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Role of Dopamine D3 receptor in the regulation of memory related genes

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XXVI cycle

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General introduction

Dopamine (DA) is the predominant catecholamine neurotransmitter in the brain. The major population of dopaminergic neurons in the brain are synthesized by mesencephalic neurons in the substantia nigra (SN) and ventral tegmental area (VTA). DA neurons originate in these nuclei and project to the striatum, cortex, limbic system and hypothalamus. Through these pathways, DA affects many physiological functions, such as the control of coordinated movements and hormone secretion, as well as motivated and emotional behaviors and cognition (Carlsson, 2001; Gainetdinov and Caron, 2003; Beaulieu and Gainetdinov, 2011; Tritsch and Sabatini, 2012).

The physiological actions of dopamine are mediated by five distinct receptor subtypes but closely related G protein-coupled receptors (GPCRs) that are divided into two major groups: the D_1-class dopamine receptors (D_1R and D_5R) (Tiberi et al.,1991) and D_2-class of dopamine receptors (D_2R, D_3R and D_4R) (Andersen et al., 1990; Niznik and Van Tol, 1992; Sibley and Monsma, 1992; Sokoloff et al., 1992; Civelli et al., 1993; Vallone et al., 2000). This classification is generally based on their structural, biochemical properties, on their ability to modulate cAMP production and the differences in their pharmacological properties. It is known that D_1-like receptors activate the Gs family of G proteins to stimulate intracellular cAMP production by adenylate cyclase (AC) (Dearry et al., 1990; Zhou et al.,1990; Grandy et al.,1991; Sunahara et al.,1991), while the D_2-like receptors coupled to the Gi family of G proteins induce inhibition of AC (Bunzow et al.,1988; Dal Toso et al.,1989; Sokoloff et al., 1990; VanTol et al.,1991; Neve et al., 2004). In contrast to the D1-class dopamine receptors, D_2 and D_3 dopamine receptors are expressed both postsynaptically on dopamine target cells and presynaptically on dopaminergic neurons (Sokoloff et al., 2006; Rondou et al., 2010) (Missale et al., 1998; Sibley et al., 1999).

D_3R are involved in reward and reinforcement mechanisms. A growing body of pharmacological evidences (Gainetdinov et al., 1996; Zapata and Shippenberg, 2002) and genetic studies in D_3 dopamine receptor knockout mice (Sibley, 1999) suggest that D_3 autoreceptors may also contribute to the presynaptic regulation of tonically released dopamine,

thereby complementing the D_2 autoreceptor's role in regulating of synthesis and phasic release of dopamine (De Mei et al., 2009). D_3R seem to influence some specific aspects of cognitive functions that are mediated by hippocampal areas (Missale et al., 1998; Sibley, 1999; Sokoloff et al., 2006).

In the first study we investigated the role of the D_3R during acquisition of a behavioral task of associative memory and we underpinned the molecular events that contribute to the enhanced cognitive performance of D_3 knockout mice (Micale et al., 2010).

The rational of our work was based on evidence indicating that $D_3^{-/-}$ mice exhibit enhanced cognitive performance in the single trial step-trough passive avoidance (PA) task as compared to wild type mice (Micale et al., 2010) and data suggesting a functional connection among D_3R, neurofibromin (NF1) and amyloid precursor protein (APP), two genes related to cognitive function (Donarum et al., 2006; Castorina et al., 2011).

The NF1 gene encodes for a large cytoplasmic tumor suppressor protein called neurofibromin, its loss results in constitutive activation of the Ras signalling pathway (Lau et al., 2000), which has been implicated both in neuronal activity and synaptic plasticity (Costa et al., 2002).

APP plays an important role in brain development, memory and synaptic plasticity (Nalivaeva and Turner, 2013). Dysfunctions in the metabolic processes of APP are widely hypothesized to underlie Alzheimer's disease (AD) (Selkoe, 2002). Indeed amyloid β-peptide (Aβ), derived by proteolytic processing of the membrane glycoprotein amyloid precursor protein (APP), lead to the formation of Aβ plaques, synapse dysfunction/loss, neuronal loss, and overall brain atrophy, which cause decline of cognitive abilities (Selkoe, 2002).

Our hypothesis was that D_3R expression could be influenced by acquisition of PA task and it was involved in transcriptional regulation of NF1 and APP.

To address this question we evaluated the mRNA and protein expression levels of D_3Rs, NF1 and APP genes in the hippocampus of both wild-type (WT) and $D_3R^{-/-}$ mice performing the PA task. Our results indicated that acquisition of behavioral task leads to increased expression levels of D_3Rs and NF1, but not of APP in hippocampus of WT mice. Since NF1 does not

change in its expression in $D_3R^{-/-}$ mice, we suggested that D_3R might be involved in trascriptional hippocampal regulation of NF1 gene.

It's well documented that under dopaminergic tone the two classes of dopaminergic receptors activate different signaling pathways, which can lead to activation of different transcription factors, like CREB, which in turn induce transcription of genes resulting in proteins underline consolidation of memory (Beaulieu and Gainetdinov, 2011). CREB is a transcription factors which plays a critical role in many function including neurogenesis, neural survival, differentiation, synaptic plasticity (Carlezon et al., 2005; Mioduszewska et al., 2003; Silva et la., 1998). The analysis of the intracellular signal transduction pathway that leads from stimulation of dopamine receptors to CREB phosphorylation is an important step toward elucidating the relationship between molecular adaptations and behavioral consequences (Dudman et al., 2003). Since the involvement of D_3R in hippocampal CREB activation of mice during PA conditioning has not investigated yet, we attempted to clarify the role of this receptor in regulating the activity of CREB during PA conditioning, and to underly the signaling pathways involved in D_3R-induced CREB activation.

To address this question, we evaluated the posphorylation levels of CREB in hippocampus of WT and $D_3^{-/-}$ mice performing PA task, and subsequently we evaluated the phosphorylation/activation levels of different protein kinase involved in CREB activation, like MAPKs. Our results showed increased phosphorylation levels of CREB and ERK in $D_3^{-/-}$ mice as compared to WT, while JNK and p-38 don't showed changes in their activation. Serine/threonine kinase Akt is also involved in CREB activation, thus we have also analyzed the phosphorylation levels of Akt in threonine 308 residue. Results reveal that its phosphorylation levels increased only in $D_3^{-/-}$ mice, while the acquisition of behavioral paradigm don't affected its activation. Data suggest that D_3Rs could modulate hippocampal CREB activation probably through phosphorylation of ERK.

Several studies show that dopaminergic receptors can modulate CREB activation through different downstream substrates, like DARPP-32 (32-kDa dopamine and cAMP-regulated phosphoprotein) (Nishi et al., 1997) via cAMP-dependent pathway and other studies show that

D_2-like receptors may also trigger a cAMP-indipendent pathway and it has been proposed that D_3R participate in D_2R cAMP-independent pathway by enhancing D_2R-mediated Akt (Thr 308) phosphoryation (Beaulieu et al., 2007). In particular when DARPP-32 is phosphorylated by PKA on Th34, inhibiting the activity of PP-1, acts in synergic manner with different protein kinases to increase the phosphorylation levels of various downstream effector proteins, among these CREB (Greengard et al., 1999). To the other hand, dephosphorylation of Akt following dopamine binding to D_2-like receptor leads to a reduction of kinase activity and a concomitant activation of its substrates glycogen synthase kinase (GSK-3β), which is negatively regulated by Akt, and this results in inhibitory effects on CREB activation (Beaulieu et al., 2005; 2007; Cross et al., 1995). Furthermore, is well known that CREB phosphorylation leads to activation of CREB responsive genes, among these BDNF (Brain-derived neurotrophic factor) or the enzyme involved in its proteolytic processing such as tPA (tissue plasminogen activator) which catalyzes the conversion of plasminogen in plasmin and leads to converting pro-BDNF in mature BDNF (Boneva and Yamashima, 2012; Ohlsson et al., 1993; Benito and Barco, 2010). However there are not evidences as regard role of D_3Rs in basal transcriptional regulation of tPA.

In the third work we hypothesized that the dopamine D_3Rs can influenced baseline tPA activity in prefrontal cortex and hippocampus, two brain region involved in learning and memory process, by modulation of Akt/CREB signaling cascade.

Results revealed that both in prefrontal cortex and hippocampus of $D_3^{-/-}$ mice, tPA, mBDNF and the expression ratio of plasmin/plasminogeno both mRNA and protein expression levels were significantly increased as compared to the WT mice. Concurrently basal Akt phosphorylation at Thr308, DARPP-32 at Thr34 and GSK3β at Ser9 were significantly increased both in the prefrontal cortex and hippocampus of $D_3^{-/-}$ mice. These data suggesting that the increased tPA activity in $D_3^{-/-}$ mice could be modulated by DARPP-32 and Akt/GSK3β signaling cascade.

Hippocampal neurofibromin and amyloid precursor protein expression in dopamine D_3 receptor knock-out mice following passive avoidance conditioning

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Abstract

Passive avoidance (PA) conditioning is a fear motivated task able to initiate a cascade of altered gene expression within the hippocampus, a structure critical to learning and memory. We have previously shown that neurofibromin (NF1) and amyloid precursor protein **(**APP), two genes implicated in cognitive function, are differentially expressed in brain of dopamine D_3 receptor knock-out mice ($D_3R^{-/-}$), suggesting that the receptor might have a role in their trascriptional regulation. Here in this study, we hypothesized that during acquisition of PA conditioning the expression of NF1 and APP genes could be influenced by D_3Rs. To address this issue, we analyzed the expression of NF1 and APP in the hippocampus of both wild-type (WT) and $D_3R^{-/-}$ mice subjected to the single trial step-through PA paradigm. Our finding demonstrated that (1) $D_3R^{-/-}$ mice exhibit increased cognitive performance as compared to wild-type (WT) mice in the step-through PA trial; (2) acquisition of PA increased D_3R and NF1, but not APP expression in WT mice hippocampus; (3) PA-driven NF1 induction in WT was abrogated in $D_3R^{-/-}$ mice and finally that (4) the heightened basal APP expression observed in naive $D_3R^{-/-}$ mice was totally reversed by acquisition of PA. In conclusion, the present finding show for the first time that both D_3R and NF1 genes are upregulated following PA conditioning and suggest that hippocampal D_3Rs might be relevant to NF1 transcriptional regulation in the hippocampus.

Keywords : dopamine D3 receptor, neurofibromin, amyloid precursor protein, passive avoidance, hippocampus

Introduction

Dopamine (DA) is a neurotransmitter with a broad array of effects in the central nervous system. The actions of DA are mediated by five distinct G-protein coupled receptors grouped into two subclasses: D_1-like (D_1R and D_5R) and D_2-like (D_2R, D_3R, and D_4R), based on their structural and pharmacological properties (Missale et al., 1998; Karasinska et al., 2005). The D_3R, cloned by Sokoloff, (1990), is an autoreceptor mainly distributed within limbic areas, as well as in brain regions critical to learning and memory, such as the hippocampus (Levant, 1998; Castorina et al., 2011)

Involvement of hippocampal D_2-like receptors in mnemonic processes has been attentioned by several research groups in human (Kaasinen et al., 2000; 2002) and rodents (Laszy et al., 2005; Izquierdo et al., 2006; Micale et al., 2010). Furthermore, it has been suggested that disturbances in hippocampal DAergic systems cause memory impairment (Gasbarri et al., 1996). However, whether hippocampal expression of D_3R is influenced by acquisition of a fear-motivated task has still not been evaluated.

Passive avoidance (PA) conditioning is a fear-motivated task able to trigger altered gene expression within the hippocampus (Izquierdo et al., 2006; Izquierdo et al., 2000; D'Agata and Cavallaro, 2003). Recently, we have shown that $D_3R^{-/-}$ mice exhibit changes in the expression of two genes related to cognitive function, namely NF1 and APP (Castorina et al., 2011; Costa et al., 2002; Marcello et al., 2008).

The NF1 gene encodes neurofibromin, a large protein with Ras GTPase activity (De Schepper et al., 2006; Guilding et al., 2007). Neurofibromin works by inhibiting excessive accumulation of the protein Ras, responsible for the increased GABA-mediated inhibition of hippocampal synaptic transmission. As such, a nonfunctional NF1 gene may ultimately lead to increased GABA activity and consequently learning deficits (Costa et al., 2002; Guilding et al., 2007). Amyloid precursor protein (APP) is a type 1 membrane glycoprotein distributed in the central and peripheral nervous system (Selkoe, 2002; Seabrook and Rosahl, 1999). APP is alternatively processed by three different proteases, α- β- and γ secretases, to produce either non-amyloidogenic or amyloidogenic Aβ fragments. These fragments may aggregate and lead to

deposition of senile plaques in the cortex and hippocampus, a hallmark of Alzheimer's disease (AD) (Moran et al., 1995; Senechal et al., 2008; DeGiorgio et al., 2002; Suh and Checler, 2002). However, despite the pathological significance of APP in AD, its well-known involvement in physiological neuronal function, such as synapse formation, axonal and dendritic outgrowth, suggest that APP may have important implications in signal transduction (De Strooper and Annaert, 2000) and memory (Marcello et al., 2008; Seabrook and Rosahl, 1999; De Strooper and Annaert, 2000). To support this, it has been reported that mice deficient in APP show a decline in memory performance which is associated with a loss of synaptic markers, further implying that APP may be critical for synaptic function and for the neuroplastic events that accompany a learning task (Dawson et al., 1999; Conboy et al., 2005).

Previously we have shown that expression levels of NF1 and APP are modified in various brain regions, including the hippocampus, of $D_3R^{-/-}$ mice, suggesting that the receptor might be implicated in the transcriptional regulation of these two memory-related genes (Castorina et al., 2011).

Herein this study, we hypothesized that during acquisition of PA the expression of NF1 and APP could be influenced by D_3Rs. We demonstrated that (1) $D_3R^{-/-}$ mice exhibited increased cognitive performance as compared to wild type (WT) mice in the step-through PA task; (2) that acquisition of PA was associated with increased D_3R and NF1, but not APP expression in the hippocampus of WT mice and finally (3) that D_3R is required for PA-driven NF1 induction.

Materials and Methods

Animals

All experiments were carried out on $D_3R^{-/-}$ and WT mice (male mice 8-12 weeks old). The animals were housed four *per* cage and fed with standard laboratory food and allowed free access to water *ad libitum*, in an air-conditioned room with a 12 h light-dark cycle. All the experimental procedures were performed during the light cycle (between 10 a.m. and 2 p.m.). $D_3R^{-/-}$ mice used in these experiments were 5th-8th generation of congenic C57BL/6J mice, and

generated by a backcrossing strategy. The genotypes of the D_3R mutant and WT mice were identified by a PCR method by using two pairs of primers flanking either exon 3 of the wild-type D_3R or the PGK (phosphoglycerate kinase 1 gene promoter) cassette of the mutated gene (Accili et al., 1996). All animals were used only once in the experiments, which were carried out according to the European Community Council Directive 86/609/EEC. Efforts were made to minimize animal suffering and to reduce the number of animals used. The rationale, design and methods of this study were approved by the Ethical Committee for Animal Research, University of Catania.

Passive avoidance test

The single trial step-through passive avoidance test was performed as previously described (Venault et al., 1986; Shirayama et al., 2002). using a passive avoidance apparatus (San Diego Instruments, Inc., San Diego, CA, USA). The apparatus was divided into two compartments by a retractable door: a lit safe compartment and a darkened shock compartment.

The experiment was carried out on male homozygous $D_3R^{-/-}$ (n=56) and WT mice (n=62). Each strain of animals was divided in four groups. The first group (naive, n=14 for $D_3R^{-/-}$ and n=17 for WTs, respectively) was maintained in the home cage. The rest of the animals experienced a 2-day behavioral training. On the first day, animals were handled by the experimenter for 2 min and then placed into the safe compartment and allowed to explore both chambers of the apparatus for 3 min. The second day, in the training trial, the second group of animals (termed 'conditioned animals', CA; n=17 for WT and n=14 for $D_3R^{-/-}$, respectively) were placed in the safe compartment with the door closed. After 2 min of acclimatization the light was turned on, the door opened and the animal was allowed to enter the dark compartment. After the mouse stepped completely with all four paws into the dark compartment, the door was closed, and a mild inescapable foot shock (0.5 mA, 2 s duration) was delivered from the grid floor. Following the shock, the mouse was removed and returned to its home cage. A third group of animals (termed 'conditioned stimulus-trained animals', CSTA; n=14 for each genotype) were placed into the safe compartment. After 2 min of acclimatization the light was turned on, the door

opened and the animal allowed to enter the dark compartment. After the mouse stepped into the dark compartment, the door was closed but no foot shock was delivered from the grid floor. Then mice returned to their home cage. The fourth group of animals (termed 'unconditioned stimulus-trained animals', USTA; n=14 for each genotype) were placed in one of the two dark compartments. They were allowed to move freely to both compartments. After 2 min of acclimatization they received an inescapable foot shock (0.5 mA, 2 s duration) and then returned to their home cage.

Six hours later, animals from each of the four experimental groups (n=6, except for naive and CA WTs, n=9) were sacrificed by cervical dislocation, hippocampi were rapidly dissected and stored at -80°C until use.

Twenty-four hours after the training trial, the remaining animals from each of the four experimental groups (n=8) performed the retention test. The animals were placed in the safe compartment with the door closed. After 2 min of acclimatization the light was turned on, the door opened and the animal was allowed to enter the dark compartment. The latency to enter the dark compartment was recorded and used as the measure of retention. Mice avoiding the dark compartment for >300 s were considered to have a step-through latency of 300 s (D'Agata and Cavallaro, 2003; Venault et al., 1986).

Measurement of D_3R, NF1 and APP levels by quantitative real time PCR

Hippocampal total RNA extracts from $D_3R^{-/-}$ (n=3 for each experimental group) and WT (n=3 for each experimental group) mice were isolated by 1 ml TRIzol reagent (Invitrogen) and 0.2 ml chloroform and precipitated with 0.5 ml isopropanol. Pellet was washed with 75% ethanol and air dried. Single stranded cDNAs were synthesized by incubating total RNA (5µg) with SuperScript III RNase H-reverse transcriptase (200 U/µl) (Invitrogen); Oligo-$(dT)_{20}$ primer (100 nM) (Invitrogen); 1 mM dNTP mix (Invitrogen), dithiothreitol (DTT, 0.1 M), Recombinant RNase-inhibitor (40 U/µl) at 42°C for 1 h in a final volume of 20 µl. Reaction was terminated by incubation of samples at 70°C for 10 min.

