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Brain Research 1028 (2004) 92–104

BRAIN RESEARCH

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Research report

Altered expression of BDNF and its high-affinity receptor TrkB in response to complex motor learning and moderate exercise

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Accepted 6 September 2004
Available online 5 October 2004

Abstract

We report that rats learning complex motor skills or exercising moderately show changes in expression of brain-derived neurotrophic factor (BDNF) and its receptor, TrkB protein, in cerebellum and motor cortex. It is now known that physical activity increases expression of some neurotrophins. We examined the time course of BDNF and TrkB expression after 1, 3, 5, 7 or 14 days in one of three conditions: (1) an "acrobatic" motor skill learning condition (AC), (2) a motor activity condition (moderately paced running on a flat track; MC) and (3) an inactive social-only control (SC) that served as a baseline group. Expression levels of BDNF and TrkB were evaluated by measuring relative optical density of the immunocytochemical reaction product. In cerebellar molecular layer, expression of BDNF correlated significantly with time spent in AC and MC over the first 7 days of training and remained elevated after 14 days of AC but not of MC. Changes in TrkB protein expression in cerebellar molecular layer mirrored those for BDNF during the first 7 days of training, but subsequently its expression subsided to the control level. In motor cortex, a significant increase in BDNF and TrkB protein expression was detected in the upper layers after 14 days in AC. Increased expression of BDNF, but not of TrkB, was observed in upper motor cortical layers after 14 days of MC. These data indicate that complex motor learning and moderate physical activity with little learning produce different effects on the expression pattern of BDNF and its receptor and may have implications for neural plasticity arising from such experiences.

Theme: Neural basis of behavior
Topic: Neural plasticity

Keywords: Cerebellum; Motor cortex; Neurotrophin; Plasticity; Immunocytochemistry; Rat

1. Introduction

Neurotrophins, a class of small growth factor proteins, are involved in neuronal survival, growth, differentiation and axon/dendrite extension and branching during development (for review, see Refs. [4,6,28,50,53,58]). The neurotrophin family includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3), and neurotrophin 4/5 (NT4/5) [4,7]. Neurotrophins bind with two types of receptors: the tropomyosine receptor kinase (Trk) receptors (TrkA, TrkB, TrkC) and the neurotrophin receptor p75 [4,14,18,64,75]. There is growing evidence that in adulthood neurotrophins are essential for maintenance of CNS neuron processes [23] and for plasticity of neuronal connections, specifically for experience-dependent modification of synapses and dendrites and for LTP [33,49,85].

That BDNF plays an important role during development is demonstrated by the fact that the survival rate of

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doi:10.1016/j.brainres.2004.09.003

BDNF$^{-/-}$ knockout mice is low, and those animals that do survive do not live longer than 3 weeks and have severe deficiencies in coordination and balance (including ataxic gate) [16,22,38]. The BDNF$^{-/-}$ animals have excessive sensory ganglia degeneration [22,38], extensive loss of cerebellar granule cells and stunted growth of Purkinje neurons [74]. In the adult CNS, a significant number of studies have demonstrated widespread expression of BDNF mRNA and protein, as well as the TrkB receptor [15,25,41,68,86,87]. BDNF is the most abundant neurotrophin with the highest levels of expression in cerebral cortex, hippocampus, thalamus, hypothalamus and cerebellum [15,24,68,87]. TrkB receptor protein is also widely expressed in adult brain: in cerebral cortex, hippocampal CA and dentate gyrus, and cerebellar Purkinje and granule neurons [9,27,51,52,86].

Previous studies have demonstrated that physical activity (running) and learning (water maze spatial task learning, radial arm training, contextual learning) increased the expression of BDNF mRNA and protein in task-relevant areas (hippocampus, cortex, cerebellum, spinal cord) [30–32,37,42,59,60,63]. Interruption of the running activity led to a significant and specific decrease of BDNF and TrkB mRNA expression (no changes in expression of other neurotrophins and their receptors were observed) [84]. After a year in a complex environment, animals had significantly elevated levels of BDNF protein in hippocampus, cortex and hindbrain (including cerebellum and pons) compared to cage housed controls [34,67,81]. The same activities are known to increase morphological plasticity in the brain: synaptogenesis and dendritic outgrowth occur as a result of environmental complexity and learning [5,39,43,44,46,82], while angiogenesis can be increased by physical exercise [5,35].

The possibility that exercise and learning may regulate BDNF mRNA expression in different ways has been addressed by Kesslak et al. [42] in a hippocampus-dependent task, the Morris water maze. In that study, animals that learned the task had significantly higher levels of BDNF mRNA in the hippocampal formation 3 days after beginning training sessions, while yoked control animals that swam without a platform present did not. The authors commented that the stress response in the yoked control animals could be a significant factor preventing an increase in BDNF mRNA expression.

The present study examined the relative roles of brain activity driven by moderate physical exercise and by learning in the regulation of BDNF and TrkB protein expression in the cerebellum and motor cortex. Previous work has shown that motor skill learning triggers synaptogenesis in motor cortex and cerebellum [5,43,44], while exercise triggers angiogenesis without significant synaptogenesis in the cerebellar cortex [5]. In a follow-up report from that study, adrenal weight was recorded and there were no differences across treatment groups, and no indication of ulceration in examination of each animal's stomach lining, so stress responses seem unlikely to have contributed to the synaptic or vascular differences [35]. In the present study, we addressed the question of a possible association between the pattern of expression of neurotrophins and their receptors and the previously described patterns of learning-induced or exercise-induced brain plasticity.

2. Experimental procedures

There were three parts to this study: in the first, we evaluated the effect of 14 days of voluntary exercise or acrobat training on BDNF and TrkB protein concentration in cortex and cerebellum. In the second, we examined the time course of BDNF and TrkB expression in motor cortex and cerebellum after 1, 3, 5 or 7 days of either complex motor task learning or moderate exercise. The effect of 2 weeks of learning or exercise on expression of BDNF and TrkB protein was studied in the third part. All animal treatments and procedures were approved by the Institutional Animal Care and Use Committee at the University of Illinois at Urbana-Champaign. For all three studies at postnatal day, 30 littermates were randomly assigned to one of the three experimental conditions (described below) and were housed three per cage (one from each of the experimental conditions).

