

Ligand-dependent TrkA activity in brain impacts differently on spatial learning and long-term memory

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List of abbreviations:

NGF : nerve growth factor

APP : amyloid protein precursor

NTF : neurotrophic factor

A β : amyloid beta

AD: Alzheimer's Disease

MWM : Morris water maze

LTP : long-term potentiation

LTD : long-term depression

proBDNF : brain-derived neurotrophic factor precursor

ABSTRACT

In the CNS, the NGF receptor TrkA is expressed primarily in cholinergic neurons that are implicated in spatial learning and memory, whereas the NGF receptor p75^{NTR} is expressed in many neuronal populations and glia. We asked whether selective TrkA activation may impact differently on learning, short-term memory, and long-term memory. We also asked whether TrkA activation might impact cognition differently in wild type mice versus mice with cognitive deficits due to transgenic over-expression of mutant amyloid-precursor protein (APP mice). Mice were treated with wild type NGF (a ligand of TrkA and p75^{NTR}); or with selective pharmacological agonists of TrkA that do not bind to p75^{NTR}. In APP mice the selective TrkA agonists significantly improved learning and short-term memory. These improvements are associated with a reduction of soluble A β levels in the cortex and AKT activation in the cortex and hippocampus. However, this improved phenotype did not translate into improved long-term memory. In normal wild type mice, none of the treatments affected learning or short-term memory; but a TrkA selective agonist caused persistent deficits in long-term memory. The deficit in wild type mice was associated temporally, in the hippocampus, with increased AKT activity, increased pro-BDNF, increased NRH-2 (p75-related protein), and long-term depression. Together, these data indicate that selective TrkA activation impacts on cognition, but does so differently in impaired APP mice versus normal wild type mice. Understanding mechanisms that govern learning and memory is important for better treatment of cognitive disorders.

INTRODUCTION

Neurotrophins (NTFs) are growth factors involved in several functions in the nervous system, including survival, proliferation, differentiation and synaptic plasticity (Skaper, 2008). Nerve Growth Factor (NGF), the first described NTF, acts through two distinct receptors, TrkA and p75^{NTR}. In the CNS, TrkA receptors are almost exclusively expressed in the cholinergic neurons of the cortex, septum, and basal forebrain. In contrast, p75^{NTR} are widely expressed in many neuronal populations as well as in glia and reactive astrocytes. Ligand-dependent activation of TrkA mediates signals that are generally thought to be neuroprotective, while ligand-dependent activation of p75^{NTR} mediates a complex set of signals often associated with neurodegeneration (Skaper, 2008). For that reason, growth factors that bind to both receptors cause effects that are difficult to predict and control.

The possible involvement of NGF in AD was suggested after the observation that NGF has neurotrophic actions on basal forebrain cholinergic neurons (BFCNs), whose degeneration is at the basis of the cholinergic deficit and cognitive decline in Alzheimer's disease (AD). Indeed, studies in humans (Counts et al., 2004; Mufson et al., 2002); and aged rats (Saragovi, 2005) have shown that phenotypic silencing of TrkA expression correlates with neuronal atrophy. Thus, inappropriate TrkA expression or activity seems to be a prelude of neuronal death and cognitive impairment. On the other hand, there are no significant changes in NGF or p75^{NTR} expression in AD (Mufson et al., 2003).

Indirect evidence of a role for TrkA in cognition is shown by the fact that blocking TrkA function results in withdrawal of cortical cholinergic boutons in the normal adult rat (Debeir et al., 1999) and accelerates neurodegeneration in mice with cholinergic deficits (Capsoni et al., 2010). More direct evidence is shown by the fact that cognition was restored by treatment of

cognitively impaired aged rats with wild type NGF or with selective TrkA agonists (Bruno et al., 2004). Indeed, various tools to deliver wild type NGF have been and continue to be tested in human clinical trials for AD patients (Tuszynski et al., 2005).

However, wild type NGF can bind to both TrkA and to p75^{NTR}; and in mice lacking proper TrkA signaling the activation of p75^{NTR} can cause accumulation of A β peptides (Capsoni et al., 2010). This is a concern because in human AD there is also poor TrkA expression or signaling (Counts et al., 2004; Mufson et al., 2002). As a further concern, there have been no studies using neurotrophic intervention in mice over-expressing mutant human APP gene (APP mice, that accumulate A β peptides and form plaques) as a model of AD. Additionally, there have been no studies of neurotrophic intervention in the learning or the memory of wild type mice as a model of a “normal” cognitive state. The aims of the present paper are to examine the impact of selective TrkA activation on short-term and long-term memory in APP-mice and in wild type mice.

MATERIALS AND METHODS

Transgenic mouse model.

Animals used in this study were 4-5 months-old heterozygous transgenic mice that over-express the Swedish (670/671_{KM→NL}) and Indiana (717_{V→F}) mutations of human APP gene under the control of the platelet-derived growth factor- β promoter, on a C57BL/6J background (APP_{Swe,Ind}, line J20) (Mucke et al., 2000). Wild type (wt) littermates were used as normal controls. All experiments were approved by the Animal Ethics Committee of the Montreal Neurological Institute and were in compliance with the guidelines of the Canadian Council on Animal Care. Every effort was made to minimize animal suffering. Mice had access to water and food *ad libitum*, and their body weight was monitored before and after the treatments, with no significant changes observed.

Drug delivery to the CNS by osmotic minipumps.

We used an Alzet model 1002 osmotic minipump (Durect Corporation, ALZET Osmotic Pumps, Cupertino, CA 95015-0530) that accommodated 100 μ L of solution and continuously delivered the respective drugs for a period of 2 weeks, and were prepared following manufacturer instructions. Minipumps were implanted subcutaneously and connected through cannulas to the left lateral cerebral ventricle, through cannulas at the following stereotaxic coordinates from bregma: -1.0 mm lateral and -0.22 mm posterior on the left, according to the Atlas of Paxinos and Watson. Dental cement was used to fix the cannulas to the skull. After surgery, animals were injected subcutaneously with 0.2 mg/kg buprenorphine to ameliorate any pain. Correct location of the cannulas in the lateral ventricle was verified post-mortem for each mouse, and was a pre-requisite for inclusion in the study.

Test compounds and experimental groups.

Artificial cerebrospinal fluid (aCSF)(150 mM NaCl; 1.8 mM CaCl₂; 1.2 mM MgSO₄; 2 mM K₂HPO₄; 10 mM Glucose, 0.001% mouse serum, pH 7.3) was used as drug vehicle. Treated APP and wt mice received a total dose of the following agents, over two weeks. As much as possible, all groups included comparable number of males and females, and control wt mice were littermates. Control groups of APP and wt mice received vehicle. Quality controls to validate purity and activity were performed on all compounds, *ex vivo* using cultured neurons and *in vivo* in other models of neurodegeneration (Bai et al., 2010a).

