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Effects of brain-derived neurotrophic factor on dopaminergic function and motor behavior during aging

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Abstract

Brain-derived neurotrophic factor (BDNF) is critical in synaptic plasticity and in the survival and function of midbrain dopamine neurons. In the present study, we assessed the effects of a partial genetic deletion of BDNF on motor function and dopamine (DA) neurotransmitter measures by comparing (*Bdnf*^{+/−}) with wildtype mice (WT) at different ages. *Bdnf*^{+/−} and WT mice had similar body weights until 12 months of age; however, at 21 months, *Bdnf*^{+/−} mice were significantly heavier than WT mice. Horizontal and vertical motor activity was reduced for *Bdnf*^{+/−} compared to WT mice; but was not influenced by Age. Performance on an accelerating rotarod declined with age for both genotypes and was exacerbated for *Bdnf*^{+/−} mice. Body weight did not correlate with any of the three behavioral measures studied. DA neurotransmitter markers indicated no genotypic difference in striatal tyrosine hydroxylase (TH), dopamine transporter (DAT), or vesicular monoamine transporter 2 (VMAT2) immunoreactivity at any age. However, DA transport via DAT (starting at 12 months) and VMAT2 (starting at 3 months) as well as KCl-stimulated DA release were reduced in *Bdnf*^{+/−} mice and declined with age suggesting an increasingly important role for BDNF in the release and uptake of DA with the aging process. These findings suggest that a BDNF expression deficit becomes more critical to dopaminergic dynamics and related behavioral activities with increasing age.

Keywords

BDNF; aging; dopamine; uptake; striatum; mice

Introduction

Aging is associated with progressive, albeit sometimes subtle, functional and structural deterioration of neural systems, affecting both motor and memory functions (Barbeau 1973;

Kluger *et al.* 1997; Kokmen *et al.* 1977; Newman *et al.* 1985; Richards *et al.* 1993; Volkow *et al.* 1998). Motor dysfunction and deterioration of dopamine (DA) systems have also been reported for animal models of aging (Ingram 2000). Evidence for age-related DA dysfunction includes (1) reductions in both tyrosine hydroxylase (TH) and the DA transporter (DAT) in the SN of aged rodents (Himi *et al.* 1995), (2) reduced DA uptake in aged Rhesus monkeys (Dejesus *et al.* 2001), (3) reduced DA release in aged F344 rats (Hebert & Gerhardt 1998), (4) reduction in DA and glutamine receptor binding in aged mice (Ossowska *et al.* 2001), (5) reduced striatal DAT and VMAT2 activity (Hebert & Gerhardt 1999; Cruz-Muros *et al.* 2008), and (6) marked increase in the response of aged substantia nigra (SN) DA neurons to neurotoxins (Cass *et al.* 2002). Moreover, alterations in many of these DA markers are associated with a conspicuous decrease in spontaneous locomotion in aged rodents (Hebert & Gerhardt 1997; Watanabe *et al.* 1991) although the specific factors mediating these age-related changes have not been fully elucidated.

DA neurons depend on continuous support from growth factors. Withdrawal or reduction of these growth factors, primarily glial cell line-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF), increases the vulnerability of DA neurons to external stressors and toxins especially with increasing age (Yurek & Fletcher-Turner 2000; Yurek & Fletcher-Turner 2001). BDNF is involved in the development of motor coordination and the survival and maintenance of nigral DAergic neurons (Do *et al.* 2007; Strand *et al.* 2007). The expression of BDNF and its high affinity receptor, TrkB, has been shown to be decreased with normal aging and in patients with Alzheimer's and Parkinson's disease (Murer *et al.* 2001; Nagatsu & Sawada 2007; Silhol *et al.* 2005). These findings indicate a relationship of BDNF to the degeneration of SN neurons in PD, but do not establish whether the BDNF reduction is a result of, or a contributor to, the degenerative process.

BDNF null mutations confer severe neurological dysfunction on newborn pups, resulting in death within the first 2–3 weeks postpartum (Liebl *et al.* 1997). Mice heterozygous for the BDNF allele (*Bdnf*^{+/−}) exhibit alterations in cortical postsynaptic responses (Abidin *et al.* 2006), behavioral alterations related to anxiety, appetite, and motor function (Lyons *et al.* 1999), reduced hippocampal serotonergic innervations (Luellen *et al.* 2007) and altered mRNA expression of TH in the SN and opioid peptides in the striatum (Saylor *et al.* 2006). Studies also document that *Bdnf*^{+/−} mice exhibit (a) an enhanced aging-related decline of beam walking, spontaneous locomotion activity thought to be mediated by nigrostriatal dopaminergic functions, (b) higher total DA levels in caudate-putamen, (c) reduced K⁺ stimulated DA release from *ex vivo* slice preparations, (d) decreased neurotoxic response to methamphetamine, and (e) no changes in DAT-ligand binding (Disshon & Dluzen 1997; Dluzen *et al.* 2001; Dluzen *et al.* 2004; Dluzen *et al.* 1999; Joyce *et al.* 2004). To date, however, there are no reports of studies examining altered presynaptic DAergic function resulting from reduced BDNF expression in terms of altered motor function, DAT and VMAT2 functional activities and synaptic DA levels and K⁺ stimulated DA release in freely moving animals. Therefore the purpose of the current study was to assess possible differences in motor function (horizontal and vertical activity, accelerating rotarod) along with markers of nigrostriatal DAergic function in *Bdnf*^{+/−} and WT mice during aging. The study included the use of striatal and substantia nigra DAergic immunohistochemical markers, extracellular basal and K⁺ evoked striatal DA levels using microdialysis, and functional activities of DAT and VMAT2 from striatal synaptosomes.

Materials and Methods

Animals

Pairs of *Bdnf*^{+/−} mice were generously provided by L. Tessarollo (National Cancer Institute, Frederick, Maryland). They were generated by replacing the first coding exon with the

pGKneo recombination cassette (Liebl *et al.* 1997) and have been subsequently backcrossed five times onto a C57BL/6J genetic background. Upon arrival to our animal facility, they were genotyped (backcrossed 4 times) to be 95% C57BL/6J and 5% 129S4/SvJa (service provided by Jackson labs) and were maintained at this level by heterozygous crosses. Genotyping was performed as previously described (Liebl *et al.* 1997). These *Bdnf*^{+/-} mice, carrying a single null allele for BDNF were viable, fertile, and appear to have a normal life span. 12 breeding pairs supplied all the male *Bdnf*^{+/-} and WT littermates utilized in these studies. All mice were group-housed in a temperature- and humidity- controlled room and maintained on a 12-h light/dark cycle with food and water available *ad libitum*. Body weight was monitored throughout the lifespan. All procedures used on animals in this study were in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Medical University of South Carolina Institutional Animal Care and Use Committee.

