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. *In vitro* evidence supports that these targets may include brain-derived neurotrophic factor (BDNF) and its receptor, tropomyosin receptor kinase B (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. We hypothesized that this 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 also examined BDNF mRNA expression at an intermediate age (8-10 weeks). 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:

Zinc transporter; synaptic zinc; zincergic neuron; SLC30A3; zinc signaling; neurotrophin

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 BDNF and TrkB levels are 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 a total loss of vesicular zinc throughout the CNS. In terms of total zinc content, this corresponds with a reduction of about 20% in the cortex and 20-40% in the hippocampus; these regions exhibit similar total zinc concentrations (Cole et al., 1999; Lee et al., 2002; Adlard et al., 2010).

To date, several studies have examined BDNF or TrkB levels in ZnT3 KO mice, 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 elevated 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 is 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 by comparing wild type (WT) and ZnT3 KO mice of both sexes at two different ages within the same study, examining BDNF and TrkB levels in both the hippocampus and neocortex.

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 60 mice. The initial Western blotting and ELISA experiments used 40 mice, including WT and ZnT3 KO, male and female, and young (5-week-old) and mature (12-

week-old) animals, resulting in eight experimental groups (n = 5 for each). Additional ELISA and qRT-PCR experiments used 20 mice, including male and female animals of both genotypes (8-10 weeks of age), resulting in four experimental groups (n = 5 for each).

2.3 Sample collection and protein extraction

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.

For certain experiments, samples were acidified during preparation to increase the detection of BDNF (Okragly & Haak-Frendscho, 1997). The acidification procedure was adapted from Helgager et al. (2014) and was performed after the samples spent 1 h on ice, but prior to centrifugation. The pH of the samples was adjusted to <3.0 using 1 M HCl, and the samples were left for 20 min at room temperature. The pH of the samples was then adjusted to ~7.4 using 1 M NaOH.

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) that has been validated to show good selectivity for mature BDNF over proBDNF (Polacchini et al., 2015). Within each experiment, 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 for non-acid treated samples; 200 µg for hippocampus, 400 µg for neocortex for acid treated samples) 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 10-18 min (depending on the experiment) 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 using a microplate reader (Wallac 1420 Victor^2, Perkin Elmer Life Sciences; or FilterMax F5 Multi-Mode Microplate Reader, Molecular Devices, depending on the experiment). BDNF concentrations were determined based on a standard curve and converted to pg of BDNF per mg of total protein.

2.6 RNA isolation and qRT-PCR

Total RNA was isolated from frozen tissue using the Allprep RNA/DNA Mini kit (Qiagen), as per the manufacturer's protocol. Total RNA concentration and purity was determined using a Nanodrop 2000 (Thermo Fisher Scientific). Purified total RNA (2 µg) was reverse transcribed to cDNA using oligo(dT)$_{20}$ of the Superscript III First-Strand Synthesis Supermix kit (Invitrogen), as per the manufacturer's protocols. All primers for the target gene and reference genes were purchased from IDT (Coralville, IA). Primers were designed by an in-house technician using Primer3 (http://bioinfo.ut.ee/primer3/) to span exon-exon junctions.

To assess gene expression, 10 ng of cDNA with 0.5 µM of each of the forward and reverse primers for the target or reference genes and 1× SYBR Green FastMix with Rox was subjected to qRT-PCR using a CFX Connect Real-Time PCR Detection System (Bio-Rad). A standard curve to determine the PCR efficiency was prepared by serial dilution of cDNA from pooled control samples from 95.24 ng to 1.5625 ng. A no-template control was also subjected to qRT-PCR per gene. The thermocycling conditions were as follows: initial denaturation at 95 °C for 3 min, 40 cycles of denaturation at 95 °C for 15 s and annealing for 30 s at the optimal annealing temperature (see Supplemental Table 1), followed by a melt curve data collection at 65 °C for 5 s plus 0.5 °C per cycle until 95 °C was reached. Samples were tested in duplicate. Relative target gene expression was determined by normalizing to two housekeeping genes, CycA and Ywhaz (Bonefeld et al., 2008), using the $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001).

2.7 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 2.

