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MOLECULAR BASIS OF HIPPOCAMPAL SYNAPTIC PLASTICITY AND MEMORY: THE ROLE OF DOPAMINE D3 RECEPTORS

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INDEX

General introduction

BACKGROUND

Memory serves as the mind's recording framework. From the day of birth, the brain receives a continuous influx of information and stores an astonishing quantity of data, that shapes our identity over time. To guarantee this dynamic and complex process, memory relies on a sophisticated interplay between neurons that enable the acquisition, encoding, storage, and retrieval of information. At a cellular level, the foundation of memory is driven by synaptic plasticity, a mechanism based on the synapses ability to adjust the strength of their connections in response to activity. Within this context, Long-Term Potentiation (LTP) reflects a strengthening of synaptic efficacy, while Long-Term Depression (LTD) represents it's weakening (for a review see Bliss & Collingridge, 1993; Hawkins et al., 1993). Within the hippocampus, one of the main brain regions enrolled for memory formation, LTP is divided in two distinct phases, the early-LTP (E-LTP) and late-LTP (L-LTP). Electrophysiological E-LTP represents a transient form of synaptic plasticity, typically induced by a single high-frequency

stimulation train (HSF). This phase lasts approximately one hour, does not require de novo protein synthesis, and from a behavioural perspective correspond to short-term memory (STM). In contrast, L-LTP is induced by three, or more, HFS, persisting at least three hours. This prolonged phase is dependent on new protein synthesis and is associated with the consolidation of long-term memory (LTM) (Frey et al., 1993; Huang & Kandel., 1994; Huang et al., 1996). These functional changes occurring at the synapses rely upon molecular pathways that involve neurotransmitters, membrane receptors, second messengers, transcription factors and, ultimately, plasticity-related proteins (Kandel, 2012).

Among the molecular pathways sustaining LTP, cyclic nucleotides play a pivotal role. The best characterized are cAMP and cGMP, produced by adenylyl and guanylate cyclases respectively and degraded by phosphodiesterases (PDEs). Both act through their downstream kinases, PKA and PKG, to phosphorylate CREB, drive gene transcription, and promote the synthesis of plasticity-related proteins required for synaptic remodeling. While cAMP/PKA signaling is essential for the late maintenance of LTP and long-term memory consolidation (Frey

et al., 1993; Huang et al., 1994; Nguyen & Kandel, 1996), the NO/cGMP/PKG pathway, once thought to be restricted to early plasticity, has also been implicated in late-LTP and memory persistence (Boulton et al., 1995; Son et al., 1996; Lu et al., 2002; Prickaerts et al., 2002). Dysregulation of these pathways is linked to impaired plasticity, deficits in memory consolidation, and cognitive decline in aging and Alzheimer's disease (Bollen et al., 2014; Ricciarelli et al., 2018).

Dopamine D3 receptors (D3-Rs) are metabotropic receptors belonging to the D2-like family of dopamine receptors including D2, D3 and D4 subtypes. They are negatively coupled to adenylyl cyclase (AC) and lead to a decrease of cAMP levels and inhibition of PKA (Griffon et al., 1997). D3-Rs are expressed at both pre- and post-synaptic level and are highly sensitive to dopamine. Presynaptic D3-Rs have been demonstrated to regulate dopamine release through a negative feedback mechanism, preventing a further release of dopamine (Stahl, 2017). Moreover, D3-Rs may be localized on GABA interneurons where they inhibit GABAergic transmission via pre- and post-synaptic mechanisms regulating, in turn, glutamate and dopamine transmission (Chen et al., 2006; Lemercier et al., 2016). Thus, genetic deletion or

pharmacological inhibition of D3-Rs can increase dopamine release through different mechanisms.

D3-Rs are mainly expressed in nucleus accumbens, striatum, VTA, substantia nigra, hypothalamus and prefrontal cortex where they regulate social, emotional, and motivational processes (Sokoloff and Le Foll, 2016). Therefore, a variety of studies have focused on their role in the pathophysiology and treatment of schizophrenia, depression, and drug addiction (Pich and Collo, 2015). Also, they have been studied in the context of Parkinson's disease for their effects on locomotor activity (Young et al., 2020). Remarkably, although less abundant than D2 subtype, D3-Rs have a high affinity for dopamine so that slight modifications of their function lead to significant variation of synaptic transmission making them an interesting pharmacological target. For instance, some antipsychotics showed a very high and preferential affinity to D3-Rs resulting in a unique pharmacodynamic profile that has been exploited for the treatment of negative symptoms, mood disorders and cognitive deficits in schizophrenia (Calabrese et al., 2020).

The key function of dopamine in memory has been established but the role of D3-Rs remains elusive. Although expressed in the

hippocampus, a brain area mainly involved in memory formation and consolidation, D3-Rs involvement in synaptic plasticity and memory as well as in disorders characterized by memory loss and cognitive impairment is not entirely understood. Previous behavioral studies in animal models have shown that blockade or knockout of D3-Rs exerts pro-cognitive effects (Glickstein et al., 2005; Laszy et al., 2005; Loiseau and Millan, 2009; Xing et al., 2010) whereas activation of these receptors impairs spatial learning, object recognition, associative learning, episodic memory, attention, and working memory (Ukai et al., 1997; Watson et al., 2012; Nakajima et al., 2013), Accordingly, in humans, activation of D3-Rs have been shown to impair cognitive performance, whereas their blockade seems to have pro-cognitive effects (Gross et al., 2013).

AIM OF THE THESIS

The main aim of this thesis was to investigate the molecular basis underlying synaptic plasticity and memory in the hippocampus, with a particular focus on the role of D3 receptors in hippocampal synaptic plasticity and memory in physiological conditions. In addition, I aimed to study whether D3-Rs blockade might rescue the synaptic and memory impairment occurring during physiological aging.

THESIS ORGANIZATION AND MY CONTRIBUTION

The present thesis is divided into different sections, as follows:

In *Chapter 1* I discuss one of the projects I conducted during my PhD program, which examined the physiological role of hippocampal dopamine D3 receptors. In particular, we demonstrated that in adult wild-type (WT) mice, both pharmacological blockade and genetic deletion of D3Rs converted E-LTP into L-LTP via the cAMP/PKA pathway and promoted the transition from short- to long-term memory, effects primarily mediated by postsynaptic mechanisms. In aged WT mice, D3R blockade reversed deficits in LTP, memory, and plasticity-related protein expression, while genetic deletion prevented age-related cognitive decline. I contributed to this work by conducting behavioral studies (from design to analyses of results) and participating in the revision of the manuscript. I presented data from this work as oral communication at the 17th Annual Meeting of Young Researchers in Physiology (May 2024; Catania) and the 74th National Congress of the Italian Society of Physiology (Sept. 2024; Rome), and as poster presenter at the Society of Neuroscience (Oct. 2024; Chicago).

In *Chapter 2* I describe the behavioral techniques that I applied in this project, focusing on experimental paradigms commonly used to evaluate hippocampus-dependent learning and memory in rodents. I will discuss the modifications I introduced to standard protocols, aimed at reducing variability, improving accuracy of data collection, and ensuring a more robust interpretation of synaptic and cognitive outcomes.

In *Chapter 3* I present a literature review summarizing current knowledge on the interaction between the NO/cGMP/PKG/CREB pathway and amyloid-β (Aβ) in both the healthy and diseased brain. The study highlights the synaptic mechanisms underlying the physiological interplay between Aβ and the cGMP pathway, and how this balance is disrupted in AD, offering new insights into AD pathophysiology. In addition, we discuss evidence from preclinical and clinical studies supporting the enhancement of cGMP signaling as a potential therapeutic strategy for AD. I contributed to this work performing bibliography research and participating in the conceptualization and writing of the manuscript.

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Chapter 1: Blockade of dopamine D3 receptors improves hippocampal synaptic function and rescues age-related cognitive phenotype

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ABSTRACT

Dopamine D3 receptors (D3Rs) modulate neuronal activity in several brain regions including the hippocampus. Although previous studies reported that blocking D3Rs exerts pro-cognitive effects, their involvement in hippocampal synaptic function and memory in the healthy and aged brain has not been thoroughly investigated. We demonstrated that in adult wild type (WT) mice, D3R pharmacological blockade or genetic deletion as in D3 knock out (KO) mice, converted the weak form of long-term potentiation (LTP1) into the stronger long-lasting LTP (LTP2) via the cAMP/PKA pathway, and allowed the formation of long-term memory. D3R effects were mainly mediated by post-synaptic mechanisms as their blockade enhanced basal synaptic transmission (BST), AMPAR-mediated currents, mEPSC amplitude, and the expression of the post-synaptic proteins PSD-95, phospho(p)GluA1 and p-CREB. Consistently, electron microscopy revealed a prevalent expression of D3Rs in post-synaptic dendrites. Interestingly, with age, D3Rs decreased in axon terminals while maintaining their levels in post-synaptic dendrites. Indeed, in aged WT mice, blocking D3Rs reversed the impairment of LTP, BST, memory, post-synaptic protein expression, and PSD length. Notably, aged D3-KO mice did not

exhibit synaptic and memory deficits. In conclusion, we demonstrated the fundamental role of D3Rs in hippocampal synaptic function and memory, and their potential as a therapeutic target to counteract the age-related hippocampal cognitive decline.

INTRODUCTION

Dopamine plays a crucial role in various cognitive functions, including memory, and alterations in dopaminergic transmission have been associated with age-related cognitive decline (Berry et al., 2016; Hemby et al., 2003; Kaasinen et al., 2000; Shohamy & Wimmer, 2013). In this context, we focused on the metabotropic dopamine D3 receptors (D3Rs) belonging to the D2-like family, which are negatively coupled to adenylyl cyclase (AC), resulting in a reduction of cAMP levels and the inhibition of PKA (Robinson & Caron, 1997). Despite being less abundant than the D2 subtype, dopamine exhibits a higher affinity for D3Rs rather than D2Rs (reviewed in Maramai et al., 2016), so even slight modifications in their function can result in significant changes in synaptic transmission, making them an intriguing target for pharmacological interventions. Therefore, they have been investigated in the pathophysiology and

treatment of schizophrenia, depression, drug addiction, and locomotor disorders (reviewed in Kiss et al., 2021).

D3Rs are expressed in different brain areas, including the hippocampus (Li & Kuzhikandathil, 2012; Meador-Woodruff et al., 1994), which is mainly involved in memory formation and consolidation. Both a direct and indirect interaction between D3Rs and glutamatergic synapses has been demonstrated, suggesting their role in the modulation of excitatory signals (Sokoloff et al., 2013). However, their contribution to hippocampal synaptic plasticity and memory in the healthy brain and during aging is not entirely understood. Previous studies demonstrated that a selective pharmacological blockade of D3Rs improved social and recognition memory (Millan et al., 2007; Sigala et al., 1997; Watson et al., 2012), and that D3R genetic deletion enhanced cognitive functions (Leggio et al., 2021; Micale et al., 2010; Xing et al., 2010). Similarly, in humans, activation of D3Rs impaired cognitive performance, whereas their blockade exerted cognitive enhancing effects (Gross et al., 2013).

Based on these findings, here we investigated the role of D3Rs in modulating hippocampal excitatory synaptic function and

cognition in the healthy brain and during aging in mouse models. We have studied the effects of the pharmacological blockade or genetic deletion of D3Rs, and whether targeting of D3Rs could offer potential avenues for addressing age-related cognitive decline. Furthermore, we have conducted an electron microscopic study of the CA1 area of the hippocampus to analyze the subcellular and synaptic distribution of D3Rs.

MATERIALS AND METHODS

2.1 Animals

We used WT mice (C57BL/6 J; RRID:IMSR_JAX:000664) purchased from The Jackson Laboratory. D3R−/− mice (D3-KO) were generated by Accili et al. (1996) and subsequently backcrossed to C57BL/6J mice to obtain a congenic colony after 10th–12th generations. Colonies were established in the animal facilities at University of Catania, Università Politecnica delle Marche and Università Cattolica del Sacro Cuore. The housing conditions were controlled, maintaining stable hygrometric and thermic conditions (50%; 21°C ± 1°C) on a 12-h light/dark cycle with ad libitum access to food and water.

Animal care, handling, and procedures were carried out in accordance with national and European Community Council

Directives (2010/63/UE) and were approved by the Italian Ministry of Health (226/2021-PR, 40A31.N.ZUK, 623/2022-PR). The experiments complied with the ARRIVE guidelines and were conducted to minimize animal suffering.

We used sex-balanced mice for electrophysiological recordings, behavioral experiments, and western blotting, and male mice for electron microscopy. Animals were used at 4–8 months (adult) and 18–22 months (old) according to our scientific work plan.

2.2 Drugs

Hippocampal slices were treated with NGB-2904 (Sigma-Aldrich, 1 μM), SB-277011A (Sigma-Aldrich, 10 μM), NKY-80 (Abcam, 10 μM), and Rp-8-Br-cAMPS (Biolog, 20 μM). NGB-2904 and Rp-8-Br-cAMPS were dissolved in DMSO, whereas other drugs in bidistilled water. Aliquots were diluted in ACSF to the desired final concentration right before electrophysiological experiments. For behavioral studies, we dissolved NGB-2904 in a solution containing DMSO and Tween 20, and then diluted in saline solution (0.9% NaCl) to a dose of 3 mg/kg in a final volume of 200 μL for intraperitoneal (ip) administration. This dose was selected according to a previous

work showing a pro-cognitive effect of NGB-2904 (Huang et al., 2015).

2.3 Electrophysiology

Electrophysiological recordings were performed on hippocampal CA3-CA1 synapses. Field recordings were performed and analyzed as previously described (Gulisano et al., 2019) to assess basal synaptic transmission (BST), LTP, paired-pulse facilitation (PPF), and post-tetanic potentiation (PTP). Patch-clamp recordings were performed and analyzed as previously described (Aceto et al., 2022; Leggio et al., 2019), with minor modifications, to study excitatory post-synaptic currents (EPSC), AMPA/NMDA ratio, and miniature EPSCs (mEPSCs). See Supplementary Methods for a detailed description.

2.4 Electron microscopy

2.4.1 Immunoperoxidase and pre-embedding procedures

Mice were deeply anesthetized and perfused with saline and a fixative mixture. Brains were post-fixed, cut into 50 μm parasagittal sections, and processed for pre-embedding studies. Sections were incubated with anti-D3 primary antibodies

(1:100; ADR-003, RRID:AB_2039830; Alomone) (Castro-Hernández et al., 2015; Figure S1) and secondary biotinylated antibodies, followed by avidin-biotin peroxidase complex and 3,3'diaminobenzidine tetrahydrochloride (DAB). Immunostained sections were then post-fixed, dehydrated, embedded, and cut in ultrathin sections (~60 nm) for electron microscopy (Melone et al., 2019). Data were collected from CA1 stratum radiatum, and D3 immunoreactive profiles were studied. Microscopical fields were acquired and analyzed to obtain quantitative data on the density of D3 positive profiles and their subcellular distribution at asymmetric synapses. See Supplementary Methods—Data S1 for a detailed description.

2.4.2 Electron microscopy of hippocampal slices

Hippocampal slices were fixed right after electrophysiological recordings, embedded, and sectioned according to the same protocol applied for pre-embedding studies (Gulisano et al., 2019; Melone et al., 2019). Synaptic domains were identified based on established criteria, and quantitative analysis of vesicle pool, number of docked vesicles, area of spines, length of the post-synaptic density (PSD), and the proportion of

perforated synapses was carried out. See Supplementary Methods—Data S1 for a detailed description.

2.5 Western blot on hippocampal slices

Hippocampal slices (n = 3 from n = 4 mice/group) were homogenized in lysis buffer (62.5 mM Tris–HCl pH 6.8, 3% SDS) in the presence of phosphatase (Phosphatase Inhibitor Cocktail, Calbiochem) and protease inhibitors (Protease Inhibitor cocktail, Sigma), and sonicated three times for 1 min (Acquarone et al., 2019; Gulisano et al., 2019). Protein concentrations were determined by Pierce BCA protein assay kit (Thermo Fisher Scientific) and equal amounts of proteins (30 μg) were diluted in Laemmli buffer, boiled, and resolved by SDS-PAGE. The following primary antibodies were incubated overnight at 4°C all diluted 1:1000: rabbit anti-PSD-95 (Abcam #18258), mouse anti-pGluA1Ser845 (Cell Signaling #8084), mouse antiGLuA1 (Millipore #2263), rabbit anti-pCREBS133 (Millipore #06–519), mouse anti CREB (Thermo Fisher Scientific #MA1-083), and mouse anti-GAPDH (Abcam #9484). ECL signals were acquired and analyzed with the UVITEC imaging system (Cambridge Alliance). Densitometric analysis was performed with UVItec software after

normalization with loading controls. Results are expressed as fold change versus vehicle-treated WT slices, which were considered equal to 1. Molecular weights for immunoblot analysis were determined using Precision Plus proteinTM standards (Biorad).

2.6 Behavioral studies

Open Field, Novel Object Recognition (NOR) and Location (NOL) (Tropea et al., 2021; Tropea, Sanfilippo, et al., 2022), and Morris Water Maze (Gulisano et al., 2018) were performed as previously described. For NOR and NOL, mice underwent the training session (T1), and after 24 h, the testing session (T2) to assess memory retention. We used two different protocols varying in the duration of exposure to the two familiar objects during T1: (i) a short protocol (T1 = 3 min), which is normally insufficient to trigger long-term memory (LTM) formation; (ii) a long protocol (T1 = 10 min) to evaluate LTM.

Stereotaxic surgery for intrahippocampal cannula implantation was performed as described in Tropea, Torrisi, et al., 2022. Customized home-made cannulas were implanted in dorsal hippocampi under anesthesia, and mice recovered for at least 6–8 days. NGB-2904 was bilaterally infused 15 min before the T1

phase of NOR. See Supplementary Methods—Data S1 for a detailed description.

2.7 Statistics

All experiments were performed in blind with respect to treatment or genotype. Data were expressed as mean ± standard error mean (SEM). The level of significance was set at $p < 0.05$. Statistical analysis was performed by Systat 9, GraphPad Prism Software 9.5.1, and SigmaPlot software 14.0. Based on preliminary analyses of normal distribution by Shapiro–Wilk normality test, we performed: (i) ANOVA for repeated measures to analyze LTP, PTP, PPF, BST, and MWM latency; (ii) one-way ANOVA with Bonferroni's post-hoc correction for open field, NOR and NOL in aged mice, MWM probe test; (iii) two-tailed t-test when comparing two samples; (iv) one sample t test to compare D with zero in NOR and NOL; (v) Kruskal–Wallis One Way Analysis of Variance on Ranks with Student–Newman–Keuls for multiple comparison for western blot analyses. Given the non-normal distribution of electron microscopical data, as assessed by D'Agostino and Pearson normality test, we used nonparametric contingency analysis with Fisher's test for pre-embedding electron microscopy data and nonparametric

Kruskal–Wallis test and Dunn's multiple comparison test for electron microscopy measures obtained from hippocampal recorded slices.

RESULTS

3.1 D3R blockade or genetic deletion enhances hippocampal plasticity

To examine the impact of D3R blockade on hippocampal synaptic transmission and plasticity we performed electrophysiological studies at CA3-CA1 synapses. We first investigated the role of D3Rs in the weak form of long-term potentiation (LTP1), also known as early LTP, a transient modification in synaptic strength evoked by a weak tetanic stimulation (1 TBS) that requires additional reinforcement to be converted into a more persistent and stable LTP2.

Experiments of field recordings confirmed that 1 TBS induced LTP1 in vehicle-treated slices from adult WT animals (Gulisano et al., 2019), whereas a treatment with the D3R antagonist NGB-2904 for 15 min before 1 TBS was sufficient to elicit LTP2 comparable to that obtained by a strong tetanic stimulation (3 TBS) (Figure 1a). Blocking D3Rs with NGB-2904 further enhanced 3TBS-induced potentiation (Figure 1a).

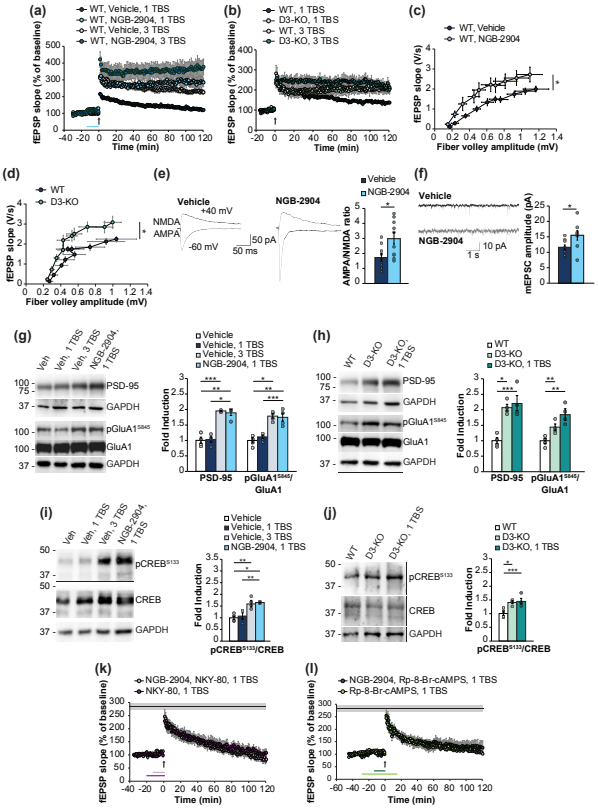

Figure 1 - Effects of D3R blockade on hippocampal synaptic function. (a) NGB-2904 (1 μM for 15 min before a weak tetanic stimulation, 1 TBS) converted LTP1 into LTP2 ($F(1,12) = 75.341$, $p < 0.0001$, $n = 7/7$) and enhanced potentiation induced by a strong tetanic stimulation (3 TBS) ($F(1,13) = 4.745$, $p = 0.048$, $n = 7$). (b) D3-KO slices developed LTP2 after 1 TBS ($F(1,11) = 10.194$, $p = 0.009$, $n = 7/7$). No differences were found in 3

TBS-induced LTP2 in D3-KO vs. WT slices ($F(1,9) = 1.507$, $p = 0.251$, $n = 6/6$). (c) fEPSP slope measured during BST increased in NGB-2904-treated slices compared with vehicle ($n = 10/13$; $F(1,21) = 4.416$, $p = 0.048$ vs. vehicle), whereas no difference was found in fiber volley amplitude ($F(1,21) = 0.304$, $p = 0.587$). (d) fEPSP slope measured during BST increased in slices from D3-KO mice compared with WT ($n = 10/12$; $F(1,20) = 5.020$, $p = 0.037$), whereas no difference was found in fiber volley amplitude ($F(1,20) = 0.049$, $p = 0.828$). (e) Representative traces of AMPA- and NMDA-receptor-mediated EPSCs and bar graph of AMPA/NMDA ratio after 10 min incubation with vehicle or NGB-2904 (1 μM) ($t(18) = 2.774$; $p = 0.012$; $n = 10/10$). (f) Representative traces and graph showing that the peak amplitude of mEPSCs increased in slices incubated with NGB-2904 ($t(15) = 2.333$, $p = 0.034$). (g) Representative WB images of PSD-95, GluA1, and pGluA1 (cropped images based on MW) performed in lysates from slices treated for electrophysiology stored at 1 min after tetanus ($n = 3$ slices/lane). GAPDH was used as loading control. On the right, bar graph showing the increase of PSD-95 ($p = 0.046$) and pGluA1 ($p < 0.001$) expression in WT slices treated with NGB-2904 + 1 TBS. (h) PSD-95 ($p = 0.049$) and pGluA1 expression ($p = 0.009$) increased in D3-KO slices in basal conditions and after 1 TBS ($p < 0.001$; $p = 0.009$). (i) pCREB expression increased in WT slices treated with NGB-2904 + 1 TBS ($p = 0.005$) and (j) D3-KO slices in basal conditions ($p = 0.038$) and after 1 TBS ($p < 0.001$). (k) NKY-80 (10 μM, 20 min before tetanus) prevented the NGB-2904-induced LTP2 ($F(1,12) = 84.83$, $p < 0.0001$, $n = 7$). Shaded area with dotted line corresponds to mean ± SEM of last point of potentiation induced by NGB-2904 + 1 TBS shown in Figure 1a. (l) Rp-8Br-cAMPs (20 μM, 30 min before and 15 min after tetanus) prevented the NGB-2904-induced LTP2 ($F(1,12) = 52.806$, $p < 0.0001$ vs. NGB-2904 + 1 TBS, $n = 7$). Data expressed as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. [Correction added on 4th October after first online publication: Figure 1 has been replaced as it had some material and data copying errors.]

