BRAIN-DERIVED NEUROTROPHIC FACTOR AND TRKB LEVELS IN MICE THAT LACK VESICULAR ZINC: EFFECTS OF AGE AND SEX

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ABSTRACT

In certain neurons, zinc ions are stored in synaptic vesicles by a membrane transport protein called zinc transporter 3 (ZnT3). Vesicular zinc can then be released synaptically to modulate a number of targets, potentially including brain-derived neurotrophic factor (BDNF) and its receptor, tropomyosin receptor kinase B (TrkB). There is *in vitro* evidence that zinc can interact directly with the BDNF protein; interact with enzymes that cleave BDNF from its precursor, proBDNF; and exert BDNF-independent effects on TrkB. But the effects of vesicular zinc on BDNF and TrkB in the intact brain are not clear. Studies of mice that lack ZnT3 – and, as a result, vesicular zinc – have shown abnormalities in BDNF and TrkB levels, but results have been mixed and are therefore difficult to interpret. This discrepancy might be caused, at least in part, by differences in the age and sex of mice used in previous studies. In the present study, we measured BDNF and TrkB protein levels in two brain regions, the hippocampus and neocortex, comparing between wild type and ZnT3 knockout mice of both sexes, and including both young (5-week-old) and mature (12-week old) animals. We found that, regardless of age or sex, levels of BDNF and TrkB (truncated or full-length) did not differ between wild type and ZnT3 knockout mice. There was a sex difference in BDNF expression, however; in both hippocampus and neocortex, BDNF levels increased with age in female mice but not in male mice. And male mice had greater neocortical levels of full-length TrkB than did female mice. We conclude that, at least in naïve mice housed under standard laboratory conditions, vesicular zinc is not involved in the processing of BDNF, and elimination of vesicular zinc does not affect BDNF or TrkB levels.

Keywords:

Synaptic zinc; zincergic neuron; SLC30A3; zinc signaling; Western blot; enzyme-linked immunosorbent assay (ELISA)

Abbreviations:

BDNF: brain-derived neurotrophic factor; ELISA: enzyme-linked immunosorbent assay; KO: knockout; MMP: matrix metalloproteinase; TrkB: tropomyosin receptor kinase B; TrkB.T: truncated TrkB; WT: wild type; ZnT3: zinc transporter 3.

Declarations of interest:

None.

1. INTRODUCTION

The divalent cation zinc has essential biological functions throughout the body, including in the brain. Though most zinc in the brain is tightly-bound in protein structures, a portion exists in a "free" (unbound or loosely-bound) state, making it available to participate in signaling functions (reviewed by McAllister & Dyck, 2017). The concentration of extracellular free zinc is relatively low, in the nanomolar range (Frederickson et al., 2006a), and the cytosolic concentration is even lower, in the picomolar range (Colvin et al., 2010). However, in certain regions of the brain and spinal cord, a considerable pool of free zinc is stored in the synaptic vesicles of neurons (Pérez-Clausell & Danscher, 1985). This vesicular zinc can be released in an activity-dependent manner (Assaf & Chung, 1984; Howell et al., 1984; Aniksztejn et al., 1987), elevating the extracellular free zinc concentration to – by most estimates – the low micromolar range (Frederickson et al., 2006b), though hundreds of micromolar might be achievable with intense stimulation.

One intriguing target of zinc is brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family (Barde et al., 1982). Like other excreted peptides, BDNF is produced in the cell soma as a larger precursor protein. Pre-proBDNF is cleaved intracellularly into proBDNF (32 kDa). ProBDNF is then cleaved, by furin or other proprotein convertases, to produce mature BDNF (14 kDa), which forms an active homodimer (Mowla et al., 2001). BDNF is anterogradely transported to axon terminals in dense core vesicles (Conner et al., 1997; Michael et al., 1997; Fawcett et al., 1997; Dieni et al., 2012), from where it can be released along with the cleaved pro-domain (Kohara et al., 2001; Matsumoto et al., 2008). Uncleaved proBDNF can also be released and processed extracellularly, by plasmin or matrix metalloproteinases (MMPs), into mature BDNF (Lee et al., 2001; Gray & Ellis, 2008; Nagappan et al., 2009; but see Matsumoto et al., 2008). In addition to pre-synaptic release, BDNF is also stored post-synaptically in dendrites and spines, and it can be released to act as a retrograde or autocrine signal (Wong et al., 2015; Harward et al., 2016; Choo et al., 2007).

Once released, BDNF exerts effects through tropomyosin receptor kinase B (TrkB), allosterically dimerizing these receptors and inducing their kinase function (Klein et al., 1991). This activates several signaling cascades, including the Ras, Rac, PI3-kinase, and PLC-γ1 pathways (Reichardt, 2006). Truncated variants of TrkB are also expressed, with unique cytoplasmic domains that lack catalytic kinase function (Klein et al., 1990), though the T1 variant has its own BDNF-dependent signaling pathway that results in intracellular calcium release (Rose et al., 2003). When expressed in the same cells, truncated TrkB forms heterodimers with full-length TrkB and inhibits its function (Eide et al., 1996).

There is *in vitro* evidence that zinc can increase BDNF mRNA expression (I. Hwang et al., 2011), act directly on the BDNF protein (Ross et al., 1997; Post et al., 2008; Travaglia et al., 2013), activate extracellular enzymes that cleave BDNF from its precursor (J. Hwang et al., 2005; I. Hwang et al., 2011; Poddar et al., 2016), and transactivate TrkB through BDNF-independent mechanisms (Huang et al., 2008;

Huang & McNamara, 2012). However, uncertainty remains about how vesicular zinc interacts with BDNF in the intact brain. One way of studying this is to examine how the BDNF-TrkB pathway is affected in mice that lack the dedicated vesicular zinc transporter, zinc transporter 3 (ZnT3) (Palmiter et al., 1996; Wenzel et al., 1997). Elimination of this protein results in total loss of vesicular zinc (Cole et al., 1999). Several studies have used this approach, but they have produced mixed results. Adlard et al. (2010) found that hippocampal proBDNF levels, but not BDNF or TrkB levels, are reduced in ZnT3 knockout (KO) mice at 3 months of age. By 6 months, both proBDNF and TrkB levels are reduced. Similarly, Nakashima et al. (2011) showed that TrkB mRNA levels in barrel cortex are reduced in 2-month-old male ZnT3 KO mice. On the other hand, Helgager et al. (2014) showed that ZnT3 KO mice, aged 3-6 months, have normal levels of hippocampal TrkB but increased BDNF, resulting in increased TrkB phosphorylation. And Yoo et al. (2016) found that 5-week-old male ZnT3 KO mice have increased levels of TrkB in the hippocampus and cortex, increased mature BDNF and proBDNF in the cortex, and increased mature BDNF but decreased proBDNF in the hippocampus.

One obstacle to synthesizing these results are the differences in the age and sex of the mice examined, as well as differences in the brain regions assessed. Our goal in the present experiment was to address this limitation. We examined hippocampal and neocortical protein levels of BDNF and TrkB, comparing wild type (WT) and ZnT3 KO mice of both sexes at two different ages within the same study.

