Ligand-Dependent TrkA Activity in Brain Differentially Affects Spatial Learning and Long-Term Memory

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ABSTRACT

In the central nervous system, the nerve growth factor (NGF) receptor TrkA is expressed primarily in cholinergic neurons that are implicated in spatial learning and memory, whereas the NGF receptor p75NTR is expressed in many neuronal populations and glia. We asked whether selective TrkA activation may have a different impact on learning, short-term memory, and long-term memory. We also asked whether TrkA activation might affect cognition differently in wild-type mice versus mice with cognitive deficits due to transgenic overexpression of mutant amyloid-precursor protein (APP mice). Mice were treated with wild-type NGF (a ligand of TrkA and p75NTR) or with selective pharmacological agonists of TrkA (a) and p75NTR. In APP mice, the selective TrkA agonist significantly improved learning and short-term memory. These improvements are associated with a reduction of soluble Aβ 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 brain-derived neurotrophic factor precursor, increased neurotrophin receptor homolog-2 (p75-related protein), and long-term depression. Together, these data indicate that selective TrkA activation affects 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 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 neurotrophin, acts through two distinct receptors, TrkA and p75NTR. In the central nervous system, TrkA receptors are almost exclusively expressed in the cholinergic neurons of the cortex, septum, and basal forebrain. In contrast, p75NTR is widely expressed in many neuronal populations and in glia and reactive astrocytes. Ligand-dependent activation of TrkA mediates signals that are generally believed to be neuroprotective, whereas ligand-dependent activation of p75NTR 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 Alzheimer’s disease (AD) was suggested after the observation that NGF has neurotrophic actions on basal forebrain cholinergic neurons, whose degeneration is at the basis of the cholinergic deficit and cognitive decline in AD. Indeed, studies in humans (Muf-
son et al., 2002; Counts et al., 2004) 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<sub>NTR</sub> 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 the 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 patients with AD (Tuszynski et al., 2005).

However, wild-type NGF can bind to both TrkA and to p75<sub>NTR</sub>, and in mice lacking proper TrkA signaling, the activation of p75<sub>NTR</sub> can cause accumulation of Ab peptides (Capsoni et al., 2010). This is a concern because in human AD, there is also poor TrkA expression or signaling (Mufson (Capsoni et al., 2010). This is a concern because in human AD, there is also poor TrkA expression or signaling (Mufson et al., 2003). Indirect evidence of a role for TrkA in cognition 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). 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).

Materials and Methods

Transgenic Mouse Model. Animals used in this study were 4- to 5-month-old heterozygous transgenic mice that overexpress the Swedish (670/671<sub>KM</sub>−<sub>NL</sub>) and Indiana (717<sub>V</sub>−<sub>f</sub>) mutations of human APP gene under the control of the platelet-derived growth factor β promoter on a C57BL/6J background (APP<sub>sw</sub>,Ind, line J20) (Mucke et al., 2000). Wild-type (wt) litter mates 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 Central Nervous System by Osmotic Minipumps. We used an Alzet model 1002 osmotic minipump (ALZET Osmotic Pumps; Durect Corporation, Cupertino, CA) that accommodated 100 μl of solution and continuously delivered the respective drugs for a period of 2 weeks and were prepared following the manufacturer’s 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. 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 postmortem for each mouse, and was a prerequisite for inclusion in the study.