To provide genetic evidence of the involvement of D3Rs in synaptic transmission and plasticity, we performed a series of

additional experiments by using D3 knock out mice (D3-KO) and found that D3-KO slices were capable to develop a LTP2 after 1 TBS (Figure 1b), whereas 3 TBS-induced LTP2 was comparable to that evoked in WT slices (Figure 1b).

Because LTP involves both pre- and post-synaptic modifications, we examined pre-synaptic forms of plasticity while blocking LTP inductive mechanisms with the NMDA antagonist (2R)-amino-5-phosphonovaleric acid (APV). We observed that NGB-2904 did not alter the presynaptic component of post-tetanic potentiation (PTP; Figure S2a) or paired-pulse facilitation (PPF; Figure S2b), suggesting that presynaptic mechanisms were not involved.

We then analyzed basal synaptic transmission (BST) and found that blockade of D3Rs increased fEPSP slopes obtained during input–output recordings in NGB-2904-treated compared to vehicle-treated slices (Figure 1c, Figure S2c) and in D3-KO compared to WT slices (Figure 1d, Figure S2e). To better understand whether the BST enhancement was due to pre- and/or post-synaptic modifications we analyzed afferent volley amplitude, an index of pre-synaptic firing, that was unchanged,

confirming that D3R blockade mainly acted via a post-synaptic mechanism (Figure 1c,d, Figure S2d,f).

As a further insight, we conducted electrophysiological experiments using whole-cell patch-clamp recordings in hippocampal slices (Ripoli et al., 2014). We studied the input–output properties of AMPAR- and NMDAR-mediated excitatory post-synaptic currents EPSCs in response to fixed-intensity test stimuli. Analysis of the AMPA/NMDA ratio indicated an increase of the AMPAR-mediated synaptic responses following treatment with the D3R antagonist NGB-2904 (Figure 1e), thus suggesting an enhancement of synaptic strength.

This effect was further confirmed by assessing miniature excitatory post-synaptic currents (mEPSCs) that exhibited an increase in mEPSC amplitude after the extracellular administration of NGB-2904 (Figure 1f, Figure S2g). No differences were observed in mEPSC frequency (Figure S2h,k), rise time (Figure S2i,l), and decay time (Figure S2j,m), confirming that D3R blockade primarily induced post-synaptic modifications.

3.2 D3R blockade induced modifications in post-synaptic protein expression

Since electrophysiological data indicated a primary involvement of post-synaptic D3Rs, we investigated the effect of D3R blockade on post-synaptic machinery. We therefore assessed post-synaptic protein expression performing western blot (WB) experiments on the same hippocampal slices used for LTP electrophysiological recordings (Gulisano et al., 2019). We first evaluated the levels of PSD-95, whose expression strongly correlates with synaptic strengthening, and observed a significant increase in its expression in slices treated with NGB-2904 paired with 1 TBS (+89%; Figure 1g), comparable to that found in vehicle-treated slices that underwent a strong tetanization (+96%; Figure 1g). The increase of PSD-95 expression was also evident in D3-KO slices that received 1 TBS (+121%; Figure 1h) and 3 TBS (+98%; Figure S3a). Interestingly, a similar increase was present in D3-KO slices that did not receive tetanic stimulation (+113%; Figure 1h).

Since PSD-95 modulates the trafficking and localization of glutamate receptors, we examined the expression of AMPA receptor subunit GluA1 phosphorylated at Ser845 (pGluA1), known to influence synaptic strength and plasticity. In hippocampal slices treated with NGB-2904 and 1 TBS, the expression of pGluA1 increased and was comparable to that

found in vehicle-treated slices that underwent a strong tetanization (+75% and + 79%, respectively; Figure 1g). We observed the same increase in D3-KO slices after 1 TBS (+76%; Figure 1h), 3 TBS (+68%; Figure S3b), but also in basal conditions (+44%; Figure 1h).

Since D3Rs are known to be negatively coupled to AC (Robinson & Caron, 1997), their blockade was expected to increase the cAMP/PKA signaling pathway, leading to CREB phosphorylation, which is associated with LTP2 and memory formation (Teich et al., 2015). Consistently, CREB phosphorylated at Ser133 (p-CREB) increased in slices treated with NGB-2904 and 1 TBS (+66%; Figure 1i), as well as in D3-KO slices treated with either 1 TBS, 3 TBS, or vehicle alone (+45%, +61%, +39%; Figure 1j, Figure S3b).

To further investigate the influence of D3Rs on the cAMP/PKA/CREB intracellular signaling pathway, we performed a series of LTP recordings in slices from WT animals treated with NGB-2904 paired with inhibitors of the cAMP pathway. Our experiments demonstrated that both the inhibition of adenylyl cyclase by NKY-80 (Figure 1k) and the inhibition of PKA by Rp-8-Br-cAMPS (Figure 1l) prevented NGB-2904

from converting LTP1 into LTP2, thus confirming that the LTP enhancement induced by D3R blockade was cAMP/PKA dependent. Control experiments showed that NKY-80 or Rp-8-Br-cAMPS did not affect LTP evoked by 1 TBS (Figure 1k,l).

3.3 D3Rs ultrastructural localization in hippocampal CA1 area

Prompted by the pronounced effects of D3R antagonism on hippocampal synaptic plasticity, we evaluated the localization of D3Rs in the hippocampus, focusing on the CA1 area, where electrophysiological recordings were performed. Light microscopy of intact adult CA1 revealed diffuse D3R immunoreactivity (IR) in stratum oriens (so), pyramidalis (pyr), and radiatum (sr) characterized by neuronal- and glial-like positive cells, positive profiles, and small positive (+) puncta dispersed in the neuropil (Figure 2a,a″).

Pre-embedding electron microscopy of pyr-sr layers confirmed the presence of immuno-positive products in the cytoplasm of neurons and astrocytes, deciphered the dendritic, axonal, and glial nature of D3R+ profiles, and unveiled the expression of D3Rs in axon terminals, small distal dendrites, post-synaptic dendrites, and distal astrocytic processes in close relationship with synaptic elements (Figure 2b–j).

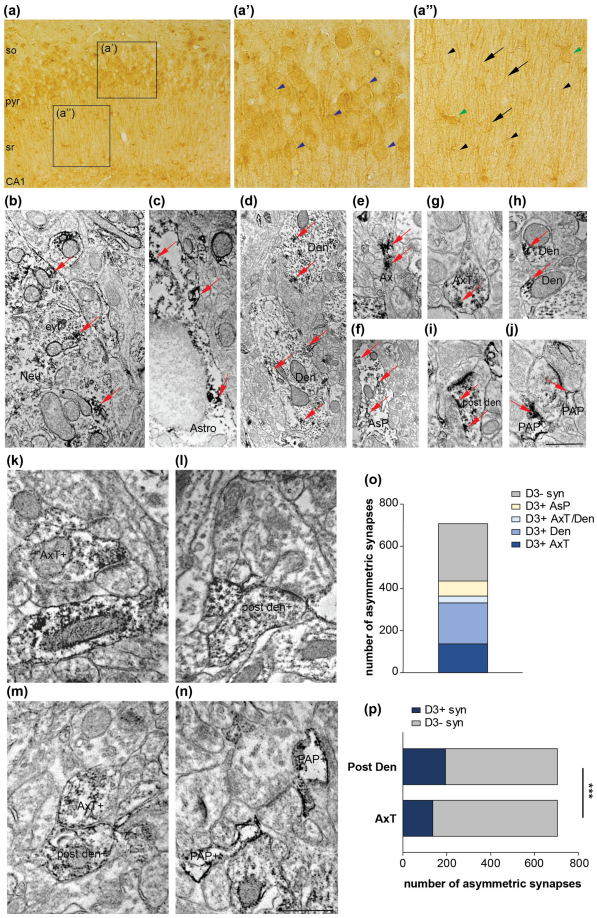

Figure 2 - Cellular and subcellular localization of D3Rs in CA1 adult hippocampus. (a) Light microscopy of D3R immunoreactivity (IR) in stratum oriens (so), pyramidalis (pyr), and radiatum (sr) unveiled neuronal- and glial-like positive cells (blu arrowheads in a' and green arrowheads in a", respectively), positive profiles [arrows in (a")], and small positive (+) puncta [black arrowheads in (a")]. (b–j) Pre-embedding electron microscopy of pyr-

sr layers identified immuno-positive electron dense products (examples of them marked with red arrows in all panels) in the cytoplasm (cyt) of neurons [(Neu in (b)], astrocytes [Astro in (c)], dendrites [Den in (d)], axons [Ax in (e)], proximal glial processes [AsP in (f)], axon terminals [AxT in (g)], small distal dendrites [den in (h)], post-synaptic dendrites [post den in (i)], and peri-synaptic astrocytic processes [PAP in (j)] in close relationship with synaptic elements. (k–n) Pre-embedding microscopy showed that at asymmetric synapses, D3Rs localize at axon terminals [AxT+ in (k)], post-synaptic dendrites [post den+ in (l)], at both AxT and post den (m), and in pre-synaptic astrocytic processes [PAP+ in (n)] in close relationship with synaptic domains. (o) Graph illustrating the proportion between positive synaptic elements and negative synapses. Out of 704 asymmetric synapses, 137 (19.5%) were positive at AxT (D3+ AxT), 194 (27.6%) at post-synaptic dendrites (D3+ Den), 33 (4.7%) at both AxT and post-synaptic dendrites (D3+ AxT/Den), 68 (9.6%) at peri-synaptic astrocytic processes (D3+ AsP), and 272 (38.6%) were negative (D3− syn). (p) Comparison between pre-synaptic (AxT) and post-synaptic domains (Post Den) showed that in D3 positive synapses (D3+ syn), D3Rs were mainly expressed in the post-synaptic dendrites (19.5% vs. 27.6%, $p = 0.0004$, Fisher's exact test). Scale Bar: 200 μm for (a), 120 μm for (a')–(a"), 500 nm for (b)–(n). ***$p \leq 0.001$.

We next estimated quantitatively the degree of expression of D3Rs in synaptic domains of asymmetric synapses in sr. D3Rs were detectable in axon terminals (Figure 2k,o,p), post-synaptic dendrites (Figure 2l,o,p), in both AxT and post-synaptic dendrites (Figure 2m,o), and in distal astrocytic processes (Figure 2n,o) in close proximity to synaptic domains. Analysis of D3R distribution between synaptic domains of positive asymmetric synapses revealed that D3R expression was higher in the post-synaptic dendrites than in axon terminals (Figure 2p).

3.4 D3R blockade or genetic deletion induce long-term memory formation

Our results indicated that D3R blockade induces the synaptic changes underlying LTP and CREB phosphorylation, intracellular signaling events that play an essential role in the molecular mechanisms underlying memory formation. Thus, we examined whether blocking D3Rs exerted a cognitive-enhancing effect. We used modified protocols of NOR and NOL tasks with a short training phase (T1) that, normally, is sufficient to induce short-term memory lasting up to 6–8 h (Blokland & Sesia, 2023) but does not allow to discriminate between the familiar and the novel object after a 24-h interval testing phase (Bollen et al., 2014; Gulisano et al., 2019; Palmeri et al., 2017).

Vehicle-treated mice did not discriminate between objects or their positions after a 24-h interval (Figure 3a), whereas mice treated with intraperitoneal (ip) administration of NGB-2904 showed a high discrimination index (Figure 3a). We then evaluated the effect of D3R blockade on LTM. To this end, mice were trained for a longer time in T1 (10 min) that ensures memory formation after 24 h. Under these conditions, vehicle-treated and NGB-2904-treated animals exhibited similar

discrimination indices (Figure 3a) in both the NOR and NOL tests.

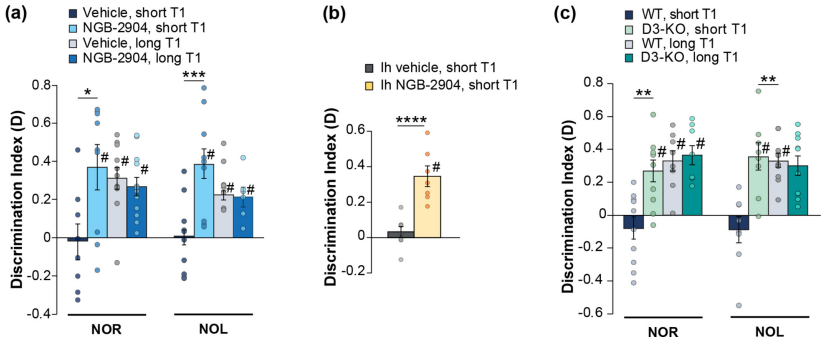

Figure 3 - D3R blockade or genetic deletion stimulates long-term memory formation. (a) A short training (3 min in T1) in NOR and NOL did not allow long-term memory (LTM) formation in vehicle-treated mice (discrimination index D = 0 NOR: $p = 0.869$, $n = 8$; NOL: $p = 0.989$, $n = 10$) bur was sufficient for LTM formation in NGB-2904-treated WT animals ($D \neq 0$ NOR: $p = 0.011$, $n = 9$; NOL: $p = 0.001$, $n = 10$). Mice treated with vehicle or NGB-2904 formed LTM if exposed to a long T1 (10 min) ($D \neq 0$ NOR: $p < 0.0001$, $n = 11$; NGB-2904 $p < 0.0001$, $n = 12$; NOL: Vehicle $p < 0.0001$, $n = 8$; NGB-2904 $p = 0.009$, $n = 6$). Planned comparison confirmed the difference between vehicle- and NGB-2904-treated mice that underwent a short T1 in NOR ($F(3,36) = 4.364$), $p = 0.01$; Bonferroni's $p = 0.012$) and NOL ($F(3,30) = 6.977$, $p = 0.001$; Bonferroni's $p = 0.001$). (b) Intrahippocampal injections (Ih) of NGB-2904 induced LTM after a short T1 ($D \neq 0$: $p = 0.001$, $n = 7$). Two-group comparison confirmed that treatment affected D ($t(13) = 5.107$, $p < 0.0001$). (c) D3-KO mice that underwent a short T1 formed LTM in NOR ($D \neq 0$; $p = 0.003$, $n = 10$) and NOL ($p = 0.004$, $n = 8$). A long T1 evoked LTM in both WT and D3-KO mice ($D \neq 0$ in NOR: WT $p = 0.001$, $n = 8$; D3-KO $p < 0.0001$, $n = 8$; NOL: WT $p < 0.0001$, $n = 9$; D3-KO $p = 0.001$, $n = 10$). Planned comparison confirmed the difference between WT and D3-KO mice that underwent a short T1 in NOR ($F(3,32)$

= 10.163, $p < 0.0001$; Bonferroni's $p = 0.002$) and NOL ($F(3,31) = 9.077$, $p < 0.0001$; Bonferroni's $p = 0.001$).Data expressed as mean ± SEM. *$p < 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$; # difference from 0.

Considering that systemic ip treatment with NGB-2904 can block D3Rs in various brain areas, we investigated the role of hippocampal D3Rs in memory formation by administering NGB-2904 via intrahippocampal injections. Our findings revealed that mice treated with NGB-2904 (injected 15 min before the short T1) spent a longer time exploring the novel object in T2 compared to vehicle-treated mice (Figure 3b), confirming the cognitive-enhancing effect of hippocampal D3Rs blockade.

The same results were obtained in D3-KO mice that were capable to form memory when tested with a NOR and a NOL task after a short T1 (Figure 3c), whereas presented normal recognition and spatial memory after a long T1 (Figure 3c).

Total exploration time was not affected by ip or intrahippocampal treatment with NGB-2904 (Figure S4a–c) or D3-R genetic deletion (Figure S4d,e). Furthermore, treatment and genotype did not modify the Open Field results

(Figure S4f,g), suggesting that mouse exploratory behavior did not influence memory performance.

No significant differences were detected when analyzing data obtained with NOR, NOL and OF tests in males versus females (Figure S5).

3.5 D3R levels and D3R expression at excitatory synapses are age-dependent

Our next goal was to investigate the role of D3Rs in aging and whether the positive effect of D3R blockade on synaptic plasticity and memory could be exploited against the age-related cognitive impairment. To accomplish this, we utilized animal models of physiological aging, specifically WT mice aged 18–22-month-old, previously shown to exhibit a deficit of LTP and memory (Palmeri et al., 2013).

We first studied whether aging modified the expression and localization of D3Rs at the hippocampal level. Quantitative ultrastructural analysis of D3 IR of sr from adult and aged mice revealed that D3+ profiles in aged mice were reduced by ~41% (Figure 4a–c).

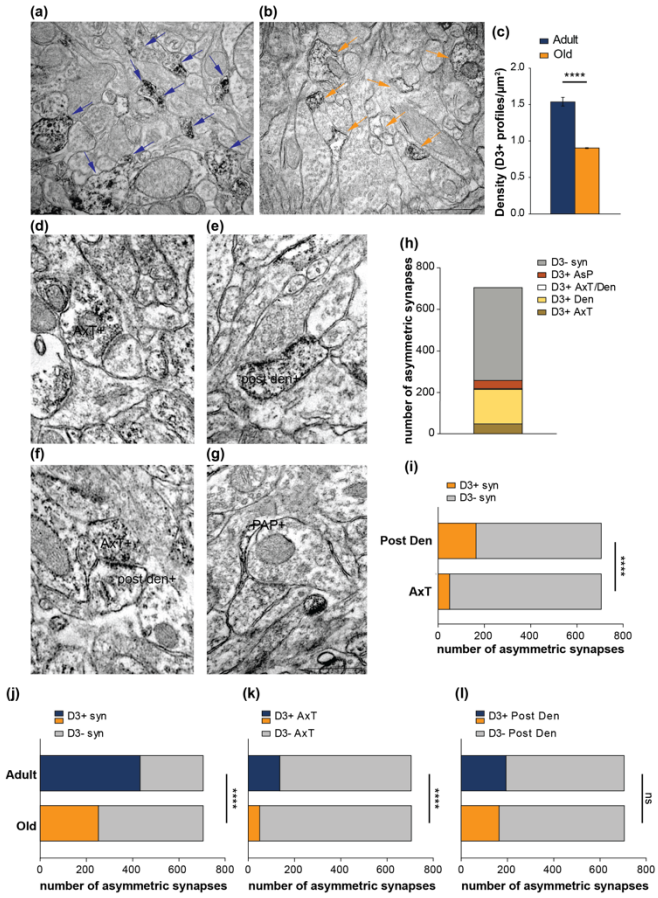

Figure 4 - Modifications of D3Rs expression and distribution in hippocampi from aged mice. (a, b) Representative examples of electron microscopical fields immunoreacted for D3R displaying several positive profiles (with dark electrodense products) marked arrows from adult (blu arrows) and aged (orange arrows) mice. (c) Graph reporting the comparison between the density of D3+ profiles/µm2 of adult (1.5 ± 0.05 profiles/µm2; from 167 microscopical fields/3 mice) and aged mice (0.9 ± 0.03 profiles/µm2; from 173 microscopical fields/3 mice. (d–g) Pre-embedding

microscopy showed that D3Rs are expressed in axon terminals [AxT+ in (d)], post-synaptic dendrites [post den+ in (e)], at both AxT and post den (in f), and in distal astrocytic processes [PAP+ in (g)] touching synaptic domains. (h) Graph illustrating the proportion between positive synaptic elements and negative synapses [out of 708 asymmetric synapses, 48 (6.8%) were positive at AxT, 166 (23.4%) at post den, 4 (0.6%) at both AxT and post den, 39 (5.5%) at peri-synaptic astrocytic processes (AsP), and 451 (63.7%) were negative]. (i) Comparison between pre-synaptic (AxT) and post-synaptic (post den) domains showed that in D3+ synapses of aged mice, D3R localization was predominantly post-synaptic (6.8% vs. 23.4%, $p < 0.0001$, Fisher's exact test). (j–l) Graphs representing the comparison between adult and aged hippocampal synapses indicated a general reduction of D3+ synapses [(j) 61.4% vs 36.3%, $p < 0.0001$, Fisher's exact test)] and that the level of pre-synaptic expression of D3R was significantly lower in aged mice [(k), 19.5% vs 6.8%, $p < 0.0001$, Fisher's exact test]. Conversely, the degree of post-synaptic expression was quite comparable between adult and aged mice [(l), 27.6% vs. 23.4%, $p = 0.077$, Fisher's exact test). Scale bar: 500 nm. ****$p \leq 0.0001$.

In line with results obtained in adult mice (see Figure 2), at excitatory synapses, D3Rs were localized at axon terminals, post-synaptic dendrites, both axon terminals and post-synaptic dendrites, and peri-synaptic astrocytic processes in close relationship with synaptic domains (Figure 4d–h). As in adult mice, quantitative analysis revealed that in aged mice the expression of D3Rs was higher in the post-synaptic dendrites than in axon terminals (Figure 4i). The comparison of D3R expression at asymmetric synapses between adult and aged mice

revealed a reduction of positive synapses in aged mice (Figure 4j). Interestingly, the lowered expression of D3Rs in aged synapses was caused by the reduction of D3Rs at pre-synaptic sites but not at post-synaptic dendrites (Figure 4k,l).

3.6 D3R blockade or genetic deletion rescues the age-related synaptic and memory impairment

To assess whether D3R blockade might rescue the age-related cognitive phenotype, we first studied LTP in hippocampal slices and found that a perfusion with the D3R antagonist NGB-2904 restored the age-related impairment of potentiation (Figure 5a). To further confirm the positive effect of D3R blockade, we used another D3R antagonist, SB-277011A, which also normalized LTP in slices from aged WT mice (Figure 5a). We then evaluated LTP in hippocampal slices from aged D3-KO animals and found that they did not exhibit the expected age-related impairment in LTP but a normal potentiation after a strong tetanus (Figure 5b). Because D3R blockade or genetic deletion increased BST in slices from adult animals (see Figure 1c,d), we also studied whether acting on these receptors could exert a positive effect on BST in old animals. A pharmacological blockade with NGB-2904 (Figure 5c) or the genetic deletion in D3-KO models

(Figure 5d) not only restored the age-related impairment of BST but improved fEPSP responses when compared with slices from adult animals, without changing afferent volley (Figure S6a,b).