Wild type NGF: recombinant wild type NGF (>99% pure, a kind gift of Genentech) was used at a total dose of 2 µg (NGF-2 group), and 20 µg (NGF-20 group) over a two-week period. The dose was based on extrapolation of the maximal effective doses published that are non-toxic in rats (Bruno et al., 2004).

Selective TrkA agonist mutant NGF (NGF-C): A mutant NGF, herein coded NGF-C for double-blinded studies, contains mutations in K32A-K34A-E35A. This mutant NGF was reported as a TrkA specific, p75 receptor negative protein (Ibáñez et al., 1992) and has been well characterized and utilized by several laboratories since then. Recombinant NGF-C was produced as described previously (Luo and Neet, 1992). Purification was performed using a protocol that produces >98% pure protein (cation exchange chromatography followed by immunoaffinity on an anti-NGF mouse monoclonal antibody). The yield of the purified protein was less than 1 mg per 5 L of culture, which was sufficient for the experiments reported herein. NGF-C does not bind p75^{NTR} but activates TrkA *ex vivo* (Ibáñez et al., 1992) and *in vivo* (Bai et al., 2010a). In the present work we used 5 µg NGF-C. The dose was based on extrapolation of the published effective dose that is non-toxic in rats (Bai et al., 2010a), and from pilot studies. The aim was to

achieve an effective dose of NGF-C at concentrations lower than the highest dose of wild type NGF used (NGF-20). NGF-C doses were also limited by the availability of this reagent.

Selective TrkA agonist small molecule (D3 peptidomimetic). D3 is a peptidomimetic small molecule agonist of TrkA that does not bind to p75^{NTR} (Maliartchouk et al., 2000). D3 does not bind to or activate TrkB or TrkC receptors either. D3 has been shown to activate TrkA in neuronal cultures as well as in vivo, and to afford neuroprotection in three models of neurodegeneration: cognitively impaired aged rats (Bruno et al., 2004), optic nerve axotomy (Bai et al., 2010a) and glaucoma (Bai et al., 2010a; Shi et al., 2007). D3 is a partial TrkA agonist that can also potentiate TrkA signals activated by sub-optimal levels of NGF (Maliartchouk et al., 2000) because its binding site is non-overlapping with NGF. D3 was applied at 10 µg (D3-10 group) or at 40 µg (D3-40 group). D3 doses used were based on an extrapolation of the effective doses in rats reported in the literature (Bruno et al., 2004).

Morris water maze task (MWM).

After two weeks of treatment, mice were subjected to the MWM as adapted for APP transgenic mice (Deipolyi et al., 2008). The MWM consisted of 8 days of training in a pool, divided virtually in 4 quadrants landmarked with visual cues on the surrounding wall, and filled with opaque water. In the first 3 days of training, the platform was held visible in one of the quadrants, 1 cm above the water, to exclude animals with visual or motor deficits. For the next five days (learning), the visual cues were moved and the platform was placed in a different quadrant and was submerged ~1 cm below the water level. Each day, escape latencies were recorded from 3 different directions for each mouse, with an inter-trial time not exceeding 45 minutes. In the last day of training, a minimum of two hours were allowed for mice to rest, after

which the platform was removed and mice were allowed to freely swim for 60 seconds (probe trial 1, short-term memory, STM).

One day after the first probe trial, pumps were removed under isoflurane anesthesia. Two weeks later, mice from D3-10, NGF-2, NGF-20 and NGF-C groups underwent a second probe trial (probe trial 2, long-term memory, LTM), after which they were euthanized, perfused with saline, and their brains collected and processed for biochemical work, as described below.

In the case of D3-40 treatment, three separate groups were constituted. Mice from the first group performed MWM training and were euthanized just after probe trial 1, and their brains were processed for ELISA quantification. Mice from the second D3-40 group also performed a MWM training, and underwent a probe trial 1 and a probe trial 2 two weeks later. To test the persistence of the behavioral effect after D3-40 treatment, mice were subjected to a second MWM, which was done 23 days after the original MWM was completed. Therefore, the visual cues were moved and the platform was hidden in a different quadrant than in the first experiment, and 5 days of training were allowed for mice to find the hidden platform in this new location. This was done to avoid confusion of the mice that may have remembered the original training (Bruno et al., 2004). A probe trial was then performed on day 5 (probe trial 1a), at least 2 hours after the last training. Another probe trial was performed one week later (probe trial 2a), after which mice were euthanized. A third D3-40 group, constituted only of wt mice (D3-treated and vehicle-treated). These mice were euthanized after probe trial 2, and their brains processed for electrophysiological studies.

All learning data are expressed as time (in seconds) to reach the hidden platform \pm SEM, while probe trials data are expressed as percentage of time and distance spent in the quadrant where the platform was previously hidden \pm SEM.

ELISA for soluble A β .

Mice from one D3-40 group were sacrificed just after the probe trial 1, in order to correlate the gain in memory with changes in A β and other proteins. Brain fraction containing soluble A β was extracted as previously described (Nicolakakis et al., 2008). Cortex or hippocampus were homogenized by sonication in a buffer containing 20 mM tris buffer, 1 mM EGTA, 1 mM EDTA, 250 mM sucrose, and protease inhibitors. Soluble A β was extracted by centrifugation (100,000 x g, 60 min, 4°C) using diethyl acetate (0.4% in 100mM NaCl), and A β_{1-40} and A β_{1-42} levels, expressed as nanomoles per gram of protein, were measured as described by the manufacturer (BioSource International).

Biochemical analyses.

Subcellular fractions from cortex, hippocampus and basal forebrain area were prepared for biochemical work. In brief, tissue was homogenized in ice-cold lysis buffer (containing 15 mM tris; pH7.6; 0.25 M sucrose; 1 mM PMSF; 2 mM EDTA; 1 mM EGTA; 10 mM Na₃VO₄; 25 mM NaF; 10 mM Na₄P₂O₇·10H₂O; and protease inhibitor tablet). Homogenates were first centrifuged at 800 x g for 5 min, to remove nuclei and large debris. The resulting supernatant was centrifuged (10,000 x g; 30 min) to obtain the cytosolic fraction (supernatant) and the crude synaptosome fraction (pellet). The latter was resuspended in a buffer containing 1% triton X-100 and 300 mM NaCl, and then centrifuged (16,000 x g; 30 min) to obtain the triton-soluble (containing the cytosolic fraction of synaptosome) and triton-insoluble (P2 fraction, synaptosome) fractions. The P2 fraction was dissolved in 1% SDS, and protein levels in different fractions were assessed using Bio-Rad protein assay protocol.

Western blots.

For measuring biochemical signals, phospho-AKT was used because it is a validated surrogate marker downstream of TrkA. Because growth factor-dependent protein phosphorylation in vivo is generally short-lived (Bai et al., 2010b), mice were sacrificed 12 hours after acute drug treatment and tissue samples were collected. Treatment consisted of ICV administered D3 2 µg dose (n=3 wt group and n=3 APP group). This is comparable to the dose of D3 40 µg over a 15-day period (2.66 µg/day). Control groups received aCSF (n=3 wt group and n=4 APP group).