Test Compounds and Experimental Groups. Artificial cerebrospinal fluid (aCSF) (150 mM NaCl, 1.8 mM Ca<sub>Cl</sub><sub>2</sub>, 1.2 mM MgSO<sub>4</sub>, 2 mM K<sub>HPO</sub><sub>4</sub>, 10 mM glucose, and 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 2 weeks. As much as possible, all groups included a 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, South San Francisco, CA) was used at a total dose of 2 μg (NGF-2 group) and 20 μg (NGF-20 group) over a 2-week period. The dose was based on extrapolation of the maximal effective doses published that are nontoxic 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 (Ibañez et al., 1992) and has been well characterized and used 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<sub>NTR</sub> but activates TrkA ex vivo (Ibañez et al., 1992) and in vivo (Bai et al., 2010a). In the present work, we used 5 μg of NGF-C. The dose was based on extrapolation of the published effective dose that is nontoxic 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<sub>NTR</sub> (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 (Shi et al., 2007; Bai et al., 2010a). D3 is a partial TrkA agonist that can also potentiate TrkA signals activated by suboptimal levels of NGF (Maliartchouk et al., 2000) because its binding site is nonoverlapping with NGF. D3 was applied at 10 (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. After 2 weeks of treatment, mice were subjected to the Morris water maze (MWM) task as adapted for APP transgenic mice (Deipolyi et al., 2008). The MWM consisted of 8 days of training in a pool, divided virtually into four 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 three different directions for each mouse, with an intertrial time not exceeding 45 min. In the last day of training, a minimum of 2 h was allowed for mice to rest, after which the platform was removed and mice were allowed to freely swim for 60 s (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 2 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 h after the last training. Another probe trial was performed 1 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 were processed for electrophysiological studies.

All learning data are expressed as time (in seconds) to reach the hidden platform ± S.E.M., whereas probe trials data are expressed as a percentage of time and distance spent in the quadrant in which the platform was previously hidden ± S.E.M.

**ELISA for Soluble Aβ.** Mice from one D3-40 group were sacrificed just after the probe trial 1 to correlate the gain in memory with changes in Aβ and other proteins. Brain fraction containing soluble Aβ was extracted as described previously (Nicolakakis et al., 2008). Cortex or hippocampus were homogenized by sonication in a buffer containing 20 mM Tris buffer, 1 mM EDTA, 250 mM sucrose, and protease inhibitors. Soluble Aβ was extracted by centrifugation (100,000g, 60 min, 4°C) using diethyl acetate (0.4% in 100 mM NaCl), and Aβ1-40, and Aβ1-42, levels, expressed as nanomoles per gram of protein, were measured as described by the manufacturer (BioSource International, Camarillo, CA).

**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, pH 7.6, 0.25 M sucrose, 1 mM phenylmethylsulfonyl fluoride, 2 mM EDTA, 1 mM EGTA, 10 mM Na3VO4, 25 mM NaF, 10 mM Na2PO4·10H2O, and protease inhibitor tablet). Homogenates were then centrifuged at 800g for 5 min to remove nuclei and large debris. The resulting supernatant was centrifuged (10,000g for 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 (16,000g for 30 min) to obtain the Triton-soluble (containing the cytosolic fraction of synaptosome) and Triton X-100-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 (Bio-Rad Laboratories, Hercules, CA).

**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 h after short-term drug treatment, and tissue samples were collected. Treatment consisted of intracerebroventricularly administered D3 2-μg dose (n = 3 wt group and n = 3 APP group). This is comparable with 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, and their tissues were dissected and solubilized in detergent (Bai et al., 2010b). To quantify proteins of interest (p-AKT, p75NTR, NRH-2, and pro-BDNF) 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-polyacrylamide gel electrophoresis. To quantify CTP-β, 50 μg of cytosolic fraction was loaded in tricine/SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto nitrocellulose membrane, and blots were incubated overnight with primary antibodies against CTP-β (mouse 6E10 antibody; BioSource International), rabbit anti-p-p75NTR (Promega, Madison, WI), and rabbit anti-pro-BDNF (Alomone Labs, Jerusalem, Israel), or antibodies to total AKT and p-AKT (Cell Signaling Technology, Danvers, MA). Rabbit anti-NRH-2 antibodies were kindly provided by Dr. Phillip Barker (Montreal Neurological Institute). Mouse anti-β-actin (Sigma-Aldrich, St. Louis, MO) was used as internal control for loading. Blots were incubated with horseradish peroxidase-conjugated secondary antibodies (1:2000; Jackson Immunoresearch Laboratories Inc., West Grove, PA), and proteins were visualized with enhanced chemiluminescence (ECL Plus kit; GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) using a PhosphorImager (Scanner STORM 860; GE Healthcare) followed by densitometric quantification with ImageQuant 5.0 (GE Healthcare).

