following the HFS protocol [9].

Despite the number of studies performed in one or another strain subtype, differences between inbred and outbred strains have been, and unfortunately remain, poorly investigated. Few anatomical differences were identified in inbred C57BL/6 when compared with outbred NMRI, the most striking differences being a higher density of both the mossy fiber layer (MF) and excitatory dentate granule cells, as well as longer infrapyramidal MF projections [21–23]. In a way, these anatomical differences are in accordance with the highest TBS-induced LTP magnitude observed in C57BL6 mice. According to the hippocampus tri-synaptic loop model, the CA1 neuronal response, as we measured it, should be shaped by both the dentate gyrus (DG) and MF, which, respectively, act upstream as either a preprocessor or detonator [24,25].

Conversely, these anatomical differences are at odds with a similar level of HFS-induced LTP. However, this similarity could easily be explained by the common hippocampal neurochemical profile and histochemical pattern between strains of mice [26]. Nevertheless, this argument should be taken with caution since not all neurotransmission systems have been investigated so far. For instance, the GABAergic system is clearly differently involved in the two LTP conditioning protocols [27]. Contrary to HFS, TBS-induced LTP closely relies on pyramidal cell disinhibition, which is dependent on GABAergic neurotransmission. The feedforward inhibition of pyramidal cells (“priming”) that occurs

early during induction of LTP is then suppressed through the auto-inhibition process for about a second. With respect to 200 ms inter-burst intervals, these ensure maximal postsynaptic pyramidal cell depolarization, hence resulting in the highest LTP magnitude. Therefore, the efficacy of TBS-induced LTP (LTP magnitude) relies on the degree of facilitation that can be estimated through the measurement of the area under the curve (AUC) during the five bursts in the first train of stimulation. Interestingly, we showed that the higher exhibited TBS-LTP magnitude in C57BL/6 mice was consistent with a higher degree of facilitation across bursts compared to NMRI. Unfortunately, a clear-cut conclusion is dampened by the few comparative studies regarding the genetic background's influence on neurotransmission systems, including the GABAergic one. Since the outcome of GABAergic inhibition could be interpreted multi-fold depending on the receptors targeted (either all chloride channels by picrotoxin, post-synaptic GABA_A-Rs or pre- or post-synaptic GABA_B-Rs), further comparative studies may benefit from the investigation of GABA-R densities and their morphology as well as their functional properties (i.e., conductance, membrane resistance, capacitance, etc.).

Ultimately, our work also raises the question of whether or not LTP induced artificially could be considered a model for learning mechanisms. In other words, whether or not the slight discrepancies in *ex vivo* hippocampal synaptic plasticity between strains affect behavioral performance, especially when spatial cognitive functions are involved. Unfortunately, comparisons between behavioral studies are scarce, and results remain partly divergent. To the best of our knowledge, four studies have so far compared spatial memory performances (Morris water maze) in C57BL/6 versus NMRI mice (see Table 1). Most often, a similar level of performance (both during the learning phase and probe test) is described [28–31], thus in agreement with *ex vivo* results for HFS-LTP. In previous research, when notable, the discrepancy was in favor of NMRI mice, which displayed a faster learning rate [30,31]. This last result contrasts with the higher level of TBS-LTP observed in C57BL/6 mice. The MWM test already proved to be sensitive to D-AP5, an NMDA-R antagonist (D,L-2-amino-5-phosphonovaleric acid) also known to induce an LTP deficit [32,33]. Consequently, this test is appropriate to compare memory performance between two strains in line with their electrophysiological pattern. However, the aversive character of water should be kept in mind, as it could interfere with the result as soon as a strain-different sensitivity to stress can be highlighted [34]. In addition, dissociation between memory performances and the LTP level has been described several times [35,36]. Although useful to catch underlying molecular mechanisms of the memory process, a direct correlation of LTP level (either *ex vivo* or *in vivo*) with learning performance (as just done) is not always true and is often a too simplistic way of thought. Neither are there only two types of LTP, nor only one brain structure involved in learning and memory processes.

4. Materials and Methods

4.1. Animals

All experiments were carried out in accordance with the European Community guidelines (2010/63EU) and the French law on animal experimentation. Electrophysiological experiments were conducted on dorsal hippocampal slices obtained from adult male mice aged 3–6 months from either C57BL6/Rj or NMRI strains (respectively, 28 ± 0.8 g and 29 ± 0.3 g) (purchased from Janvier Labs, France). Mice were housed in groups of 8 in standard polycarbonate cages, with food and water given *ad libitum*. The animal facility was under a reversed 12:12 light–dark cycle (light off at 7am), with a controlled environment in temperature (22 ± 1 °C) and hygrometry ($55 \pm 10\%$).

4.2. Electrophysiological Recordings

As previously described [37], transverse hippocampal slices (400 μ m thick) were prepared using a tissue chopper (McIlwain®). Briefly, field excitatory postsynaptic potentials

(fEPSPs) were recorded from the CA1 area using glass micropipettes following stimulation of the Schaffer collateral axons with the bipolar tungsten electrode (Figure 4A). fEPSPs are commonly the first-in-use method to enable rapid and easy sampling of population synaptic responses resulting from glutamatergic transmission within hippocampal slice preparations [38].

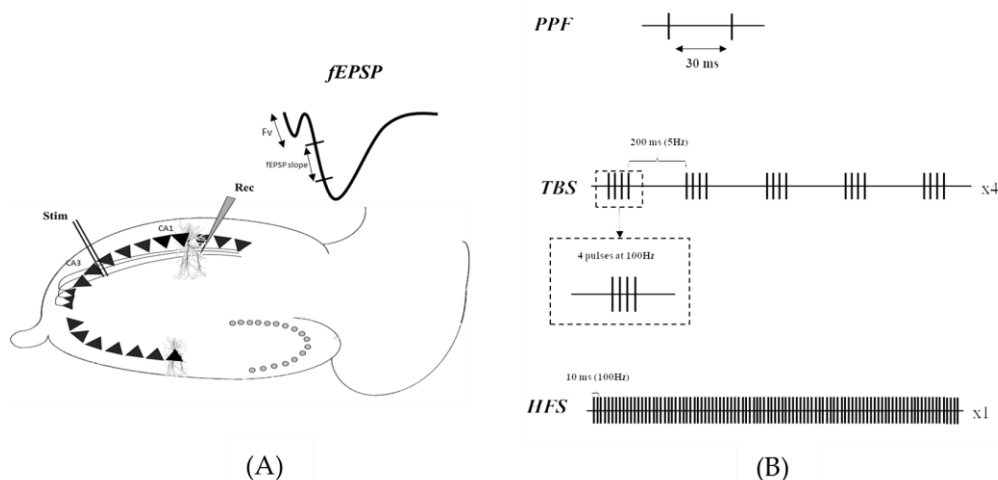


Figure 4. (A) Experimental setup, showing stimulating (Stim) and recording (Rec) electrodes placed in the stratum radiatum of the Schaffer collateral and CA1 area, respectively. (B) Stimulation pattern of either HFS or TBS used to induce LTP.

Basal synaptic transmission was assessed through input/output (I/O) curves consisting of stimulation of increasing intensities (600, 800 and 1000 μ A).

In addition, pre-synaptic changes were investigated using the paired-pulse facilitation (PPF) protocol, described as two electrical stimuli applied at 30 ms intervals (Figure 4B). Stimulation intensity was set to elicit a first fEPSP slope of 0.05 mV/s. This experimental procedure allows us to avoid bias in the interpretation of the results due to a difference in the basal synaptic transmission level.

The ability of the CA3-CA1 synapse to undergo long-term plasticity was assessed through LTP recordings. The stimulation intensity was set to elicit an fEPSP slope of 0.1 mV/s. Following a stable 15 min baseline (0.1 Hz test pulse), LTP was induced using either HFS (100 pulses at 100 Hz) or TBS (5 bursts at 5 Hz consisting of 4 pulses at 100 Hz—and repeated 4 times at 0.1 Hz, Figure 4B) protocols.

4.3. Data Analyses

An index of synaptic efficacy (ISE) corresponding to the fEPSP/Fv ratio was calculated to compare basal synaptic transmission based on I/O curves. PPFs were analyzed using a facilitation index determined as the ratio of the slope of the second fEPSP to the slope of the first one (fEPSP2/fEPSP1).

The last 15 min of LTP recordings, reflecting its magnitude, were used for statistical analysis. In addition, for the TBS protocol, the trapezoidal method was used to measure the area under curve (AUC) of responses of the first five bursts. We then evaluated the potentiation of each burst relative to the first one (AUC_n/AUC₁) to assess the efficacy of the TBS conditioning. On-line acquisition and off-line analyses of bursts AUC and fEPSP slopes were performed using WinLTP® software [39]. A fixed 1 ms cursor was placed just after the fiber volley, thus allowing the software to calculate the fEPSP slope along the linear zone [40].

4.4. Statistical Analyses

ANOVA for repeated measures was used to compare I/O curves with the last 15 min of LTP recordings between groups. PPF and the medians of the last 15 min of LTP recordings of each group were compared using the Mann–Whitney U test. In addition, a univariate test was used to compare the medians of the last 15 min of LTP magnitude of each group, with the value of 100% (Wilcoxon signed-rank test). Finally, the CVs were compared using a one-way ANOVA. All statistical analyses were performed using R® software and graphs were drawn using GraphPad Prism software version 8 (GraphPad Software Inc., San Diego, CA, USA).

5. Conclusions

To conclude, Yilmazer-Hanke stated that, “studying a certain strain for neuronal correlates of learning and memory mainly tells us something about this particular strain, but not necessarily about this species or other species” [41]. In line with this way of thought, our results underline the importance of the choice of mice strains before starting experiments and, above all, invite us to pay attention to the mice’s backgrounds when interpreting the results. Our results encourage the use of C57BL/6 background mice (which exhibit a higher magnitude of TBS-LTP) to investigate the alteration of synaptic plasticity, whereas NMRI strains would be more appropriate when LTP enhancement is expected. Furthermore, the intra-strain level of individual variability (higher either in C57BL/6 or in NMRI for, respectively, the magnitude of TBS- or HFS-LTP) should also be considered when designing a research protocol.

Author Contributions: The study was designed by T.F. and P.S.-B. The experiments were carried out by P.L., J.-M.B. and C.M.R. Data analysis was performed by C.M.R. Original draft preparation was carried out by C.M.R. Editing was performed by M.L., E.E. and T.F. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Convention Industrielle de Formation par la Recherche (CIFRE) fellowship (N°2018/0833) from the Association Nationale de la Recherche et de la Technologie (ANRT) as well as by Normandy University and Normandy Regional Health Agency.

Institutional Review Board Statement: The animal study was performed in accordance with French and European Economic Community guidelines for the care and use of laboratory animals (2010/63/UE).

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in the study are publicly available. The data can be found here: [10.6084/m9.figshare.19596610] accessed on 14 April 2022.

Conflicts of Interest: The authors declare no conflicts of interest.

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Summary

➤ Is CA1 hippocampal synaptic plasticity similar between NMRI outbred mice stock and C57BL/6Rj inbred strains?

This second study allowed to highlight differences in LTP magnitude between an outbred stock and an inbred strain according to the protocol used for LTP induction.

HFS-induced LTP were similar between the two strains. However, TBS-induced LTP magnitude was significantly higher in C57BL/6Rj mice as compared to NMRI ones.

The origin of such differences remains to be elucidated but could eventually be supported by anatomical and neurochemical differences to some extent. Above all, these results support that the effects of RS67333 could be investigated in mice from C57BL/6Rj mice background as previously described, with potentially a more pronounced effect of RS67333.

COMPLEMENTARY RESULTS

Results from [Article 2](#), argue in favor of the use of C57BL/6 related genetic background when reduced LTP levels are expected. In [Article 1](#), we demonstrated an interplay between 5-HT₄Rs activation and the GABAergic system leading to a decrease of TBS-induced LTP in NMRI mice. In order to confirm the involvement of the GABAergic system in the effects of 5-HT₄Rs on TBS-LTP, we resorted to optogenetic technique using transgenic mice constructed from C57BL/6 background. Transgenic mice expressed photosensitive Cl⁻ inhibitory channel (Archaeorhodopsin, ArchT) transgene specifically in GABAergic interneurons that were inactivated upon a laser exposure with determined wavelength.

Complementary methods

Mice were obtained following successive breedings of two transgenic mouse lines as described in details in the [Material and Methods](#) section as well as in [Appendix A](#). The expression of ArchT in hippocampal GABAergic interneurons was confirmed by immunofluorescent assay ([Appendix C](#)).

The setup for extracellular recordings was then adapted (lasers, protections) and experimental conditions (laser intensity, effects of long-lasting exposure *etc*) were tested. Due to the difficulties in obtaining mice of generation of interest (genetic defect inducing breast involution and fertility issues), we used mice from both sexes, aged of 4-7 months.

The involvement of GABAergic transmission in the RS67333-induced decrease of TBS-LTP was assessed through the silencing of GABAergic neurons during the TBS stimulation (induction) concomitant with bath application of RS67333 as previously described ([Article 1](#)). We expected (1) TBS-LTP to be reduced after RS67333 application (no laser) to confirm the effect of RS67333 are similar across strains (2) the laser exposure leading GABAergic inhibition to prevent or limit the RS67333-induced decrease of LTP.

Complementary results

First of all, in experimental conditions closed to that described in [Article 1](#) (RS67333 10μM for 15 mins prior TBS, no laser exposure), 5-HT₄Rs activation tends to a reduction - even if not significant (number of samples too low)- of LTP magnitude as compared to control condition (Laser OFF, control aCSF) ([Table 10](#)). This supports that 5-HT₄Rs activation by bath application of agonist has close effects on TBS-LTP in both NMRI mice and in mice with C57BL/6 genetic background.

Experimental results

Table 10: Summary of TBS-LTP magnitude upon optogenetic GABAergic silencing and following 5-HT₄Rs stimulation. Data are expressed as median \pm IQ.

	LASER OFF	LASER ON
Control aCSF	165% (146,228)	190% (123,217)
RS67333 10 μ M	149% (140,166)	177% (141,183)

Laser exposure in the absence of 5-HT₄Rs agonist (ON control conditions) resulted in non-significant increased of LTP magnitude as compared to the condition with no laser. Based on previous studies such increase of LTP magnitude was expected (Chapman et al., 1998; Grover & Yan, 1999).

Under laser stimulation, the effects of RS67333 were attenuated so that the magnitude of LTP increased to a level close to the control condition (ON control) (**Figure 21**).

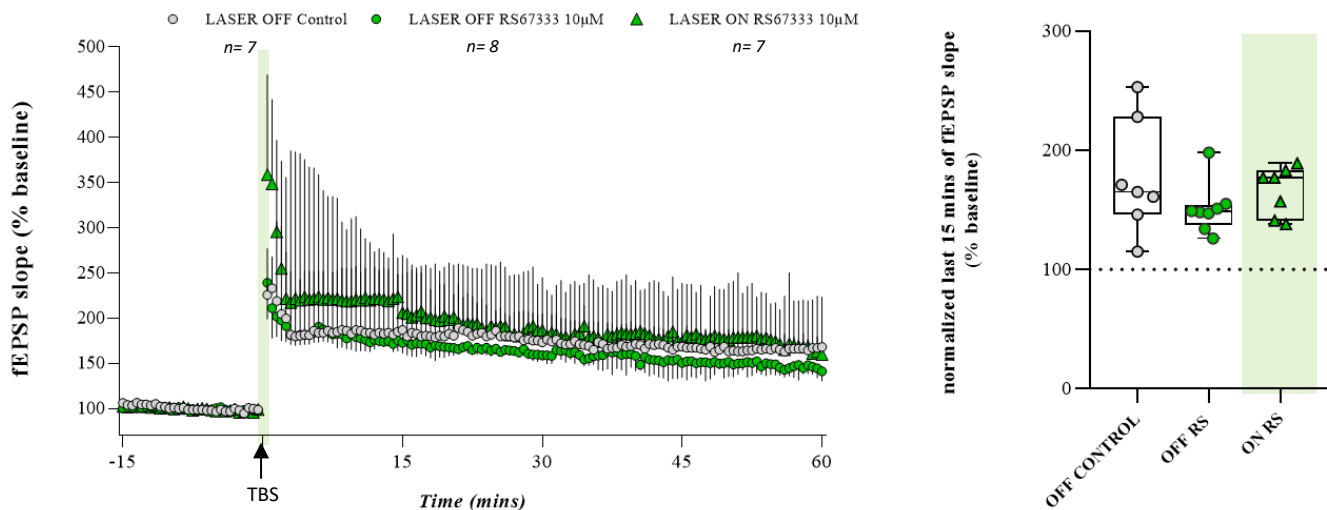


Figure 21: Effects of RS67333 under optogenetic silencing of GABAergic neurotransmission on TBS-induced LTP. Data are expressed as median \pm IQ. Green bar represents the duration of laser exposure for optogenetic inhibition of GABAergic interneurons. Arrow marks the time when conditioning stimulation (TBS) was applied. Time course of fEPSP slope after TBS protocol under optogenetic inhibition of GABAergic interneurons (left) and median of normalized last 15 mins of recordings after TBS (right) in the different conditions: Laser OFF in standard aCSF (OFF control), laser OFF combined with bath application of RS67333 10 μ M (OFF RS) and laser ON combined with RS67333 10 μ M (ON RS).

Altogether these results encourage the hypothesis of an involvement of the GABAergic system in the effects of 5-HT₄Rs activation on LTP. It is important to keep in mind that these results are preliminary and would benefit from supplementary recordings to soften the high variability displayed, which could have masked differences

ARTICLE 3

ARTICLE 3 : Beneficial effects of 5-HT₄R_s activation in mice: a transversal approach, from memory to its hippocampal correlates

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In preparation

Contextual overview:

The beneficial effects of 5-HT₄R_s agonists on memory performance have been shown in a number of cognitive tests. Albeit some of the cellular and molecular effects of 5-HT₄R_s described could be attributable to the observed beneficial effects, mechanisms at work still remain elusive. Indeed, the neurobiological correlates of memory have never been investigated in the same conditions in which the 5-HT₄R_s agonists was shown to display pro-cognitive and anti-amnesic effects (*i.e* systemic administration at 1 mg/kg). Hence, for the first time we attempted to identify a direct relationship between behavioral outcomes and hippocampal function. To this end, we probed the effects of RS67333 administered intraperitoneally at 1 mg/kg on the “where”, “what”, “when” components on ELM in healthy mice. Using the same conditions of administration of RS67333, we investigated its effects on:

- *in vivo* qEEG in hippocampal CA1 area of mice during exploratory behavior
- *ex vivo* CA3-CA1 hippocampal plasticity
- Hippocampal neurotransmitter levels

This third study was performed in the aim to address the following questions:

- **What are the hippocampal-dependent domains of episodic memory that could benefit from 5-HT₄R_s activation?**
- **Are the beneficial effects of 5-HT₄R_s supported by changes in hippocampal functioning?**

Beneficial effects of 5-HT₄Rs activation in mice: a transversal approach, from memory to its hippocampal correlates

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Abstract

Type 4 serotonin receptors (5-HT₄Rs) have earned a place in the sun as a promising therapeutic target for the treatment of memory disorders. Indeed, both pro-mnesiant and anti-amnesiant effects of 5-HT₄Rs activation have been repeatedly described in rodents and more recently pro-cognitive effects were identified in healthy volunteers. Despite some hypotheses have been raised, mechanisms at work still remain to be elucidated. A better understanding of the underpinning mechanisms would help to extend the beneficial effects of pharmacological 5-HT₄Rs stimulation - *so far limited to the fields of Alzheimer's and Major depressive disorders as central nervous system diseases* - to additional brain pathologies such as Parkinson's and Schizophrenia. These disorders are characterized by early decline in episodic memory that are associated with alterations of hippocampal functioning. Hence, we herein addressed such mechanistic issue through a transversal approach. We investigated the effects of systemic administration of the 5-HT₄Rs agonist RS67333 on different functions of hippocampal-dependent episodic-like memory and its neurobiological correlates such as hippocampal synaptic plasticity as well as plasticity-related brain oscillations and neurotransmitters. We identified location and novelty discrimination as two domains of episodic memory that could benefit from 5-HT₄Rs activation. Besides, while hippocampal theta power was increased, the magnitude of long-term potentiation was reduced in a frequency-dependent manner. These changes were accompanied by reduced levels of excitatory neurotransmitter glutamate in the hippocampus. Overall, our results support that the beneficial effects of 5-HT₄Rs activation on memory are intimately linked to changes in hippocampal synaptic plasticity. The latter are likely due to the observed variations in neurotransmitter levels and dependent oscillatory rhythms that are relevant for plasticity processes.

Running title: Mechanisms underlying beneficial effects of 5-HT₄Rs activation on memory.

Keywords: 5-HT₄Rs, RS67333, object recognition memory, temporal order memory, location discrimination, hippocampus, synaptic plasticity, electrophysiology, neurotransmitter, mice

Introduction

Episodic memory impairments are common symptoms of the broad spectrum of neurodegenerative and psychiatric conditions - such as *Alzheimer's disease (AD)*, *Parkinson's disease (PD)* and *Major Depressive Disorder (MDD)* or *Schizophrenia (SZ)* respectively) - and are often related to molecular, cellular and downstream functional hippocampal alterations (Roux et al., 2021). The promises of type 4 serotonin receptors (5-HT₄Rs) as a therapeutic target against cognitive disorders still continue to gain prominence especially since they were identified in brain regions intimately related to learning and memory - and more especially the hippocampus. This growing interest is supported by a large number of preclinical studies reporting beneficial effects of 5-HT₄Rs activation on cognition (Hagena & Manahan-Vaughan, 2017). For instance, administration of 5-HT₄Rs agonists such as RS67333 in rodents repeatedly exerted pro-memory or anti-amnesic effects in recognition memory paradigms (Freret et al., 2012a; Hotte et al., 2012; Lamirault & Simon, 2001; Levallet et al., 2009; Moser et al., 2002; Quiedeville et al., 2015).

Recently, clinical studies led in healthy volunteers described similar pro-cognitive effects of pharmacological 5-HT₄Rs stimulation (de Cates et al., 2022). Among the cognitive domains that benefit from 5-HT₄Rs activation, the recognition memory (RM) was described as particularly sensitive to the effects of prucalopride, a 5-HT₄Rs partial agonist (de Cates et al., 2021, 2022; Murphy et al., 2020). However, despite the growing literature that consent on the use of 5-HT₄Rs activation as an interesting strategy to improve memory, there is a lack of understanding on the underlying neurobiological correlates. Still, although sometimes controversial, the effects of 5-HT₄Rs stimulation have been evaluated hippocampal long-term potentiation (LTP) (Roux et al., 2021) - an activity-dependent strengthening of synaptic transmission which is the main molecular and cellular substrate of memory processes (Martin et al., 2000). The patterns of electrical stimulation used to experimentally elicit LTP are largely inspired from the two main naturally occurring brain oscillations observed in animal's and human's electroencephalogram (EEG) named as Theta θ - (4-8Hz) and Gamma γ - (>30Hz) that trigger synaptic plasticity (Colgin, 2020). Interestingly, the 5-HT₄Rs agonist BIMU-8 was found to increase the release of neurotransmitters such as serotonin (5-HT) (Ge & Barnes, 1996; Licht et al., 2010) and acetylcholine (ACH) (Consolo et al., 1994; Mohler et al., 2007; Siniscalchi et al., 1999) that are relevant to cognition and which pace upstream brain oscillations and subsequent plasticity. Furthermore, 5-HT₄Rs agonists were shown to promote the synthesis or activity of plasticity-related proteins such as brain-derived neurotrophic factor (BDNF) (Lezoualc'h & Robert, 2003; Pascual-Brazo et al., 2012), cyclic adenosine monophosphate (cAMP) (Ishii et al., 2019; Moser et al., 2002; Tesseur et al., 2013) and cAMP response element binding protein (CREB) (Ishii et al., 2019; Pascual-Brazo et al., 2012).

Using a multiscale approach, we here aimed at investigating the hippocampal mechanisms involved in the beneficial effects of 5-HT₄Rs activation on episodic memory. We first assessed the effects of systemic administration of the 5-HT₄Rs agonist RS67333 in a battery of hippocampal cognitive tasks assessing the three components of episodic-like memory in rodent, *i.e.* the “*where*” using the location discrimination task (LD), the “*what*” using the novel object recognition task (NOR) and the “*when*” using the temporal order memory task (TOM). Meanwhile, in order to identify the neurobiological processes underlying the behavioral effects of 5-HT₄Rs activation, the effects of acute systemic treatment with RS67333 were investigated at the hippocampal level using *in vivo* EEG recordings, as well as at the cellular level through *ex vivo* functional synaptic plasticity measurements. Finally, from a molecular standpoint, hippocampal neurotransmitters were quantified.

Materials and methods

Animals

Experiments were performed on 9-week-old C57BL/6Rj male mice (Janvier Labs, France). Total number of animals in each testing procedure and their repartition in each group is provided in **Figure 1**. Mice were housed in groups of 5-8 within standard polycarbonate cages, with food and water *ad libitum* and maintained in a regulated environment ($22\pm 1^\circ\text{C}$) under 12h reversed light/dark cycle (light on from 7 pm to 7 am). Animals were acclimatized to the facility and handled one week before the beginning of the experiments.