Aliquots of cDNA (400 ng) from WT and $D_3R^{-/-}$ mice hippocampi and external standards at known amounts (purified PCR products, ranging from 10^2 to 10^8 copies) were amplified in parallel reactions, using primer pairs indicated in table 1. mRNA levels of the reference gene, 18S ribosomial subunit, were measured in each amplification. Each PCR reaction contained 0.5 μM primers, 1.6 mM $MgCl^{2+}$, 1X Light Cycler-FastStart DNA Master SYBR Green I (Roche Diagnostic). Amplifications were performed using the Light Cycler 1.5 instrument (Roche Diagnostic) with the following program setting : (I) cDNA denaturation (1 cycle: 95°C for 10 min); (II) quantification (45 cycles: 95°C for 10 s, 60°C for 30 s, 72°C for 7 s); (III) melting curve analysis (1 cycle: 95°C for 0 s, 65°C for 15 s, 95°C for 0 s); (IV) cooling (1 cycle: 40°C for 30 s). Quantification was obtained by comparing the fluorescence emitted by PCR products at unknown concentration with the fluorescence emitted by external standards at known concentration. For this analysis, fluorescence values, measured in the log-linear phase of amplification, were estimated with the second derivative maximum method using Light Cycler Data Analysis software. PCR products specificity was evaluated by melting curve analysis.

To assess the different expression levels we analyzed the mean fold change values of each sample, calculated using the comparative Ct method (Schmittgen and Livak, 2008). The Ct represents the number of cycles needed to detect a fluorescence above a specific threshold level and it is inversely correlated to the amount of nucleic acids template present in the reaction. The ΔCt was calculated by normalizing the mean Ct of each sample to the mean Ct of the reference gene measured in the same experimental conditions. For the quantification of each gene we considered the naive WT mice group as the positive sample (calibrator sample). The ΔΔCt of each sample was then calculated by subtracting calibrator ΔCt to sample ΔCt. The formula $2^{-\Delta\Delta Ct}$ was used to calculate fold changes. Baseline measurements for each calibrator sample were set to 1.

Table 1: Primer sequences.

Forward and reverse primers were selected from the 50 and 30 region of each gene mRNA. The expected length of each PCR amplification product is indicated in the right column

Gene	*Forward*	*Reverse*	*bp length*
NF1Acc#NM_010897.2	TTCGATACACTTGCGGAAAC	CACATTGGCAAGAGCCATAG	114
APPAcc# NM_007471	GGTTCTGGGCTGACAAACAT	CAGTTTTTGATGGCGGACTT	102
Dopamine D3 receptor Acc# NM_007877	GGGGTGACTGTCCTGGTCTA	AAGCCAGGTCTGATGCTGAT	110
Ribosomal protein 18S Acc# NM_011296.2	GAGGATGAGGTGGAACGTGT	GGACCTGGCTGTATTTTCCA	115

Western blot analysis

Crude extracts from WT (n=3 for each experimental group) and $D_3R^{-/-}$ (n=3 for each experimental group) mice hippocampi were prepared by homogenizing samples in a buffer containing 20 mM Tris (pH 7.4), 2 mM EDTA, 0.5 mM EGTA; 50 mM mercaptoethanol, 0.32 mM sucrose and a protease inhibitor cocktail (Roche Diagnostics) using a Teflon-glass homogenizer and then sonicated twice for 20 sec using an ultrasonic probe, followed by centrifugation at 10.000 g for 10 min at 4 °C. Protein concentrations were determined by the Quant-iT Protein Assay Kit (Invitrogen). Sample proteins (30 μg) were diluted in 2X Laemmli buffer (Invitrogen, Carlsbad, CA, USA), heated at 70°C for 10 min and then separated on a Biorad Criterion XT 4-15% Bis-tris gel (Invitrogen) by electrophoresis and then transferred to a nitrocellulose membrane (Invitrogen). Blots were blocked using the Odyssey Blocking Buffer (Li-Cor Biosciences). Immunoblot analysis was performed by using a rabbit polyclonal antibody raised against amino acids 1-50 of D_3R of human origin (sc-9114, Santa Cruz

Biotechnology Inc), a rabbit polyclonal antibody raised against amino acids 676-695 of APP of human origin (A8717, Sigma), a rabbit polyclonal antibody raised against peptide mapping within the C-terminus of neurofibromin of human origin (sc-67, Santa Cruz Biotechnology Inc) and a rabbit polyclonal antibody raised against amino acids 210-444 of β-tubulin of human origin (sc-9104, Santa Cruz Biotechnology Inc). All primary antibodies were diluted 1:200, while the secondary antibody (goat anti-rabbit IRDye 800nm, cat #827-06905; Li-Cor Biosciences) was used at 1:20000. Blots were scanned with an Odissey Infrared Imaging System (Odyssey). Densitometric analyses of Western blot signals were performed at non-saturating exposures and analyzed using the ImageJ software (NIH, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html). Values were normalized to β-tubulin, which served as loading control. No signal was detected when the primary antibody was omitted (data not shown).

Tissue preparation for immunohistochemical staining

Brains from decapitated mice (naive WT, n=3; CA WT, n=3) were removed and stored for at least 24 h in 4% formaldehyde at 4°C before dehydration and embedding in paraffin. Ten-micrometer-thick sections were cut, mounted on glass slides, kept overnight at 37°C, and then at room temperature until use. Prior to immunohistochemical staining, the sections were dewaxed in xylene and rehydrated through graded alcohols. They were then rinsed in 0.1M Tris–HCl buffered saline (TBS, pH 7.4) and treated with 3% hydrogen peroxide (H_2O_2) in PBS for 10 min to reduce endogenous peroxidase activity.

Immunohistochemical analysis

Immunohistochemical analysis was performed in accordance with the standard ABC method. To reduce nonspecific staining, sections were treated with 5% bovine serum albumin (BSA) and 3% goat serum in TBS for 1 h. Sections were then incubated with a rabbit polyclonal antibody raised against amino acids 1-50 of D_3R of human origin (sc-9114, Santa Cruz Biotechnology Inc). The antibody was diluted in TBS containing 3% normal goat serum (NGS),

1% BSA, and 0.25% Triton X-100. After several rinses in TBS, the sections were incubated with a 1:200 diluted biotinylated goat anti-rabbit IgG for 1 h at room temperature. To visualize the immunoreaction sites in tissues, the sections were then rinsed and treated with reagents from an ABC Kit for 1 h at room temperature. The sections were rinsed in TBS and incubated with 0.025% 3,3-diaminobenzidine (DAB) plus 0.33% H_2O_2 in TBS for 10 min. Then, Tris buffer was added to stop the DAB reaction. The stained sections were dehydrated through graded alcohols, cleared in xylene, and covered with neutral balsam. All sections were examined and images were taken with a light microscope (Axiovert, Carl Zeiss Inc) equipped with a digital color camera. The images were further processed using Adobe Photoshop software.

Statistical analysis

One-way analysis of variance (ANOVA) was used to compare differences among three or more groups followed by Tukey *post-hoc* test to evaluate statistical significances. A level of $p<0.05$ was accepted as indicative of significant difference.

Results

Cognitive performance of WT and $D_3R^{-/-}$ mice in the passive avoidance test

Mice were trained using a behavioral protocol, the single trial step-through passive avoidance test, known to require hippocampus-dependent learning (Venault et al., 1996). In these experiments, conditioned animal (CA) were trained to avoid moving from the lighted to the darkened section of a conditioning chamber by delivering a foot-shock when they entered the darkened section. Control mice included untrained (naive) animals, and animals exposed to the unconditioned (USTA) or the conditioned (CSTA) stimulus. To verify that the trained mice in fact learned the passive avoidance (PA) task, learning was assessed in a comparable group of animals by evaluating the latency of step-through in the retention test. Twenty four hours after the one-trial training period, only CA in either genotype learned to associate stepping through the darkened chamber with the foot shock (Fig. 1) ($F_{7,63}=40.90$; *** $p<0.001$ vs naïve and

CSTA WT mice, # p<0.05 vs USTA WT mice, §§§ p<0.001 vs naïve CSTA and USTA $D_3^{-/-}$ mice) (D'Agata and Cavallaro, 2003). Furthermore, CA $D_3R^{-/-}$ mice exhibited a better behavioral response as compared to CA WTs in the retention test (Fig. 1) (++ p<0.01 vs CA WT, One-Way ANOVA followed by Tukey-Kramer *post-hoc* test).

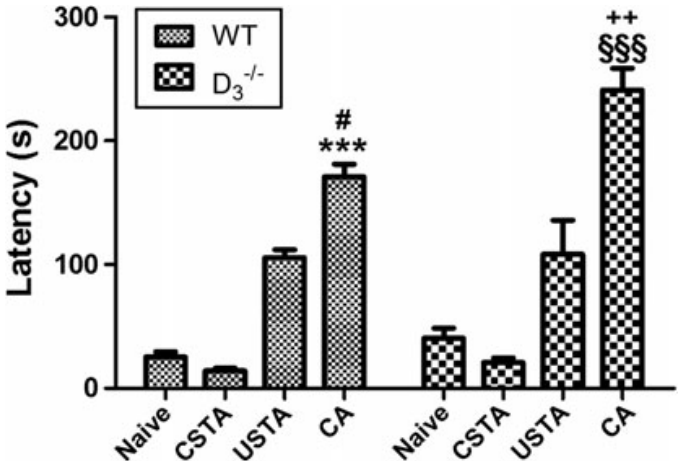

Figure 1. Cognitive response of WT and $D_3R^{-/-}$ animals in the passive-avoidance paradigm

Cognitive response of WT and $D_3R^{-/-}$ animals in the passive avoidance paradigm. Conditioned animals (CA) were trained to avoid moving from the lighted to darkened section of a conditioning chamber by the delivery of the foot shock when they entered the darkened section. Control mice included untrained (naive) animals, and animals exposed to the conditioned (CSTA) or unconditioned (USTA) stimulus. The values (time in seconds taken for re-entering the dark box measured in the retention test performed 24 h after the learning trial) are the means ± S.E.M. of WT (n=8 *per* group) and $D_3R^{-/-}$ mice (n=8 *per* group) (*** p<0.001 vs naïve and CSTA WT mice, # p<0.05 vs USTA WT mice, §§§ p<0.001 vs naïve CSTA and USTA $D_3^{-/-}$ mice, ++ p<0.01 vs CA WT mice, One-Way ANOVA followed by Tukey-Kramer *post-hoc* test).

D_3R expression in the hippocampus of WT mice after the acquisition of passive avoidance trial

To evaluate whether acquisition of PA influenced hippocampal D_3R expression in WT mice, we performed both quantitative real-time PCR and Western blot analyses 6 h after the training task,

an interval of time sufficient to observe changes at protein level. To exclude the potential involvement of non-learning based, state-dependent changes such as arousal or stress factors which could affect gene or protein expression, CSTA mice (mice that were subjected to the same experimental procedure as trained animals with the exception that they did not receive the associative stimulus, i.e. the inescapable footshock) and USTA mice (mice that received only the inescapable footshock stimulus) were included as further control groups. Comparative analyses with control groups demonstrated that acquisition of PA in CA WT animals significantly increased D_3R expression both at mRNA ($F_{3,23}$=41.89, ***p<0.001 vs naïve, CSTA and USTA) and protein levels ($F_{3,11}$=12.83, ***p<0.001 vs naïve, CSTA and USTA) (Fig. 2 A-C).

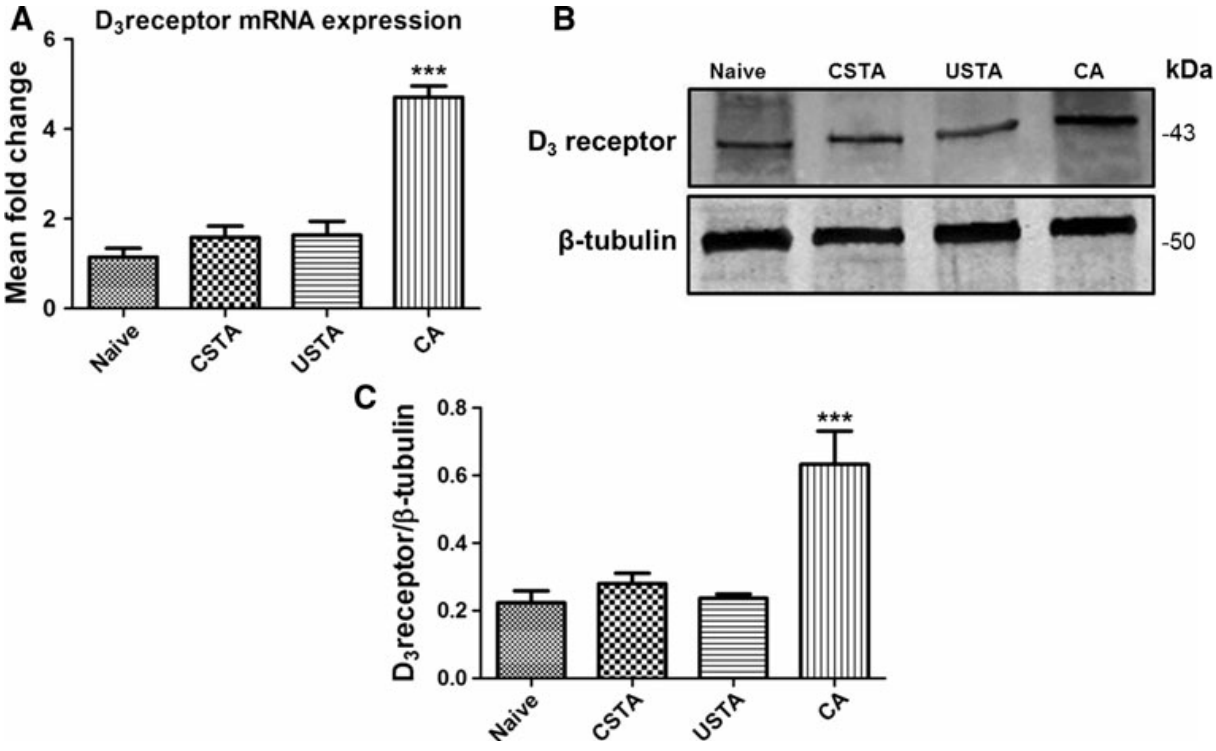

Figure 2. D_3R mRNA and protein expression in the hippocampus of WT mice after acquisition of the passive avoidance trial

(**A-C**) Quantitative real-time PCR and Western blot analyses showing increased D_3R mRNA and protein expression in the hippocampus of WT mice 6 h after the acquisition of the passive avoidance trial (CA) with respect to naïve, CSTA and USTA animals. (**A**) Results are presented as mean fold changes of controls (Naive, CSTA and USTA, n=3 *per* group) and conditioned

animals (CA n=3) ± S.E.M. Relative fold changes of D_3R expression were normalized to the endogenous ribosomal protein 18S (housekeeping gene) and then calculated using the comparative ΔCt method. Baseline expression levels of the control group were set to 1. Experiments were performed four times independently, each run in duplicate. (**B**) Representative immunoblots containing 30µg of tissue homogenates (n=3 hippocampi *per* group) were incubated using a rabbit polyclonal D_3R antibody and scanned with an Odyssey Infrared Imaging System, as described in Materials and Methods section. (**C**) Bar graph showing bands intensity ratios normalized to β-tubulin which were obtained using the ImageJ software and are expressed as mean ± S.E.M from at least three independent determinations. *** $p<0.001$ vs Naïve, CSTA and USTA WT mice, as determined by One-Way ANOVA followed by Tukey-Kramer *post-hoc* test.

Hippocampal D_3R immunolocalization in WT mice subjected to PA conditioning

To determine hippocampal D_3R distribution before and after acquisition of PA conditioning immunohistochemical analyses were carried out in brain sections of both naive and CA WT mice. Naive mice sections served as control. As shown in Fig. 3, no evident changes in the distribution of D_3Rs between the two mice groups were apparent in the hippocampal regions examined (CA1, CA2, CA3 and dentate gyrus, respectively). However, the weak D_3R signal intensity observed in hippocampal regions of naive WT mice was remarkably increased by PA acquisition in CA1-CA3 fields, but not in the dentate gyrus of CA mice (Fig. 3).

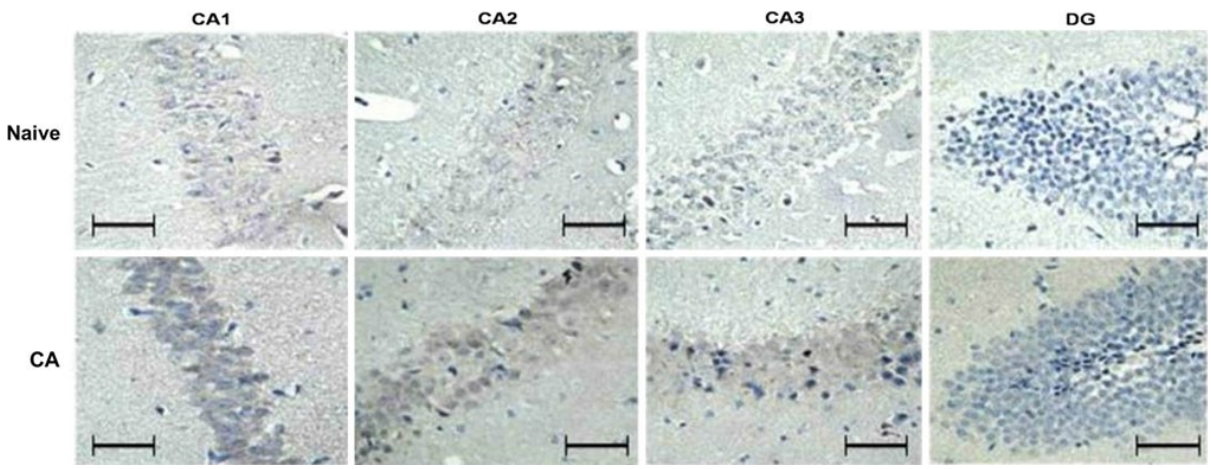

Figure 3. D_3R distribution in the hippocampus of naive and trained WT mice. Representative photomicrograhs showing D_3R immunoreactivity in specific hippocampal brain regions (CA1-CA2-CA3 and dentate-gyrus, respectively) of WT mice before (naïve) and after acquisition (CA) of the passive avoidance task. No apparent changes in receptor distribution are visible between the two groups. However, the weak D_3R positiveness in naive animals is clearly increased following the conditioning trial almost in every hippocampal region examined (CA1, CA2 and CA3 fields), except the dentate gyrus. Scale bar = 40μm. Images were taken from different brain sections of naive and CA WT animals and examined under a light microscope (Axiovert, Carl Zeiss Inc) equipped with a digital color camera.

NF1 and APP expression in the hippocampus of WT and $D_3R^{-/-}$ mice after the acquisition of passive avoidance trial

To establish whether acquisition of PA differentially influenced NF1 and APP mRNA and protein expression levels either in the presence or absence of D_3Rs, quantitative real-time PCR and Western blot analyses were performed in hippocampi of both WT and $D_3R^{-/-}$ mice from the four experimental groups (detailed in Materials and Methods section).