Experimental Design

At 3-, 12-, and 21-months of age, independent groups of male WT and *Bdnf*^{+/-} mice (N=8 per group) were assessed for motor function via open-field locomotor testing and accelerating rotarod. Upon completion of these tasks, the mice were weighed, anesthetized, and the brains extracted and bisected. One hemisphere was post-fixed in 4% paraformaldehyde for 48 hrs and then processed for immunohistochemistry. The other hemisphere was frozen at -80°C and used for BDNF tissue levels. A second cohort of male 3-month-old and 12-month-old WT and *Bdnf*^{+/-} mice (N=8 per group) were used to assess basal extracellular DA, as well as K⁺-stimulated DA release in the dorsal striatum. At the end of the microdialysis experiment, the mice were sacrificed and brains were stored in 4% paraformaldehyde for probe placement verification. DAT and VMAT2 activities were measured from fresh striatum obtained from an independent cohort of 3-month 12-month and 21-month old male WT and *Bdnf*^{+/-} mice. The numbers of mice from the different Genotype × Age groups included in different experiments are summarized in Table 1.

Locomotor Activity Testing

Independent groups of male *Bdnf*^{+/-} and WT mice were tested at 3, 12, and 21 months of age (N=8 per genotype per age) to evaluate the effects of a partial BDNF deletion on motor activity across most of the lifespan. Horizontal (total distance traveled) and vertical (rearing frequency) activity were assessed for one hour using a Digiscan Animal Activity Monitor system (Omnitech Electronics Model RXYZCM(8) TAO, Columbus, OH) contained in light- and sound- controlled chambers. The details of the apparatus have been described previously (Halberda *et al.* 1997). On the day of testing, the mice were transferred from the animal colony into the laboratory in groups of six and individually tested for one hour in a darkened environment. Data were collected in 15 min intervals for one hour at the same time of day (8 am to 12 pm)

Accelerating Rotarod

Motor coordination was evaluated with an accelerating rotarod (Ugo Basile, Verese, Italy) procedure. Mice from each of the six groups created by the Genotype × Age experimental independent group design (N=8 per group) were tested at 3, 12, and 21 months of age. The cross-sectional design employed allowed naive mice at each of the three ages to be behaviorally tested and then sacrificed for morphology and biochemistry. Using a rotarod procedure adapted from Rosas, et al. (Rosas *et al.* 1997), mice were initially trained to remain on the rotarod for 10 min at a set speed of 4 rpm one day prior to actual testing. They were then tested for three consecutive days on their ability to remain on the rotarod at increasing rotation speeds of 4, 8, 16, 24, 32, and 40 rpm. During the daily tests, mice were tested for a maximum of 5 min at each rotation speed with 5-min rest periods in their home

cage between each rotation speed. Results are expressed as Kaplan-Meier survival curves representing the probability for mice of the different genotype/age groups remaining on the rotarod as time increased with fall time collapsed across the increasing rotational speeds. Statistical comparisons are described in the Statistical Methods section. In addition to determining motor coordination, examination of performance over the three-day period provided an assessment of potential genotypic difference in motor learning.

Enzyme Linked Immunosorbent Assay (ELISA)

BDNF protein levels were assessed using Promega BDNF ELISA kits (Promega, Madison, WI) according to standard laboratory protocols (Boger *et al.* 2006). The striatum was harvested from one hemisphere, and placed in pre-weighed Eppendorf tubes, weighed, and kept at -80°C until tested. Samples were homogenized and centrifuged in 0.5 ml lysate buffer (Promega, Madison, WI) and aliquoted. Ninety-six-welled flat-bottom NUNC-Immuno maxisorp ELISA plates were incubated with carbonate-coating buffer containing captive neurotrophic factor antibodies (Promega) overnight at 4°C . A standard curve was created from serial dilutions of known concentrations of the protein provided in the Promega kit. Duplicate samples were incubated for 6 hr at room temperature, and with secondary antibody followed by an antibody conjugated to HRP. A TMB/peroxidase solution was used as the chromogen to visualize the reaction product. The reaction was terminated with 1N HCl, and the optical density measured at 450 nm (Albeck *et al.* 2003). Using these kits, BDNF can be quantified in the range of 7.8–500 pg/ml. BDNF was expressed as pg/mg wet tissue.

Immunohistochemistry

For immunohistochemical analysis, mice from each genotype at each age were sacrificed using an overdose of isoflurane. The brains were quickly removed, post-fixed in paraformaldehyde (4%) for 48 hours, and then transferred to 30% sucrose in 0.1M phosphate buffered saline (PBS) for at least 24 hours before sectioning. As described above, one hemisphere was utilized for ELISA assays, while the other hemisphere from each mouse was utilized for immunohistochemical studies. The striatum was sectioned on a cryostat (Microm, Zeiss, Thornwood, NY, USA) at $45\text{ }\mu\text{m}$. Every 6th section throughout the striatal region and every 3rd section from the midbrain (for TH only) was processed for free-floating immunohistochemistry using a rabbit polyclonal antibody against tyrosine hydroxylase (TH, 1:5000 dilution, Pel-Freeze Inc., Roger, AZ, USA), a rat monoclonal antibody against the dopamine transporter (DAT, 1:2000 dilution, Millipore Corp., Temecula, CA), or a rabbit polyclonal antibody against vesicular monoamine transporter 2 (VMAT2, 1:2000 dilution, Millipore Corp., Temecula, CA). Immunohistochemistry was performed according to our standard protocol (Granholt *et al.* 1997). Briefly, free-floating serial sections were treated with H_2O_2 , methanol, and 0.01 M Tris buffer saline (TBS, pH 7.6; 1:2:7 respectively) for 15 minutes to quench endogenous peroxidase activity. Sections were permeabilized in TBST (Tris buffer with 0.9% NaCl and 0.25% Triton X-100) and treated for 20 minutes with sodium *m*-periodate (0.1 M) in TBS. To block non-specific binding sites, sections were incubated in 10% normal goat serum (NGS, Sigma-Aldrich, St. Louis, MO) in TBST for 30 minutes at room temperature. Sections were incubated with the primary antibody (1:1000) in TBST with 3% NGS at room temperature for 24 hrs. Following washing in TBST, sections were incubated with an appropriate secondary antibody, biotin-conjugated goat anti-rabbit (TH, VMAT2) or goat anti-rat (DAT) IgG (1:200, Vector Labs, Burlingame, CA) and incubated subsequently in an avidin-biotin complex (ABC kit, Vector Labs, Burlingame, CA). The reaction was developed by staining with VIP (Vector Labs, Burlingame, CA), which yields a purple reaction product. Prior to the use of each antibody, different titers were assessed to determine the optimal dilution to prevent saturation of antibody. Furthermore, all sections from all mice were processed simultaneously and rigorously timed

to avoid saturation of reaction product. Sections were mounted on glass slides and cover-slipped with DPX.

Semi-quantitation of Immunohistochemistry

Semi-quantitation of striatal TH, DAT, and VMAT2 immunoreactivity from 3-, 12-, and 21-month-old mice (N=8 per genotype per age) WT and *Bdnf*^{+/-} mice was conducted. Briefly, staining intensity of TH immunostaining in the dorsolateral striatum was determined using NIH Image® Software to measure a gray scale value within the range of 0 – 256, where 0 represents white and 256 black. A template covering the medial as well as lateral dorsal striatum was created and used on all brains similarly, and images were captured with a Nikon Eclipse E-600 microscope, an Olympus-750 video camera system, and a Dell Pentium III computer. Measurements were performed blinded from 5–6 sections per brain and then averaged to obtain one value per subject. Staining density was obtained when background staining was subtracted from mean staining intensities on every 6th section through the striatum.

