



Exploring the relationship between serotonin and brain-derived neurotrophic factor: analysis of BDNF protein and extraneuronal 5-HT in mice with reduced serotonin transporter or BDNF expression

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Abstract

Serotonin (5-HT) has been proposed to promote neuronal plasticity during the treatment of mood and anxiety disorders and following neurodegenerative insult by altering the expression of critical genes including brain-derived neurotrophic factor (BDNF). In this study, mice with constitutive reductions in the serotonin transporter (SERT) or BDNF were investigated to further assess the functional relationship between serotonin neurotransmission and BDNF expression. Using a modified extraction procedure and a commercial enzyme-linked immunosorbant assay, 50% decreases in BDNF protein in hippocampus, frontal cortex and brain stem were confirmed in 4-month-old mice lacking one copy of the BDNF gene (BDNF^{+/-}). By contrast, 4-month-old male and female mice with partial (SERT^{+/-}) or complete (SERT^{-/-}) reductions in SERT expression showed no differences in BDNF protein levels compared to SERT^{+/+} mice, although male SERT knockout mice of all genotypes had higher BDNF levels in hippocampus, frontal cortex, and brain stem than female animals. Microdialysis also was performed in BDNF^{+/-} mice. In addition to other phenotypic aspects suggestive of altered serotonin neurotransmission, BDNF^{+/-} mice show accelerated age-related degeneration of 5-HT forebrain innervation. Nevertheless, extracellular 5-HT levels determined by zero net flux microdialysis were similar between BDNF^{+/+} and BDNF^{+/-} mice in striatum and frontal cortex at 8–12 months of age. These data illustrate that a 50% decrease in BDNF does not appear to be sufficient to cause measurable changes in basal extracellular 5-HT concentrations and, furthermore, that constitutive reductions in SERT expression are not associated with altered BDNF protein levels at the ages and in the brain regions examined in this study.

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1. Introduction

Depression and anxiety disorders are psychiatric illnesses commonly treated by serotonin reuptake inhibiting drugs

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(SRIs) suggesting an underlying dysfunction in the serotonin (5-HT) system or other neurotransmitter systems or circuits modulated by 5-HT (Hen, 1996; Gingrich and Hen, 2001; Nestler et al., 2002). SRIs inhibit the serotonin transporter to prevent the uptake of 5-HT from the extracellular signaling space; however, elevated extraneuronal 5-HT levels in serotonergic projection fields and the efficacy of SRIs for relieving the symptoms of depression and anxiety take weeks to develop fully in response to continuous administration of these drugs (Kreiss and Lucki, 1995; Duman et al., 1997; Hervas and Artigas, 1998; Trillat et al., 1998; Malagie et al., 2001; Nestler et al., 2002). These observations have led to the hypothesis that SRIs in particular, and antidepressants in general, act by evoking adaptive changes in extracellular signaling and subsequently, postsynaptic signal transduction and gene expression. In particular, studies have linked chronic antidepressant treatment with changes in the expression of the neuronal trophic, brain-derived neurotrophic factor (BDNF) (Nibuya et al., 1995, 1996; Duman et al., 1997; Zetterstrom et al., 1999; Coppell et al., 2003). For example, Nibuya et al. found that chronic treatment of rats with a variety of antidepressants (SRIs, tricyclics, monoamine oxidase inhibitors and atypical antidepressants) elevates BDNF mRNA in hippocampal and cortical brain regions (Nibuya et al., 1995, 1996).

Neurotrophic factors are endogenous soluble proteins that regulate the survival, growth, morphological plasticity, and synthesis of new neurons for differentiated function (Hefti et al., 1993). The neurotrophin family in mammals is composed of four known proteins: BDNF, nerve growth factor, neurotrophin-3 and neurotrophin-4. BDNF is a 27 kDa homodimeric protein whose signaling actions are mediated via the tyrosine kinase B (trkB) receptor. Furthermore, BDNF is the most abundant neurotrophic factor in brain with the highest levels of mRNA and protein found in hippocampus and frontal cortex (Altar et al., 1997; Conner et al., 1997).

In addition to acting as a trophic factor, BDNF is thought to modulate other signaling molecules including the monoamine, amino acid and peptide neurotransmitters (Lindsay et al., 1994; Kreiss and Lucki, 1995; Duman et al., 1997; Siuciak et al., 1997; Dluzen et al., 1999, 2002; Goggi et al., 2002; Nestler et al., 2002). Indirect evidence suggests that BDNF can augment serotonergic neurotransmission (Mamounas et al., 1995, 2000; Siuciak et al., 1996, 1997; Goggi et al., 2002). BDNF infused directly into the brain is known to influence the survival and function of serotonergic neurons, affect the turnover ratio of 5-HT versus its major metabolite 5-hydroxyindoleacetic acid (5-HIAA) and potentiate activity-dependent release of 5-HT (Mamounas et al., 1995; Siuciak et al., 1996; Goggi et al., 2002). In addition, Siuciak et al. have linked BDNF with depression by altering animal behavioral thought to model depression via central administration of BDNF, the latter producing an antidepressant-like effect (Siuciak et al., 1997). This further emphasizes a potential role for BDNF in the mechanism of action of antidepressants; however, the molecular mechanisms

by which BDNF might modulate the 5-HT system are still unknown.

To further investigate the effects of reduced serotonin uptake on neurotransmission, gene expression and behavior, mice with a targeted disruption of the serotonin transporter (SERT) gene have been produced (Bengel et al., 1998). Mice lacking both copies of the SERT gene (SERT^{-/-}) show a complete loss of SERT protein expression and functional serotonin uptake, resulting in increased extracellular 5-HT levels (Bengel et al., 1998; Fabre et al., 2000b; Fedele et al., 2001; Montanez et al., 2003) (see also accompanying manuscript by T.A. Mathews et al.). SERT^{-/-} mice also display a phenotype characterized by the absence of locomotor stimulation in response to the substituted amphetamine, 3,4-methylenedioxymethamphetamine (MDMA), reduced aggressive behavior and an increase in stress responsiveness that is manifest as heightened anxiety-related behavior (Bengel et al., 1998; Li et al., 1999; Murphy et al., 2001; Holmes et al., 2002, 2003). Moreover in humans, a 40% reduction in SERT expression driven by a promoter polymorphism has been correlated with an increase in anxiety-related personality traits and, recently, to enhanced susceptibility to stress related major depressive episode (Lesch et al., 1996; Greenberg et al., 2000; Caspi et al., 2003).

Mice with genetically controlled-reductions in the expression of BDNF have also been generated (Liebl et al., 1997). Mice lacking both copies of the BDNF gene (BDNF^{-/-}) die shortly after birth; however, mice with one functional copy of the gene (BDNF^{+/-}) are viable (Liebl et al., 1997; Lyons et al., 1999). BDNF^{+/-} mice develop a phenotype characterized by increased aggressive behavior at 2.5–4.5 months of age and hyperphagia at 3–11 months, both of which have been associated with dysfunction in the 5-HT system (Lyons et al., 1999). In addition, a blunted *c-fos* response to the serotonin-releasing amphetamine, dexfenfluramine (d-fen) in 3–6-month-old mice and significant changes in 5-HT receptor (1A, 1B, 2A and 2C) mRNA levels in 6–9-month-old animals were found in various brain regions in BDNF^{+/-} mice (Lyons et al., 1999). In BDNF^{+/-} mice >12 months of age, an accelerated loss of serotonergic innervation to the forebrain and decreased total tissue levels of 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) have been observed in hippocampus and frontal cortex. Together these data, as well as those from many other studies, suggest a complex modulatory relationship between BDNF and the serotonin system.

In the present investigation, SERT knockout mice were utilized to determine specifically whether long-term SERT-mediated changes in serotonergic signaling modulate BDNF protein expression. To investigate serotonergic regulation of BDNF levels, BDNF protein was extracted from regions of the mouse brain using a procedure optimized to yield high recovery from tissue. Extracts were then analyzed using a commercial enzyme-linked immunosorbent assay (ELISA). Since chronic administration of SRIs has been shown to increase BDNF mRNA levels, we hypothesized that genetically-induced reductions in serotonin uptake would,

similarly, lead to increased expression of BDNF, particularly in the hippocampus and frontal cortex.

We also investigated BDNF^{+/-} mice to ascertain whether reductions in BDNF expression alter 5-HT neurotransmission. In BDNF^{+/-} mice 8–12 months of age, we employed *in vivo* zero net flux microdialysis to investigate regional changes in extraneuronal 5-HT levels. In this case, a 50% reduction in BDNF expression (Kolbeck et al., 1999) was hypothesized to be associated with decreased extracellular 5-HT levels. Reduced serotonergic neurotransmission was theorized to underlie phenotypic alterations in BDNF^{+/-} mice and to precede the accelerated age-related loss of serotonergic forebrain innervation occurring in these mice.