We found that NF1 mRNA expression, as well as its gene product neurofibromin, were significantly upregulated in the hippocampus of CA WTs in comparison with naive, CSTA or USTA mice ($F_{7,47}$ = 17.4 ***p<0.001 vs naive, CSTA and USTA mRNA levels; $F_{7,23}$= 113.4 ***p<0.001 vs naive, CSTA and USTA protein levels, respectively) (Fig. 4 and 5). Interestingly, PA-driven increase in gene and protein expression was completely abrogated in $D_3R^{-/-}$ mice (Fig. 4 and 5), suggesting that D_3R might be necessary for the transcriptional regulation of NF1.

In contrast to NF1, neither APP mRNA nor protein levels were affected by acquisition of the PA trial in WT mice, whereas the heightened basal expression observed in naive (Castorina et al., 2011) CSTA or USTA $D_3R^{-/-}$ mice was significantly reduced by acquisition of the avoidance task ($F_{7,47}$ = 28.03 §§§p<0.001 vs naive, CSTA and USTA mRNA levels; $F_{7,23}$= 41.66 §p<0.05 vs naive protein levels, respectively) (Fig. 4 and 5).

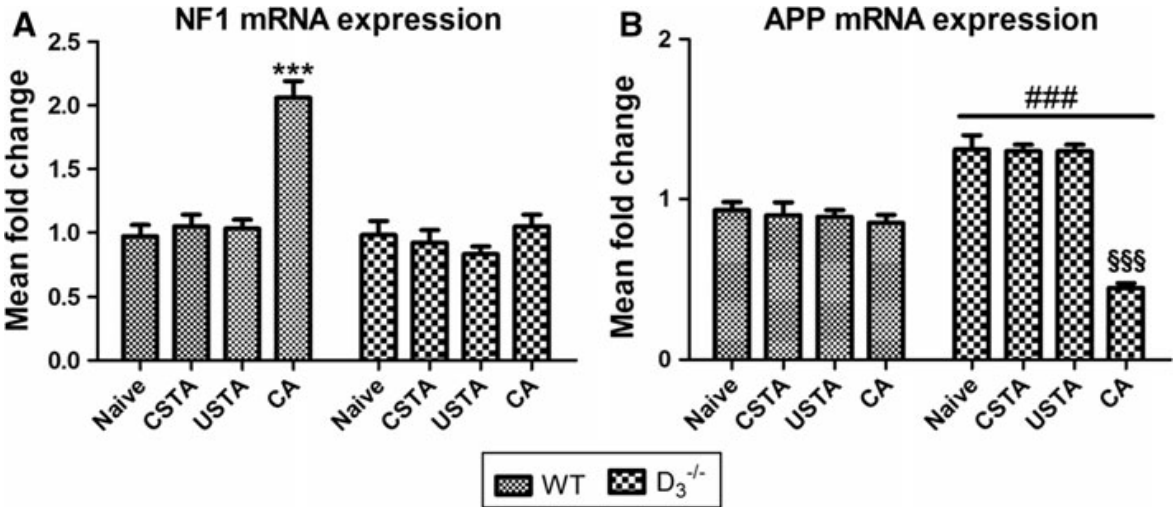

Figure 4. NF1 and APP mRNA expression in the hippocampus of WT and $D_3R^{-/-}$ mice after acquisition of the passive avoidance trial

Data obtained for quantitative real-time PCR analyses showing NF1 and APP mRNA expression in the hippocampus of WT mice 6h after the acquisition of the passive avoidance trial (CA) as compared to naïve, CSTA or USTA mice. For more details on experimental groups refer to the corresponding "Materials and Methods" subsection. Results are presented as mean fold changes of WTs (n=3 *per* group) and $D_3R^{-/-}$ (n=3 *per* group) ± S.E.M. Relative fold changes of either NF1 (**A**) or APP (**B**) genes were normalized to the endogenous ribosomal protein 18S (housekeeping gene) and then calculated using the comparative Ct method. Baseline expression levels of the control group (Naive WT) were set to 1. Experiments were performed four times independently, each run in duplicate. *** $p<0.001$ vs Naïve, CSTA and USTA WT mice, ### $p<0.001$ vs WT mice, §§§ $p<0.001$ vs Naïve, CSTA and USTA $D_3R^{-/-}$ mice, as determined by One-Way ANOVA followed by Tukey-Kramer *post-hoc* test.

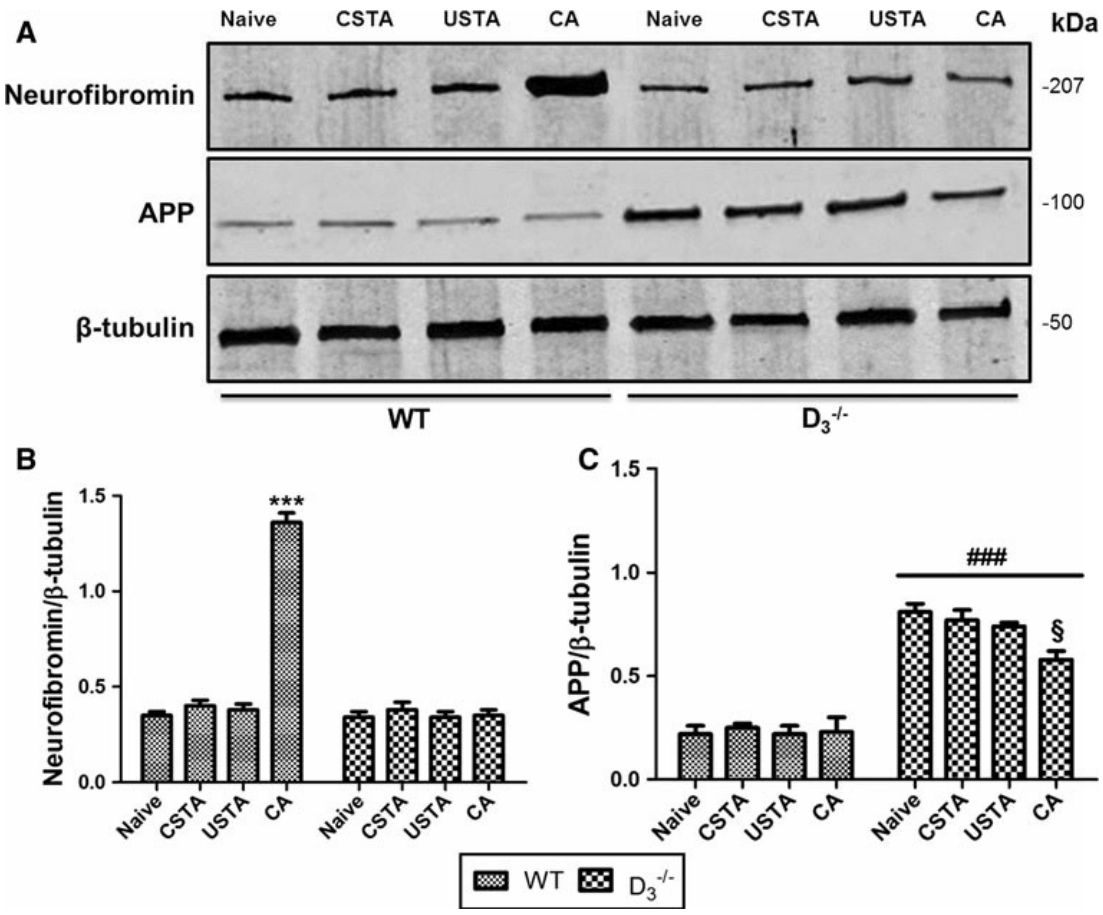

Figure 5. Neurofibromin and APP protein expression in the hippocampus of WT and $D_3R^{-/-}$ mice after acquisition of the passive avoidance trial

(**A**) Representative immunoblots containing 30µg of tissue homogenate (n=3 hippocampi *per* group) were incubated using rabbit polyclonal antibodies raised against both neurofibromin and APP and scanned with an Odyssey Infrared Imaging System, as described in the corresponding "Materials and Methods" subsection.

(**B-C**) Bar graphs showing relative bands intensities normalized to β-tubulin were obtained using the ImageJ software and are expressed as mean ± S.E.M. *** $p<0.001$ vs Naïve, CSTA and USTA WT mice, ### $p<0.001$ vs WT mice; § $p<0.05$ vs Naïve $D_3R^{-/-}$ mice, as determined by One-Way ANOVA followed by Tukey *post-hoc* test.

Discussion

The rationale of the present study was based on previous evidence indicating that $D_3R^{-/-}$ mice exhibit enhanced cognitive performance in the single trial step-through passive avoidance (PA) task as compared to WTs (Micale et al., 2010) and on our recent observation showing that expression levels of both NF1 and APP genes are modified in various brain regions, including the hippocampus, of mice lacking D_3R (Castorina et al., 2011). Since PA is known to initiate a cascade of altered gene expression in the hippocampus (Izquierdo et al., 2000), we first hypothesized that D_3R expression might be affected by acquisition of the trial and subsequently, that receptor could be involved in the transcriptional regulation of these two memory-related genes.

As shown in Fig. 1, results obtained from the PA behavioral paradigm are in agreement with previous data showing that the genetic inactivation of D_3R ameliorates the learning processes of rodents subjected to several experimental cognitive paradigms. The mechanisms underlying the enhanced cognitive performance are not fully understood, even though the involvement of this receptor in the control of AChergic transmission has been suggested (Glickstein et al., 2005; Lacroix et al., 2006; Millan et al., 2007). However, the potential interaction with AChergic systems may be just part of a bigger puzzle, since the involvement of endocannabinoid/endovanilloid systems have also been proposed (Micale et al., 2010). Therefore, it is likely that acquisition of a memory-related task involves co-activation of a multitude of systems, which thereby initiates a broad array of transcriptional changes in genes associated with maintenance of synaptic function and/or neuronal remodelling. In the present study we have focused our attention on NF1 and APP genes, both of which have been shown to play a significant role in cognition and memory performance (Costa et al., 2002; De Schepper et al., 2006; Guilding et al., 2007; Dawson et al., 1999; Conboy et al., 2005). Converging data obtained through qPCR, Western blot and immunohistochemistry revealed that hippocampal D_3R expression and immunoreactivity are significantly increased following acquisition of PA (Fig. 2 and 3), suggesting the hippocampal DA levels might be increased soon after the learning event and consistent with D_3R autoreceptor function (Collo et al., 2012). Interestingly, NF1 but

not APP expression mirrored PA-driven increase in D_3R mRNA and protein levels (Fig. 2-5), which was abrogated in trained $D_3R^{-/-}$ mice, supporting a role of the receptor on gene transcriptional activity, at least in the hippocampus.

Previous studies have indicated that the NF1 gene acts as an inhibitory regulator of Ras, involved both in GABA-mediated inhibition of hippocampal synaptic transmission (Costa et al., 2002) and in the activation of signaling cascades that regulate neuronal outgrowth during both early- and late-phase LTP (Guilding et al., 2007). These evidences, together with our finding, suggest that NF1 expression might be under the control of D_3R to exert either facilitatory/inhibitory actions on synaptic function following acquisition of the cognitive task.

As opposite to NF1 data, APP expression was unchanged in WT mice following PA acquisition, but was significantly increased in naive $D_3R^{-/-}$ mice and totally reversed after the acquisition of the behavioral task (Fig. 4 and 5). This result is consistent with our previous evidence showing that APP levels are thoroughly augmented in several brain regions of mice lacking D_3R (Castorina et al., 2011), even though it does not explain why expression levels were significantly reduced by acquisition of the avoidance task (Fig. 4 and 5). Unfortunately, a plausible explanation for the latter result could not be attributed directly to D_3R, although it is possible that genetic inactivation of the receptor has profound effects on PA-driven regulation of APP expression, possibly through the involvement of alternative molecular mechanisms. In agreement with this hypothesis, a study performed using NF1 knock-out mice proposed that APP and neurofibromin form a binding complex that interacts with D_3Rs and that their dysfunctional cellular trafficking due to the primary gene defect might explain the cognitive deficits observed in these murine models (Donarum et al., 2006). It is therefore possible that the imbalanced APP expression observed in $D_3R^{-/-}$ mice both before and after the training trial might involve the disrupted interaction between the NF1/APP complex and the receptor, although it remains unclear whether it could have repercussions of gene transcriptional activity. However, our study was limited to the evaluation of gene expression profile during the acquisition of PA, a specific fear conditioning paradigm. Using different behavioral tests beside fear-associated

ones should be warranted to enhance the conclusiveness of these finding with respect to associative memory.

In conclusion, the present study provides novel insights to better comprehend the relevance of hippocampal D_3R in the transcriptional regulation of NF1 and APP genes following the acquisition of the PA task.

Acknowledgements

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Increased hippocampal CREB phosphorylation in dopamine D3 receptor knockout mice following passive avoidance conditioning

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Abstract

Dopamine D_3 receptors (D_3Rs) are implicated in synaptic plasticity and memory processes. Previously we have shown that D_3Rs mediate inhibitory effects on learning, since D_3R knockout ($D_3^{-/-}$) mice display enhanced performance in the passive avoidance task (PA). Formation of new memories is known to require *de novo* synthesis of proteins related to synaptic function through the activation of signalling pathways including the mitogen-activated protein kinases (MAPKs) and activation of the nuclear transcription factor cAMP response element binding protein (CREB). However, there are no clear indications regarding the specific involvement of D_3Rs in the activation of these signalling cascades after acquisition of PA. Therefore, in this study we assessed whether phosphorylation levels of several MAPKs, Akt and CREB were differentially affected by PA in both wild-type (WT) and $D_3^{-/-}$ mice hippocampi. Animals were divided in naive, unconditioned stimulus trained, conditioned stimulus trained and conditioned animals. Phosphorylation of extracellular signal-regulated kinase 1/2 (ERK 1/2), c-Jun-N-terminal kinase (JNK) and p38, as well as of Akt and CREB were determined. Acquisition of PA significantly increased pCREB levels both in WT and $D_3^{-/-}$ mice. The extent of PA-driven increase in pCREB levels was significantly higher in mice lacking D_3Rs. Similarly, pERK 1/2 was further augmented in trained $D_3^{-/-}$ mice as compared to trained WTs, whereas JNK and p38 phosphorylation was not affected neither by PA nor by genetic background. Finally, Akt activation was observed in $D_3^{-/-}$ mice, but not in response to PA. In conclusion, these data

supports the notion that D_3Rs might modulate CREB phosphorylation after acquisition of PA, probably via activation of ERK signaling.

Keywords : dopamine D_3 receptor, cAMP/CREB signalling, passive avoidance

Introduction

Dopamine (DA) is a monoamine neurotransmitter involved in regulation of multiple functions in the central nervous system (CNS) and periphery, including locomotion, endocrine regulation, emotional behaviours and cognition (Carlsson, 2001; Gainetdinov and Caron, 2003; Zhou and Palmiter, 1995; Beaulieu and Gainetdinov, 2011). DA system is part of a network that plays very important roles in cognitive function, in which multiple DA receptor subtypes contribute to different aspects of learning and memory. It has been shown that DA functions are mediated by two different classes of GPCRs (Beaulieu et al., 2005), D1-like (D_1R and D_5R) and D2-like (D_2R, D_3R and D_4R), based on their structural and pharmacological properties (Missale et al., 1998; Karasinska et al., 2005). Although evidence suggests that, a the five DA receptors, the D_1R plays a dominant role in modulating synaptic plasticity and memory process, other DA receptor subtypes including the D_3 receptor (D_3R) seem to be involved in cognitive functions (El-Ghundi et al., 2007). In fact, many studies have demonstrated that the D_3R could be a critical modulator of normal DAergic function, and consequently cognition (Nakajima et al., 2013) and that it is also involved in synaptic plasticity and memory processes (Laszy et al., 2005) and in regulation of gene expression (Castorina et al., 2011). D_3R exhibits sustained high affinity for DA, suggesting that D_3R *in vivo* may be occupied by endogenous DA for extended periods of time, leading to its high spontaneous activation (Richtand et al., 2001; Vanhauwe et al., 2000).

In last decade many findings showed that mice genetically deficient in D_3R show regular emotional behavior (Chourbaji et al., 2008) and increased cognitive flexibility in the attentional set-shifting task and enhanced cognitive performance in the single trial step-through passive

avoidance (PA) task as compared to WTs mice (Glickstein et al., 2005; Micale et al., 2010; D'Amico et al., 2013).

The cognitive effects mediated by D_3Rs may be attributable to activation of the cAMP-PKA/CREB signaling in the hippocampus (Nakajima et al., 2013). CREB is a nuclear transcription factor which is thought to play a key role in learning and memory process (Xing et al., 2010). CREB-dependent signaling associated with memory impairment is well-documented in literature; for example studies have shown that loss of CREB signalling in rodent hippocampus is implicated in age-related spatial memory impairment (Brightwell et al., 2004; Mouravlev et al., 2006). CREB mediated gene expression is dependent on its phosphorylation at Ser133 via a number of signaling pathways that involve the activation of protein kinases, among these the MAPKs (Josselyn and Nguyen, 2005). Activation of MAPK, including the extracellular signal-regulated kinase (ERK), c-Jun-N-Terminal kinase (JNK), and p38 transmits signals from a variety of extracellular stimuli to the nucleus (Schaeffer et al., 1999). The ERK signaling has been implicated in hippocampal synaptic plasticity, spatial learning and memory (English et al., 1997; Blum et al., 1999; Selcher et al., 1999) and recent findings also suggest that JNK and p38 might play a role in synaptic plasticity (Brust et al., 2007; Brust et al., 2008). Moreover, *in vivo* studies revealed that D2 class receptors also exert their action in a cAMP-independent manner by promoting the dephosphorylation-inactivation of serine/threonine kinase Akt on its regulatory Thr308 residue (Beaulieu et al., 2004).

The serine/threonine kinase Akt is also involved in the activation of CREB (Beaulieu et al., Accili et al., 1996). It is clear that D_3Rs may influence cognition by regulating CREB signalling in the hippocampus (Xing et al., 2010), however no data exist about this relationship during the formation of associative learning. Given the involvement of D_3Rs in memory processes and in regulating the activity/phosphorylation of several signalling molecules, we used $D_3^{-/-}$ and WT mice to further elucidate the relative contribution of this receptor in regulating CREB phosphorylation following the acquisition of PA, as well as the major underlying molecular mechanism involved.

Our data points to D_3Rs as negative regulators of CREB activity in the hippocampus, as a targeted D_3R deletion further reinforces PA-driven increases in the phosphorylation state of this transcription factor, probably through the involvement of ERK-CREB signalling.