For LD task in operant touchscreen chambers, mice were submitted to mild food-restriction (MRF) protocol to maintain them at 85-95% of their original body weight (Bouët et al., 2007) during the whole duration of the task. All experiments were approved by the regional ethics committee (Comité d'Ethique NOrmandie en Matière d'EXpérimentation Animale, CENOMEXA; agreement numbers: 21467 and 29543), in compliance with the European directive 2010/63/EU for animal experiments.

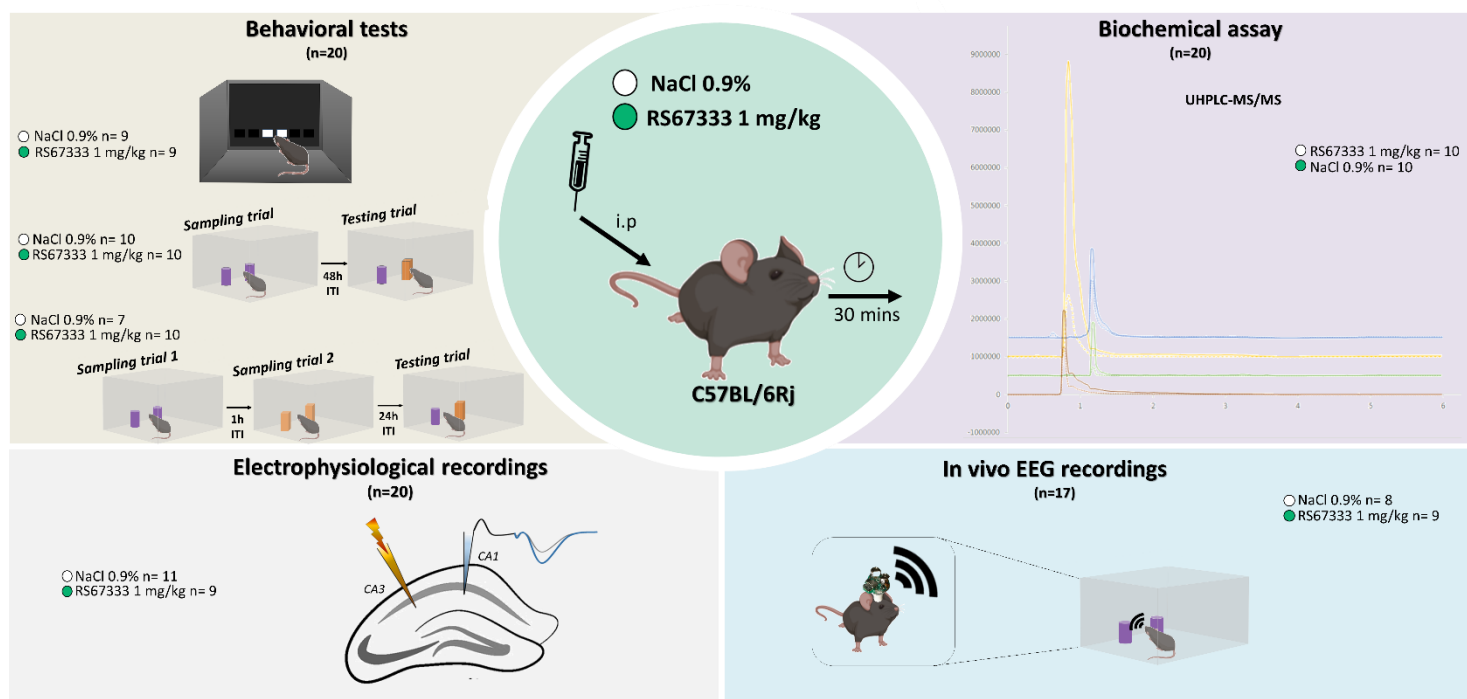


Figure 1: Graphical overview of the experimental design for the evaluation of systemic administration of RS67333 1 mg/kg on the hippocampal function.

Pharmacological treatments

RS67333 HCl (*1-(4-Amino-5-chloro-2-methoxyphenyl)-3-(1-butyl-4-piperidinyl)-1-propanone*) was purchased from Tocris® and dissolved in physiological saline (NaCl 0.9%). Acute systemic RS67333 treatment (1 mg/kg (Fontana et al., 1997) or NaCl 0.9% (vehicle group) was injected intraperitoneally (i.p.) 30 min before the beginning of the sampling phase 1 for NOR and TOM tests, before the probe sessions of the LD task, or before hippocampi collection for *ex vivo* experiments (electrophysiology and neurotransmitters quantification).

Behavioral tasks

Location discrimination task (LD)

Apparatus

LD task was carried out in sound-and light-attenuating boxes (Campden Instruments Ltd., Leics, UK). Each chamber was equipped with a touchscreen (25x31x5cm) and a reward magazine located at the opposite wall. The touchscreen chambers also contained LED light-bulbs (located at both the top of the chambers and inside the reward magazines), infra-red-light beams (at the front of the screen and inside the reward magazine), a reward dispenser, a ventilation fan and a tone generator. The touchscreen was covered by a black plastic mask (28x24cm) delimitating 1 x 6 response windows (3cm $w \times$ 2cm h , 1 cm apart) where visual stimuli (white square 25x25 pixels) were displayed to the mouse. For each trial, a nose poke to the visual stimulus (at the correct location depending on the stage of the task) resulted in liquid reward delivery (creamy strawberry yogurt, Yoplait®, France), accompanied by a tone (1s, 3 kHz) and magazine illumination as a conditioned reinforcer. Each trial automatically started after a 3s inter-trial interval (ITI). Alternatively, a nose poke response to the other (“incorrect”) visual stimulus resulted in a time out period (5s) indicated by a house-light illumination. Mice were exposed to the apparatus 5 days per week. The different protocols were monitored by dedicated software which also generated the raw data (ABET II Touch Campden Instruments U.K.). General overview of the system and of the protocol is displayed in **Figure 2A**.

Pre-training

Detailed protocol used in this study is available elsewhere (Delotterie et al., 2014; Oomen et al., 2013). Mice first went through general touchscreen pre-training consisting in progressive stages of: (1) *Habituation* (1 day, 30 min) in which mice received the reward for head entry to the magazine, (2) *Initial Touch*, in which the visual stimulus was displayed on the screen and the amount of reward was dispensed 3x after a nose-poke on the screen or 1x in the absence of nose-poke, (3) *Must Touch*, in which the visual stimulus was displayed on the screen and the reward was delivered only after a nose-poke (4), *Must Initiate*, in which mice had to initiate a trial by head entry into the reward magazine, and (5) *Punish Incorrect (PI)*, in which an incorrect responses (nose-poke outside the stimulus) was followed by lighting off the whole chamber for 5s. The visual stimulus was displayed again on the same location until a correct response was made. The criterion for moving from one stage to the next one was the completion of 30 trials within 60 min in stages 2, 3 and 4. For the PI stage, the criterion to completion was set to 77% of correct responses on 2 consecutive days.

Intermediate training

Mice were then trained to discriminate between two identical visual stimuli localized at an “intermediate” distance of separation (two unlit windows left between, *i.e.* illumination of the 2nd and 5th windows). Only one visual stimulus was rewarded (for example, the left one). Once 7 out of 8 trials correct responses were obtained (session criterion), the rewarded visual stimulus location was reversed (for example, the right stimulus was then rewarded). To limit over-performance, the session automatically ended up after 4 reversals. Otherwise, the session ended up when 60 trials were completed or when 60 min had elapsed. The first rewarded stimulus location was randomized between mice and changed at every daily session. Once the mouse reached at least one time the session criterion in 3 out of 4 consecutive sessions (whatever the number of reversals completed per session), mice advanced to the

probe test of the LD task. Based on their performances, mice were then assigned to one of the two treatment groups such that each group required approximately the same number of sessions to fulfill the intermediate training.

Probe test

The probe sessions consisted in the presentation of the two identical visual stimuli with either large separation referred as the “easy level” (1st and 6th windows illuminated) or by small separation termed as “hard level” (3rd and 4th windows illuminated). A total of 12 probe sessions were performed consisting in 6 sessions of each level of difficulty, alternated every 2 days. The correct stimulus location was counterbalanced within each difficulty level. When changing the level of difficulty (*i.e.* “easy” to “hard”), the last location of the correct stimulus (*i.e.* “easy, right”) was re-used for the next session (*i.e.* “hard, right”). The session criterion of 7 correct responses over 8 consecutive trials was applied and a maximum of 4 reversals was allowed. The session automatically ended up after 4 reversal or after the completion of 60 trials or when 60 min had elapsed. The mean number of trials to reach the session criterion (1 reversal) was calculated for each group and each level of difficulty and used as the primary outcome to assess the LD performances. A score of 60 trials (*i.e.* the maximum number of trials allowed within a session) was attributed to mice that did not reach the criterion before the end of the session but has a total number of trials higher than the mean number of trials to complete the criterion. Mice that did not reach the criterion and did not have a total number of trial higher than the mean number of trials to criterion of their group had no value. number of trials. A velocity index (Number of trials/Session duration) was calculated and the reward collection latency was analyzed to evaluate the general motivational state of mice during the task.

Spontaneous object exploration tasks

General procedure

Spontaneous object exploration tasks were performed in a matte grey square open-field (30x30x20cm) illuminated at 30 Lux at the center. Each task began with a short habituation period to the empty open-field 24h before (Leger et al., 2013b). During the following sampling and testing trials, two objects were fixed at 8 cm from the back wall of the open-field. The objects and the open-field were carefully washed with ethanol 70% to eliminate olfactory cues between each trial. Each object (lego tour, sand-filled falcon and cell culture flask) was available in triplicate and was previously tested for absence of innate preference (Leger et al., 2013b). The position (left/right) and the nature of the objects were randomized between mice. During each trial, the time spent to explore each object was manually measured. Exploratory behavior was considered when the mouse was actively sniffing the object at a distance below 2 cm. Chewing and climbing on the objects was not considered as exploratory behavior. For each trial, mice were allowed to freely explore the objects until a criterion of 20s of cumulated exploration of both objects was reached. Mice failing to reach this criterion within 10min were excluded from the analysis. The time elapsed before reaching the 20s criterion was used as an index of motivation/locomotion.

Novel object recognition (NOR) test

As previously described (Leger et al., 2013b), mice were allowed to explore one set of two identical objects (sampling trial 1). After a 48h-ITI (testing trial), mice were exposed to a third copy of one of the objects presented before (familiar object) and a novel object (**Figure 2A**). Based on the innate preference of mice for novelty, animals

were expected to spend more time exploring the novel object relative to the familiar one. The time spent to explore the novel object was used to assess novelty discrimination performances.

Temporal order memory (TOM) test

Based on the protocol described before (Barker & Warburton, 2011), mice were first allowed to explore one set of two identical objects (sampling trial 1), followed 1h later by another set of two identical objects (sampling trial 2). In a subsequent testing trial (24h-ITI), animals were exposed to a copy of one object from the sampling trial 1 (“old” object) and a copy of one object from the sampling trial 2 (“recent” object) (**Figure 3A**). Based on their ability to remember the order in which objects have been presented, animals were expected to spend more time to explore the less recent object encountered. The time spent to explore the old object was used to assess recognition performances for recency.

To confirm that TOM performances are strictly related to recency memory and not to a novelty effect (Barker et al., 2019), *i.e.* due to a forgetting of the object previously encountered, a NOR task was performed using the same ITI. Confirming the expectations, intact NOR performances were observed (*Supplementary figure*).

Extracellular recordings

As previously described in details (Lecouflet et al., 2021), electrophysiological experiments were conducted on transverse hippocampal slices (400µm thick) using a tissue chopper (McIlwain®). Field excitatory postsynaptic potentials (fEPSPs) were recorded from the CA1 area following stimulation of the Schaffer collateral axons with bipolar tungsten electrode. Following a stable 15min baseline (0.1Hz test pulse), LTP was induced using either HFS (100 pulses at 100Hz) or TBS (5 bursts at 5Hz constituted of 4 pulses at 100Hz - and repeated 4 times at 0.1Hz) protocols. The last 15min recordings, as an indicator of LTP magnitude, were used for statistical analysis.

Quantitative electroencephalographic (qEEG) recordings

Under isoflurane anesthesia (5% for induction and 2% for maintenance, under 100% O₂), mice were placed on a stereotaxic frame. After a skin incision to expose the skull surface, two holes (~0.5mm in diameter) were drilled over the right fronto-parietal cortex (1) and the left occipital cortex (2) until reaching the dura matter. Two stainless steel screws were implanted epidurally on position 1 and 2 and served as reference and anchor screws respectively. For hippocampal recordings, a third whole was drilled according to the stereotaxic coordinates (AP: +2.0, ML:+1.5, DV: -2.0 from Bregma) of the CA1 area of the hippocampus determined using the Mouse Brain Atlas (Paxinos & Franklin, 2019). The depth electrode consisting of a pair of twisted insulated platinum iridium wire was inserted into the hole until reaching the CA1 area. Electrodes (epidural and depth) were fitted into a 10-hole head connector and whole assembly was secured on the skull with dental cement.

The wounds were sutured and animals were placed individually to their home cages. Mice were given 5 mg/kg s.c. carprofen (Rimadyl®, non-steroidal anti-inflammatory drug) during surgery and for 48h post-surgery. After a resting period of 2 weeks minimum, animals were connected to the telemetric transmitter (RodentPACK, emka technologies) to check the quality of the signals. Field potential were sampled at 500Hz and band-pass filtered between 1 and 100Hz.

Data were analyzed off-line using ecgAUTO software (emka Technologies). First, artifact removal was performed by visual inspection. Then, a Fast Fourier Transform (FFT) automatically performed by the software was used to

compute EEG power spectrum density in sub-frequency bands including 4-8Hz (theta band) and 32-64Hz (gamma band). Spectra power were calculated from epochs of 8.20s duration with 85% overlap and hamming window of 1s and notch filter at 50Hz. Spectral power density (PSD, in μV^2) was calculated. PSD were averaged over periods of either 1 or 10min. The percentage of change in power after treatment was calculated relative to baseline activity.

Hippocampal neurotransmitter quantification

According to the method described elsewhere (Party et al., 2019), Ultra-High-Performance Liquid Chromatography coupled to tandem Mass Spectrometry (UHPLC-MS/MS) analyses were performed on isolated hippocampi to quantify glutamate (GLUT), acetylcholine (ACH), γ -gamma-amino butyric acid (GABA) and serotonin (SER) concentrations. Briefly, hippocampi were homogenized in formic acid aqueous solution (2%) before been injected into the column (Raptor Biphenyl). Lower limits of neurotransmitter (NT) quantification in the injected solutions were 0.002ng/g for GLUT, 0.025ng/g for GABA, 25.000ng/g for ACH and 3.000ng/g for SER calculated for 25mg of sample (7 μ L injection volume).

Data analyzes

Data are represented as mean \pm standard error of the mean (SEM) or as median (min-max) when assumptions of normality and homogeneity of variance were unmet. All graphs were done using Prism 8 (GraphPad Software, In., La Jolla, CA). Statistical difference was set at $p < 0.05$. Statistical analyzes were performed using R[®] software as follows:

LD task. The number of sessions to complete the pre-training and intermediate training was analyzed through two-way ANOVA and Mann-Whitney *U* test respectively. ANOVA-type repeated measurements was used to compare the number of trials to reach the criterion between groups during the probe sessions.

Spontaneous object recognition tests. Because the times spent to explore objects within a single trial were interdependent (ending trial criterion of 20s), time spent exploring the left object (sample trial) and time spent to explore either the novel or the old object (testing trial) were compared to the chance level of exploration (*i.e.* 10s) through Univariate *t*-test (NOR) or Wilcoxon signed-rank test. Trial duration was analyzed using unpaired *t* test (NOR) or Mann-Whitney *U* test (TOM) for group comparison.

Electrophysiological recordings. ANOVA-type for repeated measurements and Mann-Whitney *U* test were performed to compare LTP magnitude between groups. Univariate *t* tests were performed to compare the percentage of change from baseline activity (theoretical value = 0).

Quantitative electroencephalographic recordings. Univariate *t* tests were performed to compare the percentage of change of theta and gamma power from baseline (theoretical value = 0).

Neurotransmitter levels. Comparisons of NT levels were performed through Student's *t* test.

Results

RS67333 enhanced pattern separation performance in the LD task

The mean number of sessions to complete the pre-training and the intermediate training did not differ between groups [two-way ANOVA ($F_{1,50}=0.01$, $p=0.91$) and Mann-Whitney U test, $p=0.73$ respectively (**Figures 2B et 2C**)], suggesting similar baseline performances in vehicle and RS67333-treated groups.

During probe sessions, the mean number of trials per session to reach the criterion was significantly lower in the RS67333-treated group as compared to the vehicle-treated group [group effect ($F_{1,10}=5.35$, $p=0.02$); **Figure 2D**]. Neither the velocity completion index nor the reward collection latencies statistically differed between groups [two-way ANOVA: no group effect ($F_{(1,10)}=0.38$, $p=0.54$), no effect of the level of difficulty ($F_{(1,10)}=0.26$, $p=0.61$) and no interaction ($F_{(1,10)}=3.04$, $p=0.08$), **Figure 2E**; no group effect ($F_{(1,20)}=0.06$, $p=0.81$), no effect of the level of difficulty ($F_{(1,20)}=0.10$, $p=0.75$) and no interaction ($F_{(1,20)}=0.07$, $p=0.79$) **Figure 1F**], suggesting similar motivation level to accomplish the task.

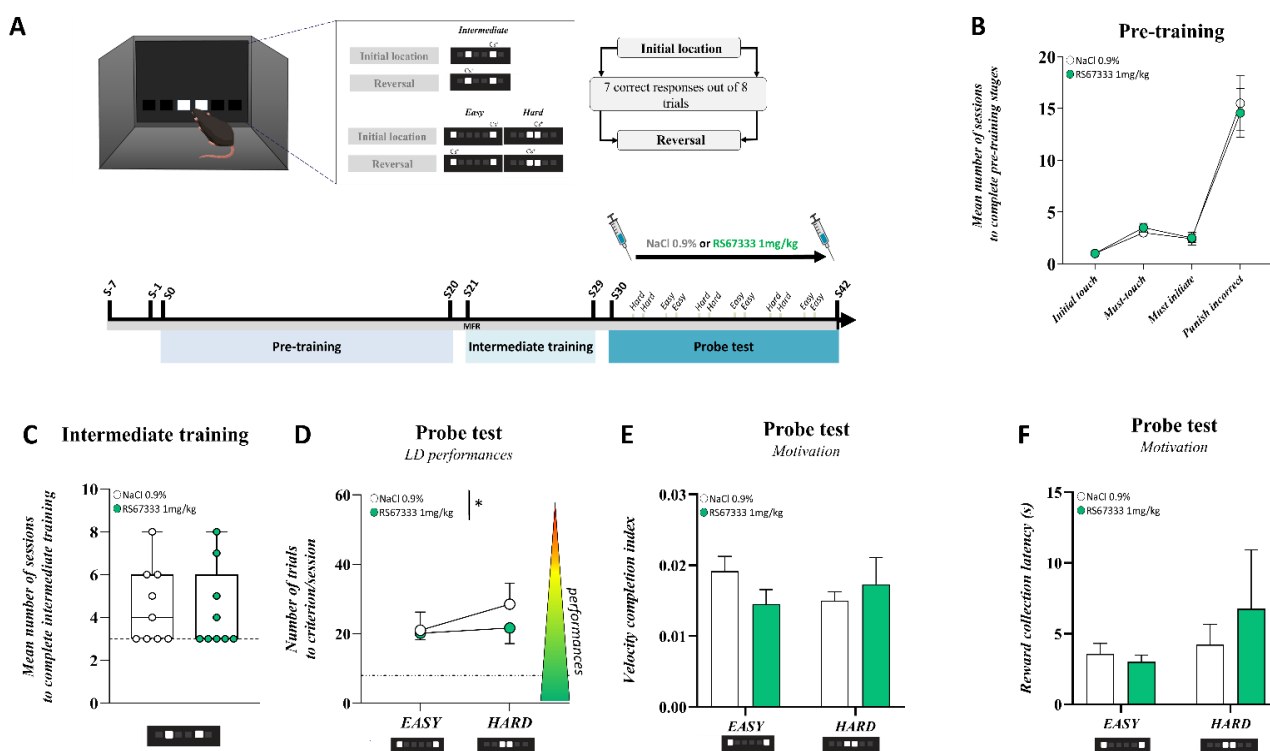


Figure 2: Effect of acute systemic administration of RS67333 (1mg/kg) on LD performance.

(A) Experimental design of the LD task. (B) Number of sessions to complete each stage of the pre-training in both the vehicle group ($n=9$) and the RS67333-treated group ($n=9$). Data are represented as mean \pm SEM (C) Number of sessions to complete the intermediate training. Data are represented as median \pm interquartile (D) Number of trials to reach the criterion before the session ends in “easy” and “hard” level of difficulty in the probe test. Data are expressed as median \pm interquartile (E, F) Velocity completion index and reward collection latency respectively during the probe test in both “easy” and “hard” level of difficulty to indirectly assess motivation.

RS67333 reinforced memory trace persistence recognition memory of novelty, but not recency.

Novel object recognition test

During the sampling trial, mice did not show any preference for one of the two objects presented, whatever the group considered as the time spent to explore the left object was not different from chance level of 10s ($p=0.71$ and $p=0.27$ for vehicle and RS67333-treated group respectively, Univariate t test versus 10s chance level; **Figure 3A**). After a 48h-ITI, RS67333-treated mice, but not vehicle-treated ones, spent significantly more time to explore the novel object ($p=0.03$ and $p=0.34$, respectively, Univariate t test, **Figure 3B**).

Any potential bias due to locomotion and/or motivation-like behavior was assessed based on the measurement of the time spent to reach the 20s criterion (trial duration). There was no difference between groups neither during sampling nor during testing trials ($p=0.73$ and $p=0.80$ respectively, Unpaired t test, **Figure 3C**).

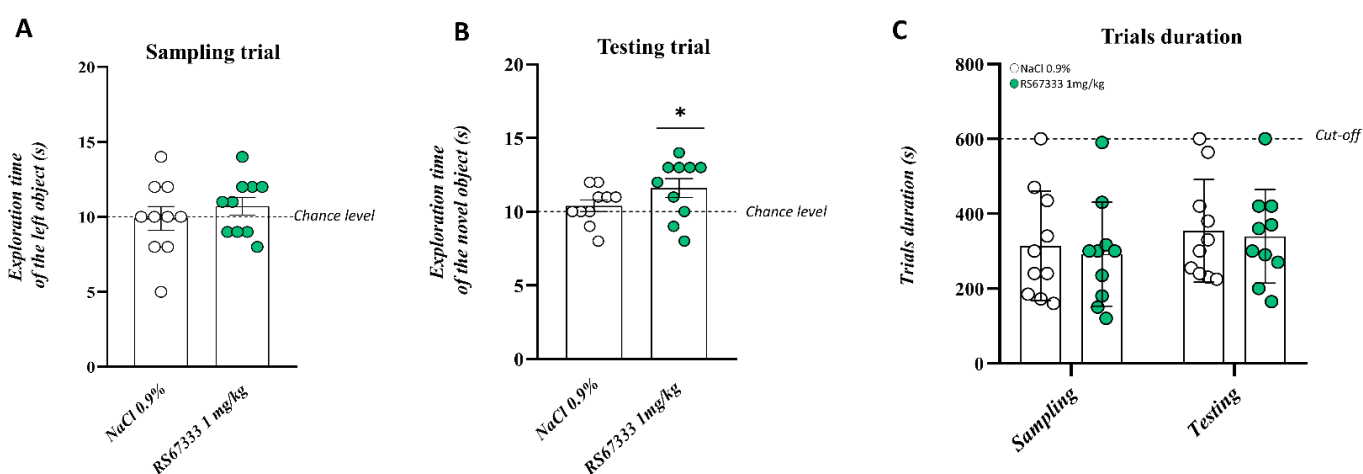


Figure 3: Effects of acute systemic administration of RS67333 (1mg/kg) on NOR performance.

Data are represented as (mean ± SEM). (A) Exploration time of the left object during sampling trial for both vehicle ($n=10$) and RS67333-treated group ($n=10$). (B) Exploration time of the novel object during testing trial for both groups ($*p<0.05$, Univariate t test). (C) Time to reach 20s of total exploration (trials duration) at each trial for both groups.

Temporal order memory test

Animals from the vehicle and RS67333-treated groups explored equally the two objects during sampling session 1 ($p=0.13$ and $p=0.13$, Wilcoxon signed-rank test respectively) and sampling session 2 ($p>0.99$ for both groups, Wilcoxon signed-rank test, **Figure 4A**). Following a 24h-ITI, mice did not significantly spend more time to explore the old object, whatever the treatment group ($p>0.99$ and $p=0.68$ respectively, Wilcoxon signed-rank test, **Figure 4B**). Mann-Whitney U test for unpaired data revealed that the time to complete the 20s of total exploration criterion did not differ between groups during the sampling trial 1 ($p=0.12$). However, mice from RS67333-treated group completed the 20s of exploration faster than the vehicle-treated group during the sampling trial 2 ($p=0.04$). The duration of the testing trial did not differ between groups ($p=0.22$ Mann-Whitney U test, **Figure 4C**).