2.1. Behavioral methods for the time course study of BDNF and TrkB expression

Fifty Long–Evans female rats (30 days old) were housed in standard cages, three per cage with food and water ad libitum and a 12:12 h light/dark cycle. Rats were handled daily for a week before the start of training and on day 30 were assigned to one of three conditions: an acrobatic condition (AC) where rats had to learn to traverse an elevated obstacle course consisting of chains and rope ladders, narrow bridges and an elastic cord, pathways with unstable obstacles etc. (for detailed description of the obstacle course, see Ref. [48]); a motor condition (MC) where they were run on a 6-m-long flat oval elevated track with a transparent Plexiglas floor and solid non-transparent walls for the same amount of time that the AC littermate rats spent on the obstacle course; and an inactive social cage baseline condition (SC) where they were handled daily in order to control for the handling of the animals in the AC and MC groups. AC and MC rats were given three consecutive trials per day without a significant interval between trials and the length of the training period was either 1, 3, 5, or 7 days (described in detail in Ref. [48]). Animals received equal amount of prodding during acrobat training or running on the track. Time to complete the task and number of errors (an error was defined as an obvious slip, foot fault or other apparent loss of locomotor control) were recorded for AC animals. Distance (number of track laps) was recorded for MC animals. Two durations were chosen for the SC group—1 and 7 days.

2.2. Behavioral methods for the study of the effect of 2 weeks of leaning/activity on BDNF and TrkB expression

As levels of BDNF and TrkB expression had not returned to baseline after 1 week, a second experiment was run using a 2-week duration of learning and exercise. Long–Evans rats (12 female rats, about 35 days old) were divided into three groups and housed three per cage (one from each condition): acrobatic condition (AC), motor condition (MC), and social cage condition (SC) as above. The groups were trained for 14 days continually in the manner described above (the duration of training and the number of trials per day were previously demonstrated to be sufficient to produce morphological changes in both motor cortex and cerebellum, see Refs. [43,44]). AC and MC rats were trained three times consecutively per day, and the time that the AC rats took to complete the course was measured as well as the number of slips or falls they made during traversal of the course. MC rats ran for the amount of time AC rats took to complete the acrobatic course, and the number of laps they completed during that time was recorded.

2.3. Behavioral methods for the protein concentration analysis after 2 weeks of leaning/activity on BDNF and TrkB expression

Long–Evans rats (15 female rats, about 35 days old) were divided into three groups (five animals in each group): acrobatic condition (AC), motor condition (MC), and social cage condition (SC) as above. The groups were trained for 14 days continually in the manner described above. After completion of training, animals were sacrificed by rapid decapitation and cerebral hemispheres and cerebelli were quickly dissected for further protein analysis (see Western blot analysis section).

2.4. Western blot analysis

The frontal half of the cerebral cortical hemispheres (containing motor cortex) and entire cerebellar hemispheres (excluding the vermis) from 15 rats, described in the preceding paragraph, were individually homogenized in 1 ml of 50 mM Tris buffer (pH 7.2, 4 °C) containing 20 mM EDTA, 0.1 mM sodium orthovanadate, 20 μg/ml of aprotinin, 10 μg/ml of leupeptin, and 0.1 mg/ml of phenylmethylsulfonyl fluoride. Protein concentrations were determined using the BCA protein assay (Pierce, Rockford, IL). Homogenates were mixed in proportion 3:2 with sample buffer containing 4% SDS, 250 mM Tris, 3 mM EDTA, 20% glycerol, 5% β-mercaptoethanol and 0.05% bromophenol blue, pH 8.0, and boiled for 5 min. For each condition, 25 μg of protein was loaded into each well on polyacrylamide gel (8% SDS-polyacrylamide gel was used for TrkB and 12% gel—for BDNF protein determination). After separation by electrophoresis, protein samples were electrotransferred onto nitrocellulose in a transfer buffer (25 mM Tris/192 mM glycine/0.02% SDS/ 20% methanol) for 90 min at a constant current (200 mA). Additional protein binding sites on the nitrocellulose were saturated by incubation in 10 mM Tris-buffered saline with 0.1% Tween-20 (TBST; pH 7.4) containing 5% dry milk powder for 1 h at room temperature. After a short wash with TBST, blots were incubated with either BDNF (1:2000, polyclonal rabbit IgG, Santa Cruz Biotechnology, Santa Cruz, CA) or TrkB (1:3000, polyclonal goat IgG, Chemicon International) antiserum overnight at 4 °C. After rinsing three times with buffer, membranes were incubated with peroxidase-conjugated secondary antibody (1:2000 dilution, antirabbit HRP for BDNF and antigoat HRP for TrkB, Vector Laboratories) during 1 h at room temperature. Bands were developed on autoradiographic film by chemiluminescence using an ECL kit (Amersham, Arlington Heights, IL). The film signals were scanned using a high-resolution scanner and the relative optical density (ROD) of scanned images was measured using AIS 3.0 software (Imaging Research, Ontario, Canada). A ratio of BDNF and TrkB expression in experimental condition (MC or AC) to inactive cage control was determined and these values compared.

2.5. BDNF and TrkB immunocytochemistry

Two hours after completion of training, rats were anesthetized with sodium pentobarbital (100 mg/kg) and perfused through the aorta with 4% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.2). After perfusion, the brains were carefully removed from the skull and placed in buffered sucrose solutions of increasing (10–30%) concentration until they sank. The cerebellum was sectioned on a Reichert cryostat microtome at 50 μm (parasagittal sections) and motor cortex was sectioned coronally at 50 μm. Both areas were sectioned serially and all sections were collected and stored in a refrigerator in cryoprotectant solution containing ethylene glycol at −10 °C until needed.

