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Brain-derived neurotrophic factor (BDNF) overexpression in the forebrain results in learning and memory impairments

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ABSTRACT

In this study we analyzed the effect on behavior of a chronic exposure to brain-derived neurotrophic factor (BDNF), by analysing a mouse line overexpressing BDNF under the αCaMKII promoter, which drives the transgene expression exclusively to principal neurons of the forebrain. BDNF transgenic mice and their WT littermates were examined with a battery of behavioral tests, in order to evaluate motor coordination, learning, short and long-term memory formation. Our results demonstrate that chronic BDNF overexpression in the central nervous system (CNS) causes learning deficits and short-term memory impairments, both in spatial and instrumental learning tasks. This observation suggests that a widespread increase in BDNF in forebrain networks may result in adverse effects on learning and memory formation.

Introduction

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family of growth factors, which is widely expressed throughout the mammalian brain, together with its high affinity receptor TrkB (Lewin and Barde, 1996; Thoenen, 1995), and plays a crucial role in the development, maintenance and function of the CNS (Bonhoeffer, 1996; Huang and Reichardt, 2001; Huang and Reichardt, 2003). In addition, in the past decade, accumulating data indicate that neuronal activity regulates BDNF transcription, transport of BDNF mRNA and protein into dendrites and activity-dependent secretion of BDNF that in turn modulate synaptogenesis, synaptic plasticity and memory formation (Bekinschtein et al., 2007b; Lu et al., 2007; Poo, 2001; Soule et al., 2006; Tyler et al., 2002).

A unique feature among the neurotrophin family is BDNF activity-dependent secretion, as a mixture of proBDNF and mature BDNF (Egan et al., 2003; Lu, 2003; Pang and Lu, 2004; Pang et al., 2004). Compelling evidence indicates that, by binding to its high affinity receptor TrkB, mature BDNF plays a critical role in both early and late forms of long-term potentiation (E-LTP and L-LTP, respectively). On one side, Ca^{2+} and NMDA glutamate receptor dependent BDNF secretion, upon tetanic stimulation, facilitates maintenance of E-LTP (Aicardi et al., 2004; Balkowiec and Katz, 2002; Figurov et al., 1996; Gartner and Staiger, 2002; Gottschalk et al., 1998; Hartmann et al., 2001; Lever et al., 2001; Rex et al., 2006; Yano et al., 2006). On the other, both genetic and pharmacological blockade of BDNF and TrkB signaling results in L-LTP impairments, particularly upon theta burst stimulation or forskolin application (Korte et al., 1995; Korte et al., 1996; Pang et al., 2004; Patterson et al., 1996; Minichiello et al., 2002; Minichiello et al., 1999; Patterson et al., 2001). Moreover, at least in certain conditions, BNDF has been shown to elicit by itself a form of synaptic plasticity, termed BDNF-LTP (Kang and Schuman, 1995; Kang and Schuman, 1996; Messaoudi et al., 2002; Ying et al., 2002). This is a form of late LTP involving brief, local application of BDNF which is transcription-dependent and requires Arc synthesis (Messaoudi et al., 2007).

From a behavioral perspective, several studies have shown that BDNF and TrkB are implicated in learning and memory by directly examining their role in a variety of learning paradigms in rodents (Bekinschtein et al., 2007b; Bekinschtein et al., 2008; Lu et al., 2007; Yamada et al., 2002). BDNF expression was found to be increased in the hippocampus of rats, following Morris Water Maze (MWM) (Kesslak et al., 1998), radial arm maze (Mizuno et al., 2000), passive avoidance (Ma et al., 1998) or contextual fear conditioning (Hall et al.,

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2000; Rattiner et al., 2004b) and gene ablation of either BDNF or TrkB resulted in learning impairments (Linnarsson et al., 1997; Minichiello et al., 1999). Accordingly, a single intra-hippocampal BDNF administration in adult rats improves performance in the MWM test (Cirulli et al., 2004). Furthermore, pre-training infusions of either anti-BDNF antibodies or antisense BDNF oligonucleotides caused impaired spatial learning and memory in rats, as assessed in the MWM (Mu et al., 1999), the radial arm maze (Mizuno et al., 2000), fear conditioning (Lee et al., 2004; Rattiner et al., 2004a; Rattiner et al., 2005) or passive avoidance (Alonso et al., 2005; Alonso et al., 2002; Tyler et al., 2002; Bekinschtein et al., 2007a; Ma et al., 1998).

A key aspect that has recently started to be investigated is the role of proBDNF in plasticity and memory formation. In humans, a val66met polymorphic substitution in the 5′ pro-region of the human BDNF protein results in hippocampal-dependent episodic memory impairments that are linked to proBDNF deficits in localizing to secretory granules or synapses (Egan et al., 2003). In addition, strong evidence suggests that tissue-plasminogen activator (tPA), a protein whose secretion is also upregulated during L-LTP and learning, through activation of plasmin, converts the proBDNF precursor into mature BDNF and that this proteolytic step is necessary for L-LTP expression (Pang et al., 2004). Together with the observation that, at least in the hippocampus, a substantial fraction of the total BDNF secreted extracellularly is proBDNF (Farhadi et al., 2000; Mowla et al., 2001; Mowla et al., 1999) and that its selective binding to the low affinity receptor p75NTR may facilitate neuronal apotosis (Lee et al., 2001; Teng et al., 2005) and possibly other neuronal functions such as long-term depression (LTD), a contrasting bidirectional regulation of synaptic plasticity and memory formation has been suggested for the mature and precursor forms of BDNF (Lu, 2003).

Unfortunately, very little work has so far been performed on animal models in which BDNF levels have been increased chronically in the brain. Since the relevance of BDNF as potential therapeutic molecule to treat a number of brain disorders, from Parkinson's and Huntington's diseases to depression and substance abuse, this aspect deserves to be evaluated in detail (Chao et al., 2006). In the only available study, a widespread overexpression of BDNF in mice, under the control of the ubiquitous β-actin promoter, resulted in learning deficits in passive avoidance, increased neuronal excitability and susceptibility to seizures (Croll et al., 1999). Such finding, highly relevant in our opinion for the future clinical use of BDNF but largely neglected in the literature, has largely been confirmed by the present work. Here we have analyzed a mouse line, which overexpresses BDNF specifically in the forebrain structures (Huang et al., 1999). We found, quite surprisingly, significant impairments in both short and long-term memory formation.

Materials and methods

Animals

All mice used in this work were maintained on a C57Bl/6 genetic background (more than 10 generations) from Charles River Laboratories. Mice were kept 5 or 6 per cage in a SPF environment, with 50% relative humidity, a temperature of 21 ± 1 °C and on a 12 h light-dark schedule, with food and water available *ad libitum*. Littermates were weaned around day P21 and genotyped for the presence of the BDNF transgene, using the forward primer 5′-TCAGTCAAGCCGGTTCTC-3′ and the reverse primer 5′-AGTCCGCGTCCTTATGGT-3′. In this work, transgenic BDNF and WT control mice were littermates of 9–14 weeks of age and only naive males were used. For the behavioral analysis, mice were handled for 1 week before experiments and all experiments were performed in a low luminosity environment (20–25 lx) and carried out blind with respect to genotype. All animal procedures were conducted according to EC guidelines (EC Council Directive 86/609, 1986) and to the Italian legislation on animal experimentation (Decreto L.vo 116/92).

BDNF protein quantification by ELISA

Mice were sacrificed by cervical dislocation and decapitated. Brains were quickly removed and microdissected by use of a stereotactic microscope. Samples from the selected regions were collected and the BDNF protein quantification was determined by use of the BDNF E_{max} ImmunoAssay System (Promega), according to the manufacturer's instructions. Briefly, 96-well plates were incubated overnight at 4 °C with anti-BDNF monoclonal antibody, diluted 1:1000 in carbonate coating buffer. Plates were blocked with 1X Block and Sample buffer for 1 h at room temperature. Samples and standards (0–500 ρg/ml) were added to the plates and incubated for 6 h, followed by incubation with a 1:500 anti-human BDNF polyclonal antibody, overnight at 4 °C. Anti-IgY horseradish peroxidase conjugate, diluted 1:200, was then added to each well and plates were incubated for 2 h at room temperature. Plates were developed by incubation 15 min at room temperature with 100 μl of TMB One Solution. The reaction was stopped by adding 100 μl of 1N HCl and absorbance was measured at 450 nm within 30 min. BDNF protein quantification for each structure was performed in duplicate, and mean BDNF levels were reported in ng/g of weight tissue. The results for the BDNF protein levels were expressed as mean ± SEM, for both genotypes. Statistical analysis between genotypes for each structure analysed consisted on a two-tail student's *t*-Test.