At the endpoint, mice were saline-perfused, their tissues dissected and solubilized in detergent (Bai et al., 2010b). To quantify proteins of interest (p-AKT, p75^{NTR}, NRH2, and proBDNF) or control proteins (actin or total AKT) 20 µg of cytosolic fraction each from cortex, hippocampus and basal forebrain area were loaded in glycine/SDS-PAGE. To quantify CTF-β 50 µg of cytosolic fraction were loaded in tricine/SDS-PAGE. Proteins were transferred onto nitrocellulose membrane, and blots incubated overnight with primary antibodies against CTF-β (mouse 6E10 antibody; BioSource International), rabbit anti-p75^{NTR} (Promega) and rabbit anti-pro-BDNF (Alomone Labs), or antibodies to total AKT and p-AKT (Cell Signaling). Rabbit anti-NRH2 antisera was kindly provided by Dr. Phillip Barker (Montreal Neurological Institute). Mouse anti-β-actin (Sigma Aldrich) was used as internal control for loading. Blots were incubated with horseradish peroxidase-conjugated secondary antibodies (1:2000; Jackson ImmunoResearch), and proteins visualized with enhanced chemiluminescence (ECL Plus kit; GE Healthcare) using a PhosphorImager (Scanner STORM 860; GE Healthcare), followed by densitometric quantification with ImageQuant 5.0 (Molecular Dynamics).

Electrophysiological recording in hippocampal slices.

Groups of vehicle-treated and D3-40-treated wt mice were used, and hippocampal slices were prepared as described (Wong et al., 2007), 3 weeks after the end of treatment. At this time D3-40-treated wild type mice exhibited impaired long-term memory. Briefly, under deep anesthesia, brains were rapidly removed and coronal slices (350 μ m thickness) were cut in hyperosmotic, ice-cold and carbogenated (bubbled by 95% O₂/5% CO₂ to maintain the pH at 7.4) solution (in mM: 252 sucrose, 2.5 KCl, 4 MgCl₂, 0.1 CaCl₂, 1.25 KH₂PO₄, 26 NaHCO₃ and 10 glucose) using a Vibratome. Freshly cut slices were placed in an incubating chamber with carbogenated aCSF (~310 mOsmol/L) consisting of (mM): 125 NaCl, 2.5 KCl, 1 MgCl₂, 2 CaCl₂, 1.25 NaH₂PO₄, 26 NaHCO₃, and 25 glucose. Slices were recovered at 32°C for one hr and subsequently maintained at room temperature. Carbogenated aCSF containing bicuculline methbromide (5 μ M) to block GABA_A receptor-mediated inhibitory synaptic currents was used to perfuse slices in all recordings. Postsynaptic responses, evoked by stimulating the Schaffer collateral-commissural pathway via constant current pulses (0.08 ms) delivered through a tungsten bipolar electrode (FHC), were recorded from the hippocampal CA1 region, amplified by a Multiclamp 700B (Axon) and stored in a PC for offline analysis using Clampfit (Axon). Field excitatory postsynaptic potential (fEPSP) was evoked at 0.05 Hz and detected by an aCSF-filled glass electrode placed in the stratum radiatum of the hippocampal CA1 region. Long-term plasticity of fEPSP such as long-term potentiation (LTP) and long-term depression (LTD) were induced by high-frequency (100 Hz, 100 pulses) and low-frequency (1 Hz, 900 pulses) tetanus, respectively. Recording and analysis of electrophysiological data were performed blind to the identity and treatment of the mice.

Statistical analysis.

All data are expressed as mean \pm SEM (except for ELISA, expressed as mean \pm SD). For multiple-group comparisons, one-way ANOVA followed by Newman-Keuls post-hoc multiple comparison test was used. Student's *t*-test was used for two-group comparisons (GraphPad Prism 4, San Diego, CA, USA). $p < 0.05$ was considered significant.

RESULTS

Comparison of control APP mice versus wt mice in the MWM

The wt mice performed significantly better than the APP mice in both the learning and memory components of the MWM, as expected. In the learning component the wt mice learned rapidly to locate the hidden platform. The APP littermates displayed longer latency, despite no visual deficits (see training portion in the 3 days of visible platform, **Figures 1A 1B**), and no swimming deficits (data not shown). In the memory component, herein termed the “probe trial”, the APP mice spent significantly less time and traveled shorter distances in the target quadrant compared to wt mice. This is indicative of memory impairment (**Figures 1C 1D**).

NGF does not improve learning in APP mice, and does not affect wt mice.

Treatment with NGF (2 µg dose) did not improve the spatial learning deficits observed in APP mice, and had no effect in wt mice (**Figure 1A**). A higher dose of NGF (20 µg) did not improve the learning performance of APP mice, and had no effect in wt mice either (**Figure 1B**). Moreover, the performance of APP and wt mice in probe trials 1 and 2 were not affected by either doses of NGF (**Figures 1C, 1D**). Overall, our findings indicate that ICV infusion of NGF had no effect on spatial learning or on memory, irrespective of whether the mice are cognitively impaired or not.

TrkA selective agonist NGF mutant (NGF-C) improves learning but not memory in APP mice

We next tested the hypothesis that the mutant NGF-C, which is a selective TrkA agonist that does not bind to p75^{NTR}, might have different properties *in vivo* when compared to wild type NGF.

APP mice treated with NGF-C exhibited a trend towards improved performance in MWM learning compared to vehicle-treated APP mice (**Figure 2A**). Note that wild type NGF

did not exhibit this trend even when delivered at 4-times higher doses. NGF-C–treated APP mice showed no improvement in probe trials testing for memory (**Figure 2B**).

Similar treatment of wt mice with NGF-C showed no changes in learning or in memory (**Figures 2A, 2B**).

Because the pharmacokinetics and the stability of wild type NGF and NGF-C proteins are likely to be similar, these behavioral data suggest that selective targeting of TrkA may have some benefit in disease states.

The partial TrkA agonist D3 improves learning and short-term memory (STM) in APP mice.

Because of the small improvement seen with NGF-C, we then tested the effect of peptidomimetic D3 which is a proteolytically stable small molecule selective partial agonist of TrkA.

APP mice treated with D3 (10 µg, hereafter D3-10) had significantly improved spatial learning at all days of training, compared versus vehicle-treated APP mice (**Figure 3A**). In fact, in the learning phase APP mice treated with D3-10 performed almost the same as wt mice. However, the improved learning of APP mice did not extend into improved STM (probe trial 1) (**Figure 3B**). Similar treatment of wt mice with D3-10 showed no changes in learning or in STM (**Figure 3A, 3B**). At this dose of D3 there was no effect on LTM (probe trial 2, data not shown). APP remained impaired and wt remained normal.