Evenly spaced cerebellar sections containing PML (every third section, seven sections total) and sections containing M1 and M2 motor areas of cerebral cortex (every sixth section, 11 sections total) (according to the Paxinos rat brain atlas [66]) were selected and washed in several changes of PB. Non-specific binding was blocked with 5% normal goat serum, 2% bovine serum albumin (BSA) and 0.2% Triton X-100 in 0.1 M PB (pH 7.3) for an hour. After this, the free-floating sections were incubated with primary antibodies against BDNF (polyclonal rabbit IgG, recognizes both longer proBDNF and shorter mature BDNF protein, 1:2000, Santa Cruz Biotechnology) and TrkB (raised against synthetic peptide–amino acid residues at C terminus, does not recognize truncated TrkB receptor—full length only, polyclonal rabbit IgG, 1:2000, Santa Cruz Biotechnology) overnight at 4 °C. The primary antibodies have been previously shown to label BDNF [13,23,88] and TrkB [86] specifically. To verify the specificity of labeling with primary antibodies, “no primary” controls were included in

each run (seventh section in the cerebellum series and 11th section in the motor cortex series). None of the other incubation steps were omitted nor altered. There was no labeling of any structures in these sections.

Next day, the sections were washed in washing solution containing 1.5% normal goat serum in 0.1 M PB. The sections were then placed in secondary goat antirabbit IgG (1:1000, Vector) for 1 h and then washed with washing solution. The sections were incubated for 1 h at room temperature in avidin–biotin–peroxidase complex (ABC, Vector) to amplify the signal. The ABC was washed off with two changes of 0.1 M PB followed by 0.05 M Tris buffer solution. Localization of binding sites was visualized by a histochemical reaction of the peroxidase with 0.003% hydrogen peroxide in the presence of 0.05% 3,3′ -diaminobenzidine (DAB) in Tris buffer. After washing with phosphate buffer, the sections were mounted on gelatin-coated slides. After drying, slides with sections were dehydrated and coverslipped.

2.6. Image analysis

The level of BDNF and TrkB protein expression within different layers of the cerebellum (the molecular layer, granule cell layer, dentate nucleus) and of the motor cortex (Layers I–III and IV–VI) was evaluated by densitometry. In order to minimize the effect of variability in the immunostaining procedure, the brain sections from all experimental conditions were processed simultaneously. All the slides were coded prior to densitometry, such that the experimenter was not aware of the experimental condition of individual animals. First, the sections were scanned using a Umax Powerlook3000 Flatbed Scanner via Adobe Photoshop 5.5. Next, the relative optical density (ROD) of scanned images was measured using AIS 3.0 software (Imaging Research). To collect the data, a probe of constant area (circle with 50 μm diameter) applied in an unbiased manner equidistantly within the layer of interest in cerebellum or motor cortex (stratified sampling). Five to seven measurements of each cerebellar layer and six to eight measurements of each cortical layer were taken, and 6–10 sections were used for each animal (Fig. 1). During data analysis, the readings from each layer in each section were averaged first, then normalized using the white matter readings for that particular section. Normalized data was averaged within areas of interest (e.g., upper motor cortex, molecular layer of PML) across sections for each animal.

2.7. Statistical analysis

The relative optical density readings from different layers were normalized with white matter readings and the data was further transformed as percent of change from the baseline (SC) level. The data from the two SC durations–1 and 7 days–were pooled together because there was no difference in BDNF and TrkB expression on those two days.

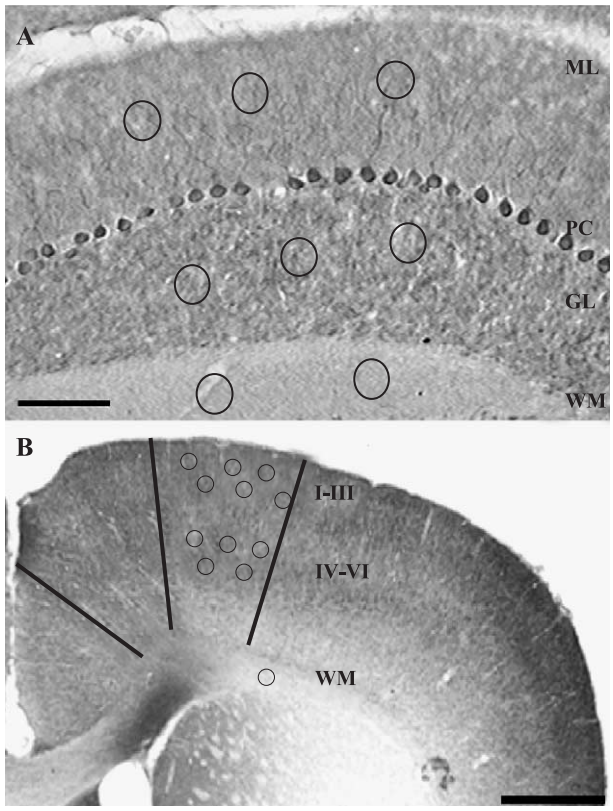

Fig. 1. Low-power views of BDNF immunoreactivity in paramedian lobule of cerebellum (A) and in motor cortex (B). Circles depict the sampling areas for densitometric measurements. Molecular layer (ML), Purkinje cell layer (PC), granule cell layer (GL), white matter (WM). Scale bar=100 μm in panel A and 800 μm in panel B.

Statistical analysis was run for each brain area and antibody to verify it; for example, BDNF expression in cerebellar molecular layer was not significantly different in SC1 and SC7 animals ($F_{1,8}$=6.23, p=0.64). Data was assessed by two-way ANOVA (TRAINING×DURATION) for the time-course study followed by Tukey's post hoc test for comparison of acrobat training vs. motor exercise at all time points. In addition, linear regression analysis was used to evaluate the relation between training DURATION and level of BDNF/TrkB expression across the first 7 days of training. One-way ANOVA followed by Tukey's HSD test was performed to compare the effect of 14 days of acrobat or motor exercise training with SC animals. The SPSS (SPSS, Chicago, IL) statistical package was used for analysis. The level of significance was set at $p<0.05$. All data are reported as mean±S.E.M.