Materials and Methods

Animals

All experiments were carried out on $D_3R^{-/-}$ and WT mice (male mice 8-12 weeks old). The animals were housed four *per* cage and fed with standard laboratory food and allowed free access to water *ad libitum*, in an air-conditioned room with a 12 h light-dark cycle. All the experimental procedures were performed during the light cycle (between 10 a.m. and 2 p.m.). $D_3R^{-/-}$ mice used in these experiments were 5th-8th generation of congenic C57BL/6J mice, and generated by a backcrossing strategy. The genotypes of the D_3R mutant and WT mice were evaluated by PCR analysis by using two pairs of primers flanking either exon 3 of the wild-type D_3R or the PGK (phosphoglycerate kinase 1 gene promoter) cassette of the mutated gene (Accili et al., 1996). Each strain was divided into four groups, as previously described by D'Agata and Cavallaro (2003) and sacrificed by cervical dislocation six hours after the training trial, hippocampi were rapidly dissected and stored at -80°C until use (D'Amico et al., 2013). All animals were used only once in the experiments, which were carried out according to the European Community Council Directive 86/609/EEC. Efforts were made to minimize animal suffering and to reduce the number of animals used. The rationale, design and methods of this study were approved by the Ethical Committee for Animal Research, University of Catania.

Passive avoidance test

The single trial step-through passive avoidance test was performed as previously described by D'Amico et al. (2013) using a passive avoidance apparatus (San Diego Instruments, Inc., San Diego, CA, USA). The apparatus was divided into two compartments by a retractable door: a lit safe compartment and a darkened shock compartment.

The experiment was carried out on male homozygous $D_3R^{-/-}$ and WT mice. Each strain of animals was divided in four groups. The first group (naive) was maintained in the home cage. The rest of the animals experienced a 2-day behavioral training. On the first day, animals were handled by the experimenter for 2 min and then placed into the safe compartment and allowed to explore both chambers of the apparatus for 3 min. The second day, in the training trial, the second group of animals (termed 'conditioned animals', CA) were placed in the safe compartment with the door closed. After 2 min of acclimatization the light was turned on, the door opened and the animal was allowed to enter the dark compartment. After the mouse stepped completely with all four paws into the dark compartment, the door was closed, and a mild inescapable foot shock (0.5 mA, 2 s duration) was delivered from the grid floor. Following the shock, the mouse was removed and returned to its home cage. A third group of animals (termed 'conditioned stimulus-trained animals', CSTA) were placed into the safe compartment. After 2 min of acclimatization the light was turned on, the door opened and the animal allowed to enter the dark compartment. After the mouse stepped into the dark compartment, the door was closed but no foot shock was delivered from the grid floor. Then mice returned to their home cage. The fourth group of animals (termed 'unconditioned stimulus-trained animals', USTA) were placed in one of the two dark compartments. They were allowed to move freely to both compartments. After 2 min of acclimatization they received an inescapable foot shock (0.5 mA, 2 s duration) and then returned to their home cage.

Six hours later, animals from each of the four experimental groups were sacrificed by cervical dislocation, hippocampi were rapidly dissected and stored at -80°C until use. These hippocampi were used in our experimental protocols. Twenty-four hours after the training trial, the remaining animals from each of the four experimental groups performed the retention test. The animals were placed in the safe compartment with the door closed. After 2 min of acclimatization the light was turned on, the door opened and the animal was allowed to enter the dark compartment. The latency to enter the dark compartment was recorded and used as the measure of retention. Mice avoiding the dark compartment for >300 s were considered to have a step-through latency of 300 s (D'Amico et al., 2013; D'Agata and Cavallaro, 2003).

Western blot analysis

Crude extracts of WT (n=3 for each experimental group) and $D_3R^{-/-}$ (n=3 for each experimental group) mice hippocampi were dissected according to the mouse brain atlas (Paxinos et al. 2001) and prepared by homogenizing samples in a buffer containing 20 mM Tris (pH 7.4), 2 mM EDTA, 0.5 mM EGTA; 50 mM mercaptoethanol, 0.32 mM sucrose and a protease inhibitor cocktail (Roche Diagnostics) and phosphatase inhibitor (PhosSTOP, Roche Diagnostic) using a Teflon-glass homogenizer and then sonicated twice for 20 sec using an ultrasonic probe, followed by centrifugation at 10.000 g for 10 min at 4 °C. Protein concentrations were determined by the Quant-IT Protein Assay Kit (Invitrogen). Then, protein samples were separated by electrophoresis as previously described (Scuderi et al., 2013). Briefly, sample proteins (40 µg) were diluted in 2X Laemmli buffer (Invitrogen, Carlsbad, CA, USA), heated at 70°C for 10 min and then separated on a Biorad Criterion XT 4-15% Bis-tris gel (Invitrogen) by electrophoresis and then transferred to a nitrocellulose membrane (Invitrogen). Blots were blocked using the Odyssey Blocking Buffer (Li-Cor Biosciences). Immunoblot analysis was performed by using a rabbit anti-phospho-CREB (AB3442, Millipore, 1:500), rabbit anti-CREB (04-218, Millipore, 1:1000), mouse ERK1/2 monoclonal antibody (sc-135900, Santa Cruz, 1:200), rabbit p-ERK1/2 (sc-16982, Santa Cruz, 1:200), mouse total Akt (C67E7, Cell Signaling, 1:1000), mouse phspho-Akt (C31E5E, Cell Signaling, 1:1000), rabbit p-p38 (sc-17852-R, Santa Cruz, 1:200), rabbit p38α/β (sc-7149, Santa Cruz, 1:200), mouse p-JNK (sc-6254, Santa Cruz, 1:200), rabbit JNK (sc-571, Santa Cruz 1:200). The secondary antibody goat anti-rabbit IRDye 800CW, (cat #926-32211; Li-Cor Biosciences), goat anti-mouse IRDye 680CW, (cat#926-68020D; Li-Cor Biosciences) was used at 1:20000, 1:30000 respectively. Blots were scanned with an Odissey Infrared Imaging System (Odyssey). Densitometric analyses of Western blot signals were performed at non-saturating exposures and analyzed using the ImageJ software (NIH, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html). Values were normalized to β-tubulin, which served as loading control. To assess phosphorylation / activity state we normalized the expression of phospho-proteins over pan-proteins. No signal was detected when the primary antibody was omitted (data not shown).

Statistical analysis

Data are reported as mean ± S.E.M. One-way analysis of variance (ANOVA) was used to compare differences among groups and statistical significance was assessed by Tukey-Kramer post-hoc test. The level of significance for all statistical tests was $p \leq 0.05$.

Results

Enhanced phosphorylation of p-CREB in hippocampus of $D_3R^{-/-}$ mice trained with passive avoidance test

In order to evaluate whether cognitive response of WT and $D_3R^{-/-}$ animals in the passive avoidance paradigm affected CREB activation we performed Western blot analyses in WT and $D_3R^{-/-}$ animals that underwent a passive avoidance task according to a protocol described in our previous work (D'Amico er al., 2013). As seen in Fig. 1, expression levels of p-CREB were significantly increased following passive avoidance conditioning in WT mice (CA) as compared to control groups ($F_{7,23}$=74,89, *p<0.05 or ***p<0.001 vs Naïve WT). Moreover, the degree of hippocampal CREB phosphorylation was significantly higher in $D_3R^{-/-}$ conditioned animals with respect to either untrained WT or $D_3R^{-/-}$ mice (Naïve animals) (***p<0.001 vs Naïve WT, ###p<0.001 vs Naïve $D_3R^{-/-}$; Fig. 1 A-B). This result suggests that learning-dependent activation of hippocampal CREB is favored in $D_3R^{-/-}$ mice.

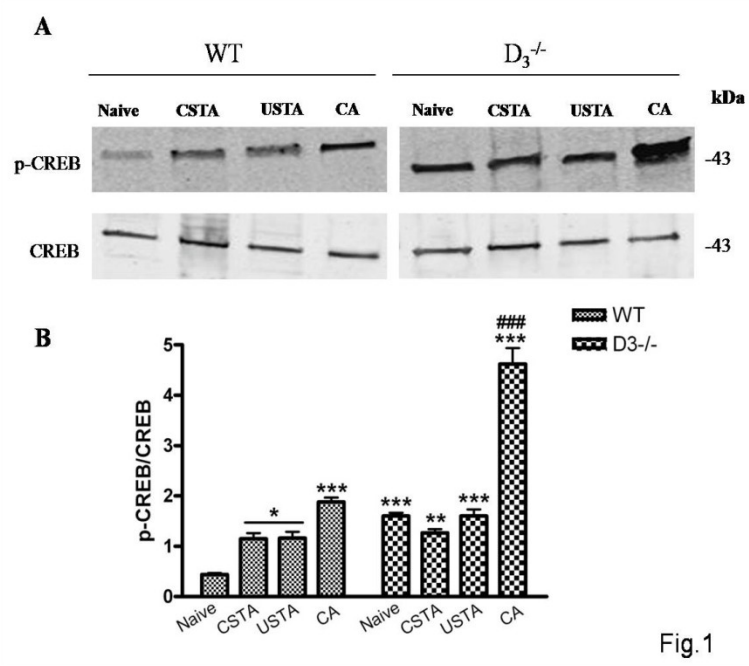

Figure 1. CREB phosphorylation in the hippocampus of WT and $D_3^{-/-}$ mice subjected to the passive avoidance task.

(A) Representative immunoblots obtained using 40μg of homogenates from WT and $D_3^{-/-}$ mice hippocampus in conditioned animals (CA) and in control mice that included untrained (naive) animals, and animals exposed to the conditioned (CSTA) or unconditioned (USTA) stimulus.

(B) The bar graph shows the results of three independent experiments. Protein levels are expressed as arbitrary units obtained after normalization to β-tubulin, which was used as loading control. Data are expressed as mean ± S.E.M. *p<0.05, **p<0.01 or ***p<0.001 vs Naïve WT, ### p<0.001 vs Naïve, CSTA and USTA $D_3^{-/-}$ as determined by One-Way ANOVA followed by Tukey post-hoc test.

MAPKs activation in the hippocampus of WT and of $D_3^{-/-}$ mice after passive avoidance training

The involvement of D_3Rs in the regulation of signaling pathways related to the formation of new associative memories was evaluated by performing Western blot analyses on ERK, which is known to be involved in hippocampal synaptic plasticity and memory formation (English and Sweatt, 1997; Blum et al., 1999). As shown in Fig. 2 there was a significant activation of ERK in the hippocampus of either in WT mice subjected to the step-through PA and heightened activity was also identified in $D_3R^{-/-}$ naïve when compared to respective control groups

($F_{3,15}$=37,91, *p<0.05 or ***p<0.001 vs Naïve WT). Interestingly, the degree of ERK activation was significantly higher in the hippocampus of $D_3R^{-/-}$ conditioned animals as compared to the $D_3R^{-/-}$ naïve mice (###p<0.001 vs Naïve $D_3R^{-/-}$ **;** Fig. 2 A-B). Since that other two MAPKs, JNK and p38, are involved in synaptic plasticity (Brust et al., 2007; Moult et al., 2008), to further investigate the roles of D_3R in MAPK signaling in associative memory formation, we also examined JNK and p38 phosphorylation levels in the hippocampus of WT and $D_3R^{-/-}$ mice after PA conditioning. As shown in Fig. 3, no significant changes in activity levels could be observed neither for p-JNK ($F_{3,11}$=0,8620) nor p-p38 ($F_{3,11}$=0,2253) in both WT and $D_3R^{-/-}$ animals compared to baseline (Naïve) (Fig.3), suggesting that activation of MAPKs after PA training is specific for ERK.

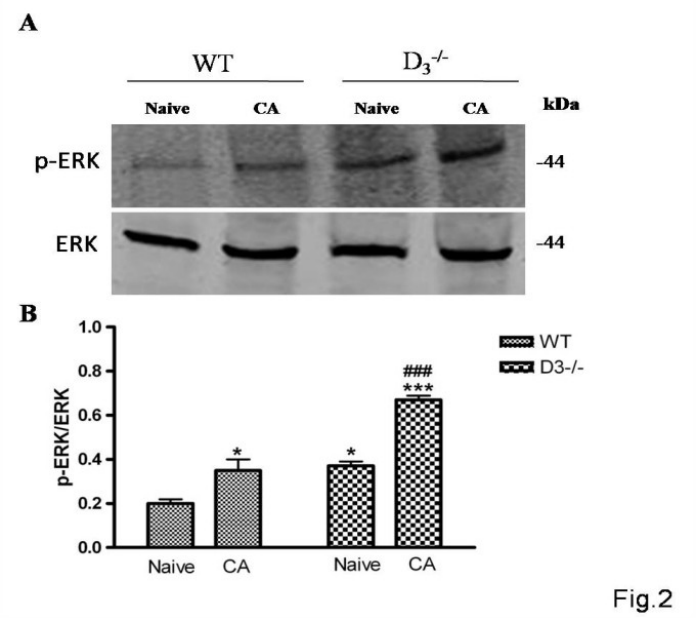

Figure 2. Hippocampal MAPK activaty is specific for ERK in WT and $D_3^{-/-}$ mice after PA acquisition

(A) Representative immunoblots obtained using 40μg of homogenates from WT and $D_3^{-/-}$ mice hippocampus.

(B) The bar graph shows the results on three independent experiments. Protein levels are expressed as arbitrary units obtained after normalization to β-tubulin, which was used as loading control. Data are expressed as mean ± S.E.M. *p<0.05 or ***p<0.001 vs Naïve WT, ### p<0.001 vs Naïve $D_3^{-/-}$ as determined by One-Way ANOVA followed by Tukey post-hoc test.

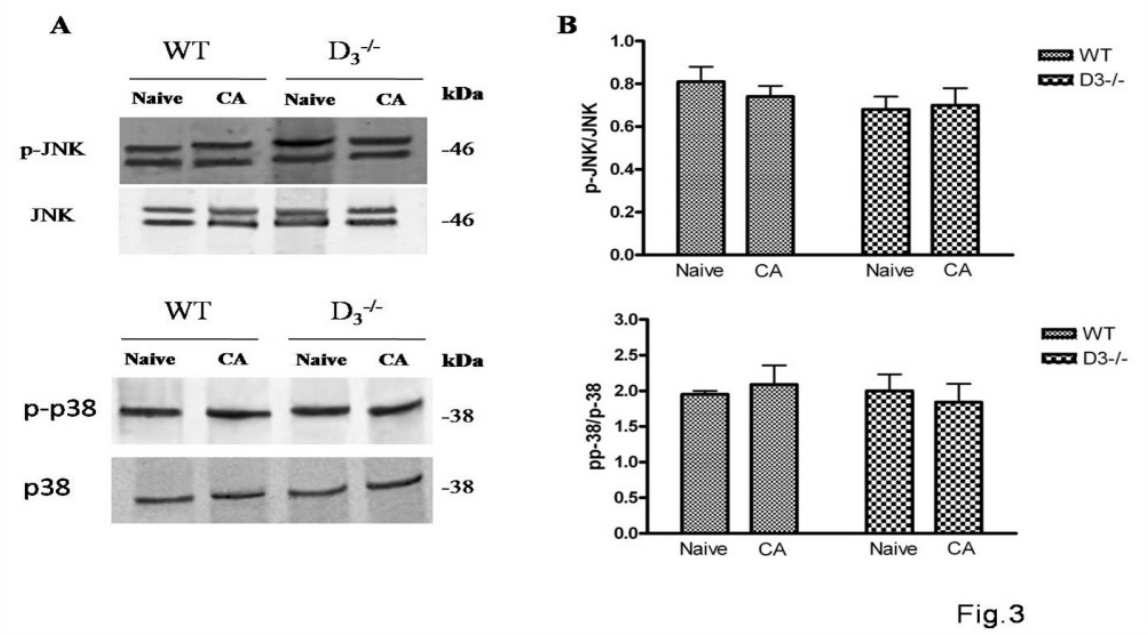

Figure 3. MAPK activation in the hippocampus of WT and $D_3^{-/-}$ mice after PA training.

(A) Representative immunoblots obtained using 40μg of homogenates from WT and $D_3^{-/-}$ mice hippocampus.

(B) The bar graph shows the results on three independent experiments. Protein levels are expressed as arbitrary units obtained after normalization to β-tubulin, which was used as loading control. Data are expressed as mean ± S.E.M. and determined by One-Way ANOVA followed by Tukey post-hoc test.

$D_3R^{-/-}$ mice exhibit increased phosphorylation of Akt which is not dependent on the acquisition of the passive avoidance trial

D2-class receptor (D2R,D3R,D4R) couple to Gαi/0, thus downregulating cAMP production and PKA activity (English and Sweatt, 1997; Blum et al., 1999). Previous study have demonstrated that prolonged stimulation of D2 class receptor leads to specific dephosphorylation/inactivation of the serine/threonine kinase Akt on its regulatory threonine 308 (Thr308) residue (Beaulieu et al., 2004). Therefore, in the attempt to estabilish the relative contribution of D_3Rs on Akt activation after PA conditioning, we compared the phosphorylation levels of Akt at its Thr308 residue in WT and $D_3R^{-/-}$ mice both in basal condition or after acquisition of the task. Results demonstrated that Akt phosphorylation at Thr308 was significantly increased in the

hippocampus of naïve $D_3R^{-/-}$ mice, confirming recently published data in literature (Castorina et al., 2013) and in CA animals as compared to WT ($F_{3,15}$=25,90, **p<0.01 or ***p<0.001 vs Naïve and CA WT, Fig.4). This result suggests that Akt activation might be correlated to the absence of the receptor and not to this specific type of conditioning.

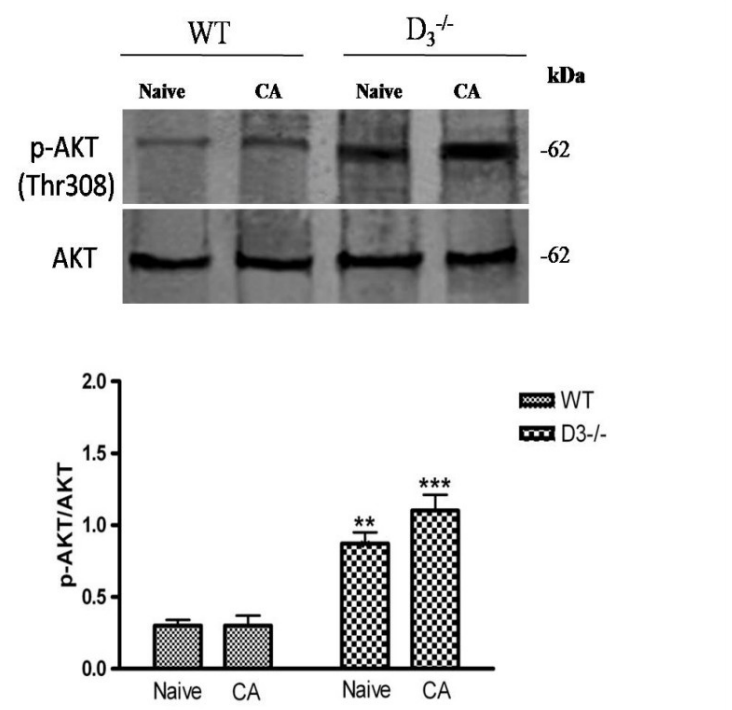

Fig.4

Figure 4. AKT phosphorylation at the Thr308 residue in the hippocampus of WT and $D_3^{-/-}$ mice after PA training.

(A) Representative immunoblots obtained using 40μg of homogenates from WT and $D_3^{-/-}$ mice hippocampus.

