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Research Report

Exercise differentially regulates synaptic proteins associated to the function of BDNF

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ABSTRACT

We explored the capacity of exercise to impact select events comprising synaptic transmission under the direction of brain-derived neurotrophic factor (BDNF), which may be central to the events by which exercise potentiates synaptic function. We used a specific immunoadhesin chimera (TrkB-IgG) that mimics the BDNF receptor, TrkB, to selectively block BDNF in the hippocampus during 3 days of voluntary wheel running. We measured resultant synapsin I, synaptophysin, and syntaxin levels involved in vesicular pool formation, endocytosis, and exocytosis, respectively. Synapsin I is involved in vesicle pool formation and neurotransmitter release, synaptophysin, in the biogenesis of synaptic vesicles and budding, and syntaxin, in vesicle docking and fusion. Exercise preferentially increased synapsin I and synaptophysin levels, without affecting syntaxin. There was a positive correlation between synapsin I and synaptophysin in exercising rats and synapsin I with the amount of exercise. Blocking BDNF abrogated the exercise-induced increases in synapsin I and synatophysin, revealing that exercise regulates select properties of synaptic transmission under the direction of BDNF.

1. Introduction

Exercise has the capacity to induce hippocampal synaptic plasticity, prominently enhancing synaptic efficacy (Farmer et al., 2004; Vaynman et al., 2003) and the expression of molecules implicated in learning and memory (Farmer et al., 2004; Vaynman et al., 2003, 2004). These molecular changes may comprise the ability of exercise to impact behavioral plasticity, i.e. to improve learning and memory (Fordyce and Wehner, 1994; Kramer et al., 1999) and reduce the mental decline associated with aging (Laurin et al., 2001). Specifically, exercise-induced synaptic plasticity in the hippocampus, a site critical for learning and memory, may sub-serve the ability of exercise to enhance hippocampal-dependent cognitive function.

Exercise alters molecules implicated in learning and memory functions, distinctly increasing hippocampal brain derived neurotrophic factor (BDNF; Molteni et al., 2004; Neeper et al., 1995; 1996; Vaynman et al., 2004). BDNF modulates synaptic-plasticity in the adult brain (Lo, 1995), and has the capacity to modify synaptic function in the hippocampus by modulating the efficacy of neurotransmitter release (Kang and Schuman, 1995). However, it remains to be determined if exercise impacts events constructing

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vesicular release properties and the role of BDNF in these events.

In the present study, we selected a group of molecules involved in synaptic transmission, i.e., synapsin I, synaptophysin, and syntaxin, which have distinct actions on vesicle clustering, endocytosis, and exocytosis, respectively, and which may comprise main events characterizing synaptic function during exercise. Synapsin I is a member of a family of terminal specific phosphoproteins involved in synaptic vesicle clustering and release, which mediates synaptic transmission (Jovanovic et al., 1996). Synaptophysin is a specific component of the membrane of presynaptic vesicles, and possibly important for the biogenesis of synaptic vesicles, vesicle budding, and endocytosis (Daly et al., 2000; Tartaglia et al., 2001). Syntaxin, localized to the presynaptic plasma membrane, plays a crucial role in the docking and fusion of vesicles during neurotransmitter release (McMahon and Sudhof, 1995). We used a specific immunoadhesin chimera (TrkB-IgG) that mimics the BDNF receptor TrkB to selectively block the function of BDNF and measured the protein levels of synapsin I, synaptophysin, and syntaxin in the hippocampus following 3 days of exercise. Our results demonstrate that exercise uses BDNF to selectively modulate the levels of synapsin I, and synaptophysin but not that of syntaxin in the hippocampus.