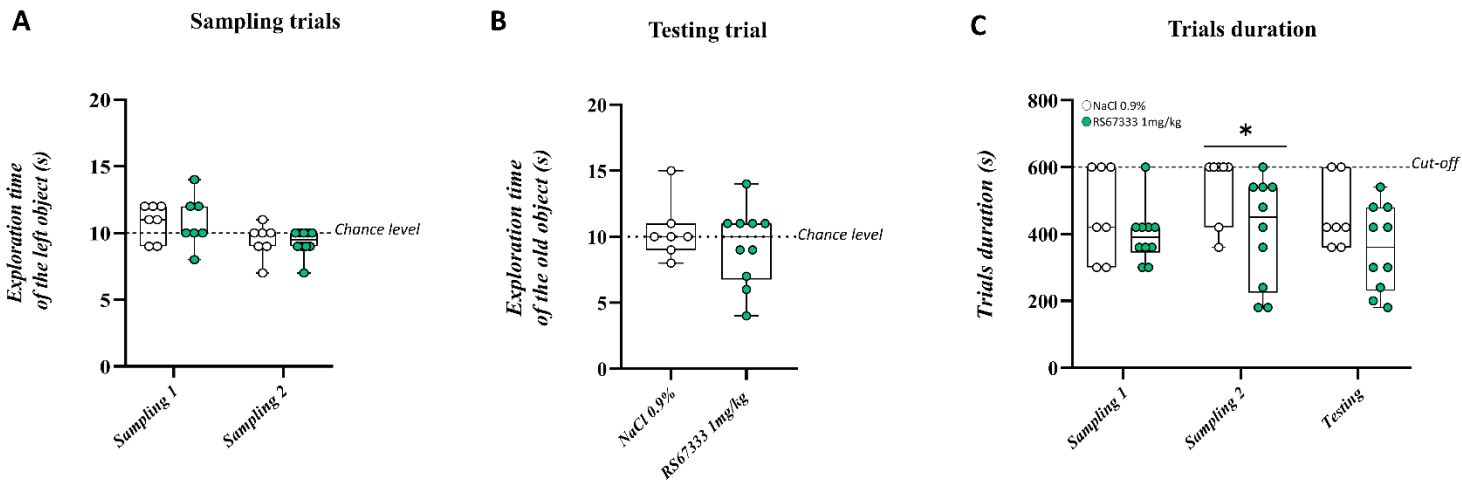


Figure 4: Effects of acute systemic administration of RS67333 (1mg/kg) on TOM performance.

Data are represented as (median \pm interquartile). (A) Exploration time of the left object during sampling trial for both vehicle ($n=7$) and RS67333-treated group ($n=10$). (B) Exploration time of the novel object during testing trial for both groups (C) Time to reach 20s of total exploration (trials duration) at each trial for both groups.

5-HT₄R_s activation by RS67333 reduced *ex vivo* LTP and increased theta power recorded *in vivo* in CA1 hippocampal subfield.

Ex vivo functional synaptic plasticity experiments revealed that TBS stimulation induced a stable and robust LTP in both groups (173 ± 13 % of baseline and 155 ± 8 % of baseline in vehicle and RS67333-treated groups, respectively). The fEPSP slopes during the last 15min did not significantly differ between groups [ANOVA-type for repeated measurements: no group effect ($F_{(1, \infty)}=0.68, p=0.41$), no time effect ($F_{(8.71, \infty)}=0.79, p=0.62$) and no interaction ($F_{(8.71, \infty)}=0.86, p=0.56$); **Figure 5A**]. When using HFS stimulation, a stable and robust LTP was also observed in both groups (153 ± 6 % of baseline and 132 ± 5 % of baseline in vehicle and RS67333-treated groups, respectively). Statistical analysis revealed a significant lower tetanus-induced LTP in RS67333-treated group (Mann-Whitney test, $P=0.02$). The fEPSP slopes during the last 15min was additionally significantly reduced in RS67333-treated group [ANOVA-type for repeated measurements: group effect ($F_{(1, \infty)}=6.1, p=0.01$), no time effect ($F_{(7.27, \infty)}=0.46, p=0.87$) and no interaction ($F_{(7.27, \infty)}=0.87, p=0.53$); **Figure 5B**].

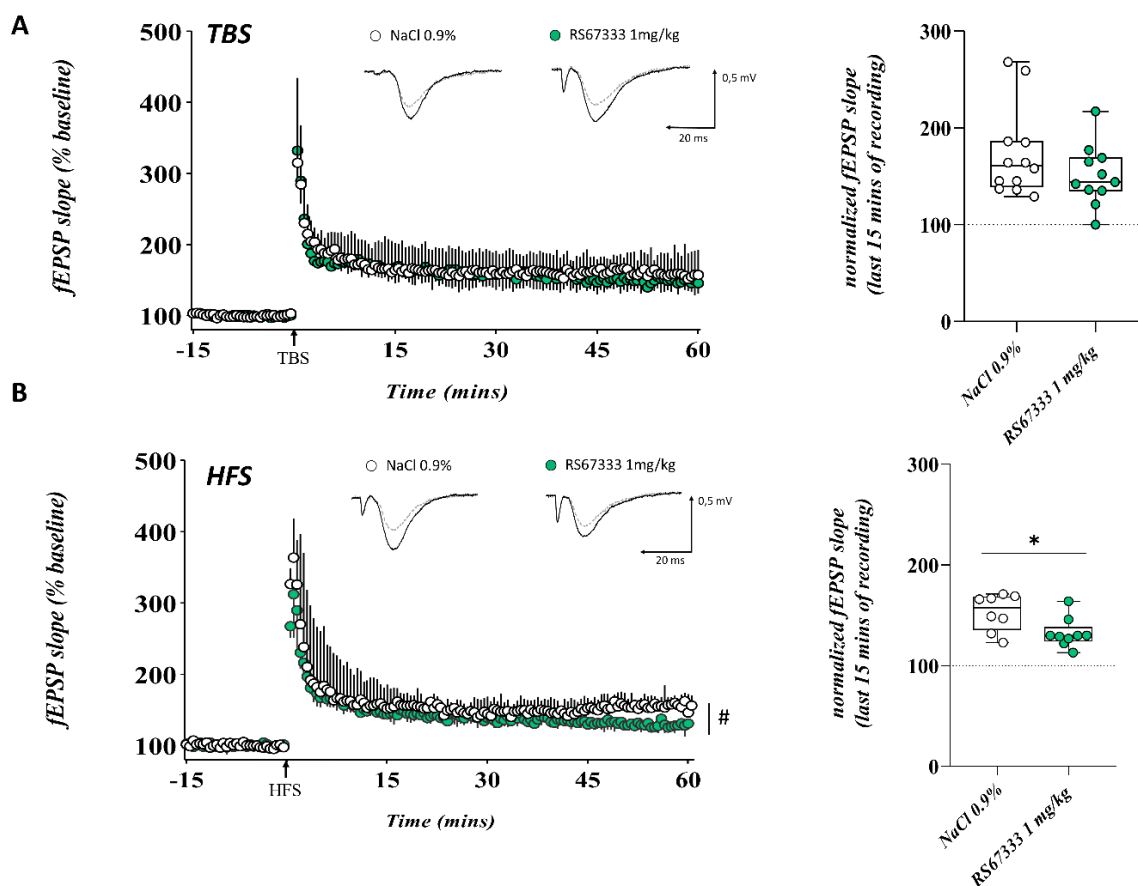


Figure 5: Effects of acute systemic administration of RS67333 (1 mg/kg) on hippocampal LTP induced by HFS and TBS. (A) Time course of fEPSP slope after TBS-induced LTP in slices from vehicle mice ($n = 12$ slices/ $n = 7$ mice) and RS67333-treated mice ($n = 11$ slices/ $n = 10$ mice) (left) and corresponding last 15 min of fEPSP slope (right). (B) Time course of fEPSP slope after HFS-induced LTP in slices from vehicle mice ($n = 8$ slices/ $n = 7$ mice) and RS67333-treated mice ($n = 9$ slices/ $n = 8$ mice) (left) and corresponding last 15 min of fEPSP slope (right). Data are expressed as median \pm interquartile. Arrow marks the time when conditioning stimulation was applied. Insets show representative traces of fEPSP before (dashed line) and after (full line) conditioning stimulation. # $p < 0.05$, ANOVA-type for repeated measures; * $p < 0.001$ and Mann–Whitney U test.

In vivo quantitative EEG recordings showed that theta power was significantly increased in the RS67333-treated group ($p = 0.03$, Univariate t test) while it remained unchanged in vehicle-treated group in comparison with the baseline activity ($p = 0.07$, Univariate t test). Gamma power was significantly increased as compared to baseline in both vehicle and RS67333-treated group ($p = 0.02$ and $p < 0.01$ respectively, Univariate t tests; **Figure 6**).

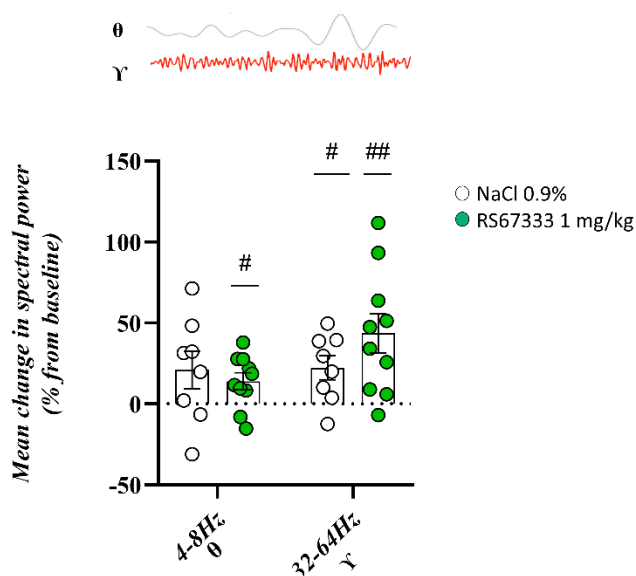


Figure 6: Effects of acute systemic administration of RS67333 (1mg/kg) on theta and gamma frequency bands.

Data are represented as mean \pm SEM. Percentage of change in theta and gamma powers relative to baseline in both vehicle (n=8) mice and RS67333 treated mice (n=10). # p <0.05; ## p <0.01 Univariate t test.

5-HT₄Rs activation by RS67333 reduced glutamate levels in hippocampus

A significant decrease in hippocampal levels of GLUT was observed in RS67333-treated group in comparison with the vehicle one (Student's t test, p =0.01). Meanwhile, the concentration of the other neurotransmitters (GABA, ACH, SER) did not significantly differ between groups (p = 0.06, p = 0.56, p = 0.86 respectively) (**Table 1**).

Table 1: Effects of acute systemic administration of RS67333 (1 mg/kg, n=10) on hippocampal neurotransmitters concentrations (mean \pm SEM). Student's t test: * p <0.05 compared to vehicle-treated group (NaCl 0.9%, n= 10).

	Glutamate (μ g/g)	GABA (μ g/g)	Acetylcholine (μ g/g)	Serotonin (ng/g)
NaCl 0.9%	6.8 \pm 0.35	4.4 \pm 0.28	3.9 \pm 0.29	0.3 \pm 0.03
RS67333	5.7 \pm 0.22 [*]	3.8 \pm 0.22	4.1 \pm 0.24	0.3 \pm 0.01

Discussion

Through a transversal approach, we explored the effects of 5-HT₄Rs activation by a systemic administration of RS67333 (1 mg/kg, i.p) on different hippocampal processes sustaining EM. We identified the location and novelty discrimination as two cognitive domains of EM that could benefit from 5-HT₄Rs activation. The enhancement of performance in these two functions are accompanied by changes within the hippocampal function; from plasticity-related frequency bands and neurotransmitters to downstream synaptic plasticity.

Deriving from the Cambridge Automated Neuropsychological Test Associated Battery (CANTAB) which is validated as a diagnostic tool for cognitive impairments in patients (J. Talpos & Steckler, 2013) as well as for the effects of cognitive enhancers (Haider et al., 2006), touch-screen based cognitive assays have been developed in rodents to test the efficacy of pharmacological ligands (Graf et al., 2018; J. C. Talpos et al., 2009). Here, we investigated for the first time the effects of a 5-HT₄Rs agonist on LD performances in mice. Particularly solicited in such task, the pattern separation (PS) is a crucial function that allows accurate encoding of overlapping stimuli into separate events (Yassa et al., 2011). We showed that RS67333 administration before probe sessions improved LD performances, without having any influence on motivation and/or locomotion. This suggests an overall enhancement of PS ability following 5-HT₄Rs activation. This is consistent with the observation that 5-HT₄Rs agonists are known to boost brain derived neurotrophic factor (BDNF) synthesis (Pascual-Brazo et al., 2012) which has in turns been demonstrated to be critical to PS (Bekinschtein et al., 2013). Moreover, PS function has been repeatedly shown to rely on the dentate gyrus (DG) subregion (Schmid et al., 2019; Treves & Rolls, 1994). The DG is considered within the canonical tri-synaptic circuit as the main entrance of the input flow stemming from the entorhinal cortex. Consequently, granule cells (GC) have the particularity to exhibit low excitability and strong feedforward and feedback inhibition enabling to maintain a competitive network for only the most salient inputs being processed. As 5-HT₄Rs were found to be predominantly expressed on dentate GC (Tanaka et al., 2012), the beneficial effects of RS67333 on PS ability may be related to GC activity changes. In keeping with this view, intracerebroventricular (icv) administration of RS67333 was reported to inhibit basal excitatory synaptic transmission in the DG (Kulla & Manahan-Vaughan, 2002) and curtailed LTP in the DG of anaesthetized rats (Kulla & Manahan-Vaughan, 2002; Marchetti et al., 2004; Twarkowski et al., 2016). Hence, 5-HT₄Rs activation may contribute to the maintenance of GC in low excitability state to limit the initiation of mnemonic process to very strong inputs. Further experiments would thus be necessary to confirm the modulatory role played by 5-HT₄Rs on GC activity.

Other fundamental aspects of EM are the ability to remember the order in which events have been experienced as well as the nature of the event experienced, *i.e.* the “when” and “what” components of EM. The efficacy of RS67333 to improve these two features of EM in natural forgetting condition was evaluated in the TOM and the NOR tests, respectively. By using a 1h-24h ITI in the TOM test, we confirmed that the vehicle-treated mice naturally lost their ability to discriminate the less recent *versus* the most recently encountered object. Our results additionally showed that administration of RS67333 before the first sampling trial, *i.e.* the encoding phase of TOM, was not able to improve discrimination performances in the testing trial. To ensure that our conditions reflected memory for order without being interfered with familiarity, we assessed discrimination performances of novel object and the less recent object using the same protocol as in TOM (Barker et al., 2019).

We found that recognition memory for novelty was intact in these conditions (*supplementary results*). To our knowledge, the effects of pro-cognitive pharmacological agents and especially serotonergic ligands have never been investigated in such TOM task. Additional experiments with varying experimental conditions would deserve to be performed in order to confirm whether RS67333 has no effect on TOM or whether another design would allow to emphasize pro-cognitive effects. Indeed, several protocols have been described for assessing TOM in rodents. However, there are inconsistencies as regard to the consequence of varying the number of items in sequence, the length of sample exposure time and of ITIs between sampling/retention phases on performances which make the choice of the optimal experimental design difficult (Barker et al., 2019; Hatakeyama et al., 2018). In the NOR experiment, by using a longer retention ITI (48h) than those previously described (*i.e* 4h, 6h; Freret et al., 2012a; Lamirault & Simon, 2001), we showed that RS67333 injected before acquisition extended the recognition memory trace for novelty while a natural forgetfulness occurred in the vehicle group at this delay. Of note, the time to complete the criterion of 20s of exploration (trial length) was similar between group, suggesting no differences in motivational state of the animals. These results are interesting in view of recent clinical data showing that both a single dose and 6-days treatment with the 5-HT₄Rs agonist prucalopride, improves memory accuracy in healthy volunteers in several hippocampal-dependent cognitive tasks that are closed to the NOR protocol (de Cates et al., 2021; Murphy et al., 2020).

Interestingly, it appears from our results that 5-HT₄Rs activation differently affects two cognitive processes (TOM and NOR) both supported by CA1 hippocampal subfield. The CA1 subfield is believed to act as a temporal separator through time cells that fire at a specific moment during cognitive task (Manns et al., 2007) and act as a mismatch detector thanks to back-projections to CA3 recurrent which allow examination of the contents of memory to determine if the stimulus has or has not previously been experienced (Gilbert et al., 2001; Kesner & Rolls, 2015). However, despite a deeper knowledge on the cellular dynamics for RM for novelty and TOM is definitely needed, it is clear that these two computations operate under distinct processes as shown by the intact novelty recognition performances observed in the protocol adapted from the TOM test. Nonetheless, it is now clear that 5-HT₄Rs agonists exert fine tuning of CA1 pyramidal cells activity. Electrophysiological data previously reported an increase in hippocampal CA1 neurons excitability following 5-HT₄Rs activation (Mlinar et al., 2006). This was demonstrated to be achieved by reduction in the cyclic adenosine-monophosphate (cAMP) mediated after-hyperpolarization (AHP) that follows action potentials (Ansanay et al., 1992) as well as activation of hyperpolarization-activated (*I_h*) currents (Bickmeyer et al., 2002). On a more functional standpoint, it has been shown that 5-HT₄Rs can influence hippocampal synaptic plasticity including the CA1 subfield (Roux et al., 2021). Over the three existing studies, one reported enhanced LTP (Matsumoto et al., 2001), while it was found unchanged in a second study (Kemp & Manahan-Vaughan, 2005) and decreased following TBS or unchanged after HFS induction in the third one (Lecouflet et al., 2021). Despite these observations, the behavioral effects of 5-HT₄Rs activation have never been correlated to plasticity - as well as plasticity-related processes - using experimental conditions that sustain pro-cognitive effects (*i.e* acute systemic *i.p.* administration, 30 mins prior to the cognitive task). Therefore, we assessed the effects of systemic administration of a pro-cognitive dose of RS67333 on electrophysiological measures within the hippocampus, consisting on *in vivo* qEEG and *ex vivo* functional synaptic plasticity recordings. As the beneficial effects of 5-HT₄Rs agonism have been repeatedly reported in CA1-dependant tasks such as NOR task (Darcet et al., 2016; Freret et al., 2017b; Hotte et al., 2012; Lamirault & Simon, 2001; Levallet et al., 2009; Moser et al., 2002; Quiedeville et al., 2015), we chose to focus our investigations on

the CA3-CA1 subfields, representing the most characterized hippocampal pathway in the literature.

We found that the magnitude of LTP induced by HFS protocol was significantly reduced following acute treatment with RS67333 while LTP induced by TBS remained unaffected. As regard qEEG recordings, we found that gamma power was increased in both vehicle and RS67333-treated groups whereas theta power was increased only following RS67333 treatment in awake mice. These results constitute a first approach in the characterization of the effects of 5-HT₄Rs stimulation on EEG rhythms and are consistent with previous studies reporting enhanced cortical theta power in rats after administration of approved drugs against dementia (*i.e.* donepezil, rivastigmine, memantine) (Ahnaou et al., 2014; Drinkenburg et al., 2015). Nevertheless, the interpretation of our results needs to be taken cautiously. Indeed, theta rhythm has also been shown to relate to diverse functions such as arousal and locomotion speed. Further, the effects of RS67333 on theta-gamma coupling during high cognitive demand task would be of worth since such process is known to be altered in both mouse model of AD (Zhang et al., 2016) and AD patients (Goodman et al., 2018). The opposite effects exerted by RS67333 on LTP measurements (reduction) and theta-gamma powers (enhancement) in CA1 subfield may rely to the different processes involved. Indeed, in one case (qEEG measures) we addressed the effects of RS67333 on the generation of theta-gamma rhythms whereas on the other case (LTP measurements) we rather artificially reproduced these rhythms to mirror the downstream cellular response induced. Also, based on the literature, our LTP results are inconsistent with the 3 preexisting studies conducted on CA1 hippocampal area and involving 5-HT₄Rs. Several major differences can explain these discrepancies. First, we administrated the agonist systemically while it was either bath applied (Lecouflet et al., 2021) or injected directly in the target region (Kemp & Manahan-Vaughan, 2005; Matsumoto et al., 2001). Hence, the final concentration at the site of interest would be expected to be different and consequently receptor occupancy could be dissimilar. According to its pharmacological profile (R. Eglén, 1995), 10 μ M of bath application of RS67333 should occupy ~90% of 5-HT₄Rs whereas only ~25% would be occupied after systemic administration of RS67333 at 1mg/kg (Nirogi et al., 2013). Second, in our conditions (*ex vivo* measurements) the hippocampus was free of influence of any surrounding brain area that are susceptible to respond to 5-HT₄Rs stimulation in contrast to the studies of Matsumoto *et al* and Kemp and Manahan-Vaughan *et al*. In fact, interaction with non-CA1 or more broadly hippocampal 5-HT₄Rs likely contribute to the difficulty of interpreting the results. For instance, it was shown that systemic activation of 5-HT₄Rs induces behavioral changes whereas terminal manipulation of 5-HT₄Rs activity in the CA1 did not impact the behavior (Teixeira et al., 2018). Lastly, the stimulation paradigm used to induce LTP is critical to the interpretation of the results. Indeed, the cellular and molecular processes involved in TBS and HFS differ consistently. On the one hand, the in-between key difference of HFS and TBS protocols relies on circuitry priming by TBS. When followed by a second burst at 200ms interval (corresponding to interneurons refractory period) –the feed-forward inhibition (disinhibition process) is suppressed and allows maximal post-synaptic depolarization (Larson & Munkácsy, 2015). On the other hand, HFS is considered as strong stimulation which induces maximal glutamate release concurrent with fatigue of the synapse. Hence, TBS is believed to be more economical than HFS for LTP induction. Indeed, while TBS exploits endogenous circuit properties to maximize NMDA-Rs activation with the least amount of afferent stimulation; the excessive glutamate release induced by HFS leads to maximal recruitment of NMDA-Rs but with minimal activation – “*more is not necessarily better!*” (Larson & Munkácsy, 2015).

Beyond, we found a reduction in hippocampal glutamate levels of RS67333-treated mice. Hence, the reduced HFS-induced LTP observed in RS67333-treated mice likely occurred secondary to reduced levels of

hippocampal glutamate. In keeping with the considerations mentioned above, HFS would appear more sensitive to glutamate variations than TBS. Moreover, HFS patterns may engage additional pathways that would be sensitive to disruptions that have little effect on TBS-related LTP. For instance, trains of 200 Hz stimulation were reported to activate voltage-dependent calcium channels (VDCC) as well as synaptic NMDA-Rs-mediated calcium fluxes (Larson & Munkácsy, 2015). Moreover, the main molecular actors involved in HFS (cAMP, PKA) are common to 5-HT₄Rs signaling cascade (Lynch, 2004). Consistently with the observed decrease in glutamate levels, the 5-HT₄Rs agonist prucalopride was found to indirectly reduce bursts of AMPA-receptor-mediated currents in the CA3 auto-associative network of the hippocampus, by altering glutamatergic transmission (Chen et al., 2020). The authors claimed that it may support a greater signal-to-noise ratio of relevant stimuli, which is in line and strengthens the hypothesis of beneficial lowering of hippocampal network excitability for learning and memory improvement. Indeed, it is becoming increasingly evident that out of the range (excessive or insufficient) network excitability and subsequent plasticity could be deleterious to learning and memory processes. Following up with this idea, scaling down the hippocampal circuit through the activation of 5-HT₄Rs may be beneficial for memory accuracy, making any stimulus more salient.

Conclusion and perspectives

Our results strongly support a role for 5-HT₄Rs agonists in the maintenance of a competitive network that limits interferences during encoding by selecting the most salient information. This is reflected by improvements in PS and non-spatial recognition memory for novelty. These behavioral outcomes are supported by changes at the hippocampal level including a decrease in excitatory neurotransmission which is likely the cause of the observed reduced LTP. Our work thus reinforces the interest of 5-HT₄Rs activation as a new strategy of interest both to improve the cognitive domains particularly vulnerable to physiological and/or pathological decline, *i.e.* EM, and to exert disease-modifying effects by acting on upstream pathological drivers (glutamatergic transmission and downstream synaptic plasticity) of numerous CNS diseases (AD, PD, SCHIZ, MDD) for which hippocampal-dependent memory disorders are common symptoms. Such thorough understanding of 5-HT₄Rs effects on the hippocampal function may provide a major breakthrough to develop new personalized approach using 5-HT₄Rs – based therapies.

Acknowledgments : Thanks to the “Plateforme de Recherche et d’Innovation en spectrométrie de masse et métabolomique” (PRISMM) in Caen, for performing the neurotransmitter quantification. Many thanks to Gérald Née and Stacy Largillière who helped to realize touch-screen experiments. We warmly thank Céline Rondeau and Mathilde Martineau for performing the surgery for qEEG recordings.

Author Contributions: The study was designed by T.F, M.L and C.M.R. The experiments were carried out by C.M.R. Data analysis was performed by D.Z and C.M.R. Original draft preparation was carried out by C.M.R. Editing was performed by M.L., E.E. and T.F. All authors have read and agreed to the published version of the manuscript.

Funding : This work was supported by the Convention Industrielle de Formation par la Recherche (CIFRE) fellowship (N°2018/0833) from the Association Nationale de la Recherche et de la Technologie (ANRT).

Institutional Review Board Statement: The animal study was performed in accordance with French and European Economic Community guidelines for the care and use of laboratory animals (2010/63/UE).

Conflicts of Interest: The authors declare no conflicts of interest.

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Summary

➤ What are the hippocampal-dependent domains of episodic memory that could benefit from 5-HT₄Rs activation?