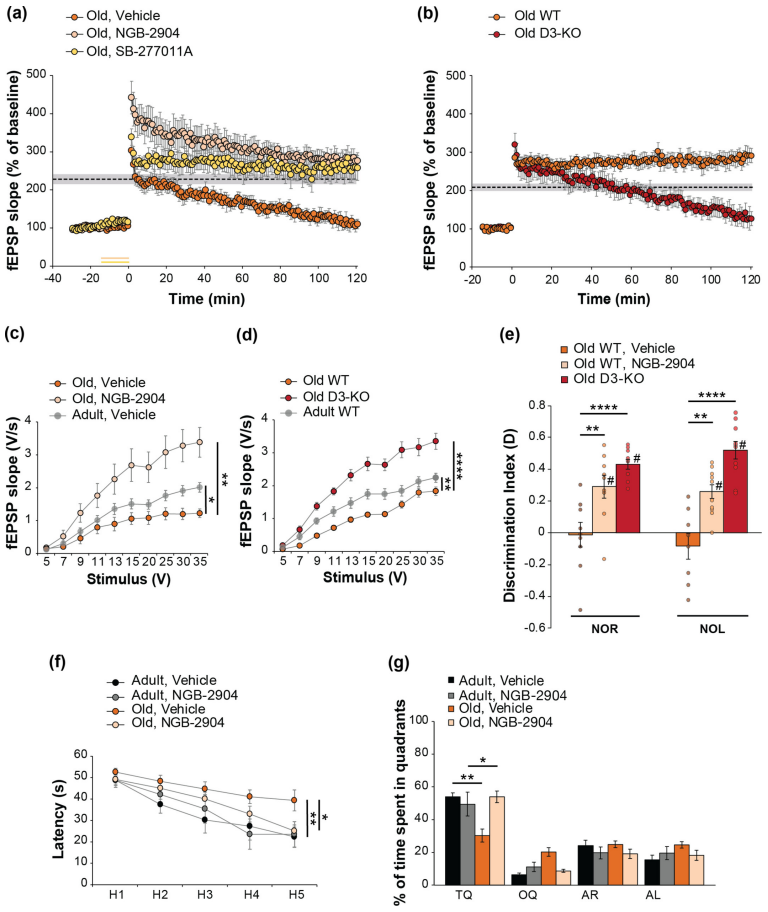

Figure 5 - The blockade or genetic deletion of D3Rs rescues the age-related synaptic and memory impairment. (a) The LTP impairment in slices from old wild type mice (n = 6) was restored by the D3-antagonists

NGB-2904 or SB-277011A (n = 8/6; $F(1,12) = 17.878$, $p = 0.001$; $F(1,10) = 10.836$, $p = 0.008$). Shaded area with dashed line = mean ± SEM of the last LTP recording in vehicle +3 TBS shown in Figure 1a. (b) Slices from aged D3-KO mice did not exhibit LTP impairment (n = 8/9, $F(1,15) = 11.196$, $p = 0.004$). Shaded area with dashed line = mean ± SEM of the last LTP recording in WT + 3 TBS shown in Figure 1b. (c) The age-related impairment of BST ($F(1,21) = 5.01$, $p = 0.036$) was rescued by NGB-2904 ($F(1,18) = 11.438$, $p = 0.003$, n = 10/10). (d) Slices from D3-KO did not present the impairment of BST ($F(1,19) = 51.531$, $p < 0.0001$, n = 11/10). (e) The age-related impairment of memory ($D = 0$ NOR: $p = 0.981$, n = 10; NOL: $p = 0.341$; n = 8) was rescued by NGB-2904 ($D \neq 0$ NOR: $p = 0.004$, n = 9; NOL: $p < 0.0001$, n = 10) or in D3-KO mice (NOR: $p < 0.0001$, n = 10; NOL: $p < 0.0001$, n = 10). Planned comparisons confirmed the rescuing effect of NGB-2904 (Bonferroni's $p < 0.01$) and D3R deletion ($p < 0.0001$). (f) The age-related decrease of latency ($F(1,17) = 7.983$, $p = 0.012$, n = 10/9) was rescued by NGB-2904 ($F(1,19) = 4.792$, $p = 0.041$, n = 11). (g) Aged mice treated with NGB-2904 spent more time in TQ ($p < 0.0001$). Planned comparisons confirmed that the age-related memory impairment was rescued by NGB-2904 ($p = 0.001$). Data expressed as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$; # difference from 0. AR, adjacent right; AL, adjacent left; OQ, opposite quadrant; TQ, target quadrant.

Thus, D3R pharmacological blockade or genetic deletion had a protective effect against the age-dependent impairment of hippocampal synaptic transmission and plasticity.

Next, we performed the behavioral test of NOR and NOL on aged WT mice treated with ip injections of the D3R antagonist NGB-2904 and on D3-KO animals to understand whether D3R blockade or genetic deletion could rescue the age-related

memory impairment. NGB-2904-treated and D3-KO aged mice both presented normal recognition memory in a NOR test, as they spent more time in exploring the novel compared with the familiar object resulting in a restored D index (Figure 5e). We obtained the same results in a NOL test where aged mice treated with NGB-2904 and aged D3-KO presented a normal D index due to the higher time of exploration for the object located in a novel position compared to the old one (Figure 5e). Total exploration time was mot modified by treatment or genotype in NOR and NOL (Figure S6c). The Open field test confirmed that exploratory behavior was unaffected in old WT treated with NGB-2904 and D3-KO animals (Figure S6d,e).

Consistent with findings in adult mice, we observed no sex differences in aged WT mice, animals treated with NGB-2904, or in D3-KO models (Figure S7).

The effect induced by D3R blockade was also verified with the Morris Water Maze test, a behavioral test widely used to assess spatial learning and reference memory in rodents. Aged mice showed an impairment of spatial memory as they need a higher time (latency) to reach the hidden platform during the spatial learning test compared to adult controls (Figure 5f), but a

treatment with NGB-2904 was capable to rescue this deficit (Figure 5f). Then, we assessed reference memory with the probe test that evaluated the amount of time spent in each quadrant of the maze after removing the platform. For each experimental group, we first compared the time spent in the TQ, where the platform was located during training, with other quadrants to verify whether mice retained reference memory. We found that adult mice treated with vehicle or NGB-2904 spent significantly more time in the TQ compared with other quadrants (Figure 5g). Conversely, aged mice treated with vehicle spent a similar amount of time in the four quadrants indicating an impairment of reference memory that was rescued after treatment with NGB-2904 (Figure 5g). Planned comparison indicated that adult mice treated with vehicle or NGB-2904 and aged mice treated with NGB-2904 spent a similar amount of time exploring the TQ, whereas aged mice treated with vehicle spent less time exploring the TQ (Figure 5g). A visible platform trial did not reveal any significant difference in the time to reach the platform among the 4 groups of mice (Figure S6f).

3.7 D3R blockade or genetic deletion elicits post-synaptic modifications underlying the rescue of age-induced synaptic-impairment

Based on the prevalent post-synaptic effect of D3Rs in healthy adult mice, we investigated whether the ability of D3R blockade to restore synaptic function in aged mice relied upon changes in post-synaptic protein expression. We performed WB experiments on hippocampal slices that previously underwent electrophysiology and found that the pharmacological blockade of D3Rs by NGB-2904 or the genetic deletion as in D3-KO animals restored the age-related impairment of PSD-95 (+117% and + 86%, respectively; Figure 6a,b), pGlua1 (+63% and + 61%, respectively; Figure 6c,d), and p-CREB (+66% and + 97%, respectively; Figure 6e,f).

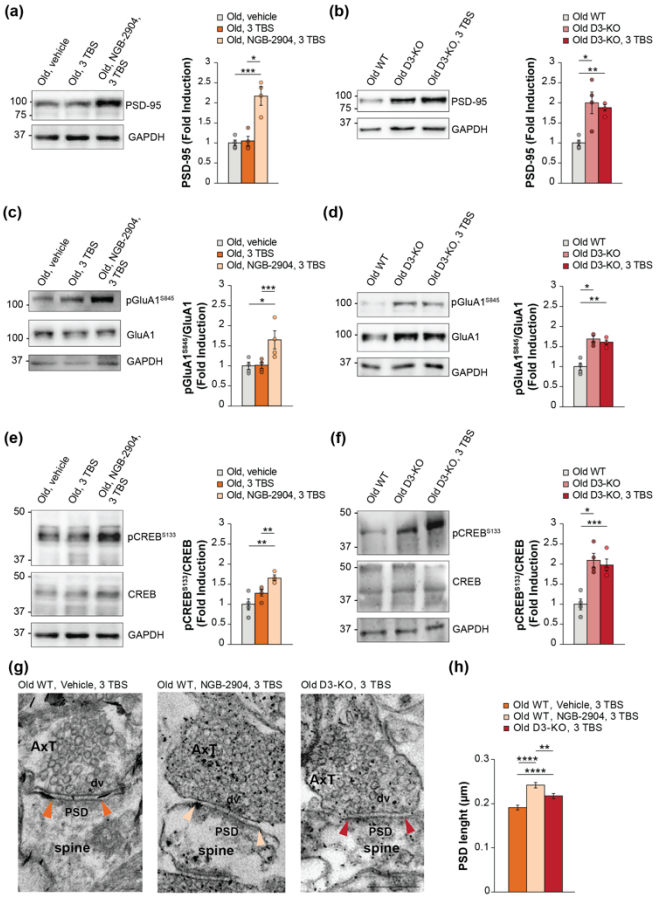

Figure 6 - D3R blockade or genetic deletion rescues the age-related impairment of post-synaptic proteins and PSD length. (a) Representative images of WB experiments of PSD-95 (cropped images based on MW) performed on hippocampal slices treated for electrophysiological experiments stored at 1 min after treatment (n = 3 slices/lane). Bar graph showing that a treatment with NGB-2904 rescued PSD-95 expression in slices from aged WT mice that underwent a strong tetanic stimulation (p = 0.049). GAPDH was used as loading control. (b) Slices from aged D3-

KO mice presented an increase of PSD-95 expression either in basal conditions ($p = 0.017$) or after 3 TBS ($p = 0.004$) vs. old WT. (c) NGB-2904 rescued pGluA1 expression in slices from aged WT mice that underwent a strong tetanic stimulation ($p < 0.001$). (d) Slices from aged D3-KO mice presented an increase of pGluA1 expression either in basal conditions ($p = 0.029$) or after 3 TBS ($p = 0.001$) versus old WT. (e) NGB-2904 rescued pCREB expression in slices from aged WT mice ($p = 0.009$). The same lane for GAPDH is shown here and in panel (a) (loading control for PSD-95). (f) Slices from aged D3-KO mice presented an increase of pCREB expression either in basal conditions ($p = 0.029$) or after 1 TBS ($p = 0.001$) versus old WT. (g) Representative asymmetric axo-spinous synapses of CA1 stratum radiatum from hippocampal slices from aged mice previously treated for electrophysiological recordings. Colored arrowheads point to PSD edges for each synapse from different experimental conditions. Asterisks mark docked vesicles (dv) at the active zone of the axon terminals (AxT). (h) PSD length analysis reveals an increase of PSD in WT+ NGB-2904+ 3TBS and in D3-KO + 3TBS compared to WT + 3TBS ($p < 0.0001$ and $p = 0.0006$, respectively; Kruskal–Wallis, Dunn's test for multiple comparison) as a difference between WT + NGB-2904 + 3TBS and D3-KO + 3TBS ($p = 0.0048$; see Table S1 for the complete detailed analysis). Data are expressed as mean ± SEM. Scale bar: 200 nm. ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

These results were corroborated by EM studies evaluating ultrastructural synaptic modifications in tetanized hippocampal slices where D3Rs were antagonized or deleted. By investigating specific ultrastructural changes (i.e., vesicle pool, number of docked vesicles, area of spines, PSD length, and percentage of perforated synapses; Table S1) (Gulisano et al., 2019) at axo-spinous synapses of CA1 stratum radiatum, we observed an increase of PSD length in tetanized slices treated

with the D3R antagonist NGB-2904 and in those from D3-KO mice (Table S1; Figure 6g,h), indicating that synapses display plasticity-induced changes at the post-synaptic site following D3R blockade or deletion.

DISCUSSION

In this work we showed that pharmacological blockade or genetic deletion of D3Rs enhanced hippocampal synaptic transmission and plasticity, as well as the expression of post-synaptic plasticity-related proteins, and memory in adult mice. Notably, our findings also evidenced that these effects were predominantly mediated by post-synaptic mechanisms, in line with the higher presence of D3Rs on post-synaptic dendrites assessed through an EM study of the CA1 area of the hippocampus. Building upon these insights, we investigated whether targeting D3Rs could offer potential avenues for addressing age-related cognitive decline. We have indeed demonstrated that D3R blockade restored the normal cognitive phenotype, and that aged D3-KO animals showed no signs of plasticity and memory deficits. A summary of the effect of D3R blockade on synaptic plasticity and memory in adult and old mice is illustrated in Figure 7.

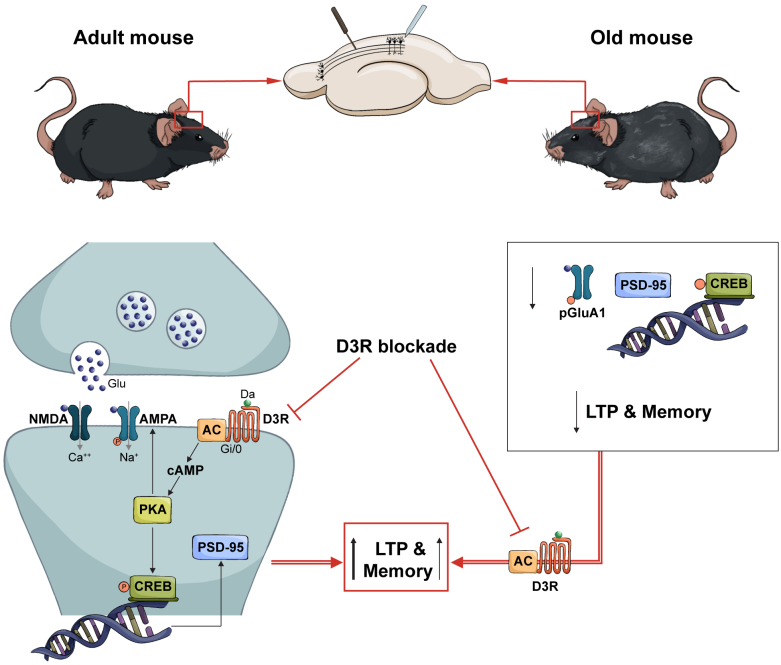

Figure 7 - Effects of D3R blockade on synaptic plasticity and memory in adult and aged mice. Picture depicting the main results of our manuscript. In adult mice, D3R blockade operated through the cAMP/PKA pathway, leading to increased expression of phopsho(p)-CREB, p-GluA1 (AMPA receptor subunit), and PSD-95. This results in structural modifications that promote long-lasting LTP and memory formation. In old mice, D3R blockade normalized the decreased expression of post-synaptic proteins and reversed the impairment of LTP and memory. NMDA = N-methyl-D-aspartate receptors; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors: Glu, glutamate; Da, dopamine; AC, adenylyl cyclase.

Our first aim was to study the impact of D3R blockade on synaptic plasticity and transmission in hippocampal glutamatergic synapses, essential for memory formation. We found that NGB-2904, a D3R antagonist, converted LTP1 into LTP2 and further enhanced LTP2. Notably, this conversion was also evident in D3-KO mice, which naturally exhibited LTP2 following a weak tetanic stimulation. Few other studies have investigated the effect of D3R modulation on hippocampal transmission and plasticity. For example, it has been reported that D3R agonists increased LTP via GABAergic depression (Swant et al., 2008; Swant & Wagner, 2006), with both inhibition and stimulation of PKA preventing these effects (Swant et al., 2008). However, although we cannot exclude that D3R agonists might exhibit a positive effect on plasticity depending on concentrations, doses, and duration of treatment employed, previous literature suggests that D3R stimulation impairs, while D3R blockade improves memory (reviewed in Kiss et al., 2021). This is in line with our data clearly indicating that blocking D3Rs enhanced synaptic transmission and plasticity via the cAMP/PKA pathway. Indeed, perfusion with either an adenylyl cyclase or a PKA antagonist prevented NGB-2904 from converting LTP1 into LTP2. The results align with

the recognized function of D3Rs that decrease cAMP and subsequently lessen PKA activity (Robinson & Caron, 1997). Consequently, blocking D3Rs would stimulate the cAMP/PKA pathway leading to an increase of hippocampal LTP, as extensively demonstrated.

Building on electrophysiological findings, we also thoroughly investigated the localization and distribution of D3Rs in the hippocampus. Light microscopy demonstrated a widespread presence of D3Rs across all hippocampal strata, aligning with previous studies that explored D3R localization in the hippocampus of rodents (Li & Kuzhikandathil, 2012), and humans (Meador-Woodruff et al., 1994). Here, EM ultrastructural studies provided a detailed sub-localization of D3Rs within the hippocampal CA1 region, revealing a high expression of D3Rs in excitatory asymmetric synapses in the pyr-sr layers, with a differential distribution of D3 IR among post-synaptic dendrites, axon terminals, and astrocytic processes. Interestingly, D3 IR was more prevalent in post-synaptic dendrites than in axon terminals. This aligns with previous studies placing D3Rs in the post-synaptic dendrites of excitatory synapses in the nucleus accumbens (Sokoloff

et al., 2013), where they modulate glutamatergic transmission (Liu et al., 2009).

Our functional data converged with EM findings, suggesting that D3 antagonism primarily impacts post-synaptic mechanisms as supported by the increase of fEPSP and mEPSC amplitude, and AMPA currents with no changes in pre-synaptic indices (i.e., PTP, PPF, afferent volley, and mEPSC frequency). Consistently, we found an increased expression of post-synaptic proteins such as PSD-95, pGluA1, and pCREB in slices treated with the D3R antagonist paired with a weak tetanic stimulation as well as in slices from D3-KO animals, even in basal conditions. These findings fit with the well-known role of post-synaptic proteins in synaptic and memory functions as phosphorylation of GluA1 at Ser845 boosts AMPA-R conductance and trafficking, mediating LTP by a PKA-dependent mechanism (reviewed in Kessels & Malinow, 2009). PSD-95, a post-synaptic density constituent, regulates AMPA-R incorporation and positioning during hippocampal plasticity and memory, impacting AMPA-R-mediated synaptic currents (Ehrlich & Malinow, 2004). Interestingly, it has been previously demonstrated that D3Rs interact with Ca2+/calmodulin-dependent protein kinase II in the PSD of glutamatergic neurons leading to a stimulation of

AMPA-R GluA1 subunit (Liu et al., 2009), initiating intracellular signaling crucial for long-lasting LTP and memory, including CREB phosphorylation. Consistently, we found that pharmacological blockade or genetic D3R deletion enhanced pCREB aligning with previous studies demonstrating increased pCREB in D3-KO mice neuronal cultures treated with nicotine (Mutti et al., 2022), and in hippocampal tissues of D3-KO mice trained with passive avoidance test (D'Amico et al., 2013). In this context, the basal increase in plasticity-related proteins mediated by D3R blockade might represent a crucial event for synaptic tagging. In fact, after a weak tetanic stimulation, synapses should enter a receptive state that, when aligns with the production of plasticity-related proteins, supports the maintenance of LTP (Wang et al., 2010).

Taken together, our data showed that the blockade of D3Rs induced molecular and functional modifications at the synapse that are crucial for LTM formation. Consequently, we investigated their role in memory processes. We found that acute treatment with the D3Rs antagonist NGB-2904 was able to induce LTM, exerting a robust pro-cognitive effect on recognition and spatial memory. Interestingly, similar results were obtained with D3-KO animals, that were capable to

naturally form LTM when exposed to a short training phase. These findings are in line with previous works demonstrating that the blockade or knockout of D3Rs exerts pro-cognitive effects (reviewed in Kiss et al., 2021). However, considering that the precise contribution of these receptors to hippocampal memory processes has not been thoroughly investigated, we injected the D3R antagonist NGB-2904 selectively into the hippocampus and found the same effects evidenced with a systemic treatment, proving that the blockade of hippocampal D3Rs enhanced memory. Notably, the blockade of D3Rs did not modify the total exploration time in NOR and NOL tests, nor the time spent in the center during the open field test. This suggests that D3R blockade did not affect movement or thigmotaxis, the latter being an indicator of unconditioned anxiety-like behavior. This is particularly significant considering that D3Rs are implicated in several brain functions, including emotional behavior and locomotion (reviewed in Sokoloff & Le Foll, 2017). Further studies are needed to better understand these aspects and their influence on memory.

Based on data obtained in the healthy mouse brain, we decided to investigate the effects of D3R blockade during aging and to exploit it as a possible strategy to counteract age-related

cognitive decline. First, we evaluated if aged hippocampi presented a modification of D3R quantity and distribution at hippocampal level. We demonstrated a significant decline of D3+ profiles within the hippocampal CA1 area, in agreement with earlier findings highlighting a significant age-related decline of D3R mRNAs in CA1 pyramidal neurons in human post-mortem brain tissues (Hemby et al., 2003). Interestingly, D3R expression remained unaltered in the post-synaptic dendrites but decreased at pre-synaptic sites. This imbalance might lead to reduced regulation or control over the quantity or timing of dopamine release and could heighten sensitivity or responsiveness of the receiving neuron to dopamine signaling thus altering downstream signaling pathways that influence synaptic transmission and plasticity.

In this context, the positive effects of D3R blockade on age-related cognitive decline could stem from restoring this imbalance. Specifically, treatment with two distinct D3R antagonists rescued impairment in synaptic transmission and plasticity in slices from aged mice. Conversely, D3-KO animals appeared resistant to aging, displaying no impairment in synaptic plasticity and transmission. NGB-2904 also completely restored the impairment of recognition memory and spatial

learning and memory in aged mice, whereas D3-KO mice did not show any of the age-related memory impairment. These results are consistent with a previous work demonstrating that aged D3-KO mice maintained intact spatial memory compared to aged-matched WT (Xing et al., 2010). Furthermore, they align with other studies showing the pro-cognitive effect of D3Rs in models of cognitive impairment (Laszy et al., 2005; Millan et al., 2007; Neill et al., 2016; Torrisi et al., 2017; Zimnisky et al., 2013).

Considering that previous studies reported sex differences in D3-KO behavior (Klinker et al., 2017; Liu et al., 2017), and given the disproportionate impact of cognitive decline in the hippocampus on the aging female population, we analyzed our behavioral data for potential sex differences. However, neither adult nor old animals exhibited any sex differences, whether they were WT, treated with NGB-2904, or D3-KO mice. It is important to note that our analysis was conducted on existing sex-balanced data divided by sex. Given the relatively low numbers of males and females, this could potentially mask some differences and might deserve further investigations.

In line with prior research, aged mice exhibited a decline in post-synaptic proteins associated with synaptic strengthening and memory, including pGluA1-Ser845 (Henly and Kilkinson, 2013), PSD-95 (Zarate et al., 2021), and pCREB-Ser133 (Palmeri et al., 2013). Corroborating our electrophysiological and behavioral findings, we observed that treatment with NGB-2904 restored their expression in hippocampal slices from aged mice following tetanization. Intriguingly, slices from D3-KO animals exhibited elevated levels compared to age-matched WT mice, even without tetanic stimulation. Furthermore, EM performed on hippocampal slices from aged WT mice showed that pharmacological blockade of D3Rs induced structural modifications at the post-synaptic site consisting in an increase of PSD lenght, a known factor underlying functional long-lasting synaptic potentiation.

In conclusion, our data contribute to clarifying the role of D3Rs at hippocampal glutamatergic synapses and suggest the potential use of D3R antagonists as pharmacological agents for treating or preventing memory loss associated with physiological aging and neuropsychiatric disorders characterized by memory decline, holding high translational applicability.