Microdialysis Surgical Procedures

At 3- and 12-months of age, a separate cohort of *Bdnf*^{+/-} and WT male mice (N=8 per group) were anesthetized using a ketamine (150 mg/kg) and xylazine (3 mg/kg) cocktail and placed in a stereotaxic instrument (Stoelting, Wood Dale, IL). Unilateral guide cannulae (CMA/7; CMA Microdialysis, Sweden) were aimed at the dorsal striatum (relative to Bregma: AP +0.8, ML +1.5, and DV -2.5; Franklin and Paxinos, 1997) and secured directly to the skull using a light-cured resin system (Kerr Corporation, Orange, CA).

In Vivo Microdialysis Study

The day before dialysate collection, a microdialysis probe (CMA/7; 1mm active membrane) was lowered into the guide cannula and mice were perfused at 0.1 µL/min overnight with filtered artificial cerebral spinal fluid (aCSF: 7.4 mM glucose, 3.0 mM KCl, 140 mM NaCl, 0.5 mM MgCl₂, 1.2 mM CaCl₂, 1.2 mM Na₂HPO₄, 0.3 mM NaH₂PO₄). The next day, the perfusion flow of aCSF was increased to 1.0 µL/min. After 2 hrs, dialysis samples were collected every 15 min into vials containing 2.5 µL of 750 mM perchloric acid diluted to a final concentration of 150 mM by the sample. Following the collection of 6 basal dialysate samples, 60 mM KCl-aCSF (7.4 mM glucose, 60 mM KCl, 83 mM NaCl, 0.5 mM MgCl₂, 1.2 mM CaCl₂, 1.2 mM Na₂HPO₄, 0.3 mM NaH₂PO₄) was perfused for 15 min, after which 3 mM KCl-aCSF was perfused for an additional 4 dialysate collections.

Dopamine Measurement by High-Performance Liquid Chromatography

Dialysate samples were injected by an autosampler (ESA model 542, Chelmsford, MA). DA was separated from the sample with a reverse-phase column (HR 80, ESA, Chelmsford, MA) using the mobile phase (150 mM NaH₂PO₄, 4.76 mM citric acid, 50 µM EDTA, 10% methanol, 2.5 mM SDS, 17% acetonitrile, pH 5.6) at a flow rate of 0.9 ml/min. The column was kept in an oven at 30°C and DA detected using coulometric detection. Three electrodes were used: a guard cell (400 mV), a reduction analytical electrode (100 mV), and an oxidation analytical electrode (220 mV). Peaks were recorded, and the area under the curve was measured by a computer running ESA Chromatography Data System. DA concentrations were determined by comparing peak areas to an external standard curve.

Probe Placement Verification

After concluding the microdialysis experiment, the mice were sacrificed and brains removed and stored in 4% paraformaldehyde for 48 hrs. After transfer to 30% sucrose for 24 hrs,

coronal sections (45 μ M) were mounted and stained with Cresyl violet. Probe placement was determined according to Franklin and Paxinos (2001).

Synaptosome Preparations

At 3-, 12-, and 21-months of age, independent groups of male WT and *Bdnf*^{+/-} mice (N=8 per group) were rapidly decapitated, and the brains were collected in ice-cooled dishes. The striatum was rapidly dissected and collected in 10 volumes (wt/vol) of cold 0.32 M sucrose. The tissue was homogenized using a Teflon-glass homogenizer under chilled water and centrifuged at 1000 $\times$ g for 15 min at 4 °C. The resulting supernatant was centrifuged at 15,000 $\times$ g for 25 min and the pellet was washed by resuspending in 0.32 M sucrose (Samuvel *et al.* 2005). The synaptosomes were suspended in regular Krebs-Ringer-HEPES (KRH) buffer pH 7.4 (120 mM NaCl, 4.7 mM KCl, 2.2 mM CaCl₂ 10 mM HEPES, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 5 mM Tris, and 10 mM D- glucose) saturated with 95% O₂/5% CO₂. Protein concentration was determined by DC protein assay (BioRad) using bovine serum albumin as standard. The synaptosomal preparation was used immediately for experiments. The striatum was pooled from 3 animals in such a way that all of the experiments were repeated at least three times.

Preparation of Synaptic Vesicles from Striatal Synaptosomes

Synaptic vesicles from synaptosomes were prepared as previously described (Sandoval *et al.* 2002). Synaptosomal fractions obtained from striatal tissue as described above were suspended in cold water (1 mg protein/ml) containing phosphatase inhibitor (okadaic acid, 1 μ M) and other protease inhibitors (1 μ g/ml aprotinin, 1 μ g/ml leupeptin, 1 μ M pepstatin and 250 μ M phenylmethylsulfonyl fluoride). This solution was passed through a 20 gauge needle and incubated for 30 min at 4 °C with continuous shaking. HEPES and potassium tartate, pH 7.5 were added slowly to the mixture to the final concentration of 25 mM and 100 mM, respectively. The lyzed synaptosomal fraction was centrifuged at 22,000 g for 30 min at 4 °C. The supernatant containing the synaptic vesicular fractions was further concentrated by a MgSO₄ (1 mM) salt-out method followed by centrifugation at 100,000 g for 1 hr. The pellet represents vesicular fractions used for VMAT2 analysis (Sandoval *et al.* 2002).

DAT Activity Assay

DA uptake was performed as described previously (Samuvel *et al.* 2005). Briefly, 20 μ g of synaptosomes were incubated for three minutes in 250 μ l of KRH buffer pH 7.4 containing 0.1 mM ascorbic acid and 0.1 mM pargyline and 40 nM [³H]DA. Synaptosomes were pre-incubated with the DAT inhibitor nomifensine (100 μ M) or GBR12909 (0.2 μ M) at 37 °C for 10 min followed by the addition of [³H]DA to determine the nonspecific DA uptake. Uptake was terminated with the addition of 3 ml ice-cold PBS followed by rapid filtration using vacuum 10 mm Hg over 0.3% polyethylenimine coated GF-B filters on a Brandel Cell Harvester. Filters were washed rapidly with 5 ml cold PBS and radioactivity bound to filter was counted using a liquid scintillation counter. Nonspecific uptake was defined as the uptake in the presence of DAT blocker and subtracted from the total accumulation to yield specific DAT mediated DA uptake. Mean values of specific uptake $\pm$ SEM of at least three separate experiments were determined. Our initial optimizing assay conditions for nomifensine (100 μ M) or GBR12909 (0.2 μ M) sensitive DAT activity in striatal synaptosomes showed linear DA uptake with respect to uptake time (1 to 8 min) and protein concentration (5 to 30 μ g) using 50 nM labeled DA. Therefore, we utilized a 5 min uptake time using 20 μ g synaptosomal proteins in all subsequent DAT assays.