We demonstrated that systemic administration of RS67333 at a dose which have previously shown beneficial effects on memory is effective at enhancing location discrimination and novelty recognition, but not memory for order. These results suggest beneficial effects of 5-HT₄Rs activation on pattern separation and recognition memory for novelty which respectively represent the “where” and “what” component of episodic memory. These functions are believed to be supported by the DG and the CA1 areas of the hippocampus respectively.

➤ Are the beneficial effects of 5-HT₄Rs supported by changes in hippocampal functioning?

Beyond the fact that pattern separation and recognition memory for novelty are believed to mainly rely on the hippocampus, we found that CA3-CA1 hippocampal synaptic plasticity was unaffected following TBS but was reduced when using the HFS protocol. Interestingly, plasticity-related theta rhythm was found to be increased in the CA1 area whereas the levels of glutamate were reduced following systemic administration of RS67333.

Our results suggest that 5-HT₄Rs activation may reduce hippocampal activity to a level that allows maintaining a competitive network which becomes active only when a stimulus is relevant enough, hence improving memory encoding accuracy and subsequent better recall performance.

COMPLEMENTARY RESULTS

Assessment of anxiety-related, exploratory, and motivation-like behavior

A number of variables are susceptible to influence animal's performance while performing a behavioral task. Particularly, touch-screen-based assays are sensitive to stress due to the confined and lightened environment. They also require good locomotor capabilities as animals navigate between the touch-screen and the reward magazine. Further, motivation to accomplish the task is crucial and needs to be carefully maintained and monitored during the whole study. To get free of any bias related to these variables and ensure that the observed improvement of PS ability in the RS67333- treated group was purely dependent on cognitive aspects, we collected additional data to evaluate anxiety-like behavior, locomotion and motivation.

Although motivation state was maintained to a certain level by MFR along the experiments, a velocity completion index was calculated and the latency to collect the reward was extracted from raw data ([Article 3](#)).

Supplementary methods

Anxiety-like behavior was assessed in the Elevated Plus Maze (EPM) after the last sessions of the LD task according to the protocol described (Leger et al., 2015). Briefly, 30 mins following RS67333 systemic injection, mice were placed at the center of the EPM and were allowed to freely explore the maze for 5 mins. The time spent in each zone (center, open arms, closed arms) was collected as well as the number of rears and crossing to the closed arms. The maze was lightened at 30 lux at the center. In addition, as an indirect indicator stress and locomotor activity, exploratory activity was assessed using the open-field (OF) test as described elsewhere (Seibenhener & Wooten, 2015). Thirty minutes after RS67333 systemic administration, mice were allowed to freely explore the OF for 10 minutes. The test was performed in total dark (0 lux), using infrared light detection.

Supplementary results

Elevated Plus Maze

Over a 5 minutes' period, animals from the vehicle-treated group spent the majority of their time in the closed arms (**Figure 22A**) and alternated between the closed arms (reflected by the number of crossings in **Figure 22B**). This led to very few time spent in the center of the maze. They did not go to the open arms (*i.e.* the anxiogenic zone of the maze). These results give the basal level of anxiety of the animals. There was no difference in the time spent in each zone of the maze between the vehicle and RS6733-treated group suggesting similar basal level of anxiety.

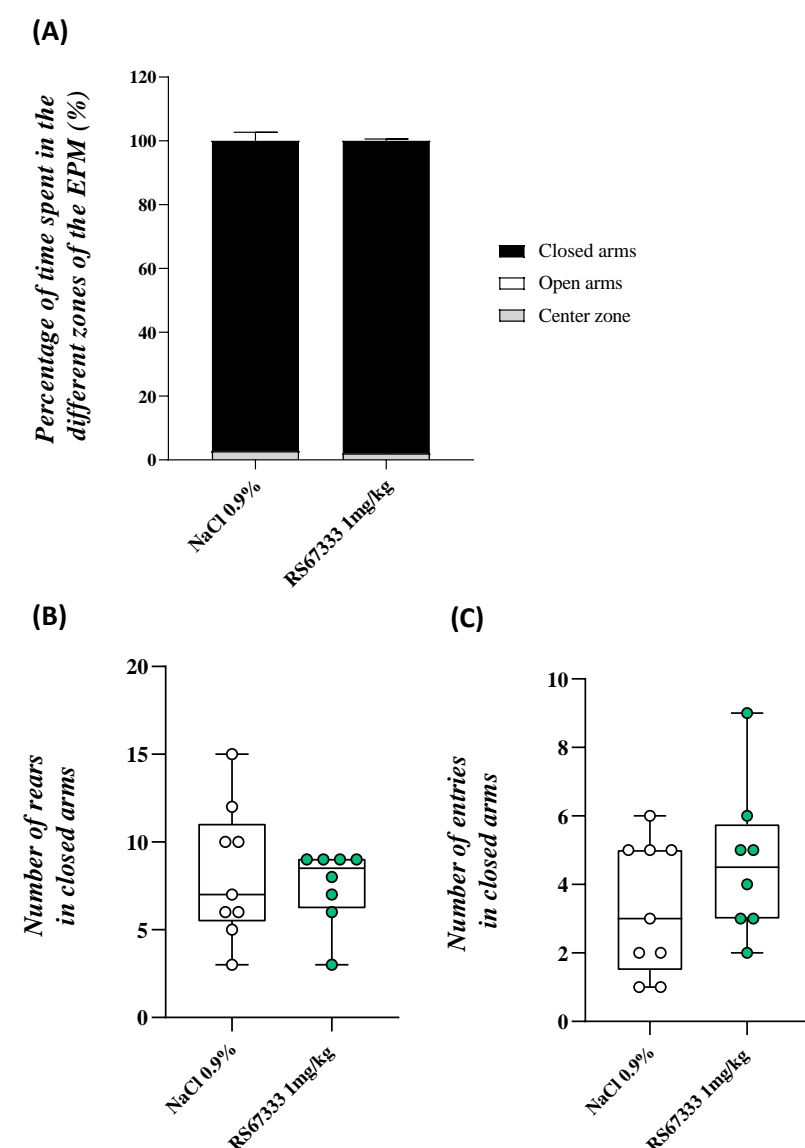


Figure 22: Effect of systemic administration of R67333 in the EPM test.

Data are expressed as median \pm IQ range. Time spent in each area of the EPM (A), number of rears in the closed arms (B) and number of entries in the closed arms (C) for both the vehicle ($n=9$) and RS67333-treated mice ($n=8$).

Open field test

In the OF test, animals from the vehicle and RS67333-treated group spent similar time at the center of the arena (*i.e.* anxiogenic zone) and had similar frequency of entries in this zone (**Figures 23A and 23B**). In addition, the mean velocity as well as total distance traveled in the arena (**Figures 23C and 23D**) were found to be similar in both groups suggesting that locomotion is comparable between groups. The pattern of activity depicted in **Figure 26E** supports that animals from both groups have comparable level of anxiety-like behavior.

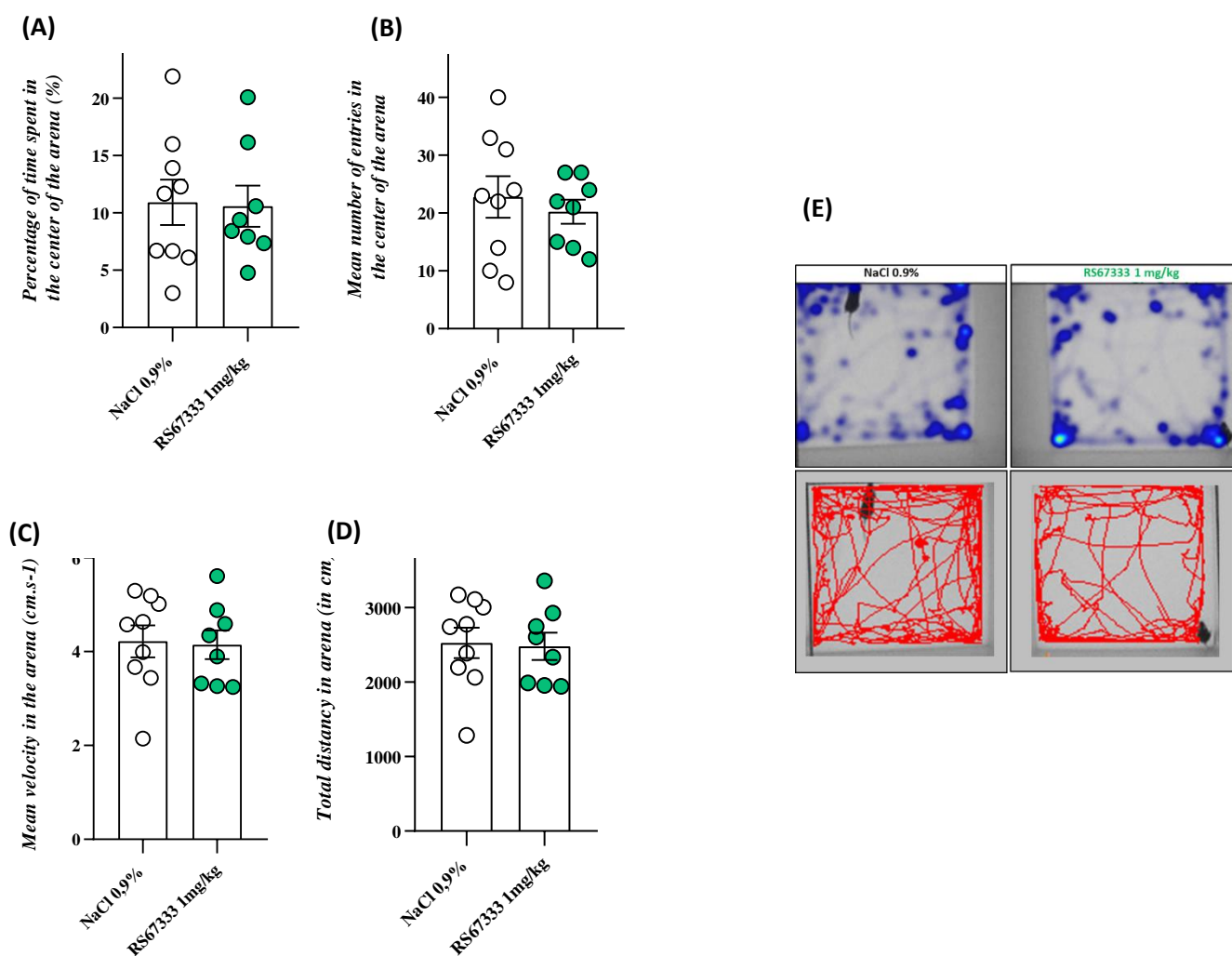


Figure 23: Effects of systemic administration of RS67333 in the OF test.

Data are expressed as mean \pm SEM. Effects of RS67333 at 1 mg/kg on the time spent the center of the arena (*i.e.* the most anxiogenic zone) (**A**), the number of entries in the center of the arena (**B**) the mean velocity in the arena (**C**) and on the total distance in the arena. (**D**) for both the vehicle ($n=9$) and RS67333- treated mice ($n=8$). Patterns of activity following administration of vehicle or RS67333 (1mg/kg) are depicted in figure (E).

Altogether these results reveal no differences in anxiety-like behavior and locomotion between the vehicle and RS67333-treated group. This suggest that (1) the enhancement of performances induced by RS67333 observed in the LD task - and by transposition in the NOR test - is purely dependent on cognitive processes as it is not biased by more pronounced anxiety or locomotor issue in the vehicle group (2) the absence of beneficial effects of 5-HT₄Rs activation in the TOM test should not be due to alteration in locomotion and/or basal level of anxiety.

DISCUSSION

Discussion

Episodic memory processes are highly sensitive to both normal and pathological ageing (such as AD, PD and MDD). Associated memory decline is often related to hippocampal dysfunction with cellular and structural alterations. Therefore, targeting the hippocampal functions to prevent and/or to limit the progression of the disease represents a relevant strategy. Of most interest, preclinical and clinical studies reviewed in **Article 1** report beneficial effects of 5-HT₄Rs activation on cognitive impairments related to the hippocampal function. However, further investigations still remain to be done to identify and understand molecular and cellular mechanisms that sustained those beneficial effects. Hence, the prime objective of this thesis was to **investigate mechanisms at work by focusing on the hippocampal function**. Within this framework, three lines of perspectives were discussed as follows:

- **To which extent 5-HT₄Rs activation is beneficial to the different domains of episodic-like memory.**
- **How much hippocampal synaptic plasticity mechanisms are involved in the behavioral effects of 5-HT₄Rs activation.**
- **Hypothesis and theories of the underlying mechanisms.**

We further opened the discussion on the future of 5-HT₄Rs as part of multi-target drug ligand (MTDL) strategy development against memory disorders related to hippocampal dysfunction.

5-HT₄Rs activation in the different domains of episodic-like memory: the 3W's.

The effects of acute systemic pharmacological stimulation of 5-HT₄Rs (RS67333) were investigated in three different hippocampal-dependent behavioral tasks reflecting the different facets of episodic-like memory ([Article 3](#)). These results are summarized in [Table 11](#).

Table 11: Summary of the effects of RS67333 on the different features of episodic-like memory in healthy mice.

Treatment	LD task ("Where?")	NOR test ("What?")	TOM test ("When")
RS67333 1 mg/kg (i.p -30min)	✓	✓	✗

➤ The **"where"** component of episodic memory was assessed through the **LD task**. This task is representative of **PS function**, which relies on DG functioning (Rolls, 2013). We demonstrated for the first time that **RS67333 (1mg/kg)** - administrated 30 min before probe sessions - **improved discrimination performances (PS capacities)**.

Indeed, to the best of our knowledge, the effects of 5-HT₄Rs agonists on PS ability have never been investigated before. However, the observed enhancement of PS capacity is consistent with known effects of 5-HT₄Rs agonists in the DG subfield. For instance, increasing the level of expressions of certain transcription factors (CREB) (Ishii et al., 2019) and neurogenesis are both considered as essential to PS (Clelland et al., 2009). Regardless, previous work of Graf and collaborators (Graf et al., 2018) have evaluated the effects of other pharmacological agents - with various mechanisms of action but all described as cognitive enhancers - on PS abilities using touch-screen-based assays. In their study, none of the six drugs tested - *rolipram (PDE4 inhibitor)*, *memantine (NMDA-R antagonist)*, *donepezil (acetylcholinesterase inhibitor)*, *nicotine (nACh receptor agonist)* and *xanomeline (M1/M4 preferring muscarinic agonist)* - were reported to be efficient in normal cognitive condition. In contrast, rolipram improved LD performances in scopolamine-induced deficit conditions. Therefore, we further assessed the potential of RS67333 to reverse scopolamine induced deficits. In our conditions, scopolamine alone increased the number of trials to criterion, suggesting impaired PS capacities. Unfortunately, RS67333 was not able to reverse this pharmacologically induced deficit. The absence of beneficial effects of RS67333 is not likely to be attributed to differences in motivation (similar session length, latency to reward collection), but could potentially be explained by impulsivity behavior caused by scopolamine. Indeed, scopolamine is known to induce attentional deficits (Klinkenberg & Blokland, 2010) and was shown to induced hyperactivity-like behavior ([Appendix D](#)). Hence a possible explanation could be that the reversal of scopolamine-induced deficits is more sensitive to drugs that improve attentional processes rather (which are not generally addressed within the scope of 5-HT₄Rs ligands) than purely mnemonic. Perhaps the refinement of the dose of scopolamine could limit such effects as well as peripheral effects that also include mydriasis that can eventually contribute to lower performances in visual based-tests. It is also possible that the deficits induced by scopolamine were too pronounced to be reversed by RS67333 due to the chronicity of scopolamine injections. Indeed, the ability of

RS67333 to reverse scopolamine-induced deficits has been essentially demonstrated in acute or sub-chronic conditions (Freret et al., 2017a; Lelong et al., 2003).

Hereafter, for the evaluation of the “what” and “when” components, **we used spontaneous object recognition paradigms**, based on the attraction of rodents by novel/less recent objects (Dere et al., 2005).

➤ The “**what**” component of episodic memory was assessed through the **NOR** test. **Administration of RS67333 (1mg/kg) 30 min before the sampling session improved object recognition after a 48h retention delay.** This result is consistent with previous data showing enhanced NOR performances following 5-HT₄R activation, but with a quite shorter inter-trial interval (ITI, 4h-24h) (Darcet et al., 2016; Freret et al., 2012b; Hotte et al., 2012; Lamirault & Simon, 2001; Levallet et al., 2009; Moser et al., 2002; Quiedeville et al., 2015). Interestingly, there is increasing evidence that argue for a particular role played by the CA1 hippocampal area according to the ITI used in the NOR. More precisely, CA1 area appears to be particularly involved during long ITI (*i.e.* >20 min) (Ásgeirsdóttir et al., 2020). Taken together, this confirms that the beneficial effects of RS67333 on cognition are supported by the hippocampal function and suggest that the CA1 area may be particularly involved in these effects.

➤ The “**when**” component of episodic memory was assessed through the **TOM** task. This task assesses animal ability to distinguish **past experiences in the order that they occurred**. In contrary to the NOR test, **RS67333 (1 mg/kg) administrated 30 min before the first sampling session, did not improve discrimination performances in the TOM task.**

To the best of our knowledge, this is the first reported experience of pharmacological modulation (with 5HT₄R agonists - or any other cognitive enhancers) in this task. We showed that RS67333 did not influence the temporal aspect of episodic-memory. This absence of effect can be discussed in regards to the distinct cellular processes and brain structures engaged differentially in each component. Indeed, albeit the CA1 area seems to be at the cornerstone of both novelty detection and TOM, the information may be conveyed and integrated under distinct pathways. In contrast to non-spatial object recognition processes which may be more purely dependent on CA3-CA1 pathways, the dynamics of time cells, which fire at specific moments within a cognitive task or experience in the CA1 are governed by medial entorhinal cortex (MEC) inputs (Robinson et al., 2017) where 5-HT₄R have not been identified (Tanaka et al., 2012). Moreover, it is possible that bridging timely spaced cues may rather require self-sustained activity for extended periods (Cox et al., 2019) in contrast to the overall reduced hippocampal activity we put forward in the following sections of the manuscript. Finally, the variety of experimental conditions in which TOM test can be performed (*i.e.* number of item sequences, ITI), should also be considered.

More complex and integrated experiments could include the evaluation of the effects of RS67333 in a task which simultaneously assesses object memory for “what”, “where” and “when” as described by Dere and collaborators (Dere et al., 2005).

Mini summary

- **5-HT₄Rs** activation has the potential to **improve PS and prolonge RM for novelty**. In this regards their activation constitutes an **interesting therapeutic lead** in **brain diseases** for which EM dysfunction represents a hallmark (such as AD, and SCHIZ).
- **5-HT₄Rs activation** does **not** seem to **have a role in natural forgetting condition of TOM**. Confirmation by further experiments is needed as well as a deeper understanding in neurobiological substrates of TOM.

Memory improvement and 5-HT₄Rs activation: a matter of plasticity?

Hippocampal spike timing-dependent synaptic plasticity – the main cellular and molecular substrates of learning and memory (Nicoll, 2017)- is orchestrated by dynamic electrical hippocampal rhythms, which co-exist in different frequency domains. The most extensively studied rhythms in the rodent hippocampus are the theta and gamma bands. Both rhythms have been reported to be highly involved in cognitive processes. Interestingly, 5-HT₄Rs have been shown to interact with several molecular actors (5-HT, GLUT, GABA), and there is an intimate relationship between neurotransmitter tones and EEG activity and stemming brain network communication (plasticity and connectivity) (Drinkenburg et al., 2015).

Therefore, **we wondered whether the observed behavioral effects of RS67333 could be supported by changes in hippocampal oscillations, as well as downstream synaptic plasticity.** In this framework, we evaluated the effects of 5-HT₄Rs activation on (1) *in vivo* hippocampal rhythms through qEEG and on (2) *ex vivo* hippocampal synaptic plasticity induced by patterns which mimic endogenous oscillations. All electrophysiological recordings (*in vivo* EEG and *ex vivo* synaptic plasticity) were performed within the CA1 area of the hippocampus. The CA1 area was shown to be involved in beneficial effects of RS67333 (as shown by the NOR test) and is the most characterized and accessible area for electrophysiological recordings as compared to the DG.

Effects of 5-HT₄Rs activation on in vivo EEG rhythms

Alongside with the development of EEG recordings technique in mice at Porsolt's laboratory, we first explored the effects of acute administration of RS67333 on qEEG. In a resting state, RS67333 did not show any effect neither on the theta, nor on gamma bands (*data not shown*). Conversely, when EEG was performed while mice were exploring a novel environment, a slight - *but not statistically significant* - difference was observed. Indeed, gamma power was significantly increased in both vehicle and RS67333 groups, compared to baseline level. This is consistent with previous studies showing that CA1 hippocampal gamma powers were increased under behavioral demand (Montgomery & Buzsáki, 2007). These first result support the idea of dynamic variations of pharmacological effects on EEG frequency bands between the two experimental conditions (active *versus* resting states). Such state-specific variation of the effects of pharmacological substances have already been described elsewhere (Hansen et al., 2019). Further, we found that theta power was increased in RS67333 treated group (compared to baseline), whereas it remained unchanged in the vehicle group. The effects of cognitive enhancers and/or approved drugs against memory disorders (*i.e* donepezil and memantine) have previously been investigated on cortical EEG (Ahnaou et al., 2014) and reported similar increase of theta power but to our knowledge, this is the first time the effects of 5-HT₄Rs agonists are described on hippocampal rodent EEG. Although the role of theta and gamma in cognition has been largely acknowledged, whether increase or decrease in one or another frequency band is predictive of enhanced memory performances is still arguable.

Most often, increase power either in cortical theta or gamma band of frequencies is related to impaired memory performances. Such results have been reported either in AD patients (Mably & Colgin, 2018) but also in animal models of amnesia either genetically induced (for review see Mehak et al., 2022) after administration of cognitive

disruptors such as scopolamine (Sambeth et al., 2007). Reciprocally, a decrease of theta power was found to support novelty detection and better memory encoding performances in epileptic patients (Lin et al., 2017) and healthy animals (Jeewajee et al., 2008).

Curiously enough increasing gamma rhythms by optogenetic reverses memory impairments and is associated with successful encoding and retrieval in a mouse model of AD (Etter et al., 2019) as well as in AD patients (Benussi et al., 2022). Besides, the cognitive enhancer Donepezil was found to increase even more the theta power when co-administered with scopolamine in rats (Sambeth et al., 2007). Anyhow, those results confirm that EEG is extremely sensitive to fluctuations of memory performances and to the animal's state. Moreover, these observations mainly stem from cortical EEG studies which may slightly differ from EEG recorder in depth (*i.e.* hippocampal EEG).

Unfortunately, we were not able to measure EEG simultaneously while animal was performing behavioral task (see below).

Limitations: *This experiment is part of the methodological development of the wireless EEG technique in mice. In a first step, we used miniaturized internal implants thought to give better signal quality and permitting to maintain mice in group-housed condition. Unfortunately, due to poor signal quality, we finally had to adapt a rat telemetry system to the mouse.*

*This customized telemetry system came out with relatively good quality EEG signals, but due to their weight and volume, they were not compatible with spontaneous exploration and thus did not allow to perform simultaneously any behavioral tests (such as NOR and TOM) as initially planned. Future studies should certainly benefit from new internal implants (Lundt et al., 2016). Regardless, the time spent on method development reduced our opportunity to further analyze discrete EEG features as much as we desired. Indeed, brain oscillations in the rodent hippocampus (either theta or gamma) relate not only to memory performance, but also to running speed independently of explicit memory tasks (Trimper et al., 2017). Therefore, our results deserve to be analyzed by precisely identifying EEG epochs associated with a particular behavior (exploration / walking / sniffing / etc..). Further, cross-frequency-coupling (CFC) where the phase of slower frequencies (*i.e.* theta) is coupled to the amplitude of faster frequencies (*i.e.* gamma) would also constitute an interesting additional analysis. In fact, the hippocampal theta-gamma coupling reflects normal memory processes in both rodents and humans (Alekseichuk et al., 2016; Tort et al., 2008) and inversely, disturbance in such coupling is predictive of memory impairments across species (Goodman et al., 2018; Zhang et al., 2016).*

Effects of 5-HT₄R_s activation on *ex vivo* synaptic plasticity

Synaptic plasticity is a cellular response to upstream oscillations in particular in the theta and gamma range. Effects of 5-HT₄R_s pharmacological activation (either RS67333 or SC53116) on electrophysiological standpoint have been investigated *in vivo*, but so far results are controversial (Kemp & Manahan-Vaughan, 2005; Matsumoto et al., 2001). To get free of any extrahippocampal influences, we investigated the effects of 5-HT₄R_s activation on CA1 synaptic plasticity using ***ex vivo* hippocampal slices**. This pioneer experiment was performed with **RS67333**, either **directly apply into the bath (Article 2)** or **after systemic administration (Article 3)**. For both studies, dose (10 μM and 1 mg/kg), route and time of administration (30 min before slicing and i.p. respectively) were chosen in regards to literature data showing pro-cognitive effects of RS67333 (Hagena & Manahan-Vaughan, 2017).

Table 12: Summary of the effects of RS67333 on CA3-CA1 hippocampal LTP.