2. Materials and methods

2.1. SERT knockout mice

SERT^{+/+}, SERT^{+/-} and SERT^{-/-} mice on a CD1 × 129S6/SVev background (Bengel et al., 1998) were housed in groups of three to four per cage with food and water *ad libitum* (12-h light/dark cycle). SERT knockout mice were acquired via heterozygote brother-sister matings. Mice used for these experiments were from the F10-F12 generations. Adolescent mice at the time of weaning (3–4-weeks-old) were ear tagged and the terminal 2–3 mm of their tails were clipped for genotype identification by polymerase chain reaction (PCR) amplification of a region of exon 2 of the SERT gene.

2.2. BDNF knockout mice

BDNF^{+/+} and BDNF^{+/-} mice were generated as described previously (Liebl et al., 1997). Mice were backcrossed for 10–12 generations onto a C57BL/6 genetic background. BDNF^{+/-} mice were bred and genotyped in the laboratories of Drs. Laura Mamounas and Ernest Lyons at the Johns Hopkins Medical Institutions and Dr. Lino Tessarollo at the National Cancer Institute Center for Cancer Research. BDNF knockout mice were transferred to the Pennsylvania State University at 3–5 months of age and group housed by sex (2–5 animals/cage) in a temperature and humidity controlled room under an automatic 12-h light/dark cycle with food and water *ad libitum*. In all cases, experimental protocols strictly adhered to National Institutes of Health Animal Care guidelines and were approved by the Pennsylvania State University Institutional Animal Care and Use Committee.

2.3. Original extraction procedure for BDNF from mouse brain tissue (Promega Co.)

Mice were killed by cervical dislocation and frontal cortex, brain stem and bilateral hippocampi were rapidly dissected and placed on dry ice followed by storage at -70°C . At the

time of analysis, samples were removed from the freezer, weighed and 200 μL Promega lysis buffer (137 mM NaCl, 20 mM Tris-HCl (pH 8.0), 1% Tergitol type NP40, 10% glycerol, 1 mM PMSF (α -toluenesulfonyl fluoride), 10 $\mu\text{g}/\text{mL}$ aprotinin, 1 $\mu\text{g}/\text{mL}$ leupeptin, and 0.5 mM sodium vanadate) was added to each sample. Samples were sonicated (Virtis Virsonic, Virtis Company, Gardiner, NY with a microtip at power level 4 and pulses at 1 s intervals for 15 s). Samples then were centrifuged at $16,000 \times g$ for 30 min at 4°C . One hundred microliter aliquots of the resulting supernatants were removed and diluted with 400 μL of DPBS buffer (137 mM NaCl, 2.68 mM KCl, 1.47 mM KH_2PO_4 , 8.1 mM Na_2HPO_4 (pH 7.35), 0.9 mM $\text{CaCl}_2 \cdot \text{H}_2\text{O}$, 0.5 mM $\text{MgCl}_2 \cdot \text{H}_2\text{O}$). Samples were acid treated with 20 μL of 1 N HCl to decrease the pH to ~ 2.5 , followed by incubation at room temperature for 15 min. Samples then were neutralized with 20 μL 1 N NaOH.

2.4. Modified extraction procedure for BDNF from mouse brain tissue

Tissue samples were obtained and stored as described above. Prior to analysis, samples were removed from the freezer and weighed. Lysis buffer—(100 mM PIPES (pH 7), 500 mM NaCl, 0.2% Triton X-100, 0.1% NaN_3 , 2% BSA, 2 mM EDTA- $\text{Na}_2 \cdot 2\text{H}_2\text{O}$, 200 μM PMSF (frozen in isopropanol), 10 μM leupeptin (frozen separately in deionized water), 0.3 μM aprotinin (frozen separately in 0.01 M HEPES (pH 8) and 1 μM pepstatin (frozen separately in DMSO)) (LeMaster et al., 1999; Pollock et al., 2001)—was then pipetted into each tube (2 mL for unilateral hippocampus, bilateral frontal cortex or unilateral brain stem bisected on the midline). Samples were homogenized as described above, after which an additional 2 mL of lysis buffer was added to the samples and they were resonicated. Samples were split and one-half of each sample was spiked (increasing the concentration of BDNF by 250 pg/mL to determine percent recovery). Samples were centrifuged for 30 min at $16,000 \times g$ at 4°C . Supernatants were then removed and frozen at -70°C until analysis.

2.5. Studies on *trkB* and phosphatase

Hippocampal samples were obtained, stored, lysed and homogenized as described in Section 2.4 with the exception that 2% BSA was omitted from the lysis buffer. Following the second homogenization step, 3–500 μL aliquots (groups A, B, and C) were removed from each sample. Twenty-five microliter aliquots of lysis buffer were added to the samples in group A (control). Three microliter aliquots of 250 $\mu\text{g}/\text{mL}$ *trkB* antibody (Transduction Laboratories #T16020) and 22 μL of lysis buffer were added to the samples in group B (*trkB*) and 25 μL aliquots of alkaline phosphatase (Sigma, #P-4252) were added to the samples in group C. All samples then were centrifuged at $16,000 \times g$ for 30 min at 4°C .

2.6. BDNF enzyme-linked immunosorbant assay

The Promega BDNF Emax ImmunoAssay System was employed to measure the amount of BDNF in each sample (Promega Co., Madison, WI). Each well of a 96-well polystyrene plate was incubated overnight at 4 °C with 100 μ L anti-BDNF monoclonal antibody (mAb) diluted 1:1000 in carbonate coating buffer (25 mM sodium bicarbonate and 25 mM sodium carbonate, pH 9.7). Unadsorbed mAb was removed and plates were washed once with TBST wash buffer (20 mM Tris–HCl (pH 7.6), 150 mM NaCl and 0.05% (v/v) Tween 20). Just prior to blocking, tissue extracts were removed from the freezer and allowed to come to room temperature. Plates were blocked using 200 μ L Promega 1X Block and Sample buffer followed by incubation for 1 h at room temperature. Plates were then washed using TBST wash buffer. One hundred microliter of each sample or standard (1000, 750, 500, 400, 300, 200, 100, 0 pg/mL) were added in triplicate to the plates. Plates were incubated for 2 h with shaking (~600 rpm) at room temperature. Plates were then washed five times with TBST wash buffer. Anti-human BDNF polyclonal antibody (pAb) (100 μ L diluted 1:500 in 1X Block and Sample) was added to each well and plates were incubated for 2 h with shaking (~600 rpm) at room temperature. Plates were washed again five times using TBST wash buffer. Anti-IgY horseradish peroxidase conjugate (100 μ L diluted 1:200 in 1X Block and Sample) was then added to each well and plates were incubated for 1 h with shaking (~600 rpm) at room temperature. Plates were emptied again and washed using TBST wash buffer. Finally, plates were developed using 100 μ L Promega TMB One Solution and the reaction was stopped using 100 μ L 1 N HCl. Absorbance was measured at 450 nm. BDNF levels are reported in ng/g wet weight tissue (ng/g, ww) \pm S.E.M.

2.7. Surgery to implant guide cannulae for microdialysis

Adult male mice (30–40 g) were anesthetized with Avertin administered in a volume of 20 mL/kg, by the intraperitoneal (ip) route (Papaioannou and Fox, 1993). The eyes were protected with sterile ophthalmic ointment (NLS Animal Health, Baltimore, MD). The skin over the skull was shaved, sterilized with Betadine and alcohol, incised and the exposed skull was cleaned and dehydrated with 10% H₂O₂. Mice were placed on a stereotaxic frame equipped with a mouse palate adapter and a burr hole was drilled (1 mm diameter). A guide cannula for a CMA/7 microdialysis probe (CMA/Microdialysis, Chelmsford, MA) was implanted into the striatum or frontal cortex using coordinates determined from mouse atlases (Slotnick and Leonard, 1975; Franklin and Paxinos, 1997), and refined by empirical determination (coordinates relative to Bregma for striatum: A + 0.6, L-1.8, V-2.5 and for frontal cortex: A + 2.1, L-0.6, V-1.5). The skin and exposed skull surrounding the guide cannula were sealed with a fast drying two-part epoxy (Loctite, Fastneal

State College, PA) that held the cannula in place. Immediately following surgery, mice were individually housed and allowed to recover for 3–5 days prior to dialysis. After dialysis, mice were sacrificed by cervical dislocation and brains were removed for histological confirmation of probe placement.