2. Results

2.1. *Effect of blocking BDNF action during exercise on vesicle associated proteins*

Quantitative analysis of Western blot assay revealed that 3 days of exercise significantly ($P < 0.05$) increased synapsin I levels in the hippocampus (133 ± 12%) compared to sedentary/cytC controls (Fig. 1A). Fig. 1A shows a significant difference between the Exc and Sed group, Exc and Exc/IgG group, and Exc and the sed/IgG group ($P < 0.05$). Exercise significantly ($P < 0.05$) increased synaptophysin in the hippocampus (202 ± 25%) above sedentary/cytC controls (Fig. 1B), but did not significantly alter syntaxin levels (106 ± 14%) from sedentary/cytC controls (Fig. 1C). Fig. 1B shows significant changes between the Exc and sed control, Exc and Exc/IgG, and Exc and Sed/IgG ($P < 0.05$). Blocking the action of BDNF effectively prevented the exercise-induced increases in synaptic plasticity markers, by reducing synapsin I (133 ± 12% to 58 ± 4%), below sedentary control levels (Fig. 1A) and synaptophysin by (202 ± 25% to 119 ± 21%) to approach control levels (Fig. 1B). Blocking BDNF action during the sedentary condition significantly ($P < 0.05$)

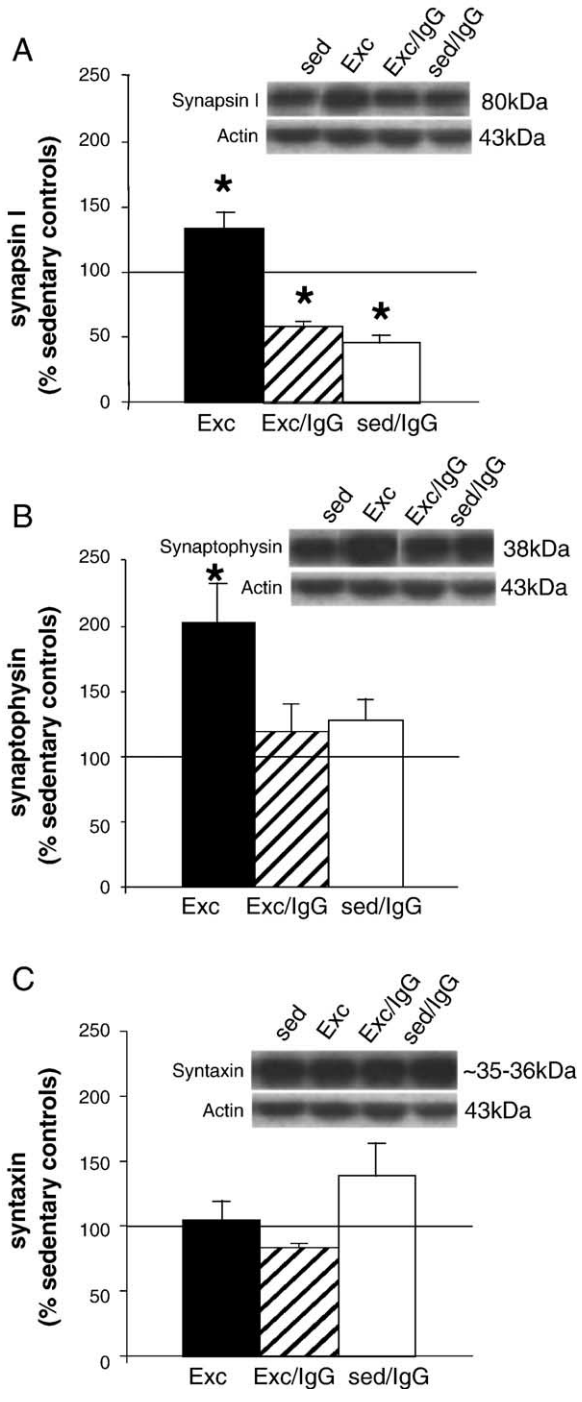


Fig. 1 – Effect of exercise and blocking BDNF action on (A) synapsin I, (B) synaptophysin, and (C) syntaxin proteins in the hippocampus. Exercise significantly increased the expression of synapsin I and synaptophysin (without affecting the expression of syntaxin). Blocking BDNF action inhibited the upregulation of synapsin I and synaptophysin proteins induced by exercise in the hippocampus. Blocking BDNF action during the sedentary condition reduced synapsin I levels below sedentary/cytC controls but did not significantly alter synaptophysin and syntaxin levels. Representative immunoblots for synapsin I, synaptophysin, and syntaxin of each group are shown to the right of the graph, with actin as an internal standard control. Each value represents the mean ± standard error of the mean (SEM) (*$P < 0.05$; ANOVA). Exc ($n = 5$) = exercise/cytC, Exc/IgG ($n = 6$) = exercise/TrkB-IgG, Sed/IgG ($n = 6$) = sedentary/TrkB-IgG. Sedentary/cytC ($n = 5$) controls are represented by the 100% horizontal line, such that the statistical significance of protein levels for exercise animals is represented compared to this line.

reduced synapsin I levels (45 ± 5%) below sedentary/cytC controls (Fig. 1A), but did not significantly alter synaptophysin (127 ± 17%; Fig. 1B) and syntaxin (140 ± 25%; Fig. 1C) levels.