SDS-PAGE and western blotting

WT and BDNF transgenic mice were decapitated and the brain was rapidly removed and placed on ice. Hippocampus, striatum, cortex and cerebellum were dissected and frozen on liquid nitrogen. Tissues were homogenized in a lysis buffer (150 mM NaCl, 20 mM Hepes, 1 mM MgC, 1 mM EDTA) and western blotting was performed. Proteins (10 μg) were separated on 12% Tris-Glicine gel at a constant voltage of 100 V and then transferred to Hybond-P PDVP membranes (Amersham Biosciences) at a constant voltage of 100 V for 1 h. Blots were blocked in 5% non-fat milk in a Tris buffered saline solution containing 1% Tween 20 (TBS-T) and this TBS-T solution was used for all subsequent washes. Primary and secondary antibodies were diluted in TBS-T containing 5% non-fat milk and were used at the following concentrations: 1:5000 anti-BDNF (AP1779SP, Chemicon), 1:1000 anti-GAPDH (Sigma) and used as previously described (Barnes and Thomas, 2008). HRP-conjugated secondary antibodies were used at room temperature for 1 h in 5% milk in TBS plus 0.5% Tween 20 followed by immunological detection with ECL Plus (Amersham Bioscience). The immunoblots were exposed with Hyperfilm, scanned and analyzed with ImageJ software to measure the integrated optical density of the bands.

Immunohistochemistry

WT and BDNF transgenic mice (five for each genotype) were anaesthetized and perfused via intracardial infusion of ice-cold 4% PFA (dissolved in 0.1 M Na_2HPO_4/NaH_2PO_4 buffer, pH 7.4). Brains were rapidly extracted, post-fixed overnight and transferred to 25% buffered sucrose for 24 h. Coronal sections were cut at 30 μm thickness on a freezing microtome and stored in a cryoprotective solution at −20 °C until they were processed for immunohistochemistry as previously described (Valjent et al., 2000). Free-floating sections were incubated with a primary antibody against Phospho-p44/42 Map Kinase (1:200, Cell Signalling) overnight at 4 °C. Sections were then incubated with biotinylated goat anti-rabbit IgG (1:200, Vector Labs) for 2 h. Detection of the bound antibodies was carried out using a standard peroxidase-based method (ABC-kit,

Vectastain, Vector Labs), followed by DAB staining. Quantification of p-ERK positive neurons was performed for different regions of the Hippocampus (DG, CA3, CA2, CA1) and Cortex (Cingulate Cx: Cin Cx; Prefrontal Cortex: Pf Cx) in 2 sections per mouse, bilaterally. Sample areas were visualized under a 20× objective in a Leica DM IRB microscope by a blind investigator to condition and genotype and counts per region were averaged across the sections using the ImageJ software.

Accelerating Rota-Rod

The apparatus (Ugo Basile Instruments, Comerio, Italy) consists on a rotating cylinder, covered with textured rubber to provide grip. Mice walk forward on the rotating cylinder, at speeds increasing from 4 to 40 rpm over a 6 min cut-off testing session. Latencies in falling off the cylinder were measured over 3 consecutive daily sessions, 3 trials per session (Biffi et al., 2004).

Locomotor activity

The apparatus (Ugo Basile Instruments, Comerio, Italy) consists in a 90×40×45 cm activity box containing UV photoelectric beams encompassing the whole length of the cage, with a line of photocells 2 cm above the floor to measure horizontal movements, and another line located 6 cm above the floor to measure vertical movements. Spontaneous locomotor activity and exploratory behavior were assessed by individually placing the mice in the locomotor boxes for 10 min: horizontal and vertical activity was measured as the total number of beam disruptions during the monitoring period. A daily session was performed, during 3 consecutive days (Mazzucchelli et al., 2002).

Passive avoidance

The apparatus (Panlab s.l., Barcelona, Spain) consists of two chambers: a white strongly illuminated chamber, separated by a door from a black dark chamber, which has a floor grid that permits the passage of electric foot shocks (Pittenger et al., 2006). The task began by placing the mouse in the illuminated compartment and then opening the door between the chambers, after a period of exploration. When the mouse entered the dark compartment after a short latency, the door was closed and a single electric foot shock of 0.10 mA was administered, for 2.0 s. We performed the passive avoidance test in two different conditions: in the first condition we used an apparatus in which the size of the white, illuminated compartment was approximately twice as large as that of the black, dark compartment, that mice were allowed to explore for 10.0 s before door opening. In the second condition we used a white compartment approximately of the same size than the black one, which mice were allowed to explore for 3.0 s. After reintroducing the same mice in the same illuminated chamber, after 1 h or 24 h, we determined the latency of time spent in the illuminated side, as a measure of short- and long-term memory, respectively. For both cases, the acquired memory was recalled after 10 days.

Eight-arm radial maze

Animals were food restricted for a week before the test and maintained at 80% of their initial weight throughout the test. The apparatus consisted in a plastic maze with 8 identical arms, numbered 1 to 8, radiating from a central platform. A series of external cues were placed outside the maze for spatial orientation. At each daily trial, a food pellet reward was placed at the end of each arm and the mouse was placed on the centre of the maze; the trial finished when the mouse visited all the 8 baited arms and the overall latency to complete the task was recorded. During each trial, two parameters were scored: total number of errors and total number of correct visits. An error corresponds to re-entering a previously visited arm and the number of correct visits corresponds to the number of arms visited before scoring an error. We performed one trial per day during 12 consecutive days and a recall trial after 10 days (Brambilla et al., 1997; Olton et al., 1978).

Morris water maze (MWM)

We performed the hidden platform version of the MWM test (Brambilla et al., 1997; Morris et al., 1982). A large water tank of 120 cm of diameter was filled with white opaque water at 20 °C and divided into four quadrants of equal area arbitrarily named Northeast, Southeast, Southwest and Northwest. The water tank was in a room with two- and three-dimensional visual cues in the walls. An escape platform of 11.4 cm of side was submerged 1 cm below the water surface and placed in the center of the Southeast quadrant. The platform was maintained in this position for all the swim trials through the test. Mice were trained to swim to the platform in 2 daily trials, with a 30 min interval, during 10 consecutive days. Each trial was initiated at one of four different starting positions at the outer edge of the pool and the swimming path of each mouse was video recorded. Upon reaching the platform, each mouse was allowed to rest for 20 s on it. The trial finished when the mouse found the platform or when 60 s had elapsed. If the mouse did not reach the platform within 60 s, it was guided to the platform and allowed to rest for 20 s on it. After each trial each mouse was returned to its home cage where it rested until the next trial. On the day 5 and on the day 10, 30 min before the daily trials of hidden platform, each mouse was tested in a probe trial in which the hidden platform was removed and the swimming path of each mouse was video recorded over 60 s. A recall probe trial was performed after 10 days. Different parameters were scored as an average of the 2 daily trials and for each probe trial, using Noldus EthoVision 3.0 platform, a video tracking system for automatic recording of movement. For each trial we measured: latency to reach the platform, in sec, total distance swam to the platform, in cm, and average swim speed, in cm/s. For each probe trial we measured the total amount of time spent in each quadrant, in sec, and the total amount of time spent in the platform zone, in sec.

Statistical analysis

All results were expressed as mean ± SEM and all statistical analysis made use of the Statistical Package for Social Sciences (SPSS 13.0 software). For behavioral tests, removal of outliers was performed by a Blox plot analysis. Analysis of differences between groups consisted on a two-way analysis of variance (ANOVA) for repeated measures, considering both within- (time; time × genotype) and between-subjects (genotype) effects, followed by post-hoc comparisons (Scheffe test). Results were considered significant when $p<0.05$. For BDNF level determination and body weight, a two-tailed *t*-Test was used.