3. RESULTS

3.1 BDNF and TrkB levels - Western blot analysis

3.1.1 BDNF

Levels of BDNF in brain tissue samples from WT and ZnT3 KO mice, including both male and female and young (5-week-old) and mature (12-week-old) animals, were first determined from Western immunoblots. We selected an antibody against BDNF (polyclonal anti-BDNF N-20; Santa Cruz Biotechnology, Inc.) that was used by Yoo et al. (2016) to show altered BDNF levels in ZnT3 KO mice. The specificity of this antibody for proBDNF and mature BDNF has previously been characterized using hippocampal tissue from conditional BDNF KO mice (Matsumoto et al., 2008). It should be noted, though, that due to potential batch-to-batch variability, it cannot be concluded with certainty that the results of this characterization would apply to the batch used in the present experiment. As a positive control, we confirmed the ability of the antibody to recognize recombinant BDNF and proBDNF (Figure 1A). In brain lysates (Figure 1B), the antibody detected three bands between approximately 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 BDNF, though the positions were not identical, as can be seen in Figure 1A. The other two bands could represent 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. One possibility is that these bands also represent intermediate cleavage products or alternative isoforms of proBDNF; previous work has shown a 28-kDa truncated form of proBDNF (Mowla et al., 2001). It is also possible that the band around 28-kDa could result from dimerization of BDNF (Radziejewski et al., 1992), though this is unlikely given that samples were prepared under denaturing conditions. Finally, it is possible that one or both of these bands resulted from non-specific binding of the antibody. 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.

In the hippocampus, mature BDNF levels, measured by densitometry (Figure 2A), did not differ between WT and ZnT3 KO mice [$F(1,32) = 2.69$, $p = .111$], nor was there a difference based on the sex

[$F(1,32) = 0.01$, $p = .924$] or age [$F(1,32) = 0.68$, $p = .416$] of the mice. Levels of BDNF in the neocortex (Figure 2B) also did not differ between genotypes [$F(1,32) = 0.92$, $p = .345$], though, unlike in the hippocampus, neocortical levels of BDNF were found to be higher in the 12-week-old mice compared to the 5-week-old mice [main effect of age: $F(1,32) = 11.56$, $p = .002$]. No significant effect of sex was observed [$F(1,32) = 2.85$, $p = .101$].

3.1.2 TrkB

Levels of TrkB were also assessed by Western blotting (example in Figure 1B). 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).

In the hippocampus, TrkB levels did not differ between WT and ZnT3 KO mice [$F(1,32) = 1.60$, $p = .215$], nor did they differ based on sex [$F(1,32) = 0.47$, $p = .496$] or age [$F(1,32) = 0.34$, $p = .566$] of the mice (Figure 3A). Likewise, hippocampal TrkB.T levels (Figure 3B) did not differ based on genotype [$F(1,32) = 1.49$, $p = .231$], sex [$F(1,32) < 0.01$, $p = .958$], or age [$F(1,32) < 0.01$, $p = .959$]. In contrast, TrkB levels in the neocortex (Figure 3C) were significantly higher in male mice than in female mice [$F(1,32) = 5.13$, $p = .030$]. However, there was no difference between genotypes [$F(1,32) = 1.28$, $p = .267$] or effect of age [$F(1,32) = 0.89$, $p = .353$]. Finally, levels of TrkB.T in the neocortex (Figure 3D) did not differ based on genotype [$F(1,32) = 1.29$, $p = .265$], sex [$F(1,32) = 0.10$, $p = .757$], or age [$F(1,32) = 0.08$, $p = .777$].

3.2 BDNF levels – ELISA analysis

While the results of our Western blotting analysis indicated some differences in BDNF/TrkB levels based on age or sex, the analysis produced no evidence that levels differed between WT and ZnT3 KO mice. However, this analysis was limited by the semi-quantitative nature of the data generated by Western blotting. It was also limited by the uncertain identity of the bands on our blots, particularly regarding the BDNF experiments. Specifically, we did not conduct a negative-control experiment to confirm that the band assumed to represent mature BDNF in our tissue samples was not the result of non-specific binding by the antibody. Therefore, to provide a second assessment, we also measured hippocampal and neocortical BDNF concentrations by ELISA, using a kit that is selective for mature BDNF (Polacchini et al., 2015). This also allowed us to provide a quantitative assessment of BDNF levels.

By this method, we were still unable to detect a difference in hippocampal BDNF levels between WT and ZnT3 KO mice [$F(1,32) = 0.36$, $p = .552$; Figure 4A]. Interestingly, we did observe 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).

The results from the neocortex (Figure 4B) mirrored those from the hippocampus. Levels of BDNF, measured by ELISA, did not differ between WT and ZnT3 KO mice [$F(1,32) = 0.35$, p = .557], whereas age exerted a significant effect that differed between males and females [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 BDNF levels increased significantly with age in females (p = .022; Bonferroni-corrected).

BDNF levels measured by our ELISA analysis were somewhat low relative to levels presented in previous reports (e.g., Helgager et al., 2014). This could be due to methodological aspects of the sample preparation. In particular, acidification of tissue samples has been shown to increase the detection of BDNF by ELISA (Okragly & Haak-Frendscho, 1997). Therefore, we conducted an additional ELISA experiment using hippocampal and neocortical samples that were acidified during processing. Tissue samples were taken from mice at an intermediate age (i.e., 8-10 weeks) relative to the two age groups tested in the experiments above.