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SUPPLEMENTARY INFORMATION

Blockade of dopamine D3 receptors improves hippocampal synaptic function and rescues age-related cognitive phenotype

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SUPPLEMENTARY METHODS

Electrophysiology

Electrophysiological field recordings

Extracellular field recordings were conducted on 400 μm transverse hippocampal slices as described previously (Gulisano et al., 2019). After sectioning using a manual tissue chopper, the slices were placed in a recording chamber and perfused (1–2 ml/min) with ACSF solution containing (in mM) 124 NaCl, 4.4 KCl, 1 Na2HPO4, 25 NaHCO3, 2 CaCl2, 2 MgCl2, and 10 glucose, maintained at 29°C and continuously bubbled with 95%

O2 and 5% CO2. After 120 minutes of recovery, field excitatory post-synaptic potentials (fEPSPs) were recorded in CA1 stratum radiatum (sr) using a glass electrode filled with ACSF in response to Schaffer collateral stimulation by a bipolar tungsten electrode.

Basal synaptic transmission (BST) was measured by stimulating with a series of increasing voltage pulses (from 5 to 35 V) to select healthy slices for recordings. Before LTP induction, baseline responses were recorded every minute using a voltage that evoked a response of 35% of the maximum evoked response in BST. We used a theta-burst stimulation (TBS) protocol to induce LTP where each tetanus consisted in 3 trains of 10 bursts at 100 Hz, with five pulses per burst repeated at 5 Hz. We have studied LTP1 and LTP 2, frequently referred to as early-phase LTP and late-phase LTP, corresponding to forms of LTP that are, respectively, independent of and dependent on de novo protein synthesis (Bliss et al., 2018). To induce LTP1 a single TBS train (weak tetanic stimulation) was delivered, while for LTP2, three TBS trains were delivered with a 15-second intertrain interval (strong tetanic stimulation). fEPSP slopes were normalized to the first 15 minutes of baseline recordings.

In additional experiments we evaluated the presynaptic component of post-tetanic potentiation (PTP) and paired-pulse facilitation (PPF) in the presence of the NMDA receptor antagonist (2R)-amino-5-phosphonovaleric acid (APV; 50 μM) applied for 45 minutes before recordings. For PTP, we delivered three 10-burst trains similar to those used to produce LTP. For PPF, paired pulses were delivered with varying time intervals (10, 20, 30, 40, 50, 100, 200, 500, and 1000 ms), and the percentage of the synaptic response of the second stimulus against the first delivered stimulus was recorded. All the recordings were performed and analyzed using pClamp10.

Patch-clamp recordings

Brain slices were prepared as previously described (Aceto et al., 2022), with minor modifications. Slices (350 μm thick) were cut on a vibratome (VT1200S; Leica Microsystems, Germany) and immediately transferred to an incubation chamber held at 32°C and filled with a recovery solution. After 30 min, slices were transferred to a second incubation chamber held at 32°C and filled with ACSF containing (in mM): 124 NaCl, 3.2 KCl, 1.2 NaH2PO4, 1 MgCl2, 2 CaCl2 2, 26 NaHCO3 and 10 glucose, pH 7.4. Slices were equilibrated at RT for at least 45 min and then transferred to a submerged recording chamber constantly

perfused with heated ACSF (32°C). Slices were constantly bubbled with 95% O2/5% CO2. Neurons of the CA1 area were visualized under DIC infrared illumination. Stimulation of the SC was obtained by means of a current stimulus isolator (WPI, Worcester, MA, USA), connected to a bipolar concentric stimulating electrode (FHC, Bowdoin, ME, USA) which was positioned in contact with the SC pathway. Patch pipettes had a resistance of 4–6 MΩ when filled with an internal solution containing (in mM): 145 K-gluconate, 2 MgCl2, 10 HEPES, 0.1 EGTA, 2.5 Na-ATP, 0.25 Na-GTP, 5 phosphocreatine, pH adjusted to 7.2 with KOH. For AMPA/NMDA ratio experiments, the internal solution contained (in mM): 135 CsCH3SO3, 10 HEPES, 8 NaCl, 0.25 EGTA, 2 MgCl2, 4 Mg-ATP, 0.3 Na-GTP, 5 phosphocreatine, pH adjusted to 7.3 with NaOH. After establishing a gigaseal, the patch was broken by applying negative pressure to achieve a whole-cell configuration. A series resistance lower than 15 MΩ was considered acceptable and monitored constantly throughout the entire recording. For evoked and spontaneous Excitatory Post-synaptic Currents (EPSC) measurements, neurons were held at −70 mV and electrical stimuli were delivered to the SC. To obtain the AMPA/NMDA currents ratio, stimuli of identical amplitude

were delivered at holding potentials of −70 and +40 mV, as described (Ahmad et al., 2012), with a frequency of 0.05 Hz; 50 μM picrotoxin (PTX) was added to the bath. The identity of evoked EPSCs was confirmed at the end of the recordings by adding 10 μM of the selective AMPA receptor blocker 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX) to the bath. For miniature EPSCs (mEPSCs) measurements, 0.5 μM tetrodotoxin and 50 μM PTX were applied to the bath. Recordings were performed in cells from slices incubated (10-20 min) with either vehicle or NGB-2904 (1 μM). Recordings were performed using a Multiclamp 700B/Digidata 1550A system (Molecular Devices, Sunnyvale, CA, USA) and digitized at a 10,000 Hz sampling frequency. All the electrophysiological recordings were analyzed using the Clampfit 10.6 software (Molecular Devices). AMPA receptor-mediated EPSC amplitude was calculated as the difference between the peak response and the baseline. NMDA-receptor-mediated EPSC amplitude was calculated as the amplitude 50 ms (Ahmad et al., 2012) after the response onset. For mEPSC frequency analysis, a template was constructed using the “Event detection/create template” function, as described (Leggio et al., 2019). Then, mEPSCs were detected using the “Event

detection/template search" function and the result inspected for false positives. For mEPSC amplitude analysis, all the waveforms detected during a single recording using template analysis were averaged and the amplitude calculated.

Electron microscopy

Animals and tissue preparation

Three 8-month-old, three 21-month-old male C57BL/6 mice, and four 7-8-month-old mice (two WT and two D3-KO) mice were anesthetized with an intraperitoneal injection of chloral hydrate (300 mg/kg) and perfused transcardially with a flush of saline solution, followed by 4% freshly depolymerized paraformaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer (PB; pH 7.4). Brains were removed, post-fixed in the same fixative (for 48 hrs) and cut on a Vibratome in 50 μm serial parasagittal sections, which were collected in PB until processing.

Immunoperoxidase and pre-embedding procedures

Sections were pre-treated with sodium borohydride for 20 min to prevent non-specific binding, treated with H2O2 (1% in PB; 30 min) to remove endogenous peroxidase activity, rinsed in PB and pre-incubated in 10% normal donkey serum (NDS, 1 hr).

According to (Requie et al., 2022), sections were then incubated in a solution containing anti-D3 primary antibodies (1:100; ADR-003, RRID:AB_2039830; Alomone, rabbit primary antibodies raised against a synthetic peptide corresponding to AA 15-29 within the extracellular N-terminus sequence of rat D3 receptor validated by the lack of immunoreactivity in D3 knock-out mice; Castro et al., 2015; Figure S1); 2 hr at room temperature [RT] and overnight at 4°C. The following day, sections were rinsed 3 times in PB and incubated first in 10% NDS (15 min) and then in a solution containing secondary biotinylated secondary antibodies (1:300; Jackson; 1.5 hr at RT). Sections were subsequently rinsed in PB, incubated in avidin-biotin peroxidase complex (ABC Elite PK6100, Vector), washed several times in PB, and incubated in 3,3'diaminobenzidine tetrahydrochloride (DAB; 0.05% in 0.05 M Tris buffer, pH 7.6 with 0.03% H2O2). Method specificity was verified by substituting primary antibodies with PB. As previously described (Melone et al., 2019) after completion of immunoperoxidase procedures, sections were post-fixed in 1% osmium tetroxide in PB for 45 m and contrasted with 1% uranyl acetate in maleate buffer (pH 6.0; 1 h). After dehydration in ethanol and propylene oxide, sections were embedded in

Epon/Spurr resin (Electron Microscopy Sciences, Hatfield, PA, USA), flattened between Aclar sheets (Electron Microscopy Sciences) and polymerized at 60°C for (48 hrs). Chips including sr of CA1 were selected by light-microscopic inspection, glued to blank epoxy and sectioned with an ultramicrotome (MTX; Research and Manufactoring Company Inc., Tucson, AZ, USA). The most superficial ultrathin sections (~60 nm) were collected and mounted on 300 mesh nickel grids, stained with Sato's lead and examined with a Philips EM 208 and CM100 electron microscopes coupled to a MegaView-II high resolution CCD camera (Soft Imaging System). To minimize the effects of procedural variables, all material from 8 and 21 mice was processed in parallel.

Electron microscopy of hippocampal slices

Hippocampal slices (n = 2 for each condition from 8 old animals) were immersed (within ~ 30 s after 120 min of electrophysiological recording) in a solution containing 4% paraformaldehyde and 0.5% glutaraldehyde in phosphate buffer (PB) and then stored for 12 weeks at 4°C in the same fixative solution. Next, slices were embedded procedure according to previous studies (Gulisano et al., 2019; Melone et al., 2019) and the protocol applied for pre-embedding procedures described

above. A small block of slices containing CA1 sr was selected and sectioned with an ultramicrotome; 18-20 ultrathin sections (~60 nm) for each CA1 block were mounted on 200 mesh copper grids, contrasted, and examined.

Data collection and electron microscopical analysis of D3 pre-embedded material

All data were obtained from sr CA1 of immunoreacted sections. D3 immunoreactive profiles were studied in ultrathin sections from the surface of the embedded blocks. Quantitative data derived from the analysis of microscopic fields (10–12 ultrathin sections/animal) selected and captured at original magnifications of 25,000x-30,000x. Microscopical fields containing positive processes were randomly selected. Acquisition of microscopical fields of 8- and 21-months-old mice was performed under blinded conditions (167 fields/3 mice from 8-month-old animals and 173 fields/3 mice from 21-month-old mice). For the analysis of the density of D3 positive profiles (characterized by the presence of immunoreactive electron dense products), subcellular compartments were identified according to well-established criteria (Peters et al., 1991). For quantifying D3 distribution at asymmetric synapses, the identification of synaptic domains was based according to

previous studies (DeFelipe et al., 1999; Requie et al., 2022; Melone et al., 2019). Pre-synaptic axon terminals were characterized by the presence of clear and round vesicles nearby the pre-synaptic density (active zone) associated with a synaptic cleft displaying electrodense material, post-synaptic elements showed a prominent post-synaptic density typical of the asymmetric synapses (PSD), and astrocytic profiles in close relationship with neuronal synaptic elements, were identified based on their typical irregular outlines and the paucity of cytoplasmic components (with the exception of ribosomes, glycogen granules and various fibrils).

Data collection and electron microscopical analysis of slices

Electron microscopy was performed on hippocampal slices from aged WT treated with three TBS, with NGB and three TBS, and on hippocampal slices from aged D3KO mice treated with three TBS. Quantitative analysis of vesicle pool, number of docked vesicles, area of spines, length of the post-synaptic density (PSD), and the proportion of perforated synapses was carried out as described (Babits et al., 2016; Bourne et al., 2013; Gulisano et al., 2019). Randomly selected electron microscopical fields of the sr (WT+3TBS n = 99, WT+NGB+3TBS n = 100, D3KO+3TBS n = 99) with at least an identifiable axo-spinous

synapse were acquired at original magnification of 44,000x-46,000x.

Sr axo-spinous synapses were identified according to the criteria used for D3-prembedding studies described above. The distinction between vesicle pools (which comprise the reserve vesicle pool) and docked vesicles (which are thought to be part of the readily releasable pool), was performed as described (Bourne et al., 2013; Gulisano et al., 2019). Briefly, vesicle pools were determined by counting the total number of small vesicles (∼50 nm) per terminal; the docked vesicles pool by identifying and counting the vesicles touching the membrane of the pre-synaptic active zone. Spines profile area and PSD length were measured by Image J software tools measurement (Babits et al., 2016; Gulisano et al., 2019). Spine profiles and PSD were traced along the membranes and measured; PSD length corresponded to the distance between the edges of PSD. Perforated synapses were identified based on the presence of a discontinuous PSD (Babits et al., 2016). Ultrathin sections and microscopical features of axo-spinous synapses were examined and analyzed blinded.

Behavioral studies

Hippocampal cannulas implantation

Mice underwent stereotaxic surgery for intrahippocampal cannulas implantation. After anesthesia with tiletamine + zolazepam (60 mg/kg) and medetomidine (40 μg/kg), mice were implanted with a 26-gauge guide cannula into the dorsal part of the hippocampi (coordinates from bregma: posterior = 2.46 mm, lateral = 1.50 mm to a depth of 1.30 mm). The cannulas were fixed to the skull with acrylic dental cement (RelyXTM Unicem, 3M), and mice were allowed to recover for a minimum of 6-8 days. Fifteen minutes before the training phase (T1) of the novel object recognition test, mice were bilaterally infused with NGB-2904 (1 μM) in a final volume of 1 μl over 1 min, using a microsyringe connected to the cannulas via polyethylene tubing. During infusion, animals were handled gently to minimize stress. After infusion, the needle was left in place for another minute to allow diffusion. In some animals, after behavioral studies, a solution of 4% methylene blue was infused for localization of infusion cannulas (Tropea et al., 2022a).

Open field

Open Field was performed in an apparatus of our design and construction, consisting of an arena (45 × 45 × 40 cm) made by white matte polymethyl methacrylate non-reflective panels illuminated by a perpendicular light source located 65 cm from the floor (Tropea et al., 2022b). A webcam, connected to the computer, was fixed on the top of the apparatus. Each mouse was placed in the arena and allowed to freely explore for 5 min. We scored the percentage of time spent in the center and the number of entries into the center.

Novel Object Recognition and Location

Novel Object Recognition (NOR) and Novel Object Location (NOL) tests were performed as previously described (Gulisano et al., 2019; Tropea et al., 2021) using the same apparatus utilized for open field experiments. After 5 days of habituation to the arena, objects, and i.p. injections, mice underwent the training session (T1), and after 24 hrs, the testing session (T2) was conducted to assess memory retention. We used two different protocols: i) to assess whether D3R blockade enhanced cognition in young mice we employed a “short protocol” (T1 = 3 minutes, T2 = 5 minutes), which is known to induce short-term memory that typically fades after about 6-8 hours (Blokland and

Sesia, 2023). Consequently, when testing memory after 24 hours, the animals normally cannot distinguish between the familiar objects presented during T1 and the new object in T2; ii) to evaluate long-term memory, we used a “long protocol” (T1 = 10 minutes, T2 = 10 minutes) since the longer exposure during training allows for memory retention that can be assessed after 24 hours. The “long protocol” has been utilized to determine whether D3R blockade could restore memory in old animals or further enhance memory formation in young mice. In both protocols, during T1, mice were placed in the arena and allowed to explore two identical objects. In T2, for NOR, mice were presented with two different objects, a "familiar" one (i.e., the one used in T1) and a "novel" one; for NOL, mice were presented with an object located as in T1, and the other in a different location. For both tests, animal exploration - defined as the mouse pointing its nose toward the object from a distance not > 2 cm - was measured in T2. We analyzed discrimination (D) index, “exploration of novel object minus exploration of familiar object/total exploration time”, and total exploration time. Mice with a total exploration time < 5 s were excluded from analysis.

Morris Water Maze

Morris Water Maze was performed as previously described (Gulisano et al., 2018) in a plastic maze filled with water (25°C) made opaque by the addition of nontoxic white paint. The submerged platform was located in the south-west quadrant and spatial cues were placed on the 4 cardinal points of the maze. Spatial learning was assessed over 5 days (2 daily sessions, 3 trials of 1 min each). Mice started from a randomly chosen quadrant, and the time taken to reach the hidden submerged platform (latency) was recorded. The 6th day, to evaluate reference memory retention, the probe test was performed (one session, 4 trials of 1 min each). The maze was divided into 4 quadrants (target quadrant [TQ] previously containing the platform, adjacent left, adjacent right, and opposite quadrant) and the percent of time spent in each quadrant was recorded and analyzed with a video tracking system (Netsense srl, Catania, Italy). Visual, motor, and motivation skills were assessed by measuring the time taken to reach a visible platform randomly positioned in different places and marked with a green flag (2 daily sessions, 3 trials of 1 min each). For the analyses, results from trials performed in each session were averaged.

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SUPPLEMENTARY FIGURES

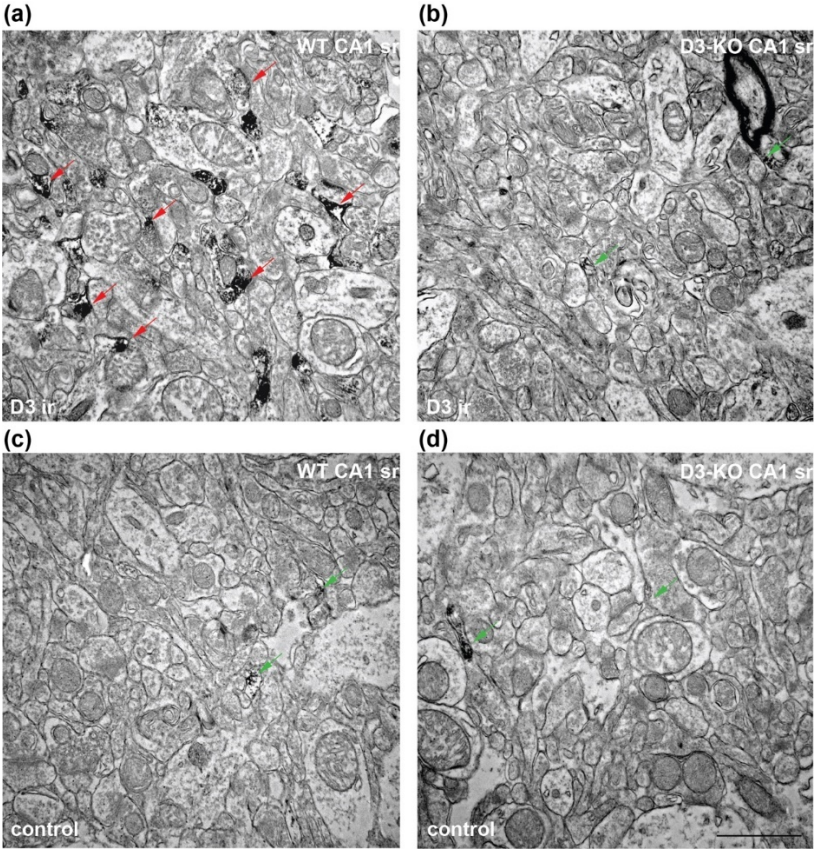

FIGURE S1 – Specificity of D3 immunoreactivity in CA1 stratum radiatum. (a) Electron microscopy of pre-embedded immunoreacted ultrathin sections revealed D3 immunoreactivity in slices from WT (red arrows) but not **(b)** D3-KO mice. Sporadic electron-dense products (green arrows) dispersed in the neuropil processes were found in D3-KO and **(c-d)** control sections reacted in parallel with the omission of D3 primary antibody. Tissue preparation, immunoperoxidase processes with the D3 primary antibody, (ADR-003, RRID:AB_2039830; Alomone), and pre-embedding methods were described in Material and Methods. Scale bar: 1 μm.

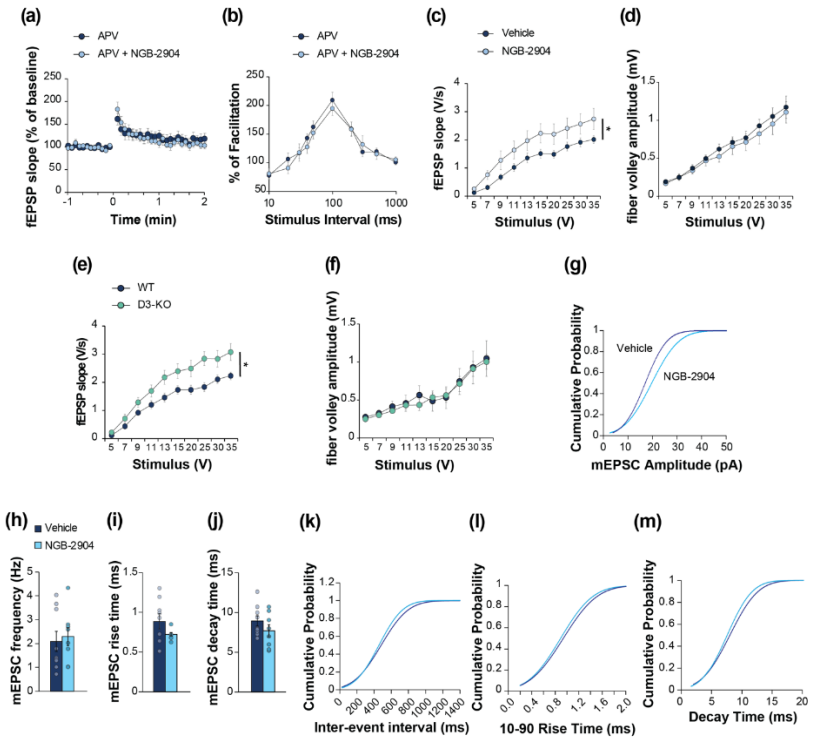

FIGURE S2 – D3R blockade did not affect presynaptic forms of hippocampal synaptic transmission and plasticity. (a) Perfusion of hippocampal slices with the D3R antagonist NGB-2904 (1 μM) for 15 min before tetanus did not modify post-tetanic potentiation ($F(1,20) = 0.11$, $p = 0.743$, $n = 10/12$). **(b)** Perfusion with NGB-2904 in the presence of the NMDA receptor antagonist (2R)-amino-5-phosphonovaleric acid (APV; 50 μM) did not modify paired pulse facilitation ($F(1,19) = 0.434$, $p = 0.518$, $n = 11/10$). **(c)** Basal synaptic transmission (BST) shown in Figure 1C plotted as fEPSP slope and **(d)** fiber volley amplitude versus stimulus intensity in NGB-

2904- and vehicle-treated slices. **(e)** BST shown in Figure 1D plotted as fEPSP slope and **(f)** fiber volley amplitude in slices from D3-KO and WT animals. **(g)** Cumulative frequency distributions for mEPSC amplitude in the experimental conditions shown in Figure 1f. **(h)** Bar Graph showing that NGB-2904 did not affect mEPSC frequency ($t(15) = 0.354$, $p = 0.727$), **(i)** rise time ($t(15) = 1.556$, $p = 0.140$) and **(j)** decay time ($t(15) = 0.244$, $p = 0.232$). **(k)** Cumulative probability graphs for mEPSC inter-invent intervals, **(l)** rise time and **(m)** decay time. Data expressed as mean ± SEM. *$p < 0.05$.

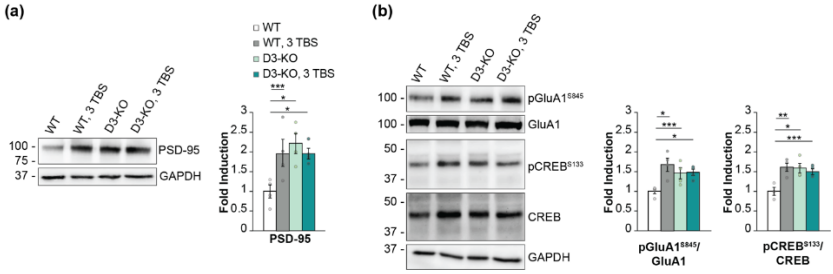

FIGURE S3 – Expression of post-synaptic proteins PSD-95, pGluA1 and pCREB in slices from D3-KO mice that underwent 3 TBS. (a) Representative WB images of PSD-95 (cropped images based on MW) performed in lysates from slices treated for electrophysiology stored at 1 min after 3 TBS tetanus (n = 3 slices/lane). GAPDH was used as loading control. On the right, bar graph showing the increase of PSD-95 expression in D3-KO slices in basal conditions ($p = 0.046$) and after 3 TBS ($p = 0.012$) as well as in WT slices after 3 TBS ($p < 0.001$). **(b)** pGluA1 and pCREB expression increased in D3-KO slices in basal conditions ($p < 0.001$; $p = 0.046$) and after 3 TBS ($p = 0.012$; $p = 0.046$) as well as in WT slices after 3 TBS ($p = 0.016$; $p = 0.002$). Data expressed as mean ± SEM. *p< 0.05; ***p< 0.01; ***p< 0.001.