3. Results

3.1. Behavioral measures

AC animals significantly improved their performance on the acrobat course by the 5th day of training as measured by the mean daily latency to complete the task (Fig. 2A) and by

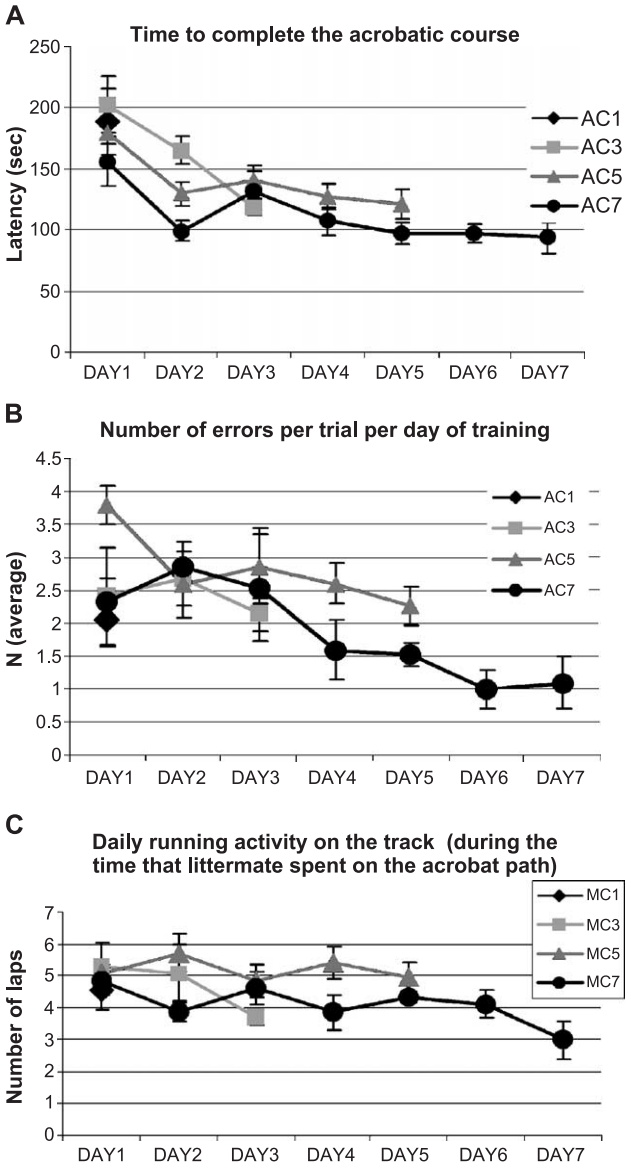

Fig. 2. (A) Performance on the acrobat complex motor task was assessed by the average latency for each rat to complete the task on each of the test days, and then the data were averaged per group (N=6 rats for each group). (B) Number of errors (slips, foot faults) per trial per day of training. (C) Average daily running activity for MC rats. MC rats spent exactly the amount of time on the flat track that was required by its AC littermate to complete the acrobat course. The length of one lap was equal to the length of the acrobat course. Each point on the graphs represents mean±S.E.M.

the number of errors per trial (Fig. 2B). Daily running activity (distance traveled) of the MC littermates during the training was three- to sixfold higher than that of the AC rats (a lap on the track equaled the length of the acrobat course) (Fig. 2C).

3.2. Western blot analysis of BDNF and TrkB protein levels in cortex and cerebellum

We used protein samples from the frontal half of the cortex and from cerebellum to evaluate changes in BDNF and TrkB expression in Western blot analyses after exercise and motor learning. Antibodies against BDNF recognized both precursor BDNF (band at about 30 kDa) and mature BDNF. For densitometry, only 14 kDa bands corresponding to mature BDNF were used. Antibodies against TrkB consistently recognized a single band on the blot at about 140 kDa. Results of densitometry suggested that BDNF protein in motor cortex (but not in cerebellum—data not shown) was elevated in comparison with control after 14 days of acrobat learning or exercise ($F_{2,13}$=2.31, p<0.05) (Fig. 3A). Levels of TrkB protein were not significantly different in either motor cortex (Fig. 3B) or cerebellum (data not shown) after the same conditions. We concluded that (1) the changes in BDNF and/or TrkB protein expression might have occurred earlier (within the first week of exercise) and be transient; and that (2) changes could be area- or layer-specific and could have been too subtle for Western blot detection. Thus, we used a semiquantitative immunocytochemical approach to further study the effects of complex motor skill learning and exercise on BDNF and TrkB expression.

3.3. Cerebellum: time course of the effect of learning and motor activity on BDNF expression measured immunocytochemically

3.3.1. Molecular layer

An ANOVA on TRAINING×DURATION revealed a significant effect of only the DURATION of learning or exercise and not of the type of training on BDNF expression with no interaction ($F_{3,36}$=4.842, p=0.008) during the first 7 days. Post hoc analysis using Tukey's test further indicated that both complex motor learning and exercise increased the level of BDNF expression in the molecular layer of cerebellar PML after 5 and 7 days for MC and 7 days for AC (Fig. 4). Separate linear regression analysis of the differences between the AC or MC values and the SC baseline values (repeated at each time point) revealed significant overall increases in BDNF expression in the PML molecular layer following both motor skill training and physical exercise (AC: $F_{1,21}$=24.21, p<0.001; MC: $F_{1,21}$=14.05, p<0.01). BDNF expression levels in cerebellar molecular layer tended to increase across days of both AC and MC. Hence, both learning and exercise increased BDNF expression over the first week. The second experiment, using a two-week duration of exercise and learning, revealed that a significant effect of learning was maintained ($F_{1,11}$=8.24, p=0.009), whereas the effect of exercise was no longer statistically significant at 2 weeks (Fig. 5).