(B) The bar graph shows the results on three independent experiments. Protein levels are expressed as arbitrary units obtained after normalization to β-tubulin, which was used as loading control. Data are expressed as mean ± S.E.M. **p<0.01 or ***p<0.001 vs Naïve WT, as determined by One-Way ANOVA followed by Tukey post-hoc test.

Discussion

Learning and memory are integrative brain functions based on neural plasticity, which involve a variety of molecules such as neurotransmitters, neurotrophins, and their receptors as well as second messengers and protein kinases (Kudo et al., 2004). Futhermore, acquisition and consolidation of new memories depend upon induction of gene expression and subsequent protein synthesis (Davis and Squire, 1984). The DAergic system has been implicated in cognitive function through animal and human research, including studies of molecular genetics and neuroimaging (Bäckman et al., 2006; Cole et al., 2012). Unlike D_2R receptors, D_3Rs can be stimulated by tonic DA levels in the brain due to their high affinity for DA (Sokoloff et al., 1990) and may attenuate any effects of DA fluctuation related to phasic DA release. Accordingly, small changes in the number or function of D_3R may lead to dramatic effects on synaptic transmission, suggesting that this receptor could be implicated in cognition (Nakajima et al., 2013). Numerous animal studies have explored the cognitive effects of pharmacological interventions targeting D_3R. In recent years, the performance of $D_3R^{-/-}$ mice was tested using various cognitive tasks, such as the two-choice perceptual discrimination test, social novelty discrimination, the step-through passive-avoidance test, T-maze and the delayed alternation test, thus showing that this receptor subtype might contribute to ameliorate certain aspects of cognitive performance, including associative learning (Chourbaji et al., 2008; Glickstein et al., 2005; Micale et al., 2010; D'Amico et al., 2013; Xing et al., 2010; Watson et al., 2012). Furthermore a recent study showed that D_3R-regulated CREB signalling in the hippocampus may be involved in age-associated cognitive alterations (Xing et al., 2010), and other findings showed a close relationship between behavioural performance and CREB phosphorylation in hippocampus after contextual fear and PA conditioning (Kudo et al., 2004). In particular the transcription factor CREB is required for hippocampus-depenent long term potentiation (LTP) (Balschun et al., 2003) and many papers suggest that several memory-associated signaling molecules are related to phosphorylation of CREB (Dubynina and Dolotov, 2009).

Several studies have suggested that D_3R activation might mediate negative effects on cognitive performance, based on the ameliorated responses obtained on knockout animal models in

different behavioural paradigms (Micale et al., 2010; D'Amico et al., 2013; Xing et al., 2010). The biochemical explanation appears to rely on the capacity of D_3Rs to inhibit adenylate cyclase and modulate MAPKs which could in turn regulate CREB activity (Yan et al., 1999). However, whether D_3R are involved in the activation of CREB in the hippocampus of mice performing the PA trial remained to be investigated. To address this issue, in the present study we focused our interest on elucidating whether D_3Rs contribute to differentially influence the activity of specific signalling pathways related to memory performance in mice subjected to the PA task, with particular emphasis on the evaluation of CREB phosphorylation and the potential underlying signalling pathways involved.

Many signaling molecules are known to have key roles in learning and memory process. ERK and CREB molecules are involved in memory retrieval in several brain regions (Abel and Lattal, 2001; Nader, 2003; Suzuki et al., 2004; Viosca et al., 2007), and it is widely accepted that the ERK signaling pathway is crucial for memory retrieval (Viosca et al., 2007; Barros et al., 2000; Barros et al., 2003; Izquierdo et al., 2001). Hippocampal MAPK/ERK has a central role in several different memory types including fear memory in contextual fear conditioning and inhibitory passive avoidance (Izquierdo et al., 2001; Leon et al., 2010; Sweatt et al., 2004; Davis and Laroche, 2006; Giovannini, 2006). Szapiro et al., 2000 have also suggested that the activity of the MAPK pathway in hippocampal CA1 region is important for memory retrieval, and other works have shown increased ERK phosphorylation in the hippocampus of mice following the retention trial of a passive avoidance task (Kim et al., 2012). In agreement with these previous reports, we observed increased hippocampal CREB and ERK phosphorylation in $D_3R^{-/-}$ subjected to PA conditioning. Our findings suggested that D_3R is involved in the conditioning process, possibly through the modulation of hippocampal ERK-CREB signaling (Fig.1,2). Other two MAPKs implicated in synaptic plasticity process, were also considered: JNK and p38. Their expression was not affected neither with respect to genetic background nor in response to the trial (Fig.3).

Since the serine/threonine kinase Akt is a downstream target of DA receptor signaling and that previous studies have shown that $D_3R^{-/-}$ mice display enhanced striatal Akt activation under

basal condition (Castorina et al., 2013), we also sought to evaluate the phosphorylation levels of Akt Thr308 between WT and $D_3R^{-/-}$ mice following the PA task. The results showed that changes in phosphorylation state were related to genotype, but no difference could be attributed to the conditioning process (Fig.4).

In conclusion, the reported finding suggest that D_3R inactivation might improve cognitive performance in mice through the induction of hippocampal CREB activity, preferentially via ERK signaling. However, considering that CREB is a transcription factor whose activity is dependent on a broad range of upstream signaling molecules and its wide spectrum of downstream targets have not been entirely characterized, future efforts should be warranted to clearly establish how CREB activity could influence more complex aspects of cognition that involve other brain areas in which D_3R have also been identified, as the prefrontal cortex and striatum.

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Dopamine D_3 receptor deletion increases tissue plasminogen activator (tPA) activity in prefrontal cortex and hippocampus

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Abstract

Considerable evidence indicate that dopamine (DA) influences tissue plasminogen activator (tPA)-mediated proteolytic processing of the precursor of brain derived neurotrophic factor (proBDNF) into mature BDNF. However, specific roles in this process for the dopamine D_3 receptor (D_3R) and the underlying molecular mechanisms have yet to be fully characterized. In the present study, we hypothesized that D_3R deletion could influence tPA activity in the prefrontal cortex and hippocampus. Using D_3R knockout ($D_3^{-/-}$) mice, we show that receptor inactivation is associated with increased tPA expression/activity both in prefrontal cortex and, to a greater extent, in the hippocampus. Augmented tPA expression in $D_3^{-/-}$ mice correlated with increased BDNF mRNA levels, plasmin/plasminogen protein ratio and conversion of proBDNF into mature BDNF, as well as enhanced tPA and mature BDNF immunoreactivity, as determined by qRT-PCR, immunoblot and immunohistochemistry. In addition, when compared to wild-type controls, $D_3^{-/-}$ mice exhibited increased basal activation of the canonical cAMP/PKA-driven Akt/CREB signaling cascade, as determined by the increased Akt phosphorylation both at Thr304 and Ser473 residues, of DA and cAMP regulated protein of 32kDa (DARPP-32) at Thr34 and a phosphorylation state-dependent inhibition of glycogen synthetase kinase-3β (GSK-3β) at Ser9, a substrate of Akt whose constitutive function impairs normal cAMP-response element binding protein (CREB) transcriptional activity through phosphorylation at its Ser129 residue. Accordingly, CREB phosphorylation at Ser133 was significantly increased in $D_3^{-/-}$ mice, whereas the GSK-3β-dependent phosphorylation at Ser129 was diminished. Altogether, our finding reveal that mice lacking D_3Rs show enhanced tPA

proteolytic activity on BDNF which may involve, at least in part, a potentiated Akt/CREB signaling, possibly due to hindered GSK-3β activity.

Keywords : dopamine D3 receptor; BDNF; tissue plasminogen activator; hippocampus; prefrontal cortex

Introduction

The neurotransmitter dopamine (DA) exerts a multitude of effects in the central nervous system (CNS). Its actions are mediated by five distinct G-protein coupled receptors grouped into two subclasses: D_1-like (D_1R and D_5R) and D_2-like (D_2R, D_3R, and D_4R), based on their structural and pharmacological properties (Missale et al., 1998; Karasinska et al., 2005). In particular the D_3R, cloned by Sokoloff, (1990), which is coupled to $G\alpha_{i/0}$ proteins to inhibit adenylyl cyclase activity, has the highest affinity for DA and is expressed in limbic areas, but it is also detectable in other brain regions critical to learning and memory, such as the prefrontal cortex (Levant et al., 1998; Sokoloff et al., 1992, 2006; Cho et al., 2010; Castorina et al., 2011).

In recent years, by taking advantage of genetically engineered mice lacking functional D_3Rs, we and others have attempted to partly unravel the specific involvement of this receptor in non-learning and learning-based behaviours (Chourbaji et al., 2008; Xing et al., 2010; Micale et al., 2010; D'Amico et al., 2013); these studies revealed that D_3R knockout ($D_3^{-/-}$) mice show regular emotional behaviour (Chourbaji et al., 2008) and spatial learning abilities (Xing et al., 2010), whereas they display enhanced acquisition and retention of fear-associated memories in the passive avoidance test (Micale et al., 2010; D'Amico et al., 2013).

Pharmacologically, current knowledge on the varying affinities for DA of the five receptor subtypes and their coupling to different G-proteins has supported the notion that higher affinity D_3Rs, in contrast to other D_2-class receptors, are primarily activated by basal level tonic DA rather than by phasic bursts to reduce both basal cAMP production (Wall et al., 2011) and activation of downstream cAMP/PKA-regulated substrates, including the DA and cAMP regulated protein of 32kDa (DARPP-32) (Nishi et al., 1997) and the Akt/glycogen synthetase

kinase-3β (GSK-3β) signaling cascade (Beaulieu et al., 2005, 2008; Cho et al., 2010). Interestingly, both DARPP-32 and the Akt/GSK-3β pathway are commonly engaged by DAergic neurons to modulate cAMP-response element binding (CREB) protein transcriptional activity, a point of convergence of many signaling pathways mediating increased synaptic activity (Nishi et al., 1997; Beaulieu et al., 2007; Benito and Barco, 2010). More specifically, DARPP-32 is a cytosolic protein that, when phosphorylated by PKA on Thr34, inhibits protein phosphatase 1 (PP-1) dephosphorylating activity on CREB at Ser133 (Yan et al., 1999; Greengard et al., 1999). Similarly, Akt, when phosphorylated by PKA at Thr308, blocks GSK-3β inhibitory activity on CREB through phosphorylation at Ser9 (Beaulieu et al., 2004, 2005). Inactivation of PP-1 by DARPP32 or of GSK-3β by Akt both facilitate CREB function, whereas their activation impairs it.

Activated CREB induces the expression of CREB-responsive genes, including neutrophins mediating retrograde signaling such as the precursor of brain derived neutrophic factor (proBDNF) (Boneva and Yamashima, 2012) or enzymes involved in its proteolytic processing, such as the tissue type plasminogen activator (tPA) (Ohlsson et al., 1993; Benito and Barco, 2010).

tPA is a serine protease that catalyzes the conversion of plasminogen to plasmin and whose best-known function is as thrombolytic enzyme (Bugge et al., 1996; Teesalu et al., 2002). tPA is also expressed by many types of neurons in the developing and adult brain (Melchor and Strickland, 2005) and is highly expressed in regions involved in learning and memory (Seeds et al., 1995; Salles and Strickland, 2002). In the CNS, extracellular tPA elicits its proteolytic function by converting proBDNF to its mature form (Pang et al., 2004), which then interacts with tyrosine kinase receptors to directly induce CREB phosphorylation (Numakawa et al., 2010). Interestingly, tPA is transcriptionally regulated by CREB (Ohlsson et al., 1993; Benito and Barco, 2010) and its expression is augmented upon exogenous treatment with D_1R agonists (Ito et al., 2007) or drugs of abuse such as cocaine or methamphetamine (Yamada et al., 2005), whereas it is oppositely regulated by D_2R preferring agonists (Ito et al., 2007), suggesting that D_2-like receptor subtypes might mediate an inhibitory effect of DA on CREB-driven tPA gene

expression. Unfortunately, to our knowledge, evidence showing the specific contribution of D_3Rs in the transcriptional regulation or activity of tPA is currently missing. Therefore, in the present study, by using D_3R knockout ($D_3^{-/-}$) mice, we determined whether receptor deletion influences baseline tPA expression/activity in two specific brain regions involved in learning and memory, the prefrontal cortex and hippocampus. In addition, we attempted to elucidate the potential underlying mechanisms mediated by D_3R to regulate CREB activity on tPA. Our results indicate that D_3R deletion increases tPA proteolytic activity on proBDNF and suggest that in the absence of the receptor, the combined increase in DARPP-32 and impairment of GSK-3β activity might contribute to potentiate the Akt/CREB signalling cascade to promote tPA gene expression and proteolytic function.

1. Experimental Procedures

1.1 Animals

All experiments were carried out on $D_3^{-/-}$ and WT mice (male mice 8-12 weeks old). The animals were housed four *per* cage and fed with standard laboratory food and allowed free access to water *ad libitum*, in an air-conditioned room with a 12 h light-dark cycle. All the experimental procedures were performed during the light cycle (between 10 a.m. and 2 p.m.). $D_3^{-/-}$ mice used in these experiments were 5th-8th generation of congenic C57BL/6J mice, and generated by a backcrossing strategy. The genotypes of the D_3R mutant and WT mice were identified by a PCR method by using two pairs of primers flanking either exon 3 of the wild-type D_3R or the PGK (phosphoglycerate kinase 1 gene promoter) cassette of the mutated gene (Accili et al., 1997). All animals were used only once in the experiments, which were carried out according to the European Community Council Directive 86/609/EEC. Efforts were made to minimize animal suffering and to reduce the number of animals used. The rationale, design and methods of this study were approved by the Ethical Committee for Animal Research, University of Catania.

1.2 Quantitative real time polymerase chain reaction

Total RNA extracts from prefrontal cortices and hippocampi of WT and $D_3^{-/-}$ mice (n=3-5 for each genotype) were isolated using 1 ml TRIzol reagent (Invitrogen) and 0.2 ml chloroform and precipitated with 0.5 ml isopropanol. Pellet was washed with 75% ethanol and air dried. Single stranded cDNAs were synthesized by incubating total RNA (5µg) with SuperScript III RNase H-reverse transcriptase (200 U/µl) (Invitrogen); Oligo-$(dT)_{20}$ primer (100 nM) (Invitrogen); 1 mM dNTP mix (Invitrogen), dithiothreitol (DTT, 0.1 M), Recombinant RNase-inhibitor (40 U/µl) at 42°C for 1 h in a final volume of 20µl. Reaction was terminated by incubation of samples at 70°C for 10 min.

Aliquots of cDNA (400ng) from prefrontal cortex and hippocampus of WT and $D_3^{-/-}$ mice and external standards at known amounts (purified PCR products, ranging from 10^2 to 10^8 copies) were amplified in parallel reactions, using primer pairs indicated in Table 1. mRNA levels of the reference gene, 18S ribosomial subunit, were measured in each amplification. Each PCR reaction contained 0.5 µM primers, 1.6 mM $MgCl^{2+}$, 1X Light Cycler-FastStart DNA Master SYBR Green I (Roche Diagnostic). Amplifications were performed using the Light Cycler 1.5 instrument (Roche Diagnostic) with the following program setting : (I) cDNA denaturation (1 cycle: 95°C for 10 min); (II) quantification (45 cycles: 95°C for 10 s, 60°C for 30 s, 72°C for 7 s); (III) melting curve analysis (1 cycle: 95°C for 0 s, 65°C for 15 s, 95°C for 0 s); (IV) cooling (1 cycle: 40°C for 30 s). Quantification was obtained by comparing the fluorescence emitted by PCR products at unknown concentration with the fluorescence emitted by external standards at known concentration. For this analysis, fluorescence values, measured in the log-linear phase of amplification, were estimated with the second derivative maximum method using Light Cycler Data Analysis software. PCR products specificity was evaluated by melting curve analysis.

To assess the different expression levels we analyzed the mean fold change values of each sample, calculated using the comparative Ct method (Schmittgen and Livak, 2008). The Ct represents the number of cycles needed to detect a fluorescence above a specific threshold level and it is inversely correlated to the amount of nucleic acids template present in the reaction. The ΔCt was calculated by normalizing the mean Ct of each sample to the mean Ct of the reference

gene measured in the same experimental conditions. For the quantification of each gene we considered the WT mice group as the positive sample (calibrator sample). The ΔΔCt of each sample was then calculated by subtracting calibrator ΔCt to sample ΔCt. The formula $2^{-\Delta\Delta Ct}$ was used to calculate fold changes. Baseline measurements for each calibrator sample were set to 1.

Table 1: Primer sequences

Forward and reverse primers were selected from the 5' and 3' region of each gene mRNA. The expected length of each PCR amplification product is indicated in the right column.

Gene	Forward	Reverse	bp length
Tissue plasminogen activator (tPA) Acc# NM_008872.2	GCCTGTCCGAAGTTGCAGCGA	TGCTGTGCTCCACGTGCCTC	184
Brain derived neutrophic factor (BDNF) Acc# NM_007540.4	CGAGTGGGTCACAGCGGCAG	GCCCCTGCAGCCTTCCTTGG	160
Ribosomal protein 18S Acc# NM_011296.2	CCTGCGAGTACTCAACACCA	CTGCTTTCCTCAACACCACA	110

1.3 Western blot analysis

Prefrontal cortices and hippocampi from WT and $D_3^{-/-}$ mice (n=3-5 for each genotype) were dissected according to the mouse brain atlas (Paxinos and Franklin, 2001). Tissues were then homogenized in an ice-cold lysis buffer containing 20 mM Tris (pH 7.4), 2 mM EDTA, 0.5 mM EGTA; 50 mM mercaptoethanol, 0.32 mM sucrose, a protease inhibitor cocktail (Roche Diagnostics) and a phosphatase inhibitor (PhosSTOP, Roche Diagnostic) using a Teflon-glass homogenizer and then sonicated twice for 20 sec using an ultrasonic probe, followed by centrifugation at 10.000 g for 10 min at 4 °C. Protein concentrations were determined by the Quant-iT Protein Assay Kit (Invitrogen). Protein samples were then separated by electrophoresis as previously described (D'Amico et al., 2013). Briefly, sample proteins (30 μg)

were diluted in 2X Laemmli buffer (Invitrogen, Carlsbad, CA, USA), heated at 70°C for 10 min, separated on a Biorad Criterion XT 4-15% Bis-tris gel (Invitrogen) by electrophoresis and then transferred to a nitrocellulose membrane (Invitrogen). Blots were blocked using the Odyssey Blocking Buffer (Li-Cor Biosciences). Immunoblot analyses were performed using antibodies that recognize tPA (1:500, sc-15346, Santa Cruz Biotechnology), plasminogen-plasmin (1:400, sc-25546, Santa Cruz Biotechnology), proBDNF (1:500, sc-65513, Santa Cruz Biotechnology), mature BDNF (1:500, sc-20981, Santa Cruz Biotechnology), phospho-Akt (Thr308) and (Ser473) (1:1000, C31E5E and D9E, Cell Signaling), total Akt (1:1000, C67E7, Cell Signaling), phospho-DARPP-32 (Thr34) and total DARPP-32 (1:1000, D29E8 and 19A3, Cell Signaling), phospho-GSK-3β (Ser9) and total GSK-3β (1:800, D85E12 and 27C10, Cell Signaling), tyrosine hydroxylase (1:500, sc-14007, Santa Cruz Biotechnology), phospho-CREB (Ser133) and (Ser129) (1:300, sc-7978 and sc-101662, Santa Cruz Biotechnology), total CREB (1:300, sc-186, Santa Cruz Biotechnology) and β-tubulin (1:500, sc-9104, Santa Cruz Biotechnology Inc). Secondary antibodies (goat anti-rabbit IRDye 800CW, cat #926-32211 and goat anti-mouse IRDye 680CW cat #926-68020; Li-Cor Biosciences) were used at 1:20000 and 1:30000, respectively. Blots were scanned with an Odyssey Infrared Imaging System (Li-Cor Biosciences). Densitometric analyses of Western blot signals were performed at non-saturating exposures and analyzed using the ImageJ software (NIH, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html). Values of non-phosphorylated proteins were normalized to β-tubulin, which served as loading control, whereas values from phosphorylated proteins were normalized to their respective total protein levels. Background correction values were subtracted from each lane to minimize the variability across membranes. No signal was detected when the primary antibody was omitted (data not shown).