2. METHOD

2.1 Animals

All protocols were approved by the Life and Environmental Sciences Animal Care Committee at the University of Calgary and followed the guidelines for the ethical use of animals provided by the Canadian Council on Animal Care. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to *in vivo* techniques, if available. Mice were housed in temperature- and humidity-controlled rooms maintained on a 12:12 light/dark cycle (lights on during the day). Food and water were provided *ad libitum*. WT and ZnT3 KO mice, on a mixed C57BL/6×129Sv genetic background, were bred from heterozygous pairs. Offspring were housed with both parents until postnatal day 21, at which point they were weaned and housed in standard cages (28 × 17.5 × 12 cm with bedding, nesting material, and one enrichment object) in groups of 2-5 same-sex littermates.

2.2 Experimental design

Brain tissue was collected from 40 mice. These included WT and ZnT3 KO, male and female, and young (5-week-old) and mature (12-week-old) mice, resulting in eight experimental groups (n = 5 for each).

2.3 Sample preparation

Mice were briefly anaesthetized with isoflurane and killed by decapitation. The brain was rapidly extracted, and the neocortices and hippocampi were dissected. The neocortical samples contained primarily posterior cortex, to avoid including striatum in the sample. The extracted tissue was frozen on dry ice and stored at -80 °C.

For protein analysis, tissue samples were placed in chilled RIPA buffer (Millipore-Sigma, 50 mM Tris-HCl, 150 mM NaCl, 0.25% deoxycholic acid, 1% NP-40, 1 mM EDTA; hippocampus: 200 µl; neocortex: 400 µl) containing protease and phosphatase inhibitors (Thermo Scientific Halt Protease and Phosphatase Inhibitor Cocktail, EDTA-Free) and homogenized using a bead lyser (5 min, 50 Hz). The lysates were placed on ice for 1 h and then centrifuged at 4 °C (15 min, 12,000 *g*). The supernatants were collected, protein concentrations were determined by Bradford assay (Bio-Rad), and the supernatants were stored at 20 °C until further analysis.

2.4 Western blotting

Protein samples were heated at 95 °C for 3 min in sample buffer containing 2% 2-mercaptoethanol. Samples were separated by electrophoresis on 12% SDS-PAGE gels (30 µg of protein per lane) and transferred to PVDF membranes (Bio-Rad). The membranes were blocked for 1 h in Odyssey tris-buffered saline (TBS) blocking buffer (LI-COR Biosciences) and incubated overnight at 4 °C in Odyssey blocking buffer containing one or more of the following primary antibodies: rabbit anti-BDNF (1:1000; Santa Cruz Biotechnology, Inc.; sc-546); rabbit anti-TrkB (1:1000, Cell Signaling Technology; #4603); mouse anti-beta actin (1:2000; Abcam; ab8224). Blots were washed 3 × 10 min in TBS containing 0.1% Tween-20 (TBST), incubated for 1 h in Odyssey blocking buffer containing the secondary antibodies (1:10,000 anti-rabbit IRDye 800, 1:15,000 anti-mouse IRDye 680; LI-COR Biosciences), and again washed 3 × 10 min in TBST. Blots were imaged using an Odyssey infrared imaging system (LI-COR Biosciences) and quantified by densitometry using ImageJ (https://imagej.nih.gov/ij/index.html). Levels of BDNF or TrkB were normalized to the level of beta-actin.

2.5 Enzyme-linked immunosorbent assay (ELISA)

BDNF levels were also quantified using a sandwich ELISA kit (Aviscera Bioscience, SK00752-01) with good selectivity for mature BDNF over proBDNF (Polacchini et al., 2015). The plates used were from the same lot and were run simultaneously. Samples were assayed in duplicate (protein loaded per well: 500 µg for hippocampus, 800 µg for neocortex) following the provided instructions. Briefly, samples were incubated for 2 h in a 96-well plate precoated with a monoclonal antibody against BDNF. The plate was then incubated for 2 h with the biotinylated detection antibody, followed by a 1 h incubation with streptavidin-HRP conjugate. Tetramethylbenzidine (TMB) substrate solution was added,

and colour was developed for 18 min before addition of the stop solution (0.5 M HCl). All incubations were conducted at room temperature on an orbital shaker. Absorbance was measured at 450 nm for 0.1 s using a microplate reader (Wallac 1420 Victor^2; Perkin Elmer Life Sciences). BDNF concentrations were determined based on a standard curve and converted to pg of BDNF per mg of total protein.

2.6 Statistical analysis

Statistical analyses were conducted using IBM SPSS Statistics (Version 24). Unless otherwise specified, data were analyzed by three-way analysis of variance (ANOVA), with sex (male vs. female), age (5 week vs. 12 week), and genotype (WT vs. ZnT3 KO) as factors. Significant interactions were followed-up with Bonferroni-corrected simple effects tests using the pooled error term, unless equality of variances could not be assumed (Levene's test: $p < .05$), in which case non-pooled error terms were used. All ANOVA results, including non-significant interactions, are summarized in Supplemental Table 1.

3. RESULTS

3.1 Hippocampus

Levels of BDNF in the hippocampus were first quantified by densitometry on Western immunoblots (Figure 1). The antibody against BDNF detected three bands between 11-kDa and 18-kDa; the lower molecular weight band was assumed to be mature BDNF, as its position most closely resembled that of recombinant human BDNF (Alomone Labs, #B-250). The other bands were presumed to be intermediate cleavage products of proBDNF, as suggested by Chacón-Fernández et al. (2016). Two more bands were also apparent, around 25-kDa and 28-kDa. These bands could also represent intermediate products, or they could have resulted from non-specific binding of the antibody. Previous work has shown a 28-kDa truncated form of proBDNF (Mowla et al., 2001). There was, at most, a very faint band around 32-kDa, where proBDNF would be expected. Given previous estimates that mature BDNF levels are over 10 times greater than proBDNF levels in the hippocampus (Matsumoto et al., 2008; Dieni et al., 2012), it is possible that proBDNF levels were too low to detect robustly. Mature BDNF levels, measured by densitometry (Figure 2A), did not differ based on sex [$F(1,32) = 0.01$, $p = .924$], age [$F(1,32) = 0.68$, $p = .416$], or genotype [$F(1,32) = 2.69$, $p = .111$].

To quantitatively assess BDNF, we also measured BDNF concentrations by ELISA (Figure 2B), using a kit that is selective against mature BDNF. Here, we found that age had differing effects on male and female mice [age × sex interaction: $F(1,32) = 7.95$, $p = .008$]. Specifically, BDNF levels did not differ between 5-week-old and 12-week-old males ($p = .151$), but in females, BDNF levels increased significantly with age ($p = .034$; Bonferroni-corrected). Overall, no significant effect of genotype was observed [$F(1,32) = 0.36$, $p = .552$]. Though this pattern of results differed somewhat from the results obtained by Western blotting, the BDNF levels quantified by the two methods were significantly correlated [$r(38) = .49$, $p = .001$].

Levels of TrkB were also assessed by Western blotting (Figure 1). The antibody against TrkB detected two bands; one around 135 kDa, assumed to be full-length TrkB, and one around 90 kDa, assumed to be truncated TrkB (TrkB.T). TrkB.T appeared to be more abundant than the full-length version, consistent with previous findings (Fryer et al., 1996). TrkB levels (Figure 3A) did not differ based on sex [$F(1,32) = 0.47$, $p = .496$], age [$F(1,32) = 0.34$, $p = .566$], or genotype [$F(1,32) = 1.60$, $p = .215$]. Likewise, TrkB.T levels (Figure 3B) did not differ based on sex [$F(1,32) < 0.01$, $p = .958$], age [$F(1,32) < 0.01$, $p = .959$], or genotype [$F(1,32) = 1.49$, $p = .231$].