The selective improvement in learning but lack of effect on STM caused by D3 in APP mice was reminiscent of the data produced by NGF-C polypeptide. Given the positive effect of D3-10 on the spatial learning deficit in APP mice, we predicted that a higher D3 dose (40 µg or D3-40) would produce a stronger improvement.

In APP-mice treatment with D3-40 significantly improved spatial learning in all training days, to levels comparable to wt mice (**Figure 3C**). On days 6 and 8 of training, the mean escape latency of D3-40-treated APP mice was significantly lower than the vehicle-treated APP control group. D3-40 treatment also improved spatial memory in APP-mice. In probe trial 1, D3-40-treated APP mice swam longer distances in the target quadrant (**Figure 3D**) (significant versus the vehicle-treated APP group, and not different from wt mice groups). When time spent in the target quadrant was evaluated, D3-40-treated APP mice was not statistically different from vehicle-treated wt mice control group. In wt mice D3-40 had no effect on the spatial learning or STM performance (**Figure 3C, 3D**).

Together, these data indicate that treatment of APP mice with selective TrkA agonists can either improve spatial learning (NGF-C), or both spatial learning and STM (D3), in a dose-dependent manner. Moreover, the fact that selective TrkA agonists did not affect the learning and STM of wt mice indicates that these compounds do not have measurable toxic effects on non-diseased brains. Lack of toxicity is further documented below.

The partial TrkA agonist D3 increases p-AKT in cortex and hippocampus and decreases cortical A β levels in APP mice.

We tested several biochemical parameters to attempt correlations with the treatment that improve learning and memory, namely D3.

Cortical levels of A β 1-40 and A β 1-42 were measured immediately after probe trial 1 of D3-40-treated APP mice, which had shown gains in STM. Cortical levels of A β 1-40 and A β 1-42 were significantly lower in D3-40-treated APP mice, compared to vehicle-treated APP mice (52% and 62% decrease respectively, **Figure 3E**; $p < 0.05$). This decrease was accompanied by a corresponding increase in the levels of the APP C-terminal fragment-beta (CTF- β) (40 %

increase, **Figure 3F**; $p < 0.05$). Lack of CTF- β processing into A β suggests an inhibition of cortical γ -secretase activity after D3-40 treatment. In contrast, D3-40 had no effect on A β levels in the hippocampus (**Figure 3E**), suggesting that the effect is localized to cortex.

To confirm activation of TrkA receptors, we tested the PI3K/AKT pathway because it is a valid surrogate marker. Significantly increased p-AKT was detected in the cortex and the hippocampus (but not in the nucleus basalis area) of D3-treated APP mice, compared to vehicle-treated APP mice (**Figure 4**).

Thus, there appears to be a temporal association between a dose-dependent ligand activation of TrkA causing activation of p-AKT in cortex and hippocampus, and a decrease in toxic A β peptides in cortex presumably through the inhibition of γ -secretase. These events are associated with improvements in learning and STM.

Effects of the partial TrkA agonist D3 on long-term memory (LTM)

A second probe trial was done 2 weeks after probe trial 1, to assess D3-treatment on the LTM of APP mice or wt mice.

D3-40-treated APP mice performed worse in probe trial 2 than they did in probe trial 1 (**Figure 5A**). Both the time and the distance in the target quadrant were significantly lower compared to wt control. These data suggest that the learning and STM improvement observed for D3-40-treated APP mice during probe trial 1 is not long-lasting.

Surprisingly, the normal LTM in wt mice treated with D3-40 was significantly compromised (**Figure 5A**). In probe trial 2, the D3-40-treated wt mice appeared to be impaired compared to vehicle-treated wt mice. Since the D3-40-treated wt mice exhibited normal learning and STM (see MWM and probe trial 1), this unexpected finding was explored.

D3-40-treatment affects long-term memory, but not learning and short-term memory in wt mice.

The D3-40-treated wt mice had LTM impairment, thus we wished to verify whether their learning capacity was still intact.

A second MWM training was performed using the same D3-40-treated or vehicle-treated wt mice groups (**Figure 5B**). This was done 23 days after the original probe trial 2 that showed impairment, and 45 days after drug delivery was completed. In this second MWM training, we changed the visual cues and target quadrant to avoid confusion in the mice that may have retained some LTM.

The learning capacity of D3-40-treated wt mice was intact, and was identical to that of vehicle-treated wt mice (**Figure 5B**). Moreover, the D3-40-treated wt mice had normal STM (**Figure 5C**, probe trial 1a), as demonstrated by comparable time and distance traveled in the target quadrant to those of control vehicle-treated wt mice. Normal learning and STM in this paradigm suggests that the drugs, given 50 days before, did not cause general toxicity.

However, when the D3-40-treated wt group was tested in probe trial 2a one week after probe trial 1a, they again performed significantly worse than the vehicle-treated group. The time and distance in the target quadrant of D3-40-treated mice were significantly worse than vehicle-treated wt mice (**Figure 5C**, probe trial 2a, * $p < 0.05$).

Together these data show that D3-40 treatment in APP-mice improves learning/STM but not LTM; and that D3-40 treatment in wt mice does not alter normal learning/STM but persistently impairs LTM.

The partial TrkA agonist D3 increases p-AKT in hippocampus of wild type mice.

Because these are wt mice, with normal working memory, we sought to examine the signals that might be relevant to a persistent impairment of LTM.

As done before for APP-mice, analyses of p-AKT from D3-treated wt mice showed a significant increase in the hippocampus, compared to vehicle-treated wt mice. However, no significant change of p-AKT was observed in the cortex or in the basal forebrain (**Figure 6**). Thus, in wt brains, there appears to be a paradoxical association between acute hyperactivation of AKT pathways in hippocampus and selective LTM impairment, without causing effects in learning and STM.

D3-40-treatment increases long-term depression in wt mice.

To further address the mechanism by which D3-40 treatment induced a decline in the LTM in wt mice, we recorded long-term potentiation (LTP) and long-term depression (LTD) in the CA1 layer of hippocampal slices (**Figure 7A**), immediately after probe trial 2, a time at which D3-40 treated wt mice exhibit impaired LTM. There were no differences in LTP for D3-40-treated and vehicle-treated wt mice. However, there was a significant increase in LTD in D3-40-treated, compared to vehicle-treated wt mice.

To address possible mechanisms by which LTD may be increased, we evaluated pro-BDNF and p75^{NTR} expression, because these two molecules have been shown to induce LTD (Woo et al., 2005). The levels of pro-BDNF were significantly higher in the hippocampus of D3-40-treated mice, but we found no change in p75^{NTR} expression between the two groups (**Figure 7B**). However, we found an increased expression of NRH2, a p75-related protein, in the hippocampus (Figure 7B) and the cortex (data not shown) of D3-40-treated, compared to vehicle-treated wt mice.