We found a significant positive correlation between hippocampal synapsin I protein levels and running distance (km) over a 3-day period ($r = 0.9$, $P < 0.05$; Fig. 2A). Although we found a positive trend between synaptophysin levels and running distance ($r = 0.67$, $P = 0.22$; Fig. 2B), we found a significant positive correlation between synapsin I and synaptophysin levels in the exercise condition ($r = 0.91$, $P < 0.05$; Fig. 2C).

3. Discussion

The benefits of exercise on CNS health and the preponderance of BDNF in mediating these benefits are becoming well accepted. Exercise impacts a cavalcade of synaptic and cognitive functions, from increasing synaptic efficacy (van Praag et al., 1999) to enhancing memory and cognition in the adult (Fordyce and Wehner, 1994; Kramer et al., 1999) and even senescent brain (Laurin et al., 2001). However, the changes at the hippocampal synapse, which may sub-serve the ability of exercise to impact neuronal and cognitive plasticity, have remained for the most part obscure and poorly understood. Here, we show that exercise uses a BDNF-mediated mechanism to change specific properties of the hippocampal synapse at the presynaptic membrane by selectively increasing the protein levels of synapsin I and synaptophysin without changing syntaxin levels.

3.1. *BDNF modulates the exercise-induced changes in select vesicle associated proteins*

Blocking BDNF action has been shown to contribute to both synaptic fatigue and the decrease of synaptic vesicle proteins (Pozzo-Miller et al., 1999). As a result, we investigated whether BDNF mediates the levels of synapsin I, synaptophysin, and syntaxin during exercise. We found that blocking BDNF action completely abrogated the exercise-induced increase in synapsin I levels (Fig. 1A). Thus, in addition to regulating exercise-induced synapsin I mRNA levels (Vaynman et al., 2004) and the phosphorylation state of synapsin I (Jovanovic et al., 2001), BDNF seems to regulate synapsin I protein levels during exercise.

Synapsin I tethers synaptic vesicles (SV) to each other and to the actin cytoskeletal net, thus functioning to maintain a reserve pool of vesicles in the vicinity of the active zone needed for proper synaptic transmission. In fact, inhibiting synapsin I reduces both the SV reserve pool and neurotransmitter release (Hilfiker et al., 1999). A substantial localized SV reserve pool helps to prevent vesicular run down during high frequency stimulation (Pieribone et al., 1995). Our results show that synapsin I protein levels in the hippocampus increase in proportion to the amount of exercise (km) in individual animals (Fig. 2A). Thus, increasing synapsin I levels may be needed to sustain the release of