3.2 Neocortex

Levels of BDNF in the neocortex, as assessed by Western blotting, were higher in the 12-week-old mice compared to the 5-week-old mice [main effect of age: $F(1,32) = 11.56$, $p = .002$; Figure 4A]. No significant effect of sex [$F(1,32) = 2.85$, $p = .101$] or genotype [$F(1,32) = 0.92$, $p = .345$] was observed.

BDNF concentrations in the neocortex were also assessed by ELISA (Figure 4B). This produced a somewhat different pattern of results than did Western blotting, though a similar pattern to the ELISA results observed in the hippocampus. Age had differing effects on male and female mice [age × sex interaction: $F(1,32) = 6.60$, $p = .015$]. BDNF levels did not differ between 5-week-old and 12-week-old males ($p = .355$), but in females, BDNF levels increased significantly with age ($p = .022$; Bonferroni-corrected). Overall, no significant effect of genotype was observed [$F(1,32) = 0.35$, $p = .557$]. Unlike for the hippocampus, the neocortical BDNF levels measured by ELISA and by Western blotting were not significantly correlated [$r(38) = .18$, $p = .266$].

TrkB levels in the neocortex were significantly higher in male mice than female mice [$F(1,32) = 5.13$, $p = .030$; Figure 5A]. There was no effect of age [$F(1,32) = 0.89$ $p = .353$] or genotype [$F(1,32) = 1.28$, $p = .267$]. Levels of TrkB.T (Figure 5B) did not differ based on sex [$F(1,32) = 0.10$, $p = .757$], age [$F(1,32) = 0.08$, $p = .777$], or genotype [$F(1,32) = 1.29$, $p = .265$].

4. DISCUSSION

Previous examinations of BDNF and TrkB levels in the brains of ZnT3 KO mice have produced seemingly discrepant results. The objective of the present experiment was to determine whether differences in age or sex of the mice tested might account for the discrepancy. The primary finding was that elimination of vesicular zinc, by genetic inactivation of ZnT3, did not affect hippocampal or neocortical levels of BDNF or TrkB, regardless of whether mice were male or female, young or mature. Thus, our results do not explain the discrepancy in the literature – to some extent, they add to it.

Our results are in closest agreement with those of Adlard et al. (2010), who found that hippocampal TrkB levels do not decline in ZnT3 KO mice until somewhere between 3-6 months; older than the mice examined in our study. They also found that mature BDNF levels are normal in ZnT3 KO mice at least up to 6 months of age. Our results are in partial agreement with Helgager et al. (2014), who

reported that overall hippocampal TrkB levels are normal in mature ZnT3 KO mice – though TrkB phosphorylation is enhanced. However, they also reported that hippocampal BDNF levels are elevated, for which we found no evidence. Our results correspond least closely with those of Yoo et al. (2016), who found elevated levels of both mature BDNF and TrkB (most likely truncated TrkB, based on their figure 4.3B) in 5-week-old ZnT3 KO mice.

It is worth noting that the present results are consistent with our own previous, unpublished findings. Specifically, we have found that ZnT3 KO mice show abnormal hippocampal BDNF levels following enriched housing (Chrusch et al., unpublished) or chronic fluoxetine treatment (Boon et al., unpublished), but in both experiments there was no difference between control WT and control ZnT3 KO mice. Together with the present results, this suggests that under "normal" conditions (e.g., standard housing, no drug treatment), vesicular zinc does not contribute to BDNF production or processing. But vesicular zinc might be necessary for the BDNF "boost" induced by certain experiences. How might this work?

First, assume that mature BDNF is the dominant BDNF product in the adult brain and that proBDNF levels are relatively minimal, for which there is some evidence (Matsumoto et al, 2008; Dieni et al., 2012). Apparently, under normal conditions the brain has adequate enzymatic capacity to process most proBDNF into mature BDNF intracellularly, and this is not dependent on vesicular zinc. Under certain conditions that increase BDNF gene expression – such as environmental enrichment or chronic antidepressant treatment (Nibuya et al., 1996; Zajac et al., 2010; Tsankova et al., 2006) – proBDNF production will be increased, which may exceed the enzymatic capacity to process proBDNF, leading to it building-up within vesicles. It is unlikely that zinc interacts with BDNF within vesicles, as BDNF is localized to dense core vesicles (Michael et al., 1997; Dieni et al., 2012) and zinc is found in clear, round vesicles (Pérez-Clausell & Danscher, 1985).

More likely is that zinc interacts with BDNF in the extracellular space, since both are secreted in response to neuronal activity (Frederickson et al., 2006b; Matsumoto et al., 2008; Nagappan et al., 2009; Wong et al., 2015). A build-up of proBDNF within vesicles would lead to increased proBDNF secretion. ProBDNF can be cleaved by extracellular MMPs (Lee et al., 2001), protease enzymes that require zinc to perform their catalytic function. Zinc, which promotes the activity of these enzymes at a (probably) physiologically-relevant concentration of 10 μM (J. Hwang et al., 2005), might increase the processing of proBDNF into mature BDNF within the synaptic cleft. Interestingly, MMP activity also appears to promote the expression of tissue plasminogen activator (I. Hwang et al., 2011), which, by activating plasmin, also increases the extracellular capacity to cleave proBDNF into mature BDNF (Pang et al., 2004). Alternatively, it is possible that vesicular zinc acts upstream of BDNF gene expression; this could be tested by examining whether ZnT3 KO mice show increased BDNF mRNA expression in response to environmental enrichment or antidepressant treatment. There is *in vitro* evidence that exposure to zinc can

increase BDNF mRNA expression in cortical neurons, through an unknown mechanism that is dependent on MMPs (I. Hwang et al., 2011). Vesicular zinc release has also been shown to activate the mitogen-activated protein kinase (MAPK) pathway presynaptically in the hippocampal mossy fibers (Sindreu et al., 2011). One downstream target of MAPK is cyclic AMP response element-binding protein (CREB) (Impey et al., 1998), which can activate BDNF gene transcription (Tao et al., 1998).

Although we found no effects of eliminating vesicular zinc on BDNF or TrkB levels in the present experiment, there were unexpected effects of age and sex. For BDNF levels, the results obtained by Western blotting and by ELISA were somewhat divergent; we tend to favour the ELISA results, for two reasons. One is that ELISA provides quantitative estimates of protein levels, based on a standard curve of known BDNF concentrations, versus estimates of relative differences from the Western blots. The other is that samples were assayed in duplicate for the ELISA, versus only once for the Western blots. The clearest result, as it was observed in both the hippocampus and neocortex, was that BDNF levels increased with age – from 5 weeks to 12 weeks – in female mice, but not in male mice. Other researchers have previously noted that BDNF levels increase over the first few postnatal weeks in mice and rats (Katoh-Semba et al., 1997; Kolbeck et al., 1999; Silhol et al., 2005; Yang et al., 2009). It is possible that BDNF expression peaks slightly later in females, which could explain why levels continue to increase after 5 weeks, whereas in male mice they apparently do not. We also observed that full-length TrkB levels were higher in male mice than in female mice, though only in the neocortex.

5. CONCLUSION

The results of the present experiments support the conclusion that – contrary to previous reports (Helgager et al., 2014; Yoo et al., 2016) – BDNF protein levels in the hippocampus and neocortex are not affected by the absence of vesicular zinc in naïve mice housed under standard laboratory conditions. This conclusion is consistent with our own previous findings, and it supports a more complex model in which vesicular zinc, if it does modulate BDNF processing, only does so under certain conditions, such as environmental enrichment. We found no evidence that differences in age or sex of the mice tested could explain discrepant findings in the literature on how BDNF and TrkB levels are affected in ZnT3 KO mice; this discrepancy is, therefore, likely the result of other unidentified methodological factors.