We confirmed that acidifying the samples dramatically increased the levels of BDNF that were detected by ELISA (compare Figure 4B to 4C). Likely, this was due to increased extraction of BDNF from the tissue, but also in part to a loss of some protein from the sample, as we noticed the formation of a considerable amount of precipitate when samples were acidified, and also noted that protein content, as determined by Bradford analysis, tended to be lower in the acidified samples than the non-acidified samples (data not shown). Therefore, BDNF likely accounted for a larger proportion of the protein remaining in the sample, causing more BDNF to be detected per unit of protein loaded in the assay.

The increase in detected BDNF levels was so large that, even when less protein was loaded than in our previous ELISA experiment, many of the measured values from the hippocampal samples fell outside the standard curve of the assay. Therefore, these data could not be used for analysis. The values measured from the neocortical samples (Figure 4C) were within the standard curve, however, and so were analyzed for differences based on the genotype and sex of the mice, using a two-way ANOVA. One sample (WT-female) was excluded from this analysis because the measured level of BDNF was very low (< 10 pg/mg of protein) relative to the other acid treated samples (range from 100-300 pg/mg of protein). Again, we were unable to detect a difference in BDNF levels between WT and ZnT3 KO mice [$F(1,15) = 0.08$, p = .781]. There was also no difference between males and females [$F(1,15) = 0.01$, p = .937].

3.3 BDNF mRNA levels – qRT-PCR analysis

Taken together, the results from the above analyses indicate that BDNF protein levels are not abnormal in ZnT3 KO mice. We next sought to examine whether the same would apply at the level of BDNF mRNA. Samples were collected from mice at an intermediate age relative to the 5-week-old and 12-week-old groups described above (i.e., 8-10 weeks), and submitted to qRT-PCR analysis for BDNF

mRNA expression. Differences based on genotype or sex were analyzed by two-way ANOVA. In the hippocampus (Figure 5A), there was no difference in BDNF mRNA levels between WT and ZnT3 KO mice [$F(1,16) = 0.52$, $p = .481$], nor was there a difference between males and females [$F(1,16) = 0.65$, $p = .432$]. Likewise, in the neocortex (Figure 5B), there was no difference in BDNF mRNA levels between genotypes [$F(1,16) = 0.01$, $p = .932$] or between sexes [$F(1,16) = 1.43$, $p = .250$]. In summary, a lack of vesicular zinc throughout life resulted in no effect on BDNF levels, in terms of either protein or mRNA, in the hippocampus and neocortex.

4. DISCUSSION

Previous examinations of BDNF levels in the brains of ZnT3 KO mice have produced seemingly discrepant results. The objective of the present study 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, regardless of whether mice were male or female, young or mature. This was true for both protein and mRNA levels of BDNF. Moreover, the same applied to protein levels of the BDNF receptor, TrkB (mRNA levels for TrkB were not assessed in the present study). 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 mature BDNF levels are normal in ZnT3 KO mice at least up to 6 months of age. They also found that hippocampal TrkB levels do not decline in ZnT3 KO mice until somewhere between 3-6 months; older than the mice examined in the present study. When considered along with our previous finding that TrkB mRNA expression in barrel cortex is reduced in ZnT3 KO mice at 2 months of age (Nakashima et al., 2011), it appears that decreased TrkB gene expression precedes a decrease in TrkB protein levels by at least a month. 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.

A possible explanation for the disparity in findings is the genetic background of the ZnT3 KO mice used. To our knowledge, all ZnT3 KO mice reported on to date were on a mixed C57BL/6×129Sv background strain and derive from the same original source: the laboratory of Dr. Richard Palmiter, where ZnT3 was first identified and ZnT3 KO mice were first generated (Palmiter et al., 1996; Cole et al., 1999). Our colony, initially established from mice generously provided by Dr. Palmiter, has been independently maintained over many years and mouse generations; long enough that genetic drift may

have rendered them a different substrain from the colonies maintained by other groups (e.g., Yoo et al., 2016) or by the Jackson Laboratory (#005064), from which mice were obtained for the experiments conducted by Helgager et al. (2014). To test this possibility, the present experiments could be repeated using mice freshly obtained from the Jackson Laboratory.

One caveat to our conclusions is that our results were obtained using mice in which ZnT3 has been deleted from the germline. Thus, these mice lack ZnT3 in all cell types and throughout all of life, including development. The lack of cell specificity is not a major concern for the present experiments, but the lack of temporal specificity is an important issue, as it raises the possibility that compensatory changes could arise to counteract the effects of eliminating ZnT3, masking the effects that vesicular zinc is actually exerting in the normal brain. It is possible that more acute elimination of vesicular zinc, as could be achieved using conditional ZnT3 KO mice, would reveal different or greater effects on BDNF, as well as on the many other known or hypothesized targets of zinc. The eventual availability of such mice will greatly strengthen the field of zinc neurobiology by allowing for such possibilities to be addressed experimentally.