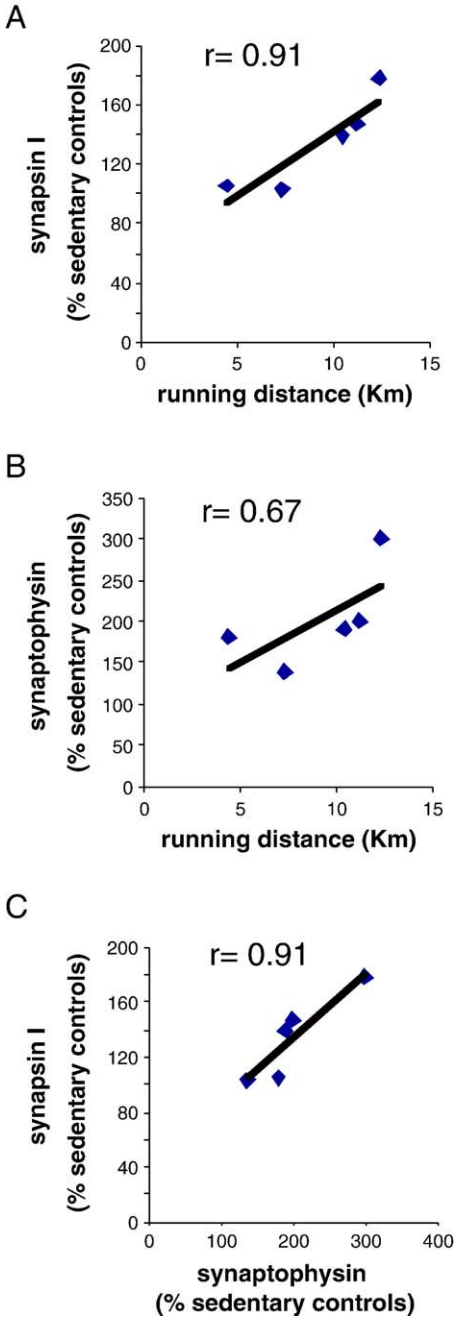


Fig. 2 – Association between synapsin I, synaptopysin, and running distance (km) over a 3-day period for individual animals. (A) Significant positive correlation between hippocampal synapsin I protein levels and running distance ($r = 0.91$, $P < 0.05$), showing that there was a tendency for animals that ran longer distances to have the highest levels of synapsin I protein. (B) Nonsignificant correlation between synaptophysin and running distance ($r = 0.67$, $P = 0.22$). (C) Significant positive correlation between hippocampal synapsin I and synaptophysin protein levels ($r = 0.91$, $P < 0.05$), showing that there was a tendency for animals with the highest synapsin I levels to have the highest synaptophysin levels.

neurotransmitter under high levels of activity, imposed by conditions, like exercise, that necessitate a large expenditure of energy at the synapse.

We found that exercise significantly increased the levels of synaptophysin above sedentary controls and that blocking BDNF action was sufficient to abrogate the exercise-induced increase in synaptophysin levels (Fig. 1B). This is consistent with the finding that BDNF knockout mice have a reduction in synaptophysin in hippocampal synaptosomes (Pozzo-Miller et al., 1999). Synaptophysin is a major integral protein on synaptic vesicles. Synaptophysin acts as a key protein in the biogenesis of synaptic vesicles from cholesterol (Thiele et al., 2000) and may possibly facilitate membrane retrieval during vesicle recycling. It has been postulated that the ability of synaptophysin to form oligomers may promote membrane curvature to facilitate synaptic vesicle budding (Thiele et al., 2000). In fact, studies at the giant squid axon have implicated synaptophysin in rapid clathrin-independent vesicle endocytosis at the active zone (Daly et al., 2000). The retrieval of synaptic vesicle proteins by endocytosis, in order to generate fusion competent synaptic vesicles, is important for rapid neurotransmission, especially in the CNS where the endocytotic rate exceeds that of nonneuronal cells by at least tenfold (Betz and Wu, 1995). There is little evidence to indicate the involvement of synaptophysin in vesicle docking, although it is tightly associated to synaptobrevin (Edelmann et al., 1995), considered a required component of vesicle docking (Pevsner et al., 1994).

