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

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Running title : D_3R, NF1 and APP in classic 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 D3 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 [1,2]. 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 [4,5].

Involvement of hippocampal D_2-like receptors in mnemonic processes has been attentioned by several research groups in human [6,7] and rodents [8,9,10]. Furthermore, it has been suggested that disturbances in hippocampal DAergic systems cause memory impairment [11]. 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 [9,12,13]. Recently, we have shown that $D_3R^{-/-}$ mice exhibit changes in the expression of two genes related to cognitive function, namely NF1 and APP [5,14,15].

The NF1 gene encodes neurofibromin, a large protein with Ras GTPase activity [16,17]. 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 [14,17].

Amyloid precursor protein (APP) is a type 1 membrane glycoprotein distributed in the central and peripheral nervous system [18,19]. 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) [20,21,22,23]. 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 [24] and memory [15,19,25]. 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 [26,27].

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 [5].

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 [28]. 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 [29,30] 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 [13, 29].

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 [31]. 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.

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 [29]. 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)[13]. 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).

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).

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).

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 [5] 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).

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 [10] 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 [5]. Since PA is known to initiate a cascade of altered gene expression in the hippocampus [12], 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 [32,33,34]. 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 [10]. 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 [14,16,17,26,27]. 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 [35]. 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 [14] and in the activation of signaling cascades that regulate neuronal outgrowth during both early- and late-phase LTP [17]. 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 [5], 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 [36]. 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.

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References

1. Missale C, Nash SR, Robinson SW, Jaber M, Caron MG. (1998) Dopamine receptors: from structure to function. Physiol Rev 78:189-225.
2. Karasinska JM, George SR, Cheng R, O'Dowd BF. (2005) Deletion of dopamine D1 and D3 receptors differentially affects spontaneous behaviour and cocaine-induced locomotor activity, reward and CREB phosphorylation. European Journal of Neuroscience 22: 1741–1750. doi:10.1111/j.1460-9568.2005.04353
3. Sokoloff P, Giros B, Martres MP, Bouthenet ML, Schwartz JC. (1990) Molecular cloning and characterization of a novel dopamine receptor (D3) as a target for neuroleptics. Nature 347:146-51. doi:10.1038/347146a0
4. Levant B. (1998) Differential distribution of D dopamine receptors in the brains of several 3 mammalian species. Brain Res. 800:269-274. doi:10.1016/S0006-8993(98)00529-0
5. Castorina A, Leggio GM, Giunta S, Magro G, Scapagnini G, Drago f, D'Agata V. (2011) Neurofibromin and amyloid precursor protein expression in dopamine D3 receptor knock-out mice brains. Neurochem Res 36:426–434. doi:10.1007/s11064-010-0359-0
6. Kaasinen V, Vilkman H, Hietala J, Någren K, Helenius H, Olsson H, Farde L, Rinne J. (2000) Age-related dopamine D2/D3 receptor loss in extrastriatal regions of the human brain. Neurobiol Aging 21:683-8. doi:10.1016/S0197-4580(00)00149-4
7. Kaasinen V, Kemppainen N, Någren K, Helenius H, Kurki T, Rinne JO. (2002) Age-related loss of extrastriatal dopamine D(2) -like receptors in women. J Neurochem 81:1005-10. doi:10.1046/j.1471-4159.2002.00895
8. Laszy J, Laszlovszky I, Gyertyán I. (2005) Dopamine D3 receptor antagonists improve the learning performance in memory-impaired rats. Psychopharmacology (Berl) 179:567-75. doi:10.1007/s00213-004-2096-z
9. Izquierdo I, Bevilaqua LR, Rossato JI, Bonini JS, Medina JH, Cammarota M. (2006) Different molecular cascades in different sites of the brain control memory consolidation. Trends in Neurosciences 29:496-505. doi:10.1016/j.tins.2006.07.005
10. Micale V, Cristino L, Tamburella A, Petrosino S, Leggio GM, Di Marzo V, Drago F. (2010) Enhanced cognitive performance of dopamine D3 receptor "knock-out" mice in the step-through passive-

avoidance test: assessing the role of the endocannabinoid/endovanilloid systems. Pharmacol Res. 61:531-6. doi:10.1016/j.phrs.2010.02.003