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FIGURE CAPTIONS

Figure 1. Example Western blots for BDNF and TrkB. The antibody against BDNF detected numerous bands, assumed to be intermediate cleavage products of BDNF or non-specific labeling. A band around 13-kDa was assumed to represent mature BDNF. The antibody against TrkB detected two variants; full-length TrkB and a truncated form of TrkB (TrkB.T).

Figure 2. Effects of age, sex, and ZnT3 status on BDNF protein levels in the hippocampus. **A.** BDNF levels, quantified by densitometry from Western blots and normalized to actin levels, were not affected by age, sex, or genotype. **B.** BDNF levels, quantified by ELISA, were greater in mature (12-week-old) female mice than in young (5-week-old) female mice, but did not differ between young and mature male mice. There was no difference between WT and ZnT3 KO mice. Error bars represent SEM.

Figure 3. Effects of age, sex, and ZnT3 status on TrkB protein levels in the hippocampus. **A.** TrkB levels, quantified by densitometry from Western blots and normalized to actin levels, were not affected by age, sex, or genotype. **B.** TrkB.T levels were similarly unaffected by age, sex, or genotype. Error bars represent SEM.

Figure 4. Effects of age, sex, and ZnT3 status on BDNF protein levels in the neocortex. **A.** BDNF levels, quantified by densitometry from Western blots and normalized to actin levels, were greater in mature (12-week-old) mice than in young (5-week-old) mice. There was no effect of sex or difference between genotypes. **B.** BDNF levels, quantified by ELISA, were greater in mature female mice than in young female mice, but did not significantly differ between young and mature male mice. There was no difference between WT and ZnT3 KO mice. Error bars represent SEM.

Figure 5. Effects of age, sex, and ZnT3 status on TrkB protein levels in the neocortex. **A.** TrkB levels, quantified from Western blots and normalized to actin levels, were greater in male mice than in female mice. There was no effect of age, or difference between WT and ZnT3 KO mice. **B.** TrkB.T levels were not affected by age, sex, or genotype. Error bars represent SEM.

REFERENCES

Adlard PA, Parncutt JM, Finkelstein DI, Bush AI (2010) Cognitive loss in zinc transporter-3 knock-out mice: a phenocopy for the synaptic and memory deficits of Alzheimer's disease? J Neurosci 30: 1631-1636.

Aniksztejn L, Charton G, Ben-Ari Y (1987) Selective release of endogenous zinc from the hippocampal mossy fibers in situ. Brain Res 404: 58-64.

Assaf SY, Chung SH (1984) Release of endogenous Zn^{2+} from brain tissue during activity. Nature 308: 734-736.

Barde YA, Edgar D, Thoenen H (1982) Purification of a new neurotrophic factor from mammalian brain. EMBO J 1: 549-553.

Chácon-Fernández P, Säuberli K, Colzani M, Moreau T, Ghevaert C, Barde YA (2016) Brain-derived neurotrophic factor in megakaryocytes. J Biol Chem 291: 9872-9881.

Choo M, Miyazaki T, Yamazaki M, Kawamura M, Nakazawa T, Zhang J, Tanimura A, Uesaka N, Watanabe M, Sakimura K, Kano M (2017) Retrograde BDNF to TrkB signaling promotes synapse elimination in the developing cerebellum. Nat Commun 8: 195.

Cole TB, Wenzel HJ, Kafer KE, Schwartzkroin PA, Palmiter RD (1999) Elimination of zinc from synaptic vesicles in the intact mouse brain by disruption of the ZnT3 gene. Proc Natl Acad Sci U S A 96: 1716-1721.

Colvin RA, Holmes WR, Fontaine CP, Maret W (2010) Cytosolic zinc buffering and muffling: their role in intracellular zinc homeostasis. Metallomics 2: 306-317.

Conner JM, Lauterborn JC, Yan Q, Gall CM, Varon S (1997) Distribution of brain-derived neurotrophic factor (BDNF) protein and mRNA in the normal adult rat CNS: evidence for anterograde axonal transport. J Neurosci 17: 2295-2313.

Dieni S, Matsumoto T, Dekkers M, Rauskolb S, Ionescu MS, Deogracias R, Gundelfinger ED, Kojima M, Nestel S, Frotscher M, Barde YA (2012) BDNF and its pro-peptide are stored in presynaptic dense core vesicles in brain neurons. J Cell Biol 196: 775-788.

Eide FF, Vining ER, Eide BL, Zang K, Wang XY, Reichardt LF (1996) Naturally occurring truncated trkB receptors have dominant inhibitory effects on brain-derived neurotrophic factor signaling. J Neurosci 16: 3123-3129.

Fawcett JP, Aloyz R, McLean JH, Pareek S, Miller FD, McPherson PS, Murphy RA (1997) Detection of brain-derived neurotrophic factor in a vesicular fraction of brain synaptosomes. J Biol Chem 272: 8837-8840.

Frederickson CJ, Giblin LJ, Krezel A, McAdoo DJ, Mueller RN, Zeng Y, Balaji RV, Masalha R, Thompson RB, Fierke CA, Sarvey JM, de Valdenebro M, Prough DS, Zornow MH (2006a) Concentrations of extracellular free zinc $(pZn)_e$ in the central nervous system during simple anesthetization, ischemia and reperfusion. Exp Neurol 198: 285-293.

Frederickson CJ, Giblin LJ 3rd, Balaji RV, Masalha R, Frederickson CJ, Zeng Y, Lopez EV, Koh JY, Chorin U, Besser L, Hershfinkel M, Li Y, Thompson RB, Krezel A (2006b) Synaptic release of zinc from brain slices: factors governing release, imaging, and accurate calculation of

concentration. J Neurosci Methods 154: 19-29.

Fryer RH, Kaplan DR, Feinstein SC, Radeke MJ, Grayson DR, Kromer LF (1996) Developmental and mature expression of full-length and truncated TrkB receptors in the rat forebrain. J Comp Neurol 374: 21-40.

Gray K, Ellis V (2008) Activation of pro-BDNF by the pericellular serine protease plasmin. FEBS Lett 582: 907-910.

Harward SC, Hedrick NG, Hall CE, Parra-Bueno P, Milner TA, Pan E, Laviv T, Hempstead BL, Yasuda Ryohei, McNamara JO (2016) Autocrine BDNF-TrkB signalling within a single dendritic spine. Nature 538: 99-103.

Helgager J, Huang YZ, McNamara JO (2014) Brain-derived neurotrophic factor but not vesicular zinc promotes TrkB activation within mossy fibers of mouse hippocampus in vivo. J Comp Neurol 522: 3885-3899.

Howell GA, Welch MG, Frederickson CJ (1984) Stimulation-induced uptake and release of zinc in hippocampal slices. Nature 308: 736-738.

Huang YZ, McNamara JO (2012) Neuroprotective effects of reactive oxygen species mediated by BDNF-independent activation of TrkB. J Neurosci 32: 15521-15532.

Huang YZ, Pan E, Xiong ZQ, McNamara JO (2008) Zinc-mediated transactivation of TrkB potentiates the hippocampal mossy fiber-CA3 pyramid synapse. Neuron 57: 546-558.

Hwang JJ, Park M-H, Choi S-Y, Koh J-Y (2005) Activation of the Trk signaling pathway by extracellular zinc. J Biol Chem 280: 11995-12001.