VMAT2 Activity Assay

VMAT2 activity was determined as previously described (Sandoval *et al.* 2002). Synaptic vesicles were incubated in 25 mM HEPES, 100 mM potassium tartrate, 1.7 mM ascorbic acid and 2 mM ATP-Mg, pH 7.5, in the presence of 50 nM [³H]DA at 37 C. To establish our VMAT2 assay conditions, we optimized the specificity of [³H]DA transport using synaptic vesicular fractions by (a) examining [³H]DA transport in the presence of DAT specific blocker, GBR12909 (0.1 μM) to reveal the DAT specific [³H]DA transport component, and (b) testing [³H]DA transport in the presence of reserpine and or in the absence of ATP, to isolate VMAT2 specific [³H]DA transport. DA uptake in the presence of GBR12909 and reserpine was subtracted from total uptake to obtain specific VMAT2 activity as described (Finn *et al.* 1998; Sandoval *et al.* 2002). GBR12909/reserpine-sensitive VMAT2 activity in synaptic vesicles showed linear DA uptake with respect to uptake time (1 to 5 min) and protein concentration (5 to 25 μg) using 50 nM [³H]DA. These multiple controls were adapted to ensure that the [³H]DA uptake by synaptic vesicles is mediated by VMAT2 within the linear range of time and protein concentration.

Statistical Methods

Striatal BDNF tissue concentration, body weight, total distance traveled, vertical activity, immunohistochemistry, and DAT/VMAT2 activity were analyzed with independent group 2(Genotype) × 3(Age) ANOVAs. Student-Neuman-Keul's post-hoc tests were used for group comparisons following significant Genotype × Age or Genotype × Drug Treatment Interactions. Rotarod data were analyzed as total survival time across the six rotational speeds with a maximum survival time of 1800 if the mouse did not fall during any of the six rotational speed tests. Data for the full Age × Genotype × Day factorial with, and without, adjustment for body weight were analyzed with a Cox regression analysis (STATA 9.0) with Days clustered within subjects to account for the correlated responses across the three days (Lee *et al.* 1992). The type of analysis for *in vivo* microdialysis data depended on whether the data were collected prior to (basal level), or after K⁺ -stimulation. Basal DA level data were analyzed with a 2(Genotype) × 2(Age) × 6 (Sample Interval) mixed factor ANOVA with sample interval being a repeated measure. Because the two genotypes had different basal DA levels, data collected after K⁺ -stimulation were analyzed with a 2(Genotype) × 2(Age) × 4(Sample Interval) mixed factor ANCOVA using Baseline-Sample-Interval number 6 as a covariate. Again, the Sample Interval factor was a repeated measure.

Results

Striatal BDNF concentration

BDNF protein levels in striatal tissue are summarized in Fig 1A and were significantly lower in *Bdnf*^{+/−} mice than WT littermates [Genotype: $F_{(1,32)} = 6.76$, $p \leq 0.05$]. In addition, the BDNF protein declined across age [$F_{(1,32)} = 5.53$, $p < 0.01$]. Although BDNF levels declined by ~25% in WT mice and ~40% in *Bdnf*^{+/−} mice; this apparent interaction was not statistically supported [Genotype × Age: $F_{(2,32)} = 0.19$, $p > 0.10$]

Body weight

As noted in Fig 1B, weight gain across age was greater for *Bdnf*^{+/−} than WT littermates [Genotype × Age: $F_{(2,50)} = 5.67$, $p \leq 0.01$]. Student-Neuman-Keul's post-hoc analysis revealed that 12 month-old mice weighed significantly more than 3 month-old mice, regardless of genotype ($p \leq 0.05$). Importantly, at 21 months of age, *Bdnf*^{+/−} mice weighed significantly more than either age-matched WT littermates ($p \leq 0.01$), or 12 month-old *Bdnf*^{+/−} mice ($p \leq 0.01$). Although body weight became elevated for aging *Bdnf*^{+/−} compared

to WT mice, the availability of the same number of *Bdnf*^{+/−} mice at 21 vs. 3 or 12 months of age suggests that genotype did not alter mortality rate.

Locomotor activity

The first behavior experiment completed for this study was evaluation of motor activity. Horizontal and vertical motor activity data from independent groups of WT and *Bdnf*^{+/−} mice tested in open-field locomotor activity chambers at 3, 12, and 21 months of age are summarized in 15-min intervals across hourly tests in Fig 2. Horizontal motor activity, expressed as “distance traveled” is summarized in Fig 2A and vertical activity expressed as “rearing response frequency” is summarized in Fig 2B. Data for each of the measures were analyzed with 2(Genotype) × 3(Age) × 4(15-min Interval). Both Distance traveled, Fig 2A, and Rearing Response frequency, Fig 2B, declined across the hour session [Distance Traveled: $F_{(3,19)} = 19.4$, $p < 0.001$; Rearing Responses: $F_{(3,19)} = 97.3$, $p < 0.001$]. The decline across the session was influenced by only the Age factor, and this influence of age was restricted to the rearing response measure, which declined more rapidly for the older mice [Age × Interval: $F_{(4,65)} = 3.15$, $p \leq 0.01$]. Both horizontal and vertical motor activity also declined with the Age [Distance Traveled: $F_{(2,65)} = 3.86$, $p \leq 0.03$; Rearing Responses: $F_{(2,65)} = 3.37$, $p \leq 0.04$]. Although the Genotype factor did not impact the distance traveled measure [$F_{(1,65)} = 1.02$, $p \geq 0.01$], it did influence rearing response frequency [$F_{(1,65)} = 6.27$, $p \leq 0.01$], fewer rearing responses for *Bdnf*^{+/−} mice. Finally, the pattern of change in distance traveled with age for the two genotypes suggested in Fig 2A reached only a 0.09 level of significance [$F_{(2,65)} = 2.50$, $p < 0.09$].

Since relative body weights of WT and *Bdnf*^{+/−} mice varied according to age, as noted in Fig 1, correlations of total distance traveled and vertical activity with body weights were determined within each age group. Spearman/Rho correlation analysis indicated no relationship of body weight to either of the activity measures at any of the ages tested ($p > 0.05$). Thus, the elevated body weight of *Bdnf*^{+/−} mice did not likely account for their compromised motor performance (data not shown).