Results

BDNF has been repeatedly implicated in the regulation of synaptic plasticity and cognitive functions (Bekinschtein et al., 2007b; Bramham and Messaoudi, 2005; Lu et al., 2007; Pang and Lu, 2004; Soule et al., 2006). In order to assess the effect of a sustained forebrain BDNF expression in learning and memory processing we took advantage of a mouse line in which an accelerated postnatal rise of BDNF mRNA level in the forebrain takes place (Huang et al., 1999). In this line, ectopic expression of proBDNF, the unprocessed form of this neurotrophin, is controlled by the αCaMKII promoter, which drives the transgene expression specifically to forebrain structures, including the

hippocampus, striatum, neocortex and amygdala (Mayford et al., 1996). Since expression in these forebrain structures may directly affect both behavioral performance and learning and memory we carried out a battery of tests in order to compare WT and transgenic animals at the locomotor and cognitive level.

Transgenic mice overexpress mature BDNF in most forebrain regions

In order to evaluate the BDNF protein levels in the forebrain of these mice, we dissected the following structures: striatum, hippocampus, frontal cortex, parietal cortex, occipital cortex and cerebellum and performed a BDNF specific quantitative ELISA. Cerebellum was analyzed as a control, since expression of αCaMKII driven BDNF transgene should exclusively be restricted to the forebrain. As we can see from Fig. 1A, transgenic mice showed higher BDNF immunoreactivity in all forebrain structures analyzed, as compared to WT littermates: we observed an increase of BDNF protein levels of 2.4 fold in the striatum (10.24±0.95 vs 4.18±0.32 ng/g wt tissue; $p<0.01$), 3.3 fold in the hippocampus (54.36±10.68 vs 16.67±1.66 ng/g wt tissue; $p<0.01$), 2.8 fold in the frontal cortex (71.30±15.71 vs 19.53±1.54 ng/g wt tissue; $p<0.05$), 1.8 fold in the parietal cortex (55.87±4.42 vs 31.00±2.44 ng/g wt tissue; $p<0.01$) and 2.0 fold in the occipital cortex (154.30±10.92 vs 78.67±12.46 ng/g wt tissue; $p<0.01$). However, BDNF protein levels in the cerebellum were undistinguishable between transgenic and WT mice (29.12±3.38 vs 29.41±2.03 ng/g wt tissue). Altogether, these results are in agreement with the reported data for BDNF mRNA levels in the transgenic line and confirm that our transgenic mice do overexpress BDNF proteins (Huang et al., 1999). However, this assay does not allow us to distinguish between proBDNF and mature BDNF, that are equally recognized by the antibody used in ELISA. Since the potential contrasting effects of proBDNF on mature BDNF, and considering the observed behavioral phenotype (see below), we felt important to measure the level of the precursor protein in the relevant brain structures for learning and memory. Western blot analysis on dissected brain areas, including the striatum, the hippocampus, the cortex and the cerebellum has been carried out with specific antibodies recognizing p32 proBDNF (Fig. 1B) (Barnes and Thomas, 2008). After quantification of the data, no obvious statistically significant changes were observed. Therefore, by comparing the proBDNF data with the total BDNF levels observed in the transgenic animals, we can confidently conclude that the large majority of BNDF overexpress is in the mature form and that, at least in basal conditions, the level of proBDNF is tightly regulated in the brain, as recently suggested (Matsumoto et al., 2008).

Measurement of body weight

It has been previously reported that BDNF can function as an anorectic factor, contributing to food intake and body weight regulation, likely by regulating hypothalamic function (Lyons et al., 1999; Kernie et al., 2000; Fox and Byerly, 2004). We therefore expected that in our BDNF overexpressing mouse line, the average body weight would be significantly reduced as compared with WT mice. In order to test this possibility, we weighted a considerable number of WT ($n=35$) and BDNF ($n=30$) littermate males of 9–14 weeks of age. As shown in Fig. 2A, the average body weight for WT mice is 27.47±0.76 g, while the value for BDNF mice is 25.74±0.58 g. Mean comparison, by the student's *t*-Test, indicates only a tendency, which was statistically non significant, for a reduction of body weight in the transgenics (*t*-Test, $p=0.075$). This result indicates

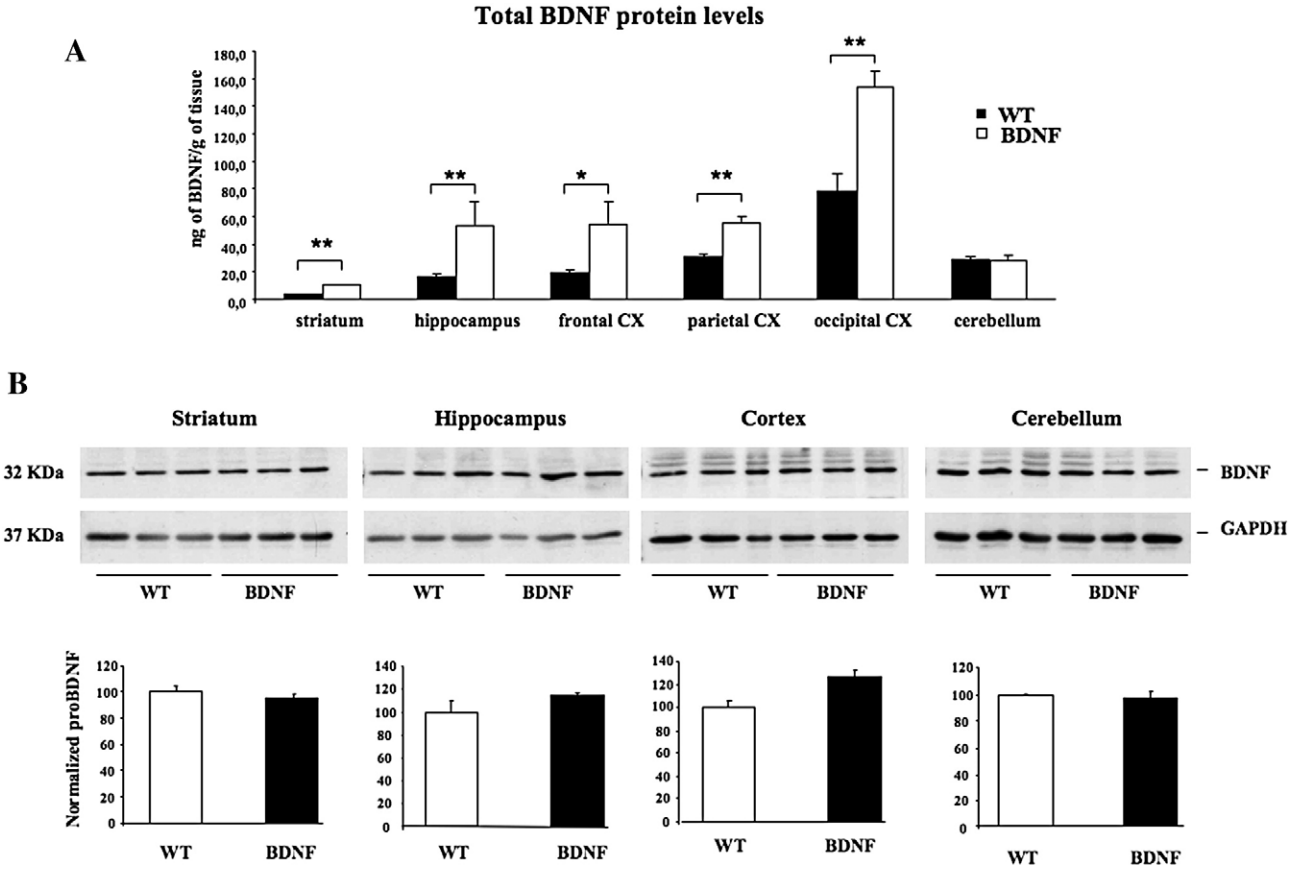

Fig. 1. Overexpression of BDNF in the transgenic mice. (A) Tissue levels of total BDNF protein, in ng of BDNF/g of wet tissue weight, in the striatum, hippocampus, frontal cortex, parietal cortex, occipital cortex and cerebellum of WT ($n=10$) and BDNF ($n=10$) mice, as analyzed by ELISA. *$p<0.05$, **$p<0.01$. (B) Protein extracts from the indicated brain areas (WT: $n=3$; BDNF: $n=3$) have been subjected to western blot analysis for p32 proBDNF and normalized for p37 GAPDH (upper panels). Quantification of data (mean±SEM) is indicated in the lower panels. Statistical analysis revealed no significant difference between genotypes for all structures analyzed.