DISCUSSION

To assess mechanisms of learning and memory in APP mice and in wt “normal” mice we studied the behavioral consequences of selectively activating TrkA; and the biochemical effects in specific regions of the brain. Three different pharmacological ligands that activate TrkA were used: wild type NGF (a ligand of TrkA and p75^{NTR}), a mutant NGF-C (only activates TrkA), and a selective partial agonist of TrkA termed D3.

Exogenous wild type NGF had no effect in the behavior of APP mice or in wild type mice, even when infused at relatively high doses. These are new and compelling data, as learning and memory had not been studied previously. However, endogenous wild type NGF is known to be essential for the maintenance of adult cholinergic neuronal phenotype (Capsoni et al., 2010; Debeir et al., 1999) and for hippocampal plasticity (Conner et al., 2009).

We therefore assessed the cholinergic neuronal phenotype by measuring protein levels of ChAT and VChAT by immunohistochemistry and western blot analyses (data not shown). We found no significant differences between treated APP and wt mice. Thus behavioral changes reported in this paper take place without any changes to the major cholinergic markers. Additionally, at the age that APP mice were tested in this study, they do not present detectable cholinergic deficits in spite of signs of cognitive deficits (Aucoin et al., 2005), so we did not anticipate detectable changes to cholinergic markers. Perhaps at later stages or older age there may be a change to cholinergic markers.

It is intriguing that wild type NGF failed to improve behavior in APP mice whereas two agents (NGF-C and D3) that exclusively activate TrkA improved learning and/or STM. We postulate that binding to p75^{NTR} may be a reason, because this is the only functional difference between NGF and NGF-C. We can exclude the trivial reasons of incorrect dose (wild type NGF

was tested at 4-fold higher doses than NGF-C), lack of activity (the same reagents were verified to be active *ex vivo* and in other models *in vivo*), and pharmacokinetics (both recombinant NGF proteins would have similar properties).

Treatment of APP-mice with TrkA agonists NGF-C and D3-10 improved learning but had no effect on memory; while D3-40 also improved STM. However, memory did not last in the long-term. Because APP mice have already an impaired LTM, the LTM impairment in D3-40-treated APP mice may be interpreted as a failure of D3 at 40 μ g to restore LTM, or as the genetically-driven neuronal injury process overcoming any short-term benefits of the drug.

In D3-40-treated mice, improved STM is associated with decreased cortical levels of soluble A β and with increased levels of CTF- β . Considering that A β fragments are produced by γ -secretase cleavage of CTF- β , our data suggest that γ -secretase activity may be reduced by D3-40 treatment. This suggestion is supported by studies showing that lack of TrkA activity causes a γ -secretase-dependent accumulation of A β in differentiated PC12 cells and in primary hippocampal neurons (Matrone et al., 2008a; Matrone et al., 2008b).

Soluble A β monomers/oligomers have been associated with memory deficits in APP mice (Cleary et al., 2005). This is consistent with our study because the memory improvement promoted by D3-40 treatment of APP mice was associated with a reduction in cortical A β levels. Moreover, treatments that do not improve memory in APP mice (e.g. D3-10 treatment) did not cause changes in A β levels in cortex or hippocampus (data not shown).

Whereas only the hippocampus appears essential during spatial memory formation (Broadbent et al., 2006), learning and STM requires, in addition to the hippocampus, the activation of cortical structures (Blum et al., 2006; Leon et al., 2010). Particularly, the entorhinal cortex, a primary input to the hippocampus, is affected early in AD patients and contributes to

the loss of STM (Kordower et al., 2001). Further, lesions of the entorhinal cortex in rodents result in deficits in acquisition and retention in the Morris water and radial arm mazes (Hardman et al., 1997). Similarly, the medial prefrontal cortex was shown to be important in the consolidation and retrieval of recent spatial memories (Leon et al., 2010). It is therefore tempting to suggest that decrease of A β in cortex of D3-40-treated APP mice may have contributed to the STM restoration in these mice. Further studies will be required to prove this point unambiguously.

Because A β *56 is an oligomeric form purported to be responsible for the memory deficit in APP mice (Lesne et al., 2006), we studied it specifically. However, we did not find any change in levels of A β *56 between D3-40-treated and vehicle-treated APP mice (data not shown). Thus, from our data in APP mice the precise role of A β *56 seems unrelated to learning and STM; but it could possibly be implicated in LTM.

One important observation is that in APP mice the D3 agonist exhibited a dose-dependent effect. The 40 μ g dose was optimal at reducing A β levels in the cortex and at restoring STM. However, in stark contrast to its positive effects in APP mice, treatment of wt mice with D3-40 affected LTM negatively. LTM regressed, notwithstanding the fact that the mice had intact learning and STM and were able to be re-trained in MWM. This was a surprising finding, and we speculate that perhaps the hyperactivation of TrkA could interfere either with memory consolidation or with the retrieval of information.

Restoration of memory in diseased animals (APP mice in the present case, cognitively impaired aged rats in previous studies) while decreasing memory in healthy animals is, however, not a totally new concept. A model supported by studies in humans and rodents states that increasing cholinergic activation would enhance neural activity for encoding information, but

leave unaltered or even impair retrieval (Hasselmo and Stern, 2006). The difference in our study is that we actually activate TrkA pathways rather than cholinergic activation.

Electrophysiological recordings performed in CA1 region of the hippocampus shed some light on the mechanism underlying LTM impairment. Compared with vehicle-treated wt mice, we detected an increase in low-frequency stimulation (LFS)-induced LTD in the CA1 region of D3-40-treated wt mice. Previous findings obtained from the young visual cortex have shown that acute NGF treatment favors long-term depression (LTD) over long-term potentiation (LTP) after high-frequency stimulation (Brancucci et al., 2004). LTP was comparable between the treated and untreated wt groups, so a decrease in LTP in the CA1 region cannot be responsible for memory regression. To our knowledge, our findings provide the first evidence that a chronic treatment of TrkA agonist produced a long-term facilitating effect on hippocampal LTD, which on behavioral testing corresponds with no short-term effects but a LTM deficit.

Recent work has shown that LTD helps to promote consolidation of spatial memory (Brigman et al., 2010; Ge et al., 2010). This may explain why D3-40-treated mice display normal learning and STM. However, LTD also plays a role in inhibition of memory retrieval (Wong et al., 2007) and this may be a reason why LTM was abolished after 2-3 weeks.

What is still unclear is how chronic TrkA activation by D3-40 facilitates LTD. There are four non-exclusive possibilities.

First, chronic TrkA activation may have trophic effects on cholinergic neurons in the septum that project to the hippocampus. This may facilitate LTD formation via increasing the cholinergic output to the hippocampus. Indeed, activation of muscarinic cholinergic receptors facilitates LTD induced by low frequency stimulation (Kirkwood et al., 1999).

Second, it is possible that chronic TrkA activation may facilitate LTD by desensitization of the TrkA signaling pathway. Previous work where TrkA was inhibited showed a significant reduction of cortical cholinergic boutons (Debeir et al., 1999); and accelerated neurodegeneration in mice with cholinergic deficits (Capsoni et al., 2010). It is also possible that increased LTD may be relayed by chronic AKT phosphorylation, as deregulation of the PI3-K/AKT pathway was shown to induce long-term synaptic depression (Guo and Zhong, 2006).