It is also worth highlighting that the present experiments focused only on experimentally-naïve mice. Thus, it cannot be ruled out that vesicular zinc contributes to regulating BDNF production, processing, or signaling under other conditions. To speculate, it is possible that under certain conditions wherein BDNF gene expression is enhanced – such as environmental enrichment or chronic antidepressant treatment (Nibuya et al., 1996; Tsankova et al., 2006; Zajac et al., 2010), zinc may become involved in the enzymatic processing of BDNF. 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 synaptic cleft, 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). Assuming that enhanced BDNF expression leads to increased secretion of proBDNF, it is possible that zinc might increase the extracellular processing of proBDNF into mature BDNF by promoting the activity of MMP enzymes. Indeed, exogenous zinc application enhances MMP activity at the (probably) physiologically-relevant concentration of 10 μM (J. Hwang et al., 2005). Furthermore, 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, under certain conditions, zinc might interact with BDNF by influencing BDNF gene expression. 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 unanticipated effects of age and sex. For BDNF levels, the results obtained by Western blotting and by ELISA were similar, though somewhat divergent; where they diverge, we favour the ELISA results for several 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. We also observed that some of our Western blotting results tended to be quite variable, especially between different blots, and even when the blots were prepared and processed in parallel and under the same conditions. For the ELISA experiments, on the other hand, all samples could be assayed on one plate, or on multiple plates from the same lot, reducing variability. An additional advantage of ELISA is that samples were assayed in duplicate, further reducing variability. 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 study support the conclusion that – contrary to previous reports (Helgager et al., 2014; Yoo et al., 2016) – BDNF protein and mRNA levels in the hippocampus and neocortex are not affected by the absence of vesicular zinc in naïve mice housed under standard laboratory conditions. Furthermore, 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.

6. ACKNOWLEDGEMENTS

The authors thank Dr. Frank Visser and the HBI Molecular Core facility for use of their equipment and for consultation, as well as Dr. Tuan Trang for use of his laboratory's plate reader and for helpful suggestions on this project. The authors also thank Melinda Wang for technical assistance.

This research was supported by an NSERC Discovery Grant (RHD). The funding source had no role in the study design; collection, analysis, and interpretation of data; or in the decision to submit the article for publication.

FIGURE CAPTIONS

Figure 1. Example Western blots. **A.** Left panel: segment from a blotting membrane probed with an antibody again BDNF. Lanes were loaded with hippocampal extracts in a descending series of amounts. Right panel: segment from the same membrane. The lane was loaded with recombinant BDNF (Alomone Labs, #B-250) and recombinant cleavage-resistant proBDNF (Alomone Labs, #B-256), both of which were recognized by the antibody against BDNF. **B.** Example Western blotting of hippocampal extracts (30 μg of protein loaded per lane). The blots were probed with antibodies against TrkB (top panel) and BDNF (middle panel), with beta-actin as a loading control (bottom panel). 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 and neocortex, as quantified by densitometry from Western blots. BDNF levels were normalized to beta-actin levels. **A.** Hippocampal BDNF levels were not affected by age, sex, or genotype. **B.** Neocortical BDNF levels were greater in mature (12-week-old) mice than in young (5-week-old) mice. There was no effect of sex, nor was there a difference between genotypes. Error bars represent SEM. $^{\#}$main effect of age, $p < .05$

Figure 3. Effects of age, sex, and ZnT3 status on TrkB protein levels in the hippocampus and neocortex, as quantified by densitometry from Western blots. BDNF levels were normalized to beta-actin levels. **A.** Hippocampal TrkB levels were not affected by age, sex, or genotype. **B.** Levels of truncated TrkB (TrkB.T) in the hippocampus were similarly unaffected by these factors. **C.** Neocortical TrkB levels were greater in male mice than in female mice, but levels were not affected by age or genotype. **D.** TrkB.T levels in the neocortex were not affected by age, sex, or genotype. Error bars represent SEM. †main effect of age, $p < .05$

Figure 4. Effects of age, sex, and ZnT3 status on BDNF protein levels in the hippocampus and neocortex, quantified by ELISA. **A.** Hippocampal BDNF levels were greater in mature (12-week-old) female mice than in young (5-week-old) female mice, but levels did not differ between young and mature males. There was no difference between WT and ZnT3 KO mice. **B.** As in the hippocampus, neocortical BDNF levels were greater in mature female mice than in young female mice, with no difference between young and mature males. There was again no difference between genotypes. **C.** Acidifying the tissue extracts during sample preparation drastically increased the detection of BDNF by the assay. However, measured BDNF levels, in neocortical extracts from mice at an intermediate age (8-10 weeks), remained unaffected by

genotype in both males and females. Error bars represent SEM. *follow-up test to significant interaction, $p < .05$