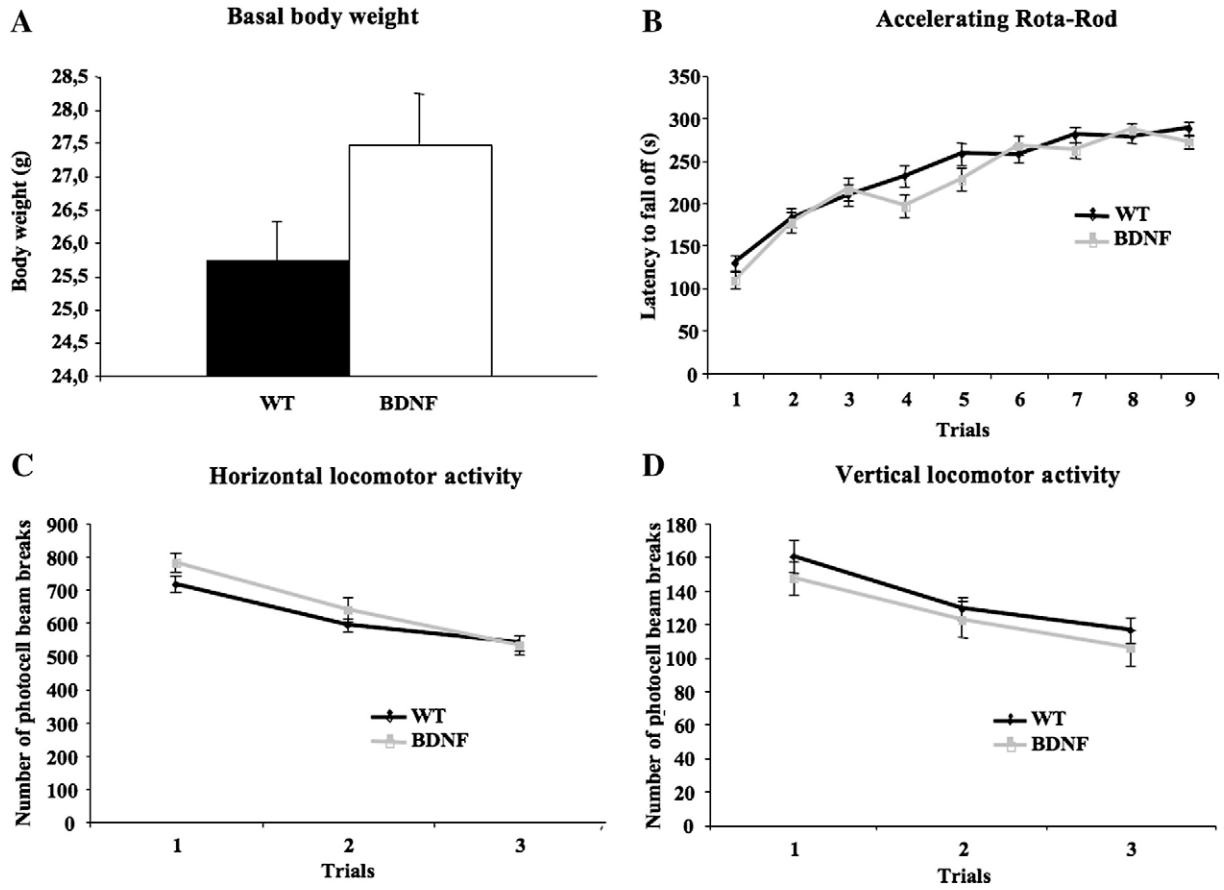

Fig. 2. (A) Basal Body weight in BDNF overexpressing mice. The mean body weight for WT mice ($n=35$) is 27.47±0.76 g and the mean body weight for BDNF mice ($n=30$) is 25.74±0.58 g. No statistically significant difference was found. (B) Analysis of motor performance of BDNF transgenics and WT mice. Latencies in falling off the Rota-Rod apparatus, during 3×3 daily sessions, for WT ($n=25$) and BDNF ($n=23$) mice. Statistical analysis shows a significant time effect ($p<0.001$, session 1 vs 9), but not a genotype or a genotype×time interaction. (C) Analysis of horizontal locomotor activity for WT ($n=30$) and BDNF ($n=26$) mice, measured by total number of photoelectric beam breaks in a 10 min daily session, during 3 consecutive days. Statistical analysis shows a significant time effect ($p<0.001$, day 1 vs 3), but not a genotype or a genotype×time interaction. (D) Analysis of vertical locomotor activity for WT ($n=30$) and BDNF ($n=26$) mice, measured by total number of photoelectric beam breaks in a 10 min daily session, during 3 consecutive days. Statistical analysis shows a significant time effect ($p<0.001$, day 1 vs 3), but not a genotype or a genotype×time interaction.

that a forebrain overexpression of BDNF may provide only a small contribution to an overall decreased body weight. Interestingly, it has also been reported that the conditional loss of BDNF in the forebrain, both in early or late adulthood, does not cause increase of body weight (Monteggia et al., 2004), as originally reported for global BDNF KO animals, further suggesting that chronic changes in BDNF expression restricted to the forebrain may not be sufficient to cause the previously reported anorectic effect.

Motor performance of transgenic mice

In order to evaluate possible performance deficits in our BDNF transgenic mice, we assayed motor coordination, balance and motor learning in the Rota-Rod task, and spontaneous locomotor activity, exploratory behavior and habituation to a novel environment in the activity boxes. Performance on the Rota-Rod test was measured by latencies in falling off the cylinder, during 3 daily trials over 3 consecutive days (Biffi et al., 2004). As shown in Fig. 2B, both genotypes (WT=25; BDNF=23) manifested a significant increase in the latency to fall, from trial 1 to trial 9 ($F_{8,368}=53.813$; $p<0.001$), demonstrating that both groups significantly improved motor coordination over time. Altogether, BDNF mice showed normal motor coordination, balance and motor learning performance since neither genotype effect ($F_{1,46}=0.001$; $p=0.993$) nor a genotype×learning interaction ($F_{8,368}=1.077$; $p=0.379$) was detected. Subsequently, horizontal and vertical activity was assayed for 3 consecutive days over a 10 min daily session (Mazzucchelli et al., 2002). As shown in the additional panels of Fig. 2, both genotypes (WT=30; BDNF=26) manifested similar performances for both horizontal (Fig. 2C) and vertical activity (Fig. 2D): no differences between genotypes for either horizontal ($F_{1,54}=1.368$; $p=0.247$) or vertical activity ($F_{1,54}=0.932$; $p=0.339$) nor genotype×time interaction for either horizontal ($F_{2,108}=1.723$; $p=0.183$) or vertical activity ($F_{2,108}=0.109$; $p=0.897$) were found. Altogether these data demonstrate that WT and BDNF mice bear no differences in spontaneous exploratory behavior. In addition, both genotypes show the expected significant reduction in horizontal ($F_{2,108}=57.027$; $p<0.001$) and vertical locomotion ($F_{2,108}=19.970$; $p<0.001$), comparing day 1 to day 3, demonstrating that all experimental groups produced a normal habituation to the test environment.

Assessment of short and long-term memory in the passive avoidance task

The strength of a memory trace has to be assessed through its retrieval, although relatively little research has focused on understanding the neurobiological processes underlying this function. To assess memory formation and retrieval we used the passive avoidance test, which consists in a single training trial (Brambilla et al., 1997; Mazzucchelli et al., 2002; Pittenger et al., 2006; Schutz and Izquierdo, 1979). We performed this test in two different versions and with the test trials performed at different time points. A Stepwise sequence of increasing footshocks from 0.02 mA to 0.50 mA

(0.02–0.05–0.10–0.20–0.50, 2.0 s shock duration) was used to determine footshock sensitivity. When each mouse showed flinching, jumping, or vocalization the sequence was terminated. In the subsequent experiments, we used the shock intensity of 0.10 mA, which was the minimal value showing a clear fear response in both experimental groups (data not shown).