We found that exercise did not significantly increase the levels of syntaxin above sedentary controls and that blocking BDNF action did not significantly alter syntaxin levels in exercise or sedentary animals (Fig. 1C). This in vivo finding is consistent with the report that BDNF does not regulate syntaxin levels in hippocampal slice preparations (Tartaglia et al., 2001). Syntaxin is a protein on the presynaptic membrane, which is a constitutive component of the SNARE complex involved in synaptic vesicle docking and fusion (McMahon and Sudhof, 1995). Thus, BDNF may not regulate the syntaxin element of vesicle exocytosis during exercise.

We found that synapsin I is associated with synaptophysin for individual animals (Fig. 2C). Concurrent changes in synapsin I and synaptophysin may be indicative of structural and functional changes in synaptic circuitry occurring during exercise. Synapsin I regulates neurite development (Melloni et al., 1994; Zurmohle et al., 1996), the formation and maintenance of the presynaptic structure (Sato et al., 2002), axonal elongation (Akagi et al., 1996), and new synaptic formation (Ferreira et al., 1998). Although synaptophysin has been associated with synaptogenesis (Bergmann et al., 1997), increases in synaptophysin likely indicate that synaptic vesicles are formed (Sarnat and Born, 1999), which could be due to increased synapse formation or increases in the number of vesicles in existing synapses. The finding that synaptic proteins such as synapsin I and synaptophysin are under the regulation of BDNF during exercise may contribute to the ability of BDNF to regulate both the number of synapses and the complexity of axonal arborization in the hippocampus. In vivo imaging experiments have visually illustrated the ability of BDNF to regulate synaptogenesis in arborizing axon terminals (Alsina et al., 2001). Indeed, studies have shown that limiting the ability of BDNF to bind to its cognate receptor by using TrkB knockout mice produces a significant reduction in axonal arborization and the number of synapses during hippocampal development (Martinez et al., 1998). Particularly, exercise may affect the DG through BDNF, as BDNF has been found to induce axonal branching of dentate granule neurons in vitro (Lowenstein and Arsenault, 1996; Patel and McNamara, 1995).

The capacity of exercise to modulate specific properties of BDNF-mediated synaptic plasticity in the hippocampus provides mechanistic support to epidemiological studies showing the effects of exercise on cognition. Importantly, the finding that exercise uses BDNF to exert changes in synaptic properties is encouraging as it brings insight critical for developing therapies to treating cognitive disorders such as Alzheimer's disease, which are correlated with synaptic dysfunction (Masliah et al., 2001).

4. Experimental procedures

4.1. *Exercise paradigm*

We chose a voluntary exercise paradigm because it simulates aspects of human behavior by enabling animals to choose how much to run. Rats (Male Sprague–Dawley rats, approximately 2 months of age, n = 5–6 animals per group) were housed in standard polyethylene cages in a 12/12 h light/dark cycle at 22–24 °C, with food and water ad libitum. Exercise rats were given access to a wheel (diameter = 31.8 cm, width = 10 cm) that freely rotated against a resistance of 100 g attached to a receiver that monitored revolutions every hour (VitalViewer Data Acquisition System software, Mini mitter company, Inc., Sunriver, OR). These studies were performed in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals, and were approved by UCLA Animal Research Committees.

4.2. *Drugs*

Recombinant human TrkB-IgG chimera (R&D System, Inc., Minneapolis, MN in powder) a highly potent and specific antagonist of BDNF (Vaynman et al., 2003) was used to block BDNF action. Sterile phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) was added to the vial to prepare a stock solution (100 μg/ml). Cytochrome C (cytC; SIGMA, St. Louis, MO) was used as a standard control for microbead injection (Vaynman et al., 2003, 2004) and was dissolved in sterile distilled water at a concentration of 100 ng/μl. Drug combination with sedentary and exercise conditions generated a total of 4 groups: exercise/TrkB-IgG (n = 6), sedentary/TrkB-IgG (n = 6), exercise/cytC (n = 5), sedentary/cytC (n = 5).