Third, proBDNF levels were significantly increased in D3-40-treated wt mice, and proBDNF can facilitate hippocampal LTD (Woo et al., 2005). The relative reduction of proBDNF processing to mature BDNF may also be important, because BDNF is required to promote LTM persistence (Rossato et al., 2009) and LTM storage (Bekinschtein et al., 2008).

Fourth, while p75^{NTR} protein did not change in cortex, hippocampus and basal forebrain; a homolog of p75^{NTR}, NRH2, increased significantly in both the cortex and hippocampus of D3-40-treated mice. Although NRH2 does not directly bind to neurotrophins it is able to form a receptor complex with TrkA to generate high-affinity NGF binding sites, suggesting that it may serve a similar function as the p75^{NTR} receptor (Murray et al., 2004). NRH2, like p75^{NTR}, uses intracellular mechanisms to regulate NGF binding to TrkA and modulate TrkA receptor signaling. The relevance of NRH2 and synaptic plasticity has not been studied, but it is likely that NRH2 may affect plasticity because its homolog p75^{NTR} influences hippocampal LTD (Rosch et al., 2005). Additional studies are obviously needed to confirm this hypothesis.

The relationship between LTD and LTM consolidation or persistence is of current interest (Medina et al., 2008). For instance, psychological stress inhibits long-term spatial memory retrieval by an LTD mechanism (Wong et al., 2007). Chronic D3-40 treatment may weaken LTM retrieval by enhancing LTD formation. Alternatively, facilitating LTD may erase

consolidated memory traces that are mediated by hippocampal LTP, so that long-term consolidation of memory cannot be established.

In summary, we found that NGF is not effective in an APP animal model of cognitive impairment. In contrast, NGF-C, a selective TrkA agonist, is partially effective in STM. Likewise, treatment with the selective D3 TrkA agonist is also effective in STM. This benefit was associated with activation of p-AKT pathways in specific locations of the brain, and with a decrease in the levels of A β in the cortex, likely through inhibition of γ -secretase. However, the deficit in memory consolidation or retrieval observed in APP mice is not resolved, because LTM is still deficient in these mice. Also, D3 at a high doses affects negatively memory consolidation or memory retrieval in wt mice, concomitant with an increase in LTD in CA1 region, perhaps through NRH2 and proBDNF.

Our results provide experimental evidence for pharmacological dissection of distinct processes related to short- and long-term memory in the healthy and diseased brain, and have implications for the treatment of individuals that have “normal cognition” versus those diagnosed with mild cognitive impairment, with advanced AD, other forms of cognitive impairment, or stress disorders.

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Author Contributions

Participated in research design: Saragovi, Hamel, Wong

Conducted experiments: Aboukassim, Tong, Chung Tse, Wong, Brahimi

Contributed new reagents or analytic tools: Woo, Neet

Performed data analysis: Aboukassim, Saragovi, Hamel, Wong

Wrote or contributed to the writing of the manuscript: Saragovi, Hamel, Aboukassim, Neet, Wong

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FOOTNOTES

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Figure 1

Wild type NGF has no effect on the learning or the memory of APP mice and wild type (wt) mice.

A) NGF at 2 µg total dose (NGF-2) had no effect on the learning of APP mice. Mean latency in APP-NGF-2 group was comparable to that in the APP-vehicle group, and both APP groups had higher latency compared to wt mice groups.

B) Treatment with 20 µg total dose of NGF (NGF-20) did not improve learning capacity of APP mice either. Mean latency was similar in both APP groups, and was higher than wt groups (day 5 and day 8, * $p < 0.05$ APP-NGF-20 vs. wt groups; day 8, * $p < 0.05$ APP-veh. vs. wt groups).

C) Probe trials for the NGF-2 group, measuring short-term memory (probe 1) and long-term memory (probe 2).

D) Probe trials for the NGF-20 group.

For C and D, Probe trial 1 was hours after completion of the MWM, Probe trial 2 was 2 weeks after completion of the MWM. NGF-2 and NGF-20 had no effect on memory deficits in APP mice, and had no effects in wt mice (* $p < 0.05$; ** $p < 0.01$). The number of animals used in probe trials for each groups are indicated in each histogram. Swim speeds were comparable between groups (data not shown). Error bars represent SEM.

Figure 2

NGF-C partially improves learning in APP mice, but not memory.

A) A trend towards better learning was observed in APP mice treated with NGF-C. Because of the elevated SEM in all days of training, the mean latency was not significantly different

between NGF-C-treated and vehicle-treated APP mice. However, in day 6, the difference between APP-vehicle and wt groups was significant (** $p < 0.01$).

B) NGF-C had no effect on memory in APP mice or wt mice. In probe trial 1, time and distance spent in the target quadrant were significantly different between wt-groups and APP groups (* $p < 0.05$; ** $p < 0.01$ APP-veh. vs wt-NGF-C; * $p < 0.05$ APP-veh. vs wt-veh.). No long-term effect in wt mice was observed with NGF-C treatment (* $p < 0.05$ APP-veh. vs. WT groups; * $p < 0.05$).

The number of animals used in probe trials for each groups are indicated in histogram. Swim speeds were comparable between groups (data not shown). Error bars represent SEM.

Figure 3

Treatment with a partial TrkA agonist improves learning and memory in a dose-dependent manner.

A) D3-10-treatment significantly improved learning in APP mice The mean latency in D3-10-treated APP group was comparable to that of observed in both wt groups, and the difference was significant compared to APP-vehicle group at day 5 (* $p < 0.05$, APP-saline group vs. all groups), day 7 (** $p < 0.01$, APP-veh. group vs both wt groups), and day 8 (* $p < 0.05$, APP-veh. vs. APP-D3 group; ** $p < 0.01$, APP-veh. group vs both wt groups). Error bars represent SEM.

B) In probe trial 1, both APP groups were comparable and were significantly different from wt groups (* $p < 0.05$; ** $p < 0.01$; ** $p < 0.01$ APP-veh. vs both wt groups). Hence while D3-10 treatment improved learning, there was no improvement to memory.

C) D3-40 treatment significantly improved learning in APP mice. At day 6 and day 8, mean latency was lower in D3-40-treated APP, compared to control APP mice (* $p < 0.05$; APP-veh. vs.

all groups). At day 5 and day 7, mean latency in APP-vehicle group was significantly higher, compared to D3-40-treated wt mice (* $p < 0.05$) and to both wt groups (* $p < 0.05$), respectively. Error bars represent SEM.