LTP magnitude	Bath perfusion	I.P. administration
HFS	=	↓
TBS	↓	=

Regardless of the experimental procedure, it should be noted that the reduction of LTP induced by RS67333 depends (1) on the stimulation paradigm used to trigger LTP (2) on the route of administration (direct bath perfusion or intraperitoneal systemic injection). Thus, when **RS67333 (10 μM)** was applied **directly to the perfusion bath, HFS-induced LTP was unchanged** (relative to control condition) whereby **TBS-induced LTP was reduced**. Conversely, after **systemic administration of RS67333 (1 mg/kg)**, **HFS-LTP was impaired** but **TBS-induced LTP remained unchanged** (relative to controls).

This frequency-dependent effect of 5HT₄R_s has been previously discussed (**Article 2** and **Article 3**). However, its opposite expression in the two studies (i.e. according to the treatment route) raises novel questions that have to be tackled.

- First of all, the use of different mice strains in the two studies should be questioned. Indeed, while the effects of 5-HT₄R_s agonist applied on bath perfusion were investigated in NMRI mice, systemic administration study was performed on C57BL/6 mice. Through a comparative approach (**Article 3**), we showed that NMRI mice displayed a weaker TBS-induced LTP than C57BL/6 mice, due to a less powerful disinhibition process. However, HFS-induced LTP revealed comparable levels in both strains. In addition, we demonstrated that 5-HT₄R_s activation by bath application of agonist led to similar effect either in NMRI strains of mice or in mouse strain constructed on a C57BL/6 background. Therefore, the difference between *ex vivo* application of RS67333 and *in vivo* systemic administration may not rely on strain differences.
- Secondly, the question of the pharmacodynamic influence has also to be handled. Indeed, receptor occupancy would be expected to be different. According to the pharmacological profile of RS67333, it is

expected that in the conditions of direct bath application the receptor occupancy would be of ~90% when applied at 10 μ M and ~50% when applied at 1 μ M. For recall, at the lowest dose of 1 μ M RS67333 did not affect LTP. Oral administration of RS67333 at 1 mg/kg resulted in 25% of 5-HT₄Rs occupancy (Nirogi et al., 2013). 5-HT₄Rs are known to be susceptible to desensitization after chronic activation (Ansanay et al., 1992). Hence, whether the decreased LTP observed in bath application conditions could be related with such downregulation in GPCR signaling would be worth of being elucidated. Receptor desensitization is unlikely to occur after a single systemic administration of RS67333. Nevertheless, how RS67333 is metabolized when systemically administered and what are the potential central effects of its metabolites have never been investigated and remains unknown.

- Thirdly, systemic administration of RS67333 prior to *ex vivo* measurements may have involved additional circuits as compared to local bath application of the agonist. In fact, the hippocampus and the prefrontal cortex (PCF) are bidirectionally connected *via* both indirect and direct pathways there is an intricate interaction between the medial prefrontal cortex (mPCF) and the hippocampus circuit during formation and retrieval of EM (Eichenbaum, 2017). Importantly, 5-HT₄Rs activation was shown to be involved in the modulation of mPCF on serotonergic neurons of the DRN – which is the main serotonergic input to the hippocampus (Faye et al., 2020; Teixeira et al., 2018). While investigating the anxiolytic properties of RS67333, it was suggested that 5-HT₄Rs share common pathway with GABAergic agonist (*i.e* diazepam) supporting an interplay between these neurotransmission systems albeit the nature of this interaction remains to be elucidated (Faye et al., 2020). Anyhow, it seems reasonable to consider that upon systemic 5-HT₄Rs stimulation, the hippocampal activity measured *ex vivo* is the result previous of dynamic interaction with neighbouring brain structures which could not have occurred when the treatment was directly applied hippocampal slices.

Besides, the cytoarchitectural organization of the hippocampus is subfield-specific and thus confers a regional specialization in cognitive functions. In this regard it is important to stress that our work mainly focused on CA1 hippocampal synaptic plasticity. Nevertheless, it would be worth to investigate the effects of 5-HT₄Rs activation within additional pathways that include PP-DG and MF-CA3 for instance. Indeed, while electrophysiological properties of each subfield are specific, the activity in one particular hippocampal region can in turn influence the activity in downstream hippocampal areas. Among *in vivo* studies, only one performed within the MF-CA3 pathway reported LTP blockade by 5-HT₄Rs activation (Twarkowski et al., 2016). These results were interpreted such as this receptor may play an important role in driving pattern separation in the DG and CA1, coupled with the suppression of (putatively) erroneous pattern completion in CA3. Two studies were performed within the DG (Kulla & Manahan-Vaughan, 2002; Twarkowski et al., 2016) with controversial results according to the way of administration. All of these studies were centered around a single hippocampal area at the time.

Given the specificity of each subfield either in terms of their position within the tri-synaptic loop or the mechanisms involved in LTP (*i.e* presynaptic LTP at MF-CA3 pathway) it appears of most interest to assess how the effects of 5-HT₄Rs activation on LTP in specific regions could shape downstream area activity/plasticity. For instance, in the case of SCHIZ, studies have led to the hypothesis of a dynamic interaction between hippocampal subfields, where

reduced activity in the DG can lead to enhanced LTP in CA3 which would be projected onto CA1 likely due to a sensitization of the targeted region to incoming stimuli thereby generating lower threshold for LTP induction (Tammimga et al., 2012). In addition, this would not be the first time that 5-HT₄ ligand are shown to display opposite effects according to the subfield of investigation. Indeed, 5-HT is generally shown to enhance LTP within the CA1 area (Cai et al., 2013) whereby being mainly inhibitory at MF-CA3 synapses (Twarkowski et al., 2016). In this view, we initiated the implementation of multi electrode array (MEA) recordings, allowing stimulation and/or recordings within different areas simultaneously.

Overall, our results point out that the effects of 5-HT₄Rs stimulation are different when investigated on the generation of EEG rhythms or in response to artificial stimulation at corresponding frequency.

Above all, our results seem to consent on an overall reduction of hippocampal synaptic plasticity following 5-HT₄Rs activation with RS67333.

This opens the discussion on **the underlying molecular and cellular mechanisms and how this could benefit to hippocampal-dependent memory performances such as PS and RM.**

Mini summary

RS67333 administered systemically **increases theta power** upon exploratory behavior.

5-HT₄Rs activation influences **hippocampal synaptic plasticity** in a **frequency-dependent** fashion within the CA1 area. The treatment route has an impact on the effects of **5-HT₄Rs** activation on **hippocampal synaptic plasticity**.

Our results support that there is a **cross-talk** between **5-HT₄Rs** and the different **neurotransmission systems engaged** in the **protocols** used for **LTP induction** and that are **relevant to learning and memory**.

There seems to be a **dissociation** between the effects of 5-HT₄Rs activation observed in the **modulation of EEG rhythms** and the **reponses to frequency-matched stimulation** paradigm induced artificially.

Hypotheses and theories

5-HT₄Rs activation regulates the excitatory/inhibitory balance through the modulation of GLUT and GABA neurotransmission

GLUT and GABA are respectively the most abundant excitatory and inhibitory brain neurotransmitters and have been both related to synaptic plasticity processes. In the CNS, the excitatory/inhibitory (E/I) balance is maintained at its equilibrium through GLUT and GABA homeostatic relationship. Therefore, it seems reasonable to assume that one, the other or both of these neurotransmission systems might be involved in the decreased hippocampal activity following 5-HT₄Rs activation that we hypothesized from our results.

Following acute systemic administration of RS67333 (1 mg/kg i.p. -30 min), we observed a decreased hippocampal GLUT levels, whereas the levels of other neurotransmitters (GABA, ACH, SER) remained unchanged. A direct link between GLUT system and 5-HT₄Rs activation has been poorly investigated so far. Nevertheless, one recent study reported that prucalopride indirectly reduced bursts of AMPA-receptor-mediated currents in the hippocampal CA3 auto-associative network, by altering glutamatergic transmission (Chen et al., 2020). The authors claimed that it may support a greater signal-to-noise ratio of relevant stimuli, which is in line and above all strengthens the hypothesis of beneficial effect of lowering hippocampal network excitability for cognitive processes.

Another possibility would involve an interaction of 5-HT₄Rs with metabotropic glutamate receptors (mGLURs), such as mGLUR1/5. These receptors are involved in synaptic plasticity processes (Niswender & Conn, 2010) and play an important role in the negative regulation of glutamate release (as auto receptors).

Stemming from the observation that bath application of 5-HT₄Rs agonist led to changes in LTP magnitude only when induced by TBS, and not HFS, it raised interrogations about the particularity of the mechanisms of action of both protocols as deeply discussed in **Articles 2 and 3**.

HFS-induced LTP is thought to be highly dependent on glutamatergic transmission as it results in maximal release of glutamate and maximal recruitment of NMDA-Rs. Thus, decrease in GLUT levels may explain the lower HFS-induced LTP magnitude in RS67333-treated group (as compared to the vehicle-treated group).

Conversely, TBS-induced LTP is rather the result of a fine regulation of GABAergic neurotransmission through disinhibition process, not necessarily maximizing the recruitment of NMDA-Rs. Therefore, the impairment observed following 5-HT₄Rs activation is likely to be related to a failure in the disinhibition process and prompted the hypothesis of an interplay between the effects of RS67333 and the GABAergic neurotransmission (**Article 2**). Indeed, we also showed that application of the GABA_A-R antagonist (bicuculline) prevented RS67333-related impairments of TBS-induced LTP. However, there was surprisingly no effect of RS67333 on evoked GABA_A-Rs currents investigated by patch-clamp. Pre-synaptic GABA_B-Rs are the main drivers of disinhibition. Their selective blockade (by CGP55845 at 1 μ M) resulted in LTP impairments similar to that observed with RS67333. Besides, when co-applied with RS67333, the pre-synaptic GABA_B-Rs antagonist did not

show either preventive or additive effects. On the contrary, the effects of RS67333 were blocked in the presence of GABA_B-Rs post-synaptic receptors antagonist (CGP35845, 1 μ M).

To confirm the contribution of GABAergic neurotransmission in the effects of RS67333 on synaptic plasticity, we designed a preliminary work using an optogenetic approach. In fact, selective silencing of all GABAergic interneurons during TBS partially restored RS67333-induced decreased LTP (**Complementary results**). These results are encouraging and would require supplementary recordings to increase the statistical power but also additional control condition (such as the testing of different laser powers). To a greater extent, investigations for the identification of the involvement of specific interneurons subsets (such as Parvalbumin positive, PV⁺) would be of prime interest. Indeed, fast-spiking PV⁺ are frequently associated with the generation of oscillatory theta rhythms in the hippocampus (Nuñez & Buño, 2021).

Previous data already suggested a cross-talk between 5-HT₄Rs and GABAergic neurotransmission system. For instance, 5-HT₄Rs activation led to activity-dependent bidirectional regulation of GABAergic signalling on pyramidal prefrontal cortex neurons (Cai et al., 2002).

More notable, an increase in GABA release was observed in guinea pig hippocampal slices after 5-HT₄Rs stimulation (Bianchi et al., 2002; Bijak & Misgeld, 1997). Nevertheless, the underlying mechanism at play still remain debated since 5-HT₄Rs mRNA were reported to not co-localize with GABAergic marker (GAD). Based on this immunohistochemistry results, authors concluded towards an absence of 5-HT₄Rs expression on GABAergic neurons (Peñas-Cazorla & Vilaró, 2015), leaving open the question of how 5HT₄Rs may modulate GABAergic function. Of note, mRNA measurements at a given time is not informative about the expression at the protein level. Indeed, while no mRNA of 5-HT₄Rs has been detected within DG interneurons, their presence was attested by electrophysiological recordings (Bijak & Misgeld, 1997). Besides, still remains open the possibility of indirect modulation by acetylcholine where 5-HT₄Rs are expressed and whose release is increased by 5-HT₄Rs activation (Consolo et al., 1994; Siniscalchi et al., 1999). Indeed, 5-HT₄Rs activation has been shown to affect electrically-evoked GABA release *via* cholinergic (M1 and M4) receptors (Bianchi et al., 2002). ACH can control the release of GABA since Ach-R have been identified on GABAergic interneurons (Van Der Zee & Luiten, 1993). Other neurotransmission system could also be at work. In fact, it has been suggested that hippocampal 5-HT₄Rs might modulate cannabinoid signaling mediated by CB1 receptors which temporarily inhibits neurotransmitters release (Nasehi et al., 2016). Authors thus showed that RS67333 tends to potentiate CB1 receptors activity, which in turns led to a negative feedback regulation of 5-HT among others as part of retrograde signaling. In addition, an interplay between 5-HT₄Rs and the cannabinoid system was observed during an object novelty detection paradigm (Nasehi et al., 2017).

Is reduced hippocampal synaptic plasticity compatible with better memory performances?

Better memory functioning is most often related to enhanced synaptic plasticity (LTP) and *vice versa*. However, under certain circumstances, this link might not be so straightforward. For instance, the genetic manipulation of key plasticity-regulating genes and cAMP signaling pathway led to spatial memory impairments whereby LTP was enhanced (Garcia-Alvarez et al., 2015). We here outline three lines of perspectives that should be considered and which could support that reduced LTP is not necessarily contradictory with better memory performances.

- E/I equilibrium

Dysfunction of the control of pyramidal neuron by GABAergic interneurons is a relatively established pathological feature of cognitive and behavioral abnormalities, notably in AD (Xu et al., 2020). In line, in a transgenic mouse model of AD (Tg2576), the number of PV⁺ GABAergic interneurons was found to be decreased as compared to aged-match WT (Huh et al., 2016) (**Appendix E**). This likely contributed to the observed reinstatement/increase of LTP whereby NOR and MWM performances were impaired.

- Saturability of LTP

Alongside with the discovery of LTP, Bliss and Lomo found that plasticity phenomenon are saturable (Bliss & Lømo, 1973). This led to the introduction of homeostatic plasticity which allows the maintenance of synaptic strength at a set level (either increased or decreased) to be changeable by subsequent neuronal activity and thus prevents saturation of LTP/LTD. For instance, both extreme or insufficient synaptic plasticity can disrupt input-specific learning rules in the hippocampus (Lee & Kirkwood, 2019).

- Metaplasticity

Metaplasticity entails a change in the physiological or biochemical state of neurons or synapses leading to modifications of the sensitivity of a population of synapses in response to afferent stimulations (Abraham, 2008). Neurons are able to sense their own excitability and trigger negative-feedback homeostatic mechanisms to counteract perturbations in synaptic activity and restrain it within a dynamic - but physiological - range. In keeping with this view, the decreased plasticity we observed following 5-HT₄Rs pharmacological activation could be the result of synaptic adaptations of upstream increased excitability. Interestingly, 5-HT₄Rs stimulation has been found to increase CA1 pyramidal neurons firing rate (Mlinar et al., 2006).

In addition, some evidences argue for a role of secreted BDNF – *which is increased by 5-HT₄Rs activation* - in driving decrease in synaptic strengths caused by extended periods of increased network activity (Turrigiano, 2008). Besides, depotentiation has been regarded as another important type of meta-plasticity. Acting as a counterbalance to the potential saturation of synaptic potentiation, DP may contribute to synaptic homeostasis. Interestingly, RS67333 prevents electrically-induced DP in the DG (Kulla & Manahan-Vaughan, 2002; Twarkowski

et al., 2016) as well as learning-induced DP in the CA1 area (Kemp & Manahan-Vaughan, 2005).

Mini summary

- 5-HT₄Rs agonists may reduce excitability and increase the threshold for LTP induction to maintain the **hippocampus as a competitive network**. But, **once established LTP is sustained to ensure the persistence of memory trace** (as reflected by DP blockade).

The future of 5-HT₄Rs-based therapies, towards Multi Target Directed Ligand (MTDL)

Clinical relevance of 5-HT₄Rs activation

From bench to bedside, our work provides new preclinical evidences (in addition to those reported in the [Review article](#)) for beneficial effects of 5-HT₄Rs in various aspects of cognition that are relevant to clinics. This is mainly supported by the following observations:

- **5-HT₄Rs activation has beneficial effects on domains of episodic memory that are early altered in patients with cognitive decline.**

We demonstrated the beneficial effects of 5-HT₄Rs stimulation on PS and RM for novelty in mice, which processes are common across species from rodents to humans, enhancing the predictiveness of such beneficial effects in humans (Yassa et al., 2011). In this line, recent clinical data showed improvement of RM in healthy subjects following prucalopride (Resolor®) intake (de Cates et al., 2021, 2022; Murphy et al., 2021). Of most interest, both RM and PS functions are early impaired in brain diseases such as AD (Ally et al., 2013; Parizkova et al., 2020), PD (Rochais et al., 2020) and SCHIZ (Conklin, 2002; Das et al., 2014) and are associated with abnormal hippocampal function (see [Review article](#)). This strongly supports the need to encourage the development of 5-HT₄Rs stimulation-based therapies in such brain diseases.

- ***Some of the effects of 5-HT₄Rs activation fit with some features of interest in current therapeutic strategies developed against memory disorders.***

Our results support that 5-HT₄Rs activation participates to overall reduction of hippocampal activity. This represents an interesting mechanism in view of pathologies such as AD and SCHIZ for which hippocampal E/I imbalance significantly contributes to the pathogenesis. Following the idea that ***“too little activation is bad, too much is even worse”***, increased NMDA-Rs activity (partly due to an excess of glutamate release) is also thought to be at play in AD and has led to the development of a therapeutic strategy based on NMDA-Rs antagonists (such as memantine). In addition, primary GABAergic dysregulation is known to be at the root of several pathologies, such as MDD, SCHIZ and AD. Inverse correlation between GLUT/GABA ratio was found in visuo-spatial working memory task in humans (Cohen Kadosh et al., 2015) suggesting that a reduction in excitability seems compatible with the improvement of memory performances. Consistently, it was recently demonstrated that high hippocampal GABA level correlate with associative learning paradigms in healthy humans (Spurny et al., 2020).

Multi Target Directed Ligand (MTDL)

In recent years, the “one gene, one target, one drug” reductionist strategy has progressively shifted towards the development of MTDL (Cavalli et al., 2008). By acting on several targets, MTDLs have the potential to display synergistic effects with limited risk of drug–drug interactions. Such an approach represents a new paradigm in drug discovery and design and appears as a promising strategy to tackle the complex aetiology of the disease, notably in the field of AD (Simone Tranches Dias & Viegas, 2014).

In this framework, in 2014, the neuroprotective effects of both 5-HT₄R activation and acetylcholine esterase (AChE) inhibition were combined for the first time into a single pleiotropic compound named donecopride. Being a structural compromise between an active group of RS67333 and of the AChE inhibitor donepezil, donecopride acts as a selective and partial (48.3%) 5-HT₄R agonist, with nanomolar potency ($K_i = 8.5$ nM) and also behaves as a mixed-type competitive inhibitor of AChE ($IC_{50} = 16$ nM) (Lecoutey et al., 2014; Rochais et al., 2015). Donecopride holds promises as both symptomatic - (*in view of its AChE inhibitory activity*) - and disease-modifying - (*in view of its 5-HT₄R agonist properties*) - therapeutic drug against AD and is currently awaiting to enter phase I of clinical trials. Indeed, it was shown to decrease the phosphorylation of neurotoxic tau protein in hippocampal neurons injured with A β and promote soluble APP α both *in vitro* and *in vivo* (Lecoutey et al., 2014). *In vivo*, donecopride demonstrated both pro-cognitive effects in healthy mice, as well as anti-amnesic properties in scopolamine-induced cognitive deficits model and in mouse models of AD (viral injection of A β and 5xFAD mouse strain) (Rochais et al., 2020).

Recently, the chemical structure of donecopride inspired the design and synthesis of new MTDL candidates associating 5-HT₄R activation, AChE inhibition and 5-HT₆R inverse agonist activity. Among the battery of synthesized compounds, one demonstrated promising results and excellent general tolerance, albeit further experiments are needed to generate pharmacokinetic data and confirm its beneficial effects in preclinical models (Hatat et al., 2019).

Similarly, the structure of RS67333 was modified for the development of a new 5-HT₄R agonist bearing antioxidant activity (Lanthier et al., 2019), which is believed to be a consequence of A β accumulation-induced mitochondrial dysfunction and ultimately a cause of neuronal death.

In this view, the confirmation of the interest of 5-HT₄R targeting by a thorough understanding of the mechanisms underlying beneficial effects of their activation represents a milestone in the journey towards the development of new MTDLs with 5-HT₄R activity against neurodegenerative and psychiatric diseases.

Conclusion & Perspectives

Our work demonstrated that the effects of 5-HT₄Rs activation involves changes in the hippocampal function. Through a behavioral approach, we confirmed the beneficial effects of 5-HT₄Rs activation on episodic-like memory and particularly in hippocampal-dependent PS and RM. Meanwhile, albeit having only slight effect on hippocampal oscillations that drive synaptic plasticity, at a cellular level, 5-HT₄Rs pharmacological stimulation tended to reduce LTP induced artificially on hippocampal slices. Stemming from additional experiments at both cellular and molecular level, different hypotheses have been put forward, including an interplay with the inhibitory GABAergic system, together with a decrease in excitatory neurotransmission through a reduction in hippocampal GLUT levels. Further experiments with GABAergic ligands assessed both *in vivo* and *ex vivo* and/or patch-clamp studies are needed, notably to determine how 5-HT₄Rs signaling can interact with one or another system, either by direct or indirect modulation.

On a functional standpoint, this could be translated into beneficial effects on hippocampal-dependent memory processes by reducing the signal-to-noise ratio and promoting filtering of the information during encoding— a function which is interestingly often attributed to PS and RM. Indeed, nowhere near all experienced events are turned into memories and thereby require the operation of filters to ensure the memorization of the most salient events, hence improving memory encoding. This assumption requires the consideration of the anatomic-functional segregation that exists along the different subfields of the hippocampus. Although we initiated such discussion to provide a functional dimension to our results, this deserves to be confirmed experimentally. It could be achieved through the use of an MEA system by recording hippocampal synaptic plasticity following 5-HT₄Rs activation in the different subfields simultaneously. Further, it should be considered that the beneficial effects of 5-HT₄Rs at the behavioral level can result from complex top-down regulations between the hippocampus and surrounding brain structures (such as the mPCF)

Altogether, this work strongly supports that the beneficial effects of 5-HT₄Rs activation on memory in rodents are at least in part supported by the hippocampus, from the molecular to the functional level. Given this, it becomes increasingly interesting to consider the targeting of 5-HT₄Rs in a broad range of cognitive disorders such as AD, PD, MDD and SCHIZ. Indeed, in addition to address the main pathological drivers of cognitive decline in these common pathologies, pharmacological stimulation of 5-HT₄Rs acts at the hippocampal level which early alterations are a core feature.

The evaluation of cognitive enhancers in healthy animals represents a crucial first step in preclinical studies but also requires to be reproduced in animal models of memory impairments induced pharmacologically and/or genetically.

Taken together, 5-HT₄Rs pharmacological stimulation is an interesting strategy in early stages of cognitive decline. Alternatively, the use of 5-HT₄Rs agonists as part of MTDL strategy for instance, represents an opportunity to widen the scope of current therapeutic strategies by featuring indirect modulation of GABAergic and glutamatergic transmission.

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APPENDIX

Appendix A

Breeding flow chart for the obtention of transgenic mice expressing ArchT in GABAergic neurons.

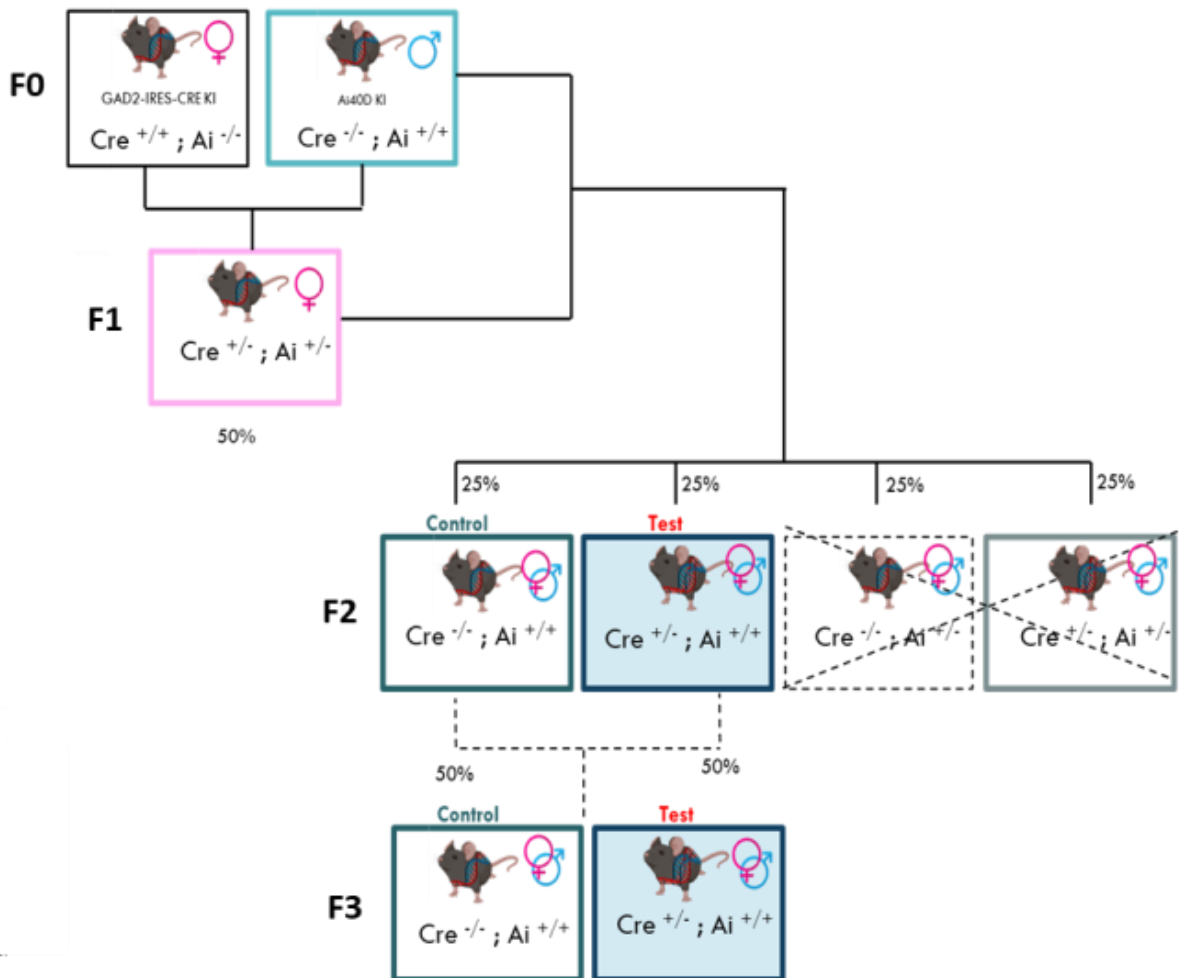


Figure 1: Breeding flow chart designed to obtain the $Ai^{+/+} Cre^{+/-}$ and $Ai^{+/+} Cre^{-/-}$ mice.