1.4 Tissue preparation for immunohistochemical staining

Brains from decapitated mice (WT and $D_3^{-/-}$, n=3 for each genotype) were removed and stored for at least 24 h in 4% formaldehyde at 4°C before dehydration and embedding in paraffin. Six-micrometer-thick coronal sections were cut, mounted on glass slides, kept overnight at 37°C,

and then at room temperature until use. Prior to immunohistochemical staining, the sections were dewaxed in xylene and rehydrated through graded alcohols. They were then rinsed in 0.1M Tris–HCl buffered saline (TBS, pH 7.4) and treated with 3% hydrogen peroxide (H_2O_2) in PBS for 10 min to reduce endogenous peroxidase activity.

1.5 Immunohistochemical analysis

Immunohistochemical analysis was performed according to the standard ABC method. To reduce nonspecific staining, sections were treated with 5% bovine serum albumin (BSA) and 3% goat serum in TBS for 1 h. Sections were then incubated with a rabbit polyclonal antibody raised against amino acids 1-90 of tPA of human origin (1:25, sc-15346, Santa Cruz Biotechnology) or with a rabbit polyclonal antibody raised against amino acids 130-247 of mature BDNF of human origin (1:50, sc-20981, Santa Cruz Biotechnology). Antibodies were diluted in TBS containing 3% normal goat serum (NGS), 1% BSA, and 0.25% Triton X-100. After several rinses in TBS, the sections were incubated with a 1:200 diluted biotinylated goat anti-rabbit IgG for 1 h at room temperature. To visualize the immunoreaction sites in tissues, the sections were then rinsed and treated with reagents from an ABC Kit for 1 h at room temperature. The sections were rinsed in TBS and incubated with 0.025% 3,3-diaminobenzidine (DAB) plus 0.33% H_2O_2 in TBS for 10 min. Then, Tris buffer was added to stop the DAB reaction. Stained sections were then dehydrated through graded alcohols, cleared in xylene, and covered with neutral balsam. All sections were examined and images were taken with a light microscope (Axiovert, Carl Zeiss Inc) equipped with a digital color camera. The images were further processed for contrast adjustments using Adobe Photoshop software.

1.6 Statistical analysis

Statistical analysis was performed using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. All values are reported as means ± SEM. Unpaired two-tailed Student *t*-test was used to compare differences between groups. *p* values less than 0.05 denote statistical significance.

2. Results

2.1 Tissue plasminogen activator and brain derived neutrophic factor mRNA expression levels are increased in the prefrontal cortex and hippocampus of $D_3^{-/-}$ mice

In order to evaluate whether D_3R deletion affected mRNA expression levels of both tissue plasminogen activator (tPA) and brain derived neutrophic factor (BDNF), we performed quantitative real-time PCR analyses using cDNA templates obtained from both prefrontal cortices and hippocampi of wild-type (WT) and $D_3^{-/-}$ mice. Comparative analysis with WT control groups demonstrated that tPA mRNA expression is significantly increased both in the prefrontal cortex (t_{10}=2.887, *p=0.017 Vs WT) and, to a greater extent, in the hippocampus of $D_3^{-/-}$ mice (t_{10}=9.332, ***p<0.001 Vs WT) (Fig. 1A). These results were paralleled by the concurrent increase of BDNF mRNAs in the same brain regions examined (Fig. 2A). Specifically, expression levels were augmented by about 1.47-fold in the prefrontal cortex (t_{10}=9.7, ***p<0.001 Vs WT) and about 1.66-fold in the hippocampus of knockout animals when compared to the corresponding brain areas in WTs (t_{10}=6.741, ***p<0.001 Vs WT).

2.2 Enhanced tPA expression in $D_3^{-/-}$ mice brain correlates with increased plasmin/plasminogen and mature BDNF/proBDNF protein ratios

Beside the well-established function of tPA as thrombolytic enzyme (Bugge et al., 1996; Teesalu et al., 2002), several evidence have proved that the protease is also detectable in brain regions crucial for memory function (Salles et al., 2002), where it catalyzes the conversion of the biologically inactive plasminogen to the active enzyme plasmin which, in turn, cleaves the precursor of BDNF into its mature form (Pang et al., 2004). Here, based on our finding indicating that both tPA and BDNF mRNA levels are increased in $D_3^{-/-}$ mice (Fig. 1A and 1B), we sought to determine whether the absence of the receptor could also reflect changes in tPA protein expression and in the conversion ratios of both plasminogen and proBDNF into their active forms. Immunoblot analyses revealed that tPA protein expression increased at significant levels in mice lacking the D_3R (pfCX, t_4=5.916, **p<0.01 Vs WT; hippocampus, t_4=8.35,

**p<0.01) (Fig. 2A and 2B). Concurrently, its proteolytic activity, measured as the expression ratio of plasmin/plasminogen and subsequently, of mature BDNF (mBDNF)/proBDNF, was also significantly augmented in both brain regions of $D_3^{-/-}$ mice with respect to WTs (plasmin/plasminogen : pfCX and hippocampus, t_4=15.086 and t_4=26.847; mBDNF/proBDNF : t_4=33.66 and t_4=32.12, respectively, ***p<0.001 Vs WT) (Fig. 2A and 2B).

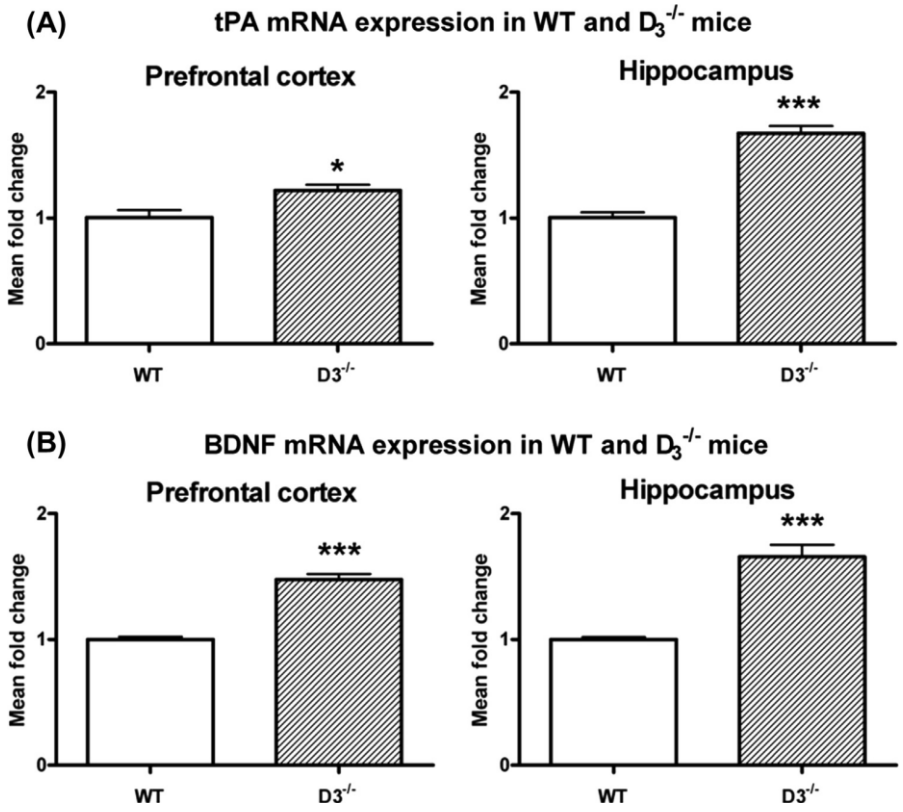

Figure 1. Tissue plasminogen activator and brain derived neutrophic factor mRNA expression levels are increased in the prefrontal cortex and hippocampus of $D_3^{-/-}$ mice

Data obtained from quantitative real-time PCR analyses indicating that tissue plasminogen activator (tPA) and brain derived neutrophic factor (BDNF) mRNA levels are increased both in the prefrontal cortex and hippocampus of $D_3^{-/-}$ mice when compared to wild-type (WT). Results are presented as mean fold changes of WTs (n=3 *per* group) and $D_3^{-/-}$ mice (n=3 *per* group) ± S.E.M. Relative fold changes of either tPA (**A**) or BDNF (**B**) genes were normalized to the endogenous ribosomal protein 18S (housekeeping gene) and then calculated using the comparative Ct method. Baseline expression levels of the control group (WT) were set to 1.

Experiments were performed three times independently, each run in duplicate. *p<0.05 or ***p<0.001 *vs* WT, as determined by unpaired two-tailed Student *t*-test.

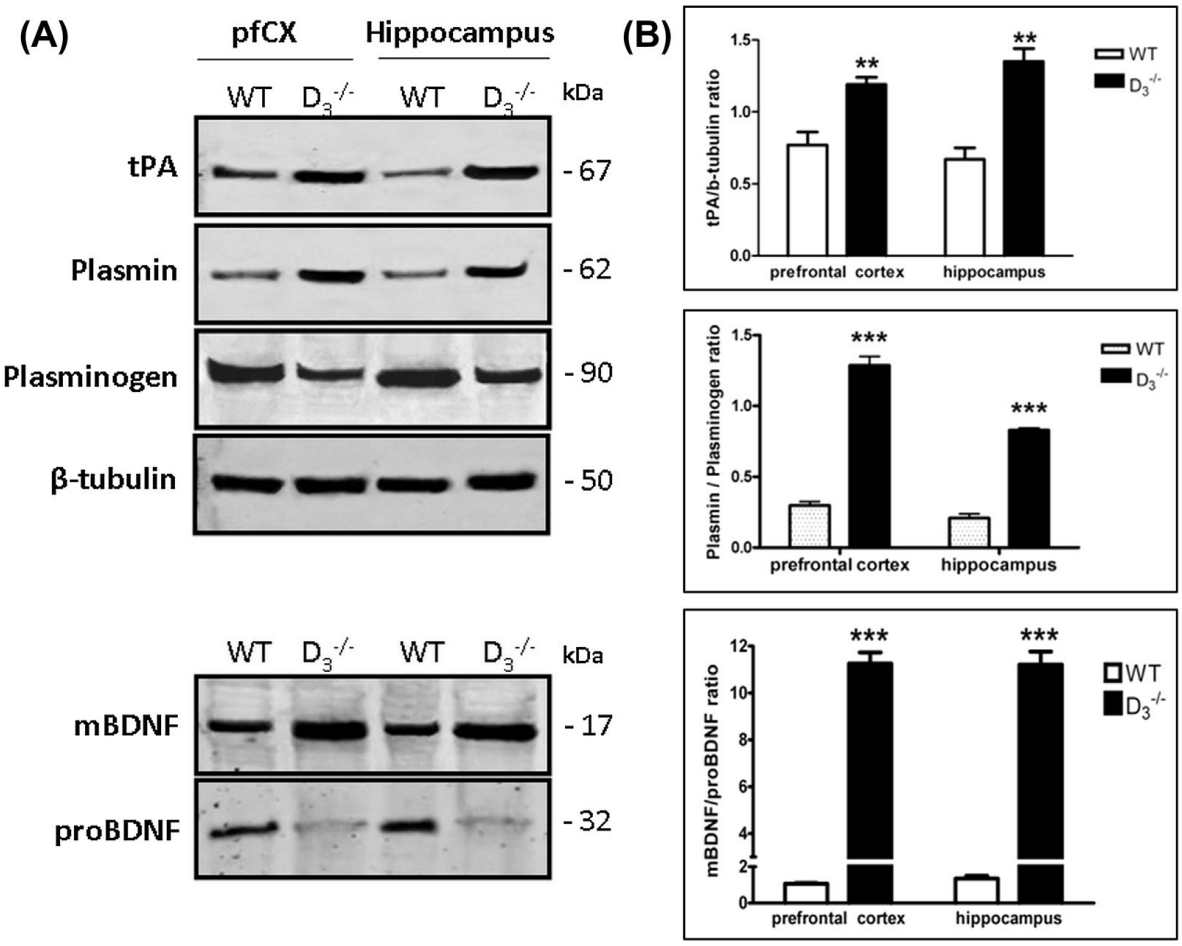

Figure 2. Enhanced tPA expression in $D_3^{-/-}$ mice brain correlates with increased plasmin/plasminogen and mature BDNF/proBDNF protein ratios

(**A**) Representative blots and densitometric analyses of bands (**B**) showing increased tPA protein expression and activity, measured as the relative conversion ratio of plasminogen into plasmin and of proBDNF into mature BDNF in the prefrontal cortex and hippocampus of both WT and $D_3^{-/-}$ mice. (**A**) 30µg of tissue homogenates (n=3 prefrontal cortices and n=4 hippocampi *per* each group) were incubated using specific antibodies and scanned with an Odyssey Infrared Imaging System, as described in Materials and Methods section. (**B**) Densitometric analyses of bands were performed using the ImageJ software and normalized values were calculated by dividing the mean optical density (OD) of bands matching the molecular weight (kDa) of cleaved proteins (plasmin and mBDNF, respectively) over the corresponding uncleaved protein

values (plasminogen and proBDNF), except for tPA which was normalized to β-tubulin, which served as loading control. Data are expressed as mean ± S.E.M., as the result of at least three independent determinations. **p<0.01 ***p<0.001 *vs* WT, as determined by unpaired two-tailed Student *t*-test.

2.3 D_3R deletion increases tPA and mature BDNF immunoreactivity in the prefrontal cortex and hippocampus

To determine whether the absence of the D_3R could also influence the distribution and/or immunoreactivity (IR) of both tPA and mature BDNF (mBDNF), immunohistochemical analyses were carried out on specific coronal brain sections, corresponding to the prefrontal cortex (Bregma +1.70mm, Paxinos and Franklin, 2001) and hippocampus (Bregma –2.06mm) of both WT and $D_3^{-/-}$ mice. WT mice sections served as control. Furthermore, coronal sections of the cerebellar cortex were uses as negative controls (Bregma –6.96mm). As shown in Fig. 3A, 3B and 3E, tPA IR was detectable both in the prefrontal cortex, hippocampus and cerebellar cortex of WT mice, and was remarkably increased in $D_3^{-/-}$ mice brain, especially in hippocampal subfields CA1 and CA2, but not in the cerebellar cortex of knockout mice. Parallel immunohistochemical analyses performed using a specific antibody that recognizes mBDNF revealed that D_3R deletion caused a robust increase in signal intensity throughout the prefrontal cortex (Fig. 3C) and the hippocampal subfield evaluated (CA1–CA3 and dentate gyrus, respectively; Fig. 3D). As expected, the absence of the receptor did not affect signal intensity in the cerebellar cortex (Fig. 3F). Conversely, no evident changes in the pattern of distribution of both tPA and mBDNF were apparent in either brain regions analyzed when comparing the two genotypes.

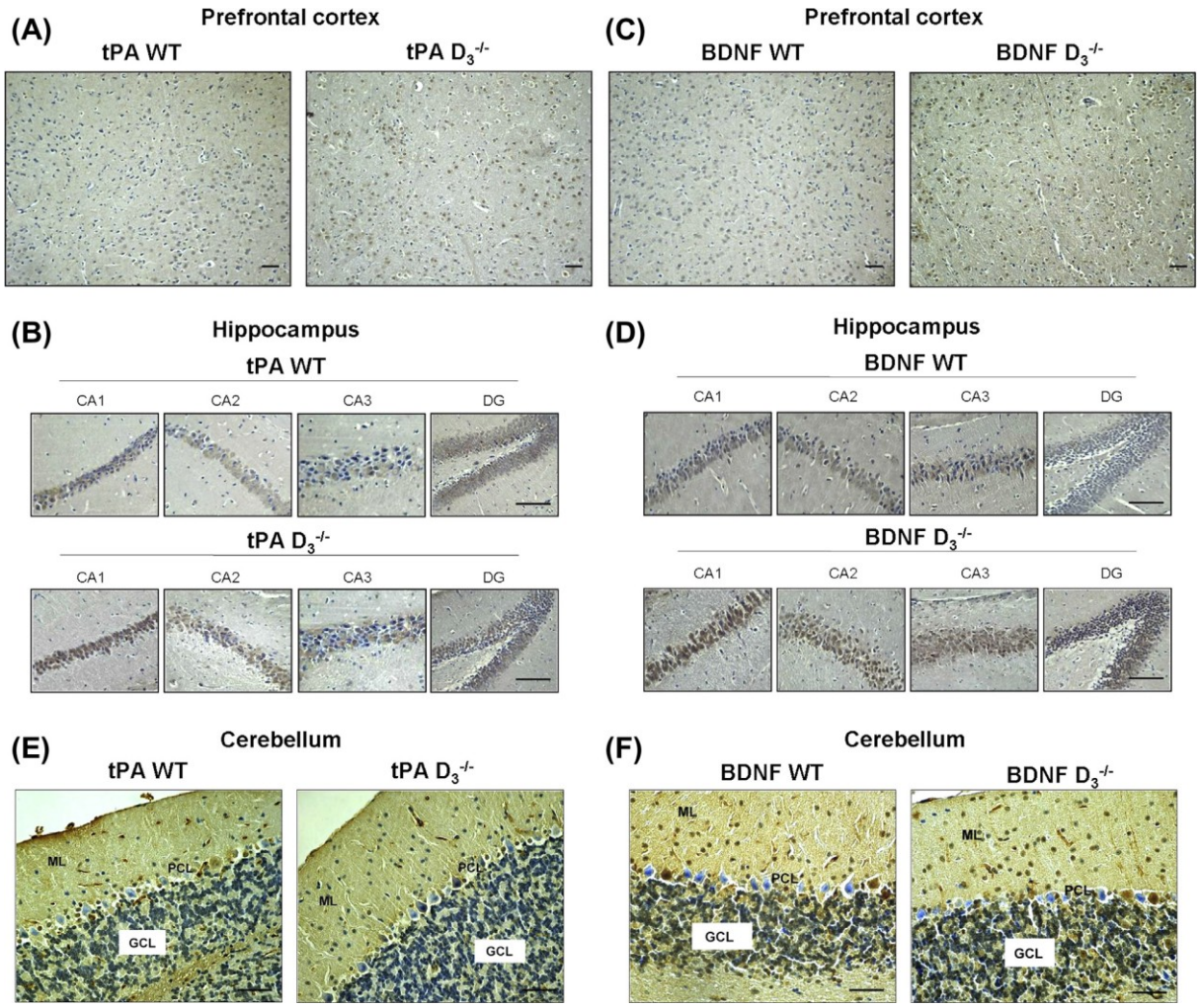

Figure 3. tPA and mature BDNF immunoreactivity in the prefrontal cortex, hippocampus and cerebellar cortex of WT and $D_3^{-/-}$ mice

Representative photomicrograhs showing tPA (**A, B, E**) and mature BDNF (**C, D, F**) immunoreactivity and distribution in brain slices corresponding to the prefrontal cortex, hippocampus (subfields CA1, CA2, CA3 and dentate gyrus [DG], respectively) and cerebellar cortex of WT and $D_3^{-/-}$ mice (Paxinos and Franklin, 2001). As clearly depicted, no apparent changes in tPA and mature BDNF localization were evident when comparing the two animal groups. Conversely, tPA signal intensity was visibly increased both in the prefrontal cortex and hippocampus of $D_3^{-/-}$ mice, particularly in hippocampal subfields CA1 and CA2. Consistently, robust mature BDNF immunoreactivity was thoroughly detected throughout the prefrontal cortex (**C**) and the hippocampal subfields (CA1–CA3 and dentate gyrus, respectively; **D**) of knockout mice. Cerebellar cortex was used as negative control, thus showing no evident

changes in the distribution and relative signal intensity of either tPA (**E**) or mature BDNF (**F**) between WT and $D_3^{-/-}$ mice. Each layer of the cerebellar cortex was labelled using the following acronyms : **ML** = Molecular Layer; **PCL** = Purkinje Cell Layer; **GCL** = Ganglion Cell Layer. Original magnification was 10x (**A** and **C**), 20x (**B** and **D**) and 40x (**E** and **F**). Scale bar = 50μm (**A-D**) and 25μm (**E-F**). Images were taken from different randomly selected brain sections and examined under a light microscope (Axiovert, Carl Zeiss Inc) equipped with a digital color camera.