2.8. Microdialysis

The night before dialysis, mice were lightly anesthetized with 80 mg/kg ketamine and 10 mg/kg xylazine injected in a volume of 8 mL/kg, ip. Dialysis probes (CMA/7, 2 mm length \times 240 μ m diameter cuprophane, 6000 MW cutoff) were inserted slowly and perfused overnight with artificial cerebrospinal fluid (aCSF) (147 mM NaCl, 3.5 mM KCl, 1.0 mM CaCl₂, 1.2 mM MgCl₂, 1.0 mM NaH₂PO₄, 25 mM NaHCO₃ (pH 7.4), modified from Trillat et al. (1997)) at a rate of 1.1 μ L/min. After a 10-h equilibration period, six baseline samples were collected at 20-min intervals and analyzed immediately by online high performance liquid chromatography with electrochemical detection (HPLC/ED; see Section 2.10 for details).

2.9. Zero net flux

Neurotransmitter recovery from the brain using in vivo microdialysis is <100% and varies between individual probes and animals. Furthermore, in vitro recovery (which is readily estimated) does not accurately reflect in vivo recovery (which is much more difficult to determine). Finally, recovery can change with alterations in active processes at the synapse. Specifically, reductions in 5-HT uptake are thought to result in decreases in the recovery of 5-HT via the microdialysis membrane (Smith and Justice, 1994). Strictly speaking, in vivo recovery cannot be determined from microdialysis measurements alone, however, it can be estimated using the method of zero net flux to determine a related quantity called extraction fraction (Yang et al., 2000; Bungay et al., 2003). Zero net flux involves infusing known concentrations of 5-HT into the microdialysis probe while measuring the 5-HT concentration in the efflux. When these two concentrations are equal, there is no net diffusion across the membrane. A plot of each concentration infused into the probe (C_{in}) versus the difference between C_{in} and the concentration exiting the probe (C_{out}) gives a line whose x -intercept is an estimate of the extracellular 5-HT concentration (C_{ext}). According to zero net flux theory, the slope is the extraction fraction (E_d) (Lonroth et al., 1987; Bungay et al., 1990; Parsons and Justice, 1994).

After collection of baseline samples, four different concentrations of 5-HT ranging from 2 to 20 nM were perfused into the microdialysis probes (Lonroth et al., 1987; Justice, 1993; Cosford et al., 1996). Each concentration was perfused for 90 min using a programmable gradient infusion pump (CMA/Microdialysis, Chelmsford, MA). Artificial cerebrospinal fluid (aCSF) was equilibrated through

the implanted microdialysis probe the night before the experiment. At the same time, aCSF containing the various concentrations of 5-HT was pumped through the dialysis tubing bypassing the animal's head directly into the sample loop for analysis. The resulting chromatograms were compared to standard curves to determine actual C_{in} . Serotonin for zero net flux infusion was diluted immediately prior to analysis in aCSF containing 200 μ M ascorbate as an antioxidant.

2.10. Chromatography

Dialysate samples (20 μ l) were injected immediately after collection onto an Advantage 100 \times 3.0 mm C₁₈ ODS-II HPLC column (Thompson Instrument Co., Chantilly, VA) for separation followed by detection by an ESA 5014B microdialysis cell ($E_1 = -175$ mV, $E_2 = +220$ mV) (ESA Coulochem II, ESA, Inc., Chelmsford, MA). A guard cell (ESA 5020) was placed in line before the injection loop and set at a potential of +350 mV. Basal in vivo levels of extracellular 5-HT are difficult to detect and quantify due to the fact that they are near the limits of detection of most chromatography systems. For the current experiments, sensitivity was improved by reducing the column diameter from 4.6 mm to 3.0 mm (the smallest column diameter that can be used with the ESA 5014B microdialysis cell due to cell volume-filling requirements). This decreased the column volume-dilution factor and band broadening. As a result, basal levels of 5-HT were more easily resolved from baseline noise and the possibility of detecting decreases in basal extracellular 5-HT existed.

The mobile phase consisted of 0.1 M monochloroacetic acid, 1.5–2.5 mM 1-octanesulfonic acid, 5.5×10^{-3} M ethylenediaminetetracetic acid, 6–12% acetonitrile and 0.3% triethylamine in a volume of 1 L (pH 2.6) (Andrews et al., 1996). Characteristic retention times for 5-HIAA and 5-HT were approximately 7 and 12 min, respectively. Peak areas were integrated using HP ChemStation software (Hewlett Packard, Waldbronn, Germany) and quantified against known standards. Concentrations are expressed in nM \pm S.E.M. Limits of detection for 5-HT were 0.1 nM (two times signal to noise).

2.11. Chemicals

Components of the mobile phase, artificial cerebrospinal fluid and neurotransmitter standards were of HPLC grade or the highest quality obtainable from Sigma–Aldrich (St. Louis, MO). All chemicals used to make the BDNF extraction buffers and TBST wash buffer were also obtained from Sigma–Aldrich. The trkB monoclonal antibody was purchased from Transduction Laboratories (Lexington, KY) and alkaline phosphatase was obtained from Sigma–Aldrich. All other chemicals used to perform ELISA assays were obtained from Promega Co.

2.12. Statistical analysis

Data were analyzed by one- or two-way analysis of variance (ANOVA) with treatment or genotype and sex as the independent variables using the Statistical Analysis System (SAS Institute, Cary, NC). Zero net flux data were initially analyzed by linear regression to generate slopes and x -intercepts for individual animals. A priori significant differences between genotypes or treatment groups and their respective control group means are indicated by t -test probabilities. All values are expressed as means \pm S.E.M. with $p < 0.05$ considered statistically significant.

3. Results

3.1. Modified procedure for extraction of BDNF protein from brain tissue is superior to the original extraction procedure

Initially, the effects of reduced SERT expression on hippocampal BDNF levels were investigated using the extraction procedure provided by Promega. Under these conditions, no differences in BDNF levels were detected with respect to genotype [$F(2,15) = 0.02$; $p = 0.98$]. BDNF levels were 19.6 ± 2.2 , 19.2 ± 2.6 and 19.6 ± 2.1 ng BDNF/g ww in SERT^{+/+}, SERT^{+/-} and SERT^{-/-} mice, respectively (Table 1). However, the amount of BDNF extracted from the tissue was low compared to some reports of BDNF levels as high as ~ 200 ng BDNF/g ww (Radka et al., 1996). Spiking the tissue with a known amount of BDNF showed that there was only 1–10% recovery of BDNF from hippocampal samples using the Promega procedure.

After investigating different conditions of pH, detergent and salt concentrations, a PIPES buffer at pH 7 with a higher salt concentration and Triton X-100 as the detergent instead of NP-40 was employed. Using this buffer, the amount of BDNF extracted from tissue samples was increased; however, no differences were detected in BDNF levels in SERT knockout mice with respect to genotype [$F(2,3) = 2.76$; $p = 0.21$]. In this case, BDNF levels were 55.4 ± 4.9 , 67.0 ± 3.2 and 51.5 ± 1.4 ng BDNF/g ww in SERT^{+/+}, SERT^{+/-} and SERT^{-/-} mice, respectively (Table 1). Since these levels were still lower than some reported in the literature, 2% BSA was added to the extraction buffer (Radka et al., 1996; Kolbeck et al., 1999; LeMaster et al., 1999). Addition of BSA further increased the amount of BDNF extracted from the tissue; however, no differences again were observed across the genotypes of SERT knockout mice [$F(2,21) = 0.24$; $p = 0.79$]. In the latter case, BDNF levels were 143 ± 13 , 161 ± 27 and 151 ± 10 ng BDNF/g ww in SERT^{+/+}, SERT^{+/-} and SERT^{-/-} mice, respectively (Table 1). Intra-assay and inter-assay variability in percent recovery using the modified extraction procedure has been found to range from ~ 70 –90% depending on the brain region and sex of the mice studied (Table 2). For the remainder of the experiments, with

Table 1

Amount of BDNF protein extracted from the hippocampus of SERT knockout mice using three different extraction procedures with the Promega BDNF Emax ImmunoAssay System

Genotype	BDNF (ng/g, ww)		
	Original extraction procedure ^a	Modified extraction procedure w/o BSA ^a	Modified extraction procedure w/BSA ^a
SERT+/+	19.6 ± 2.2	55.4 ± 4.9	143 ± 13
SERT+/-	19.2 ± 2.6	67.0 ± 3.2	161 ± 27
SERT-/-	19.6 ± 2.1	51.5 ± 1.4	151 ± 10

A three-fold increase in BDNF levels can be seen using the modified extraction procedure without BSA in the lysis buffer ($n = 2/\text{genotype}$) and an eight-fold increase in the amount of BDNF extracted from the tissue is observed using the modified extraction procedure with BSA ($n = 8/\text{genotype}$) compared to the original extraction procedure ($n = 6/\text{genotype}$).

^a Data not corrected for recovery in 4-month-old female mice.

the exception of the study on the effects of *trkB* and phosphatase, brain tissue was extracted using this modified extraction buffer with the addition of BSA and all samples were corrected for percent recovery.