**Electrophysiological Recording in Hippocampal Slices.** Groups of vehicle-treated and D3-40-treated wt mice were used, and hippocampal slices were prepared as described previously (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. In brief, under deep anesthesia, brains were rapidly removed, and coronal slices (350 μm thickness) were cut in hypothermic, ice-cold, and carbogenated (bubbled with 95% O2/5% CO2 to maintain the pH at 7.4) solution (232 mM sucrose, 2.5 mM KCl, 4 mM MgCl2, 0.1 mM CaCl2, 1.25 mM KH2PO4, 26 mM NaHCO3 and 10 mM glucose) using a Vibratome. Freshly cut slices were placed in an incubating chamber with carbogenated aCSF (~310 mOsM) consisting of 125 mM NaCl, 2.5 mM KCl, 1 mM MgCl2, 2 mM CaCl2, 1.25 mM Na2HPO4, 26 mM NaHCO3, and 25 mM glucose. Slices were recovered at 32°C for 1 h and subsequently maintained at room temperature. Carbogenated aCSF containing bicuculline methobromide (5 μM) to block GABA 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 were recorded from the hippocampal CA1 region, amplified by a Multiclamp 700B, and stored in a personal computer for offline analysis using Clampfit (Molecular Devices, Sunnyvale, CA). 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 ± S.E.M. (except for ELISA, expressed as mean ± S.D.). For multiple-group comparisons, one-way analysis of variance followed by Newman-Keuls post hoc multiple comparison test was used. Student’s t test was used for two-group comparisons (Prism 4 software; GraphPad Software Inc., San Diego, CA). 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, Fig. 1, A and B) 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 with wt mice. This is indicative of memory impairment (Fig. 1, C and D).

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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 (Fig. 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 (Fig. 1B). Moreover, the performance of APP and wt mice in probe trials 1 and 2 was not affected by either doses of NGF (Fig. 1, C and D). Overall, our findings indicate that intracerebroventricular infusion of NGF had no effect on spatial learning or on memory, irrespective of whether the mice are cognitively impaired.

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 p75NTR, might have different properties in vivo compared with wild-type NGF.

**Fig. 1.** Wild-type NGF has no effect on the learning or the memory of APP and 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 with that in the APP-vehicle group, and both APP groups had higher latency compared with 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 in 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 an hour 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 < 0.01; **p < 0.001). The number of animals used in probe trials for each groups is indicated in each histogram. Swim speeds were comparable between groups (data not shown). Error bars represent S.E.M.

**Fig. 2.** NGF-C partially improves learning in APP mice, but not memory. A, a trend toward better learning was observed in APP mice treated with NGF-C. Because of the elevated S.E.M. 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 and had no effects in wt mice (**p < 0.01; **p < 0.001). No long-term effect in wt mice was observed with NGF-C treatment (##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 S.E.M.
APP mice treated with NGF-C exhibited a trend toward improved performance in MWM learning compared with vehicle-treated APP mice (Fig. 2A). Note that wild-type NGF did not exhibit this trend even when delivered at four times higher doses. NGF-C-treated APP mice showed no improvement in probe trials testing for memory (Fig. 2B). Similar treatment of wt mice with NGF-C showed no changes in learning or in memory (Fig. 2, A and B). 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 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 with vehicle-treated APP mice (Fig. 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) (Fig. 3B). Similar treatment of wt mice with D3-10 showed no changes in learning or in STM (Fig. 3, A and B). 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.

![Figure 3](molpharm.aspetjournals.org)
In APP-mice treatment with D3-40 significantly improved spatial learning in all training days, to levels comparable with those of wt mice (Fig. 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 (Fig. 3D) and did not last as long as vehicle-treated APP mice (Fig. 5A). When time was 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 (Fig. 3, C and D).