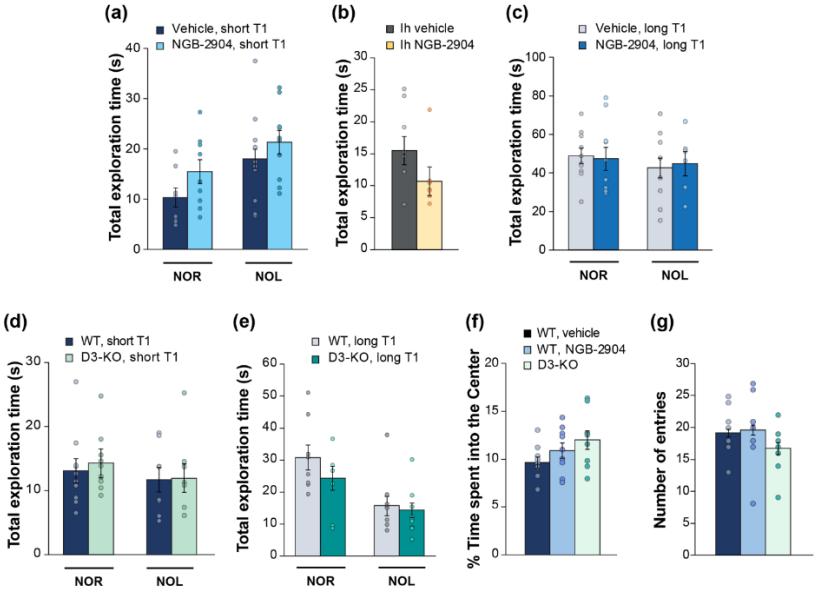

FIGURE S4 – D3R blockade or genetic deletion did not modify exploratory behavior. (a) Treatment with NGB-2904 (3 mg/kg, via i.p.) 20 min before a short training (T1) did not modify total exploration time in NOR ($t(15) = 1.659$, $p = 0.118$) or NOL tests ($t(18) = 0.884$, $p = 0.389$). **(b)** Intrahippocampal injections of NGB-2904 did not affect total exploration time ($t(13) = 1.745$, $p = 0.105$). **(c)** Treatment with NGB-2904 did not modify total exploration time in mice that underwent a long T1 (NOR: $t(21) = 0.137$, $p = 0.893$; NOL: $t(12) = 0.271$, $p = 0.791$). **(d)** Total exploration time was unchanged in D3-KO mice after a short T1 (NOR: $t(18) = 0.499$, $p = 0.623$; NOL: $t(14) = 0.078$, $p = 0.939$) or **(e)** a long T1 (NOR: $t(14) = 1.190$, $p = 0.254$; NOL: $t(17) = 0.360$, $p = 0.723$). **(f)** Open field showed no differences in WT treated with vehicle or NGB-2904 and D3-KO mice ($n = 9$ for each condition) testing in the percentage of time spent in the center compartment ($F(2,24) = 2.224$, $p = 0.13$) and **(g)** the number of entries into the center compartment ($F(2,24) = 1.114$, $p = 0.345$). Data expressed as mean ± SEM.

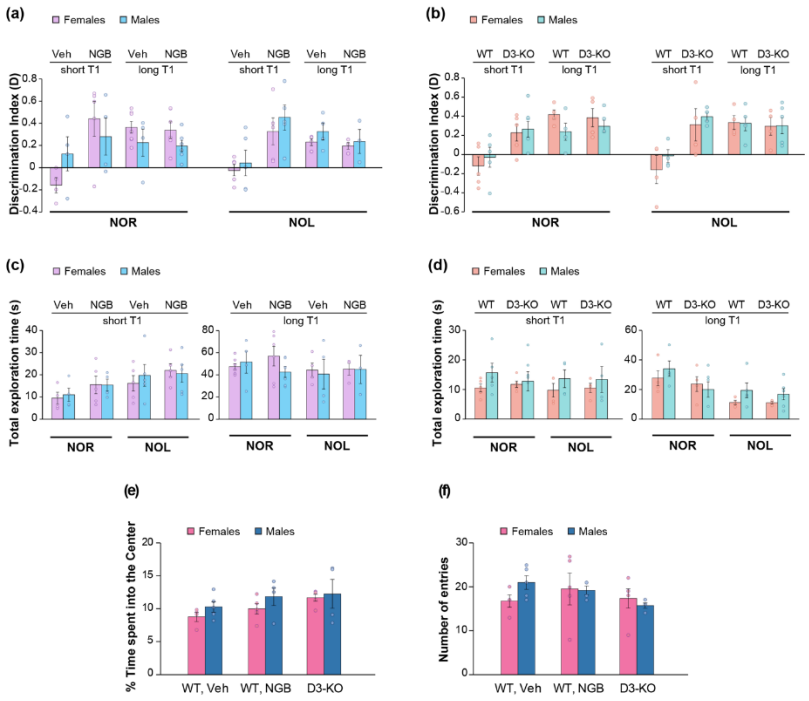

FIGURE S5 – Sex did not influence the effects of D3R blockade in behavioral studies in adult mice. (a) No sex differences were detected in discrimination index evaluated by NOR or NOL in vehicle- and NGB-2904-treated young mice that underwent a short or a long T1 (NOR short: vehicle $t(6) = 1.68$, $p = 0.144$, n = 4F/4M; NGB-2904: $t(7) = 1.69$, $p = 0.509$, n = 5F/4M; NOR long: vehicle $t(9) = 1.21$, $p = 0.256$, n = 7F/4M; NGB-2904: $t(10) = 1.69$, $p = 0.120$, n = 6F/6M; NOL short: vehicle $t(8) = 0.51$, $p = 0.622$, n = 5F/5M; NGB-2904: $t(8) = 0.74$, $p = 0.475$, n = 5F/5M; NOL long: vehicle $t(6) = 1.16$, $p = 0.289$, n = 4F/4M; NGB-2904: $t(4) = 0.35$, $p = 0.743$, n = 3F/3M; data analyses from Figure 3a). **(b)** No sex differences were detected in discrimination index evaluated by NOR or NOL in WT and D3-KO young mice that underwent a short or a long T1 (NOR short: WT $t(8) = 0.64$, $p =$

0.536, n = 5F/5M; D3-KO: t(8) = 0.58, p = 0.573, n = 5F/5M; NOR long: WT t(6) = 1.61, p = 0.159, n = 4F/4M; D3-KO: t(6) = 0.35, p = 0.736, n = 4F/4M; NOL short: WT t(6) = 0.89, p = 0.404, n = 4F/4M; D3-KO: t(6) = 0.48, p = 0.643, n = 4F/4M; NOL long: WT t(7) = 0.1, p = 0.918, n = 4F/5M; D3-KO: t(8) = 0.05, p = 0.961, n = 4F/6M; from Figure 3c). **(c)** No sex differences were detected in total exploration time evaluated during NOR or NOL in vehicle- and NGB-2904-treated young mice that underwent a short T1 (NOR: vehicle t(6) = 0.36, p = 0.725; NGB-2904: t(7) = 0.01, p = 0.99; NOL: vehicle t(8) = 0.55, p = 0.594; NGB-2904: t(8) = 0.31, p = 0.760; from Figure S4a) or a long T1 (NOR: vehicle t(9) = 0.51, p = 0.619; NGB-2904: t(10) = 1.42, p = 0.185; NOL: vehicle t(6) = 0.23, p = 0.823; NGB-2904: t(4) = 0.02, p = 0.981; from Figure S4c). **(d)** No sex differences were detected in total exploration time evaluated during NOR or NOL in WT and D3-KO young mice that underwent a short T1 (NOR: WT t(8) = 1.46, p = 0.183; D3-KO: t(8) = 2.01, p = 0.080; NOL: WT t(6) = 1.03, p = 0.341; D3-KO: t(6) = 0.608, p = 0.566; from Figure S4d) or a long T1 (NOR: WT t(6) = 0.77, p = 0.467; D3-KO: t(6) = 0.15, p = 0.880; NOL: WT t(7) = 1.42, p = 0.197; D3-KO: t(8) = 1.21, p = 0.259; from Figure S4e). **(e)** No sex differences were detected in the OF results testing the time spent in center of the arena (WT, vehicle: t(7) = 1.38, p = 0.210, n = 4F/5M; WT, NGB-2904: t(7) = 1.25, p = 0.250, n = 5F/4M; D3-KO: t(7) = 0.26, p = 0.8, n = 5F/4M; from Figure S4f) and **(f)** the number of entries into the center (WT, vehicle: t(7) = 1.93, p = 0.09; WT, NGB-2904: t(7) = 0.13, p = 0.894; D3-KO: t(7) = 0.64, p = 0.540; from Figure S4g). Data expressed as mean ± SEM. Veh = Vehicle; NGB = NGB-2904.

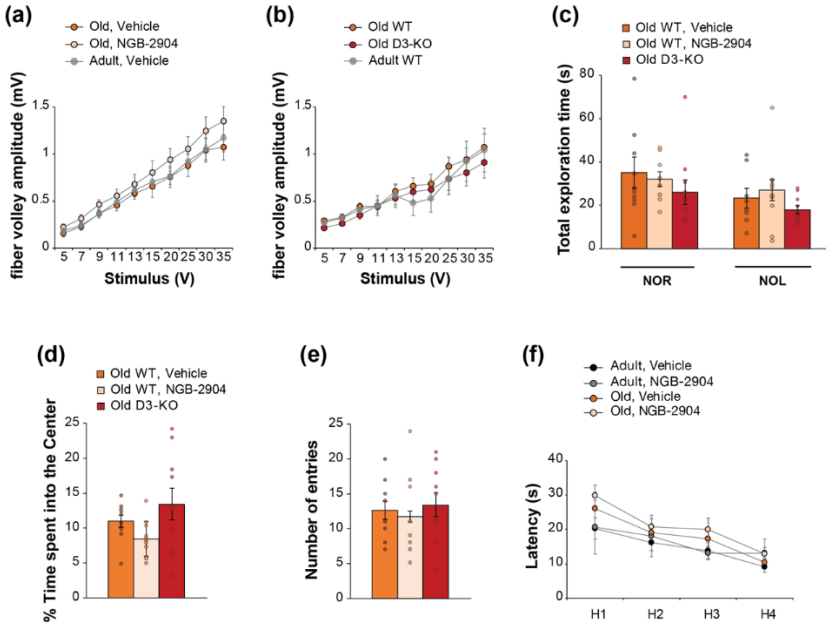

FIGURE S6 – D3R blockade or genetic deletion did not modify presynaptic transmission, exploratory behavior and mobility in aged mice. (a) No differences were found in the afferent volley amplitude assessed during basal synaptic transmission recordings between adult and old mice treated with NGB-2904 or vehicle ($F(2,30) = 0.944$, $p = 0.4$). **(b)** Afferent volley was unchanged among adult WT, aged WT or aged D3-KO mice ($F(2,28) = 0.217$, $p = 0.806$). **(c)** Total exploration time was not different among adult WT, aged WT or aged D3-KO mice in NOR ($F(2,26) = 0.57$, $p = 0.572$) and NOL ($F(2,25) = 1.177$, $p = 0.325$). **(d)** Open field test showed no differences in the three groups of mice ($n = 10/11/10$) in the time spent in the center of the arena ($F(2,28) = 3.069$, $p = 0.062$) and **(e)** in the number of entries into the center ($F(2,28) = 0.274$, $p = 0.762$). **(f)** No differences were found between adult and old mice treated with NGB-2904 or vehicle in the latency to reach the platform tested at the visible platform trial ($F(3,33) = 1.856$, $p = 0.156$). Data expressed as mean ± SEM.

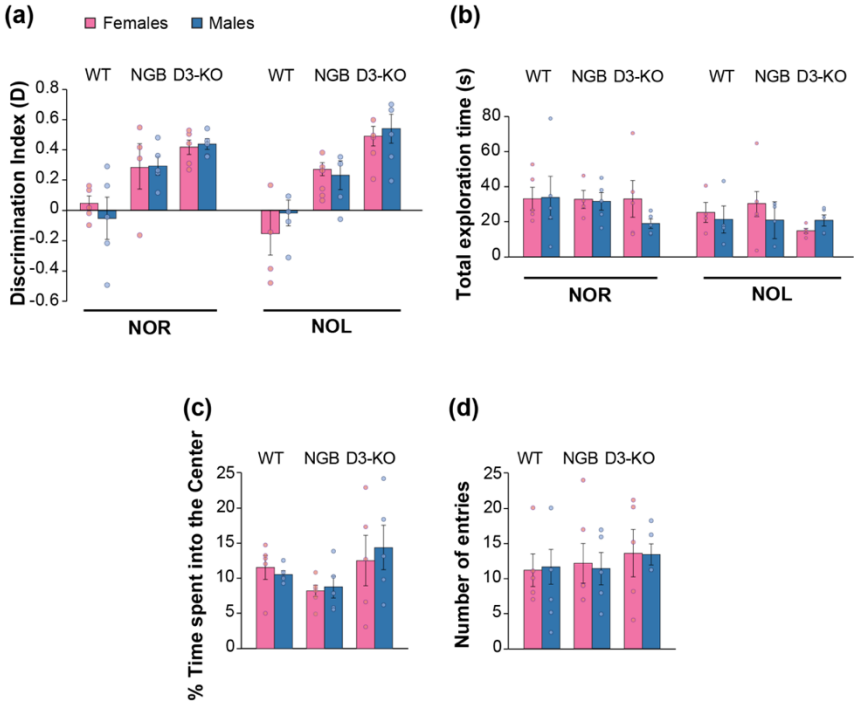

FIGURE S7 – Sex did not influence the effects of D3R blockade in behavioral studies in old mice. (a) No sex differences were detected in the discrimination index evaluated by NOR or NOL tests in vehicle- and NGB-2904-treated WT mice, as well as in D3-KO animals (NOR: WT $t(8) = 0.67$, $p = 0.519$, n = 5F/5M; NGB-2904: $t(7) = 0.07$, $p = 0.944$, n = 4F/5M; D3-KO: $t(8) = 0.33$, $p = 0.751$, n = 5F/5M; NOL: WT $t(6) = 0.8$, $p = 0.452$, n = 4F/4M; NGB-2904: $t(9) = 0.45$, $p = 0.662$, n = 7F/4M; D3-KO: $t(8) = 0.43$, $p = 0.678$, n = 5F/5M; data analyses from Figure 5e). **(b)** No sex differences were detected in total exploration time evaluated during NOR or NOL tests in vehicle- and NGB-2904-treated WT mice, as well as in D3-KO animals (NOR: WT $t(8) = 0.02$, $p = 0.984$; NGB-2904: $t(7) = 0.15$, $p = 0.879$; D3-KO: $t(8) = 1.31$, $p = 0.226$; NOL: WT $t(6) = 0.4$, $p = 0.699$; NGB-2904: $t(9) = 0.92$, $p = 0.378$; D3-KO: $t(8) = 1.72$, $p = 0.124$; data analyses from Figure S6c). **(c)** No sex differences were detected in the OF results testing the time

spent in center of the arena (WT, vehicle: $t(8) = 0.57$, $p = 0.579$, n = 5F/5M; WT, NGB-2904: $t(9) = 0.33$, $p = 0.745$, n = 6F/5M; D3-KO: $t(8) = 0.38$, $p = 0.710$, n = 5F/5M; from Figure S6d) and **(d)** the number of entries into the center (WT, vehicle: $t(8) = 1.19$, $p = 0.266$; WT, NGB-2904: $t(9) = 0.2$, $p = 0.842$; D3-KO: $t(8) = 0.05$, $p = 0.958$; from Figure S6e). Data expressed as mean ± SEM.

Experimental Condition	**WT+3T**	**WT+3T+NGB**	**D3-KO+3T**	**Statistical analyses** (P value)		
Number of asymmetric synapses (n) from 2 slices	102	103	104			
				WT+3T *vs* WT+3T+NGB	**WT+3T *vs* D3-KO+3T**	**WT+3T+NGB *vs* D3-KO+3T**
Vesicle pool (n)	57.45 ± 1.87	58.03 ± 2.01	61.87 ± 2.46	P > 0.99	P > 0.99	P > 0.99
Docked vesicles (n)	2.01 ± 0.08	2.22 ± 0.08	2.27 ± 0.09	P = 0.33	P = 0.16	P > 0.99
Area of spines (μm^2)	0.14 ± 0.01	0.13 ± 0.008	0.13 ± 0.009	P > 0.99	P > 0.99	P > 0.99
PSD length (µm)	0.191 ± 0.004	0.241 ± 0.005	0.217 ± 0.004	**P < 0.0001**	**P = 0.0006**	**P = 0.0048**
Perforated PSD	4.91 ± 2.14%	7.76 ± 2.65%	7.69 ± 2.62%	P > 0.99	P > 0.99	P > 0.99

TABLE S1 – Detailed results of electron microscopy performed on hippocampal slices recorded in the following experimental conditions: aged WT + 3 TBS (WT+3T), aged WT + 3 TBS in the presence of NGB-2904 (WT+3T+NGB), aged D3-KO + 3 TBS (D3-KO+3T). Statistical analyses performed by non-parametric Kruskal-Wallis test with Dunn's test for multiple comparison. 3 TBS = strong tetanic stimulation. Number of microscopical fields: WT+3T (n = 99); WT+3T+NGB (n = 100); D3-KO+3T (n = 99). For all parameters values are mean ± SEM.

Chapter 2: Optimizing behavioral paradigms to assess hippocampal-dependent memory in mouse models

INTRODUCTION

Rodent behavioral tests represent a cornerstone of preclinical neuroscience research, providing essential insights into the neural mechanisms of cognition and their alterations in neurological and psychiatric disorders. Over time, a wide variety of behavioral paradigms have been developed, and today more than 100 different tasks are commonly used to assess domains such as motor coordination, anxiety- and depressive-like behaviors, social interaction, reward-related processes, and cognitive functions, including learning and memory (Hånell & Marklund, 2014; Teegarden, 2012).

The hippocampus, a central component of the limbic system, plays a key role in declarative memory processes such as episodic and spatial memory (Eichenbaum, 2000). It is crucial for the consolidation of short-term information into long-term memory through dynamic interactions with cortical regions. Importantly, the hippocampus is one of the first structures to undergo functional and structural decline during aging and in

neurodegenerative diseases such as Alzheimer's disease (Rao et al., 2022). For these reasons, hippocampus-dependent tasks, such as those assessing spatial navigation or recognition memory, remain among the most widely employed paradigms in preclinical research.

Despite their widespread use, behavioral studies are notoriously sensitive to a variety of genetic and environmental factors, which can compromise reproducibility across laboratories (Crabbe et al., 1999; Lewejohann et al., 2006). Sources of variability include differences in mouse strain, housing conditions, enrichment, handling methods, and even experimenter identity or odor cues (Sousa et al., 2006; Gouveia & Hurst, 2017). Experimental settings also play a major role: illumination intensity, noise, or testing room characteristics can significantly influence performance, particularly in mice, which are nocturnal animals highly sensitive to environmental changes (Martin-Arenas & Pintado, 2014; Lauer et al., 2009). If these factors are not carefully controlled and standardized, results may be confounded by stress, anxiety, or altered baseline activity rather than reflecting genuine cognitive processes. Nevertheless, when properly conducted, behavioral tests can achieve

replicability comparable to other biological methods (Crawley, 2008).

In this chapter, I will focus on the methodological refinement of behavioral tests designed to investigate hippocampal memory in rodents. Particular attention will be given to the identification and control of experimental variables that critically influence task performance, as well as to the optimization of protocols for improved sensitivity and reproducibility. This methodological study represented an important component of the work carried out during my PhD program and enabled the refinement of the novel object recognition, novel object location, and Morris water maze tasks, which were subsequently employed in the research described in Chapter 1. By addressing these methodological challenges, behavioral paradigms can more reliably capture hippocampal-dependent learning and memory processes, thereby enhancing their translational value for the study of pharmacological mechanisms and cognitive dysfunction in aging and neurodegenerative diseases.

NOVEL OBJECT RECOGNITION AND NOVEL OBJECT LOCATION

The Novel Object Recognition (NOR) and Novel Object Location (NOL) tasks are among the most widely used behavioral paradigms in mice. They are relatively simple to perform, highly effective, and provide robust measures of hippocampus-dependent spatial memory (Vogel-Ciernia & Wood, 2014). Importantly, these tests exploit the rodent innate tendency to explore novelty, making them non-invasive and free of reinforcement or aversive stimuli, which increases their translational value and reduces stress-related confounds.

The NOR task was first described by Ennaceur and Delacour in 1988 in rats (Ennaceur & Delacour, 1988a) and has since been successfully adapted for mice (Akkerman et al., 2012; Antunes & Biala, 2012; Ennaceur, 2010; Leger et al., 2013; Lueptow et al., 2016; van Goethem et al., 2012).

Similarly, the NOL task was later developed by the same group as a test of spatial memory, using a protocol closely related to NOR (Ennaceur et al., 1997).

Methodology

The NOR task is based on the rodent natural tendency to preferentially explore novelty. The test consists of two sessions within the same context, separated by an intersession interval (ISI). In the first (familiarization) session, the animal is allowed to freely explore two identical objects. After a delay, one of the objects is replaced with a different one. In the second test session, the mouse is reintroduced to the arena and presented with both the familiar and the novel object. If memory of the familiar object is retained, the mouse will spend more time investigating the novel one.

The NOL task follows a similar structure, but here it is the spatial location of the objects, rather than their identity, that changes. During the familiarization session, the animal explores two identical objects placed in specific spatial relation to environmental cues. After the ISI, one of the objects is relocated to a different position. When reintroduced in the test session, if the animal remembers the original spatial arrangement, it will preferentially explore the displaced object.

Set Up

For the Novel Object Recognition (NOR) task, experiments were performed in a square arena (45 × 45 × 40 cm) constructed with four white matte polymethyl methacrylate non-reflective panels to avoid reflections (Figure 1). The floor of the arena was covered with a self-adhesive anti-reflective film, and illumination was provided by a perpendicular diffused light source positioned 65 cm above the floor. Based on preliminary studies, two 6 W light bulbs producing 80 lux at floor level were used. After each trial, the arena and objects were cleaned with 70% ethanol and dried with absorbent paper.

For the Novel Object Location (NOL) task, the same arena was used with one side made of transparent polymethyl methacrylate. This design allowed the display of a large green cross that served as a spatial reference point, facilitating orientation within the apparatus. The other technical parameters, including the anti-reflective floor and lighting, were identical to those used for NOR.

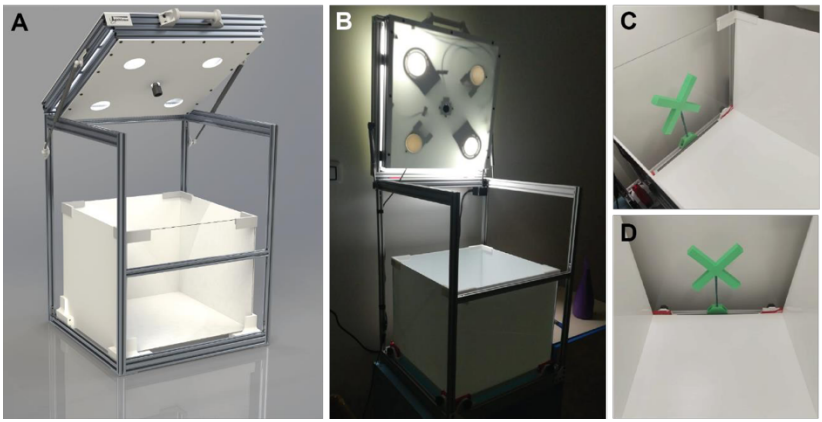

Figure 1 - Picture of the apparatus used in our laboratories. A-B) Rendered representation and real picture of the apparatus for Recognition memory. **C-D)** Pictures of the apparatus with spatial cue for NOL experiments.