11. Gasbarri A, Sulli A, Innocenzi R, Pacitti C, Brioni JD. (1996) Spatial memory impairment induced by lesion of the mesohippocampal dopaminergic system in the rat. Neuroscience 74:1037-44. doi:10.1016/0306-4522(96)00202-3
12. Izquierdo LA, Barros DM, Ardenghi PG, Pereira P, Rodrigues C, Choi H, Medina JH, Izquierdo I. (2000) Different hippocampal molecular requirements for short- and long-term retrieval of one-trial avoidance learning. Behav Brain Res 111:93-8. doi:10.1016/S0166-4328(00)00137-6
13. D'Agata V. and Cavallaro S.(2003) Hippocampal gene expression profile in passive avoidance conditioning. European Journal of Neuroscience 18: 2835-2841. doi:10.1111/j.1460-9568.2003.03025
14. Costa RM, Federov N.B, Kogan JH, Murphy G.G, Stern J, Ohno M, Kucherlapati R, Jacks T, Silva AJ. (2002) Mechanism for the learning deficits in a mouse model of neurofibromatosis type 1. Nature 415:526-530. doi:10.1038/nature711
15. Marcello E, Epis R, Di Luca M. (2008) Amyloid flirting with synaptic failure: Towards a comprehensive view of Alzheimer's disease pathogenesis. European Journal of Pharmacology 585:109–118. doi:10.1016/j.ejphar.2007.11.083
16. De Schepper S, Boucneau JM, Westbroek W, Mommaas M, Onderwater J, Messiaen L, Naeyaert JM, Lambert JL. (2006) Neurofibromatosis Type 1 Protein and Amyloid Precursor Protein Interact in Normal Human Melanocytes and Colocalize with Melanosomes. J Invest Dermatol 126:653-659. doi:10.1038/sj.jid.5700087
17. Guilding C, McNair K, Stone TW, Morris BJ. (2007) Restored plasticity in a mouse model of neurofibromatosis type 1 via inhibition of hyperactive ERK and CREB. Eur J Neurosci 25: 99–105. doi:10.1111/j.1460-9568.2006.05238.x
18. Selkoe DJ. (2002) Deciphering the genesis and the fate of amyloid beta-protein yields novel therapies for Alzheimer disease. J Clin Invest 110:1375-1381. doi: 10.1172/JCI0216783
19. Seabrook GR, Rosahl TW. (1999) Transgenic animals relevant to Alzheimer's disease. Neuropharmacology 38:1–17. doi:10.1016/S0028-3908(98)00170-1
20. Moran PM, Higgins LS, Cordell B, Moser PC. (1995) Age-related learning deficits in transgenic mice expressing the 751-amino acid isoform of human 18-amyloid precursor protein. Proc Natl Acad Sci U S A. 92: 5341-345.

21. Senechal Y, Kelly PH, Dev KK. (2008) Amyloid precursor protein knockout mice show age-dependent deficits in passive avoidance learning. Behav Brain Res 186:126–132. doi: 10.1016/j.bbr.2007.08.003

22. DeGiorgio LA, Shimizu Y, Chun HS, Kim YS, Sugama S, Son JH, Joh TH, Volpe BT. (2002) Amyloid precursor protein gene disruption attenuates degeneration of substantia nigra compacta neurons following axotomy. Brain Res 938:38–44. doi:10.1016/S0006-8993(02)02483-6

23. Suh YH, Checler F. (2002) Amyloid precursor protein, presenilins and alpha-synuclein: molecular pathogenesis and pharmacological applications in Alzheimer disease. Pharmacol Rev 54:469-525. doi:10.1124/pr.54.3.469

24. De Strooper B, Annaert W. (2000) Proteolytic processing and cell biological functions of the amyloid precursor protein. J Cell Sci 113:1857-1870.

25. De Strooper B, Annaert W. (2000) Proteolytic processing and cell biological functions of the amyloid precursor protein. J Cell Sci 113:1857-1870.

26. Dawson GR, Seabrook GR, Zheng H, Smith DW, Graham S, O'Dowd G, Bowery BJ, Boyce S, Trumbauer ME, Chen HY, Van der Ploeg LH, Sirinathsinghji DJ. (1999) Age-related cognitive deficits, impaired long-term potentiation and reduction in synaptic marker density in mice lacking the β-amyloid precursor protein. Neuroscience 90:1–13. doi:10.1016/S0306-4522(98)00410-2

27. Conboy L, Murphy KJ, Regan CM. (2005) Amyloid precursor protein expression in the rat hippocampal dentate gyrus modulates during memory consolidation. J Neurochem 95:1677–1688. doi:10.1111/j.1471-4159.2005.03484

28. Accili D, Fishburn CS, Drago J, Steiner H, Lachowicz JE, Park BH, Gauda EB, Lee EJ, Cool MH, Sibley DR, Gerfen CR, Westphal H, Fuchs S. (1996) A targeted mutation of the D3 dopamine receptor gene is associated with hyperactivity in mice. Proc Natl Acad Sci USA 93:1945–1949.