F0 mice (parental) were obtained from the Jackson Laboratories and mated together to obtain heterozygous mice for each transgene which constitute the F1 generation ($Cre^{+/-}; Ai^{+/-}$). F1 mice (males and females) were back-crossed with parental (F0) mice to obtain four different genotypes, including the negative control ($Cre^{-/-}; Ai^{+/+}$) and the testing lineage ($Cre^{+/-}; Ai^{+/+}$) expressing ArchT in GAD65-expressing cells (GABAergic interneurons). These two genotypes were maintained by successive breeding between themselves.

Appendix B

Genotyping protocol for Ai Cre mice, adapted from JAX's protocol (#9943).

Primers sequence

Table 1: Primer's sequence for Ai and Cre genes.

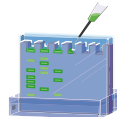
	PRIMER	SEQUENCE 5' → 3'
Ai gene	Common (A)	CTT TAA GCC TGC CCA GAA GA
	Mutant Forward (B)	TTG CAT CGC ATT GTC TGA GT
	Wild type forward (C)	AGT AAG GGA GCT GCA GTG GA
CRE gene	Common (D)	AAC AGT TTG ATG AGT GAG GTG A
	Mutant Forward (E)	CAC TGC ATT CTA GTT GTG GTT TG
	Wild type forward (F)	TCG TTG CAC TGA CGT GTT CT

PCR mix and conditions

Table 2: Preparation of the samples for amplification and amplification conditions.

REACTION A and B (Ai WT and Mutant)		REACTION C (Cre WT and mutant)	
REAGENT	FINAL CONCENTRATION	REAGENT	FINAL CONCENTRATION
ddH ₂ O	-	ddH ₂ O	-
Kapa MIX (KAPA2G fast ready mix)	1X	Kapa MIX (KAPA2G fast ready mix)	1X
Primer A	0.5µM	Primer D	0.5µM
Primer C	0.5µM	Primer E	0.5µM
DNA	1/25	Primer F	0.5µM

PCR		
TEMPERATURE (°C)	TIME	CYCLES
95°C	2mins	
95°C	20sec	
65°C – 0.5°/Cycle	15sec	10 cycles
72°C	10sec	
95°C	15sec	
60°C	15sec	30 cycles
72°C	10sec	
72°C	2mins	
4°C		



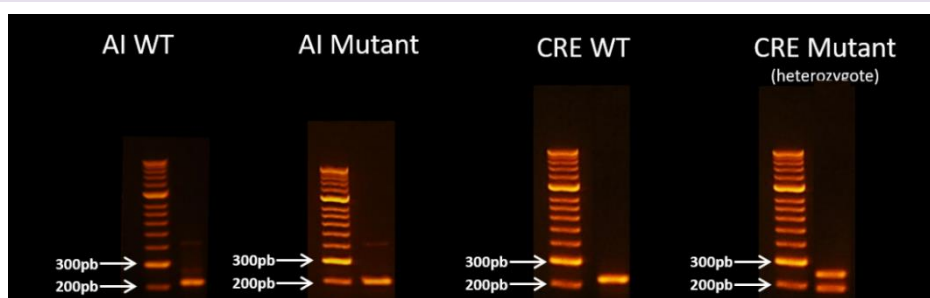
Migrate on 2% agarose gel + Ethidium Bromide (EtBr) 5% at 100V for 25 mins.

Use DNALadder 50pb as size marker (Biolabs)

Results

Table 3: Expected results of the genotyping.

	Ai gene EXPECTED RESULTS	CRE gene EXPECTED RESULTS
Mutant	~ 200 bp	176pb
Heterozygote	~ 200 bp and 216 bp	176pb and 225pb
Wild-type	216 bp	225pb



Appendix C

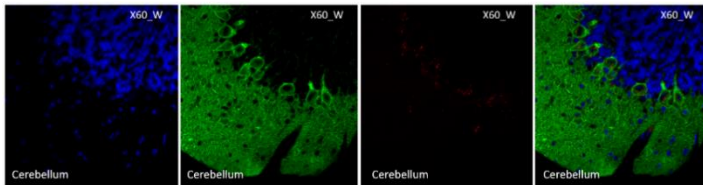
Confirmation of the expression of Arch-T in GABAergic interneurons by immune-fluorescent assay.**Table 4:** List of reagents and materials used for immunofluorescent verification of ArchT expression.

Reagents	Supplier	Reference
Mouse anti-PV	SWANT	235
Rabbit anti-GAD2	Abcam	Ab239372
Goat anti-rabbit AlexaFluor® 647	Abcam	Ab 150079
Goat anti-mouse AlexaFluor® 555	Abcam	Ab 150115
DAPI staining solution	Sigma Aldrich	CAS: 28718-90-3
PBS 0.1M	Home made (see below)	-
$Na_2HPO_4 \cdot 2H_2O$ (3.9g/L)	Fisher Scientific	CAS: 10028-24-7
$NaH_2HPO_4 \cdot 2H_2O$ (13.35g/L)	Fisher Scientific	CAS: 13472-35-0
$NaCl$ (4.5g/L)	Fisher Scientific	CAS: 7647-14-5
PBS - BSA	Sigma- Aldrich (BSA)	CAS 9048-46-8
Triton 10%	Sigma Aldrich	CAS: 9036-19-5
PB 0.1M		
Normal goat serum	Abcam	Ab7481
Immuno-mount medium	Sigma Aldrich	Fluoromount™

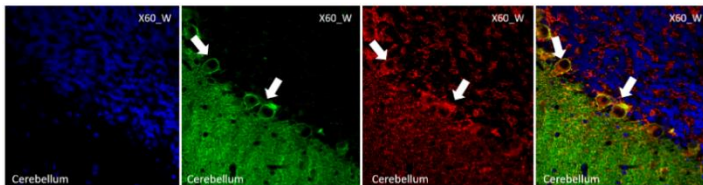
Materials	Supplier	Reference
Vibratome	Leica®	VT 1000S
Fluorescence microscope	Zeiss®	Zen (blue edition)
Camera	Hamamatsu®	

Abbreviations: **BSA:** Bovine Serum Albumin, **DAPI:** 4',6-diamidino-2-phenylindole; **PB:** Phosphate Buffer; **PBS:** Phosphate Buffered Saline; **PV:** Parvalbumin

No 1st Ab _ AlexaFluor 647



Anti-GAD67 _ AlexaFluor 647



Anti-GAD67 _ AlexaFluor 647

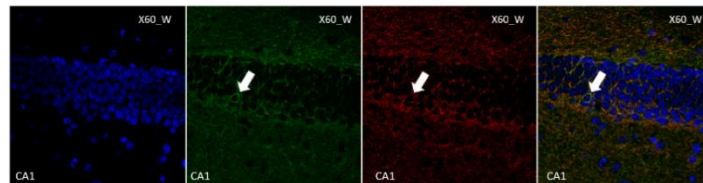


Figure 3: Verification of the expression of ArchT transgene in GABAergic interneurons of $Cre^{+/-}; Ai^{+/+}$ mice (F2 generation) by immunofluorescence. ArchT transgene was coupled to EGFP, nuclei were stained with DAPI and GABAergic interneurons were stained with antibody directed against GAD67 (pan GABA). Arrows mark neurons that co-localize for GAD67 and EGFP.

Appendix D

Evaluation of the beneficial effects of RS67333 in scopolamine-induced deficit in the mouse using the LD task.

Scopolamine is a muscarinic antagonist known for its amnesic properties making a gold standard as cognitive disruptor in a number of preclinical behavioral tests including tests performed in touchscreen chambers (Graf et al., 2018). 5-HT₄R agonists have been shown to reverse mnesic deficits induced by scopolamine in a number of tests (Freret et al., 2017a; Mastumoto, 2001).

General procedure information

Following the exact same protocol for LD task as described in [Article 3](#) and more in details in the [Materials and Methods](#) section of the manuscript, C57BL/6 mice received double injection of either **NaCl 0.9% (i.p.) followed by scopolamine at 1 mg/kg (s.c.)** or **RS67333 1 mg/kg (i.p.) prior to scopolamine at 1 mg/kg (s.c.)**, thirty (30) minutes before each probe session.

Experimental results

First of all, pharmacological induction of PS deficits by scopolamine was confirmed as shown by the increased number of trials to criterion before the session ends as compared to the vehicle group. In co-administration with scopolamine, RS67333 did not reduce either the number of trials to criterion as compared to scopolamine alone [group effect ($F_{(2, 15)} = 9.62$, $*** p < 0.001$), but no significant effect of difficulty ($F_{(1, 15)} = 0.03$, $p = 0.85$) or group \times time interaction ($F_{(2, 15)} = 1.69$, $p = 0.19$), ANOVA for repeated measurements].

These results suggest no effect anti-amnesic of RS67333 in scopolamine-induced PS deficits.

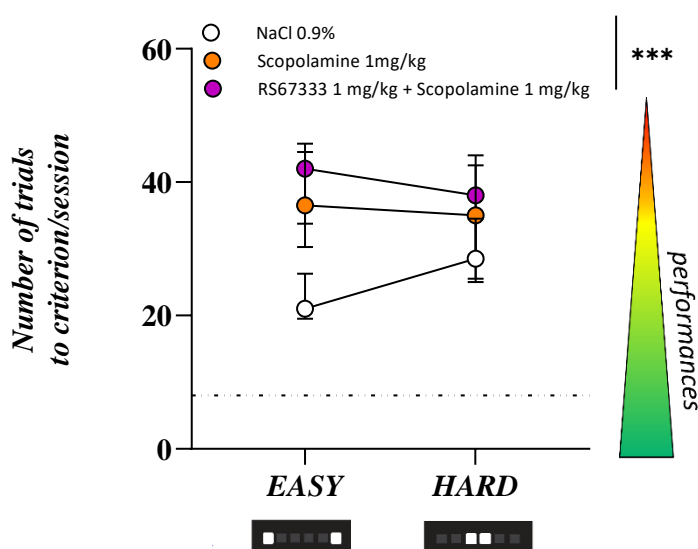


Figure 4: Number of trials to reach the criterion before the session ends in “easy” and “hard” level of difficulty in the probe test. Data are expressed as median \pm interquartile. Vehicle group (n=9), Scopolamine-treated group (n=9); RS67333+scopolamine-treated group (n=8). $***p < 0.001$, ANOVA-type for repeated measurements.

In an OF test, mice treated with scopolamine (1 mg/kg) and co-administered with RS67333 (1mg/kg) and scopolamine (1 mg/kg) exhibited thigmotaxis as shown by the reduction in the percentage of time spent at the center of the arena as compared to control [$p < 0.01$ for both scopolamine alone and RS67333 + scopolamine, One-way ANOVA followed by Dunnett's multiple comparison test) **Figure 5A**].

In addition, mice from both groups displayed increased traveled distance in the arena [($p < 0.001$ and $p = 0.01$ for scopolamine alone and RS67333 + scopolamine respectively, Kruskal-Wallis followed by Dunn's multiple comparison) **Figure 5B**] as well as increased velocity [$p < 0.001$ and $p < 0.01$ for scopolamine alone and RS67333 + scopolamine respectively, One-Way ANOVA followed by Dunnett's multiple comparison) **Figure 5C**] as compared to the vehicle group. The activity pattern depicted on **Figure 5D** allows to visually confirm such behavior.

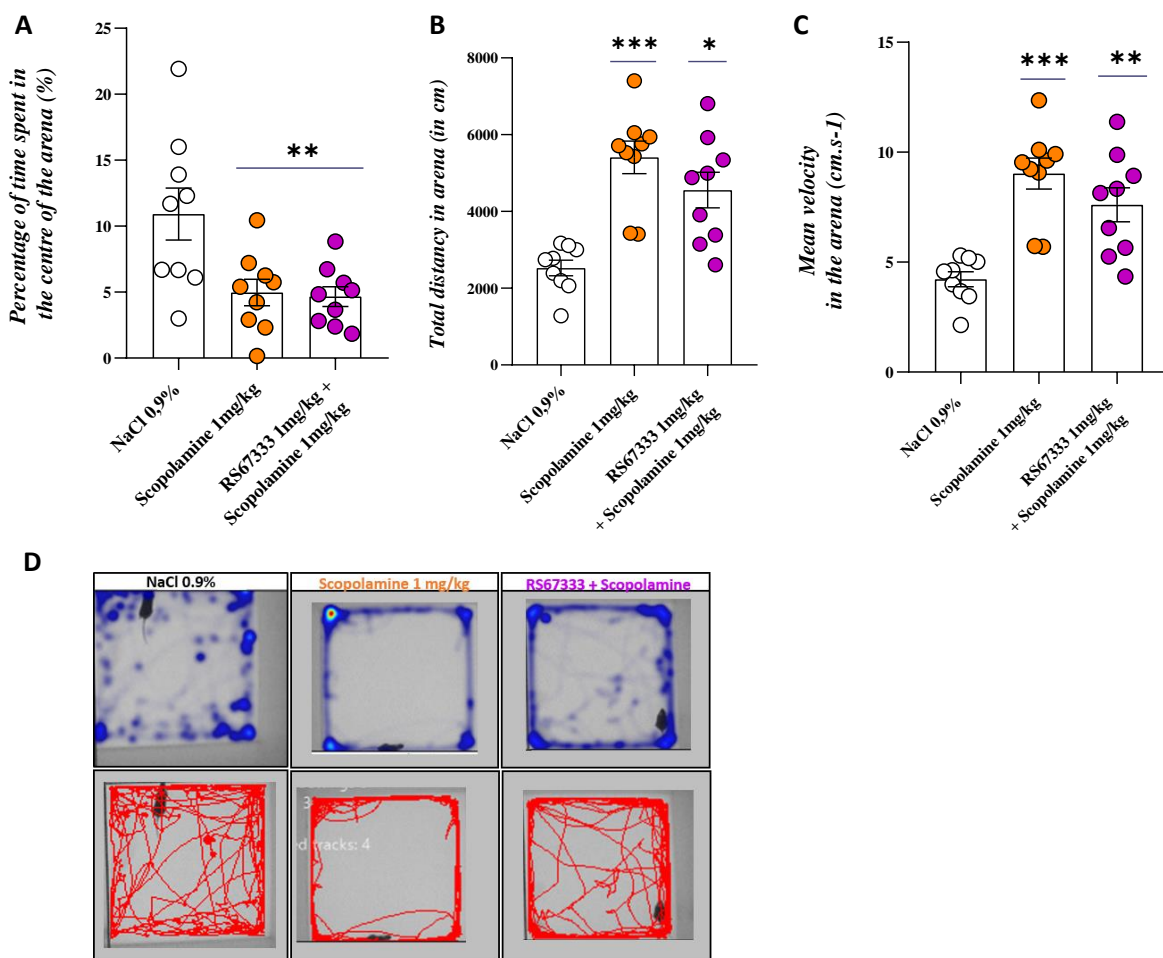


Figure 5: Evaluation of the effects of scopolamine alone and in co-administration with RS67333 in an OF test.

Data are expressed as mean \pm SEM. Effects of systemic administration of either scopolamine at 1 mg/kg ($n=9$) or in co-administration with RS67333 (1mg/kg, $n=9$) on the time spent the center of the arena (i.e the most anxiogenic zone) **(A)**, the number of entries in the center of the arena **(B)** the mean velocity in the arena **(C)** and on the total distance in the arena. **(D)** Patterns of activity following systemic treatments are depicted in figure **(E)**.

This behavior could reflect anxiogenic effect or hyper-activity. However, anxiogenic-like activity was not further confirmed by the EPM test in which there was no differences in the percentage of time spent in the open arms between groups (**Figure 6A**). No inter-group differences were found in the other parameters (number of entries in closed arms (**Figure 6B**), number of rears in closed arms (**Figure 6C**) and number of entries in opened arms (**Figure 6D**).

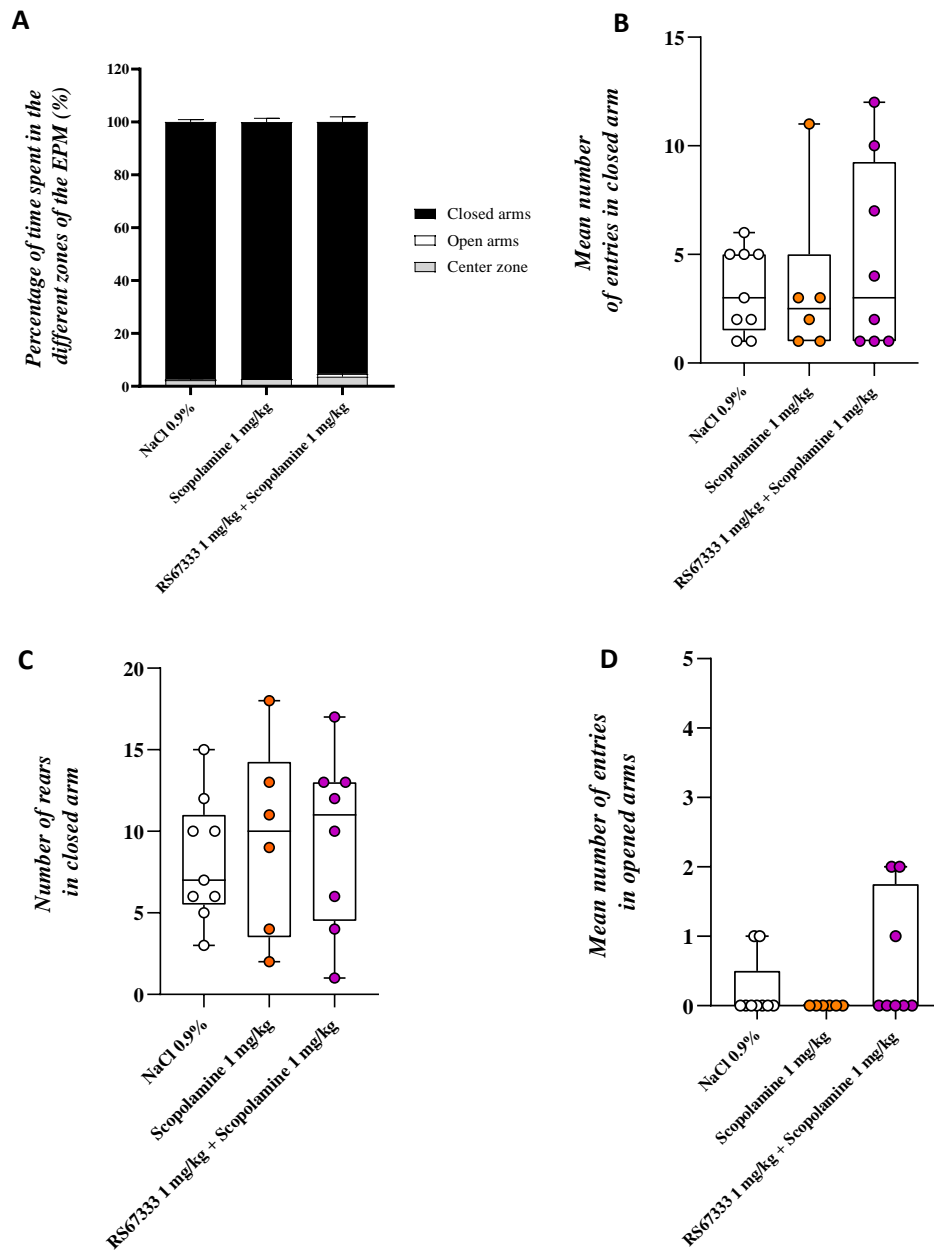
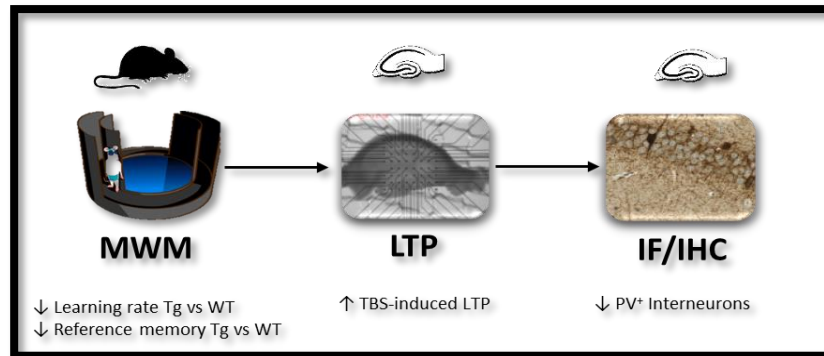


Figure 6: Evaluation of the effects of scopolamine alone and in co-administration with RS67333 in the EPM test. Data are expressed as mean \pm SEM. Effects of systemic administration of either scopolamine at 1 mg/kg ($n=6$) or in co-administration with RS67333 (1mg/kg, $n=8$) on the time spent in each area of the EPM (**A**), the number of entries in the closed arms (**B**) the number of rears in the closed arms (**C**) and the number of entries in the opened arms (**D**).

This rather suggests an excitatory effect of scopolamine which is consistent with empirical observations (Bushnell, 1987). Since hyperactivity behavior was not counterbalanced by RS67333 when co-administered with scopolamine, it could in part explain the absence of beneficial effects of RS67333 in scopolamine-induced PS deficits.

Appendix E

Characterization of spatial memory deficits and hippocampal correlates in a transgenic mouse model of AD.



General procedure information

Male transgenic mice Tg2576 aged of 17-18 months were obtained from Taconic.

We used a transversal approach to highlight memory deficits in this mouse model of AD (Hsiao et al., 1996) and investigate hippocampal correlates.

Spatial memory was assessed through Morris Water Maze (MWM) test as previously described (Vorhees & Williams, 2006). Briefly, mice were trained to learn the location of the escape platform through distinct visual cues. Spatial learning was assessed by daily sessions of 4 trials (60s) over 2 weeks (9 days) (15 min ITI) (**Figure 7**). Reference memory was then assessed through a probe-test of 60s where the platform is removed. The probe-test was performed 24h after the last learning session.

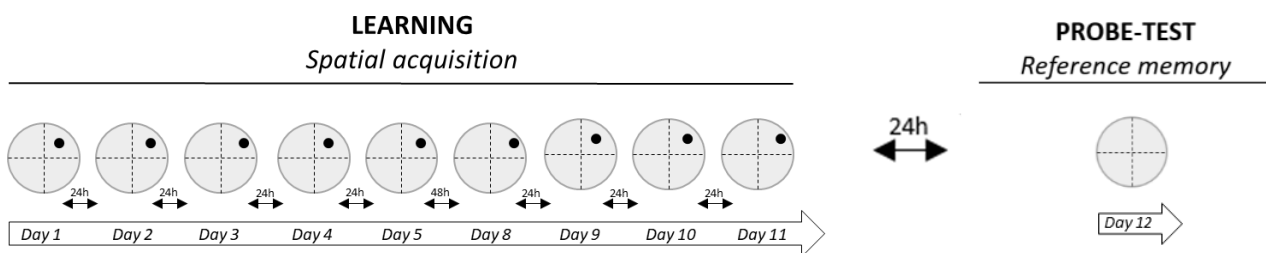


Figure 7: Experimental design for the assessment of spatial memory using the MWM in Tg2576 transgenic mice.

Then, some animals were used for the investigation of CA3-CA1 synaptic plasticity. Hippocampal slices were prepared as described along this manuscript. Extracellular recordings were performed using MEA system (MEA-2100, Multi-Channel System) (**Figure**). Both HFS and TBS protocols were used. **HFS protocol** consisted in 1 train of 100 pulses at 100Hz. **TBS protocol** consisted in 2 trains of 10 bursts at 5Hz, each composed of 4 pulses at 100Hz. Stimulus intensity was set at 50% of max slope given by I/O curve automatically determined by the software. Remaining animals were used for immuno-histochemical analyzes of the number of PV⁺ using standard 3, 3'-diaminobenzidine (**DAB**) protocol.

Table 5: List of equipments and corresponding details required for MEA experiments.