D) In probe trial 1, the distance swam in the target quadrant by D3-40-treated APP mice was comparable to that seen in both wt groups (* $p < 0.05$ APP-veh. vs. all groups). Time spent in target quadrant by D3-treated mice was comparable to wt groups, but the difference was not significant compared to APP-vehicle group (* $p < 0.05$ APP-veh. vs. WT groups). Error bars represent SEM. The number of animals used for each groups are indicated in histogram. Swim speeds were comparable between groups (data not shown).

E) ELISAs show a significant decrease of A β 1-42 or A β 1-40 in the cortex of D3-40-treated APP mice, compared to vehicle-treated mice (* $p < 0.05$), but not in the hippocampus. Error bars represent SD.

F) Expression of APP-CTF- β was significantly higher in D3-40-treated mice compared to APP-vehicle mice (* $p < 0.05$). Error bars represent SEM.

Figure 4.

Ratio of pAKT/actin in APP mice treated with D3 or control vehicle CSF.

Representative western blots from D3-treated mice (n=3) or CSF-treated mice (n=3). The summary shows average \pm SEM of 3 independent western blots for each tissue, for each mouse. Increase relative to CSF-treated mice (standardized to 1). D3 treatment results in increased p-AKT within 12 hours, in the hippocampus and cortex of APP mice. * significant $p < 0.05$.

Figure 5

Negative effect of D3-40 treatment on the long-term memory of wild type (wt) mice.

A) Probe trial 2 for D3-40-treated or vehicle-treated groups. Memory regressed in D3-40-treated wt mice, compared to the wt-vehicle group, and the memory recovery observed in D3-40-treated APP mice did not last in the long-term (** $p < 0.01$ APP-D3 vs wt-S; * $p < 0.05$ APP-veh. and wt-D3 groups vs. wt-veh. group).

B) D3-40-treated and vehicle-treated wt mice were retrained, with a completely new MWM experiment performed to train the mice. The mean latency was similar between both groups, indicating no differences in learning.

C) In probe trial 1a, time and distance spent in target quadrant by D3-40-treated wt mice was comparable to that in wt-saline mice. In probe trial 2a, the difference was again significant between wt-40-D3 and wt-vehicle groups (* $p < 0.05$).

The number of animals used in probe trial for each groups are indicated in histogram. Swim speeds were comparable between groups (data not shown). Error bars represent SEM.

Figure 6.

Ratio of pAKT/actin in wild-type (wt) mice treated with D3 or control vehicle CSF.

Representative western blots from D3-treated mice ($n=3$) or CSF-treated mice ($n=4$). The summary shows average \pm SEM of 3 independent western blots for each tissue, for each mouse. Increase relative to CSF-treated mice (standardized to 1). D3 treatment results in increased p-AKT within 12 hours, in the hippocampus of wt mice. * significant $p < 0.05$.

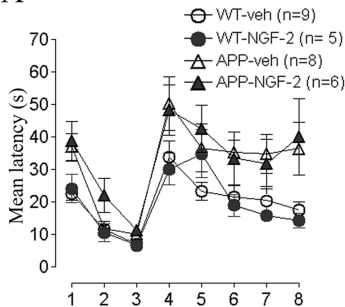
Figure 7

D3-40 treatment increases LTD in the hippocampus CA1 region of wild type (wt) mice.

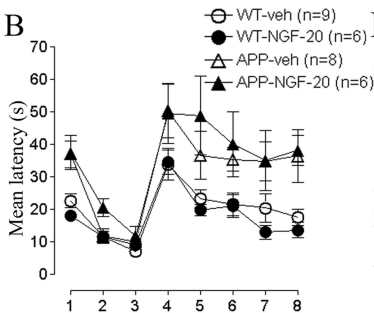
A) Upper: Scatter plots of the slope of field excitatory postsynaptic potential (fEPSP) against time on the *left* show changes in fEPSP slope recorded from the CA1 region of D3-40-treated wt and control wt (non-treated) mice before and after high frequency stimulation (HFS). Histograms on the *right* summarized the mean potentiation of fEPSP slope at 55-60 mins after HFS. Note that no change in the percentage potentiation between D3-treated and control groups was found. **Lower:** Scatter plots of fEPSP slope against time show changes in fEPSP slope after low frequency stimulation (LFS). Average depression of fEPSP at 55-60 mins after LFS in D3-treated and control wt mice is shown in the histogram on the *right*. The percentage depression recorded from D3-40-treated wt mice was significantly higher than that from control wt group (* $p < 0.05$).

B) proBDNF expression was significantly higher in the hippocampus of D3-40-treated, compared to vehicle-treated wt group (* $p < 0.05$). No significant difference in the expression of p75^{NTR} was observed in the hippocampus of both groups (here in the figure), as well as in the cortex and basal forebrain (data not shown). In contrast, the expression of NRH2, a p75^{NTR}-related protein was significantly higher in the hippocampus of p75NTR of D3-40-treated, compared to vehicle-treated wt mice (* $p < 0.05$).

A



B



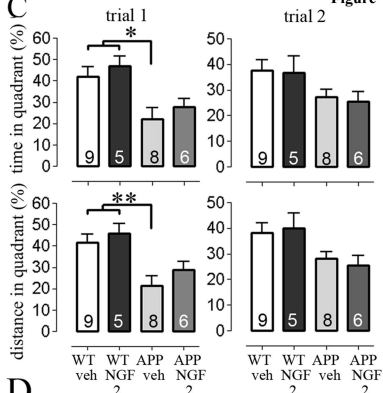
day 1 post-treatm.