Figure 5. Effects of sex and ZnT3 status on BDNF mRNA levels in the hippocampus and neocortex, as quantified by qRT-PCR. BDNF mRNA expression was normalized to the levels of two housekeeping genes, CycA and Ywhaz. **A.** In mice at 8-10 weeks of age, levels of BDNF mRNA in the hippocampus did not differ between sexes or between WT and ZnT3 KO mice. **B.** Levels of BDNF mRNA in the neocortex were similarly unaffected by sex or genotype of the mice. Error bars represent SEM.

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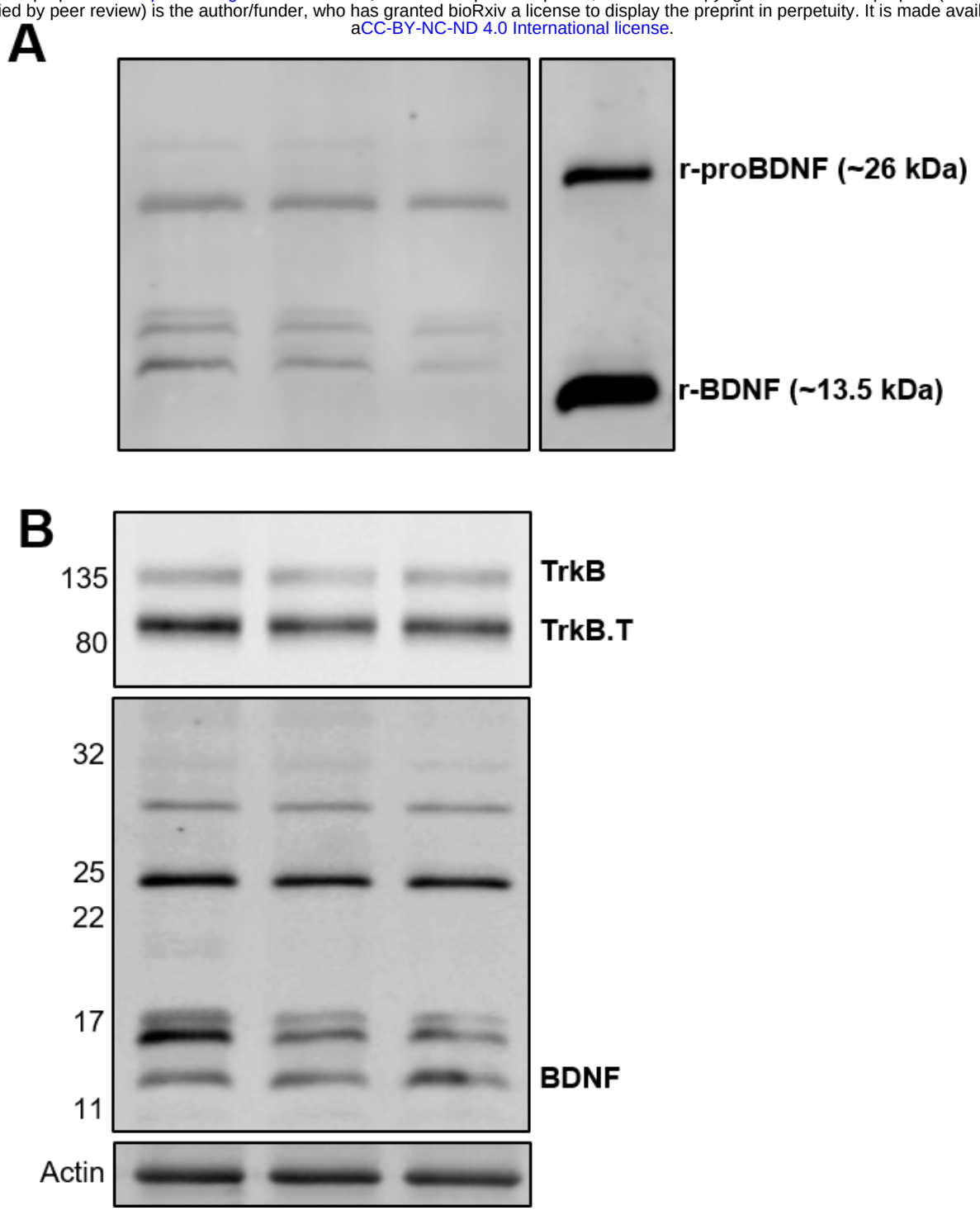