4.3. *Microbeads*

Fluorescent latex microbeads (Lumaflour Corp., Naple, FL), used as the vehicle for drug insertion into the hippocampus, were prepared by previously described methods (Vaynman et al., 2003). This consisted of coating the microbeads with each drug via passive absorbency by incubating overnight at 4 °C with a 1:5 mix of microbeads to TrkB-IgG (5 μg/μl in PBS with BSA) and cytC (100 ng/μl in sterile water).The morning after coating the microbeads, the solution was centrifuged at 14,000 × g for 30

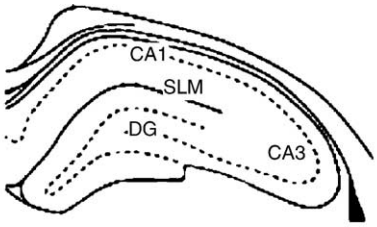
min and the microbeads were resuspended in sterile water at a 10% concentration. We have previously used the microbead injection method to successfully block the action of BDNF with TkB-IgGs on the expression of molecular targets in the hippocampus and associated cognitive function (Vaynman et al., 2003).

4.4. *Injection of drugs into hippocampus*

Exercise and sedentary rats received TrkB-IgG or the standard control cytC injection, given once prior to the 3-day running period, early in the morning such that an ample recovery time permitted all animals to begin running that same evening. We used a unilateral injection into the right hippocampus to be consistent with previous blocking experiments (Vaynman et al., 2003, 2004). We did not use the contralateral hippocampus as a control because a unilateral injection can cause changes on the contralateral side due to connecting fibers (Amaral and Witter, 1989). Animals were anesthetized by Isoflurane (2–2.5%) utilizing Mobile Laboratory Animal Anesthesia System, and positioned in a stereotaxic apparatus, used to secure the animal and to measure the sight for injection. TrkB-IgG or cytC imbedded in microbeads was injected into the right hippocampus (3.8 mm posterior to Bregma, 2.6 mm lateral to the midline, and 3.7 mm vertical from skull) using a Hamilton syringe in a volume of 2 μl over 15 min. Animals were sacrificed the morning after the last running period. Hippocampal brain tissue was quickly removed, frozen on dry ice, and stored at –70 °C until use for Western blot analysis. The location of microbead injection was verified by histological examination of selected brains, as previously described (Vaynman et al., 2003). We visually inspected all the brains at the time of dissection, such that only those showing characteristic markings of microbeads of the right hippocampus were used to examine protein levels by Western blot. The microbead injection site was additionally verified by florescence microscopy, as shown in Fig. 3, using an Olympus BX51 microscope. The location of the microbead injection is concentrated in the stratum lacunosum-moleculare (slm; Figs. 3A–C). Our microbead infusion site is consistent with our previous drug infusion protocol (Vaynman et al., 2003, 2004) as well as previous studies that evaluated the effect of inhibitors (Blum et al., 1999; Kawabe et al., 1998; Zhang et al., 2001) or exogenous BDNF (Messaoudi et al., 2002) on hippocampal function.