In both tasks, mice underwent three days of habituation (5 min/day) prior to testing. During the training phase (T1), animals were placed in the arena and allowed to explore two identical objects positioned equidistantly from the perimeter and the center for a given amount of time. Twenty-four hours later, they were tested in the retention phase (T2). In NOR, one of the familiar objects was replaced with a novel one, so that in T2 animals encountered one familiar and one novel object. In NOL, both objects were identical to those used in T1, but one was relocated to a different position (e.g., the opposite corner of the arena) (Figure 2).

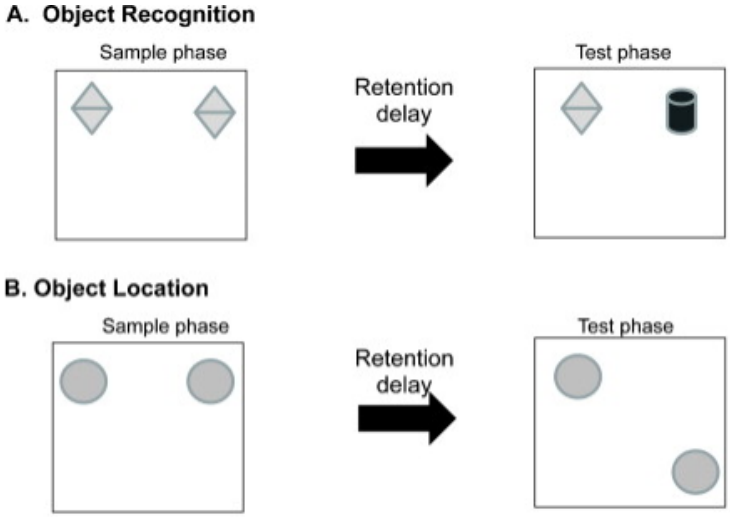

Figure 2 - Scheme of NOR and NOL procedure

In the study discussed in Chapter 1, both standard and modified protocols of the NOR and NOL tasks were applied to evaluate potential pro-cognitive effects of treatments. Two paradigms were used:

i) a modified short protocol, consisting of a 3-minute training phase (T1) and a 5-minute test phase (T2), with the retention interval extended to 24 hours. This adjustment was designed to assess whether treatments could promote persistence of memory by converting short-term into a long-term memory;

ii) a long protocol (T1 = 10 min; T2 = 10 min), classically used to assess long-term memory. This paradigm allows for the induction of a robust memory trace that remains detectable after 24 hours in mice with intact cognitive function, and was applied both to test whether memory formation could be further enhanced in young animals and to evaluate possible rescue effects in aged mice.

Data Analysis

For NOR, behavioral output was analyzed manually using computer software with stopwatch functionality. Exploration was defined as the time during which the animal directed its nose toward the object at a distance of ≤ 2 cm and/or touched it with the nose. Behaviors such as turning around or sitting on the object were not considered exploration (Ennaceur, 2010). To ensure reliability, only animals that spent at least 5 seconds in total object exploration during the test phase were included in the analysis, as shorter times may lead to inconsistent results when comparing exploration of familiar versus novel objects.

For NOL task, the same operational definition of exploration was applied.

Animals that did not reach the 5-second minimum exploration threshold were likewise excluded from the analysis to avoid unreliable discrimination between familiar and novel locations.

In both tasks, object exploration was quantified and analyzed on a personal computer. The main parameters calculated were:

Exploration levels = (Exploration time [novel object/location] × 100) / Total exploration time

NOR D.I. = (Exploration time [novel object – familiar object]) / Total exploration time

NOL D.I. = (Exploration time [novel location – familiar location]) / Total exploration time

Studied Memory

Examining in detail which brain structures support Object Location Memory (OLM) and Object Recognition Memory (ORM) is not trivial, as there is no full consensus in the scientific community. Overall, OLM and ORM appear to rely on distinct neural substrates. OLM depends critically on the hippocampus for encoding, consolidation, and retrieval (Mumby et al., 1999;

Haettig et al., 2011) and is particularly sensitive to manipulations of the dorsal CA1 region (Assini et al., 2009; Barrett et al., 2011; Vogel-Ciernia et al., 2013). In contrast, ORM engages a broader network of cortical areas, including the insular cortex (Bermudez-Rattoni et al., 2005; Balderas et al., 2008), perirhinal cortex (Winters, 2004, 2005; Balderas et al., 2008), and ventromedial prefrontal cortex (Akirav & Maroun, 2006).

The involvement of the hippocampus in ORM remains controversial. Its necessity seems to vary depending on the timing of hippocampal manipulations and the specific experimental conditions employed (Rossato et al., 2007; Balderas et al., 2008; Haettig et al., 2011; Davis et al., 2010).

These distinctions are also observed in humans. In the case of OLM, the medial temporal lobes, particularly the hippocampus, are crucial for object–location binding (Postma et al., 2008). By contrast, ORM performance in humans has been shown to rely on a broader set of regions. For example, deterioration of object memory scores has been reported in patients with lesions to the dorsolateral prefrontal cortex, posterior parietal cortex, and

hippocampus, especially in the right hemisphere (van Asselen et al., 2009).

Advantages

NOR is often preferred over NOL in behavioral studies, although both are widely used and share several advantages. These tasks are quick and easy to perform, require relatively few animals, and do not involve aversive procedures such as foot shocks or food deprivation. In fact, NOR has been shown to be much less stressful than other commonly used behavioral tests (Aguilar-Valles et al., 2006; Anisman et al., 2001; Hurst & West, 2010; Kim & Diamond, 2002; Leussis & Bolivar, 2006; Willner, 1997). Another advantage is efficiency: while tasks such as the Morris Water Maze (MWM) may require a week or longer to complete, NOR and NOL can be conducted within a much shorter timeframe.

Importantly, the conditions of the NOR task more closely resemble those used in studies of human cognition, thereby increasing its ecological validity compared to many other rodent memory paradigms. Because it is a simple visual recall task, NOR has also been successfully adapted for use in humans and

non-human primates, allowing cross-species investigation of declarative memory (Akkerman et al., 2012; Dere et al., 2007; Winters et al., 2008). Moreover, the same animals can be tested multiple times with this paradigm, provided that different objects are used, making it highly versatile. Finally, both NOR and NOL are cost-effective and can be implemented with relatively simple apparatuses.

Disadvantages

A number of critical parameters related to animal health and well-being must be considered when performing NOR and NOL tasks. Since these paradigms rely on rodents innate preference for novelty and exploration, any conditions that reduce exploratory activity can confound interpretation of the results. Such factors include stress, performance confounds, and natural differences related to strain or age (Vogel-Ciernia & Wood, 2014).

i) Animal stress. Environmental stressors within the testing room or animal facility can significantly alter task performance. To minimize stress, cages should not be changed during testing, outside odors and noise should be kept to a minimum, and

animals should be group-housed whenever possible, unless single housing is required for experimental reasons. Free access to food, water, and bedding must always be ensured. In addition, habituating mice to the testing environment is essential to reduce stress and promote natural exploratory behavior.

ii) Performance confounds. Before conducting NOR and NOL tasks, it is critical to ensure that any experimental manipulation does not impair motor or sensory functions, which could mimic memory deficits. Simple behavioral tests such as the open field can be used to assess locomotor activity and anxiety levels. In this assay, animals are placed in an empty arena under bright light, which induces anxiety; the more anxious the mice, the less time they spend in the center of the arena. This test can be performed in the same apparatus as NOR/NOL, avoiding the need for additional equipment. Furthermore, because rodents rely heavily on olfaction, it is essential to eliminate scent cues by cleaning the arena with 70% ethanol between trials.

iii) Mouse strain and age. Background strain and age are additional variables that influence performance. These protocols have been optimized for 8–12-week-old C57BL/6J mice, but strain differences in anxiety, visual discrimination, and

locomotor activity can significantly affect outcomes (Brooks et al., 2005).

iv) Spatial landmarks in NOL. In NOL, spatial landmarks are crucial to allow animals to orient in the environment and detect object displacement. To be effective, landmarks must be easily visible to the mouse, larger than its body size, but not so prominent as to distract attention away from the objects. For this reason, landmarks are best placed outside the arena, visible through a transparent panel, while the use of multiple reference points should be avoided, as they may paradoxically disorient the animal.

v) Limitations of exploration measures. A common limitation in both tasks is the assumption that differences in exploration directly reflect memory. In reality, exploration may also depend on other factors, such as intrinsic object preference. This is particularly relevant in NOR, where novel and familiar objects differ in identity, and animals may show biased interest independent of memory. In NOL, where objects remain identical and only their location changes, this confound is less pronounced.

Optimizations

Careful control of these parameters is essential to ensure that ORM and OLM performance reflects genuine memory processes rather than unrelated variables.

During my PhD program, I contributed to the validation of a novel customized apparatus designed to standardize the experimental context and improve the reproducibility of behavioral outcomes. The arena, described above (Fig. 2.1), is composed of detachable white panels that isolate the animal from external visual distractions, which can be replaced with transparent panels when spatial cues are required, as in NOL. This modular design provides flexibility, ensuring stable environmental conditions while allowing the introduction of landmarks when necessary. Importantly, the apparatus is open source, enabling reproduction at low cost and customization to meet specific experimental requirements. By controlling both lighting and contextual features, it supports more accurate and reproducible assessment of recognition and spatial memory tasks.

The choice of objects represents another crucial factor in the reliability of recognition tasks. Differences in exploration between familiar and novel objects may not necessarily reflect memory performance but rather an intrinsic preference for specific object features such as shape, color, size, or material. If, during the test phase, the novel object is inherently more attractive, animals may preferentially explore it regardless of memory. To minimize this bias, objects must be carefully selected. Previous studies have employed a wide range of shapes (pyramids, cubes, hemispheres, mugs, cans, Lego toys, PVC pipes, glass vases, candlesticks) and materials (glass, porcelain, metal, plastic, rubber, wood) (Antunes & Biala, 2012). Object size is also a critical parameter, as very small objects may be ignored, while oversized ones may alter exploration patterns. Typically, objects of comparable bulk to the animal are recommended to avoid affordance effects. Based on these considerations, I contributed to systematically assess object preference and discrimination prior to testing to prevent potential biases in the NOR task. We found that exploratory behavior is strongly influenced by object features, making systematic evaluation essential to avoid bias. Shiny materials (metal, glass), very small or very large objects, and shapes with

poor affordance reduced reliable exploration, while PLA objects of medium size and simple shapes (e.g., pyramids) proved most suitable (Tropea et al., 2022). Color also affected preference, with green objects consistently better explored. Overall, selecting objects with neutral and standardized properties is critical to ensure reliable outcomes in NOR and NOL tasks (Figure 3).

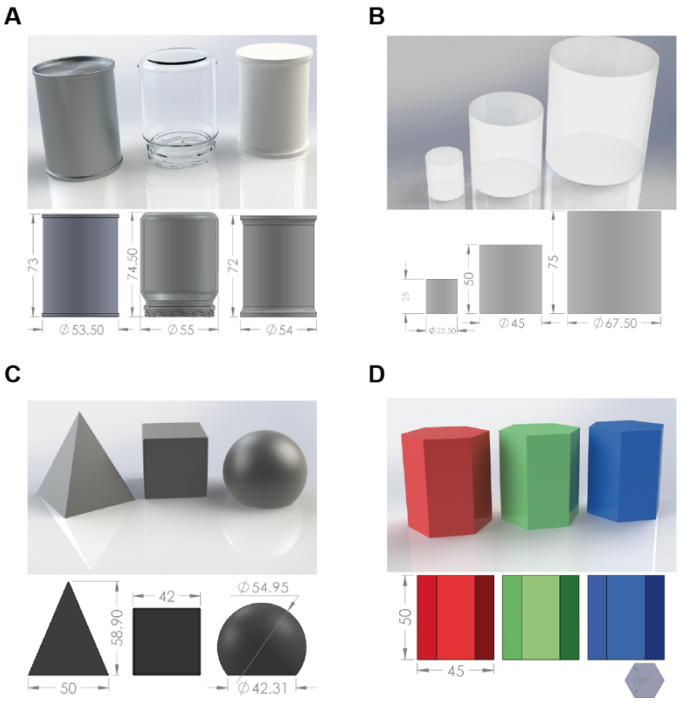

Figure 3 - Objects used to assess preference for material (A), dimension (B), shape (C) and color (D).

MORRIS WATER MAZE (MWM)

The Morris water maze (MWM), also known as the Morris water navigation task, is one of the most widely used behavioral paradigms in neuroscience to assess spatial learning and memory (D'Hooge & De Deyn, 2001). Originally developed by Richard G. Morris in 1981 (Morris, 1981), it has become a standard tool for evaluating hippocampal-dependent spatial learning and the consolidation of long-term spatial memory. Its widespread use derives from its high sensitivity to hippocampal dysfunction and its strong translational relevance, as it models key aspects of spatial navigation and memory processes that are conserved across species, including humans.

Methodology

The MWM protocol begins with a training phase, typically lasting from 2 to 10 days, during which animals learn to locate a platform hidden beneath the water surface by relying on spatial cues. In most standard protocols, mice undergo 3 consecutive days of training, with two daily sessions, each consisting of three 1-minute trials. At the start of each trial, the animal is released

from a randomly chosen quadrant, while the platform remains in a fixed location throughout the training. If a mouse fails to find the platform within 60 seconds, it is gently guided to it and allowed to remain there for 15 seconds before the next trial. Training sessions are spaced by approximately four hours to ensure that animals rely on long-term memory for platform localization.

Following training, a probe trial is conducted to assess spatial memory retention. In this phase, the platform is removed, and mice are allowed to swim freely for a fixed time period. For analysis, the pool is virtually divided into four quadrants: the target quadrant (TQ; where the platform was previously located), two adjacent quadrants (AL = left adjacent, AR = right adjacent), and the opposite quadrant (OQ). The percentage of time spent in each quadrant, particularly in the TQ, is used as an index of memory performance.

Set Up

Several articles have described the methodology of the MWM (Brandeis et al., 1989b; D'Hooge & De Deyn, 2001; Morris, 1984; Vorhees & Williams, 2006), as both apparatus and

procedures have been adapted over time. The most commonly used setup consists of: (1) a large circular plastic pool (height 75–80 cm; diameter 150–200 cm for rats, 90–120 cm for mice) filled with water to about 40 cm below the rim to prevent escape, maintained at ~25 °C, and rendered opaque (e.g., by adding non-toxic white paint, powdered milk, or synthetic opacifiers) to conceal the submerged platform; (2) a hidden platform (typically 10 × 10 cm), placed 1 cm below the water surface, usually made of Plexiglas and invisible to the swimming animal; (3) stationary distal cues, such as geometric shapes or distinct objects, positioned at the four cardinal points of the maze or on the surrounding walls and kept constant throughout testing; and (4) a ceiling-mounted camera connected to a video-tracking system and software for motion analysis (Figure 4).

Figure 4 Picture of the MWM apparatus (from Puzzo et all. 2014).

Data Analysis

During training, escape latency is recorded as the primary parameter, reflecting the time required to reach the hidden platform. In control animals, this measure typically decreases across trials. However, platform acquisition does not rely exclusively on mnemonic ability, but also on different navigation strategies: praxis strategies (sequences of movements), taxon strategies (use of proximal cues), and spatial

strategies (mapping the environment based on distal cues) (Terry, 2009). For this reason, video tracking systems should also quantify additional parameters, such as swimming path length, path directionality, and cumulative distance to the platform, which provide insights into non-mnemonic behaviors (Dalm et al., 2000). During the probe test, memory retention is evaluated by analyzing the percentage of time spent in each quadrant, with control mice typically spending 35–40% or more of their time in the target quadrant (TQ). Additional measures, such as the number of platform crossings and trajectory patterns, can further characterize spatial memory performance.

Studied Memory

The MWM is a widely used tool to study hippocampal-dependent spatial memory, but it also recruits a broader network of brain regions. Task performance has been shown to involve thalamic nuclei (Bor et al., 2011; Cholvin et al., 2013; Hamani et al., 2010; Jankowski et al., 2013; Loureiro et al., 2012; Mumby et al., 1999; Savage et al., 1997), the mammillary bodies (Aranda et al., 2008; Conejo et al., 2004; Méndez et al., 2008; Radyushkin et al., 2005; Santín et al., 1999; Vann, 2010), amygdala (Decker et al., 1995; Galliot et al., 2010; Gaskin &

White, 2013; Spanis et al., 1999), locus coeruleus (Compton et al., 1995; Khakpour-Taleghani et al., 2009; Lemon et al., 2009; Riekkinen et al., 1990), cerebellum (Lalonde, 1994; Passot et al., 2012; Petrosini, 1998; Petrosini et al., 1996; Rochefort et al., 2011), prefrontal cortex (Chiba et al., 1994; Funahashi et al., 1997; Granon & Poucet, 1995; Hannesson et al., 2004; McCarthy et al., 1996; Mogensen et al., 1995; Romanides et al., 1999), anterior cingulate cortex (St-Laurent et al., 2009; Teixeira et al., 2006; Warburton et al., 1998), and the striatum (Block et al., 1993; Devan et al., 1999; Devan & White, 1999; Whishaw et al., 1987). Damage or degeneration of these structures, frequently observed during aging and in Alzheimer's disease, leads to impairments in MWM performance (Gratwicke et al., 2013; Schliebs & Arendt, 2011; Frielingsdorf et al., 2006).

Advantages

The MWM is a widely used behavioral test because of its numerous advantages (Astur et al., 2002; McNamara & Skelton, 1993; Morris, 1984; Vorhees & Williams, 2006). It is relatively quick and easy to perform, requires no pre-training, and can be conducted with a limited number of animals. The task allows for the distinction between spatial learning and long-term spatial

memory, while also providing measures of non-spatial abilities such as visual and motor performance. The design of the apparatus minimizes olfactory interference, which is particularly relevant for hippocampal studies. Moreover, the procedure avoids highly stressful manipulations such as electric shocks or food deprivation, although the immersion in water remains an aversive stimulus.

Disadvantages

Like all behavioral tests, the role of the experimenter must be carefully considered. Ideally, testing should be conducted by a single experienced experimenter after a period of habituation handling. During the MWM, the experimenter should remain in a fixed position, or leave the room entirely, to avoid becoming an unintended distal cue.

The MWM was originally developed for rats (Morris, 1981, 1984), but since mice are now the most commonly used species in disease models, species differences must be taken into account (Lipp & Wolfer, 1998). Compared with rats, mice generally perform more poorly in this task, partly due to intrinsic behavioral traits such as thigmotaxis (swimming close to the

walls) and passivity (floating without active searching) (Vorhees & Williams, 2006). In such cases, mice can be re-tested within the same or following day, but extreme outliers should be excluded from analysis. Among wild-type strains, C57Bl/6 mice are considered the most reliable (Clapcote et al., 2005; Upchurch & Wehner, 1988; Wahlsten et al., 2005).

Environmental variables are also critical. Water temperature must be controlled, as cooler temperatures increase the aversiveness of the environment and can reduce thigmotaxis and passivity. Lighting should be soft and diffuse to minimize reflections, since rodents are nocturnal animals that rely more on olfactory than visual cues (Eichenbaum et al., 1988; Lavenex & Schenk, 1996; Reid & Morris, 1993); thus, illumination becomes a particularly important factor in this task.

General physiological and biological factors can also affect performance. Steroid hormones modulate hippocampal function and stress responsiveness (Galea et al., 2008, 2013; Lunine, 1997), underscoring the importance of minimizing stress through appropriate handling and testing conditions. If heightened anxiety is suspected, dedicated anxiety tests may be required (Gould, 2009; Walf & Frye, 2007). Infections (viral,

bacterial, parasitic) can also influence behavior, emphasizing the need for rigorous veterinary health monitoring (Braithwaite et al., 1998; Espinoza et al., 2013; Kavaliers & Colwell, 1995; Yayou et al., 1993). Finally, age is a major determinant of MWM performance, as spatial learning and memory decline with aging, partly due to changes in locomotion and exploratory drive (Bergado et al., 2011; Li et al., 2009; Palmeri et al., 2013).

Optimizations

Particular attention was given to minimizing confounding factors that could bias memory assessment. We carefully standardized lighting conditions, avoiding variations in light intensity that might alter exploratory or stress-related behavior.

Since the experimental design included drug treatments with potential effects on anxiety and locomotor activity, we systematically performed open field testing to rule out anxiety-driven or motor-related alterations.

Moreover, because aged mice often show reduced swimming speed and altered locomotion, we introduced an additional visible-platform test at the end of MWM protocol to specifically

evaluate latency and motor performance independently of spatial memory.

To further enhance the reliability of performance in aged animals, I ensured appropriate resting intervals between testing sessions, carefully monitored water temperature, and prevented mice hypothermia, thereby reducing fatigue and improving task compliance.

These methodological refinements ensured that differences observed in MWM performance reflected genuine cognitive outcomes rather than non-mnemonic variables.

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Chapter 3: Nitric Oxide/cGMP/CREB pathway crosstalk: from physiology to Alzheimer's Disease

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ABSTRACT

The nitric oxide (NO)/cGMP pathway has been extensively studied for its pivotal role in synaptic plasticity and memory processes, resulting in an increase of cAMP response element-binding (CREB) phosphorylation, and consequent synthesis of plasticity-related proteins. The NO/cGMP/CREB signaling is downregulated during aging and neurodegenerative disorders and is affected by Amyloid-β peptide (Aβ) and tau protein, whose increase and deposition is considered the key pathogenic event of Alzheimer's disease (AD). On the other hand, in physiological conditions, the crosstalk between the NO/cGMP/PKG/CREB pathway and Aβ ensures long-term potentiation and memory formation.

This review summarizes the current knowledge on the interaction between the NO/cGMP/PKG/CREB pathway and Aβ in the healthy and diseased brain, offering a new perspective to shed light on AD pathophysiology. We will focus on the synaptic mechanisms underlying Aβ physiological interplay with cGMP pathway and how this balance is corrupted in AD, as high levels of Aβ interfere with NO production and cGMP molecular signaling leading to cognitive impairment.

Finally, we will discuss results from preclinical and clinical studies proposing the increase of cGMP signaling as a therapeutic strategy in the treatment of AD.