3.2. Effects of *trkB* and phosphatase on BDNF protein levels

During the course of studies aimed at improving the analysis of BDNF protein in brain tissue, two hypotheses regarding factors that could alter recovery or detection of BDNF were tested. First, previous studies suggested that BDNF protein might bind tightly to its membrane-associated receptor protein, *trkB*, such that reduced amounts of BDNF would be extracted into the soluble portion of the homogenate (Marsh et al., 1993; Altar et al., 1994). To test this hypothesis, tissue homogenates were preincubated with a monoclonal antibody to *trkB*. Second, although specific phosphorylation sites have not been reported for BDNF (Swiss-Prot website), serine, threonine and tyrosine residues are present in the mature protein. To dephosphorylate any forms of BDNF that may not be recognized by the Promega monoclonal or polyclonal BDNF antibodies, homogenates were preincubated with phosphatase. In both cases, experiments were performed without BSA so that it would not interfere with the phosphatase reaction.

The results of these experiments are shown in Fig. 1. One-way ANOVA indicated a statistically significant difference between treatment groups [$F(2,15) = 7.10$, $p < 0.01$]; however, no differences in hippocampal BDNF levels between samples that had been incubated with the *trkB* antibody and

those prepared in its absence were detected ($p = 0.9$). On the other hand, phosphatase treatment yielded a 25% decrease in the amount of BDNF determined ($p < 0.01$). Treatment with phosphatase was hypothesized to lead to an increase in the amount of BDNF detected if phosphorylated forms of BDNF were present. Since the reverse occurred, the possibility exists that phosphatase itself interferes with the antibody recognition/binding necessary for ELISA detection of BDNF.

3.3. BDNF protein levels in BDNF^{+/-} mice are reduced by 50% in hippocampus, frontal cortex and brain stem

As a final assessment of the modified extraction procedure, BDNF protein levels were analyzed in BDNF^{+/-} mice. Kolbeck et al. previously reported a 50% decrease in protein expression in 21-day-old BDNF^{+/-} mice using mAbs developed by these investigators for use in a sandwich ELISA (Kolbeck et al., 1999). For the present experiment, the Promega BDNF Emax Immunoassay system combined with the modified extraction buffer including BSA was used to determine the total amount of BDNF protein in hippocampus, frontal cortex and brain stem in 4-month-old BDNF^{+/+} and BDNF^{+/-} mice. All samples were corrected for percent recovery. The data pictured in Fig. 2 show a significant decrease in BDNF levels on the order of 50% in hippocampus [$F(1,10) = 63.0$, $p < 0.001$], frontal cortex [$F(1,9) = 32.1$, $p < 0.001$] and brain stem [$F(1,10) = 45.2$, $p < 0.001$] in BDNF^{+/-} compared to BDNF^{+/+} mice.

Table 2

The intra- and inter-assay variability of percent recovery of BDNF in hippocampus ($n = 24$ mice or 6 assays performed on different days, respectively), frontal cortex ($n = 24$, 5), and brain stem ($n = 24$, 5) using the modified extraction procedure and the Promega BDNF Emax Immunoassay System

	Intra-assay % recovery ± SEM		Inter-assay % recovery ± SEM Female and male
	Female	Male	
Hippocampus	70 ± 3	68 ± 3	70 ± 3
Frontal cortex	93 ± 2	83 ± 3	80 ± 4
Brain stem	79 ± 2	77 ± 2	73 ± 4

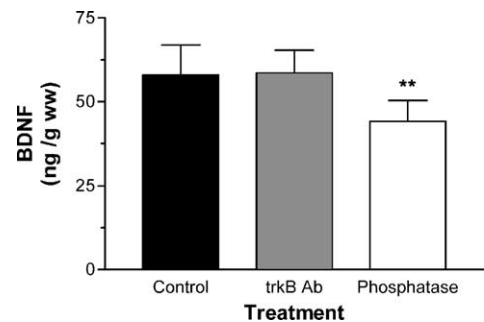


Fig. 1. BDNF protein levels in hippocampus measured by ELISA after treatment with *trkB* antibody (Ab) ($n = 6$) or phosphatase ($n = 6$) compared to control ($n = 6$). ** $p < 0.01$ for differences from control values.

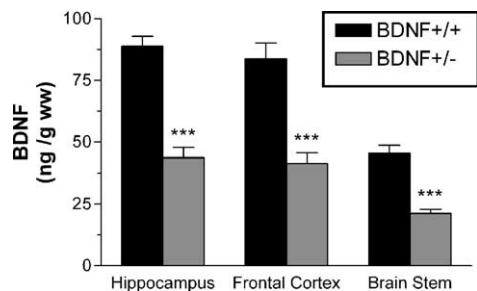


Fig. 2. Amount of BDNF protein in hippocampus ($n = 5, 7$), frontal cortex ($n = 6, 6$) and brain stem ($n = 6, 6$) quantified from BDNF^{+/+} and BDNF^{+/-} mice, respectively. *** $p < 0.001$ for differences from BDNF^{+/+} mice.

3.4. Determination of BDNF protein levels corrected for recovery in SERT knockout mice: effects of genotype and gender

After determining that the conditions outlined in the previous sections could be used to quantify differences in BDNF protein levels in mouse brain homogenates, these assay conditions were used to investigate BDNF levels in additional brain regions in female and male 4-month-old SERT^{+/+}, SERT^{+/-} and SERT^{-/-} mice. Previous studies have demonstrated that differences in receptor expression and function, and in behavior exist between female and male SERT knockout mice (Li et al., 2000; Holmes et al., 2003). The modified extraction buffer with BSA was utilized and all samples were corrected for percent recovery. BDNF protein levels in female and male SERT knockout mice were analyzed by brain region using two-way ANOVA (Fig. 3). The interaction between genotype and sex proved nonsignificant in hippocampus [$F(2,42) = 0.97, p = 0.39$], frontal cortex [$F(2,42) = 0.14, p = 0.87$] and brain stem [$F(2,42) = 1.43, p = 0.25$] indicating

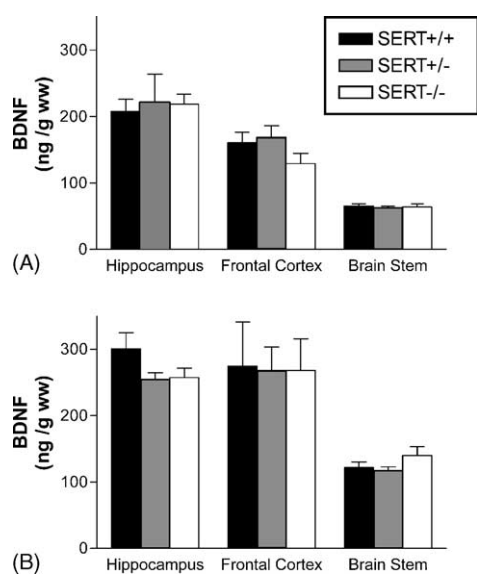


Fig. 3. BDNF protein levels measured by ELISA in (A) female and (B) male SERT^{+/+}, SERT^{+/-} and SERT^{-/-} mice ($n = 8$ for all groups).

that BDNF levels varied in a similar manner with respect to genotype and sex. The main effect of genotype was not significant in hippocampus [$F(2,42) = 0.32, p = 0.72$], frontal cortex [$F(2,42) = 0.18, p = 0.84$] and brain stem [$F(2,42) = 1.53, p = 0.23$] demonstrating that alterations in SERT expression did not appear to influence BDNF protein levels (Fig. 3). On the other hand, the main effect of sex was highly significant in hippocampus [$F(2,42) = 8.24, p < 0.01$], frontal cortex [$F(2,42) = 14.26, p < 0.0001$] and brain stem [$F(2,42) = 116.5, p < 0.0001$], the latter illustrating that overall, female mice have significantly lower levels of BDNF protein in the three brain regions examined compared to male mice ($p < 0.05$ between SERT^{+/+}, SERT^{+/-} or SERT^{-/-} female and male mice in hippocampus, frontal cortex and brain stem). Together, these results and the preliminary data shown in Table 1 indicate that constitutive reductions in serotonin transporter expression are not associated with homeostatic alterations in BDNF protein expression in brain regions associated with the mechanism of action of antidepressant drugs.

3.5. Basal dialysate levels of 5-HT in BDNF^{+/-} mice by in vivo microdialysis

To determine whether constitutive decreases in BDNF expression influence extracellular 5-HT levels, basal dialysate concentrations of 5-HT in striatum and frontal cortex were investigated in mice with reduced BDNF expression. Striatum was chosen for study initially because of its relative large size in mouse brain and its involvement in the modulation of locomotor activity and reward-related behavior. Frontal cortex was investigated due to the age-accelerated loss of 5-HT axons observed in this brain region in BDNF^{+/-} mice and its involvement in antidepressant-related changes in BDNF mRNA (Nibuya et al., 1995, 1996; Lyons et al., 1999). No statistically significant differences in dialysate 5-HT were detected in striatum where 5-HT levels were 1.2 ± 0.2 nM in BDNF^{+/+} mice and 1.0 ± 0.4 nM in BDNF^{+/-} mice [$F(1,7) = 0.2; p = 0.7$] (Fig. 4A). Similarly in frontal cortex, dialysate 5-HT in BDNF^{+/+} mice was not significantly different from that determined in BDNF^{+/-} mice (0.8 ± 0.02 nM and 1.0 ± 0.1 nM, respectively, [$F(1,5) = 1.2; p = 0.3$]; Fig. 5A). These results show that basal 5-HT levels without correction for in vivo extraction fraction in striatum or frontal cortex show no significant changes as a result of long-term decreases in BDNF protein expression.