4.5. *Western blot*

The expression of synapsin I, synaptophysin, and syntaxin was assessed by immunoblotting method in all the groups, as previously described in Molteni et al., 2004. Hippocampal tissue was homogenized in a lysis buffer containing 137 mM NaCl, 20 mM Tris–HCl pH 8.0, 1% NP40, 10% glycerol, 1 mM PMSF, 10 μg/ml aprotinin, 0.1 mM benzethonium chloride, 0.5 mM sodium vanadate. Homogenates were centrifuged, supernatants collected, and total protein concentration determined according to the Micro BCA procedure (Pierce, Rockford, IL, USA), using bovine serum albumin as standard. Equal amounts (25 μg) of protein samples were separated by electrophoresis on a 10% polyacrylamide gel and electrotransferred to a nitrocellulose membrane. Filters were blocked with 5% nonfat milk. Membranes were then incubated with anti-actin antibody (1:2000, Santa Cruz Biotechnology, Santa Cruz, CA), anti-synapsin I antibody (1:2000, Santa Cruz Biotechnology, Santa Cruz, CA), followed by anti-goat IgG horseradish peroxidase-conjugate; anti-synaptophysin antibody (1:50,000 Santa Cruz Biotechnology, Santa Cruz, CA), and anti-syntaxin antibody (1:50,000 Santa Cruz Biotechnology, Santa Cruz, CA) followed by anti-rabbit IgG horseradish peroxidase-conjugate. After rinsing with buffer, the immunocomplexes were visualized by chemiluminescence using the ECL kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ) according to manufacturer's instructions. Film signals were digitally scanned and quantified using NIH Image software, normalized for actin level.



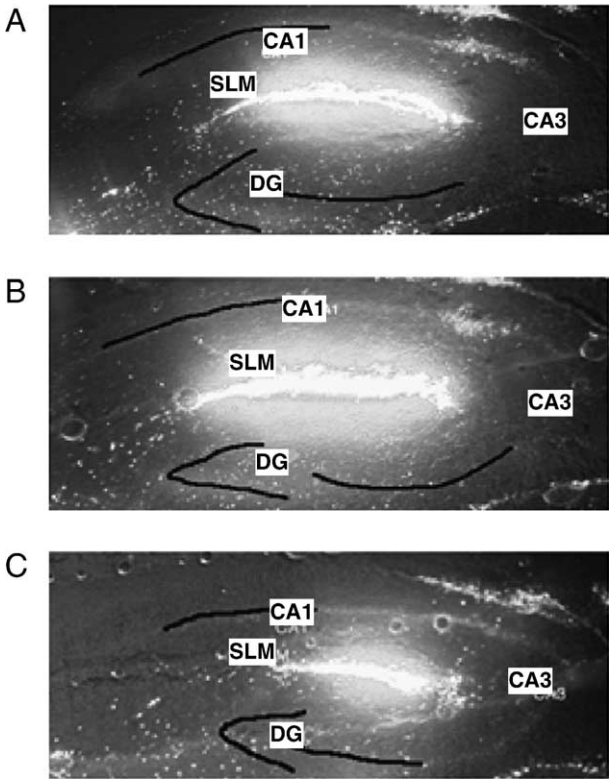


Fig. 3 – Tissue section in the saggital plane showing the site of microbead injection in the right hippocampus using fluorescence microscopy, across three sections in a single animal (approximating Bregma –3.8 to –4.5; A–C). Hippocampal areas of CA1, CA3, and dentate gyrus (DG) have been labeled and lines have been drawn in to emphasize areas CA1, CA3, DG, and stratum lacunosum-moleculare (slm) of the hippocampus in accordance with drawing shown above. Highlighted areas represent high florescence activity for the action of TrkB-IgG.

4.6. *Statistical analyses*

Actin was used as internal standards for Western blot. An analysis of variance (ANOVA) was conducted to determine differences between the four groups: exercise/TrkB-IgG (n = 6), sedentary/TrkB-IgG (n = 6), exercise/cytC (n = 5), sedentary/cytC (n = 5). Statistical differences were considered significant at $P < 0.05$. The results of exercised and sedentary rats were converted to percentages of controls for presentation in bar figures and

represent the mean ± standard error of the mean (SEM). A Fischer test was used for cross-group comparisons. To evaluate association between variables (running distance with synapsin I, running distance with synaptophysin, and synapsin I with synaptophysin), a linear regression analysis was performed on individual samples.

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