Start of learning

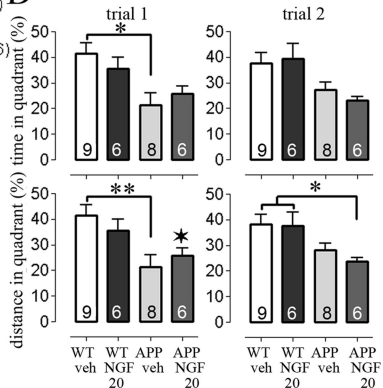
day 8

End of learning

C



D



day 8,2

Probe trial 1

day 22

Probe trial 2

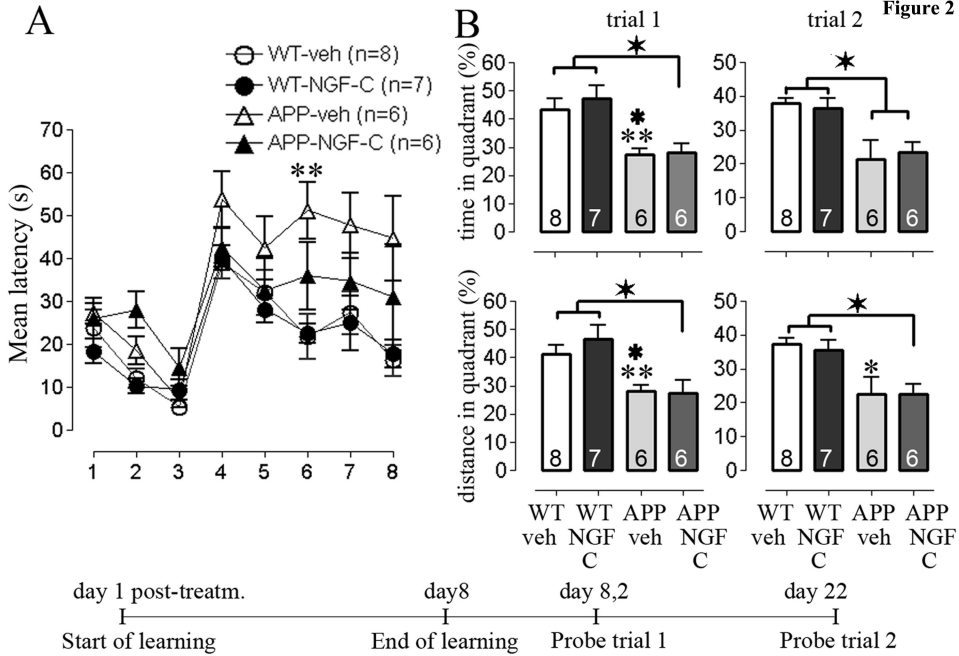


Figure 3

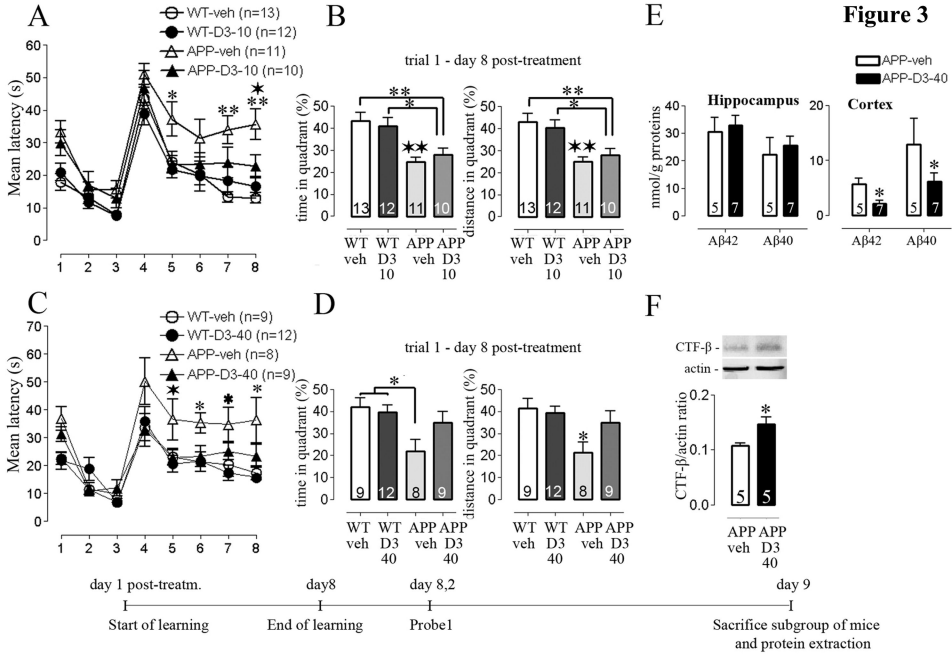
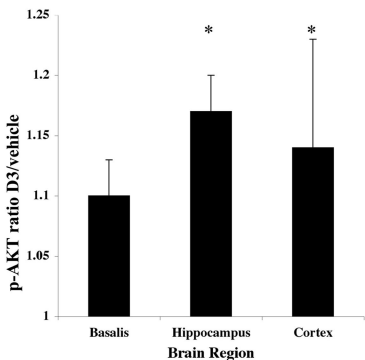
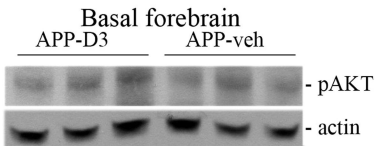
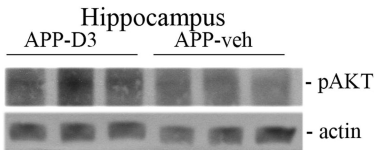
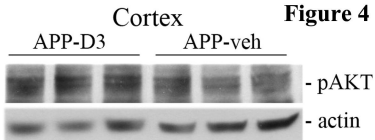
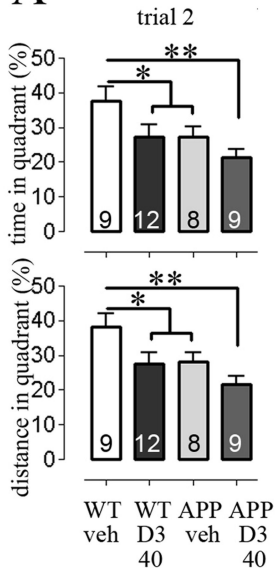
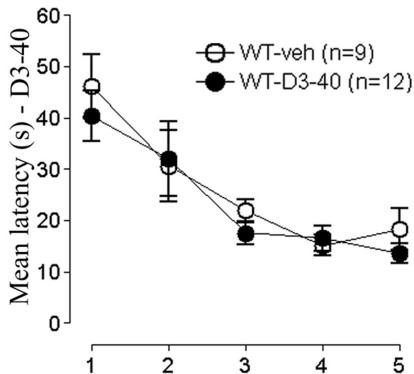


Figure 4

A

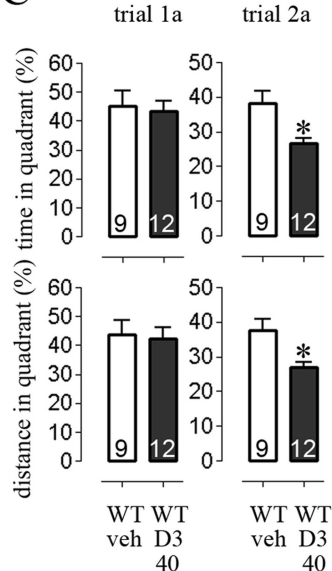
day 22 post-treatm.

Probe trial2

B

day 45

Start learning New

C

day 50.2

day 57

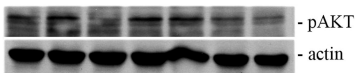
Probe trial1a

Probe trial2a

Figure 5

Figure 6

Cortex
WT-D3 WT-veh



Hippocampus
WT-D3 WT-veh



Basal forebrain
WT-D3 WT-veh

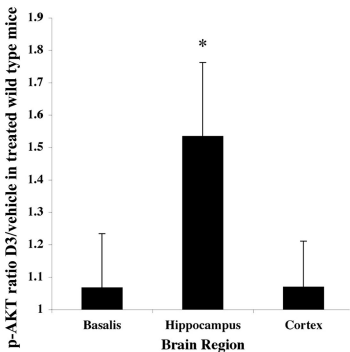
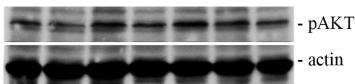


Figure 7