Accelerating rotarod performance

Since BDNF deficiency reduced horizontal and vertical activity at 21 months of age (Fig 2), we further evaluated motor behavior using accelerated rotarod performance to assess motor coordination and balance. Rotarod performance, is summarized in Figs 3A&B as Cox regression survival plots with the proportion of mice not falling during tests (ordinate) across time (abscissa), with time reflecting the seconds the animal avoided falling off the rotarod summed across the 6 rotational speeds. These data are also expressed as median survival times across rotation speeds and days for each Genotype and Age combination in Fig 3C. In general, rotarod performance improved across the three days ($C^2(2) = 9.61$, $p = 0.01$) with the most improvement occurring between day 2 and day 3; however, neither genotype nor age significantly altered this improvement. Further analysis of the genotype and age factorial across days (retaining the clustering of day within subject in the model) indicated no significant age by genotype interaction ($X^2(2) = 2.3$, $p = 0.25$). The Cox regression plots for the two genotypes across ages (Fig 3A) indicates that *Bdnf*^{+/−} mice were less able than WT controls to remain on the rotarod with increasing rotation speed ($X^2(1) = 16.7$, $p < 0.0001$). The Cox regression plots with genotypes combined for the three ages (Fig 3B) indicated a highly significant ($X^2(2) = 11.07$, $p = 0.004$) reduction in performance with increasing age. Post hoc evaluation confirmed the substantial reduction in performance between 3- vs. 12- ($p < 0.001$) or 22- ($p = 0.002$) month-old mice, with no difference between the two older groups ($p = 0.86$). Finally, Fig 3C summarizes the genotype by age interaction as median survival time. Though the age curves diverge slightly from the parallel, as noted above, the age by genotype interaction was not significant.

Adjusting the Cox regression analysis to account for the body weight differences between the two genotypes and across age did not alter any of the results noted above and body weight did not predict rotation performance ($B = -0.013(0.02)$, $p = 0.529$).

Immunohistochemistry

Our data (Fig 2 and 3) indicate the role of BDNF in the maintenance of age dependent decline of motor activity. Nigrostriatal DAergic functions have been strongly implicated in motor behavior. Notably, dysregulation of several DAergic markers have been documented in aging and several neurological disorders. Therefore, we examined whether BDNF deficiency exhibit any effects on age-dependent alterations of DAergic markers including TH, DAT, VMAT2 expressions. TH-ir, DAT-ir, and VMAT2-ir levels in the striatum of the two genotypes at 3, 12, and 21 months of age were analyzed. TH-ir levels declined with Age [$F_{(2,29)} = 6.86$, $p < 0.01$], as did DAT-ir [$F_{(2,23)} = 7.71$, $p < 0.01$] but were not influenced by Genotype or its interaction with Age. Contrary to the Age effect on striatal TH-ir and DAT-ir, VMAT2-ir was not influenced by Age, Genotype, or a Genotype $\times$ Age interaction (see Supplemental Figures 1–3).

Furthermore, the assessment of TH-ir in DA neurons of the SN at the three ages indicated no effect of Genotype, Age, or Genotype $\times$ Age Interaction (see Supplemental Figure 4).

Striatal extracellular DA and K⁺ stimulated DA release

To determine if the BDNF reduction in *Bdnf*^{+/−} mice affected *in vivo* basal synaptic DA homeostasis and evoked DA release, we conducted microdialysis on freely moving 3 and 12 months old *Bdnf*^{+/−} and WT control mice. Data for extracellular DA in the striatum during basal assessment and following stimulated release by a 60 mM KCl infusion are shown for the two genotypes in Fig 4A and C for 3-month-old mice and Fig 4B and D for 12-month-old mice. Analysis of data for basal extracellular DA levels with a 2(Genotype) $\times$ 2(Age) $\times$ 6(Baseline) ANOVA with repeated measures on the Baseline factor supported the reduced level of extracellular DA for *Bdnf*^{+/−} mice noted in Figs 4A and B [$F_{(1,22)} = 13.45$, $p < 0.001$]. Basal DA levels did not differ across Age or Baseline interval [$F_s \leq 1$], nor according to interactions of the factors. KCl-stimulated DA release data obtained from the four 15-min samples after the 60 mM KCl infusion were analyzed with a 2(Genotype) $\times$ 2(Age) $\times$ 4(Time Interval) ANCOVA. Because the genotypes had different basal levels of DA, Baseline Sample #6 was used as a co-variate for ANCOVA to account for the difference in baseline in our evaluation of KCl-stimulated DA release. Potassium stimulation increased extracellular DA during the first 15-min interval for WT and *Bdnf*^{+/−} mice, respectively, by $232 \pm 36\%$ and $197 \pm 27\%$ at 3 months and by $179 \pm 43\%$ and $166 \pm 20\%$ at 12 months. Although genotype and age did not alter the degree to which DA was elevated above basal levels, the rate at which the elevated extracellular DA declined across the four 15-min intervals differed according to genotype [$F_{(3,63)} = 4.02$, $p < 0.01$]. Follow-up analysis indicated significant differences among the four Genotype $\times$ Age groups during the first 15-min period after the KCl infusion [$F_{(3,25)} = 5.13$, $p < 0.018$]. Using the 3-month-old WT value as a base, extracellular DA levels during this time were reduced for *Bdnf*^{+/−} mice of both ages [Dunnett's test $p < 0.05$]. Additional analysis of the slopes of declining extracellular DA for the four groups indicated genotype differences with -0.43 ± 0.05 and -0.33 ± 0.05 , respectively, for 3 and 12 months old WT mice and -0.26 ± 0.05 and -0.24 ± 0.06 for the aged matched *Bdnf*^{+/−} mice. A 2(Genotype) $\times$ 2(Age) ANOVA indicated that slope depended on Genotype [$F_{(1,37)} = 7.09$, $p < 0.01$] but not Age or the interaction of the two factors.

DA uptake via DAT and VMAT2

The immunohistochemical analysis noted above indicated that total protein expression of TH and DAT declined with age, but did not differ for WT and *Bdnf*^{+/−} mice. However, microdialysis experiments revealed lower basal level of DA in the *Bdnf*^{+/−} compared to WT mice. Moreover, though KCl-stimulated DA release was similar for the two genotypes, the rate at which the elevated DA levels produced by potassium stimulation were cleared was slower for *Bdnf*^{+/−} than WT mice. These observations suggest the possibility that altered activity, but not total expression of DAT and VMAT2, may contribute to the mechanisms accounting for *Bdnf*^{+/−} mice exhibiting lower DA basal levels and reduced DA clearance rate. Thus next we examined the functional activity of DAT and VMAT2 proteins using striatal synaptosomes. The influence of partial BDNF deletion on DA uptake mediated by DAT and by VMAT2 across the three ages is summarized, respectively, in Fig 5A and B. As noted in Fig 5A, DAT activity declined progressively with age, and was lower for *Bdnf*^{+/−} mice compared to age-matched WT mice in the older age groups. Analysis of striatal DAT activity revealed a significant Genotype × Age interaction [$F_{(2,93)} = 4.29$, $p < 0.05$]. Student-Newman-Keuls comparison of group means provided statistical support for DAT-mediated DA uptake difference across each of the three age groups for both genotypes noted in Fig 5A ($p \leq 0.01$). Comparisons of genotype at each age established statistical support for lower DAT activity for *Bdnf*^{+/−} than WT mice at the 12 and 21 months time points ($p \leq 0.01$, Student-Newman-Keuls post hoc analysis), but not for the young adult mice.