INTRODUCTION

In the last decades, the gaseous molecule nitric oxide (NO) has been widely studied for its role as cellular messenger with autocrine and paracrine activities. In the CNS, NO plays a fundamental role in cell communication and has been related to several functions such as synaptic plasticity, memory formation, sensory perception, endothelium-dependent vasodilatation, immune response, modulation of hypothalamic functions such as circadian rhythm, body temperature, appetite, and hypothalamic-pituitary axes control [1,2]. NO belongs to the family of gaseous signaling molecules that, due to their gaseous lipophilic nature, can freely permeate cellular membranes and do not rely on surface receptors as in the classic neurotransmission path. Consistently with their biophysical properties, gaseous transmitters are able to act as retrograde messengers transferring information from the post- to the pre-synaptic terminal [3,4]. NO behaves in a hormetic fashion, i.e. exerts opposite actions depending upon its concentration. NO, in fact, other than being constitutively produced in neurons and

endothelial cells in physiological conditions, is released in large quantities by glial cells in response to immune stimuli leading to formation of “reactive nitrogen species” responsible of nitrosative stress inducing cellular damage. Thus, NO acts as a “double-edged sword” with neuroprotective or neurotoxic effects depending upon the dose. Nitrinergic transmission has also gained high attention as therapeutic target for a variety of CNS disorders, since NO release is elicited by glutamate [5], i.e. the main excitatory neurotransmitter in the brain, and it mediates the endothelium-dependent relaxation increasing cerebral blood flow during neuronal activity [6]. Also, NO regulates the phosphorylation of the transcription factor cAMP response element-binding (CREB), which has prosurvival activity and is required in synaptic plasticity and memory. Hence, a variety of studies investigated its role in neurodegenerative disorders characterized by cell death and disruption of neuronal communication such as Alzheimer’s disease (AD), the most common form of dementia in the elderly. Here, we will focus on the NO/cGMP/PKG pathway that leads to CREB phosphorylation at Ser133 and represents a critical event to ensure long-term potentiation (LTP) [7], a form of synaptic plasticity representing the cellular correlate of long-term

memory. This pathway is downregulated during aging and neurodegenerative disorders [8,9] and has been demonstrated to interact with Amyloid-β peptide (Aβ) and tau protein [10,11] whose increase and deposition are typical hallmarks of AD [12]. We will also discuss recent works demonstrating a crosstalk between the NO/cGMP pathway and Aβ in physiological conditions, proposing a novel perspective to interpret AD pathophysiology. Finally, we will summarize results from preclinical and clinical studies aimed at increasing cGMP signaling to counteract cognitive deficit in AD. In this context, a particular attention will be paid to phosphodiesterases (PDEs), enzymes responsible of cyclic nucleotides degradation, whose inhibition determines an enhancement of cGMP [13].

1.1.Role of the NO/cGMP/PKG/CREB pathway in synaptic plasticity and memory

NO is a lipophilic gaseous molecule with a very short half-life (4-5 s) synthesized by the conversion of the aminoacid L-arginine in L-citrulline by the enzyme NO synthase (NOS), a cytochrome P-450 type hemoprotein [14,15]. Three different isoforms of NOS have been recognized, i.e. the neuronal form (n-NOS) or type I, the endothelial form (e-NOS) or type III, and

the inducible form (i-NOS) or type II. n-NOS and e-NOS are constitutive enzymes producing NO in physiological conditions via a calcium-dependent calmodulin binding in endothelial, neuronal and glial cells. i-NOS is calcium-independent and catalyzes NO production at high concentrations in response to immunological stimuli (i.e., LPS, IFN-γ, TNF-α) in smooth muscle cells, macrophages, hepatocytes, glial and endothelial cells, being responsible of nitrosative stress and cell damage. Notably, glial cells might use iNOS to produce NO in a calcium-dependent way in response to physiological stimuli [16,17]. Thus, NO mediates vasodilation, neuronal transmission and immune response regulating a variety of physiological functions among which blood pressure, sexual function, memory formation, and inflammation [18]. In the brain, NOS is ubiquitously distributed, and the neuronal production of NO is elicited by a variety of stimuli leading to increase of calcium levels in the: i) pre-synaptic terminals, triggered by action potentials that lead to an increase of calcium through voltage-gated calcium channels; ii) post-synaptic terminals, triggered by post-synaptic neurotransmitter receptors, such as NMDA-Rs, that induce an influx of calcium. Remarkably, NO formation in the CNS might also be due to vascular e-NOS stimulation. The

NO-mediated transmission is rather different from the classic form of neurotransmission, due to the biological characteristics of NO that, being a gaseous molecule, can freely permeate biological membranes and quickly diffuse from the post- to the pre-synaptic terminal, from a neuron to another and from neurons to cells. Indeed, NO is not stored in synaptic vesicles, but synthesized at request either in the cell body and dendrites from which it spreads around to control synaptic transmission and plasticity. Once produced, NO freely diffuses across cell membranes exerting autocrine as well as paracrine effects [19] by acting on three main targets: i) transition metals such as haem iron or iron-sulphur centers modulating metalloproteins among which the heme protein soluble guanylyl cyclase (sGC) catalyzing cGMP formation; ii) protein thiol groups through S-nitrosylation that can regulate protein function by different mechanisms; iii) molecular oxygen and superoxide anions generating several NO derivatives such as peroxynitrite, a highly reactive free radical and mediator of oxidative damage. The activation of sGC leads to the formation of cGMP, a ubiquitous second messenger regulating signal transduction in response to a variety of extracellular stimuli. It activates intracellular protein kinases that, in turn, stimulate other enzymes or gene

transcription, thus mediating the cellular response to the message. Specifically, cGMP is able to activate several physiological processes by targeting: i) cyclic nucleotides gated (CNG) ion channels, mainly present in retinal photoreceptors and olfactory sensory neurons and involved in sensorial perception (for a review see [20]), but also playing a role in modulation of adult hippocampal neurogenesis [21]; ii) protein kinase G (PKGs), a family of serine/threonine protein kinases with a crucial role as signal transduction mediators [22] that, in turn, phosphorylates different substrates mediating a variety of cellular functions such as calcium homeostasis, smooth muscle contraction, platelet function, and gene expression (for a review see [23]. The capability of PKG to regulate and maintain high levels of CREB phosphorylation has been considered fundamental in synaptic plasticity and memory; iii) PDEs, that degrade cGMP in the inactive forms 5'GMP. As of now, 11 PDE families have been identified with different distribution, structural and functional properties, and capability to specifically hydrolyze cAMP (i.e. PDE4, PDE7, PDE8), cGMP (i.e. PDE5, PDE6, PDE9) or both cyclic nucleotides (PDE1, PDE2, PDE3, PDE10, PDE11) (for a review see [24]). Thus, PDE-Is prevent cAMP and cGMP degradation thereby

enhancing and/or prolonging their effects. The cGMP/PKG pathway has been demonstrated to be involved in LTP, a form of synaptic plasticity occurring at the excitatory synapses in several brain areas that has been widely studied at the hippocampus, especially in the CA3-CA1 synapses. LTP can be evoked by a high frequency stimulation and consists in an increase in amplitude of excitatory postsynaptic potentials (EPSPs) that, in turn, triggers a cascade of molecular events leading to a "long-lasting modification" of the synapse (for a review see [25]). Depending upon the intensity of the stimulation, it is possible to elicit early-LTP, a form of protein-synthesis-independent plasticity that involves a change in presynaptic neurotransmitter release and short-term kinase activity, or late-LTP, a long-lasting form of plasticity that requires de novo protein synthesis [26]. Previous studies have demonstrated that the NO/cGMP pathway is involved both in early- and late-LTP acting in the pre-synaptic terminals and in post-synaptic dendrites. On the latter, it leads to CREB phosphorylation at Ser 133 that, through the activation of histone acetyltransferases, regulates transcription of genes involved in de novo protein synthesis and dendritic spines formation to ensure the long-term changes required to form and consolidate new memories (for a review see [27]). Notably, NO

can also induce S-nitrosylation of nuclear proteins associated with CREB target genes via a Ser133independent mechanism [28]. Behavioral studies confirmed that the NO/cGMP pathway is involved in learning and memory [29–31], since NOS inhibitors or genetic deletion impaired different forms of memory in rodents [32–34] and, on the other hand, NO donors, L-Arginine or cGMP analogs improved memory [3234]. Interestingly, the NO/cGMP/PKG pathway cooperates with the cAMP/PKA cascade leading to CREB phosphorylation to ensure LTP and memory formation [7,35–39]. However, cAMP and cGMP have been reported to act during two temporally distinct phases to induce memory formation, with cGMP mediating early consolidation, and cAMP being involved in late consolidation [29,40,41]. In any case, stimulation of the cAMP/PKA or NO/cGMP pathway determines a conversion from early-LTP into late-LTP and these changes result in the conversion from short- into long-term memory.

1.2 Crosstalk between NO/cGMP/CREB pathway and Aβ in physiological conditions

Results from the studies described above, prompted us to investigate the crosstalk between the NO/cGMP/CREB pathway

and Aβ in physiological conditions. As a premise, it should be mentioned that, although Aβ has been mainly studied for its pathogenetic role in AD [42], it is also physiologically produced in the healthy brain where it exerts an antimicrobial [43], neuroprotective [44], and neuromodulatory function (for a review see [45]). At the synapse, Aβ is secreted in an activity-dependent fashion dynamically regulated by vesicle cycling [46–49], and its production increases during LTP and memory [50,51]. Indeed, endogenous Aβ is needed for memory formation since blocking its production and/or function results in an impairment of LTP and memory [50,52,53]. Several studies have demonstrated that, in physiological concentrations, Aβ acting through α7 nicotinic acetylcholine receptors (α7nAChRs) activated the MitogenActivated Protein Kinase Cascade [54,55], regulated recycling of synaptic vesicles [56], modulated glutamate, aspartate, GABA, glycine, and dopamine release [57–61], enhanced neurotransmitter release, frequency of miniature excitatory post-synaptic currents, docked vesicle number, length of postsynaptic density, and the expression of plasticity-related proteins such as phospho-CREB (p-CREB), calcium-calmodulin-dependent kinase II and BDNF [62]. These changes enhanced late-LTP [50,63] and induced a conversion of

early-LTP into late-LTP [62] Interestingly, we have previously demonstrated that upregulation of cyclic nucleotides induced a slight increase of Aβ within the picomolar physiological range in young healthy mice [51,64,65]. The increase of cAMP levels by the PDE4-I rolipram stimulates Aβ production by increasing Amyloid Precursor Protein (APP) synthesis [64,66], whereas the increase of cGMP levels by the PDE5-Is sildenafil and vardenafil did not modify APP expression but affected its processing. In particular, cGMP stimulates APP and the beta-site amyloid precursor protein cleaving enzyme 1 (BACE1) to converge in the endolysosomal compartment [51] where the more acidic environment favors amyloidogenic processing [67]. Further studies have demonstrated that cGMP regulates the subcellular localization of APP and BACE1 by inhibiting the activity of Rab5, a small GTPase associated with the plasma membrane and early endosomes, since APP-BACE1 approximation was increased in a dominant-negative Rab5 mutant [68]. As opposed to cAMP that triggers Aβ production through PKA activation [64,69] PKG seems not to be involved in the cGMP-induced Aβ production [70]. In fact, vardenafil was still capable of increasing Aβ levels in N2a cells knockdown for PKG1 and PKG2 [70]. However, the inhibition of endogenous

Aβ prevented the cAMP/PKA- and the cGMP/PKG-dependent enhancement of LTP and memory since both rolipram or vardenafil required Aβ to convert early- into late-LTP and short-term into long-term memory [51,71] The role of cyclic nucleotides in mediating the physiological effect of Aβ is not confined to the regulation of Aβ production, but involves Aβ function. Indeed, we have recently shown that the enhancement of LTP and memory induced by low pM Aβ was prevented by drugs inhibiting the NO/cGMP/PKG pathway [62]. Interestingly, hippocampal slices treated with pM Aβ presented an increased expression of nNOS, in line with previous studies demonstrating that Aβ can bind nNOS [72]. This suggests the possibility of a direct interaction between the two proteins, even if it cannot be excluded that the increased nNOS expression is a functional consequence of the Aβ-induced increase of neurotransmitter release [73]. Thus, in physiological conditions, cGMP acts both upstream of Aβ, by regulating its production, and downstream, by modulating its effects, suggesting the existence of a cGMP-Aβ-cGMP loop that regulates CREB phosphorylation to ensure LTP and memory. Figure 1 summarizes the crosstalk between Aβ and the NO/cGMP pathway in physiological conditions.

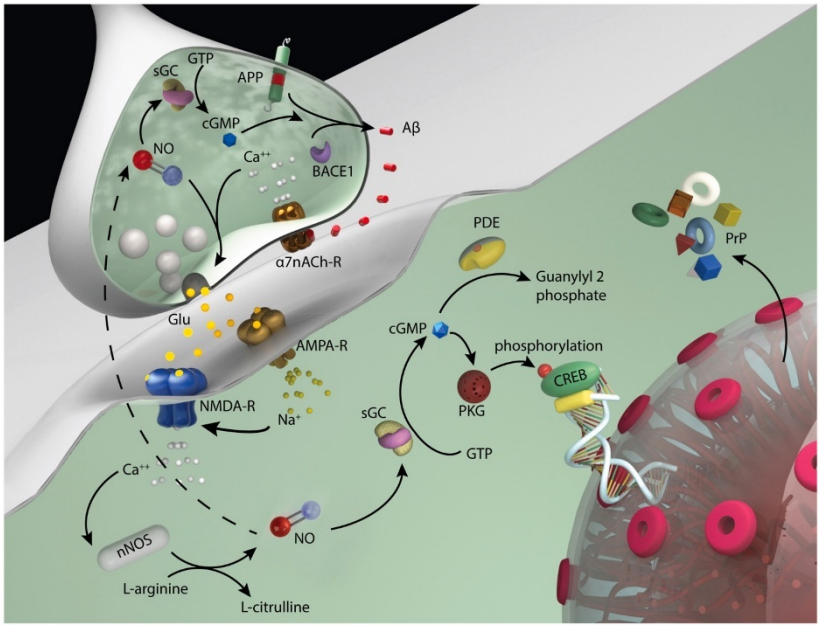

Fig. 1. – Crosstalk between Aβ and the NO/cGMP/CREB pathway in the healthy brain. In physiological conditions, cGMP facilitates APP-BACE1 approximation leading to the production of low concentrations of Aβ that, acting through α7nAChRs, enhance glutamate (Glu) release. This triggers a cascade of post-synaptic events among which the increase of the NO/cGMP/PKG signaling that, in turn, stimulates CREB phosphorylation and determines the production of plasticity-related proteins (PrP) to ensure memory formation. The capability of NO to act as a retrograde neurotransmitter maintains this cycle (dashed line).

1.3. Dysregulation of the NO/cGMP/PKG pathway in Alzheimer's disease

While in the healthy brain cGMP and Aβ cooperate to ensure LTP and memory formation, during aging and neurodegenerative disorders such as AD, this liaison is corrupted. In fact, high levels of Aβ interfere with molecular signaling pathways leading to CREB phosphorylation (for a review see [8]) and substantially contribute to the synaptic failure occurring prior to frank neuronal degeneration. Over the years, the impairment of CREB phosphorylation has been largely reported in several cellular and animal models of AD and confirmed in patients where a CREB reduction was found in prefrontal cortex [74] and hippocampus [75]. High concentrations of Aβ induced an inhibition of CREB phosphorylation in hippocampal neuronal cultures after glutamate stimulation [76] in hippocampal slices after LTP induction [77], in human neuroblastoma cell cultures [78], and in animal models of aging [65] and AD such as Tg2576 [79] and APP/PS1 mice [24,80]. Aβ also impaired neuronal expression of CREB-regulated transcription coactivator-1 (CRTC1)-dependent genes such as c-fos, BDNF and Nr4a2 which are highly related to synaptic plasticity [81]. Indeed, previous

studies showed that Aβ suppressed the activation of the CRE-containing BDNF promoter and the expression of BDNF mRNA induced by neuronal depolarization [82]. In addition, decreased expression of BDNF by oligomeric nM Aβ was linked to a reduction of CREB phosphorylation in differentiated SH-SY5Y neuroblastoma cell line [83]. These results are particularly interesting if considering that BDNF levels were reported to be downregulated in brains of AD patients [84,85] and in APP/PS1 transgenic mouse model of AD [86], with the BDNF decrease overlapping the onset of long-term memory impairment. Notably, Aβ has been demonstrated to impair each component of the NO/cGMP/PKG/CREB pathway. Both oligomeric and fibrillar forms of Aβ suppressed the NO-mediated activation of sGC leading to a decrease of cGMP production [10,87] that was paralleled by a decline of PKG expression [88]. Aβ also blocked the physiological increase in cGMP immunoreactivity occurring after LTP induction in murine hippocampal slices [77]. A decreased activity and/or expression of sGC was confirmed in superior temporal cortex of AD patients [89] and in reactive astrocytes obtained from cortical samples of AD brains [90]. Consistently, cGMP levels were reduced in cerebrospinal fluid (CSF) of AD patients compared with aged-matched controls

and, interestingly, this reduction correlated with worse performances on the Mini Mental State Examination [91,92]. In addition to the reduction of cGMP synthesis, alterations of its degradation by PDEs are likely to contribute to the impaired signaling in AD [92,93]. Other studies have focused on tau protein, whose increase and hyperphosphorylation is crucial in AD pathogenesis [94]. Although further studies are needed to investigate the crosstalk between tau and the cGMP/PKG pathway, tau is known to share common molecular mechanisms with Aβ and, for example, it has been recently demonstrated that it reduced c-Fos and Arc genes expression leading to a reduction of CREB phosphorylation, LTP and memory [11]. On the other hand, overexpression or upregulation of p-CREB by treatments stimulating cAMP or cGMP levels reduced tau hyperphosphorylation and deposition [8,11,95]. Moreover, Reynolds and colleagues have demonstrated that the nitrosylation of tau inhibits its aggregation in neurofibrillary tangles [96]. In addition, NO can regulate tau hyperphosphorylation stimulating the Phosphoinositide 3kinases (P13K)/Akt pathway resulting in the inhibition of glycogen synthase kinase 3β (GSK-3β), a key kinase involved in tau hyperphosphorylation and accumulation [95,97,98]. Thus,

a deficit of NO might be a relevant contributor in aggravating tau pathology. Notwithstanding the aforementioned studies support a positive role for the NO/cGMP pathway in AD, this topic is still controversial since NO can induce a biphasic dose-response exerting a neuroprotective role when at low concentrations and a neurotoxic effect when produced in excessive quantities (for a review see [31,99]). Consistently, a variety of studies have demonstrated that in the CNS, NO can enhance or impair synaptic function depending upon its concentration and time of application. When excessive, NO leads to the production of "reactive nitrogen species", such as peroxynitrite, inducing cell death and neurodegeneration [100]. This mechanism is mediated by iNOS that produces high concentrations of NO in response to neuroinflammation, as opposed to nNOS and eNOS that catalyze NO formation in physiological conditions. Consistently, an increase of iNOS and a decrease of nNOS and eNOS were found in animal models of aging [101], and genetic lack of eNOS in KO mice induced an increase of Aβ and cognitive dysfunction [102]. In AD, the downregulation of the NO/cGMP/CREB pathway is paralleled by an alteration in the expression of the different NOS isoforms [103,104] and aberrant S-nitrosylation of target proteins [105].

This is confirmed by the increase of iNOS expression found in postmortem brain tissue from AD patients and the higher iNOS immunoreactivity in astrocytes surrounding amyloid plaques [106,107]. Furthermore, Aβ increased the expression of iNOS and, consequently, NO production in rats neuronal-glial cultures [108], supporting the idea that the augmented expression of iNOS represents an inflammatory response induced by Aβ accumulation. On the other hand, it has been suggested that the increase of NOS activity could represent a compensatory mechanism recruited in the presymptomatic phase of AD to sustain a normal synaptic function. In fact, in young 3xTg-AD transgenic mice, showing no sign of synaptic impairment, the blockage of NO resulted in synaptic depression [109]. In this case, the continuous increase of NO may shift the pathway to toxic functions as the disease progresses, with the formation of peroxynitrate and S-nitrosylated proteins. Figure 2 summarizes how the NO/cGMP pathway is involved in AD pathophysiology.

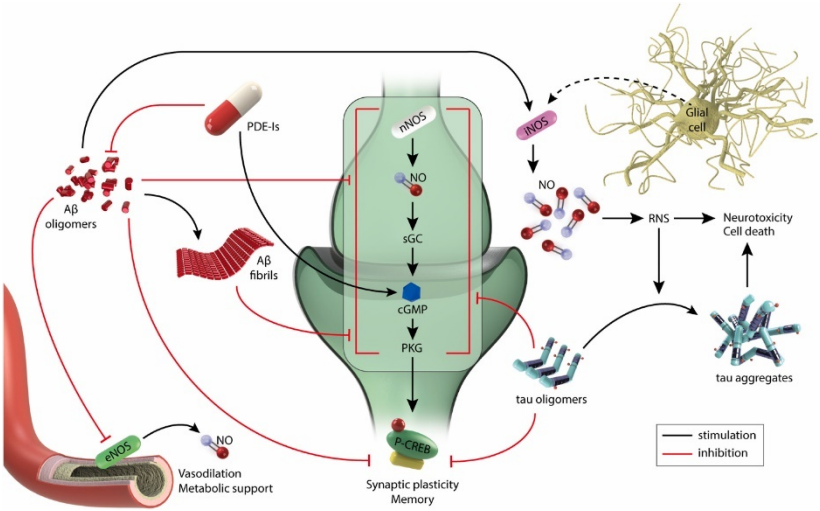

Fig. 2. – Crosstalk between Aβ and the NO/cGMP/CREB pathway in the AD brain. High concentrations of Aβ interferes with the NO signaling. The inhibition of eNOS prevents the physiological NO-mediated vasodilation. Aβ oligomers and fibrils also inhibits each component of the NOS/NO/cGMP/PKG pathway (red box) leading to and impairment of CREB-phosphorylation and therefore of synaptic plasticity and memory. Aβ also stimulates microglial iNOS that leads to the production of high concentrations of NO with the consequent production of neurotoxic reactive nitrogen species (RNS). RNS, in turn, stimulate the formation of tau aggregates that inhibits CREB phosphorylation and exert a neurotoxic effect. PDE-Is might counteract the Aβ-induced effects increasing cGMP levels.

1.4. cGMP as a pharmacological target for Alzheimer's disease treatment: preclinical and clinical studies

The extensive literature supporting a downregulation of the NO/cGMP/PKG pathway during aging and neurodegenerative disorders [24,101,110–113], has prompted researchers to identify therapeutic strategies aimed at restoring the cGMP signaling to rescue synaptic plasticity and memory deficits especially in AD. In animal models, treatment with NO donors, sGC stimulators, or cGMP-analogs reversed both the Aβ- and tau-induced detrimental effect on LTP and memory by restoring CREB phosphorylation (for a review see [114]). However, the PDE-based approach has been preferred because their high phylogenetical conservation [115] potentially confers a high degree of translatability to preclinical studies. Furthermore, PDEs are differentially expressed in different tissues [116], implicating that their physiological function can be affected by some inhibitors but not others, and their unique architecture of the active site allows for the design of selective inhibitors [117]. In this regard, although almost all PDEs are expressed in the human brain, studies on the expression of PDE5 have been controversial. Some studies reported that PDE5 mRNA expression in adult human brain is relatively low [118], but Teich

and colleagues have distinctly demonstrated that PDE5 mRNA is detectible in human brain tissue and PDE5 protein is expressed in hippocampal and cortical neurons [119]. Consistently, a 5-fold increase in expression of the PDE5A has been reported in the temporal cortex of late-stage AD patients (Braak stage V-VI) vs. controls [92]. Finally, PDEs have already been studied extensively as drug targets for a wide spectrum of pathologies, mostly for their vasoactive effects. Thus, the extensive safety and pharmacodynamic data deriving from a long-term presence on the market, pones PDE-Is as an optimal candidate for further clinical application. In fact, a variety of clinical trials have been performed on the base of extensive scientific evidence gathered from preclinical studies demonstrating that PDE-Is are able to rescue memory deficits in AD models. Here we will discuss outcomes from preclinical and clinical studies focusing on cGMP-specific and mixed PDE-Is approaches. For a summary of results from clinical trials see Table 1.