3.3.2. Dentate nucleus

Interestingly, at the same time across the initial week of experience manipulation, a significant *decrease* in BDNF expression occurred in dentate nucleus (effect of DURATION: $F_{3,36}$=3.565, p=0.027; effect of TRAINING: $F_{2,36}$=3.694, p=0.038; with no significant interaction

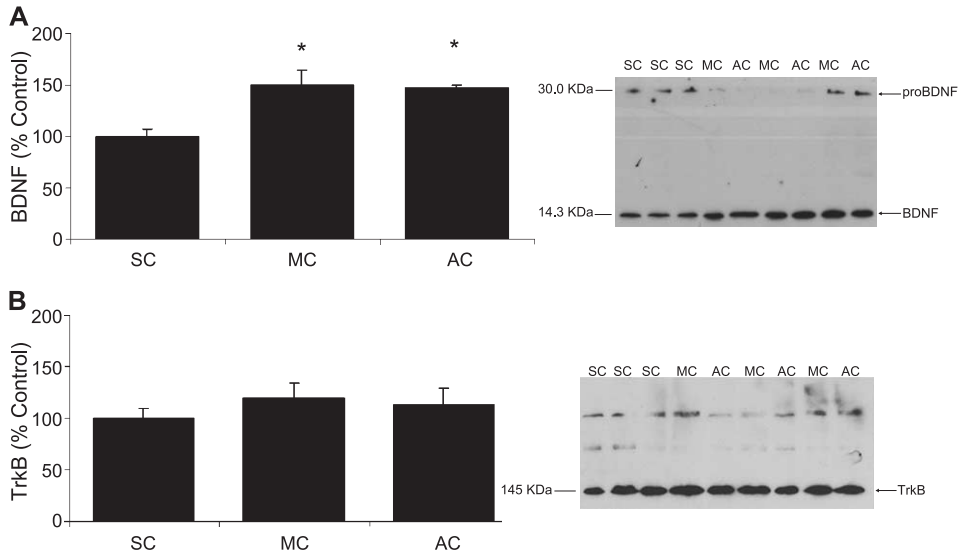

Fig. 3. (A) Relative levels of BDNF were assessed using Western blot analysis in motor cortex of rats trained on an acrobat course or run on a flat track for two weeks. Sample blot is shown for BDNF protein. Values are mean±S.E.M. *P<0.05, ANOVA and post hoc comparisons. (B) Graph showing relative levels of TrkB protein in motor cortex after 2 weeks in the MC or AC conditions. Values are mean±S.E.M.

between TRAINING×DURATION: $F_{3,36}$=1.475, p=0.243). Linear regression analysis of the difference between the AC or MC values and the SC baseline values (repeated at each time point) revealed a significant *decrease* in the level of expression of BDNF across the 7-day interval of learning or exercise experience (AC: $F_{1,21}$=7.83, p<0.05 ; MC: $F_{1,21}$=11.17, p<0.01) (Fig. 4). The experiment examining a 2-week duration of exercise and learning revealed no significant change due to either factor (Fig. 5).

3.3.3. Granule cell layer

There were no significant differences in BDNF expression in the granule cell layer across training or exercise experience. There were no statistically significant effects of a 2-week period of exercise or learning (Fig. 5).

3.4. Time course of the effect of learning and motor activity on cerebellar TrkB expression

A two-way ANOVA on TRAINING×DURATION revealed a significant effect of DURATION on TrkB expression in the molecular layer of PML ($F_{3,36}$=3.776, p=0.019), as well as of TRAINING ($F_{2,36}$=4.857, p=0.014) without a significant interaction between these parameters during the first week of training. Tukey comparisons

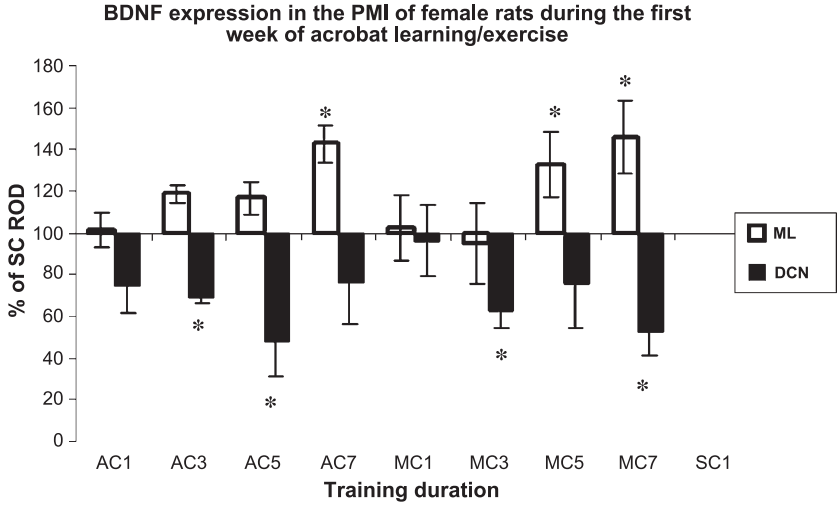

Fig. 4. Levels of expression of immunocytochemically detected BDNF protein in the paramedian lobule of cerebellum during the first week of training on AC or MC, compared to the SC baseline. Molecular layer (ML), dentate nucleus (DCN). Data for the granule cell layer is not presented as there were no statistically reliable changes. *p<0.05 (Tukey post hoc comparison). Values are mean±S.E.M. Relative optical density (ROD).

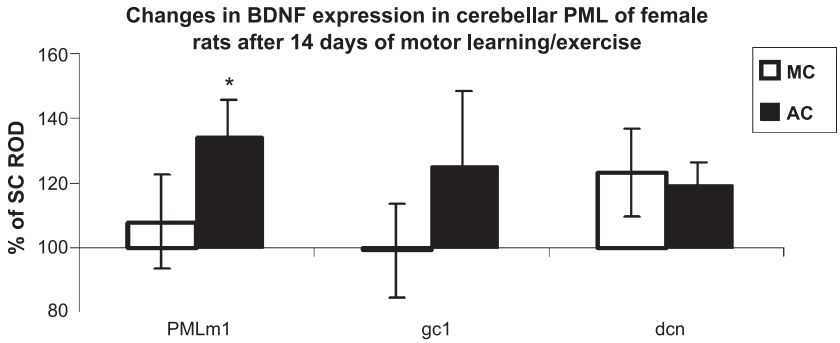

Fig. 5. Changes in the levels of immunocytochemically detected BDNF protein expression in cerebellar PML after 14 days of complex motor learning (AC) or moderate motor exercise (MC). Molecular layer (ML), granule cell layer (GCL), dentate nucleus (DCN). *p<0.05 (Tukey post hoc comparison). Values are mean±S.E.M. Relative optical density (ROD).

indicated that both complex motor learning (AC) and exercise (MC) increased the level of TrkB expression in the molecular layer of cerebellar PML after 5 and 7 days (Fig. 6). Separate linear regression analysis of the differences between the AC or MC values and the SC baseline values (repeated at each time point) revealed significant overall increases in TrkB expression in the PML molecular layer following both motor skill training and physical exercise in the cerebellar molecular layer (AC: $F_{1,21}$= 22.12, p<0.001; MC: $F_{1,21}$=12.25, p<0.01). Hence, both learning and exercise increased TrkB expression in cerebellar cortex over the first week.