29. Venault P, Chapouthier G, de Carvalho LP, Simiand J, Morre M, Dodd RH, Rossier J. (1986) Benzodiazepine impairs and beta-carboline enhances performance in learning and memory tasks. Nature 321:864-6. doi:10.1038/321864a0

30. Shirayama Y, Chen AC, Nakagawa S, Russell DS, Duman RS. (2002) Brain-derived neurotrophic factor produces antidepressant effects in behavioral models of depression. J Neurosci 22:3251-61.

31. Schmittgen TD, Livak KJ. (2008) Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 3:1101-8.

32. Glickstein SB, Desteno DA, Hof PR, Schmauss C. (2005) Mice lacking dopamine D2 and D3 receptors exhibit differential activation of prefrontal cortical neurons during tasks requiring attention. Cereb Cortex 15:1016–1024. doi:10.1093/cercor/bhh202

33. Lacroix LP, Ceolin L, Zocchi A, Varnier G, Garzotti M, Curcuruto O, Heidbreder CA. (2006) Selective dopamine D3 receptor antagonists enhance cortical acetylcholine levels measured with high-performance liquid chromatography/tandem mass spectrometry without anti-cholinesterases. J Neurosci Methods 157: 25–31. doi:10.1016/j.jneumeth.2006.03.017

34. Millan MJ, Di Cara B, Dekeyne A, Panayi F, De Groote L, Sicard D, Cistarelli L, Billiras R, Gobert A. (2007) Selective blockade of dopamine D(3) versus D(2) receptors enhances frontocortical cholinergic transmission and social memory in rats: a parallel neurochemical and behavioural analysis. J Neurochem 100:1047–1061. doi:10.1111/j.1471-4159.2006.04262

35. Collo G, Bono F, Cavalleri L, Plebani L, Merlo Pich E, Millan MJ, Spano PF, Missale C. (2012) Pre-synaptic dopamine D(3) receptor mediates cocaine-induced structural plasticity in mesencephalic dopaminergic neurons via ERK and Akt pathways. J Neurochem 120:765-78. doi:10.1111/j.1471-4159.2011.07618

36. Donarum EA, Halperin RF, Stephan DA, Narayanan V. (2006) Cognitive dysfunction in NF1 knock-out mice may result from altered vesicular trafficking of APP/DRD3 complex. BMC Neurosci. 7:22. doi:10.1186/1471-2202-7-22

Figure legends :

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).

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.

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.

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.

Table 1: Primer sequences

Gene	Forward	Reverse	bp length
NF1 Acc# NM_010897.2	TTCGATACACTTGCGGAAAC	CACATTGGCAAGAGCCATAG	114
APP Acc# NM_007471	GGTTCTGGGCTGACAAACAT	CAGTTTTTGATGGCGGACTT	102
Dopamine D_3 receptor Acc# NM_007877.	GGGGTGACTGTCCTGGTCTA	AAGCCAGGTCTGATGCTGAT	110
Ribosomal protein 18S Acc# NM_011296.2	GAGGATGAGGTGGAACGTGT	GGACCTGGCTGTATTTTCCA	115

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.