Hwang IY, Sun ES, An JH, Im H, Lee SH, Lee JY, Han PL, Koh JY, Kim YH (2011) Zinc-triggered induction of tissue plasminogen activator by brain-derived neurotrophic factor and metalloproteinase. J Neurochem 118: 855-863.

Impey S, Smith DM, Obrietan K, Donahue R, Wade C, Storm DR (1998) Stimulation of cAMP response element (CRE)-mediated transcription during contextual learning. Nat Neurosci 1: 595-601.

Katoh-Semba R, Takeuchi IK, Semba R, Kato K (1997) Distribution of brain-derived neurotrophic factor in rats and its changes with development in the brain. J Neurochem 69: 34-42.

Klein R, Conway D, Parada LF, Barbacid M (1990) The trkB tyrosine protein kinase gene codes for a second neurogenic receptor that lacks the catalytic kinase domain. Cell 61: 647-656.

Klein R, Nanduri V, Jing S, Lamballe F, Tapley P, Bryant S, Cordon-Cardo C, Jones KR, Reichardt LF, Barbacid M (1991) The trkB tyrosine protein kinase is a receptor for brain-derived neurotrophic factor and neurotrophin-3. Cell 66: 395-403.

Kohara K, Kitamura A, Morishima M, Tsumoto T (2001) Activity-dependent transfer of brain-derived neurotrophic factor to postsynaptic neurons. Science 291: 2419-2423.

Kolbeck R, Bartke I, Eberle W, Barde YA (1999) Brain-derived neurotrophic factor levels in the nervous

system of wild-type and neurotrophin gene mutant mice. J Neurochem 72: 1930-1938.

Lee R, Kermani P, Teng KK, Hempstead BL (2001) Regulation of cell survival by secreted proneurotrophins. Science 294: 1945-1948.

Matsumoto T, Rauskolb S, Polack M, Klose J, Kolbeck R, Korte M, Barde YA (2008) Biosynthesis and processing of endogenous BDNF: CNS neurons store and secrete BDNF, not pro-BDNF. Nat Neurosci 11: 131-133.

McAllister BB, Dyck RH (2017) Zinc transporter 3 (ZnT3) and vesicular zinc in central nervous system function. Neurosci Biobehav Rev 80: 329-350.

Michael GJ, Averill S, Nitkunan A, Rattray M, Bennett DL, Yan Q, Priestly JV (1997) Nerve growth factor treatment increases brain-derived neurotrophic factor selectively in TrkA-expressing dorsal root ganglion cells and in their central terminations within the spinal cord. J Neurosci 17: 8476-8490.

Mowla SJ, Farhadi HF, Pareek S, Atwal JK, Morris SJ, Seidah NG, Murphy RA (2001) Biosynthesis and post-translational processing of the precursor to brain-derived neurotrophic factor. J Biol Chem 276: 12660-12666.

Nagappan G, Zaitsev E, Senatorov VV, Yang J, Hempstead BL, Lu B (2009) Control of extracellular cleavage of proBDNF by high frequency neuronal activity. Proc Natl Acad Sci U S A 106: 1267-1272.

Nakashima AS, Butt RH, Dyck RH (2011) Alterations in protein and gene expression within the barrel cortices of ZnT3 knockout mice: experience-independent and dependent changes. Neurochem Int 59: 860-870.

Nibuya M, Nestler EJ, Duman RS (1996) Chronic antidepressant administration increases the expression of cAMP response element binding protein (CREB) in rat hippocampus. J Neurosci 16: 2365-2372.

Palmiter RD, Cole TB, Quaife CJ, Findley SD (1996) ZnT-3, a putative transporter of zinc into synaptic vesicles. Proc Natl Acad Sci U S A 93: 14934-14939.

Pang PT, Teng HK, Zaitsev E, Woo NT, Sakata K, Zhen S, Teng KK, Yung WH, Hempstead BL, Lu B (2004) Cleavage of proBDNF by tPA/plasmin is essential for long-term hippocampal plasticity. Science 306: 487-491.

Pérez-Clausell J, Danscher G (1985) Intravesicular localization of zinc in rat telencephalic boutons. A histochemical study. Brain Res 337: 91-98.

Poddar R, Rajagopal S, Shuttleworth CW, Paul S (2016) Zn^{2+}-dependent activation of the Trk signaling pathway induces phosphorylation of the brain-enriched tyrosine phosphatase STEP. J Biol Chem 291: 813-825.

Polacchini A, Metelli G, Francavilla R, Baj G, Florean M, Mascaretti LG, Tongiorgi E (2015) A method

for reproducible measurements of serum BDNF: comparison of the performance of six commercial assays. Sci Rep 5: 17989.

Post JI, Eibl JK, Ross GM (2008) Zinc induces motor neuron death via a selective inhibition of brain-derived neurotrophic factor activity. Amyotroph Lateral Scler 9: 149-155.

Reichardt LF (2006) Neurotrophin-regulated signalling pathways. Philos Trans R Soc Lond B Biol Sci 361: 1545-1564.

Rose CR, Blum R, Pichler B, Lepier A, Kafitz KW, Konnerth A (2003) Truncated TrkB-T1 mediates neurotrophin-evoked calcium signaling in glia cells. Nature 426: 74-78.

Ross GM, Shamovsky IL, Lawrance G, Solc M, Dostaler SM, Jimmo SL, Weaver DF, Riopelle RJ (1997) Zinc alters conformation and inhibits biological activities of nerve growth factor and related neurotrophins. Nat Med 3: 872-878.

Silhol M, Bonnichon V, Rage F, Tapia-Arancibia L (2005) Age-related changes in brain-derived neurotrophic factor and tyrosine kinase receptor isoforms in the hippocampus and hypothalamus in male rats. Neuroscience 132: 613-624.

Sindreu C, Palmiter RD, Storm DR (2011) Zinc transporter ZnT-3 regulates presynaptic Erk1/2 signaling and hippocampus-dependent memory. Proc Natl Acad Sci U S A 108: 3366-3370.

Tao X, West AE, Chen WG, Corfas G, Greenberg ME (2002) A calcium-responsive transcription factor, CaRF, that regulates neuronal activity-dependent expression of BDNF. Neuron 33: 383-395.

Travaglia A, La Mendola D, Magrí A, Pietropaolo A, Nicoletti VG, Grasso G, Malgieri G, Fattorusso R, Isernia C, Rizzarelli E (2013) Zinc(II) interactions with brain-derived neurotrophic factor N-terminal peptide fragments: inorganic features and biological perspectives. Inorg Chem 52: 11075-11083.

Tsankova NM, Berton O, Renthal W, Kumar A, Neve RL, Nestler EJ (2006) Sustained hippocampal chromatin regulation in a mouse model of depression and antidepressant action. Nat Neurosci 9: 519-525.

Wenzel HJ, Cole TB, Born DE, Schwartzkroin PA, Palmiter RD (1997) Ultrastructural localization of zinc transporter-3 (ZnT-3) to synaptic vesicle membranes within mossy fiber boutons in the hippocampus of mouse and monkey. Proc Natl Acad Sci U S A 94: 12676-12681.

Wong YH, Lee CM, Xie W, Cui B, Poo MM (2015) Activity-dependent BDNF release via endocytic pathways is regulated by synaptotagmin-6 and complexin. Proc Natl Acad Sci U S A 112: E4475-4484.