The granule cell layer had increased TrkB expression only after 7 days of exercise or motor learning (p<0.01) and the DCN exhibited a significant reduction of immunostaining on the third day of intervention (p<0.05) (Fig. 6).

The second experiment, using a 2-week duration of exercise and learning, revealed that effects of learning and exercise on cerebellar TrkB expression were not maintained.

3.5. Motor cortex: effect of learning or activity on BDNF and TrkB expression

No significant changes in the expression of BDNF protein were detected in the upper (I–III) and lower (IV–VI) layers of both m1 and m2 areas of motor cortex during the first 7 days of motor learning and exercise (data not shown). After 14 days of training, a significant increase in BDNF immunoreactivity was seen in the upper layers of m1 and m2 (Fig. 7). One-way ANOVA demonstrated a significant effect of TRAINING condition in upper layers of m1 ($F_{2,11}$=6.231, p=0.02) and m2 ($F_{2,11}$=6.878, p=0.015). Post hoc comparison revealed that both MC and AC rats had significantly higher expression of BDNF protein than SC rats.

There was no significant change in the expression of TrkB protein in motor cortex during the first 7 days of motor learning or exercise. As with BDNF expression, TrkB expression showed a significant increase in response to AC only after 2 weeks of daily exposure in the upper layers of m1 and m2. One-way ANOVA indicated a significant effect

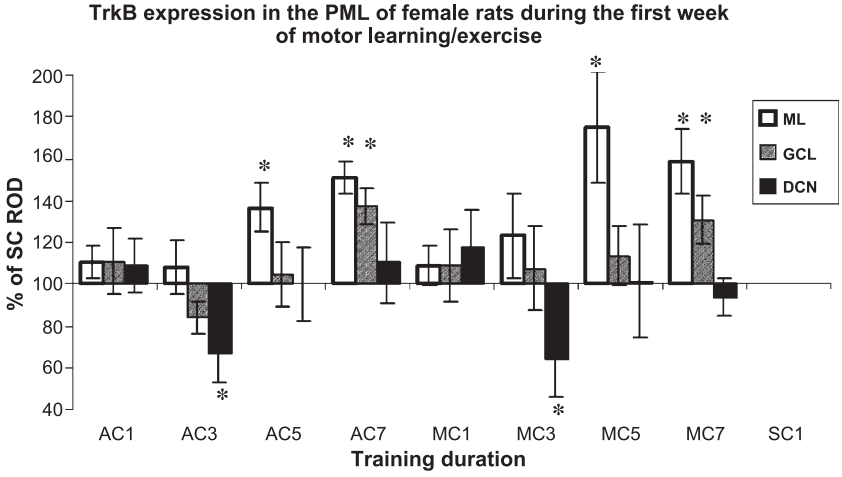

Fig. 6. Changes in the expression of immunocytochemically detected TrkB protein in the paramedian lobule of cerebellum during the first week of training on AC or MC. Molecular layer (ML), granule cell layer (GCL), dentate nucleus (DCN). *p<0.05 (Tukey post hoc comparison). Values are mean±S.E.M. Relative optical density (ROD).

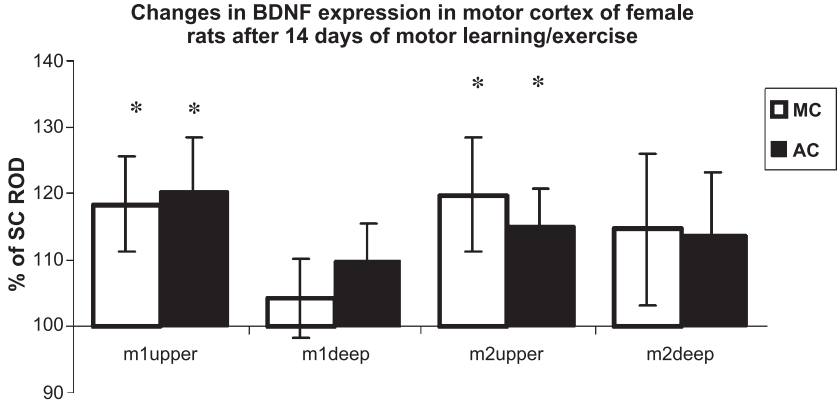

Fig. 7. BDNF protein expression in upper and lower layers of motor cortex after 14 days of training on AC or MC. *p<0.05 (Tukey post hoc comparison). Values are mean±S.E.M. Relative optical density (ROD).

of training condition in upper layers of m1 ($F_{2,11}$=5.507, p=0.027) and m2 ($F_{2,11}$=4.528, p=0.044). Post hoc comparison demonstrated that AC rats had significantly higher expression of TrkB protein than SC rats in layers I–III of the m1 and m2 areas (Fig. 8). Differences in TrkB expression between SC and MC as well as between MC and AC were not significant.

4. Discussion

Our data show that both complex motor learning and physical exercise elicit changes in the level of expression of the neurotrophin BDNF and its high-affinity receptor TrkB in cerebellum and motor cortex. These changes occur nonuniformly across time in different regions of the brain (more rapidly in cerebellum than in motor cortex) and in different subregions within the regions studied. In general, BDNF increased in expression across the first week in the cerebellar molecular layer in both learning and motor activity animals, while it decreased in an almost antiparallel fashion in the dentate nucleus. Motor cortex BDNF levels were not statistically elevated during 1 week of learning or exercise. Interestingly, BDNF protein expression remained elevated after 2 weeks of motor learning in the molecular layer of cerebellum and first became upregulated after 2 weeks of both motor learning and exercise in the upper layers of motor cortex.

TrkB expression in the molecular layer followed much the same course as BDNF, rising across 1 week of learning or physical activity. There was a slight tendency for TrkB expression to be downregulated in the dentate nucleus in both cases, but this was only statistically significant on day 3, for both learning and exercise. TrkB expression was elevated in the granule cell layer only on the 7th day of training. In motor cortex, learning did not affect TrkB receptor levels during the initial week of learning or activity, whereas learning produced an elevation of TrkB expression at 2 weeks, while physical exercise effects were still not statistically significant.