In the first experiment, WT ($n=11$) and BDNF ($n=12$) mice were subjected to the passive avoidance test in which the size of the illuminated compartment was approximately twice as large as that of the dark compartment. The test trial was performed after 24 h in order to measure long-term memory formation. The same group of animals was also subjected to a recall trial after 10 days of rest. As shown in Fig. 3A, we found a significant learning effect ($F_{2,42}=29.751$; $p<0.001$; initial latencies: WT=21.55±3.38 and BDNF=20.33±3.68; 24 h recall latencies: WT=272.89±38.62 and BDNF=143.68±29.30; 10 days recall latencies: WT=243.4±42.11 and BDNF=87.58±37.01), together with a clear genotype effect ($F_{1,21}=9.571$; $p=0.006$) and a genotype×learning interaction ($F_{2,42}=5.272$; $p=0.009$). Multiple comparisons showed no differences between genotypes in the initial latency to enter the dark compartment ($p=0.810$) but significant differences between genotypes both at 24 h ($p=0.014$) and at 10 days ($p=0.011$) were observed, This result suggests that BDNF transgenic mice may present impairments in the formation of long-term memories, which cannot be recovered at least until 10 days from learning.

To confirm our findings in a different learning context, in the second experiment, WT ($n=22$) and BDNF ($n=18$) mice were subjected to the test, in which the white compartment has been reduced in size to match the black compartment. As before, the test trial was performed after 24 h in order to evaluate long-term memory. The same group of animals was also subjected to a recall trial after 10 days. Results obtained are shown in Fig. 3B. In addition to a significant global learning effect ($F_{2,76}=6.681$; $p=0.002$), both a genotype effect ($F_{1,38}=7.950$; $p=0.008$) and a learning×genotype interaction was also significant ($F_{2,76}=4.214$; $p=0.018$). Post hoc analysis showed no genotype difference in the initial latency ($p=0.133$), but testing at either 24 h ($p=0.029$) or at 10 days ($p=0.011$) revealed a clear memory impairment in the BDNF transgenics (initial latencies: WT=18.09±1.73 and BDNF=11.87±1.07; 24 h latencies: WT=69.79±16.72 and BDNF=21.07±3.02; 10 days latencies: WT=75.13±21.10 and BDNF=11.29±1.76). In conclusion, two distinct protocols of passive avoidance consistently showed that BDNF transgenic mice possibly manifest impairments in long-term memory formation, which are preserved up to 10 days from learning.

In order to determine whether this apparent deficit in long-term memory reflects an inability to acquire the memory or an inability to consolidate the trace, we performed a third experiment including a test trial after 1 h, to measure short-term memory. WT ($n=18$) and BDNF ($n=11$) mice were subjected to the passive avoidance test in a test environment with the white and black compartments of the same size. The same group of animals also underwent two recall trials at 24 h and 10 days. As shown in Fig. 3C, a significant learning effect for both genotypes was observed ($F_{3,81}=8.700$; $p<0.001$). In addition, both a significant genotype effect ($F_{1,27}=6.289$; $p=0.018$) and a learning×genotype interaction were found ($F_{3,81}=4.487$; $p=0.006$), confirming again a significant difference between BDNF overexpressing mice and their littermate WT controls (initial latencies: WT=12.38±1.70 and BDNF=14.21±2.07; 1 h latencies: WT=80.86±22.47 and BDNF=32.60±8.76; 24 h latencies: WT=91.36±24.72 and BDNF=28.58±8.76). In this specific case though, the difference seemed to lie essentially in the 10d consolidation process. In fact, a multiple comparison analysis indicated that no differences between genotypes exist in either the initial latency to enter the dark compartment ($p=0.544$), nor in the latency at 1 h ($p=0.117$) or 24 h ($p=0.064$), with only a tendency toward significance for the latter. Instead, the difference becomes highly significant after 10 days from training ($p=0.009$). These results seem to indicate that the deficit in long-term memory stabilization in the BDNF transgenics may be linked to a partial short-term deficit that is causing a reduction in the initial

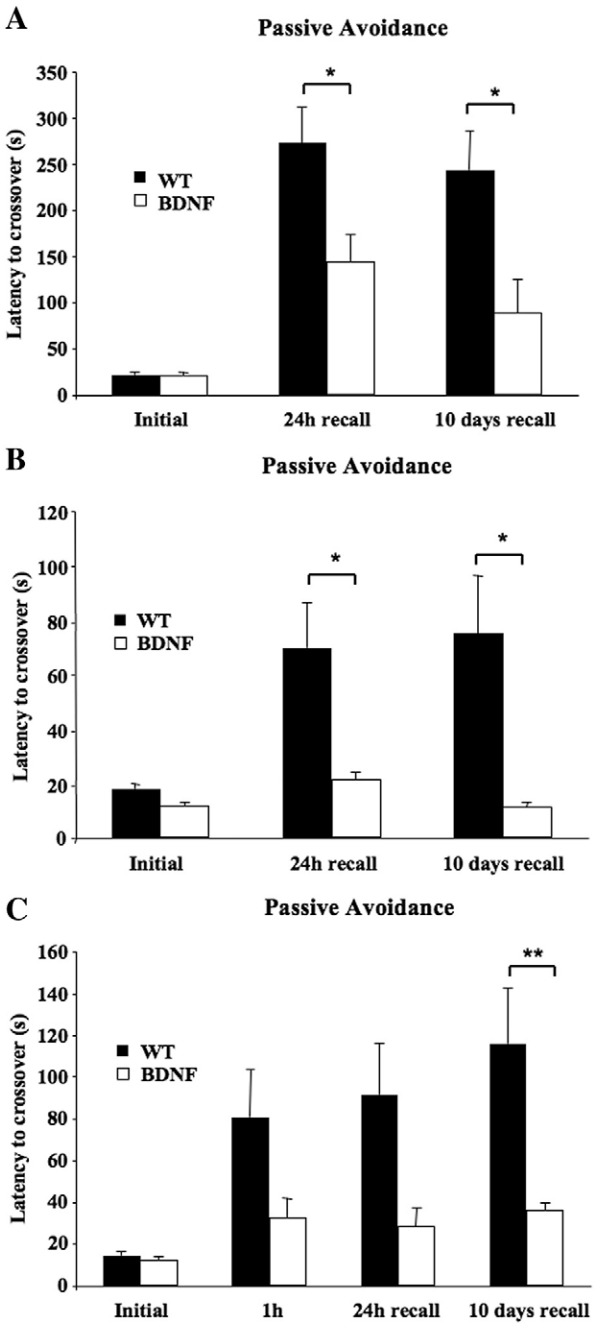

Fig. 3. Learning in passive avoidance reveals a deficit in short-term memory in the BDNF overexpressing mice. (A) Latency (in sec) to enter the dark compartment of the passive avoidance apparatus after delivery of a 0.10 mA electric foot shock. WT ($n=11$) and BDNF ($n=12$) mice were assessed in untrained conditions (Initial), 24 h after training (24 h recall) and in a recall trial performed after 10 days (10 days recall). White compartment was approximately double in size of the black compartment. (B) Latency (in sec) to enter the dark compartment of the passive avoidance apparatus after delivery of a 0.10 mA electric foot shock. WT ($n=22$) and BDNF ($n=18$) mice were assessed in untrained conditions (Initial), 24 h after training (24 h recall) and in a recall trial performed after 10 days (10 days recall). White compartment was approximately of the same size as the black compartment. (C) Latency (in sec) to enter the dark compartment of the passive avoidance apparatus after delivery of a 0.10 mA electric foot shock. WT ($n=18$) and BDNF ($n=11$) mice were assessed in untrained conditions (Initial), 1 h after training (1 h) and in a recall trial performed after 24 h (24 h recall) and 10 days (10 days recall). White compartment was approximately of the same size as the black compartment. *$p<0.05$, **$p<0.01$.

consolidation at 24 h and a more pronounced effect at 10 days. In conclusion, we demonstrated that acquisition of instrumental memory in passive avoidance is hampered in mice overexpressing BDNF in the forebrain. This impairment is likely to be a consequence of both short and long-term deficits in forming and stabilizing the memory trace.