Yang J, Siao CJ, Nagappan G, Marinic T, Jing D, McGrath K, Chen ZY, Mark W, Tessarollo L, Lee FS, Lu B, Hempstead BL (2009) Neuronal release of proBDNF. Nat Neurosci 12: 113-115.

Yoo MH, Kim TY, Yoon YH, Koh JY (2016) Autism phenotype in ZnT3 null mice: involvement of zinc dyshomeostasis, MMP-9 activation and BDNF upregulation. Sci Rep 6: 28548.

Zajac MS, Pang TY, Wong N, Weinrich B, Leang LS, Craig JM, Saffery R, Hannan AJ (2010) Wheel running and environmental enrichment differentially modify exon-specific BDNF expression in the hippocampus of wild-type and pre-motor symptomatic male and female Huntington's disease mice. Hippocampus 20: 621-636.

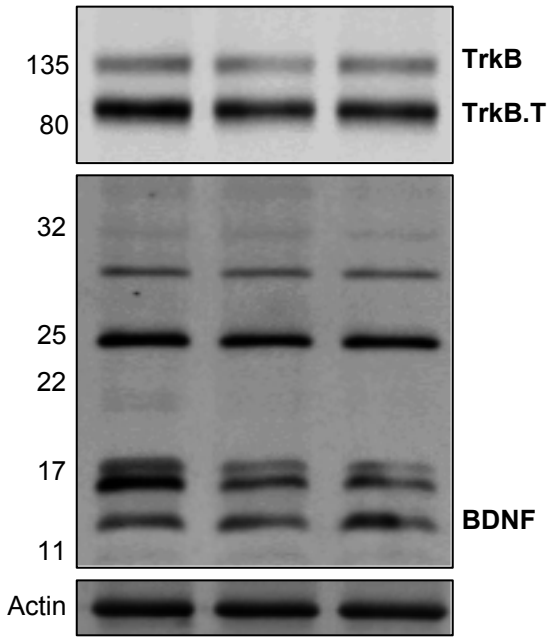

Figure 1.

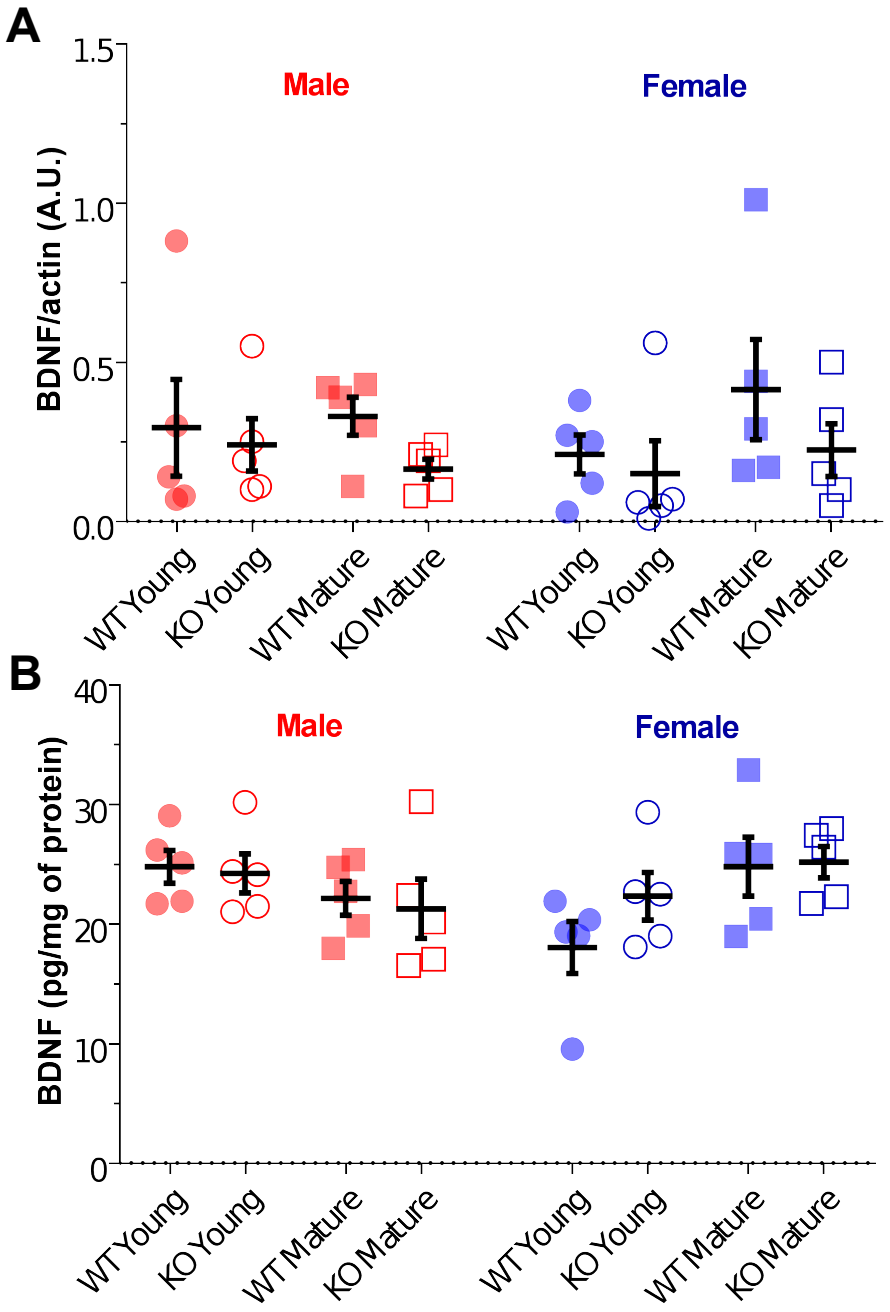
A
Male
Female
BDNF/actin (A.U.)
1.5
1.0
0.5
0.0
WT Young
KO Young
WT Mature
KO Mature
WT Young
KO Young
WT Mature
KO Mature
B
Male
Female
BDNF (pg/mg of protein)
40
30
20
10
0
WT Young
KO Young
WT Mature
KO Mature
WT Young
KO Young
WT Mature
KO Mature

Figure 2.