In addition, vesicular VMAT2-specific DA uptake (Fig 5B) was also dependent on a Genotype × Age interaction [$F_{(2,22)} = 18.38$, $p < 0.0001$]. Striatal VMAT2 activity declined with age, and was lower for *Bdnf*^{+/−} mice compared to age-matched WT mice in young adult (3 month) and mid-aged (12 month) mice. Subsequent Student-Newman-Keuls post hoc comparisons established significant reductions for 12 and 21 months compared with 3 month-old mice of both genotypes ($p \leq 0.01$). A further reduction in VMAT2 activity was evident from 12 to 21 months of age for WT ($p \leq 0.01$) but not *Bdnf*^{+/−} mice ($p \geq 0.10$).

Discussion

This study confirms reports of motor deficits for male *Bdnf*^{+/−} mice and that BDNF reduction advances the motor system decline with aging (Dluzen *et al.* 2004). The study focuses on how the aging-related decline in three different measures of motor behavior might relate to altered DA system function in a specific *Bdnf*^{+/−} mouse genotype (i.e., the exon-1 region of the BDNF gene replaced with recombinant neomycin; mice backcrossed ≥ 10 backcrosses on the B6 strain; (Liebl *et al.* 1997; Lyons *et al.* 1999). Male B6 mice with the noted BDNF gene modification in our experiments had motor function deficits that became more evident with aging, and were accompanied by functional changes in the nigrostriatal DA system. Young adult *Bdnf*^{+/−} mice were deficient on a complex motor task (rotarod), had lower basal levels of extracellular DA and a less rapid decline in DA following K⁺-stimulated release, and lower VMAT2 activity relative to WT controls. With increasing age, motor deficits for *Bdnf*^{+/−} mice were observed on less challenging tasks (open-field activity) and were accompanied by reductions in DAT-mediated DA uptake, neither of which were evident in the young mice. Reported studies include mice with different manipulations of the BDNF gene, e.g., exon-1 (Liebl *et al.* 1997; Lyons *et al.* 1999) vs exon-5 (Dluzen *et al.* 2002; Dluzen *et al.* 2004; Ernfors *et al.* 1995; Joyce *et al.* 2004; Liu *et al.* 1995) vs the entire BDNF coding region (Bennett *et al.* 1999), different inbred strains for backcrosses, e.g., B6 (Liebl *et al.* 1997) vs BALB/c (Ernfors *et al.* 1995; Liu *et al.* 1995), as well as different experimental protocols. A recent article by Crusio *et al.* (2009) and a Nature Neuroscience editorial (Crusio *et al.* 2009; Editorial 2009) reviews the importance of these factors in gene knockout experiments.

General characteristics of *Bdnf*^{+/-} mice on the C57BL/6 background

Bodyweight gain was similar for *Bdnf*^{+/-} and WT mice from 3 to 12 months of age; however, from 12 to 21 months increased by 43% for *Bdnf*^{+/-} while remaining constant for WT mice. The greater weight for the *Bdnf*^{+/-} mice is consistent with elevated fat and insulin (Kernie *et al.* 2000; Lyons *et al.* 1999) and is independent of strain background (Bartoletti *et al.* 2002; Ernfors *et al.* 1994; Liebl *et al.* 1997). Elevated body weight for the *Bdnf*^{+/-} mice occurred later in life in our study than reported by Lyons *et al.* (1999) (2–4 months). Differences in the diets used (i.e. 9% crude fat in the Lyons *et al.* (1999) study vs. 4.40% fat in our study (Harlan Teklad Rodent Diet 8604) is likely accountable (Lyons *et al.* 1999). Another possible contributing factor could be animal housing differences with single caging in the Lyons *et al.* (1999) study, and 4–5 per cage in ours which alters feeding and resultant weight gain (Lyons *et al.* 1999). The elevated bodyweights seems to require a general BDNF reduction since it did not occur when restricted to the forebrain in a conditional KO mouse (Monteggia *et al.* 2004). Elevated body weight for *Bdnf*^{+/-} mice is consistent with elevated leptin, an important regulator of feeding behavior, in the hypothalamus and increased adipocyte cell size reported for a similar mouse genotype at six months (Kernie *et al.* 2000), and also for obese humans (Caro *et al.* 1996; Friedman 1997).

Behavioral characteristics of *Bdnf*^{+/-} mice across the life span

Locomotor activity (horizontal) of *Bdnf*^{+/-} mice was comparable to that of age-matched WT controls at 3 and 12 months of age but was lower at 21 months of age. In contrast, their performance on a more challenging motor coordination task, accelerating rotarod, was deficient at 3 months of age. Such a pattern of age-related motor deficiency is common for aging humans (Bennett *et al.* 1996; Volkow *et al.* 1998). The possible contribution of elevated body weight to the motor deficits observed for *Bdnf*^{+/-} mice eliminated via correlation analysis on the locomotor activity and rotarod measures, and factoring out the weight difference on rotarod performance indicating other peripheral or central nervous system abnormalities.

The absence of a motor activity deficit for the younger *Bdnf*^{+/-} mice in our study is consistent with other reports: 1) young adult *Bdnf*^{+/-} mice on a C57BL/6 genetic background (Macqueen *et al.* 2001), 2) young female *Bdnf*^{+/-} mice on a mixed B6/SV129 background (Chourbaji *et al.* 2004), and 3) mixed sexes on a mixed BALB/C/SV129 background (Dluzen *et al.* 2001). The lower motor activity for older *Bdnf*^{+/-} mice in our study is consistent with a study using BDNF^{lacZ/neo+} mice with a different BDNF manipulation but a similar behavioral paradigm (Saylor *et al.* 2006), and with the poor performance of older, but not young, *Bdnf*^{+/-} mice on a beam walk task (Dluzen *et al.* 2004). In contrast, Kernie *et al.* (Kernie *et al.* 2000) reported hyperactivity for six-month-old *Bdnf*^{+/-} mice with normal body weight, and activity comparable to WT controls for 50% of the *Bdnf*^{+/-} mice classified as obese. These variable effects of a genetic reduction in BDNF on motor activity across different strains emphasizes the importance of considering the influence of different parental genetic backgrounds noted in the recent articles by Crusio *et al.* (2009) and the editorial in Nature Neuroscience (Crusio *et al.* 2009; Editorial 2009), as well as different behavioral testing strategies noted above when interpreting the outcome of experiments on genetically manipulated animals.

Motor coordination is one of the first behavioral components to deteriorate with normal aging (Bickford *et al.* 1992) and the poor rotarod performance of *Bdnf*^{+/-} mice suggests a role for BDNF in this decline. The poor coordination of the *Bdnf*^{+/-} mice in our experiments is consistent with a report of vertical activity and accelerating rotarod performance deficits associated with low BDNF protein expression in the cerebellum of mice in a Classic Rett syndrome model (Kondo *et al.* 2008); and with a report of low striatal and cerebellar BDNF

levels for aged rats with motor problems (Kato-Semba *et al.* 1998). In contrast, Baker *et al.* reported no rotarod performance deficit for *Bdnf*^{+/-} mice although performance of the mice declined with age (Baker *et al.* 2005). In addition, Canals *et al.* (2004) reported that although BDNF reduction alone did not alter their measure, it did enhance aging-related motor dysfunction in a double mutant mouse model of Huntington's disease (Canals *et al.* 2004). Mice used in the Baker and Canals studies were backcrossed on the BALB/c rather than the B6 strain used in our experiment and the influence of strain on open-field testing and rotarod behavior (Carola *et al.* 2002; Depino & Gross 2007; Griebel *et al.* 2000) could contribute to different outcome of these experiments. Given the influence of gender on behavioral measures (Archer 1975; Fernandes *et al.* 1999), it should also be noted that the Chourbaji *et al.* study which observed no coordination deficits in young *Bdnf*^{+/-} mice used only females (Chourbaji *et al.* 2004). Finally, the particular experimental protocol including data analysis used in rotarod assessment can influence experimental outcomes (Rustay *et al.* 2003) and differed with data from each animal across each test and each day analyzed with sensitive Kaplan-Myer survival curves in our experiment.