Assessment of spatial memory in the Eight-arm Radial Maze task

In order to extend our initial findings and provide additional evidence for possible learning impairments in the BDNF over-expressing mice, we evaluated performance in the Eight-arm Radial Maze, a sensitive measure for spatial working memory (Brambilla et al., 1997; Olton et al., 1978). Training with one trial per day during 12 consecutive days was followed by a recall session 10 days after the last trial. Three parameters were measured for each trial: number of correct visits, number of errors and latency to complete the task.

For the analysis of the number of correct visits all mice were kept (WT=35, BDNF=30), since no outliers were identified. Fig. 4A shows that a significant increase in the number of correct visits for both genotypes over the test period ($F_{12,756}=17.974$; $p<0.001$) with a learning×genotype interaction close to significance ($F_{12,756}=1.740$; $p=0.054$). However, a significant genotype effect was clearly evident ($F_{1,63}=11.160$; $p=0.001$). Multiple comparisons indicate that the main differences lie at trials 5, 7, 9 ($p<0.05$), 8 and 12 ($p<0.01$), showing a retarded learning in the BDNF transgenics. Interestingly, these differences between genotype were no longer there at the 10 days recall time ($p=0.151$).

For the analysis of errors, 35 WT and 30 BDNF mice were also considered (Fig. 4B). A decrease in the number of errors performed for both genotypes was evident (learning effect: $F_{12,756}=22.287$; $p<0.001$). Moreover, and consistently with the number of correct visits, both a significant genotype effect ($F_{1,63}=3.867$; $p=0.045$) and a learning×genotype interaction were found ($F_{12,756}=2.189$; $p=0.011$). Post hoc comparisons indicate that main differences lie at trials 4, 5, 8 and 9 ($p<0.05$). No difference between genotype was seen at the 10 days recall time ($p=0.786$). Both parameters analysed are consistent and show that BDNF mice are able to learn the spatial position of the baited arms, but less efficiently than their WT counterparts. However, these differences seem significantly attenuated when the recall trial is performed after 10 days from the last training session, indicating that the long-term memory stabilization may not be severely affected in the BDNF transgenics.

Finally, after outlier removal, 33 WT and 29 BDNF mice were considered for the analysis of latency to complete the test, which finished when all the 8 arms were visited. This parameter is not a measure for spatial memory but rather for behavioral performance. Data in Fig. 4C indicate that this parameter was found indistinguishable between genotypes ($F_{1,60}=0.637$; $p=0.428$) and no significant learning×genotype interaction was found ($F_{12,720}=0.677$; $p=0.775$), despite latency to complete the task significantly decreased for both groups over the test ($F_{12,720}=57.357$; $p<0.001$). This final control confirms that performance *per se* was not altered in the transgenics, but only the spatial working memory mechanisms.

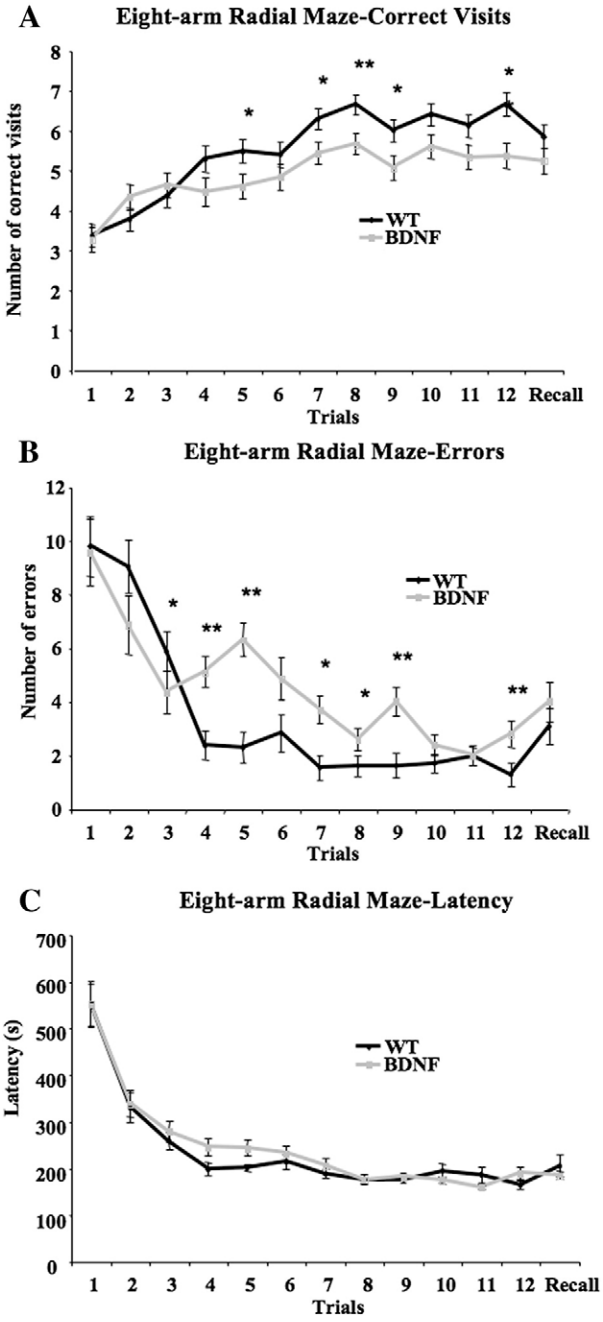

Fig. 4. BDNF overexpressing mice show a learning deficit in eight arm radial maze. Three Eight-arm Radial Maze parameters were evaluated, for 12 consecutive trials followed by the recall trial after 10 days, for WT and BDNF mice. (A) Number of correct visits for WT ($n=35$) and BDNF ($n=30$). Statistical analysis revealed a highly significant difference between genotypes over the test. (B) Total number of errors for WT ($n=35$) and BDNF ($n=30$). Statistical analysis revealed a highly significant difference between genotypes over the test. (C) Latency to finish the task for WT ($n=33$) and BDNF ($n=29$). Statistical analysis revealed no difference between genotypes over the test. *$p<0.05$, **$p<0.01$.

Assessment of spatial memory in the MWM task

Next, we wanted to test whether BDNF overexpression would affect formation of spatial reference memory in an independent test, the MWM (Brambilla et al., 1997; Morris et al., 1982). The results for WT ($n=15$) and BDNF ($n=15$) for the following parameters refer to the average of 2 daily trials, during 10 consecutive days: latency to find the platform, in sec (Fig. 5A), total distance swam, in cm (Fig. 5B), and mean velocity, in cm/s (Fig. 5C).

For the latency to reach the platform during acquisition, a significant learning effect for both genotypes ($F_{9,252}=25.495$; $p<0.001$) and a significant difference between genotypes ($F_{1,28}=4.289$; $p=0.048$) were found, but not a genotype×learning interaction ($F_{9,252}=1.522$; $p=0.140$). Multiple comparisons showed that the main differences resided on days 4, 5, 7 and 8 ($p<0.05$). These data indicate that BDNF expressing mice are generally slower in reaching the target platform in comparison to their WT controls.