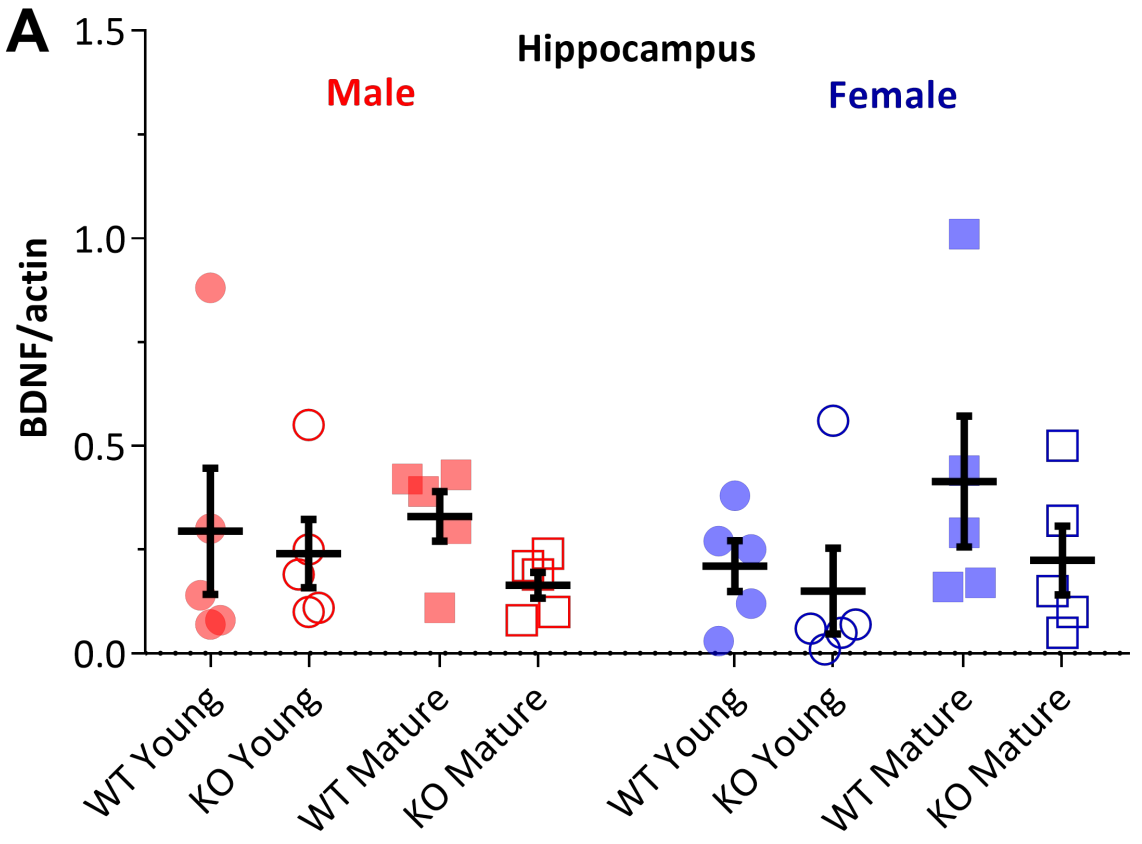

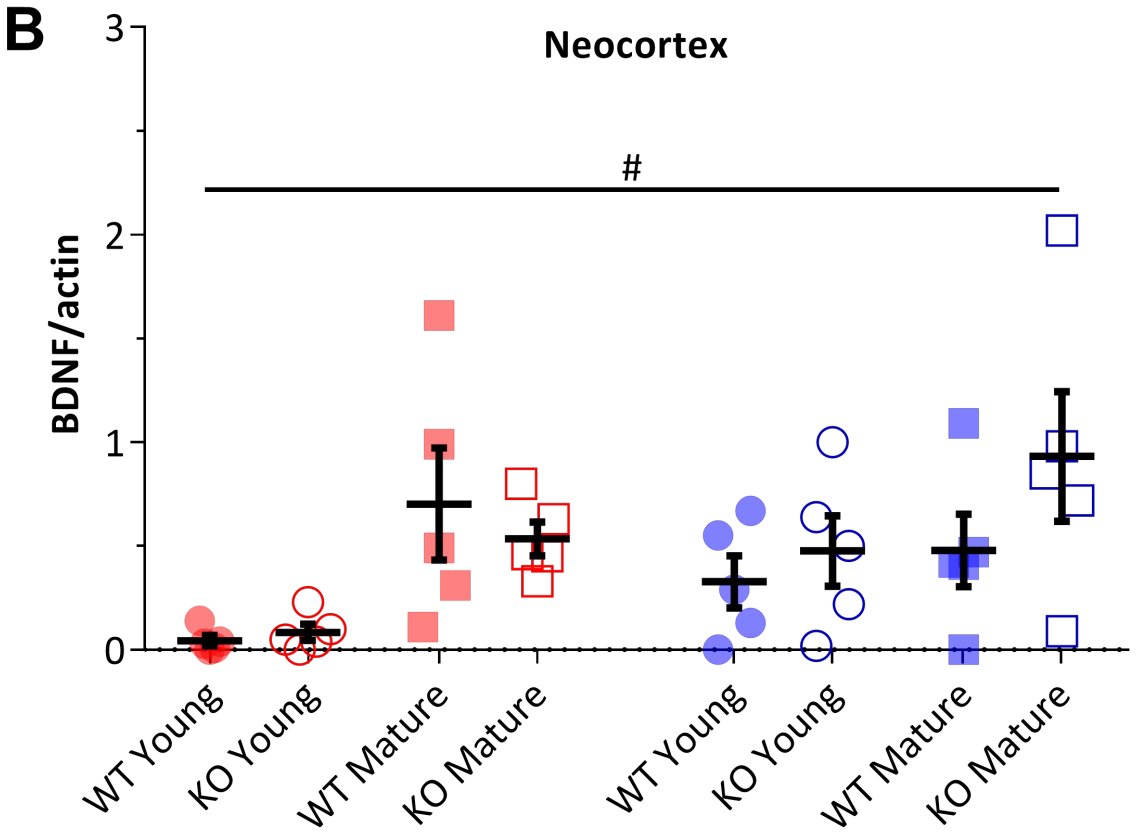

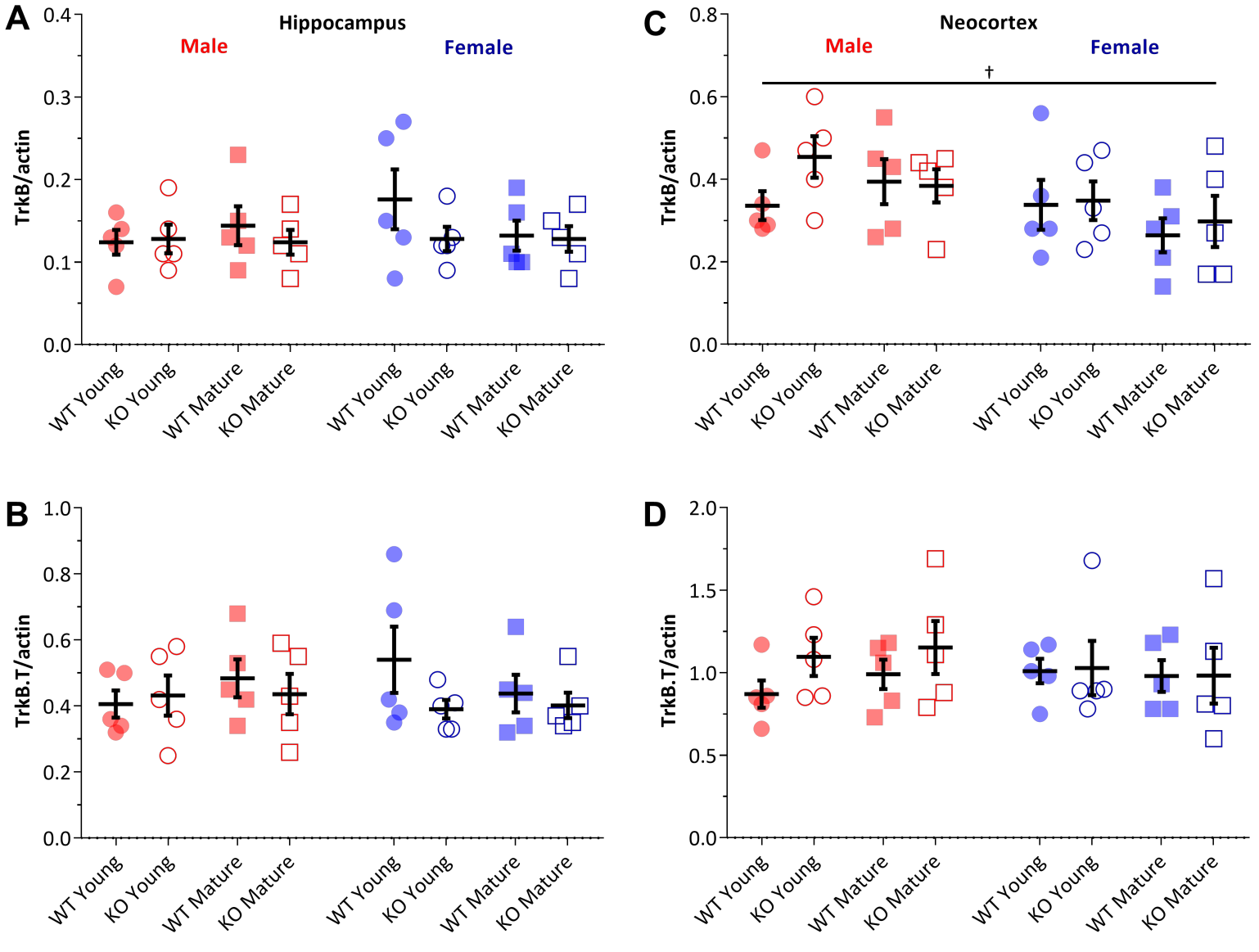
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Hippocampus
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Female
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0.1