Prior work has suggested possible developmental roles of BDNF, including mediation or modulation of the effects of

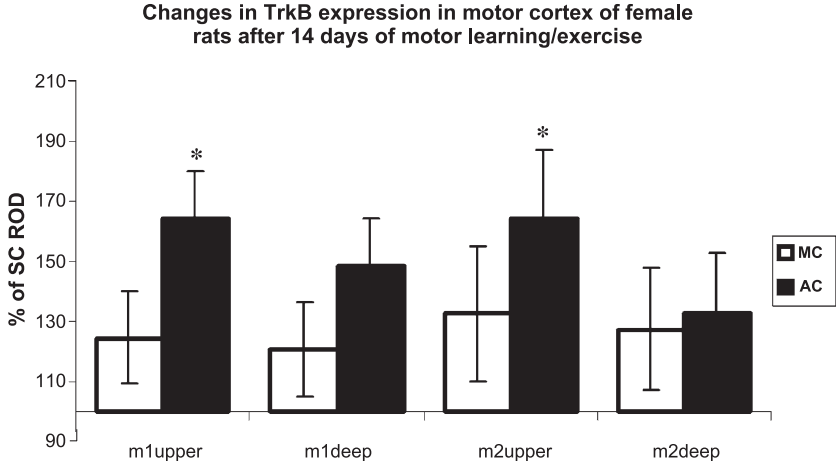

Fig. 8. TrkB protein expression in upper and lower layers of motor cortex after 14 days of training in the AC or MC conditions. *p<0.05 (Tukey post hoc comparison). Values are mean±S.E.M. Relative optical density (ROD).

experience. Expression of BDNF and its mRNA in the brain (although initially low during development) increases with maturation and either remains elevated thereafter (BDNF mRNA in neocortex, hippocampus and cerebellum) or slightly decreases (BDNF protein in neocortex, hippocampus and cerebellum) [17,24,40,41]. In addition, changes in BDNF and in some instances in TrkB expression have been proposed to play a role in activity-dependent plasticity in the adult brain (for review, see Refs. [54,57,72,79]): (1) epileptiform activity and LTP induction in hippocampus and cerebral cortex increase BDNF and NGF mRNA [21,26,36,65] and TrkB mRNA [62]; (2) light-induced upregulation of neuronal activity in visual cortex enhances BDNF mRNA expression there [12,73]; and (3) sensory stimulation of the whisker-to-barrel pathway specifically upregulated BDNF expression in layer IV of the barrel cortex (only in the stimulated barrels) [71]. Formation of declarative memory in monkey inferior temporal cortex when learning a paired-association task was accompanied by significant upregulation of BDNF protein and by an increase in the number of neurons that expressed BDNF mRNA [80]. Our findings extend the association of BDNF expression with activity and learning to the cerebellar cortex, and, in an inverse manner, to the dentate nucleus. It is of interest that the dentate (lateral) nucleus did not exhibit synapse plasticity in response to the same motor training that induced synaptogenesis in the cerebellar molecular layer [45], such that this aspect of the present results further suggests a relationship, albeit nonobligatory, between BDNF-TrkB expression and synapse activity or plasticity. These results moreover indicate an association of BDNF expression with a non-declarative form of memory involving the motor cortex and cerebellum.

Interactions between BDNF and its receptor TrkB have been shown to affect axonal and dendritic extension and synaptogenesis [8,10,55,85]. Ultrastructural demonstration of TrkB clustering at dendritic spines and excitatory-type axon terminals in hippocampus suggested that BDNF effects could be focused at synapses and modulate their activity [19]. Vicario-Abejón et al. [83] reported that neurotrophins (BDNF and NT-3) increased the number of functional synaptic connections (sevenfold) and BDNF induced formation of excitatory and inhibitory synapses in cultured neurons. BDNF overexpression greatly altered the morphology of dendrites and spines in cortical pyramidal neurons making them more unstable (turning over dendrites and spines more frequently) and significantly reducing spine density [33]. Mice lacking a functional *TrkB* gene had reduced axonal arborization and synaptic density in all subfields of the hippocampus [55]. BDNF deficit in $BDNF^{-/-}$ mutants leads (1) to impaired paired-pulse facilitation, a form of short-term plasticity, (2) to a decrease in parallel fiber to Purkinje cell synapses and (3) to a decrease in the proportion of docked vesicles in those synapses [11]. BDNF restored control numbers of axosomatic inhibitory synapses on Purkinje cells during activity blockade in cerebellar explants [76] and mediated activity-dependent density of inhibitory synapses made by postnatal hippocampal interneurons (without changing the density of excitatory synapses) [56].

In our study, animals that were learning complex motor task or moderately exercised (running on a flat track) for the same amount of time had elevated BDNF and/or TrkB expression in cerebellar PML as early as after 5 days of training, whereas it took a longer period of learning/exercising to produce detectable changes in motor cortex, where no significant changes were detected during the first 7 days of learning or training. It was reported previously that both vigorous and moderate physical exercise increased expression of mRNA for BDNF and TrkB. Neeper et al. [60] demonstrated that ad libitum access to running wheels increased BDNF mRNA in CA1 and CA4 areas of hippocampus and in layers II–III of the caudal neocortex. The level of the messenger increase was positively correlated with the distance run per night [60]. Moreover, Oliff et al. [63] repeated these results in addition to observing a significant increase in mRNA level as early as after 6 h of voluntary wheel running in CA1, CA3, and hilus.

In contrast, we did not detect a significant increase in BDNF in cerebellar molecular layer until after 5 days of moderate exercise or 7 days of learning complex motor skills. These delays may reflect the lower initial activity levels in both groups compared to those in the Neeper and Oliff studies. In any case, our results provide little evidence that *learning* potentiates the effects of physical activity on trophic factor induction and as a result on cellular plasticity in selected brain areas, as suggested, for example, by Gomez-Pinilla et al. [29]. In their study, Morris water maze learning resulted in a greater increase of fibroblast growth factor mRNA expression in hippocampus, cerebral cortex and cerebellum than swimming without learning to find the platform. In our study, there were two differential effects of learning vs. exercise after 2 weeks: (1) BDNF expression remained significantly (by 35%) increased in the cerebellar molecular layer of the AC but not the MC animals; and (2) TrkB protein expression remained elevated in upper layers of motor cortex AC (by 60%) but not in MC animals.