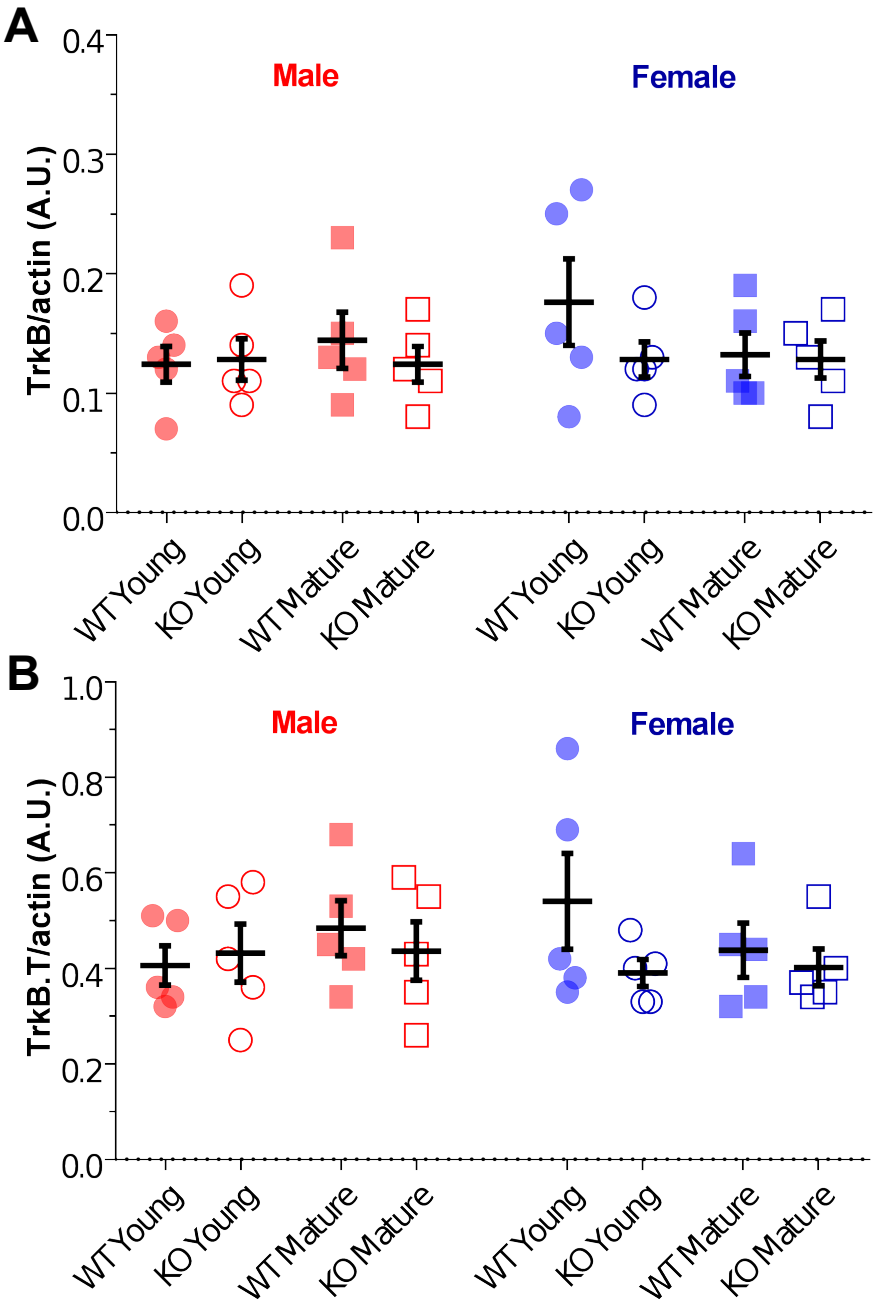
A
Male
Female
TrkB/actin (A.U.)
0.4
0.3
0.2
0.1
0.0
WT Young
KO Young
WT Mature
KO Mature
WT Young
KO Young
WT Mature
KO Mature
B
Male
Female
TrkB.T/actin (A.U.)
1.0
0.8
0.6
0.4
0.2
0.0
WT Young
KO Young
WT Mature
KO Mature
WT Young
KO Young
WT Mature
KO Mature

Figure 3.

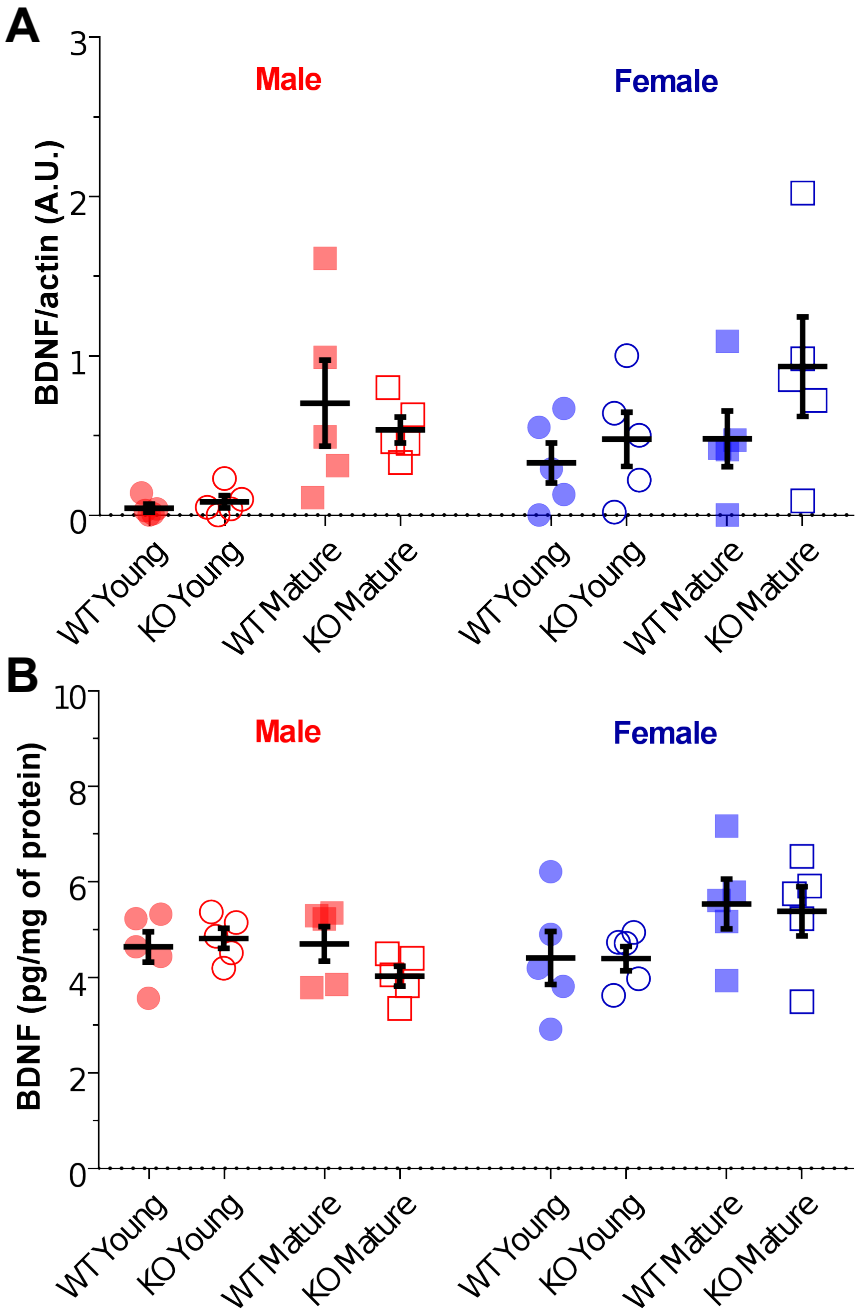
A
Male
Female
BDNF/actin (A.U.)
3
2
1
0
WT Young
KO Young
WT Mature
KO Mature
WT Young
KO Young
WT Mature
KO Mature
B
Male
Female
BDNF (pg/mg of protein)
10
8
6
4
2
0
WT Young
KO Young
WT Mature
KO Mature
WT Young
KO Young
WT Mature
KO Mature

Figure 4.