Multi Electrode Array		
Reagents or resources	Supplier	Reference/Model
Headstage	MultiChannel Sytem MCS GmbH	MEA2100-HS60
Interface	MultiChannel Sytem MCS GmbH	IFB.2
MEA well	MultiChannel Sytem MCS GmbH	60MEA200/30iR-Ti
Video microscope table	MultiChannel Sytem MCS GmbH	MEA-VMTC-1
Peristaltic perfusion pump	MultiChannel Sytem MCS GmbH	PPS2
Harp slice grid	Scop pro	HSG-MEA5-CD
Software for online acquisition	MultiChannel Sytem MCS GmbH	LTP Director
Software for offline analysis	MultiChannel Sytem MCS GmbH	LTP Analyzer

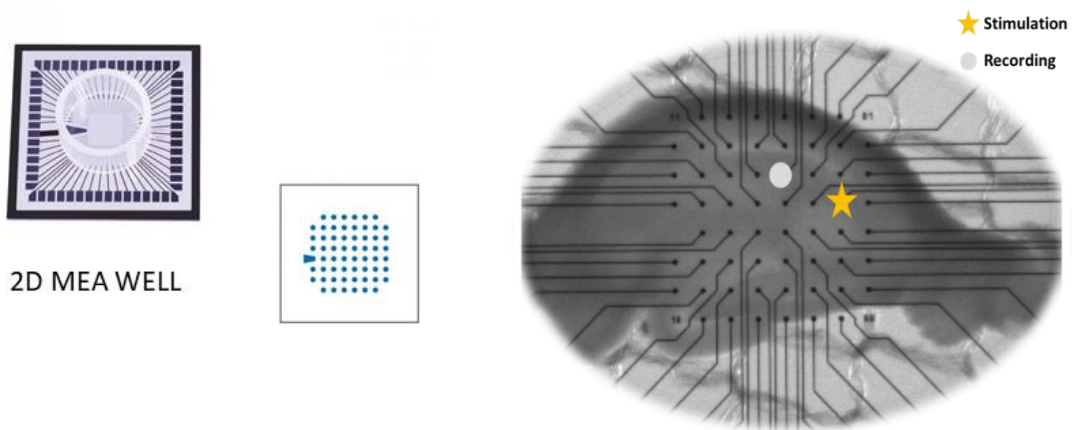


Figure 8: Overview of MEA layout for electrophysiological recordings of mouse hippocampal slice. 2D MEA well with 60 electrodes (left) and mouse hippocampal slice placed in the MEA well with typical position of stimulation and recordings indicated.

Experimental results

Morris Water Maze

LEARNING

From the 2nd day of training, WT mice learned the platform location as shown by the decay in escape latency across days (**Figure 9A**). In contrast, the escape latencies of transgenic mice were significantly higher than that of WT mice and remained stable across days indicating an absence of learning of the platform (**Figure 9A**). Similarly, Tg2576 mice swam higher distances (**Figure 9B**) than age-matched WT to find the platform, without impacting the swimming speed, thus confirming that Tg2576 mice displayed impaired learning performance.

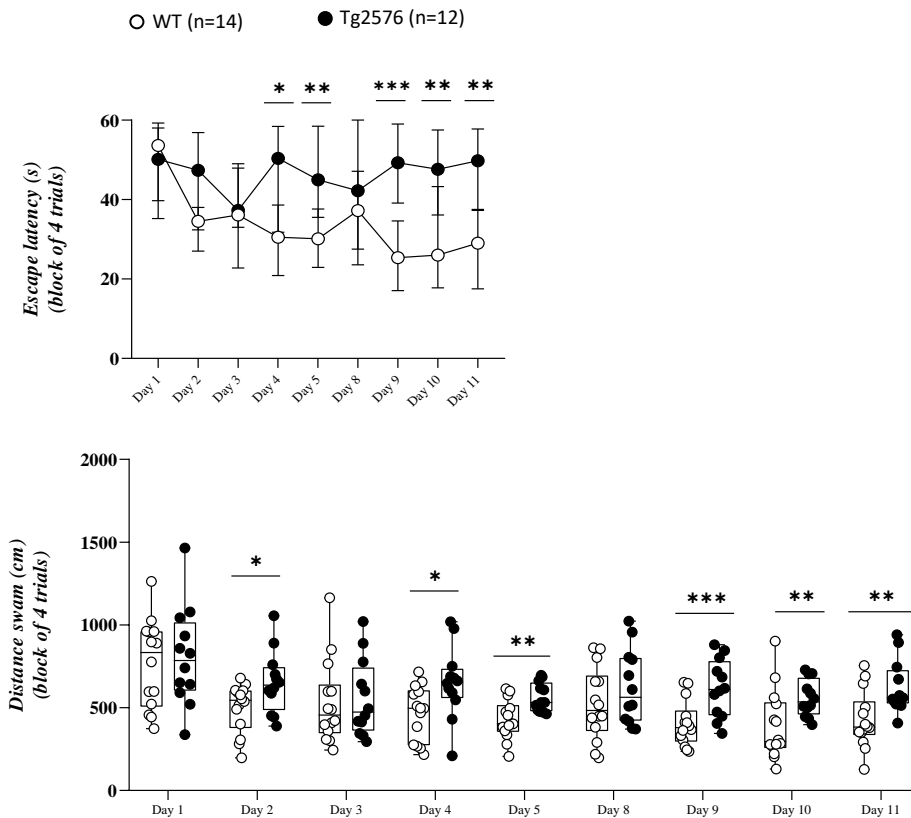


Figure 9: Evaluation of strain-differences in learning rate in the Morris Water Maze test. Data are expressed as median \pm interquartile. (A) Effect of the strain on escape latencies (B) Effect of the strain on total distance swam in the maze. * $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$ ANOVA-type for repeated measurements.

PROBE TEST

During the probe test, WT mice remembered the location of the platform as shown by the number of crossings in the target quadrant in contrary to Tg2576 mice which displayed a significant lower number of crossing in the target quadrant (**Figure 10A**). The total distance swam was similar between strains suggesting equivalent locomotion abilities (**Figure 10B**). This supports that Tg2576 mice have poor reference memory performance as compared to age-matched WT.

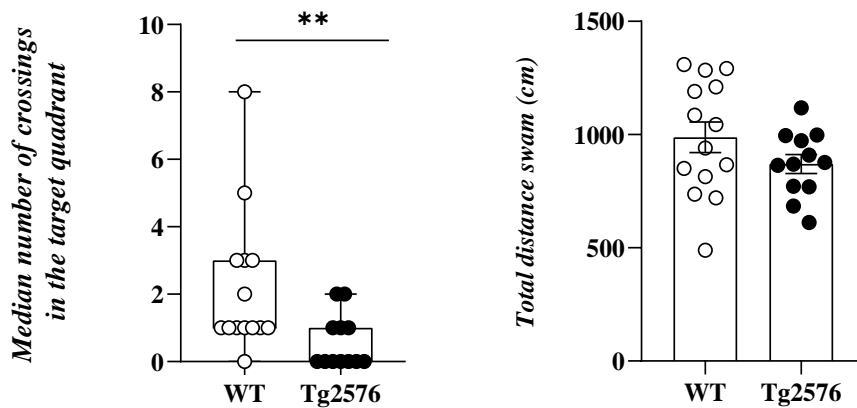


Figure 10: Evaluation of strain-differences in reference memory in the Morris Water Maze.

(A) Effects of the strain on the number of target crossings (reference memory). Data are represented as median \pm interquartile. ** $p < 0.01$, Mann-Whitney U test (B) Effects of the strain of total distance swam. Data are expressed as mean \pm SEM.

CA3-CA1 synaptic plasticity

When using the HFS protocol, LTP magnitude was similar between both WT and Tg2576 mice. Conversely, following TBS induction protocol, the magnitude of LTP was found to be higher in Tg2576 as compared to age-matched WT [ANOVA-type for repeated measurements revealed a group effect ($F_{(1, 15)} = 4.14, p = 0.012$), but no time effect ($F_{(45, 675)} = 1.03, p = 0.40$) nor interaction ($F_{(45, 675)} = 0.95, p = 0.46$) **Figure 10**].

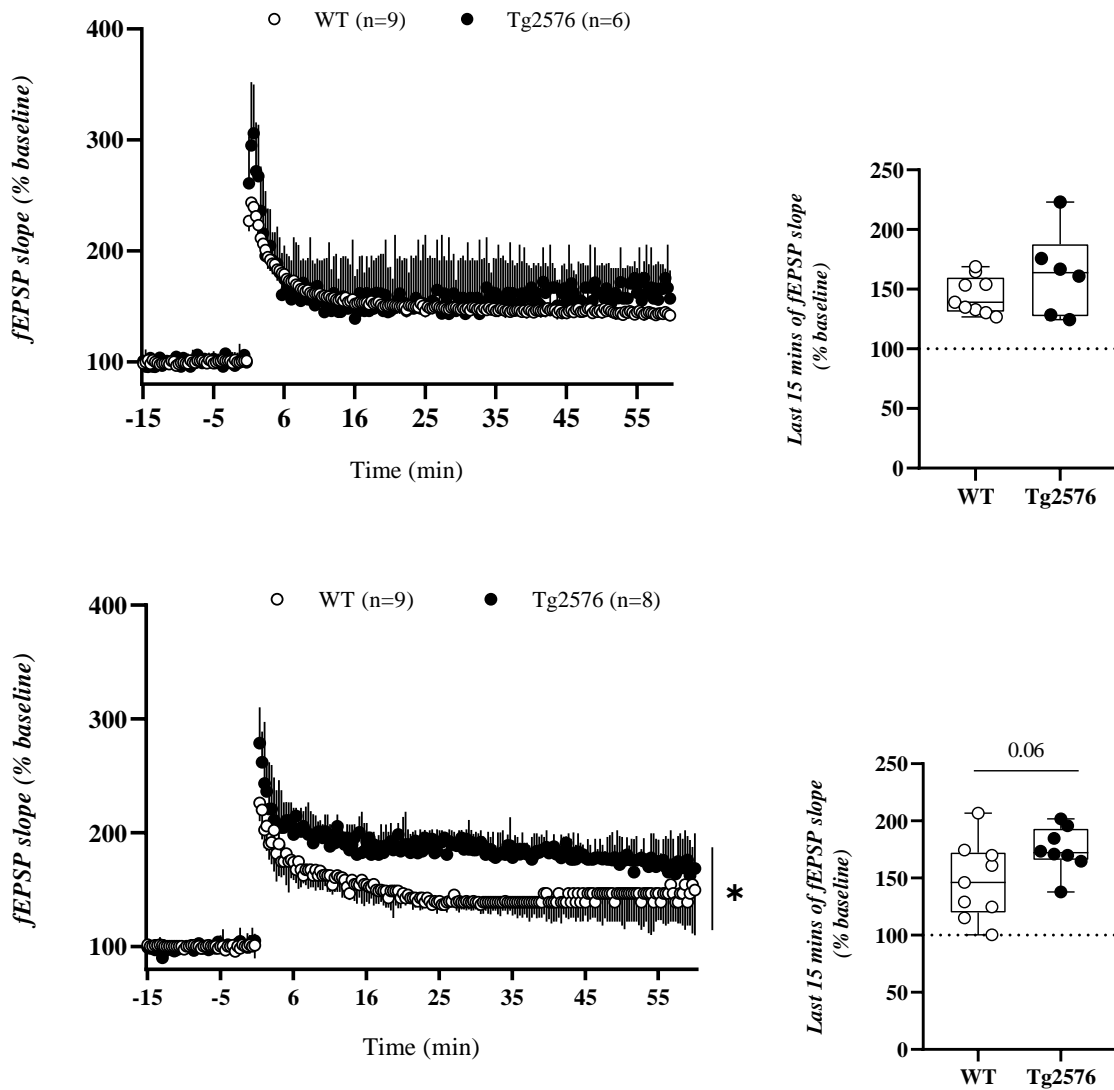


Figure 11: Effect of the strain on LTP magnitude induced by either HFS or TBS protocol. Data are represented as median \pm interquartile. Arrow marks the time when conditioning stimulation (HFS, TBS) was applied (A) Time course of the fEPSP slope following HFS stimulation protocol (left) and median of normalized fEPSP slope during the last 15 mins of the recordings (right). (B) Time course of the fEPSP slope following TBS stimulation protocol (left) and median of normalized fEPSP slope during the last 15 mins of the recordings (right). * $p < 0.05$ ANOVA-type for repeated measurements.

Immunohistochemistry

The number of Parvalbumin positive interneurons was found to be significantly reduced in the hippocampus of Tg2576 as compared to age-matched WT mice ($p = 0.02$, Unpaired student t test).

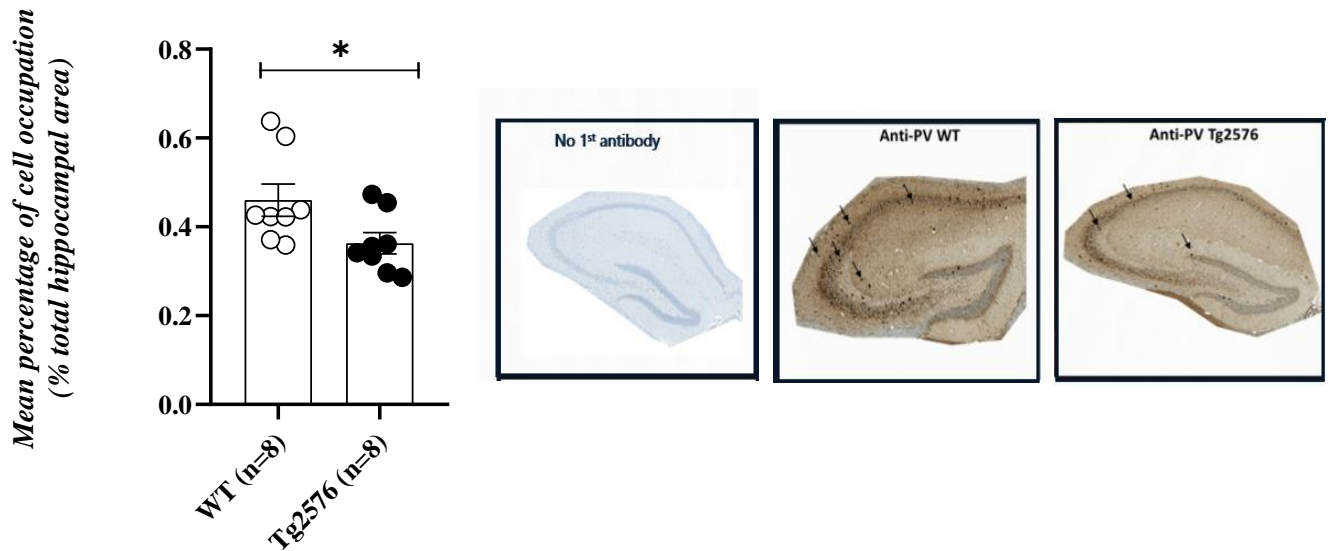


Figure 12: Effect of the strain on the percentage of parvalbumin (PV⁺).

Data are expressed as mean \pm SEM. (A) The percentage of hippocampal PV-positive cells in aged Tg2576 is significantly lower than in aged WT littermates ($*p < 0.01$, Student's t test). (B) Representative PV immunohistochemistry stains in the hippocampus of a 18 months old WT ($n = 8$) and Tg2576 ($n = 8$) mouse. PV-positive interneurons are shown in the hippocampus at a low magnification (original magnification, $\times 40$), and at a closer distance from the CA1 (original magnification, $\times 100$, pointed by arrowheads) and CA3 (original magnification, $\times 100$)

	Statistical test	Experiment	Group1	Group2	Group3	Group4	Comparison	° of freedom	F	p value	Significance
Hippocampal slices	Anova-type for repeated measurements	Optogenetic	OFF contol (n=7)	OFF RS67333 (n=8)	ON RS67333 (n=7)		Group	1	2,5	0,37	NS
							Time	4,86	3,46	0,01	*
							Group x time	1,18	6,5	0,31	NS
		Effect of mouse strain on LTP (HFS)	C57BL/6 (n=19)	NMRI (n= 13)			Group	0,75	1	0,39	NS
							Time	1,81	8,03	0,07	NS
							Group x time	0,89	8,03	0,53	NS
		Effect of mouse strain on LTP (TBS)	C57BL/6 (n=19)	NMRI (n=22)			Group	1	24,75	p<0,01	***
							Time	11	1,45	0,14	NS
							Group x time	11	0,91	0,53	NS
		Effect of mouse strain on AMPA transmission	C57BL/6 (n=20)	NMRI (n=14)			Group	1	5,32	p<0,05	*
							Time				
							Group x time				
		Effect of systemic injection of RS67333 1mg/kg on LTP (TBS)	NaCl 0,9% (n=12)	RS67333 1 mg/kg (n=10)			Group	1	0,68	0,41	NS
							Time	8,71	0,79	0,62	NS
							Group x time	8,71	0,86	0,56	NS
Effect of systemic injection of RS67333 1mg/kg on LTP (HFS)	NaCl 0,9% (n=8)	RS67333 1 mg/kg (n=9)			Group	1	6,1	0,01	**		
					Time	7,27	0,46	0,87	NS		
					Group x time	7,27	0,87	0,53	NS		
Evaluation of LTP in WT and Tg2576 mice - HFS	WT (n=9)	Tg2576 (n=6)			Group	1	0,96	0,33	NS		
					Time	4,45	0,77	0,56	NS		
					Group x time	4,45	0,69	0,62	NS		
Evaluation of LTP in WT and Tg2576 mice - TBS	WT (n=9)	Tg2576 (n=8)			Group	1	4,14	0,04	*		
					Time	6	1,03	0,4	NS		
					Group x time	6	0,95	0,46	NS		
ANOVA for repeated measurements		Effects of RS67333 10µM on LTP (HFS)	aCSF (n=8)	RS67333 10µM (n=8)			Group	1,14	1,14	0,54	NS
						Time	30,42	1,1	0,33	NS	
						Group x time	30,42	0,82	0,74	NS	
		Effects of RS67333 10µM on LTP (TBS)	aCSF (n=8)	RS67333 10µM (n=8)			Group	1,14	14,91	p<0,01	**
						Time	30,42	1,49	0,05	*	
				Group x time	30,42	1,13	0,29	NS			
Effects of RS67333 10µM + GABAA blockade on LTP (TBS)	aCSF (n=8)	RS67333 10µM + Bicuculline 10µM (n=14)	Bicuculline 10µM (n=11)			Group	2,22	0,08	0,92	NS	
				Time	30,67	1,39	0,08	NS			
				Group x time	60,67	0,81	0,84	NS			
Effects of RS67333 10µM + GABAB blockade on LTP (TBS)	aCSF (n=15)	RS67333 10µM (n=8)	CGP55845 (n=9)	RS67333 10µM + CGP55845 (n=10)		Group	3,39	8,57	P<0,001	***	
				Time	30,12	1,58	0,02	*			
				Group x time	90,12	1,48	p<0,01	**			
Effects of RS67333 10µM + GABAB blockade on LTP (TBS)	aCSF (n=8)					Group	1,16	4,15	0,059	NS	
						Time	31,5	0,76	0,08	NS	

			RS67333 10µM + CGP35845 (n=10)		Group x time	31,5	1,36	0,06	NS
	Effects of RS67333 10µM in 5-HT4R KO mice (TBS)	aCSF (n=6)	RS67333 10µM (n=7)		Group	1,11	0	0,97	NS
					Time	29,32	1,12	0,31	NS
					Group x time	29,32	1,32	0,13	NS
	Effects of the 5-HT4Rs antagonist RS39604 on the effects of RS67333 10µM on LTP (TBS)	aCSF* (n=7)	RS67333 1µM (n=12)	RS67333 10µM + RS39604 10µM (n=8)	Group	2,24	4,22	0,03	*
					Time				
					Group x time				
	Effects of RS67333 10µM on evoked IPSCs	aCSF (n=4)	RS67333 10µM (n=4)		Group	2,9	0,631	0,55	NS
					Time	39,35	1,2	0,2	NS
					Group x time	78,35	0,81	0,87	NS
	Effects of RS67333 10µM on AMPA transmission	aCSF (n=15)	RS67333 1µM (n=15)	RS67333 10µM (n=15)	Group	2,42	0,35	0,71	NS
					Stimulation intensity	2,54	0,41	0,66	NS
					Group x time	4,84	0,3	0,88	NS
	Effects of RS67333 10µM on NMDAR transmission	aCSF (n=11)	RS67333 1µM (n=11)	RS67333 10µM (n=11)	Group	2,3	0,54	0,59	NS
					Stimulation intensity	2,6	1,69	0,19	NS
					Group x time	4,6	0,28	0,89	NS
Student t test	Effects of RS67333 10µM on LTP (TBS)	aCSF (n=8)	RS67333 10µM (n=8)		Group	30,21	1,88	p<0,01	**
Univariate test	Effect of mouse strain on LTP (HFS)	C57BL/6 (n=18)			versus 100%			p<0,001	***
		NMRI (n=21)			versus 100%			p<0,001	***
	Effect of mouse strain on LTP (TBS)	C57BL/6 (n=19)			versus 100%			p<0,001	***
		NMRI (n=22)			versus 100%			p<0,001	***
	% Change in theta power (in vivo EEG recordings) - theta	NaCl 0.9% (n=8)			versus 0%			0,07	NS
	% Change in theta power (in vivo EEG recordings) - theta	RS67333 10µM (n=9)			versus 0%			0,03	*
	% Change in theta power (in vivo EEG recordings) - gamma	NaCl 0.9% (n=8)			versus 0%			0,02	*
% Change in theta power (in vivo EEG recordings) - gamma	RS67333 10µM (n=9)			versus 0%			p<0,01	**	
Mann-Whitney U test	Facilitation index	C57BL/6 (n=21)	NMRI (n=14)		Group			p<0,001	***
	Effect of mouse strain on LTP (HFS)	C57BL/6 (n=18)	NMRI (n=21)		Group			0,36	NS

	CV of HFS-LTP	C57BL/6 (n=18)	NMRI (n=21)	Group	p<0,001	***
	Effect of mouse strain on LTP (TBS)	C57BL/6 (n=19)	NMRI (n=22)	Group	p<0,001	***
	CV of TBS-LTP	C57BL/6 (n=19)	NMRI (n=22)	Group	p<0,001	***
	Facilitation across bursts	C57BL/6 (n=18)		AUC2/AUC1	p<0,01	**
		NMRI (n=21)		AUC2/AUC1	0,2	NS
		C57BL/6 (n=18)	NMRI (n=21)	Group	p<0,001	***
	Effect of systemic injection of RS67333 on LTP (TBS)	NaCl 0,9%	RS67333 1 mg/kg	Group	0,43	NS
	Effect of systemic injection of RS67333 on LTP (HFS)	NaCl 0,9%	RS67333 1 mg/kg	Group	0,02	*

Abbreviations: **aCSF**: artificial cerebrospinal fluid; **AUC**: area under the curve

Appendix G

	Statistical test	Experiment	Group1	Group2	Comparison	F	° of freedom	p value	Significance	
Behavioral studies	ANOVA-type for repeated measurements	Pro-cognitive effects of RS67333 1mg/kg i.p on LD task - probe test	NaCl 0,9% (n=9)	RS67333 1 mg/kg (n=9)	group	5,35	1	0,02	*	\$\$p<0,01, \$\$\$p<0,001, pair comparison
					difficulty	1,27	1	0,26	NS	
					group x time	0,73	1	0,39	NS	
					group	0,38	1,1	0,54	NS	
					difficulty	0,26	1,1	0,61	NS	
					group x time	3,04	1,1	0,08	NS	
					group	0,06	1,2	0,81	NS	
					difficulty	0,1	1,2	0,75	NS	
					group x time	0,07	1,2	0,79	NS	
					group	9,62	2, 15	p<0,001	***	
					difficulty	0,03	1, 15	0,85	NS	
					group x time	1,69	2, 15	0,19	NS	
					group	14,7	1	p<0,001	***	
					time	2,58	4,7	0,03	**	
			group x time	2,6	4,7	0,03	**			
		MWM performances of WT and Tg2576 mice - Learning (Escape latency)	WT (n= 14)	Tg2576 (n=12)	Group	11,75	1	p<0,001	***	
		MWM performances of WT and Tg2576 mice - Learning (Escape latency)	WT (n= 14)	Tg2576 (n=12)	Group	5,13	5	p<0,001	***	
		Pro-cognitive effects of RS67333 1mg/kg i.p on LD task - intermediate training	NaCl 0,9% (n=9)	RS67333 1 mg/kg (n=9)	Group			0,73	NS	
		Pro-cognitive effects of RS67333 1mg/kg i.p on TOM task - session duration sampling 1	NaCl 0,9% (n=7)	RS67333 1 mg/kg (n=10)	Group			0,12	NS	
		Pro-cognitive effects of RS67333 1mg/kg i.p on TOM task - session duration sampling 2	NaCl 0,9% (n=7)	RS67333 1 mg/kg (n=10)	Group			0,04	*	
		Pro-cognitive effects of RS67333 1mg/kg i.p on TOM task - session duration testing	NaCl 0,9% (n=7)	RS67333 1 mg/kg (n=10)	Group			0,22	NS	
		MWM performances of WT and Tg2576 mice - probe test (Total distance swam)	WT (n= 14)	Tg2576 (n=12)	Group			0,25	NS	
		MWM performances of WT and Tg2576 mice - probe test (number of crossings of the target quadrant)	WT (n= 14)	Tg2576 (n=12)	Group			p<0,01	**	
		Effects of RS67333 1mg/kg i.p in EPM - (number of entries in closed arms)	NaCl 0,9% (n=9)	RS67333 1 mg/kg (n=8)	Group			0,29	NS	