Target	Drug	Clinical evidence
PDE1	Vinpocetine	Improves memory in healthy adults and elderly subjects [160-162]. Ineffective in the treatment of AD [163,164].
PDE3	Cilostazol	May slow cognitive decline in MCI, mild dementia, and AD patients alone or in combination with donepezil [164-170]. Increases regional cerebral blood flow [167].
PDE5	Sildenafil	No cognitive improvement in healthy [133] and schizophrenic subjects [134]. Increases cerebral blood flow and cerebral metabolic rate of oxygen in AD patients [135]. Normalization of spontaneous neural activity in AD patients (pilot study) [121]. Reduction in the risk of AD diagnosis [121].
PDE5	Vardenafil	No significant effect in cognition in young healthy subjects [136,137].
PDE5	Tadalafil	Increases cerebral blood flow and improves cognition in patients with erectile disfunction and mild cognitive impairment [138]. No significant change of cerebral blood flow in randomized clinical trial [139].
PDE5	Udenafil	Improves cognition, executive functions, attention and working memory in erectile dysfunction patients [140, 141].
PDE9	PF-04447943	Ineffective in the treatment of AD [148].
PDE9	BI 409, 306	Ineffective in the treatment of AD [149].

Table 1. Clinical trials with cGMP-specific PDE-Is.

1.5.PDE5-Inhibitors

Some selective cGMP-specific PDE5-Is such as sildenafil (Viagra®, Revatio®) and vardenafil (Levitra®) and tadalafil (Cialis®) are considered particularly attractive because already in the market with other clinical indications, i.e., erectile dysfunction and pulmonary hypertension [120]. Indeed, the few

side effects, the long follow-up in humans in the senile population (especially for sildenafil), and the solid background relying on several preclinical studies, make them good candidates for drug repurposing. Sildenafil, originally developed as an antihypertensive drug, was eventually utilized in the treatment of erectile dysfunction which has become the leading indication with a high rate of success. Recently, it garnered again great press attention due to the results of another retrospective study analyzing a large-scale AD patient data. The authors performed a computational analysis on insurance claims data from over seven million people in the US revealing that sildenafil prescription was associated with a 69% reduction in the risk of AD diagnosis after 6 years of follow-up [121]. Sildenafil and vardenafil have been widely used in preclinical studies for neurodegenerative disorders and a variety of findings have demonstrated their capability to rescue LTP, memory and CREB phosphorylation in animal models of AD and aging [24,110,120,122,123]. Sildenafil also improved memory acquisition and consolidation in healthy animal models such as rats, mice, and macaques [29,124–126]. Other PDE5-Is such as Icariin rescued cognitive deficits in APP/PS1 mice [127] or rats treated with Aβ25-35 [128]. Furthermore, a decrease of Aβ load

and plaques and a modification of APP processing toward the non-amyloidogenic pathway were found after treatment with a variety of PDE5-Is such as sildenafil, vardenafil, icariin and yonkenafil [24,65,120,127,129]. The PDE5-I tadalafil has also shown a promising profile in AD treatment [130,131]. However, results of studies are controversial due to low blood-brain barrier (BBB) penetration of tadalafil that, indeed, failed to rescue contextual fear memory and spatial working memory in APP/PS1 mice [24]. As for clinical studies, sildenafil has been the first PDE5-I tested on central cerebral functions. In a pilot study on 6 healthy male volunteers, a single dose of sildenafil was administered and a battery of 7 different psychophysical tests was used to assess cognition, without reporting relevant effects [132]. Similarly, no cognitive improvement was observed in a double-blind, placebocontrolled study with 15 schizophrenic patients who received a cognitive test battery immediately (1 h) and 48 h after sildenafil administration [133]. In a more recent study, a single oral dose of sildenafil was able to increase cerebral blood flow and cerebral metabolic rate of oxygen in AD patients [134], indicating a clear improvement of cerebral perfusion. Furthermore, a pilot study on a small number of AD patients showed a normalization of the fractional

amplitude of low frequency fluctuations, i.e., an index of spontaneous neural activity, in the right hippocampus of patients treated with sildenafil [135]. As mentioned before, a very recent work from Fang and colleagues has shined new light on the use of sildenafil as a possible therapeutic strategy for AD based on retrospective case–control pharmacoepidemiologic analyses of insurance claims [121]. Even if it does not establish a causality link between sildenafil usage and decreased incidence of AD, this study strongly advocates a randomized controlled trial with AD patients. The PDE5 inhibitor vardenafil was tested on cognition in young healthy subjects in two different trials: double-blind placebo-controlled studies, enrolling university students who received vardenafil and underwent auditory sensory gating test 85 min later, to evaluate memory and executive functions [136,137]. However, the experimental outcome did not show any significant effect. Recently, a prospective clinical study on the PDE5-I tadalafil showed that a daily administration of tadalafil for eight weeks induced an increase of cerebral blood flow and improved cognitive function in patients with erectile disfunction and mild cognitive impairment [138]. On the other hand, newly published results of the PASTIS randomized clinical trial did not show a significant

change of cerebral blood flow but a trend of enhanced perfusion within white matter hyperintensities, suggesting possible clinical benefits [139]. Another PDE5 inhibitor, udenafil, commercially available for erectile dysfunction (ED), showed some promising results: 27 patients treated with udenafil for ED showed an improvement of general cognition and frontal execution functions, tested at the Korean version of the mini mental state examination (K-MMSE) and the Korean version of the frontal assessment battery (K-FAB) respectively [140]. Subsequently, in a double-blind, placebo-controlled study, 49 ED patients treated with udenefil for 8 weeks presented a significant increase of general cognition and attention/working memory [141].

1.6. PDE9-Inhibitors

Among cGMP-specific PDE-Is, also PDE9 has been object of some preclinical and clinical studies. BAY 73-6691 restored the Aβ-induced impairment of LTP and memory in Tg2576 mice [142]. BAY 73-6691 and other PDE9-Is, i.e. PF-04447943 and BI 409306, improved synaptic plasticity and memory in healthy rodents [143,144], restored cognition in scopolamine-induced memory impairment [144–145], and exerted a protective effect

against Aβ-induced cytotoxicity in vitro [146]. In addition, new PDE9 inhibitors seem to prevent Aβ aggregation into toxic fibrils [147]. However, when tested on humans, PDE9-Is failed to show significant effects on cognition in AD patients. A Phase 2, double-blind, placebo-controlled trial [148] was performed on mild to moderate AD patients who received the PDE9 inhibitor PF-04447943 for 12 weeks, with no significant differences reported at the Alzheimer's Disease Assessment Scale-cognitive subscale (ADAS-cog), the Neuropsychiatry Inventory, and the Clinical Global Impression (CGI)Improvement scale. A few years later, a second PDE9-I (BI 409306) was tested on patients with prodromal or mild AD in two Phase II, double-blind, placebo-controlled trials, with a 12-week drug administration. A total of 427 patients completed the studies. However, the PDE9-I treatment did not determine changes in the Neuropsychological Test Battery z-score, Clinical Dementia Rating Scale-Sum of Boxes and ADAS-Cog11 [149].

1.7. Mixed-specificity PDE-Is and combination treatments

Interestingly, treatment with drugs enhancing both cGMP and cAMP have been proven to be effective against cognitive impairment. They probably act via two different mechanisms,

cooperating to trigger CREB phosphorylation and inducing an increase of cerebral blood flow and glucose delivery to the brain. The increase of both cyclic nucleotides can be obtained with two different strategies, i.e. combining cAMP- and cGMP-specific PDE-Is (i.e. PDE4-Is and PDE5-Is), or by using mixedspecificity PDE-Is such as PDE1-, PDE2-, PDE3-, and PDE10-Is. Previous preclinical studies have demonstrated that a chronic treatment with sub-efficacious doses of the PDE5-I vardenafil combined with the PDE4-Is rolipram or roflumilast improved LTP and memory in healthy rodents [150] and AD animal models [151]. Interestingly, the cognitive enhancement found in Tg2576 persisted for 2 months beyond administration indicating a longlasting action and the potential application of these approaches as disease-modifying therapies. Furthermore, the synergistic effect of this combination therapy might allow obtaining the cognitive enhancement with few side effects due to the very low doses used. Beneficial effects have been also showed when using PDE-Is acting on both cGMP and cAMP degradation. Different studies have reported a significant improvement of neuronal plasticity and long-term memory in healthy and AD animal models treated with selective PDE1-Is and PDE2-Is [78,152–154]. PDE2 has been considered a good

candidate for AD treatment since it is highly expressed in the limbic nervous system, and its inhibition was found to rescue learning and memory performance, decrease neuroinflammation and apoptosis, and boost the PKA- and PKGdependent pathways in mice injected with Aβ or transgenic models of AD [88,154]. PDE3-Is improve cerebral circulation, reduce Aβ accumulation [155] and ameliorate memory in rodents affected by cerebral hypoperfusion [156] or aging [157]. Finally, the PDE10-I PQ-10 was found to improve the memory deficits induced by scopolamine in rodents [158]. The potential beneficial effect of mixed PDE-Is has been also studied in humans. Vinpocetine is a PDE1 inhibitor widely studied for its cognition enhancing properties in the past 30 years [159] and tested up to Phase IV trials for the treatment of cognitive impairment. Positive effects on cognition of vinpocetine were observed in healthy adults [160,161]. In elderly subjects, vinpocetine was evaluated as a nutritional supplement (Cognitex®, Life Extension) on memory impairments in a study without a placebo control, showing positive effects (ClinicalTrials.gov Identifier: NCT00719953) [162]. However, vinpocetine was not able to improve cognitive impairments in AD patients [163,164]. The PDE3-I cilostazol, approved for the treatment of intermittent claudication [165],

has been one of the earliest PDE-I investigated in clinical trials in the field of AD. In a pilot uncontrolled trial, cilostazol improved the Mini Mental State Examination (MMSE) in 10 mild to moderate AD patients treated in combination with acetylcholinesterase inhibitor donepezil for up to thirteen months [166]. In a second study, a 6 month-treatment with cilostazol prevented a further cognitive decline in elderly patients with AD and cerebrovascular disease, tested at the AD Assessment Scale-Cognitive Subscale (Japanese version), Revised Wechsler Memory Scale (logical memory-I) and Trail Making Test-A. The cilostazol group showed increased regional cerebral blood flow in the right anterior cingulate lobe, that might partially be responsible for the positive effects of cilostazol [167]. However, 6 months administration of cilostazol on mild to moderate AD patients failed to induce differences with the placebo group tested with the MMSE and the cognitive scale of the cognitive part of the AD Assessment Scale (ClinicalTrials.gov identifier: NCT0140). On the other hand, in a more recent fourth case-control study, cilostazol administered for at least twelve months in combination with acetylcholinesterase inhibitors improve the MMSE scores and clinical dementia rating sum of boxes scores [168]. Finally, two

retrospective studies on patients treated with cilostazol for cardiovascular diseases for at least 6 months found an improvement of MMSE scores in MCI patients [169] and mild AD patients as add-on to donepezil [170].

1.8. cGMP-specific PDE-Is vascular effects

In addition to the reported effect on synaptic plasticity, PDE-Is increasing cGMP levels present a marked vascular effect relaxing arterial wall, decreasing the arterial resistance, and therefore resulting in vasodilatation, and increased cerebral blood flow [171]. Indeed, sildenafil was found to increase brachial artery flow (a sensitive indicator of endothelial function), leading to increased blood flow and improvement in markers of endothelial function in diabetic patients [172,173]. Moreover, sildenafil administration decreased endothelin-1 and E-selectin levels, i.e., vascular markers which lead to vasoconstriction, and contribute to endothelial dysfunction [174,175]. A more recent study has also demonstrated an improvement of cerebral hemodynamic function and increase of cerebral oxygen metabolism in AD patients treated with sildenafil [134]. Notably, several studies have reported a significant endothelial dysfunction in patients with AD and

showed that Aβ42 toxicity properties exponentially increase in brain tissue with dysfunctional endothelium. On the other hand, a confluent endothelium limits Aβ secretion and its toxicity effect [176] (for a review see [177]). Despite the cerebrovascular effect of PDE inhibitors, animal studies showed that sildenafil can enhance cognitive functions independently of its effect on cerebral blood flow and glucose metabolism [178], mainly relying on the role of cGMP increase in the mechanisms of synaptic plasticity [24,121] Nevertheless, cGMP-specific PDEs could represent key therapeutic targets in the treatment or prevention of AD also for their ability to improve endothelial functioning, especially in patients with high risk of endothelial dysfunction.

1.9 PDE-Is side effects

Although many years of research have focused on developing PDE inhibitors for CNS disorders, there are no PDE targeting drugs currently approved in the treatment of these pathologies. The main reason for the failure in the clinical development of selective PDE inhibitors can be found in the lack of optimal efficacy, especially when compared to the expected results suggested by the strong biological rational and the studies in

animal models, and the constellation of side effects uncovered by clinical trials. Indeed, cNs are ubiquitously expressed second messengers that play a key role in the regulation of many cell functions. Therefore, drugs increasing their concentrations can result in several side-effects. For PDE5-Is, the most common side effects reported range between mild headache, flushing, dyspepsia, altered color vision, back pain and myalgias, hypotension and dizziness, rhinitis. It is noteworthy that none of the PDE5-Is are genuinely selective for the receptor PDE5, and most of the harmful effects occur due to cross-reactivity with other PDE isoenzymes. These effects are usually dose-dependent and rarely lead to treatment discontinuation, and prolonged QT or concomitant therapy with nitrates or nitroglycerin as the only absolute contraindications [119]. The PDE3 inhibitor cilostazol presents several side effects, including most commonly headache, diarrhea, abnormal stools, and since it is a quinolinone derivative also irregular heart rate and palpitations [179]. It is therefore contraindicated in patients with heart failure, as well as patients with severe hepatic or renal impairment.

CONCLUSIONS

In the last decades several preclinical studies have supported the role of the NO/cGMP/CREB pathway in synaptic plasticity and memory. The potential beneficial effect of enhancing its signaling has been demonstrated in a variety of animal models in healthy conditions, during aging and in neurodegenerative disorders such as AD. Although a recent in silico study has demonstrated that sildenafil usage was significantly associated with a marked decreased risk of AD [121], clinical trials are limited and did not give the expected results when translated into human application. This might be due to several reasons, among which the different expression and brain distribution of PDE between animals and humans; the brain selectivity and chemical properties of the PDE-Is used; the difficulty to identify the correct dose to be used in humans; the possible side effects. The search for novel PDE-Is with higher selectivity and capability to cross the BBB to be used in the elderly for chronic administration might represent a first step to overcome these limitations [180,181]. Recent drug discovery studies have contributed with novel PDE5 inhibitors, i.e. compound 7a [180] and 6c [181], that possess higher potency and selectivity, and good pharmacokinetics compared to the commercial ones,

demonstrating their potential in preclinical studies. Other studies have shown a good BBB penetration for mirodenafil that was able to rescue the AD phenotype acting on multiple signaling pathways [182]. Further studies are needed to evaluate these drugs in clinical trials. In general, it must be considered that AD is a multifactorial disease whose pathophysiology is still unclear. Therefore, it is very difficult to diagnose AD in the early stage where synaptic transmission is not yet compromised, precluding the possibility to identity the optimal time window where to start the treatment in the pre-symptomatic stage. In addition, PDE-Is and other drugs are typically tested on transgenic animal models that mimic familiar forms of AD, which do not reflect the pathological mechanisms occurring in sporadic AD (that accounts for more than 97% of AD cases). Most of the previous studies have focused on the Amyloid Cascade Hypothesis stating that the increase of Aβ is the primum movens leading to synaptic plasticity and cognitive impairment in AD. However, the failure of Aβ-target therapies and the increasing number of studies demonstrating the role of Aβ in the healthy brain have questioned this theory (for a review see [183]). Recently, we have hypothesized that when Aβ cannot exert its physiological function, it increases via a negative feedback mechanism. High

levels of Aβ, in turn, triggers the cascade of events leading to synaptic and memory dysfunction in AD. This hypothesis has been supported by our recent study demonstrating that a genetic lack or inhibition of α7nAChRs prevented Aβ to exert its physiological function thus leading to an increased expression of APP and Aβ levels resulting in an AD-like pathology [184]. Based on these results and considering that physiological Aβ is required for cGMP cognitive enhancing effect [51], PDE-Is might have a limited efficacy if Aβ-mediated plasticity is blocked downstream cGMP production, for example due to a decrease or malfunction of α7nAChRs that has been demonstrated in AD patients [185,186]. In conclusion, PDE-Is are promising drugs for the treatment of AD and other neurodegenerative disorders, but further studies are needed to better understand the crosstalk between Aβ, tau and cyclic nucleotides in physiological conditions and during neurodegenerative disorders to propose novel and safe therapeutic strategies specifically targeting the brain.

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disease-like pathology, Prog Neurobiol. 206 (2021). https://doi.org/10.1016/j.pneurobio.2021.102154.

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AUTHOR'S PUBLICATION AND AWARDS

List of Publications (reverse chronological order)

1. Tropea MR, Melone M, Li Puma DD, **Vacanti V**, Aceto G, Bandiera B, Trovato RC, Torrisi SA, Leggio GM, Palmeri A, D'Ascenzo M, Conti F, Grassi C, Puzzo D. Blockade of dopamine D3 receptors improves hippocampal synaptic function and rescues age-related cognitive phenotype. Aging Cell. 2024 Sep 5:e14291. doi: 10.1111/acel.14291. Epub ahead of print. PMID: 39236310.

2. Tropea MR, Gulisano W, **Vacanti V**, Arancio O, Puzzo D, Palmeri A. Nitric oxide/cGMP/CREB pathway and amyloid-beta crosstalk: From physiology to Alzheimer's disease. Free Radic Biol Med. 2022 Nov 20;193(Pt 2):657-668. doi: 10.1016/j.freeradbiomed.2022.11.022.

3. Maria Rosaria Tropea, Alberto Torrisi, **Valeria Vacanti**, Danilo Pizzone, Daniela Puzzo, Walter Gulisano. Application of 3D printing technology to produce hippocampal customized guide cannulas. eNeuro. 2022 Sep 13:ENEURO.0099-22.2022. doi: 10.1523/ENEURO.0099-22.2022.

Oral communications

1. **Vacanti V**, Tropea MR, Melone M, Li Puma D, Aceto G, Bandiera B, Trovato R, D'Ascenzo M, Conti F, Grassi C, Puzzo D. Blocking dopamine D3 receptors enhances hippocampal synaptic plasticity and memory via post-synaptic mechanisms. In: 74th SIF National Congress, Rome, Italy, 11-13 September 2024.

2. **Vacanti V**, Tropea MR, Melone M, Li Puma D, Aceto G, Bandiera B, Trovato R, Torrisi S, Leggio GM, Palmeri A,

D'Ascenzo M, Conti F, Grassi C, Puzzo D. Dopamine D3 receptor blockade enhances hippocampal synaptic function via post-synaptic mechanisms and rescues age-related cognitive deficits. In: 17th Annual Meeting of Young Researchers in Physiology, Catania, Italy, 22-24 May 2024.

Posters

1. **Vacanti V**, Tropea MR, Melone M, Li Puma DD, Vacanti V, Aceto G, Bandiera B, Trovato RC, D'Ascenzo M, Conti F, Grassi C, Puzzo D. The blockade of Dopamine D3 receptors improves hippocampal synaptic plasticity and memory via post-synaptic mechanisms. In Society for Neuroscience, Chicago, USA, 05-09 October 2024. (Poster Presenter)

2. Tropea MR, **Vacanti V**, Trovato RC, Puzzo D. The lack of Amyloid-beta production and function prevents the beneficial effects of phosphodiesterase inhibitors on hippocampal synaptic plasticity and memory. In Society for Neuroscience, Chicago, USA, 05-09 October 2024.

3. Tropea MR, **Vacanti V**, Trovato RC, Puzzo D. The cross-talk between Amyloid-β, α7 nicotinic acetylcholine receptors and cyclic nucleotides at the synapse: from physiology to Alzheimer's Disease. In 74th SIF National Congress, Rome, Italy, 11-13 September 2024.

4. Trovato RC, Tropea MR, **Vacanti V**, Melone M, Conti F, Puzzo D. The upregulation of GLT-1 expression rescues synaptic plasticity and memory impairment in α7 nicotinic acetylcholine receptors knock out mice. In: 74th SIF National Congress, Rome, Italy, 11-13 September 2024

5. **Vacanti V**, Tropea MR, Trovato RC, Puzzo D. Amyloid-β production and function are needed for cyclic nucleotides-mediated enhancement of synaptic plasticity and memory. In:

Federation of European Neuroscience Societies (FENS 2024) – Vienna, Austria 25-29 June 2024. (Poster Presenter)

6. Tropea MR, Melone M, Li Puma DD, **Vacanti V**, Aceto G, Bandiera B, Trovato RC, Torrisi SA, Leggio GM, Palmeri A, D'Ascenzo M, Conti F, Grassi C, Puzzo D. Dopamine D3 Receptor Blockade enhances hippocampal plasticity through post-synaptic mechanisms. In: Federation of European Neuroscience Societies (FENS 2024) – Vienna, Austria 25-29 June 2024.

7. Trovato RC, Tropea MR, **Vacanti V**, Melone M, Conti F, Puzzo D. The lack of α7 nicotinic acetylcholine receptors cause a dysregulation of glutamate reuptake underlying memory loss. In: 17th Annual Meeting of Young Researchers in Physiology, Catania, Italy, 22-24 May 2024.

8. **Vacanti V**, M.R. Tropea, M. Melone, F. Conti, D. Puzzo. The lack of α7 nicotinic acetylcholine receptors cause a dysregulation of glutamate reuptake underlying memory loss. In: 73rd SIF National Congress, Pisa, Italy, 6-8 September 2023. (Poster Presenter)

9. Tropea MR, **Vacanti V**, Gulisano W, Puzzo D. Amyloid-beta peptide is needed for cyclic nucleotide-mediated enhancement of synaptic plasticity and memory. In: 73rd SIF National Congress, Pisa, 6-8 Sept 2023.

10. Tropea MR, **Vacanti V**, Gulisano W, Puzzo D. Amyloid-beta peptide production and function is needed for cyclic nucleotides-mediated enhancement of memory. In 16th Annual Meeting of Young Researchers in Physiology. Bertinoro (FO), Jun 21-23, 2023.

11. Tropea MR, Melone M, **Vacanti V**, Centaro A, Gulisano W, Leggio GM, Conti F, Puzzo D. Inhibition of D3 receptors

rescues synaptic disfunction and memory impairment in aged and Alzheimer's disease mouse models. In: Neuroscience 2022, San Diego (CA), USA, 12-16 Nov 2022.

12. Tropea MR, Melone M, **Vacanti V**, Centaro A, Gulisano W, Leggio GM, Conti F, Puzzo D. Physiological role of dopamine D3 receptors in hippocampal synaptic plasticity and memory in physiological conditions and age-related disorders. In: 72nd SIF National Congress, Bari, Italy, 14-16 September 2022.

13. Tropea MR, Melone M, **Vacanti V**, Gulisano W, Leggio GM, Conti F, Puzzo D. Inhibition of dopamine d3 receptors improves hippocampal synaptic plasticity and memory. In: FENS Forum 2022, Paris, France, 9-13 July 2022.

14. Tropea MR, Gulisano W, Romano A, Giannino F, **Vacanti V**, Leggio GM, Puzzo D. Physiological role of dopamine D3 receptors in hippocampal synaptic plasticity and memory. In: The 39th Congress of International Union of Physiological Sciences, Beijing, China (Online) 7-11 May 2022.

Awards

1. Graduation award in Neuroscience "Umberto Scapagnini", University of Catania, 12/06/2023

2. Best Project Certificate in the psychosis constellation: from animal models to treatments of resistant psychoses. In: 20th Catania International Summer School of Neuroscience, University of Catania, 14/07/2023