The possibility that learning deficits contributed to the poor rotarod performance for *Bdnf*^{+/-} mice is unlikely because performance improved at the same rate across days for WT and *Bdnf*^{+/-} mice tested at 3 months of age in the current study. Studies focusing on more focused behavioral evaluation and perhaps location of BDNF loss in different brain regions could help clarify the impact of reducing this neurotrophic factor on motor systems.

Neuroanatomical & neurochemical characteristics of *Bdnf*^{+/-} mouse DA systems across the life span

TH-ir, DAT-ir, or VMAT2-ir in the nigrostriatal system of *Bdnf*^{+/-} mice did not differ from WT controls at any age as was previously reported for young *Bdnf*^{+/-} mice (Joyce *et al.* 2004). TH-ir was lower in striatum of older mice regardless of genotype. The aging-related decline in striatal TH-ir was accompanied by reduced DAT and VMAT2 activity, with substantial reductions at 21 months. Further, in comparison to WT controls, activity of both transporters was reduced for *Bdnf*^{+/-} mice at 12 months, and VMAT2 was also reduced at 3 months of age. Activity of DAT and VMAT2 has shown to be dynamically regulated by cellular kinases, phosphatase and transporter associated proteins. It is possible that constitutive reductions in BDNF and its receptor linked signaling cascades can affect transporter activity independent to total expressions (Jayanthi *et al.*, 2007; Krantz *et al.*, 1997). *In vivo* microdialysis focused on young adult and middle-aged mice. These experiments clearly established lower basal levels of extracellular DA for *Bdnf*^{+/-} mice regardless of age. Extracellular DA following KCl stimulation was increased above basal levels to roughly the same extent for both genotypes with DA greater for WT than *Bdnf*^{+/-} mice during the first 15-min interval. An important genotypic difference, however, was the slower return of the elevated DA level to pre-stimulus basal levels for the *Bdnf*^{+/-} compared to WT mice. Results of our *in vivo* experiment differs from the report of an *in vitro* experiment indicating that K⁺-stimulated DA release was lower in *ex vivo* nigrostriatal tissue preparations from 12 and 20 month-old *Bdnf*^{+/-} mice than from age-matched WT mice (Dluzen *et al.* 2004). Our results also differ from the results obtained from the *ex vivo* experiment in that KCl-stimulated release did not change significantly between the young adult and mid-aged mice in our experiment whereas it increased substantially in tissue from mid-aged compared to young adults in the *in vitro* report. The reasons for the different results of the two experiments are not clear but could include: 1) possible differences in neurotransmitter dynamics *ex vivo* vs *in vivo* assessments, 2) the different genetic background strains (BalbC vs C57) which have different DA characteristics, or 3) slight differences in the particular DA areas assessed. Although the slower decline of extracellular DA following KCl- stimulation for *Bdnf*^{+/-} compared to WT mice is consistent with the

lower DAT transporter activity noted for the genotype, the observed lower basal DA levels combined with lower DAT activity for the *Bdnf*^{+/−} mice remains difficult to explain. Possible explanations include some adaptive changes resulting from prolonged reduction in BDNF for the *Bdnf*^{+/−} mice. For example, the constitutive reduction in brain BDNF levels in *Bdnf*^{+/−} mice could decrease DA-synthetic pathway activity, or perhaps increase DA-degradative pathways. The higher DA levels in striatal tissue and the unaltered DA cell bodies or axons noted for *Bdnf*^{+/−} mice (Baker *et al.* 2005; Dluzen *et al.* 1999) suggest that DA synthesis or DA-neuronal loss are not likely accountable for the low levels of synaptic DA in *Bdnf*^{+/−} mice. While DAT is considered to clear DA primarily via influx/uptake process, DAT also has regulated capacity to efflux cytosolic DA to synapse (Defelice & Goswami 2007; Khoshbouei *et al.* 2004). In addition, other transporters such as the norepinephrine transporter (NET) and organic cation transporter (OCT) might contribute toward the clearance of synaptic DA (Busch *et al.* 1996; Chemuturi & Donovan 2007; Moron *et al.* 2002). Studies related to DAT-mediated DA efflux, NET and OCT3 activities and active levels of DA-degradative enzymes have yet to be conducted in *Bdnf*^{+/−} mice and are warranted for future investigations. In addition, systematic use of no net flux microdialysis approach is necessary to confirm the role of amine transporters in relevance to lower basal DA levels in *Bdnf*^{+/−} mice. Furthermore, in contrast to the decreased basal DA levels found in young and middle aged *Bdnf*^{+/−} mice, higher basal extracellular 5-HT levels in the *Bdnf*^{+/−} ventral hippocampus have been reported (Benmansour *et al.* 2008; Daws *et al.* 2007; Guiard *et al.* 2008).

DAT activity reductions from 3 to 12 months for *Bdnf*^{+/−} mice (59%) and WT mice (37%) were not accompanied by reduced striatal TH-ir. DAT knockout mouse studies indicate a direct relationship between DAT and TH expression (Jaber *et al.* 1999; Jones *et al.* 1998) with DAT depletion producing a 90–96% reduction in striatal TH-ir. Thus, maintenance of TH protein expression in the striatum appears to have a DAT activity threshold. Current data suggest that BDNF is extensively involved in the complex functions of DA release and re-uptake.

The precise relationship of the DA system deficits to the motor system deficits noted in *Bdnf*^{+/−} remain unclear, however, both the mesoaccumbens and nigrastratial systems are involved in mediating various aspects of motor function (Volkow *et al.* 1998). Clearly the advanced decline of motor function with age for *Bdnf*^{+/−} mice is consistent with the reduced levels of extracellular DA, and with the reduced DAT and VMAT2 activity, all of which suggest a less functional DA system. The aging-related decline in motor coordination is also related to striatal DA, as well as cerebellar NE (Bickford *et al.* 1992). The particular behavioral abnormalities noted and the type of *Bdnf*^{+/−} mice used in our study differed from those obtained on the *Bdnf*^{+/−} mouse type used in studies reported by Dluzen and colleagues (Dluzen *et al.* 2002; Dluzen *et al.* 1999); however, the primary conclusion are similar in that aging-related changes in DA system function and the behaviors they mediate are accentuated by reduced BDNF levels (Dluzen *et al.* 2004).