3.6. Zero net flux analysis of extracellular 5-HT levels in BDNF^{+/-} mice

The primary aim of this experiment was to determine whether changes in extracellular 5-HT levels in mice with decreased BDNF expression are of a more subtle nature such that they can only be detected by zero net flux (Gainetdinov et al., 1999; Fedele et al., 2001). Previous studies have illustrated a behavioral and biochemical phenotype in adult BDNF^{+/-} mice consistent with altered serotonergic

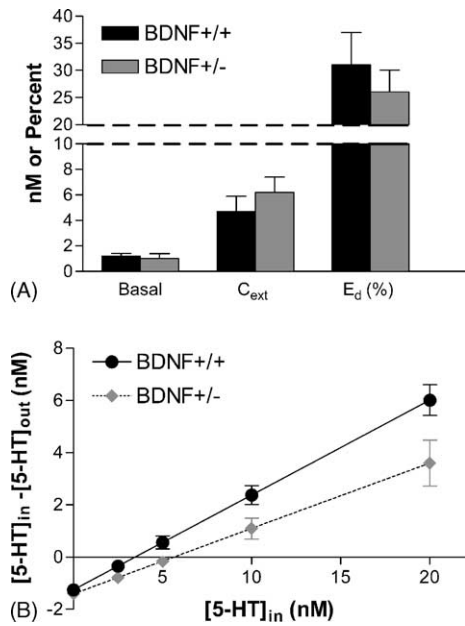


Fig. 4. In vivo microdialysis in striatum in BDNF knockout mice. (A) Mean striatal extracellular 5-HT concentrations in BDNF^{+/+} and BDNF^{+/-} mice (*n* = 6, 4, respectively) without and with correction for in vivo extraction fraction and extraction fraction expressed as percent. (B) Serotonin zero net flux regression lines in BDNF^{+/+} and BDNF^{+/-} mice.

neurotransmission (Lyons et al., 1999); therefore, we hypothesized that changes in basal 5-HT levels might underlie these phenotypic alterations. Furthermore, since there is an accelerated age-dependent loss of serotonergic forebrain innervation

in BDNF^{+/-} mice, it was hypothesized that decreased synaptic 5-HT levels in BDNF^{+/-} mice might precede eventual axonal loss. In striatum, however, no statistically significant differences were detected in extracellular 5-HT levels determined by zero net flux between BDNF^{+/+} and BDNF^{+/-} mice (4.7 ± 1.0 nM and 6.2 ± 1.0 nM, respectively, [$F(1,7) = 0.8$; $p = 0.4$]; Fig. 4A and B). In addition, extraction fractions as determined by the slopes of the zero net flux regression lines were calculated. These are thought to be sensitive to changes in the function of the serotonin transporter. BDNF^{+/+} mice showed an average E_d of 0.31 ± 0.06 compared to BDNF^{+/-} mice where E_d was determined to be 0.26 ± 0.04 ($[F(1,7) = 0.5$; $p = 0.5$]; Fig. 4A and B). Thus, changes in extracellular 5-HT levels corrected for in vivo extraction fraction do not appear to occur in striatum in BDNF^{+/-} mice at 8–12 months of age.

Extracellular 5-HT levels in frontal cortex were also determined by the method of zero net flux and were 4.1 ± 1 nM in BDNF^{+/+} mice and 5.0 ± 2 nM in BDNF^{+/-} mice (Fig. 5A and B). No statistically significant differences were observed in C_{ext} values for frontal cortex [$F(1,8) = 0.15$; $p = 0.7$]. Extraction fractions were 0.36 ± 0.06 in BDNF^{+/+} mice versus 0.26 ± 0.03 in BDNF^{+/-} mice ($[F(1,8) = 1.1$; $p = 0.3$]; Fig. 5A and B). Thus, zero net flux also revealed no changes in extracellular 5-HT levels in frontal cortex with respect to alterations in BDNF protein expression.

4. Discussion

A variety of BDNF protein concentrations in hippocampus have been reported by different investigators using the Promega BDNF Emax Immunoassay System including values ranging from 3 ng BDNF/g ww (Scaccianoce et al., 2003) to 9 ng BDNF/g ww (Angelucci et al., 2000) and as high as 15 ng BDNF/g ww (Ickes et al., 2000). On the other hand, others have reported significantly higher levels of BDNF in hippocampus using different antibodies and extraction procedures. In these cases, levels from 60 ng BDNF/g ww (Rudge et al., 1998) to 181 ng BDNF/g ww (Radka et al., 1996) and upwards of 300 ng BDNF/g ww (Kolbeck et al., 1999) have been described. Discrepancies in the amount of BDNF extracted may be related to the *pI* (9.6) of this protein (computed using the Expert Protein Analysis System of the Swiss Institute for Bioinformatics). BDNF is expected to be positively charged at physiological pH while cell membranes are negatively charged leading to possible electrostatic interactions between BDNF protein and plasma membrane fragments in tissue homogenates. This might underlie the difficulty in extracting BDNF into the soluble fraction of tissue extracts. In this study, we demonstrate an improvement in the amount of BDNF extracted from brain tissue from 20 ng BDNF/g ww using the original Promega extraction procedure to 250 ng BDNF/g ww using the modified extraction buffer with correction for recovery. Changes in the extraction solution

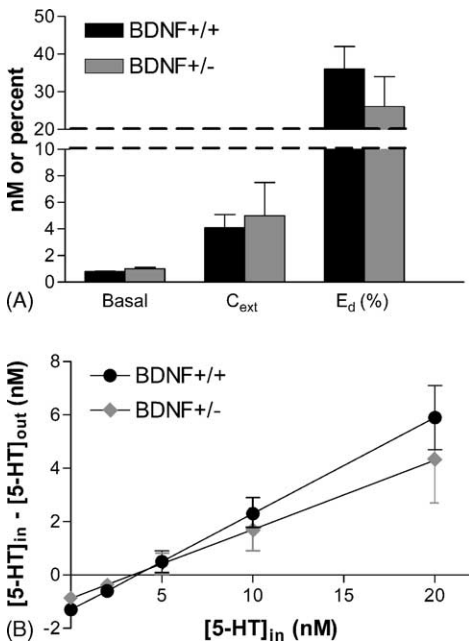


Fig. 5. In vivo microdialysis in frontal cortex in BDNF knockout mice. (A) Mean cortical extracellular 5-HT concentrations in BDNF^{+/+} and BDNF^{+/-} mice (*n* = 4, 5, respectively) without and with correction for in vivo extraction fraction and extraction fraction expressed as percent. (B) Serotonin in zero net flux regression lines in BDNF^{+/+} and BDNF^{+/-} mice.

included a higher NaCl concentration (500 mM compared with 137 mM). At relatively low salt levels, the solubility of proteins increases with increasing salt concentration, since salt stabilizes charged groups on proteins, increasing solubility in aqueous solution. Also, by adding 2% BSA to the extraction buffer, BDNF protein encounters a 'protein-like' environment in the soluble fraction for better extraction. However, this precludes reporting BDNF levels as a function of protein concentration, since the addition of BSA confounds protein determination (Lowry et al., 1951; Vitalis et al., 2002).

The Promega ELISA system with our modified extraction procedure was evaluated using BDNF knockout mice to determine if the expected 50% decrease in BDNF protein levels in BDNF^{+/-} mice compared to BDNF^{+/+} mice could be accurately detected. Both BDNF mRNA and protein levels have been determined by others in BDNF knockout mice (Ernfors et al., 1994; Korte et al., 1995; Altar et al., 1997, 2003; Kolbeck et al., 1999). In two studies, a 50% decrease in BDNF mRNA in adult BDNF^{+/-} mice was detected using Northern blot analysis in the cortex (Ernfors et al., 1994) and hippocampus (Korte et al., 1995). Protein levels in BDNF^{+/-} mice have also been reported to be decreased by ~50% using three different sandwich ELISA procedures in postnatal day-21 mice in cerebellum, cortex, hippocampus, striatum, septum, olfactory bulb, and spinal cord (Kolbeck et al., 1999), as well as in the hippocampus of adult animals (Altar et al., 1997, 2003). Using our procedure, we similarly found a 50% decrease in all brain regions studied confirming the modified extraction procedure combined with the commercially-available ELISA kit by Promega as a valid method for determining BDNF protein in mouse brain homogenates.