2.4 $D_3^{-/-}$ mice exhibit increased basal phosphorylation of Akt, DARPP-32 and GSK-3β

D_2-class receptors have been shown to inhibit the cAMP-PKA-dependent pathway (Cho et al., 2010; Wall et al., 2011). Specifically, from previous studies it has emerged that these receptors can inhibit most of DA actions either by inactivating DARPP-32 (DA and cAMP regulated phosphoprotein of Mr 32 kDa), a potent inhibitor of protein phosphatase 1 (PP-1) (Yan et al., 1999; Rosa et al., 2008) or by blocking Akt activity through dephosphorylation of its regulatory threonine 308 (Thr308) and serine 473 (Ser473) residues, which in turn, may not gate the constitutive inhibitory activity of glycogen synthase kinase-3β (GSK-3β) on CREB (Beaulieu et al., 2004, 2005). In both cases, one of the major consequences of these molecular events is the impairment of CREB, a transcriptional regulator of both tPA and proBDNF (Greengard et al., 1999; Beaulieu et al., 2004, 2005; Benito and Barco, 2010). Therefore, we attempted to establish the relative contribution of D_3Rs, which possess the highest affinity for DA, on basal activity of Akt, DARPP-32 and GSK-3β both in the prefrontal cortex and hippocampus of wild-type (WT) and $D_3^{-/-}$ mice. Results demonstrated that basal Akt phosphorylation at Thr308 was significantly increased both in the prefrontal cortex (t_4=10.19; ***p<0.001 Vs WT) and the hippocampus (t_4=5.051; **p<0.01) of knockout mice, whereas phosphorylation at Ser473 was increased at significant levels only in the hippocampus of $D_3^{-/-}$ mice (pfCX : t_4=1.342, p=0.25; hippocampus : t_4=6.344, **p<0.01) (Fig. 4A and 4B). Analogous analyses on DARPP-32 activity showed that phosphorylation at Thr34 residue was significantly increased in both brain regions of $D_3^{-/-}$ mice when compared to controls (pfCX : t_4=6.601, **p<0.01; hippocampus :

t_4=13.447, ***p<0.001) (Fig. 4C and 4D). Interestingly, a similar significant increase in phosphorylation state-dependent inhibition of GSK-3β at Ser9 residue was observed in mice lacking the D_3R (pfCX : t_4=6.601, **p<0.01; hippocampus : t_4=13.447, ***p<0.001) (Fig. 4C and 4D), suggesting that, under basal conditions, both DARPP-32- and Akt-mediated inhibitory activities on PP-1 and GSK-3β, respectively, should be increased in mice lacking functional D_3Rs.

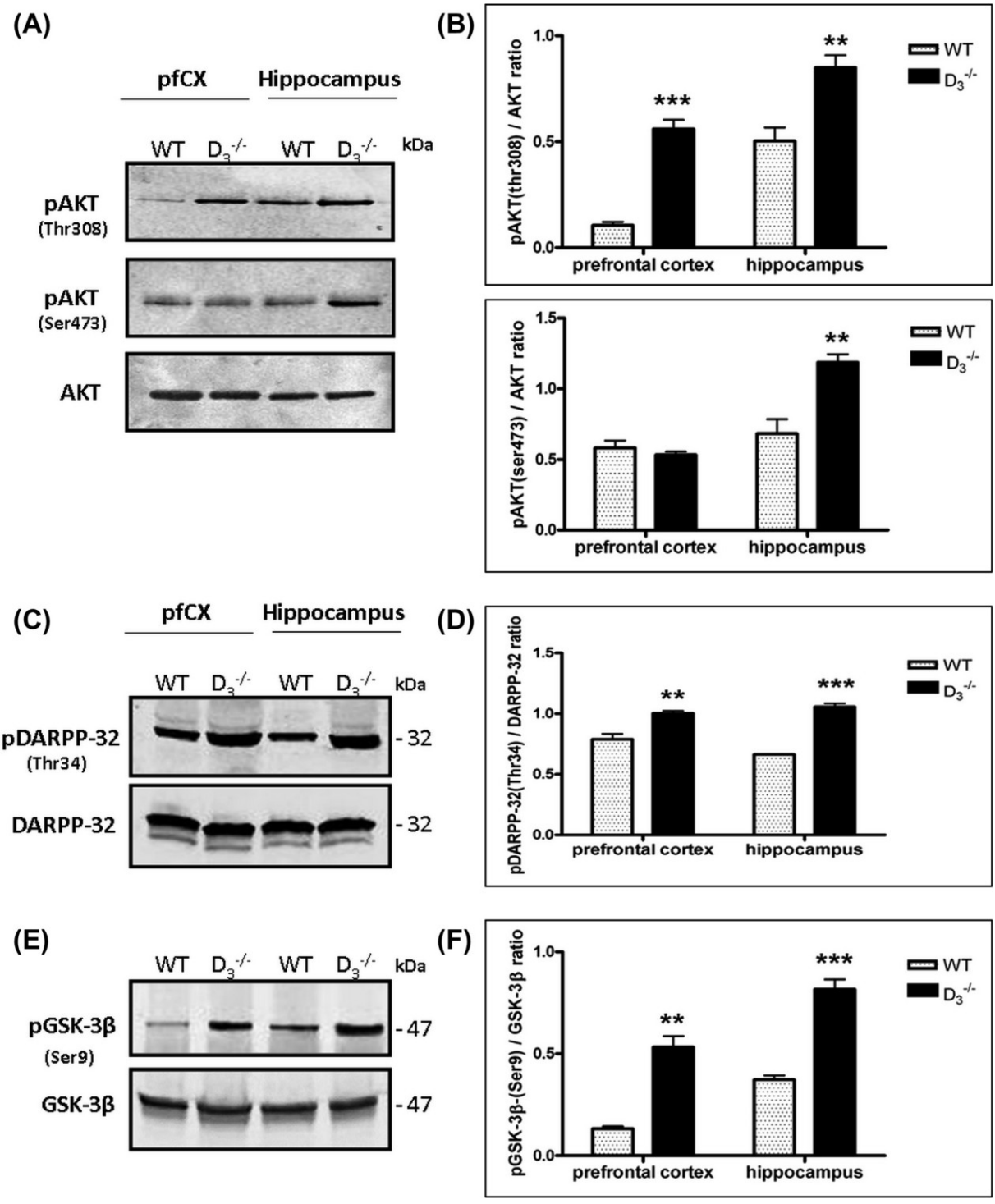

Figure 4. Increased basal phosphorylation of Akt, DARPP-32 and GSK-3β in the prefrontal cortex and hippocampus of $D_3^{-/-}$ mice

Western blots (**A**, **C** and **E**) and densitometric analyses (**B**, **D** and **F**) of phospho-Thr-308 and phospho-Ser-473 Akt (**A-B**), phospho-Thr-34 DARPP-32 (**C-D**) and phospho-Ser-9 GSK-3β (**E-F**) in extracts prepared from the prefrontal cortex and hippocampus of drug-naïve wild-types (WT) and $D_3^{-/-}$ mice. 30μg of tissue homogenates (n=4 prefrontal cortices and n=5 hippocampi *per* each group) were incubated using both phospho-specific and phospho-independent antibodies and scanned with an Odyssey Infrared Imaging System, as described in Materials and Methods section. Phospho-independent antibodies directed against respective kinases were used as loading controls. Results reported are the average relative ratios of each phosphorylated protein normalized to total protein expression ± S.E.M., obtained from three independent experiments. **p<0.01 ***p<0.001 *vs* WT, as determined by unpaired two-tailed Student *t*-test.

2.5 Opposite effects of D_3R deletion on CREB phosphorylation at Ser133 and Ser129 residues

Finally, to investigate the involvement of D_3R deletion on CREB transcriptional activity, we analyzed the expression of two CREB phospho-specific residues (Ser133 and Ser129, respectively) known to be necessary for its transcriptional activation (Grimes and Jope, 2001). We focused our attention on these phosphorylation sites since they are regulated, among other substrates, indirectly by DARPP-32 through PP-1 (Ser133) to increase CREB activity (Rosa et al., 2008) or directly by active GSK-3β (Ser129) to diminish it (Beaulieu et al., 2004), thus playing opposite roles. In addition, we analyzed the expression levels of tyrosine hydroxylase (TH), the rate-limiting enzyme of DA synthesis, to establish if neurotransmitter production could also take part in CREB activation process. As depicted in Fig. 5A and 5B, no significant differences in TH levels were found between WT and $D_3^{-/-}$ mice neither in the prefrontal cortex (pfCX : t_4=0.344, p=0.75 Vs WT) nor in the hippocampus (t_4=2.188, p=0.11), suggesting that DA synthesis might not be affected by D_3R deletion in these specific brain regions and under basal conditions.

As expected, Ser133-phospho-CREB levels were significantly increased both in prefrontal cortices (t_4=22.021, ***p<0.001 Vs WT) and hippocampi (t_4=23.356, ***p<0.001) of $D_3^{-/-}$ mice, whereas the GSK-3β-dependent Ser129-phospho-CREB protein expression was significantly diminished (pfCX and hippocampus : t_4=7.803 and 6.678, **p<0.01) (Fig. 5C and 5D).

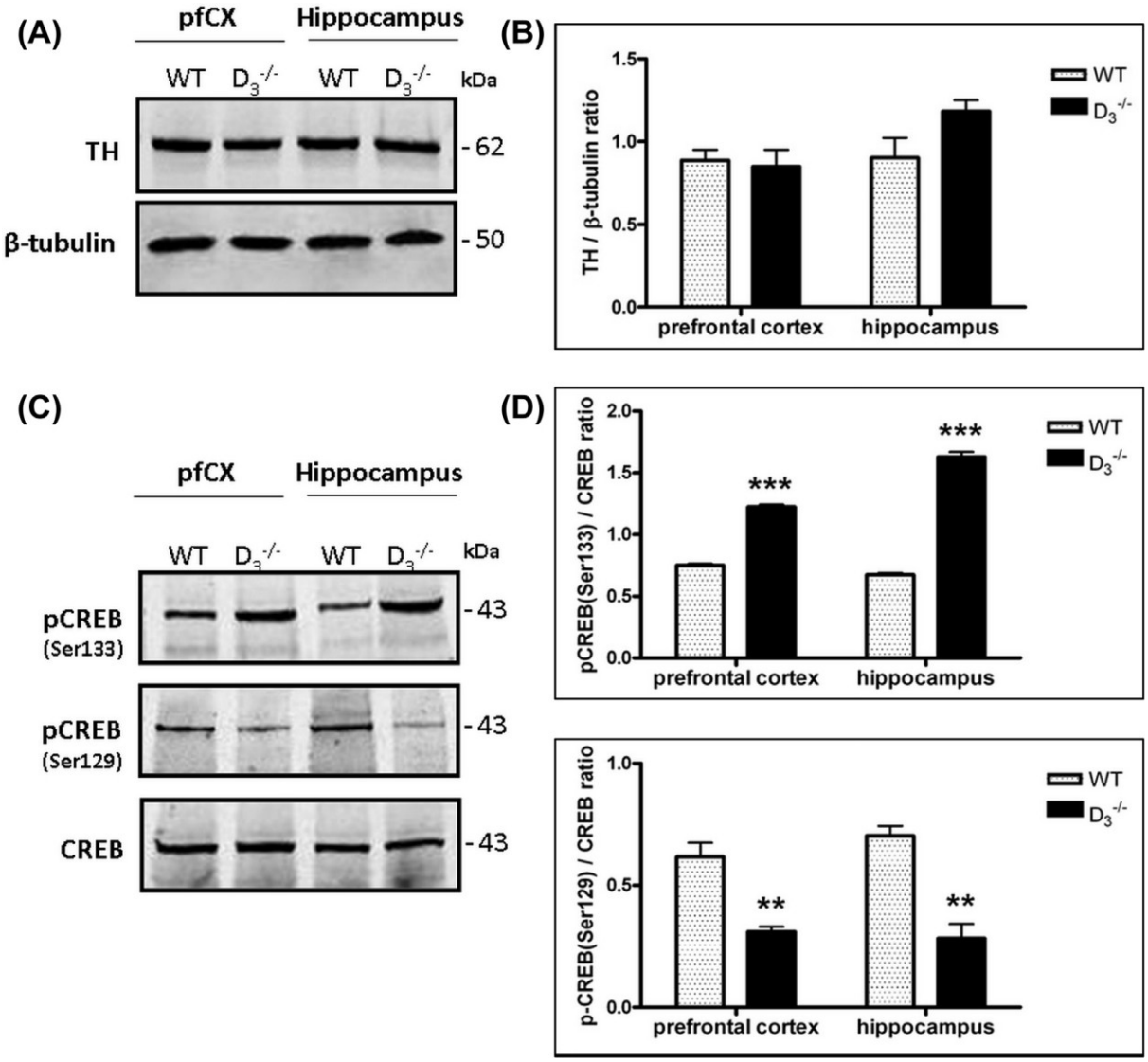

Figure 5. Basal CREB phosphorylation at Ser133 and Ser129 residues is oppositely regulated in $D_3^{-/-}$ mice, without affecting tyrosine hydroxylase expression

Representative immunoblots (**A** and **C**) and densitometric analyses (**B** and **D**) showing tyrosine hydroxylase (TH), phospho-Ser133-CREB and phospho-Ser129-CREB expression levels in tissue extracts (30µg) obtained from the prefrontal cortex (pfCX) and hippocampus of wild-type

(WT) and $D_3^{-/-}$ mice (n=3 prefrontal cortices and n=5 hippocampi *per* each group). Membranes were incubated using either a rabbit polyclonal TH antibody (sc-14007, Santa Cruz Biotechnology) or two phospho-specific primary antibodies which recognize the Serine 133 (pCREB[Ser133]) or the Serine 129 residues (pCREB[Ser129]) and then scanned with an Odyssey Infrared Imaging System, as described in Materials and Methods section. Densitometric analyses were performed using the ImageJ software and values obtained were normalized to β-tubulin (**A-B**) or to a phospho-independent antibody directed against CREB (**C-D**, sc-186, Santa Cruz Biotechnology), both of which were used as loading controls. Results are the average ratios ± S.E.M. from three independent experiments. **p<0.01 ***p<0.001 *vs* WT, as determined by unpaired two-tailed Student *t*-test.

3. Discussion

In the present work we demonstrate that D_3R deletion increases baseline expression and activity of tissue plasminogen activator (tPA), a protease involved in the proteolytic cleavage of the precursor of brain derived neutrophic factor (proBDNF) into its biologically active form, mature BDNF (mBDNF), in two brain areas involved in cognitive function, the prefrontal cortex and hippocampus. Interestingly, this represents the first study in which the D_3R is correlated to brain tPA expression/activity, especially under drug-free conditions. In fact, in the last decade, most of the attention given to these high DA affinity receptors has primarily converged towards understanding their potential involvement in addictive behaviours, with particular emphasis on the mechanisms of relapse and self-administration of drugs of abuse. However, while this direction of study is substantiated by observations on mice exposed to chronic drug treatment or in human overdose fatalities indicating increased expression of the D_3R in restricted brain regions associated to reward-related behaviors (reviewed by Newman et al., 2012), other studies undertaken on mice lacking functional D_3Rs or treated with novel and more potent D_3R antagonists have indicated that the receptor is also a key player in a number of non-pathological behaviours and functions, including some types of learning (Micale et al., 2010; Watson et al., 2012; D'Amico et al., 2013) neurogenesis (Egeland et al., 2012) and changes in gene expression

(D'Agata et al., 2009; Castorina et al., 2011). These evidence, together with the knowledge that D_3Rs possess the highest affinity for DA and are also localized in extrastriatal regions, has prompted us to investigate whether and why receptor deletion increased basal tPA gene expression and activity.

Our first finding showed that both tPA and proBDNF mRNA levels were significantly increased in the prefrontal cortex and hippocampus of $D_3^{-/-}$ mice (Fig. 1A and 1B), implying that in the absence of the receptor, their transcription could be enhanced. Furthermore, through immunoblot analyses, we were able to establish an indirect but positive relationship between increased tPA gene and protein expression and its proteolytic activity (Fig. 2A and 2B), which was measured as the relative protein conversion ratio of plasminogen into plasmin, the former being a direct target of tPA, and subsequently, of proBDNF to mBDNF by plasmin (Pang et al., 2004). Concurrently, immunolocalization studies revealed that both tPA and mBDNF IR signals were increased in the prefrontal cortex and hippocampus of $D_3^{-/-}$ mice, although their distribution did not differ between the two genotypes (Fig. 3).