In conclusion, the present study indicates that the constitutive BDNF reduction noted in *Bdnf*^{+/−} mice exacerbates the aging-related dysfunctions of motor systems and DAergic dynamics in the striatum. The accentuated aging dysfunctions were indicated in *Bdnf*^{+/−} mice; behaviorally, by lower locomotor activity and deficient performance on the accelerating rotarod, and neurochemically, by reductions in extracellular DA and activity of DAT and VMAT2 transporters. To help further elucidate the consequences of genetic manipulations on behavior and mediating neurochemistry, it will be important to attend to details of the genetic manipulation, the genetic background of the knockout mice, other biogenic amine transporters, as well as the details of the particular behavioral protocols employed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Variations in striatal BDNF levels and body weight with aging in *Bdnf*^{+/-} vs. WT mice
 (A) BDNF protein levels were lower in *Bdnf*^{+/-} than WT littermates at all ages tested with no significant effect of age. (B) Body weight (grams) depended on a Genotype × Age interaction. Post-hoc analysis indicated body weight increases for both genotypes between 3 and 12 months of age (* $p \leq 0.05$). At 21 months of age, *Bdnf*^{+/-} mice weighed more than age-matched WT mice ([#] $p \leq 0.01$) and more than 12-month-old *Bdnf*^{+/-} mice ([¶] $p \leq 0.01$).

Figure 2. Motor activity [A: Horizontal; B: Rearing Responses] of *Bdnf*^{+/-} and WT mice at 15-minute intervals during one-hour open-field activity tests at 3, 15, and 21 months of age Both Distance Traveled, Fig 2A, and Rearing Response frequency, Fig 2B, declined across the hour session. The only effect of the Genotype or Age on the decline in activity across the tests was an Age by Interval interaction for the Rearing Response measure with the decline more rapid for older mice. Both Distance Traveled and Rearing Responses declined with age. The Genotype factor was associated with reductions in Rearing Response frequency; however, did not impact Distance Traveled. Finally, the apparent difference in the pattern of change in Distance Traveled across age for the two genotypes suggested by Fig 2A reached only a 0.09 level of significance.

Figure 3. Accelerating rotarod performance was reduced for *Bdnf*^{+/−} and aging mice and was independent of body weight

Data are expressed as Kaplan-Meier survival curves representing the probability of mice within each group remaining on the rotarod collapsed across the 6 increasing rotational speeds with increasing time up to 300 sec, and across the three test days. The proportion of mice remaining on the rotarod (ordinate) with increasing time across the increasing rotation speeds (abscissa) declined more rapidly for *Bdnf*^{+/−} than for WT mice (Fig 3A), and more rapidly for 12- and 22-month-old compared to 3-month-old mice (Fig 3B). Although change in median survival times across the three ages differed somewhat for *Bdnf*^{+/−} and WT mice and were independent of body weight (Fig 3C), the different pattern was not supported by a Genotype $\times$ Age interaction.

Figure 4. Extracellular DA levels in striatum were lower for *Bdnf*^{+/-} than WT mice during basal assessment, and following KCl-stimulated release at 3 and 12 months of age

Extracellular DA levels determined from six 15-min interval basal collections are summarized for 3-month-old mice in (Fig 4A) and 12 month-old mice in (Fig 4B). Basal DA was lower for *Bdnf*^{+/-} than WT mice with no difference related to Age or its interaction with Genotype. Because basal DA differed for the two genotypes, data collected at intervals following infusion of 60 mM KCl-aCSF summarized in Figs 4C & D, respectively, for 3- and 12-month-old mice were analyzed with an ANCOVA using Baseline Sample #6 (Time=0 min) as a covariate. ANCOVA indicated that extracellular DA varied according to a Genotype $\times$ Time Interval Interaction with no effect of the Age factor. Additional analysis of the regression slopes indicated a more rapid return to basal levels for WT than *Bdnf*^{+/-} mice regardless of age.

A. DAT Activity

B. VMAT2 Activity

Figure 5. Age- and genotype-related differences in striatal DAT and VMAT2 activity in *Bdnf*^{+/-} and WT mice

DA-transport via DAT (A) and VMAT2 (B) are expressed as pmol/mg protein/min. DAT activity declined with age for both genotypes but the degree of reduction was greater for *Bdnf*^{+/-} than for WT mice (WT: * $p < 0.05$ (12 months), * $p < 0.001$ (21 months) compared with 3 months; # $p < 0.001$ compared with 12 months WT; *Bdnf*^{+/-}: ¶ $p < 0.001$ (12 months), ¶ $p < 0.001$ (21 months) compared with 3 months *Bdnf*^{+/-} ® $p < 0.001$ compared with 12 months *Bdnf*^{+/-}). The greatest reduction in DAT activity observed in *Bdnf*^{+/-} mice compared to WT mice at 12 and 21 months of age (§ $p < 0.01$ (12 months), § $p < 0.007$ (21 months) compared with 12 months and 21 months WT respectively). VMAT2 activity also declined with age for both genotypes with the extent of the reduction depending on genotype (WT: * $p < 0.001$ (12 months), * $p < 0.001$ (21 months) compared with 3 months WT; # $p < 0.05$

compared with 12 months WT; *Bdnf*^{+/−}: ¶*p* < 0.001 (12 months), ¶*p* < 0.001 (21 months) compared with 3 months *Bdnf*^{+/−}). VMAT2 activity was lower for the *Bdnf*^{+/−} at 3 and 12 months of age compared with 3 and 12 months WT (§*p* < 0.001 (3 months), §*p* < 0.001 (12 months) compared with 3 months and 12 months WT respectively).

Table 1

Numbers of animals used in each assay.

Assays	3 month WT	3 month <i>Bdnf</i> ^{+/-}	12 month WT	12 month <i>Bdnf</i> ^{+/-}	21 month WT	21 month <i>Bdnf</i> ^{+/-}
BDNF levels [#]	9	8	8	12	5	6
Body weight [#]	10	10	10	9	11	9
Horizontal activity [#]	6	6	11	10	11	9
Vertical activity [#]	6	6	11	10	11	9
Accelerating rotarod [#]	8	5	11	10	11	9
Immunohistochemistry [#]	5	6	5	5	5	4
Microdialysis [§]	7	7	7	5	ND	ND
Striatal DAT activity [¶]	12	18	15	18	18	18
Striatal VMAT2 activity [§]	4	4	4	4	6	6

[#] Separate cohort for 3, 12 and 21 month-old WT and *Bdnf*^{+/-} mice were used. Following completion of behavioral assays, brain tissues were used for immunohistochemistry and BDNF assays.

[§] Separate cohort for 3 and 12 month-old WT and *Bdnf*^{+/-} mice were used.

[¶] Separate cohort for 3, 12 and 21 month-old WT and *Bdnf*^{+/-} mice were used. DAT activity was determined from striatum of individual animals

[§] Separate cohort for 3, 12 and 21 month-old WT and *Bdnf*^{+/-} mice were used. Synaptic vesicles were prepared from pooled striatal tissue derived from three age and genotype matched animals (n=1).

ND: Not determined