When we applied this method to the measurement of BDNF in SERT knockout mice, we found no significant differences in BDNF protein levels with respect to genotype in any of the brain regions analyzed in female or male mice. We hypothesized that the increases in extracellular 5-HT occurring in these mice (Fabre et al., 2000a; Fedele et al., 2001) (see also accompanying manuscript by Mathews et al.) would be associated with elevated BDNF levels, particularly in hippocampus and frontal cortex. This hypothesis was based on previous studies proposing that serotonin transporter-mediated increases in serotonergic neurotransmission activate 5-HT_{4,6,7} receptor subtypes, which are positively coupled to adenylate cyclase and protein kinase-A. Subsequent increases in the phosphorylation of cAMP response element binding protein (CREB) are thought to upregulate BDNF expression (Nibuya et al., 1996; Duman et al., 1997, 1999).

In actuality, reports on the modulation of BDNF at the transcriptional level following chronic treatment specifically with serotonin reuptake inhibiting antidepressants have been conflicting. One study found a 20% increase in BDNF mRNA by Northern blot analysis in the hippocampus of rats when gene expression was measured 3 h after the last dose of sertraline administered for 3 weeks (Nibuya et al., 1995). A similar study found that BDNF mRNA, analyzed by Northern

blot was increased by 50% in the hippocampus of rats given fluoxetine for 10 days when gene expression was measured 18 h after the last dose of drug (Nibuya et al., 1996). This study also showed by in situ hybridization that BDNF was increased by 60% in the dentate gyrus and CA3 regions of the hippocampus with a larger increase (160%) in the CA1 region of the hippocampus (Nibuya et al., 1996). However, later studies have demonstrated a 30% decrease in BDNF mRNA expression in the dentate gyrus of rats when gene expression was measured 4 h after the last dose of fluoxetine administered for two weeks. In the same study, when gene expression was measured 24 h after fluoxetine, a 30% increase in BDNF mRNA by in situ hybridization was reported (Coppell et al., 2003). Finally, a recent study found no changes in individual BDNF mRNA splice variants in rats when fluoxetine was administered for 21 days and the animals were sacrificed 2 h after the last dose of drug (Dias et al., 2003). Furthermore, Conti et al. also demonstrated no differences in the levels of BDNF mRNA in the frontal cortex or hippocampus of mice administered 21 days of fluoxetine and killed 24 h after the last dose of drug using an RNase protection assay (Conti et al., 2002).

Thus, numerous studies on the relationship between BDNF and chronic SRI treatment have focused on assessing the modulation of BDNF at the level of transcription with mixed results. On the other hand, few investigations have been conducted on BDNF protein levels after SRI administration. In humans, serum levels of BDNF protein were reduced in unmedicated depressed patients compared to healthy controls, while BDNF protein levels measured in depressed patients receiving different types of antidepressant treatments were not significantly different from control individuals (Shimizu et al., 2003). In experimental animals, Altar et al. reported no elevations in BDNF protein levels in rats in hippocampus or frontal cortex after 14 days of fluoxetine with the animals sacrificed 3 h after the last injection of drug using another manufacturer's ELISA to quantify BDNF protein (Altar et al., 2003).

Therefore, only certain classes of antidepressants may cause a robust upregulation of BDNF and this may manifest itself principally at the level of transcription. Furthermore, SRIs which presumably act by increasing extracellular 5-HT may be weak modulators of BDNF expression. The possibility also exists that in SERT knockout mice, BDNF is only upregulated in specific subregions of the brain regions investigated and by homogenizing the entire tissue sample, we were unable to detect these subtle changes. Finally, since SERT^{-/-} and SERT^{+/-} mice most likely have increased extracellular 5-HT throughout development (Salichon et al., 2001; Vitalis et al., 2003), compensatory changes may occur in these mice including the regulation of specific receptor subtypes that are substantively different from those occurring in adult animals treated with serotonin reuptake inhibiting drugs. Thus, BDNF may be differentially regulated in SERT knockout mice versus SRI-treated animals. Interestingly, significant decreases in BDNF protein levels

were observed in female SERT knockout mice compared to male SERT knockout mice irrespective of genotype and brain region. However, other studies have reported that BDNF levels in various brain regions are higher in female rats or deer mice versus male counterparts (Kellogg et al., 2000; Turner and Lewis, 2003). Therefore, BDNF protein levels in female versus male animals may be more a function of species or strain differences than indicative of general alterations in expression with respect to sex.

In the present investigation, BDNF^{+/-} mice afforded the opportunity to examine the interactions of BDNF and the serotonin neurotransmitter system in a constitutive model of reduced BDNF expression (Nibuya et al., 1995, 1996; Zetterstrom et al., 1999). Since acute administration of antidepressants does not by itself alleviate the symptoms of depression or anxiety, many investigators have focused on studying long-term neuroadaptation in upstream and downstream signaling cascades, as well as alterations in the expression of proteins that control neuronal plasticity in an attempt to better understand the mechanisms underlying antidepressant actions. Thus far, however, most research involving altering BDNF levels directly has focused on parenchymal administration of this protein (Nibuya et al., 1995, 1996; Siuciak et al., 1996; Zetterstrom et al., 1999; Goggi et al., 2002).

The *in vivo* microdialysis data from this study demonstrate that a 50% reduction in BDNF expression does not appear to be associated with changes in basal extracellular 5-HT concentrations (with or without correction for extraction fraction) in frontal cortex or striatum. Extracellular 5-HT levels measured by microdialysis represent the overall homeostatic contributions of the release and reuptake processes. Thus, the present microdialysis data suggest the following alternate interpretations: (1) Constitutive decreases in BDNF expression have no effect to modulate serotonergic neurotransmission, (2) Changes in extracellular 5-HT in BDNF^{+/-} mice were too small to be detected even with zero net flux microdialysis methods or (3) Serotonin neurotransmitter signaling has adapted to reduced BDNF expression such that global homeostatic alterations in extracellular 5-HT levels do not occur.

Although interpretations 1 and 2 cannot be ruled out, evidence for an altered phenotype in BDNF^{+/-} mice lends support for the third interpretation. Among other aspects, BDNF^{+/-} mice are characterized by increased aggression and obesity, both of which have been associated with dysfunction in the 5-HT system (Coccaro and Murphy, 1990; Gingrich and Hen, 2001). Therefore, alterations in activity-dependent 5-HT release independent of overall changes in basal extracellular 5-HT may modulate serotonin receptor-mediated signaling in these mice. Furthermore, the hyperaggressive and hyperphagic aspects of the behavioral phenotype in BDNF^{+/-} mice are strikingly similar to 5-HT_{1B} and 5-HT_{2C} receptor knockout mice, respectively (Hen, 1996; Holmes et al., 1997). In BDNF^{+/-} mice, 5-HT_{1B} and 5-HT_{2C} mRNA is upregulated in hypothalamus, while hippocampal 5-HT_{2C} mRNA levels are downregulated (Lyons et al.,

1999). Thus, changes in 5-HT receptor expression and function in BDNF^{+/-} mice may result from the effects of decreased BDNF levels on activity-dependent processes.

In conclusion, the present study illustrates that a 50% decrease in BDNF levels is not sufficient to cause measurable changes in extracellular 5-HT levels in striatum or frontal cortex. Although increased BDNF levels have been shown to modulate 5-HT turnover and survival of serotonergic axons, we were unable to demonstrate that reductions in BDNF expression result in decreased serotonergic neurotransmission as evidenced by basal extraneuronal 5-HT levels. Lyons et al. showed that 5-HT fiber density, and 5-HT and 5-HIAA levels in tissue were not significantly altered in BDNF^{+/-} mice until they were greater than 12 months of age (Lyons et al., 1999). BDNF^{+/-} mice between 12 and 18 months show a progressive loss of 5-HT innervation to the forebrain suggesting that BDNF is involved in the maintenance of serotonergic axons throughout adult life (Lyons et al., 1999). Experiments are in progress to assess cortical, as well as hippocampal basal and K⁺-stimulated extracellular 5-HT levels in BDNF^{+/-} mice greater than 12 months of age to determine whether changes in synaptic 5-HT, instead of preceding a loss of axon density are concomitant with the neurodegenerative process. The hippocampus is of particular interest because others have demonstrated SRI-mediated neurogenesis in the subgranular zone of the hippocampus of adult animals (Santarelli et al., 2003).

Thus, BDNF^{+/-} mice provide opportunities to investigate factors modulating the age-dependent loss of serotonergic neurons and to determine whether serotonergic signaling is altered as a result. SERT knockout mice present similar prospects for examining age-dependent modulation of BDNF protein levels under conditions of altered serotonin neurotransmission.