Appendix G

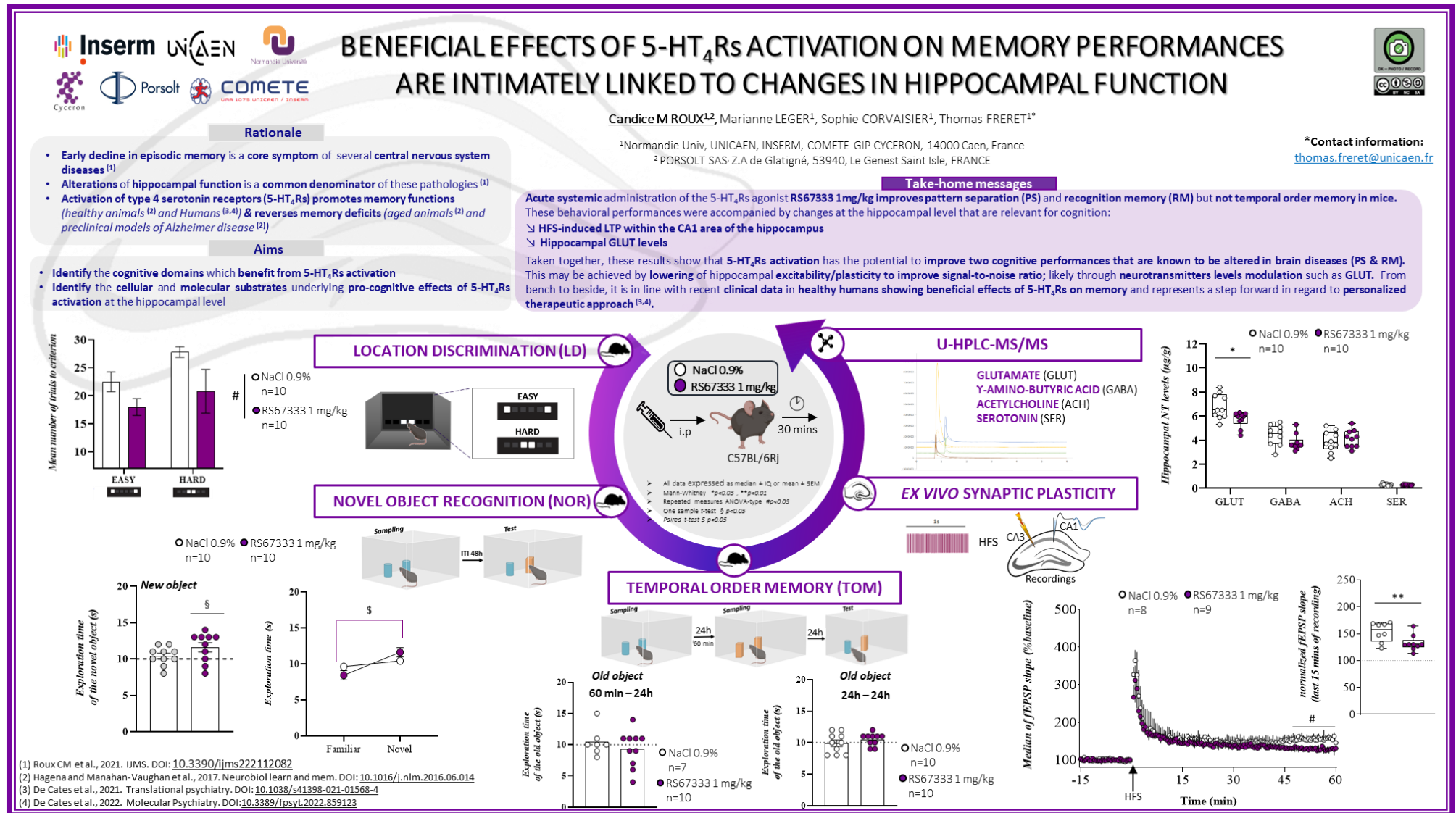
	Effects of RS67333 1mg/kg i.p in EPM - (number of entries in opened arms)	NaCl 0,9% (n=9)	RS67333 1 mg/kg (n=8)		Group	p>0,99	NS		
	Effects of RS67333 1mg/kg i.p in EPM - (number of rears in closed arms)	NaCl 0,9% (n=9)	RS67333 1 mg/kg (n=8)		Group	0,8	NS		
Wilcoxon signed-rank	Pro-cognitive effects of RS67333 1mg/kg i.p on TOM task - sampling 1	NaCl 0,9% (n=7)			exploration time left object <i>versus</i> 10s	0,13	NS		
	Pro-cognitive effects of RS67333 1mg/kg i.p on TOM task - sampling 2	NaCl 0,9% (n=7)			exploration time left object <i>versus</i> 10s	>0,99	NS		
	Effects of RS67333 1mg/kg i.p on TOM task - sampling 1	RS67333 1 mg/kg (n=10)			exploration time left object <i>versus</i> 10s	0,16	NS		
	Pro-cognitive effects of RS67333 1mg/kg i.p on TOM task - sampling 2	RS67333 1 mg/kg (n=10)			exploration time left object <i>versus</i> 10s	0,27	NS		
	Pro-cognitive effects of RS67333 1mg/kg i.p on TOM task - testing	NaCl 0,9% (n=7)			exploration time old object <i>versus</i> 10s	>0,99	NS		
	Pro-cognitive effects of RS67333 1mg/kg i.p on TOM task - testing	RS67333 1 mg/kg (n=10)			exploration time old object <i>versus</i> 10s	0,68	NS		
Two-way ANOVA	Pro-cognitive effects of RS67333 1mg/kg i.p on LD task - pre training	NaCl 0,9% (n=9)	RS67333 1 mg/kg (n=9)		group	0,01	1,5	0,91	NS
Kruskall-Wallis	Anti-amnesic effects of RS67333 1mg/kg i.p - OF test (total distance)	NaCl 0,9% (n=9)	Scopolamine 1 mg/kg \$\$\$ (n=9)	RS67333 1 mg/kg + Scopolamine 1 mg/kg \$\$\$ (n=9)	Group	p<0,001	***		\$\$\$p=0,01; \$\$\$\$p<0,001 versus NaCl 0,9% (Dunn's multiple comparison)
	Anti-amnesic effects of RS67333 1mg/kg i.p - EPM test (number of entries in opened arms)	NaCl 0,9% (n=9)	Scopolamine 1 mg/kg (n=6)	RS67333 1 mg/kg + Scopolamine 1 mg/kg (n=8)	Group	0,22	NS		
	Anti-amnesic effects of RS67333 1mg/kg i.p - EPM test (number of rears in closed arms)	NaCl 0,9% (n=9)	Scopolamine 1 mg/kg (n=6)	RS67333 1 mg/kg + Scopolamine 1 mg/kg (n=8)	Group	0,72	NS		
	Anti-amnesic effects of RS67333 1mg/kg i.p - EPM test (number of entries in closed arms)	NaCl 0,9% (n=9)	Scopolamine 1 mg/kg (n=6)	RS67333 1 mg/kg + Scopolamine 1 mg/kg (n=8)	Group	0,57	NS		
Unpaired t test	Pro-cognitive effects of RS67333 1mg/kg i.p on NOR task - session duration sampling	NaCl 0,9% (n=10)	RS67333 1 mg/kg (n=10)		Group	0,73	NS		

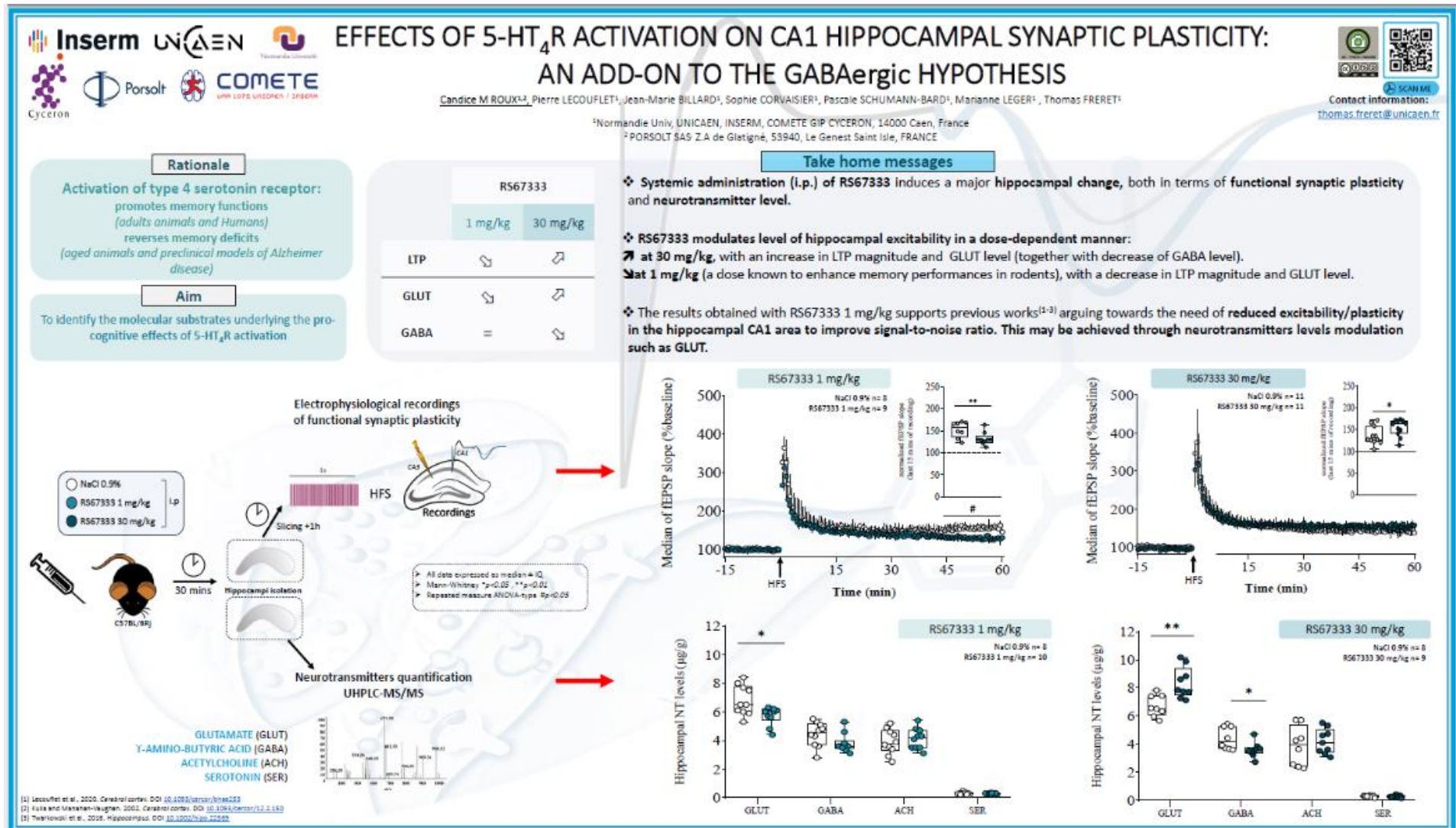
Appendix G


	Pro-cognitive effects of RS67333 1mg/kg i.p on NOR task - session duration testing	NaCl 0,9% (n=10)	RS67333 1 mg/kg (n=10)		Group	0,8	NS		
One - Way ANOVA	Anti-amnesic effects of RS67333 1mg/kg i.p - OF test (% time spent at the center)	NaCl 0,9% (n=9)	Scopolamine 1 mg/kg \$\$\$ (n=6)	RS67333 1 mg/kg + Scopolamine 1 mg/kg \$\$\$ (n=9)	Group	6,86	2, 24	p<0,01 **	\$\$\$p<0,001 versus NaCl 0,9% (Dunett's multiple comparison)
	Anti-amnesic effects of RS67333 1mg/kg i.p - OF test (mean velocity)	NaCl 0,9% (n=9)	Scopolamine 1 mg/kg \$\$\$ (n=9)	RS67333 1 mg/kg + Scopolamine 1 mg/kg \$\$ (n=9)	Group	15,16	2, 24	p<0,001 ***	
Univariate t test	Pro-cognitive effects of RS67333 1mg/kg i.p on NOR task - sampling	NaCl 0,9% (n=10)			exploration time left object <i>versus</i> 10s			0,71	NS
	Pro-cognitive effects of RS67333 1mg/kg i.p on NOR task - sampling	RS67333 1 mg/kg (n=10)			exploration time left object <i>versus</i> 10s			0,27	NS
	Pro-cognitive effects of RS67333 1mg/kg i.p on NOR task - testing	NaCl 0,9% (n=10)			exploration time novel object <i>versus</i> 10s			0,34	NS
	Pro-cognitive effects of RS67333 1mg/kg i.p on NOR task - testing	RS67333 1 mg/kg (n=10)			exploration time novel object <i>versus</i> 10s			0,03	*
	Effects of systemic administration of RS67333 1mg/kg on qEEG - theta power	NaCl 0,9% (n=8)			% change in power from baseline (versus 0%)			0,07	NS
	Effects of systemic administration of RS67333 1mg/kg on qEEG - theta power	RS67333 1 mg/kg (n=10)			% change in power from baseline (versus 0%)			0,03	*
	Effects of systemic administration of RS67333 1mg/kg on qEEG - gamma power	NaCl 0,9% (n=7)			% change in power from baseline (versus 0%)			0,02	*
	Effects of systemic administration of RS67333 1mg/kg on qEEG - gamma power	RS67333 1 mg/kg (n=10)			% change in power from baseline (versus 0%)			p<0,01	**

Appendix H

Biochemical analyzes	Statistical test	Experiment	Group1	Group2	p value	Significance
	Unpaired student's t test	Immuno-histochemical analyzes of WT versus Tg2576 mice	WT (n=8)	Tg2576 (n=8)	0,02	*
Mann-Whitney	Effects of systemic injection of RS67333 (1mg/kg) on hippocampal neurotransmitter levels	NaCl 0,9% (n=10)	RS67333 1 mg/kg (n=10)	0,56	NS	
		NaCl 0,9% (n=10)	RS67333 1 mg/kg (n=10)	0,86	NS	
				0,06	NS	
				0,04	*	









INTERPLAY BETWEEN 5-HT₄R AND GABAergic SYSTEM WITHIN CA1 HIPPOCAMPAL SYNAPTIC PLASTICITY

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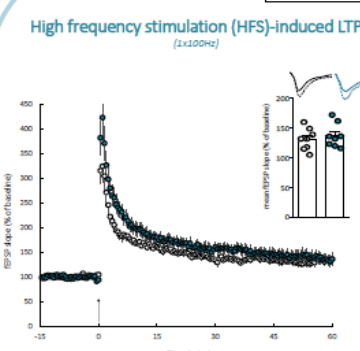
COMETE
UMR 1075 UNICAEN / INSERM

The serotonin type 4 receptor (5-HT₄R) has been described as a promising therapeutic target for learning and memory dysfunctions. Indeed, 5-HT₄R activation demonstrated promnesic effects in humans and rodents¹. Besides, anti-amnesic effects of 5-HT₄R activation have been observed either in aged animals or in pharmacologically-induced deficit condition², as well as different transgenic models of Alzheimer disease³. *In vivo* electrophysiological recordings within the hippocampus revealed a complex pattern of action of 5-HT₄R on synaptic plasticity, depending on the concerned area. In the CA1 area, results are divergent (e.g. showing either an increase⁴ or no effect⁵ on LTP after 5-HT₄ agonists application). To explore mechanisms at work, we aimed to characterize the effects of RS67333 (5-HT₄R agonist) on functional hippocampal synaptic plasticity at CA3/CA1 synapses from adult mice (NMRI, 2-3 months).

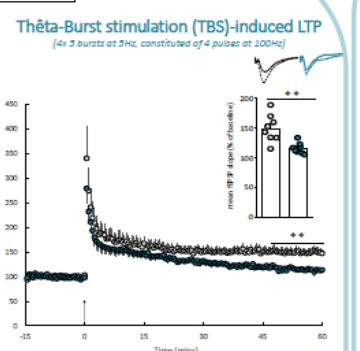
○ Control △ Bicuculline 10 μM ▲ RS67333 10 μM + Bicuculline 10 μM ▶ All data expressed as mean ± SEM
◻ RS67333 10 μM ◻ CGP55845 1 μM ◻ RS67333 10 μM + CGP55845 1 μM

EFFECTS OF 5-HT₄R ACTIVATION ON LTP

High frequency stimulation (HFS)-induced LTP
(1x100Hz)

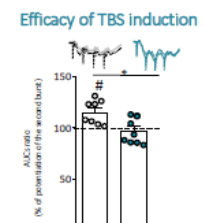


Thêta-Burst stimulation (TBS)-induced LTP
(4x 3 bursts at 5Hz, constituted of 4 pulses at 100Hz)



RS67333 (5-HT₄R agonist) did not affect HFS-induced LTP, but inhibited TBS-induced LTP ⇔ role of GABA?

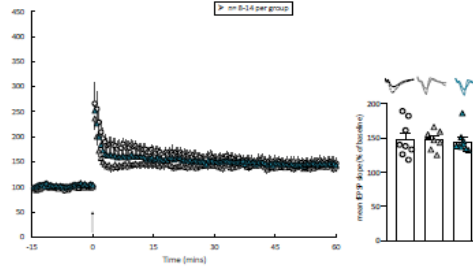
Efficacy of TBS induction



Different effect of RS67333 according to stimulation ⇔ involvement of GABAergic transmission

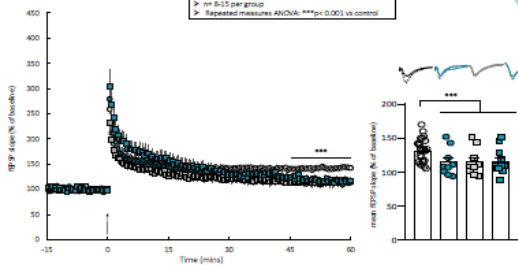
INVOLVEMENT OF GABA_AR and GABA_BR IN 5-HT₄R ACTIVATION EFFECTS ON TBS-INDUCED LTP

GABA_AR blockade (Bicuculline 10μM)



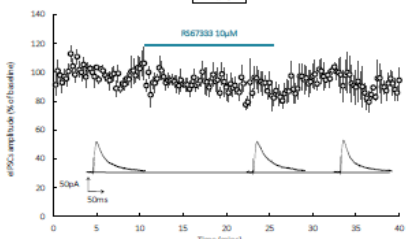
GABA_A receptors blockade suppresses 5-HT₄R activation on TBS-induced LTP

GABA_BR blockade (CGP55845 1μM)

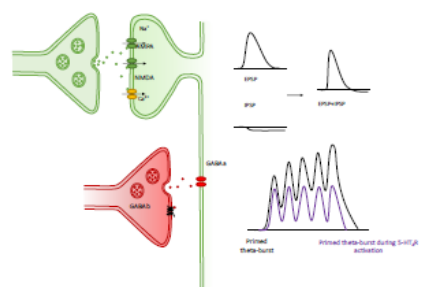


GABA_B receptors mimics the effects of 5-HT₄R activation

GABA_AR inhibitory post-synaptic currents (IPSCs)




5-HT₄R activation effects on LTP are not directly linked to GABA_AR activity since the 5-HT₄R agonist does not modify GABA_AR IPSCs



Inhibitory theta-burst during 5-HT₄R activation

Altogether, these data show a negative regulation of 5-HT₄R activation on functional plasticity induced by theta burst afferent stimulation. 5-HT₄R activation prevents the disinhibiting effect induced though theta frequency, an effect that requires interplay between 5-HT₄R and GABAergic neurotransmission within hippocampal CA1 area.

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2. Liu, A.C., De Meyer, J.H., Vermeir, S., Callaerts-Vegh, Z., Schuurkes, J.A.J., and D'Hooge, R. (2014). SSF-003392, a new 5-HT₄ receptor agonist, dose-dependently reverses scopolamine-induced learning and memory impairments in C57BL/6 mice. *Neuropharmacology*, 85, 179–189.
3. Ranganayak, K., Gnanaprakasam, P., Gnanaprakasam, S., Gnanaprakasam, S., Gnanaprakasam, S., Gnanaprakasam, S., et al. (2017). Chronic treatments with a 5-HT₄ receptor agonist decrease amyloid pathology in the entorhinal cortex and learning and memory deficits in the Tg2576 mouse model of Alzheimer's disease. *Neuropharmacology*, 126, 133–141.
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SCAN ME

ABSTRACT

Abstract

Early decline in episodic memory is a core symptom of a number of neurodegenerative and psychiatric diseases and are often associated with hippocampal alterations. While current available therapies haven't proved sufficient medical efficacy yet, type 4 serotonin receptors (5-HT₄Rs) have earned a place in the sun as a promising therapeutic target for the treatment of memory disorders. Indeed, both pro-mnesiant and anti-amnesiant effects of 5-HT₄Rs activation have been repeatedly described in rodents and their activation has been shown to display disease-modifying effects. Our work aimed at identifying the mechanisms underpinning beneficial effects of 5-HT₄Rs activation on memory to confirm the interest of their targeting as part of Multi-Target Directed Ligand development (MTDL). We focused on the hippocampal function using both *in vivo* and *ex vivo* strategies in healthy mice. First, we investigated the effects of local application of the 5-HT₄Rs agonist RS67333 on long-term potentiation (LTP) measured on isolated hippocampal slice preparation. We showed that LTP was reduced when using theta-burst stimulation protocol and we raised the existence of an interplay with GABAergic neurotransmission. Then, we investigated the effects of RS67333 when administered systemically - *at a dose previously described for having pro-cognitive effects* - on the different domains of episodic-like memory and its neurobiological correlates. We found that location and novelty discrimination were two domains of episodic memory that could benefit from 5-HT₄Rs activation. Besides, while CA1 hippocampal theta spectral power was found to be increased, the magnitude of LTP was reduced in a frequency-dependent manner. These changes were accompanied by reduced levels of glutamate, the main excitatory neurotransmitter in the hippocampus. Taken together, our results suggest that the beneficial effects of 5-HT₄Rs activation on memory are supported by overall reduction in hippocampal activity, likely driven by variations in excitatory neurotransmitter levels and oscillatory rhythms that are both relevant for synaptic plasticity and subsequent memory processes. Above all, our results strengthen the interest towards MTDL treatment involving 5-HT₄Rs agonists in a broad range of diseases of the central nervous system that are related to hippocampal alterations.

Résumé

Le déclin précoce de la mémoire épisodique est un symptôme central d'un certain nombre de maladies neurodégénératives et psychiatriques et est souvent associé à des altérations hippocampiques. Alors que les thérapies actuelles n'ont pas prouvé une efficacité suffisante, les récepteurs de sérotonine de type 4 (5-HT₄Rs) ont émergé en tant que cible thérapeutique prometteuse pour le traitement des troubles de la mémoire. En effet, de nombreuses études ont démontré les effets pro-mnésiants et anti-amnésiants de l'activation des 5-HT₄Rs et ont permis d'identifier des effets bénéfiques sur la physiopathologie des troubles de la mémoire. Nos travaux visaient à identifier les mécanismes pouvant sous-tendre les effets bénéfiques de l'activation des 5-HT₄Rs sur la mémoire afin de confirmer l'intérêt de leur ciblage dans le cadre du développement de ligands multi-cibles (Multi-Target Directed Ligand - MTDL). Nous nous sommes particulièrement intéressés à la fonction hippocampique en utilisant des stratégies *in vivo* et *ex vivo* chez des souris saines. Tout d'abord, nous avons étudié les effets de l'application locale d'un agoniste des 5-HT₄Rs, le RS67333, sur la potentialisation à long terme (PLT) mesurée sur des tranches d'hippocampe. Nous avons montré que la LTP était diminuée lors de l'utilisation d'un protocole de stimulation thêta-burst et nous avons émis l'hypothèse d'une interaction avec la neurotransmission GABAergique. Ensuite, nous avons étudié les effets du RS67333 administré par voie systémique - *à une dose précédemment décrite comme ayant des effets pro-cognitifs* - sur les différents domaines de la mémoire de type épisodique et ses corrélats neurobiologiques. Nous avons constaté que la localisation et la discrimination de nouveauté étaient deux domaines de la mémoire de type épisodique qui pouvaient être améliorés suite à l'activation des 5-HT₄Rs. De plus, alors que nous avons identifié que la puissance spectrale de la bande de fréquence thêta était augmentée dans l'aire CA1 de l'hippocampe, nous avons observé que la PLT était réduite de manière dépendante de la fréquence de stimulation dans cette même aire. Nous avons mis en évidence que ces changements étaient accompagnés d'une réduction des niveaux de glutamate, le principal neurotransmetteur excitateur dans l'hippocampe. Ainsi, l'ensemble de ces résultats confirment que les effets bénéfiques de l'activation des 5-HT₄Rs sur la mémoire sont soutenus par une diminution de l'activité hippocampique, probablement liée à des variations de quantités de neurotransmetteur et des rythmes oscillatoires impliqués dans les phénomènes de plasticité synaptique et par conséquent les processus de mémoire. Dans l'ensemble, nos résultats renforcent l'intérêt d'impliquer des agonistes des 5-HT₄Rs dans les stratégies MTDL dans un large éventail de maladies du système nerveux central qui sont liées à des altérations de l'hippocampe.