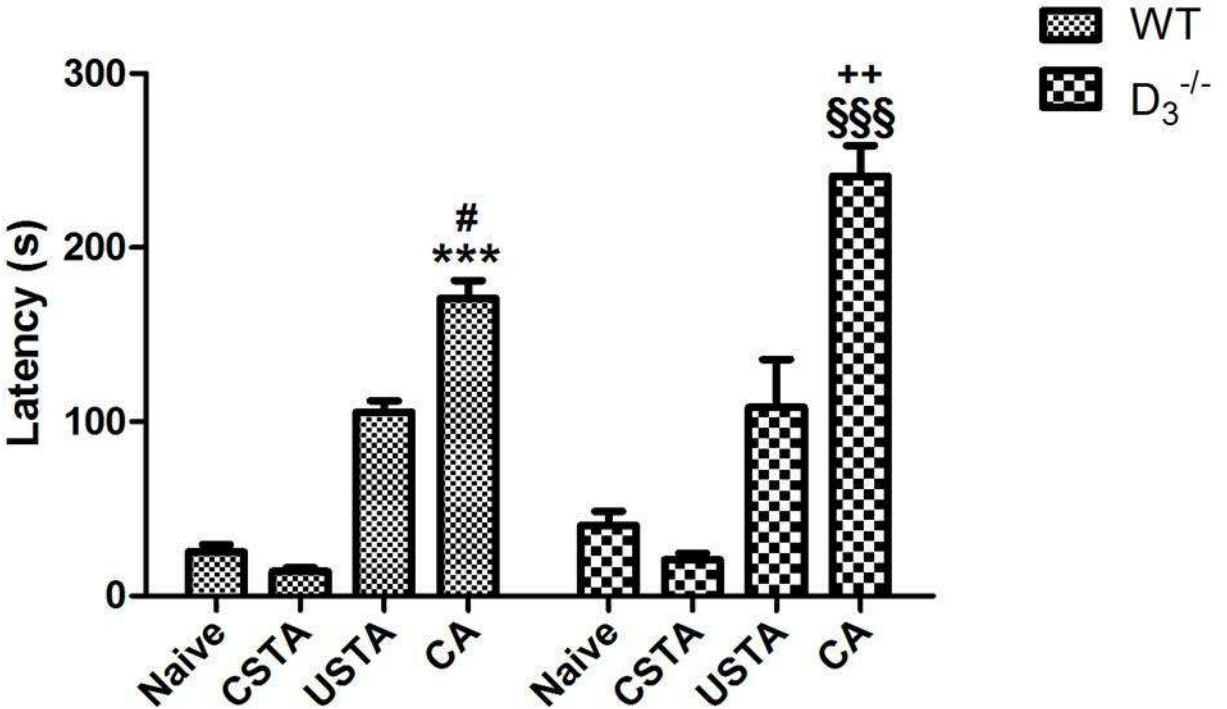
WT
D3-/-
Latency (s)
300
200
100
0
#
***
++
§§§
Naive
CSTA
USTA
CA
Naive
CSTA
USTA
CA

Fig.1

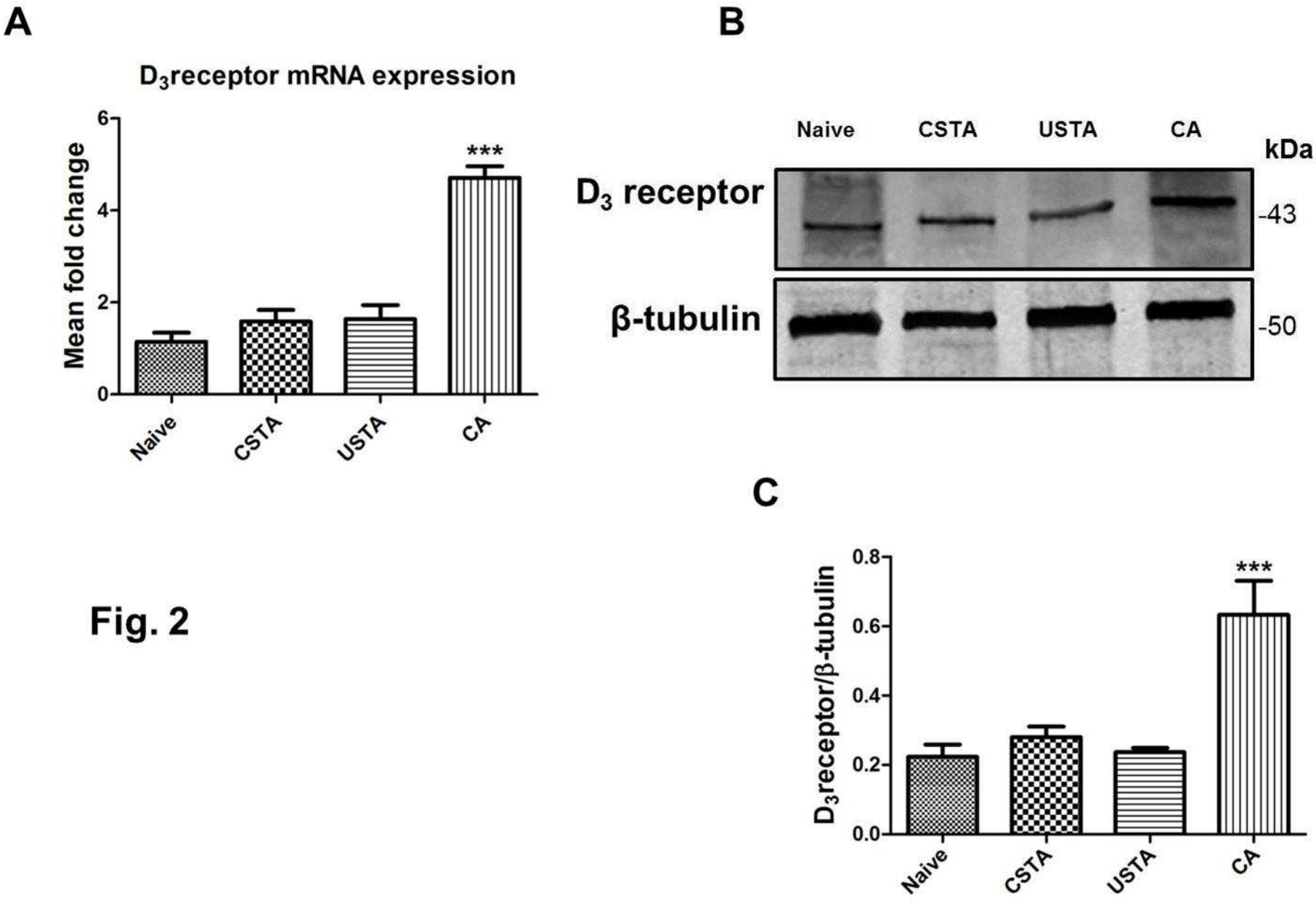
A
D3receptor mRNA expression
Mean fold change
6
4
2
0
***
Naive
CSTA
USTA
CA
B
Naive
CSTA
USTA
CA
kDa
D3 receptor
-43
β-tubulin
-50
C
D3receptor/β-tubulin
0.8
0.6
0.4
0.2
0.0
***
Naive
CSTA
USTA
CA