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References

- Altar CA, Cai N, Bliven T, Juhasz M, Conner JM, Acheson AL, et al. Anterograde transport of brain-derived neurotrophic factor and its role in the brain. *Nature* 1997;389:856–60.
- Altar CA, Siuciak JA, Wright P, Ip NY, Lindsay RM, Wiegand SJ. In situ hybridization of trkB and trkC receptor mRNA in rat forebrain and association with high-affinity binding of [¹²⁵I]BDNF [¹²⁵I]NT-4/5 and [¹²⁵I]NT-3. *Eur J Neurosci* 1994;6:1389–405.

- Altar CA, Whitehead RE, Chen R, Wortwein G, Madsen TM. Effects of electroconvulsive seizures and antidepressant drugs on brain-derived neurotrophic factor protein in rat brain. *Biol Psychiatry* 2003;54:703–9.
- Andrews AM, Ladenheim B, Epstein CJ, Cadet JL, Murphy DL. Transgenic mice with high levels of superoxide dismutase activity are protected from the neurotoxic effects of 2'-NH₂-MPTP on serotonergic and noradrenergic nerve terminals. *Mol Pharmacol* 1996;50:1511–9.
- Angelucci F, Aloe L, Vasquez PJ, Mathe AA. Mapping the differences in the brain concentration of brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) in an animal model of depression. *Neuroreport* 2000;11:1369–73.
- Bengel D, Murphy DL, Andrews AM, Wichems CH, Feltner D, Heils A, et al. Altered brain serotonin homeostasis and locomotor insensitivity to 3,4-methylenedioxymethamphetamine (“Ecstasy”) in serotonin transporter-deficient mice. *Mol Pharmacol* 1998;53:649–65.
- Bungay PM, Morrison PF, Dedrick RL. Steady-state theory for quantitative microdialysis of solutes and water in vivo and in vitro. *Life Sci* 1990;46:105–19.
- Bungay PM, Newton-Vinson P, Isele W, Garris PA, Justice JB. Microdialysis of dopamine interpreted with quantitative model incorporating probe implantation trauma. *J Neurochem* 2003;86:932–46.
- Caspi A, Sugden K, Moffitt TE, Taylor A, Craig IW, Harrington H, et al. Influence of life stress on depression: moderation by a polymorphism in the 5-HTT gene. *Science* 2003;301:386–9.
- Coccaro EF, Murphy DL. Serotonin in major psychiatric disorders. Washington, D.C.: American Psychiatric Press, Inc.; 1990.
- Conner JM, Lauterborn JC, Yan Q, Gall CM, Varon S. Distribution of brain-derived neurotrophic factor (BDNF) protein and mRNA in the normal adult rat CNS: evidence for anterograde axonal transport. *J Neurosci* 1997;17:2295–313.
- Conti AC, Cryan JF, Dalvi A, Lucki I, Blendy JA. cAMP response element-binding protein is essential for the upregulation of brain-derived neurotrophic factor transcription, but not the behavioral or endocrine responses to antidepressant drugs. *J Neurosci* 2002;22:3262–8.
- Coppell AL, Pei Q, Zetterstrom TS. Bi-phasic change in BDNF gene expression following antidepressant drug treatment. *Neuropharmacology* 2003;44:903–10.
- Cosford RJ, Vinson AP, Kukoyi S, Justice Jr JB. Quantitative microdialysis of serotonin and norepinephrine: pharmacological influences on in vivo extraction fraction. *J Neurosci Methods* 1996;68:39–47.
- Dias BG, Banerjee SB, Duman RS, Vaidya VA. Differential regulation of brain-derived neurotrophic factor transcripts by antidepressant treatments in the adult rat brain. *Neuropharmacology* 2003;45:553–63.
- Dluzen DE, Anderson LI, McDermott JL, Kucera J, Walro JM. Striatal dopamine output is compromised within +/- BDNF mice. *Synapse* 2002;43:112–7.
- Dluzen DE, Story GM, Xu K, Kucera J, Walro JM. Alterations in nigrostriatal dopaminergic function within BDNF mutant mice. *Exp Neurol* 1999;160:500–7.
- Duman RS, Heninger GR, Nestler EJ. A molecular and cellular theory of depression. *Arch Gen Psychiatry* 1997;54:597–606.
- Duman RS, Malberg J, Thome J. Neural plasticity to stress and antidepressant treatment. *Biol Psychiatry* 1999;46:1181–91.
- Ernfors P, Lee KF, Jaenisch R. Mice lacking brain-derived neurotrophic factor develop with sensory deficits. *Nature* 1994;368:147–50.
- Fabre V, Beaufour C, Evrard A, Rioux A, Hanoun N, Lesch KP, et al. Altered expression and functions of serotonin 5-HT_{1A} and 5-HT_{1B} receptors in knock-out mice lacking the 5-HT transporter. *Eur J Neurosci* 2000a;12:2299–310.
- Fabre V, Boutrel B, Hanoun N, Lanfumey L, Fattaccini CM, Demeneix B, et al. Homeostatic regulation of serotonergic function by the serotonin transporter as revealed by nonviral gene transfer. *J Neurosci* 2000b;20:5065–75.
- Fedele DE, Mathews TA, Andrews AM. Quantitative microdialysis for serotonin in striatum and frontal cortex of genetically altered mice. In: O'Connor WT, Ed. Proceedings of the Ninth International Conference on In Vivo Methods; Dublin, Ireland 2001. p. 97–8.
- Franklin KB, Paxinos G. The mouse brain in stereotaxic coordinates. San Diego, CA: Academic Press; 1997.
- Gainetdinov RR, Jones SR, Caron MG. Functional hyperdopaminergia in dopamine transporter knockout mice. *Biol Psychiatry* 1999;46:303–11.
- Gingrich JA, Hen R. Dissecting the role of the serotonin system in neuropsychiatric disorders using knockout mice. *Psychopharmacology (Berl)* 2001;155:1–10.
- Goggi J, Pullar IA, Carney SL, Bradford HF. Modulation of neurotransmitter release induced by brain-derived neurotrophic factor in rat brain striatal slices in vitro. *Brain Res* 2002;941:34–42.
- Greenberg BD, Li Q, Lucas FR, Hu S, Sirota LA, Benjamin J, et al. Association between the serotonin transporter promoter polymorphism and personality traits in a primarily female population sample. *Am J Med Genet* 2000;96:202–16.
- Hefti F, Knusel B, Lapchak PA. Protective effects of nerve growth factor and brain-derived neurotrophic factor on basal forebrain cholinergic neurons in adult rats with partial fimbrial transections. *Prog Brain Res* 1993;98:257–63.
- Hen R. Mean genes. *Neuron* 1996;16:17–21.
- Hervas I, Artigas F. Effect of fluoxetine on extracellular 5-hydroxytryptamine in rat brain: Role of 5-HT autoreceptors. *Eur J Pharmacol* 1998;358:9–18.
- Holmes A, Yang RJ, Lesch KP, Crawley JN, Murphy DL. Mice lacking the serotonin transporter exhibit 5-HT_{1A} receptor-mediated abnormalities in tests for anxiety-like behavior. *Genes Brain Behav* 2003;2:365–80.
- Holmes A, Yang RJ, Murphy DL, Crawley JN. Evaluation of antidepressant-related behavioral responses in mice lacking the serotonin transporter. *Neuropsychopharmacology* 2002;27:914–23.
- Holmes MC, French KL, Seckl JR. Dysregulation of diurnal rhythms of serotonin 5-HT_{2C} and corticosteroid receptor gene expression in the hippocampus with food restriction and glucocorticoids. *J Neurosci* 1997;17:4056–65.
- Ickes BR, Pham TM, Sanders LA, Albeck DS, Mohammed AH, Granholm AC. Long-term environmental enrichment leads to regional increases in neurotrophin levels in rat brain. *Exp Neurol* 2000;164:45–52.
- Justice Jr JB. Quantitative microdialysis of neurotransmitters. *J Neurosci Methods* 1993;48:263–76.
- Kellogg CK, Yao J, Plegler GL. Sex-specific effects of in utero manipulation of GABA(A) receptors on pre- and postnatal expression of BDNF in rats. *Brain Res Dev Brain Res* 2000;121:157–67.
- Kolbeck R, Bartke I, Eberle W, Barde YA. Brain-derived neurotrophic factor levels in the nervous system of wild-type and neurotrophin gene mutant mice. *J Neurochem* 1990;55:72–8.
- Korte M, Carroll P, Wolf E, Brem G, Thoenen H, Bonhoeffer T. Hippocampal long-term potentiation is impaired in mice lacking brain-derived neurotrophic factor. *Proc Natl Acad Sci USA* 1995;92:8856–60.
- Kreiss DS, Lucki I. Effects of acute and repeated administration of antidepressant drugs on extracellular levels of 5-hydroxytryptamine measured in vivo. *J Pharmacol Exp Ther* 1995;274:866–76.
- LeMaster AM, Krimm RF, Davis BM, Noel T, Forbes ME, Johnson JE, et al. Overexpression of brain-derived neurotrophic factor enhances sensory innervation and selectively increases neuron number. *J Neurosci* 1999;19:5919–31.
- Lesch KP, Bengel D, Heils A, Sabol SZ, Greenberg BD, Petri S, et al. Association of anxiety-related traits with a polymorphism in the serotonin transporter gene regulatory region. *Science* 1996;274:1527–31.
- Li Q, Wichems C, Heils A, Lesch KP, Murphy DL. Reduction in the density and expression, but not G-protein coupling, of serotonin receptors (5-HT_{1A}) in 5-HT transporter knockout mice: gender and brain region differences. *J Neurosci* 2000;20:7888–95.
- Li Q, Wichems C, Heils A, Van De Kar LD, Lesch KP, Murphy DL. Reduction of 5-hydroxytryptamine (5-HT)_{1A}-mediated temperature