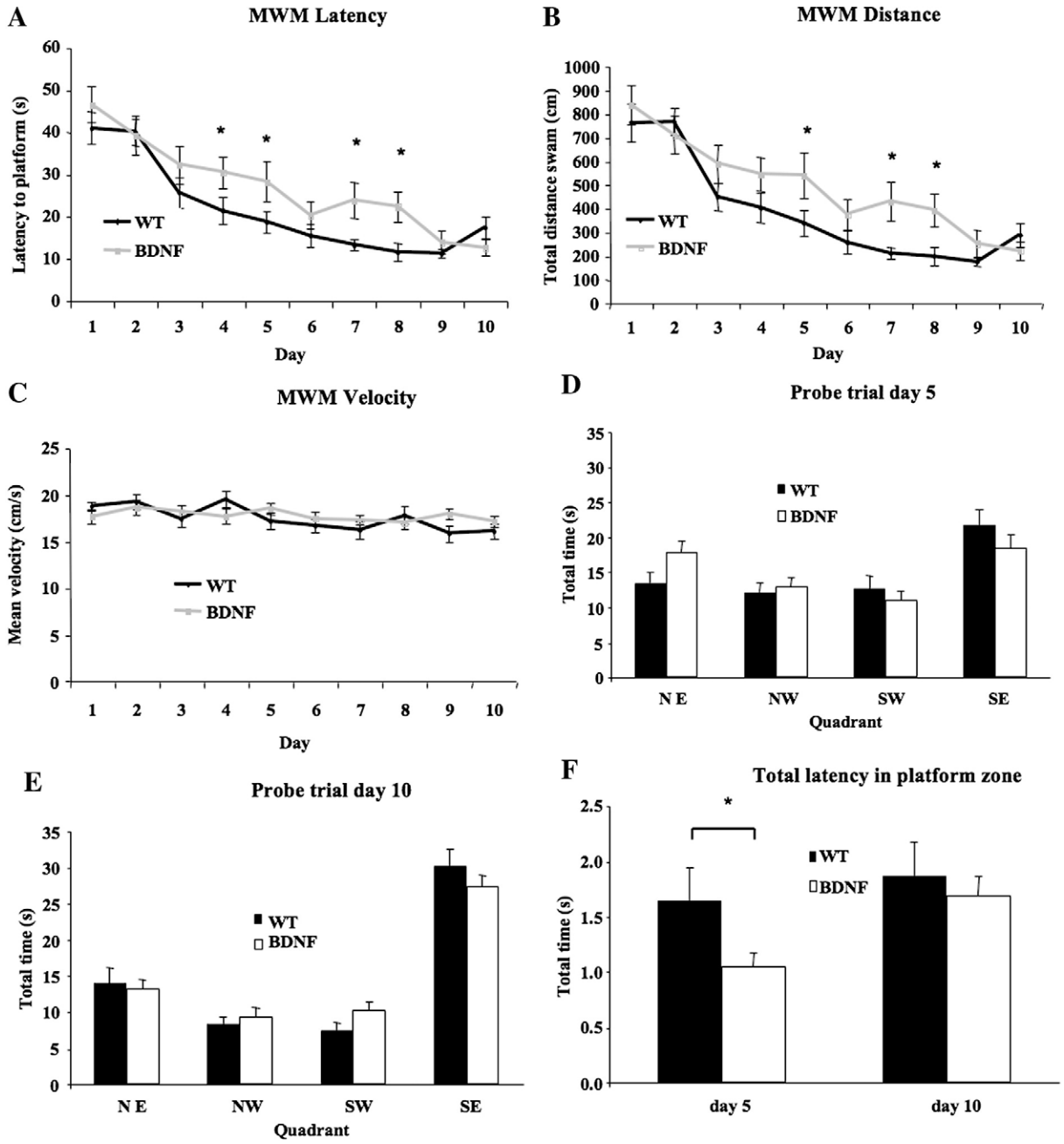

Fig. 5. Learning retardation of BDNF overexpressing mice in the Morris Water Maze. MWM parameters were measured over the 10 daily sessions, for WT ($n=15$) and BDNF ($n=15$) mice. (A) Latency, in sec, to reach the platform. Statistical analysis revealed an overall significant difference between genotypes ($p<0.05$). (B) Distance swam, in cm. Statistical analysis revealed an overall significant difference between genotypes ($p<0.05$). (C) Statistical analysis revealed no difference in the mean swim velocity between genotypes. *$p<0.05$. D–F) MWM parameters were measured for the probe trials at day 5 and day 10, for WT ($n=15$) and BDNF ($n=15$) mice. (D) Total time, in sec, spent in the quadrants Northeast (NE), Northwest (NW), Southwest (SW) and in the target quadrant Southeast (SE), for the probe trial at day 5. Statistical analysis revealed no difference between genotypes. (E) Total time, in sec, spent in the quadrants Northeast (NE), Northwest (NW), Southwest (SW) and in the target quadrant Southeast (SE), for the probe trial at day 10. Statistical analysis revealed no difference between genotypes. (F) Total time spent over the platform location, in sec. Statistical analysis revealed a significant difference between genotypes during the probe trial at day 5 ($p<0.05$). *$p<0.05$.

For the distance swam to the platform during acquisition, a significant learning effect for both genotypes ($F_{9,252}=23.007$; $p<0.001$) and a significant difference between genotypes ($F_{1,28}=5.735$; $p=0.024$) were detected, but not a learning×genotype interaction ($F_{9,252}=1.435$; $p=0.173$). Multiple comparisons showed revealed that the main differences were on days 5, 7 and 8 ($p<0.05$). Similarly to the previous parameter, these data indicate that BDNF mice had to swim a longer distance in order to reach the platform. In conclusion, both parameters demonstrate that BDNF overexpressing mice are capable of improving the ability to reach the platform position over the 10 daily sessions, since the both values for latency and distance significantly decreased through the test, but not as efficiently as WT mice. BDNF mice showed a significantly higher latency and higher distance swam to find the platform than WT mice, especially at sessions 7 and 8.

We also measured the mean swim velocity throughout the test, in order to exclude differences in navigation speed, which could account for the differences observed in the previous parameters. Importantly, we observed a significant decrease in the mean swim velocity through the test ($F_{9,252}=2.899$; $p=0.003$), but neither difference between

genotypes ($F_{1,28}$=0.240; p=0.628) nor a genotype×time interaction ($F_{9,252}$=1.843; p=0.061).

Latency and distance to reach the platform are only crude measures of performance in the water maze and by no means an accurate indicator for spatial memory deficits. Therefore, at day 5 and day 10, we performed a single probe trial in which the platform was removed, 30 min before the two daily trials of hidden platform. Total duration, in sec, spent in the quadrants Northeast (NE), Northwest (NW), Southwest (SW) and in the target quadrant Southeast (SE), for WT (n=15) and BDNF (n=15) mice, is shown in Fig. 5D, for the probe trial at day 5, and in Fig. 5E, for the probe trial at day 10. In order to simplify the statistical analysis of the data, we initially performed a one-way ANOVA with a Scheffe post hoc for multiple comparisons, considering the values for all four quadrants as independent (4 quadrants×2 genotypes×2 probe trials). We determined that the 3 non-target quadrants were statistically indistinguishable for each probe trial and therefore they were pooled for subsequent analysis (data not shown). We then performed a three-way ANOVA, considering 2 genotypes×2 quadrants×2 days (days 5 and 10), as a measure of the general tendency of the probe trials.

Learning effect was seen ($F_{1,28}$=26.396, p<.0001) as well as the quadrant effect ($F_{1,28}$=98.526, p<.0001) and the learning×quadrant interaction ($F_{1,28}$=13.453, p<.001). However, when the genotype was considered, we found a non significant effect ($F_{1,28}$=4.049; p=0.054) with only a tendency for BDNF mice to spend less time in the target quadrant than WT mice. This trend was likely due to a small difference during the first probe trial at day 5. Indeed, comparing data at day 5 only we found that both genotypes showed a significant difference between the time spent in the target quadrant versus non target quadrants ($F_{1,28}$=15.137; p=0.001) and a close to significant difference between genotypes ($F_{1,28}$=3.359; p=0.078) (Fig. 5D). At the probe trial at day 10 instead, while both genotypes showed significant differences between the time spent in the target/non target quadrants ($F_{1,28}$=94.325; p<0.001), no difference between genotypes ($F_{1,28}$=1.089; p=0.306) were found (Fig. 5E).

The previous analysis only showed a non significant trend for a retarded acquisition of the BDNF transgenics. In order to further evaluate the possibility that BDNF expressing mice might show subtle changes in their ability to locate the platform, we analyzed the total time spent on the platform zone, for both genotypes and for both probe trials (Fig. 5F). This parameter is a more sensitive measure of spatial memory, which detects mild impairments in the ability of mice to precisely locate the original platform location. A significant learning effect from probe trial at day 5 to probe trial at day 10 was observed ($F_{1,28}$=5.429; p=0.027), indicating that by day 10 both animal groups increased their precision in locating the platform position in comparison to day 5. Importantly, a significant genotype effect was observed ($F_{1,28}$=7.159; p=0.012) and a post hoc comparison revealed that the significant difference between genotypes reside on the probe trial at day 5 (p=0.014) but not at day 10 (p=0.427).

Altogether, these results show that BDNF transgenics are able to learn the task but manifest mild spatial memory impairments in comparison to WT littermates. BDNF mice seem to have a significant retarded acquisition in the MWM test, even if in the probe trials performed this impairment was found minimal. Nevertheless, the results presented here are consistent with those obtained in the Eight-arm Radial Maze test, by showing some forms of memory impairment. Importantly, since the Eight-arm Radial Maze results suggested a deficit in working memory, it is possible that the retarded spatial learning in the MWM may be also linked to such alterations, rather than a true deficit in long-term spatial memory.