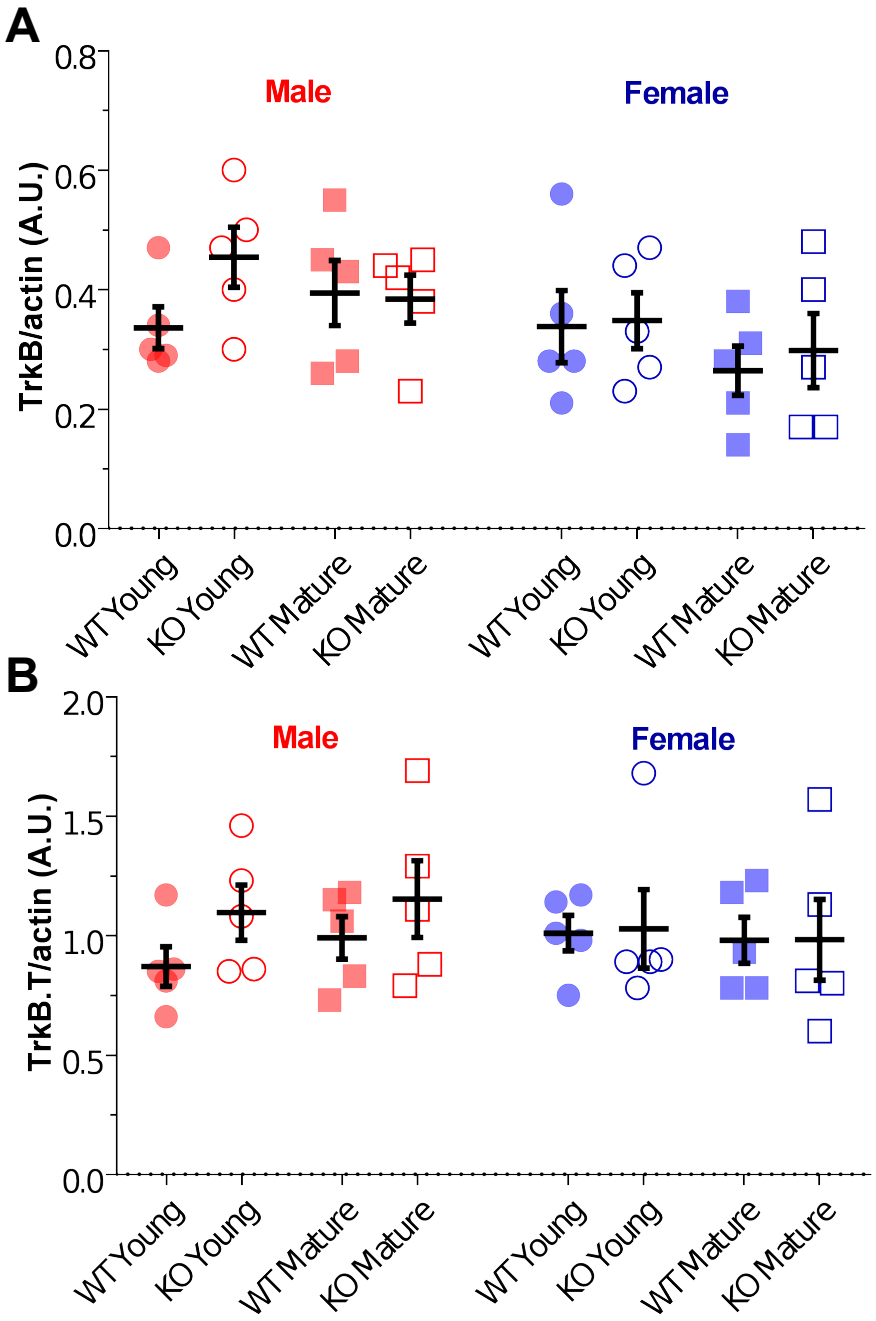
A
Male
Female
TrkB/actin (A.U.)
0.8
0.6
0.4
0.2
0.0
WT Young
KO Young
WT Mature
KO Mature
WT Young
KO Young
WT Mature
KO Mature
B
Male
Female
TrkB.T/actin (A.U.)
2.0
1.5
1.0
0.5
0.0
WT Young
KO Young
WT Mature
KO Mature
WT Young
KO Young
WT Mature
KO Mature

Figure 5.

Supplemental Table 1. ANOVA results (effects of age, sex, and genotype).

	D.F.	Age	Sex	Genotype
Neocortex				
BDNF densitometry	1, 32	***F* = 11.56, *p* = .002**	*F* = 2.85, *p* = .101	*F* = 0.92, *p* = .345
BDNF Concentration (ELISA)	1, 32	*F* = 1.54, *p* = .224	*F* = 1.92, *p* = .176	*F* = 0.35, *p* = .557
TrkB densitometry	1, 32	*F* = 0.89, *p* = .353	***F* = 5.13, *p* = .030**	*F* = 1.28, *p* = .267
TrkB.T densitometry	1, 32	*F* = 0.08, *p* = .777	*F* = 0.10, *p* = .757	*F* = 1.29, *p* = .265
Actin densitometry	1, 32	*F* = 1.75, *p* = .195	*F* = 2.00, *p* = .167	*F* < 0.01, *p* = .980
Hippocampus				
BDNF densitometry	1, 32	*F* = 0.68, *p* = .416	*F* = 0.01, *p* = .924	*F* = 2.69, *p* = .111
BDNF Concentration (ELISA)	1, 32	*F* = 0.55, *p* = .362	*F* = 0.15, *p* = .697	*F* = 0.36, *p* = .552
TrkB densitometry	1, 32	*F* = 0.34, *p* = .566	*F* = 0.47, *p* = .496	*F* = 1.60, *p* = .215
TrkB.T densitometry	1, 32	*F* < 0.01, *p* = .959	*F* < 0.01, *p* = .958	*F* = 1.49, *p* = .231
Actin densitometry	1, 32	*F* = 0.17, *p* = .680	*F* = 0.03, *p* = .869	*F* = 0.03, *p* = .860

Supplemental Table 1 (Cont.). ANOVA results (effects of age, sex, and genotype).

	Age × sex	Sex × genotype	Age × genotype	Age × sex × genotype
Neocortex				
BDNF densitometry	$F = 0.99$, $p = .327$	$F = 2.07$, $p = .160$	$F = 0.03$, $p = .858$	$F = 1.04$, $p = .316$
BDNF Concentration (ELISA)	**$F = 6.60$, $p = .015$**	$F = 0.09$, $p = .761$	$F = 0.80$, $p = .377$	$F = 0.42$, $p = .523$
TrkB densitometry	$F = 0.60$, $p = .446$	$F = 0.21$, $p = .649$	$F = 0.54$, $p = .470$	$F = 1.16$, $p = .290$
TrkB.T densitometry	$F = 0.52$, $p = .477$	$F = 1.09$, $p = .304$	$F = 0.06$, $p = .816$	$F = 0.02$, $p = .904$
Actin densitometry	$F = 0.08$, $p = .776$	$F = 0.04$, $p = .840$	$F = 0.25$, $p = .619$	$F = 0.08$, $p = .777$
Hippocampus				
BDNF densitometry	$F = 1.27$, $p = .268$	$F = 0.01$, $p = .917$	$F = 0.73$, $p = .401$	$F < 0.01$, $p = .958$
BDNF Concentration (ELISA)	**$F = 7.95$, $p = .008$**	$F = 1.26$, $p = .270$	$F = 0.62$, $p = .438$	$F = 0.45$, $p = .508$
TrkB densitometry	$F = 1.01$, $p = .322$	$F = 0.46$, $p = .504$	$F = 0.07$, $p = .795$	$F = 1.51$, $p = .228$
Truncated TrkB densitometry	$F = 1.07$, $p = .309$	$F = 0.97$, $p = .332$	$F = 0.06$, $p = .814$	$F = 1.22$, $p = .278$
Actin densitometry	$F = 1.10$, $p = .302$	$F = 0.15$, $p = .705$	$F = 0.01$, $p = .921$	$F = 1.35$, $p = .254$