- and neuroendocrine responses and 5-HT(1A) binding sites in 5-HT transporter knockout mice. *J Pharmacol Exp Ther* 1999;291:999–1007.
- Liebl DJ, Tessarollo L, Palko ME, Parada LF. Absence of sensory neurons before target innervation in brain-derived neurotrophic factor-, neurotrophin 3-, and *trkC*-deficient embryonic mice. *J Neurosci* 1997;17:9113–21.
- Lindsay RM, Wiegand SJ, Altar CA, DiStefano PS. Neurotrophic factors: from molecule to man. *Trends Neurosci* 1994;17:182–90.
- Lonnroth P, Jansson PA, Smith U. A microdialysis method allowing characterization of intercellular water space in humans. *Am J Physiol* 1987;253:E228–31.
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951;193:265–75.
- Lyons WE, Mamounas LA, Ricaurte GA, Coppola V, Reid SW, Bora SH, et al. Brain-derived neurotrophic factor-deficient mice develop aggressiveness and hyperphagia in conjunction with brain serotonergic abnormalities. *Proc Natl Acad Sci USA* 1999;96:15239–44.
- Malagie I, Trillat AC, Bourin M, Jacquot C, Hen R, Gardier AM. 5-HT_{1B} Autoreceptors limit the effects of selective serotonin re-uptake inhibitors in mouse hippocampus and frontal cortex. *J Neurochem* 2001;76:865–71.
- Mamounas LA, Altar CA, Blue ME, Kaplan DR, Tessarollo L, Lyons WE. BDNF promotes the regenerative sprouting, but not survival, of injured serotonergic axons in the adult rat brain. *J Neurosci* 2000;20:771–82.
- Mamounas LA, Blue ME, Siuciak JA, Altar CA. Brain-derived neurotrophic factor promotes the survival and sprouting of serotonergic axons in rat brain. *J Neurosci* 1995;15:7929–39.
- Marsh HN, Scholz WK, Lamballe F, Klein R, Nanduri V, Barbacid M, et al. Signal transduction events mediated by the BDNF receptor gp145trkB in primary hippocampal pyramidal cell culture. *J Neurosci* 1993;13:4281–92.
- Montanez S, Daws LC, Gould GG, Frazer A. Serotonin (5-HT) transporter (SERT) function after graded destruction of serotonergic neurons. *J Neurochem* 2003;87:861–7.
- Murphy DL, Li Q, Engel S, Wichems C, Andrews A, Lesch KP, et al. Genetic perspectives on the serotonin transporter. *Brain Res Bull* 2001;56:487–94.
- Nestler EJ, Barrot M, DiLeone RJ, Eisch AJ, Gold SJ, Monteggia LM. Neurobiology of depression. *Neuron* 2002;34:13–25.
- Nibuya M, Morinobu S, Duman RS. Regulation of BDNF and *trkB* mRNA in rat brain by chronic electroconvulsive seizure and antidepressant drug treatments. *J Neurosci* 1995;15:7539–47.
- Nibuya M, Nestler EJ, Duman RS. Chronic antidepressant administration increases the expression of cAMP response element binding protein (CREB) in rat hippocampus. *J Neurosci* 1996;16:2365–72.
- Papaioannou VE, Fox JG. Efficacy of tribromoethanol anesthesia in mice. *Lab Anim Sci* 1993;43:189–92.
- Parsons LH, Justice Jr JB. Quantitative approaches to in vivo brain microdialysis. *Crit Rev Neurobiol* 1994;8:189–220.
- Pollock GS, Vernon E, Forbes ME, Yan Q, Ma YT, Hsieh T, et al. Effects of early visual experience and diurnal rhythms on BDNF mRNA and protein levels in the visual system, hippocampus, and cerebellum. *J Neurosci* 2001;21:3923–31.
- Radka SF, Holst PA, Fritsche M, Altar CA. Presence of brain-derived neurotrophic factor in brain and human and rat but not mouse serum detected by a sensitive and specific immunoassay. *Brain Res* 1996;709:122–301.
- Rudge JS, Mather PE, Pasnikowski EM, Cai N, Corcoran T, Acheson A, et al. Endogenous BDNF protein is increased in adult rat hippocampus after a kainic acid induced excitotoxic insult but exogenous BDNF is not neuroprotective. *Exp Neurol* 1998;149:398–410.
- Salichon N, Gaspar P, Upton AL, Picaud S, Hanoun N, Hamon M, et al. Excessive activation of serotonin (5-HT) 1B receptors disrupts the formation of sensory maps in monoamine oxidase A and 5-HT transporter knock-out mice. *J Neurosci* 2001;21:884–96.
- Santarelli L, Saxe M, Gross C, Surget A, Battaglia F, Dulawa S, et al. Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. *Science* 2003;301:805–9.
- Scaccianoce S, Del Bianco P, Caricasole A, Nicoletti F, Catalani A. Relationship between learning, stress and hippocampal brain-derived neurotrophic factor. *Neuroscience* 2003;121:825–8.
- Shimizu E, Hashimoto K, Okamura N, Koike K, Komatsu N, Kumakiri C, et al. Alterations of serum levels of brain-derived neurotrophic factor (BDNF) in depressed patients with or without antidepressants. *Biol Psychiatry* 2003;54:70–5.
- Siuciak JA, Boylan C, Fritsche M, Altar CA, Lindsay RM. BDNF increases monoaminergic activity in rat brain following intracerebroventricular or intraparenchymal administration. *Brain Res* 1996;710:11–20.
- Siuciak JA, Lewis DR, Wiegand SJ, Lindsay RM. Antidepressant-like effect of brain-derived neurotrophic factor (BDNF). *Pharmacol Biochem Behav* 1997;56:131–7.
- Slotnick BM, Leonard CM. A stereotaxic atlas of the albino mouse fore-brain. Washington, D.C.: U.S. Government Printing Office; 1975.
- Smith AD, Justice JB. The effect of inhibition of synthesis, release, metabolism and uptake on the microdialysis extraction fraction of dopamine. *J Neurosci Methods* 1994;54:75–82.
- Trillat AC, Malagie I, Mathe-Allainmat M, Anmella MC, Jacquot C, Langlois M, et al. Synergistic neurochemical and behavioral effects of fluoxetine and 5-HT_{1A} receptor antagonists. *Eur J Pharmacol* 1998;357:179–84.
- Trillat AC, Malagie I, Scearce K, Pons D, Anmella MC, Jacquot C, et al. Regulation of serotonin release in the frontal cortex and ventral hippocampus of homozygous mice lacking 5-HT_{1B} receptors: in vivo microdialysis studies. *J Neurochem* 1997;69:2019–25.
- Turner CA, Lewis MH. Environmental enrichment: effects on stereotyped behavior and neurotrophin levels. *Physiol Behav* 2003;80:259–66.
- Vitalis T, Alvarez C, Chen K, Shih JC, Gaspar P, Cases O. Developmental expression pattern of monoamine oxidases in sensory organs and neural crest derivatives. *J Comp Neurol* 2003;464:392–403.
- Vitalis T, Cases O, Gillies K, Hanoun N, Hamon M, Seif I, et al. Interactions between *trkB* signaling and serotonin excess in the developing murine somatosensory cortex: a role in tangential and radial organization of thalamocortical axons. *J Neurosci* 2002;22:4987–5000.
- Yang H, Peters JL, Allen C, Chern SS, Coalson RD, Michael AC. A theoretical description of microdialysis with mass transport coupled to chemical events. *Anal Chem* 2000;72:2042–9.
- Zetterstrom TS, Pei Q, Madhav TR, Coppell AL, Lewis L, Grahame-Smith DG. Manipulations of brain 5-HT levels affect gene expression for BDNF in rat brain. *Neuropharmacology* 1999;38:1063–73.