Together, these data indicate that treatment of APP mice with selective TrkA agonists can improve either 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 nondiseased brains. Lack of toxicity is further documented below.

**The Partial TrkA Agonist D3 Increases p-AKT in Cortex and Hippocampus and Decreases Cortical Aβ 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 Aβ1–40 and Aβ1–42 were measured immediately after probe trial 1 of D3-40-treated APP mice, which had shown gains in STM. Cortical levels of Aβ1–40 and Aβ1–42 were significantly lower in D3-40–treated APP mice compared with vehicletreated APP mice (52 and 62% decrease, respectively, Fig. 3E; p < 0.05). This decrease was accompanied by a corresponding increase in the levels of the APP C-terminal fragment-β (CTF-β) (40% increase, Fig. 3F; p < 0.05). Lack of CTF-β processing into Aβ suggests an inhibition of cortical β-secretase activity after D3-40 treatment. In contrast, D3-40 had no effect on Aβ levels in the hippocampus (Fig. 3E), suggesting that the effect is localized to the 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 with vehicle-treated APP mice (Fig. 4).

Thus, there seems 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 Aβ 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 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 (Fig. 5A). Both the time and the distance in the target quadrant were significantly lower compared with 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 (Fig. 5A). In probe trial 2, the D3-40-treated wt mice seemed to be impaired compared with vehicle-treated wt mice. Because 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 (Fig. 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 with that of vehicle-treated wt mice (Fig. 4).
Moreover, the D3-40-treated wt mice had normal STM (Fig. 5C, probe trial 1a), as demonstrated by comparable time and distance traveled in the target quadrant with 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 1 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 (Fig. 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 with vehicle-treated wt mice. However, no significant change of p-AKT was observed in the cortex or in the basal forebrain (Fig. 6). Thus, in wt brains, there seems to be a paradoxical association between acute hyper-activation 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 decrease in the LTM in wt mice, we recorded LTP and LTD in the CA1 layer of hippocampal slices (Fig. 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 with vehicle-treated wt mice.

To address possible mechanisms by which LTD may be increased, we evaluated pro-BDNF and p75NTR 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 p75NTR expression between the two groups (Fig. 7B). However, we found an increased expression of NRH-2, a p75-related protein, in the hippocampus (Fig. 7B) and the cortex (data not shown) of D3-40-treated compared with 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 p75NTR), 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, because 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 (Debeir et al., 1999; Capsoni et al., 2010) and for hippocampal plasticity (Conner et al., 2009).

We therefore assessed the cholinergic neuronal phenotype by measuring protein levels of choline acetyltransferase and vesicular acetylcholine transporter 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 article take place without any changes to the major cholinergic markers. In addition, at the age that APP mice were tested in this study, they do not present detectable cholinergic deficits despite 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 p75NTR 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, whereas D3-40 also improved STM. However, memory did not last in the long-term. Because APP mice already have an impaired LTM, the LTM impairment in D3-40-treated APP mice may be interpreted as a failure of D3 at 40 μg 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 Aβ and with increased levels of CTF-β. Considering that Aβ 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 Aβ in differentiated PC12 cells and in primary hippocampal neurons (Matrone et al., 2008a,b).

Soluble Aβ 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 Aβ levels. Moreover, treatments that do not improve memory in APP mice (e.g., D3-10 treatment) did not cause changes in Aβ levels in cortex or hippocampus (data not shown).

Whereas only the hippocampus seems 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). In particular, the entorhinal cortex, a primary input to the hippocampus, is affected early in patients with AD and contributes to the loss of STM (Kordower et al., 2001). Furthermore, 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). Likewise, 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 Aβ in cortex of D3-40-treated APP mice may have contributed to the STM restoration in these mice. Further studies are required to prove this point unambiguously.