Fig. 2

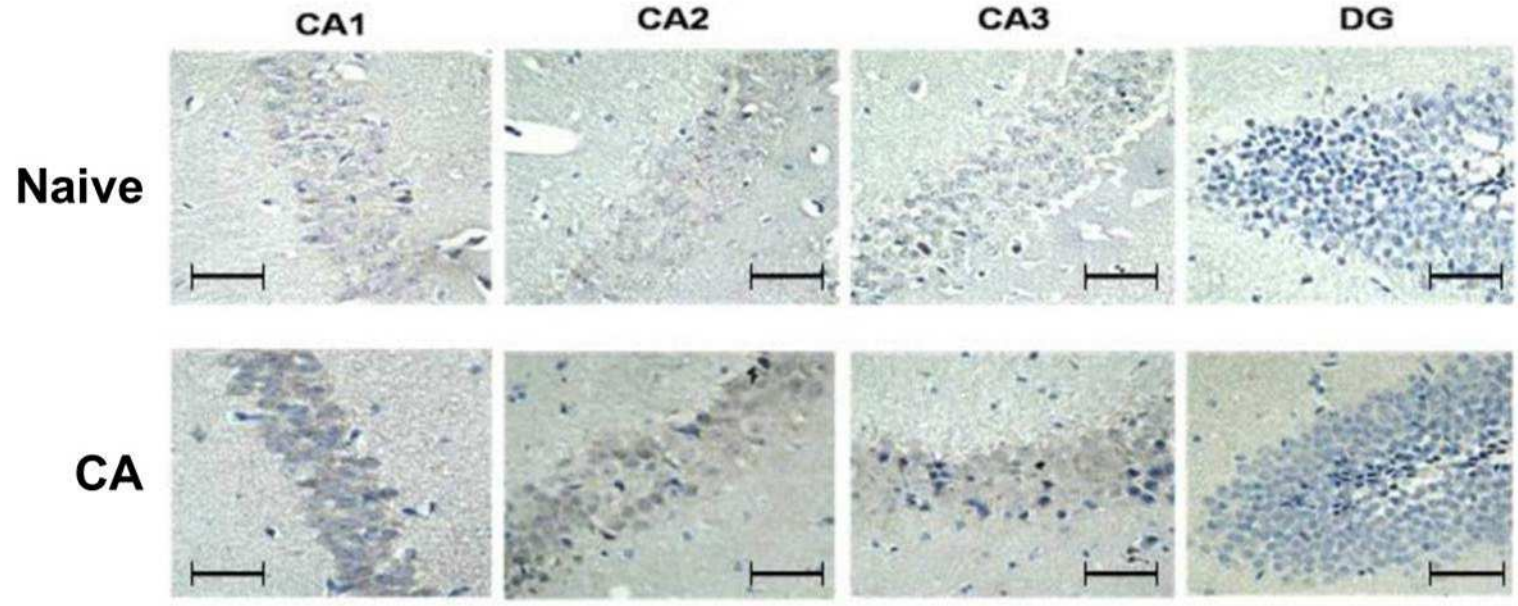

Fig. 3

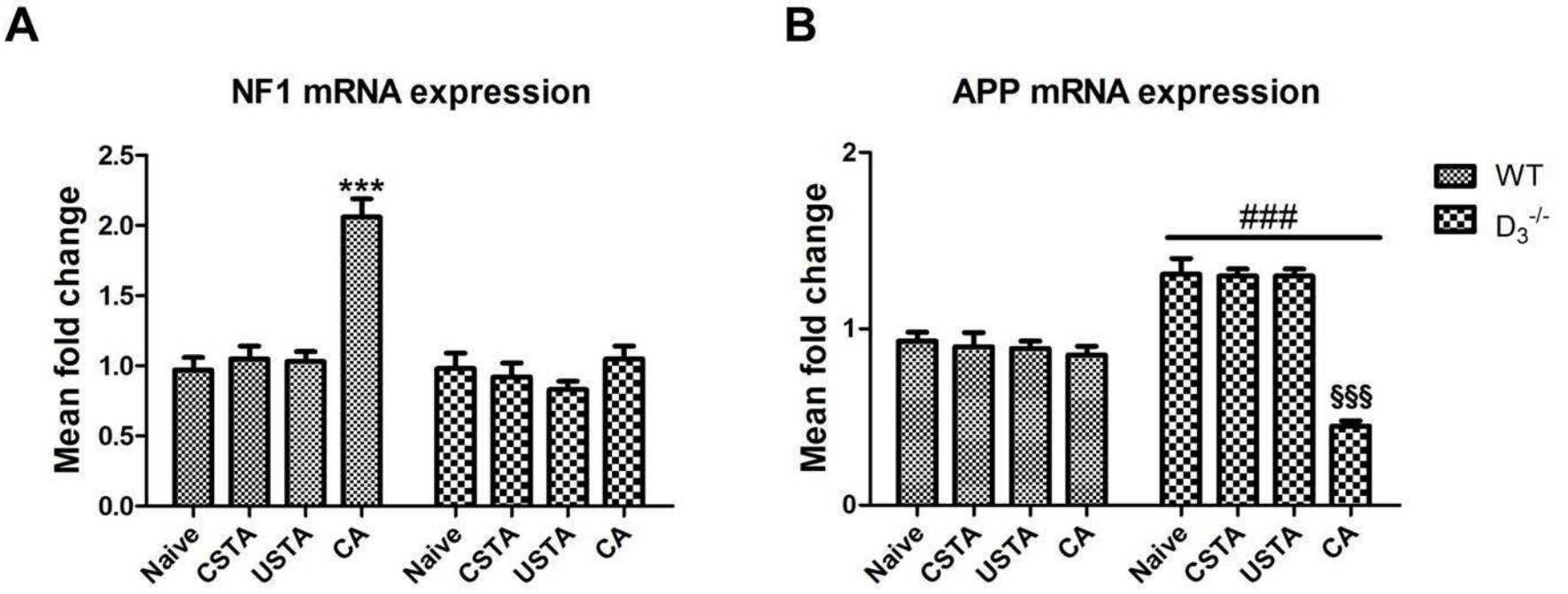
A
NF1 mRNA expression
Mean fold change
2.5
2.0
1.5
1.0
0.5
0.0
***
Naive
CSTA
USTA
CA
Naive
CSTA
USTA
CA
B
APP mRNA expression
Mean fold change
2
1
0
###
§§§
WT
D3-/-
Naive
CSTA
USTA
CA
Naive
CSTA
USTA
CA