Discussion

In this study we demonstrated that a moderate overexpression of BDNF in the forebrain results in modest but clear learning impairments in both instrumental and spatial memory tasks. These results are somewhat surprising considering the well established role of mature BDNF in promoting synaptic plasticity and memory formation. However, these data are in agreement with early observations in which BDNF was mildly overexpressed (roughly 30%) in most body tissues under the human β-actin promoter (Croll et al., 1999). In that transgenic line, memory retention in passive avoidance was found impaired in younger animals (6–8 week old) but not in older ones (6–8 month old), suggesting only transitory deficits. Importantly, a significant cognitive impairment was seen only in mutants carrying two copies of the transgene. Other non cognitive behavioral parameters were found to be normal. Although in our study we only used 2–3 month old mice, our comprehensive cognitive analysis clearly support the idea that a significant overexpression restricted to the forebrain may be sufficient to cause similarly mild deficits in learning. Importantly, our detailed analysis in passive avoidance indicates a deficit in the acquisition of short-term memory, rather than a true deficit in memory consolidation. To support their findings, Croll et al., (1999) were able to measure several electrophysiological parameters in the dorsal hippocampus, the dendate gyrus and in the entorhinal cortex, three brain structures implicated in both spatial memory and learning in the passive avoidance. Altogether, those results suggest a general membrane hyperexcitability in comparison to controls but no clear changes in either short-term or long-term plasticity, at least at the Schaffer collateral synapse. At present, we cannot provide direct evidence that synaptic transmission or plasticity is altered in our transgenic mice but certainly our behavioral data are consistent with subtle changes at this level.

What may be the cellular basis for such detrimental effect of BDNF overexpression on learning? In a recent publication it has been shown that the same BDNF line we used manifests anxiety-like behavior with a concomitant increase in spine density in the basolateral amygdala. In contrast, upon chronic stress, such overexpression appears to prevent atrophy in the hippocampus a cellular phenotype likely linked to the antidepressant effect observed in the forced swim test (Govindarajan et al., 2006). Considering these recent findings, the explanation of the observed learning deficits is likely to be a complex one, possibly involving also motivational aspects of behavior. At the molecular level, one interesting possibility is that in our transgenic animals the ratio between pro and mature BDNF may be in favor of the former, thus leading to an overall hyperactivation of the low affinity p75 receptors and a relatively lower engagement of the high affinity TrkB receptors. This possibility is particularly exciting since an abnormal activation of p75 by proBDNF could lead to synaptic alterations that may be detrimental to learning. Indeed, as recently shown, p75 receptor is involved in long-term depression (LTD) in the hippocampus and proBDNF is able to modulate LTD via p75 activation (Lu et al., 2005; Woo et al., 2005; Zagrebelsky et al., 2005; Rosch et al., 2005). Unfortunately, this possibility seems to be quite unlikely based on our results. In fact, we found, in most brain structures analyzed, very significant increases in total BDNF levels (e.g. approximately 300% in the hippocampus) without significant changes in the proBDNF protein (only 15% increase in the same structure). Therefore, the most conservative conclusion is that the large majority of BDNF produced by the transgene is in the mature form.

The hypothesis we tend to favor is instead that an excess of mature BDNF may preferentially act on inhibitory interneurons, thus leading to a general alteration of the inhibitory tone of the synaptic circuitry underlying learning. Indeed, TrkB receptors have been found in a number of interneurons in the forebrain and a number of functions have been ascribed to BNDF in non pyramidal cells, including brain diseases such as schizophrenia and epilepsy. The increased excitability observed by Croll et al (Croll et al., 1999) may suggest that a chronic BDNF expression could lead to a reduced inhibition by attenuating IPSCs, as observed in a number of interneuron types upon acute BDNF

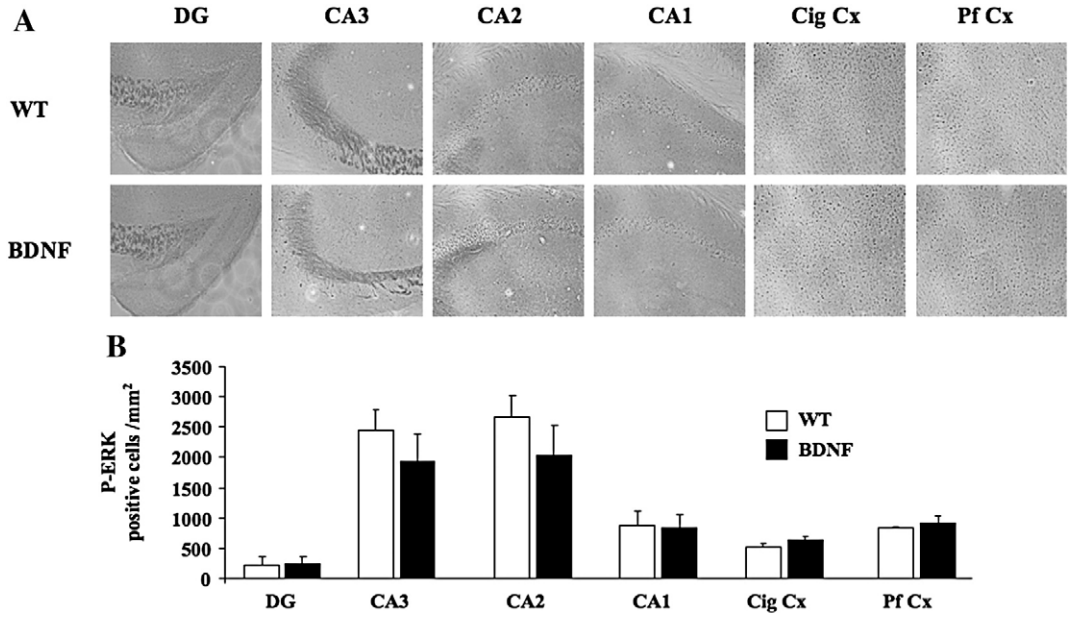

Fig. 6. Analysis of phospho-ERK induction in BDNF overexpressing mice. (A) WT ($n=5$) and BDNF ($n=5$) mice have been perfused and serial brain slices have been subjected to IHC analysis with antibodies against the phosphorylated, activated form of ERK1/2 (P-ERK). Total number/mm^2 of P-ERK positive puncta have been counted in the indicated structures (DG: dentate gyrus; CA3, CA2, CA1: dorsal hippocampus; Cig Cx: cingulated cortex; Pf Cx: prefrontal cortex). (B) Quantification of results (mean±SEM) is indicated. No significant difference between genotypes was found.

administration (for review see Woo and Lu, (2006)). In addition, as a non mutually exclusive possibility, an abnormal increase at specific excitatory synapses that in turn inhibits the main hippocampal circuitry cannot be in principle excluded. We have unsuccessfully attempted to investigate this issue by monitoring TrkB dependent signaling in the brain of the BDNF transgenics. In order to do that we measured, by immunohistochemical (IHC) techniques, the phosphorylated levels of the fully activated forms of the Extracellular-signal Regulated Kinases (ERK1/2), as a main signaling pathway readout downstream to neurotrophin receptors. However, by quantitatively comparing p-ERK levels in the wild-type and transgenic mice in different brain areas, we only observed minor and non significant changes in p-ERK levels in the transgenics (Fig. 6) This result *per se* does not exclude that some changes occur in the cell signaling properties in key brain areas for cognitive processing but actually suggests that the changes may occur in specific subsets of cells, such as a fraction of interneurons part of the hippocampal circuitry, which cannot be detected with IHC methods but rather require single cell electrophysiological analysis, an approach which goes beyond the purposes of the present work.

Altogether, with this study we believe to have demonstrated an unexpected complexity in the BDNF action in the CNS and we propose that transgenic mice overexpressing this neurotrophin may be useful animal models to study cognitive decline associated to either neurodegenerative or psychiatric diseases. Indeed, the best use of our transgenics will be in combination with either genetic or pharmacological model of a disease for which BDNF administration has been proposed to be of therapeutic value, in order to study long-term, chronic effects of this neurotrophin on the disease onset and progression.

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