In the second part of this study, experiments were performed in the attempt to provide a biochemical explanation to elucidate why, in the absence of the D_3R, basal tPA expression and activity were increased. Considering that D_3Rs, as opposite to the other D_2-class receptors, possess the highest affinity for DA and might therefore respond to low endogenous concentrations of ligand, we thought it was compelling to monitor the baseline activity levels of three major substrates belonging to the cAMP-PKA-dependent-Akt/CREB signalling pathway, the main molecular cascade modulated by D_2-class receptors to control gene expression (Beaulieu et al., 2004, 2005; Rosa et al., 2008; Cho et al., 2010; Wall et al., 2011). In line with this hypothesis, we first performed a comparative analysis of the phosphorylation levels of Akt at its regulatory threonine 308 (Thr308) and serine 473 (Ser473) residues between wild-type and $D_3^{-/-}$ mice. Then, we evaluated the activity levels of two molecules involved in the modulation of the Akt/CREB signalling pathway, namely DARPP-32 and GSK-3β, and finally assessed whether CREB activity was effectively modulated in the absence of the receptor. Intriguingly, our first finding showed that Akt phosphorylation at both regulatory residues in the prefrontal

cortex and hippocampus of $D_3^{-/-}$ mice was affected in a similar manner to that previously reported by Beaulieu et al. (2007) in the striatum, with a systematic overexpression of the Thr308 residue in both brain regions and increased phosphorylation of the Ser473 residue in the hippocampus only. In the light of these results we argued the possibility that, besides the relatively lower abundance of D_3Rs in extrastriatal regions, these receptors might also contribute to inhibit basal Akt activity with analogous mechanisms in different brain areas. In addition, we also discovered that phosphorylation levels of DARPP-32 at Thr34 and of GSK-3β at Ser9 residues were both augmented in prefrontal cortex and hippocampus (Fig. 4C-F) of knockout animals. These two cAMP-PKA-regulated substrates act through different mechanisms (Yan et al., 1999; Beaulieu et al., 2004, 2005; Rosa et al., 2008), but when phosphorylated at these specific residues they act synergistically to promote CREB transcriptional activity. For instance, increased activation of DARPP-32 through phosphorylation at Thr34 inhibits protein phosphatase-1 (PP-1)-mediated dephosphorylation of CREB at Ser133, resulting in enhanced CREB activity. Similarly, increased phosphorylation of GSK-3β at Ser9 residue by Akt blocks its constitutive inhibitory activity on CREB, which is obtained through a GSK-3β-driven phosphorylation of CREB at Ser129. Thus, the resultant is also enhanced CREB activation. Therefore, it is likely that the combined increased phosphorylation of these two important molecular substrates, independently, finally contributed to increase CREB transcriptional activity in $D_3^{-/-}$ mice. Indeed, such observation was confirmed by the evidence that knockout animals exhibited increased basal CREB phosphorylation at Ser133, concurrent with diminished phosphorylation at Ser129 (Fig. 5C and 5D), the residue phosphorylated by GSK-3β to impede normal CREB activity. Considering that CREB is responsible for the transcription of both tPA and BDNF, the increased activity of CREB observed in $D_3^{-/-}$ mice could therefore provide a plausible rationalization to explain, at least in part, the augmented transcription of both genes in the absence of the receptor (Fig. 1). However, an important aspect that should be taken into consideration, although it does not limit the conclusiveness of the presented data, is represented by the use of knockout animals. In fact, although in a previous study it has been shown that $D_3^{-/-}$ mice do not compensate for the loss of

function of the receptor with changes in the other DA receptor subtypes in the spinal cord (Zhu et al., 2008), it still remains possible that adaptive changes might affect other brain regions, including the prefrontal cortex and hippocampus. As such, an alternative explanation would be that the resultant increased tPA activity observed in $D_3^{-/-}$ mice could be the consequence of an "adapted" and potentiated D_1R signaling, the preferential regulator of BDNF synthesis in the prefrontal cortex (Xing et al., 2010), rather than be directly attributable to the lack of inhibition on the cAMP-PKA-Akt-CREB signaling caused by the absence of the receptor.

4. Conclusions

In summary, this study shows that D_3R deletion increases baseline tPA expression and activity in the prefrontal cortex and hippocampus. The proposed mechanism to explain such a heightened tPA expression in $D_3^{-/-}$ mice seems to be dependent on the cAMP-PKA-driven activation of CREB, which is under the control of both DARPP-32 and the Akt/GSK-3β signalling cascade. Considering the importance of tPA in synaptic plasticity, long-term potentiation and neurite outgrowth (Madani et al., 1999; Pang et al., 2004; Egeland et al., 2012), the present finding corroborate the proposal that either genetic or pharmacological blockade of D_3Rs might enhance certain aspects of cognitive performance (Micale et al., 2010; Egeland et al., 2012), possibly by increasing extracellular protease activity in regions critical for memory function.

Acknowledgements

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General discussion

Human learning and memory are only possible when the nervous system is functioning normally. The primary building blocks of the nervous system are neurons–specialized cells that form connections, or synapses, with specific targets. As a functional synapse is a fundamental requirement for the brain to process any task, synaptic function is tightly regulated. This regulation occurs at multiple steps, such as recruitment and assembly of molecular machinery, synapse formation and stabilization, coordinated release of neurotransmitters, downstream signaling of receptors, maintenance, plasticity, and eventual loss of the synapse (Poon et al. 2013). Synaptic plasticity is thought to be critical for learning and memory (Clopath, 2012). Neuromodulators, which alter cellular and synaptic proprieties via widespread projection (Kupfermann, 1979) have a well-known role in memory function and many modulation pathways have been targeted for memory enhancement (Floresco et al, 2011). One modulator with a well-known effect on cognition is dopamine (DA) (Stern, 2013). DA is believed to be a key neurotransmitter that mediates memory via signaling in the striatum, hippocampus, and prefrontal cortex. DA has multiple roles in cognition-related brain functions: it regulates memory, motivation, mood, motor activity, and neuroendocrine integration and it is released after novel, salient sensory, aversive, or reinforcement-relevant (reward) stimuli (Ljungberg et al. 1992; Ungless, 2004; Bromberg-Martin et al. 2010; Schultz et al. 1993).

Dopaminergic innervation in the brain can be divided into four main pathways (Beaulieu & Gainetdinov, 2011), through which dopamine plays different roles in guiding behavior in dopamine terminal regions. In particular the *mesolimbic pathway* connects ventral tegmental area to the nucleus accumbens, amygdala and hippocampus, and *mesocorticolimbic pathway* has been characterized as a region that guides associative learning (Abraham et al., 2013). Many studies looking at the role of dopamine in memory formation, consolidation and recall at a behavioral level have focused on the effects of direct hippocampal dopamine interference (Bethus et al., 2010). The presence of D_3Rs in projection regions of the *mesocorticolimbic* system suggests a potential role of these receptors in reinforcement processes, emotion and cognitive functions. Animal studies suggest that D_3Rs blockade enhances cognitive function,

while D_3Rs activation impairs cognition, which would justify clinical trials to examine the effect of D_3R antagonists on neuropsychiatric disorders (Laszy et al.,2005). Indeed there are many evidences demonstrating that $D_3^{-/-}$ mice showed better performance than WT mice in measures of selective attention (mainly olfactory) (Watson et al., 2012), aversive/associative learning (Micale et al., 2010; D'Amico et al. 2013).

A previous study of Donarum et al. (2006) suggested a functional connection between neurofibromin and the amyloid beta precursor protein/integrin/filamin complex, which is in turn related to the D_3R.

According to the amyloid cascade hypothesis, the abnormal processing of APP is the first in a series of events that then results in the neurodegenerative process underlying AD pathology (Hardy and Allsop, 1991). Characteristics of soluble Aβ oligomers are the inhibition of long-term potentiation (LTP), the electrophysiological process correlates to learning and memory; interruption of neural signalling pathways required for synaptic plasticity and neural integration; alteration of synaptic structure by decreasing dendritic spine densities; interference with receptor and channel signalling due to binding to synaptic plasma membranes and impairment of learning and memory in rodents (Haass and Selkoe, 2007; Selkoe, 2008; Arendt, 2009). Compared to control mice, APP transgenic mice in fear conditioning exhibit less freezing behavior, indicating a reduced ability to learn and store the association of an aversive with a neutral stimulus or with a certain environment (Röskam et al., 2010).

Neurofibromin, the protein product of the NF1 gene, acts as an inhibitor of Ras activity by catalyzing the hydrolysis of active GTP-bound Ras to inactive GDP-bound Ras such that in cells lacking neurofibromin, there are high levels of Ras pathway (Basu et al., 1992; Lau et al., 2000). Increased RAS pathway activity causes GABA release and subsequent diminishes synaptic plasticity in mice (Cui et al., 2008).

In many studies, using NF1 genetically engineered strains of mice and flies, investigators have successfully modeled many of the cognitive and behavioral deficits seen in children with NF1, and employed these model systems to better define the role of the NF1 protein (neurofibromin) in normal central nervous system (CNS) (Diggs-Andrews and Gutmann, 2013). NF1

heterozygous mice exhibit impaired spatial learning in the Morris water maze, a behavioral task used to assess hippocampal-based learning (Silva et al., 1997; Costa et al., 2002). Using NF1 conditional knockout (CKO) mice in which one copy of the NF1 gene was selectively inactivated in neurons or astrocytes, the consequence of reduced neurofibromin expression was evaluated in distinct cell populations during learning and memory tasks (Cui et al., 2008). A reduced NF1 expression in neurons recapitulated the learning and memory deficits was observed in NF1 heterozygous mice.

These findings clearly explicated that NF1 and APP genes are correlated to cognitive function. Based on evidences described above and due to passive (or inhibitory) avoidance is a conditioning memory that is commonly used in rodent memory tasks (Stern and Alberini, 2013), in our first study we used PA conditioning and aimed to achieve two major goals : (1) to evaluate the possible differences in D_3R expression during the acquisition of PA conditioning, (2) establish whether NF1 and APP genes could be affected by presence or absence of D_3Rs during this behavioral trial.

In agreement with data described in literature we observed that $D_3^{-/-}$ mice showed enhanced cognitive performance as compared to the WT mice (Micale et al., 2010), furthermore we showed that the expression levels of D_3R and NF1 gene were increased during the learning process in WT mice. Enhanced NF1 expression levels in trained animals were completely abrogated in hippocampus of $D_3^{-/-}$ mice, confirming the data obtained by Castorina (et al., 2011) demonstrating that in $D_3^{-/-}$ mice the hippocampal NF1 expression was unchanged as compared to WT mice. No changes either in hippocampal APP mRNA and protein expression levels were detected during behavioral trials in WT mice, while an increased APP basal expression levels was observed in $D_3^{-/-}$ mice and reversed during acquisition task. Unfortunately one good explanation for this result does not seems to be attributable to D_3Rs. In this work, in which we limited our study on the evaluation of genes expression, we provide evidences that D_3R could play a relevant role in NF1 transcriptional regulation although the precise mechanism by which D_3Rs performs this action remains unknown.

Extraordinary process has been done in the understanding of cellular and molecular mechanism that are important for memory formation in several different species and types of learning (Stern and Aleberini, 2013) and it is well documented that there are multiple pathways through which dopamine may alter signal transduction and plasticity (Abraham et al., 2013). Dopamine transmission at target synapses can induce the activation of molecular pathways leading to CREB phosphorylation and modification of synaptic strength (Dudman et al., 2003). Extensive evidences support a critical role of the pCREB in reinforcement learning (Hyman et al., 2006). Increased hippocampal CREB was found after acquiring the spatial task (Baudonnat et al., 2011) and other evidences show that dopamine $D_3^{-/-}$ mice demonstrate ameliorated age-related deficits in spatial memory process and D_3R is involved in the dysregulation of CREB activation in the aged hippocampus (Xing et al., 2010).

However there is no evidence regarding the involvement of the D_3R in the activation of CREB during a process of associative memory. In the second paper we then investigated the involvement of D_3Rs in the activation of CREB signalling cascades after acquisition of PA. In this study with the use of Ser-133 p-CREB as a marker of CREB activation (Montminy et al., 1990), the profile of activated CREB was demonstrated in $D_3^{-/-}$ mice subjected to fear-conditioning learning. The results showed increased p-CREB (Ser-133) expression levels in trained mice WT but to large extent in $D_3^{-/-}$ mice as compared to the untrained animals.

CREB is activated by different signal transduction pathway implicated in memory consolidation, for example from growth factor stimulation of tyrosine kinase receptors coupled to the activation of Ras and extracellular signal-regulated kinase (ERK); to G-protein coupled receptor (GPCR) activation coupled to activation of adenylyl cyclase, increase in cAMP, and activation of protein kinase A (PKA). All of these pathways can lead to the activation of CREB and this activation in turn can lead to the transcriptional regulation of a number of target genes that can participate in downstream synaptic changes underlying synaptic plasticity (Alberini, 2009; Alberini and Chen, 2012).

The ERK subfamily of mitogen-activated protein kinase (MAPK) contributes to crucial signal transduction systems that regulate cAMP response element-binding protein (CREB) activity,

which has a key role in long-term memory formation (Ahi et al, 2004). The early activation of ERK signaling in the hippocampus is necessary for the establishment of long term memory (Giovannini, 2006) and it has also been reported that MAPK/ERK are involved in various types of memory retrieval and extinction of step down inhibitory avoidance and auditory fear conditioning (Barros et al, 2000; Herry et al, 2006; Bonini et al, 2011).

In order to investigate the underlying molecular pathway involved in increased phosphorylation CREB in $D_3^{-/-}$ mice, we analyzed the activation of ERK1/2 in hippocampus of WT and $D_3^{-/-}$ mice performing PA task. In accordance with the data present in literature our results shown increased hippocampal ERK1/2 phosphorylation in $D_3^{-/-}$ mice during PA conditioning, while the other two MAPKs, JNK and p38 don't are affected by conditioning. Furthermore we also analyzed the expression levels of Akt (Thr 308), one downstream target of dopaminergic receptors signaling that is involved in activation of CREB. The results obtained reveal increased phosphorylation levels of Akt (Thr 308) were only correlated to the $D_3^{-/-}$ mice genotype. Based on these results, we suggested that the enhanced cognitive performance of $D_3^{-/-}$ mice during PA trials was associated to induction of CREB phosphorylation in hippocampus of mice through modulating the ERK phosphorylation that is induced during this process.

The classical view of dopamine receptor activity has focused on intracellular signaling through adenylate cyclase and cAMP activity, which is widely recognized to play central roles in learning. For example, inhibitory interactions between dopamine- and cAMP-regulated neuronal phosphoprotein (DARPP-32) and protein phosphatase-1 (PP1) are important for intracellular regulation of neural plasticity (Gould & Manji, 2005). In particular PKA induces phosphorylation of DARPP-32, which inhibits protein phosphatase 1 (PP1) leading to CREB activation at Ser133. Phosphorylation of CREB can also be induced by m-BDNF action when interacts on the tyrosine kinase (Numakawa et al., 2010). Various studies have shown that the expression of brain-derived neurotrophic factor (BDNF), a protein that can be found in the pro-BDNF, truncated-BDNF and mature-BDNF isoforms, is involved in the persistence of memory (Pang and Lu, 2004; Bekinschtein et al., 2007, 2013; Nagappan et al., 2009; Sartori et al., 2009; Leal et al., 2013). The pro-BDNF is cleaved extracellularly by plasmin protease, this enzyme is

expressed as an inactive zymogen known as plasminogen. Plasminogen activation involves another protease, the tissue plasminogen activator (tPA) (Lochner et al., 2008; Greenberg et al., 2009; Cunha et al., 2010).

In our third work in order to investigate whether the D_3Rs plays a role in modulation of others genes related to memory process, we evaluated the basal expression of tPA in hippocampus and PFC of WT and $D_3^{-/-}$ mice, analyzing its proteolitic activity through analyses of m-BDNF and plasmin. The data showed that the absence of D_3R induces an increase in the expression of tPA and mature-BDNF with concomitant enhanced in plasmin/plasimigen ratio expression levels in the hippocampus and in the PFC.

Nevertheless there are multiple pathways through which dopamine may alter signal transduction and plasticity. For example DA receptors regulate the activity of Akt (also known as protein kinase B), a serine/threonine kinase involved in various cellular functions, including cell growth and proliferation (Manning and Cantley 2007; Beaulieu et al. 2011). Glycogen synthase kinase-3β (GSK-3 β) is one effector of Akt, which is widely expressed in the brain (Leroy and Brion 1999) and implicated in processes underlying neuronal survival, development, and synaptic plasticity (Peineau et al. 2008). The inhibition of GSK-3β by Akt leading to facilitate CREB function, while the activation impairs CREB phosphorylation. Is well documented that D_2-like receptors modulates GSK-3β via a pathway that involves arrestin and inhibition of the protein kinase Akt (Beaulieu et al., 2004, 2005). Interestingly, as suggested by results obtained in D_3R knockout mice (Beaulieu et al. 2007), D_3R could also participate in Akt signaling, an hypothesis reinforced by the quick increase in Akt phosphorylation after stimulation of D_3R in various cell lines (Chen et al. 2009; Mannoury la Cour et al. 2011; Collo et al. 2012).

Considering these evidences, in the third work we also tried to understand what were the signaling pathways involved in increased expression of tPA in the absence of D_3R. To this end we have analyzed the expression of different substrates downstream PKA/Akt/CREB signaling pathway. Datas showed increased Akt, DARPP-32 and GSK-3β phospshorylation in $D_3^{-/-}$ mice, suggesting that this pathaways is involved in raised tPA expression in $D_3^{-/-}$ mice. The present finding deepens previous evidences that basal levels of phosphorylation of Akt and GSK-3β

were increased in $D_3^{-/-}$ mice and these effects have been already reported in $D_3^{-/-}$ mice by Beaulieu et al. (Beaulieu et al. 2007) who attributed it to a synergism of D_3R with the processes of Akt inhibition mediated by prolonged D_2R activation.

Conclusion

In agreement with the data present in literature we showed that $D_3^{-/-}$ mice exhibit enhanced cognitive performance during PA conditioning, and in our studies we suggested that this improving could be due to hippocampal CREB activation, through its phosphorylation at Ser133, mediated by MAPK/ERK pathway. Furthermore D_3R seems also involved in modulation of genes reletad-memory, for example plays a role in transcriptional modulation of NF1 gene during the process of memory-associative learning. Although these evidences need further investigations to clarify the mechanisms through which the D_3R is involved in modulation of some genes related to memory, this effect could be result from a series of molecular events that are enabled by absence of D_3R necessary to ensure the regulation of neuronal plasticity. Our results suggested that modulatory function of the D_3R in basal condition is mediated by inhibition of the tPA pathway PKA/GSK3β/pCREB.

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