Because Aβ*56 is an oligomeric form purported to be responsible for the memory deficit in APP mice (Lesné et al., 2006), we studied it specifically. However, we did not find any change in levels of Aβ*56 between D3-40-treated and vehicle-treated APP mice (data not shown). Thus, from our data in
APP mice the precise role of Aβ456 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-μg dose was optimal at reducing Aβ 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 retrained 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) whereas 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-induced LTD in the CA1 region of D3-40-treated wt mice. Previous findings obtained from the young visual cortex have shown that short-term NGF treatment favors LTD over 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 long-term treatment of TrkA agonist produced a long-term facilitating effect on hippocampal LTD, which on behavioral testing corresponds with no short-term effects but with an 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 to 3 weeks.

It is still unclear how long-term TrkA activation by D3-40 facilitates LTD. There are four nonexclusive possibilities. First, long-term 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

Fig. 7. D3-40 treatment increases LTD in the hippocampus CA1 region of wt mice. A, top, scatter plots of the slope of fEPSP against time on the left show changes in fEPSP slope recorded from the CA1 region of D3-40-treated wt and control vehicle-treated wt mice before and after high-frequency stimulation (HFS). Histograms on the right summarized the mean potentiation of fEPSP slope at 55 to 60 min after HFS. Note that no change in the percentage potentiation between D3-treated and control groups was found. Bottom, scatter plots of fEPSP slope against time show changes in fEPSP slope after low-frequency stimulation (LFS). Average depression of fEPSP at 55 to 60 min after LFS in D3-treated and control wt mice is shown in the histogram on the right. The percentage of depression recorded from D3-40-treated wt mice was significantly higher than that from control wt group (*, p < 0.05). B, pro-BDNF expression was significantly higher in the hippocampus of D3-40-treated compared with vehicle-treated wt group (*, p < 0.05). No significant difference in the expression of p75NTR was observed in the hippocampus of both groups (here in the figure) and in the cortex and basal forebrain (data not shown). In contrast, the expression of NRH2, a p75NTR-related protein, was significantly higher in the hippocampus of p75NTR of D3-40-treated, compared with vehicle-treated wt mice (*, p < 0.05).
of muscarinic cholinergic receptors facilitates LTD induced by low-frequency stimulation (Kirkwood et al., 1999). Second, long-term TrkA activation may facilitate LTD by desensitization of the TrkA signaling pathway. Previous work in which 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). Increased LTD may be relayed by long-term AKT phosphorylation, because deregulation of the phosphatidylinositol 3-kinase/AKT pathway was shown to induce long-term synaptic depression (Guo and Zhong, 2006). Third, pro-BDNF levels were significantly increased in D3-40-treated wt mice, and pro-BDNF can facilitate hippocampal LTD (Woo et al., 2005). The relative reduction of pro-BDNF processing to mature BDNF may also be important, because BDNF is required to promote LTD persistence (Rosato et al., 2009) and LTM storage (Bekinschtein et al., 2008). Fourth, whereas p75NTR protein did not change in cortex, hippocampus, and basal forebrain; a homolog of p75NTR, NRH-2, increased significantly in both the cortex and hippocampus of D3-40-treated mice. Although NRH-2 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 p75NTR receptor (Murray et al., 2004). NRH-2, like p75NTR, uses intracellular mechanisms to regulate NGF binding to TrkA and modulate TrkA receptor signaling. The relevance of NRH-2 and synaptic plasticity has not been studied, but it is likely that NRH-2 may affect plasticity because its homolog p75NTR influences hippocampal LTD (Rösch 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). Long-term D3-40 treatment may weaken LTD retrieval by enhancing LTD formation. On the other hand, 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 Aβ in the cortex, probably through inhibition of γ-secretase. However, the deficit in memory consolidation or retrieval observed in APP mice is not resolved because LTD is still deficient in these mice. In addition, 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 NRH-2 and pro-BDNF.

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.


Woo NH, Teng HK, Siao Cj, Chiarruttini C, Pang PT, Milner TA, Hempstead BL, and Lu B (2005) Activation of p75NTR by proBDNF facilitates hippocampal long-term depression. Nat Neurosci 8:1069–1077.

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