It has been shown recently that the effects of exercise and estrogen converge on the regulation of BDNF mRNA and protein level in the hippocampus [3]. The effect of exercise on BDNF mRNA in this brain area is dramatically reduced when estrogen is not present. Both types of estrogen receptors (α and β) are present in cerebral cortical neurons in the adulthood, and in cerebellum ERα is expressed exclusively during the development whereas ERβ is present in adult cerebellar Purkinje cells and Golgi cells [69,78]. The extent to which estrogen may play a role in the exercise-driven BDNF expression in cerebellum and motor cortex remains to be determined.

In general, these results do not point to a tight coupling or causal associations between BDNF or TrkB levels and learning-associated synaptogenesis. First, significant synap-

togenesis occurs in motor cortex during the first week of AC training [44], while no statistical elevation of BDNF or TrkB was seen in the present study, although we cannot exclude the possibility that very slightly increased levels were not detected with the subject numbers and intersubject variability in our study. Second, most synaptogenesis appears to be complete by the middle of the second week of training in AC animals [44], when training effects on BDNF and TrkB were first detected. Thus, the present findings would more closely support the involvement of neurotrophins in maintenance than in the generation of new synapses.

The BDNF in the cerebellum and motor cortex could be either (1) produced by local neurons, (2) retrogradely transported via Purkinje cell axons, or (3) anterogradely transported via parallel and/or climbing fibers. The first possibility seems to be the most likely based on the known localization of BDNF and TrkB in cerebellum and motor cortex. In the cerebellum, mRNA and BDNF-like immunoreactivity are detected in the cytoplasm of Purkinje cells and in the granule cells [20,24,41,51,61,70] and in deep cerebellar nuclei in rat [70]. Both Purkinje cells [27] and granule cells [74] express TrkB receptors as well. In cerebral cortex, BDNF-positive neurons were distributed predominantly in layers II/III, V and VI [17,24,41]. According to Pitts and Miller [68], more than 30% of neurons in these cortical layers were immunopositive for a neurotrophin and nearly 70% of neurotrophin-expressing neurons also coexpressed the high-affinity Trk receptor.

Effects of complex motor learning and exercise on BDNF and TrkB have been observed in a number of regions of the brain. It has been demonstrated previously that complex motor learning and exercise produce different effects in the brain, especially in cerebellar cortex [1,2,5]. Specifically, motor learning selectively promotes synaptogenesis whereas exercise selectively promotes angiogenesis. The molecular layer of cerebellum is known to be a site of plasticity [5,46] and structural changes accompanying acquisition of complex motor skills include an increased (compared to motor exercise) number of synapses per Purkinje cell in paramedian lobule [5,44]. Addition of parallel fiber–dendritic spine synapses on Purkinje neurons mainly contributes to this increase in overall synapse number [1,45]. Interneuron dendrite expansion has also been reported in the cerebellar molecular layer [44]. Jones et al. [39] demonstrated that motor learning caused synaptic changes in layer V of the motor cortex. In addition, motor learning has previously been found to result in an increase in synapse number per neuron in layer II/III of the motor cortex of intact adult female rats in comparison to motor exercise [43,48]. It is reasonable to suggest that the increase in BDNF and TrkB expression in cerebellar and motor cerebral cortex demonstrated in our study plays a role in these plasticity events.

The experience-dependent decreases in both BDNF and TrkB in the dentate nucleus (dentate cerebellar nucleus) were unexpected. Such simultaneous, opposite-direction increases and decreases in functionally interconnected structures do not appear to have been previously described but might reflect the sign-inverting effect of an inhibitory connection. A study of synapse numbers in the dentate cerebellar nucleus did not detect significant synaptogenesis with AC training [45], while more recent work has indicated addition of excitatory but not inhibitory (Purkinje cell input) in interpositus nucleus with eyeblink conditioning [47]. This result could reflect the nature of the relationship between cerebellar cortex and DCN: the principal cerebellar cortical afferent to the DCN is the inhibitory input of Purkinje cell axons, which may, on average, reduce activity of DCN cells. If so, this would also support the view that BDNF and TrkB expression may reflect activity more than they reflect plasticity.

The principal novelty of our findings is that both complex motor learning and motor activity affect expression of the neurotrophin BDNF in the motor cortex and cerebellum; expression of its receptor TrkB in cerebellum changes in response to both motor conditions, while in motor cortex TrkB changes only after motor skill training. Increases in cerebellar and cerebral cortices persisted longer in the complex motor skill-trained animals (BDNF in cerebellum, TrkB in motor cortex) (note that animals in our ‘exercise paradigm’ ran much less compared to previous studies such as Refs. [60] and [63]). This shows that complex motor learning may affect the neurotrophins system for a longer period than mere exercise. Complex motor learning promotes synaptogenesis in cerebellar cortex [5], and neurotrophins (and BDNF in particular) can increase the number of functional synaptic connections in hippocampus and cerebral cortex [77,83], such that it was reasonable to expect an association between BDNF/TrkB expression and AC-driven synaptogenesis in the present study. However, we did not see a selective increase in the neurotrophin expression after complex motor learning that corresponded spatially and temporally to the areas in which AC training induced synaptogenesis in previous studies. Thus, our findings suggest the involvement of the neurotrophin and its receptor is not coupled in an exclusive manner to the synaptic plasticity accompanying complex motor task learning.

Acknowledgements

We thank Amit Jamnadas, Steve Nash, Joanna Olewicz, Monika Patel and Laura Zimmerman for assistance in training animals, tissue processing, and the Beckman Institute Imaging Technology Group for use of the high-quality flatbed scanner and software to prepare illustrations.

This work was supported by AA09838 (AK) and AG10154 (WG). The work was begun when the first author

was at the University of Illinois and completed when she was at Binghamton University.

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