Fig. 4

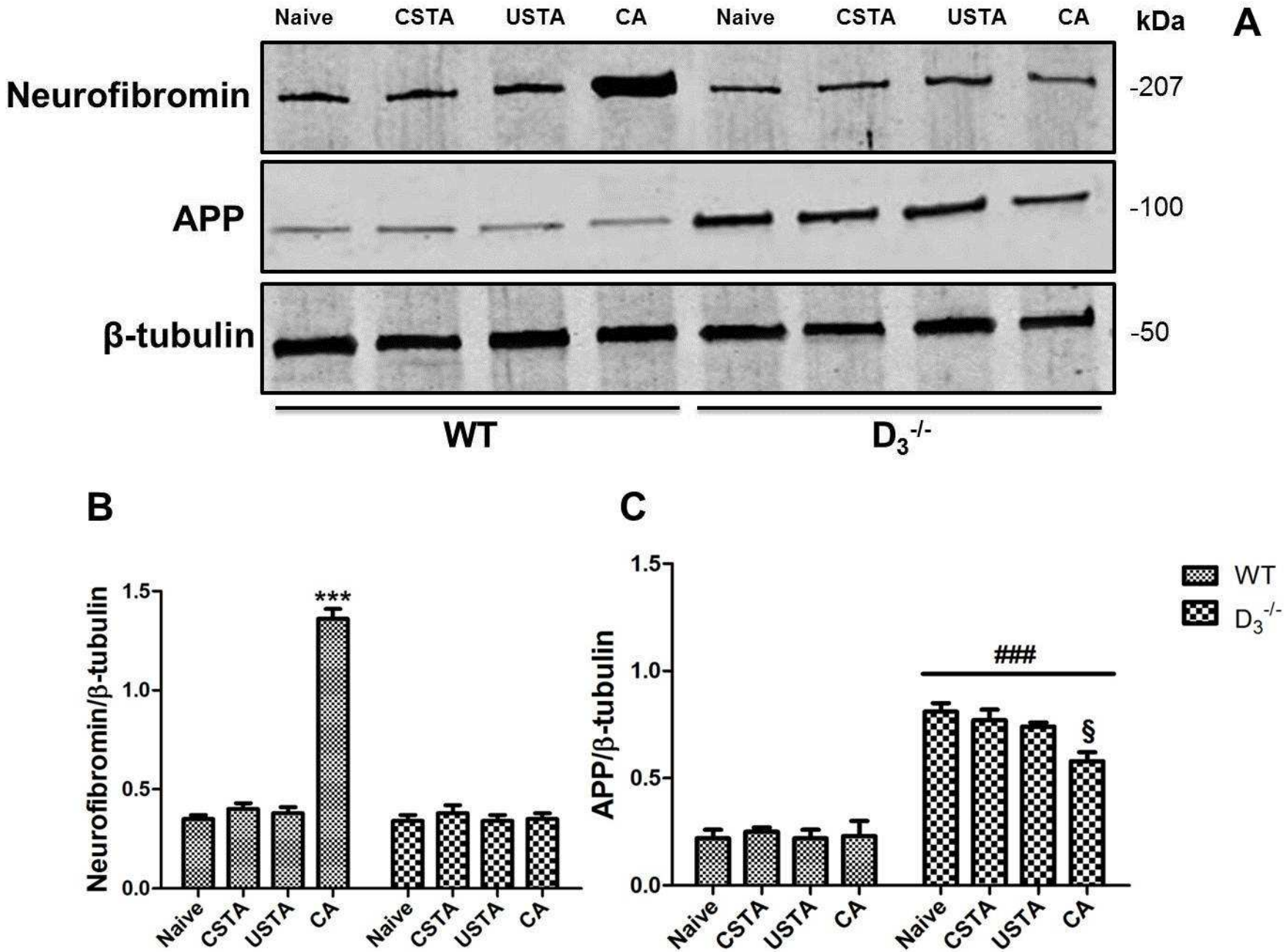
A
Naive
CSTA
USTA
CA
Naive
CSTA
USTA
CA
kDa
Neurofibromin
-207
APP
-100
β-tubulin
-50
WT
D3-/-
B
Neurofibromin/β-tubulin
1.5
1.0
0.5
0.0
***
Naive
CSTA
USTA
CA
Naive
CSTA
USTA
CA
C
APP/β-tubulin
1.5
1.0
0.5
0.0
###
§
Naive
CSTA
USTA
CA
Naive
CSTA
USTA
CA
WT
D3-/-

Fig. 5