0.2
0.3
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WT Young
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WT Young
KO Young
WT Mature
KO Mature
B
TrkB.T/actin
0.0
0.2
0.4
0.6
0.8
1.0
C
Neocortex
Male
Female
†
TrkB/actin
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D
TrkB.T/actin
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1.0
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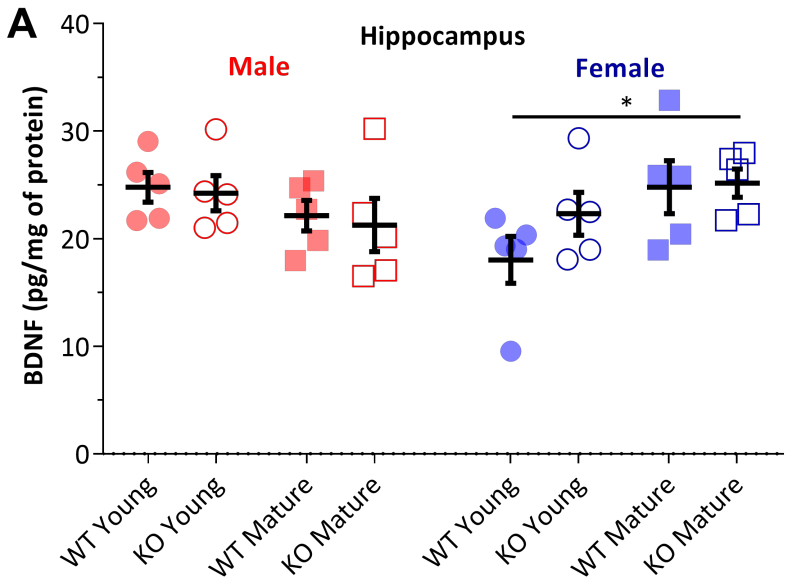
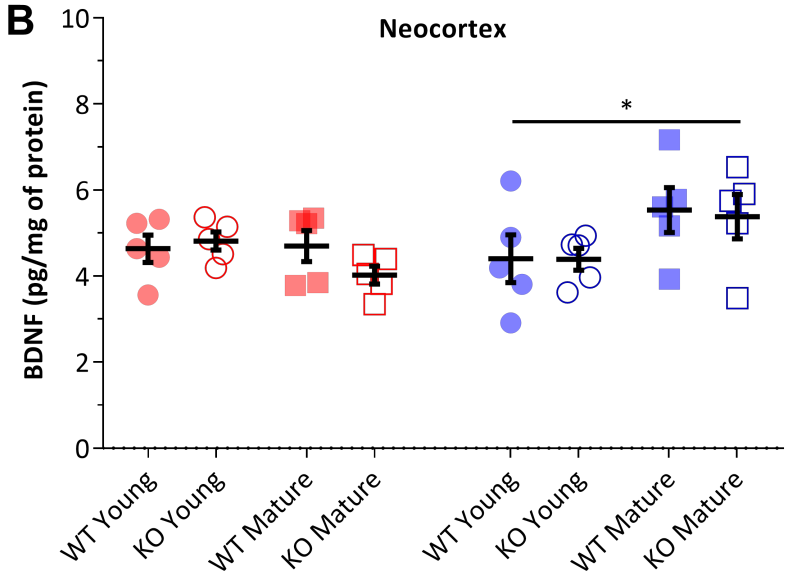
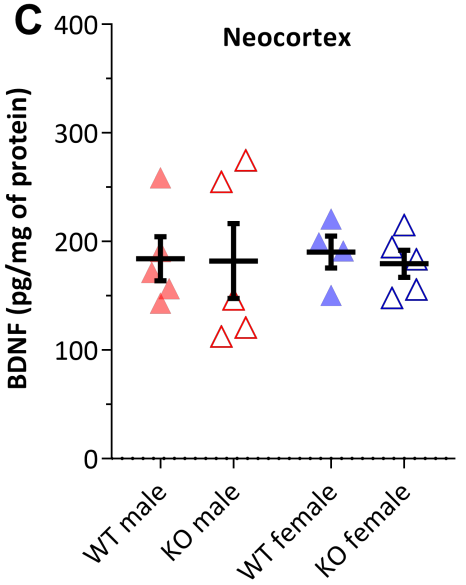
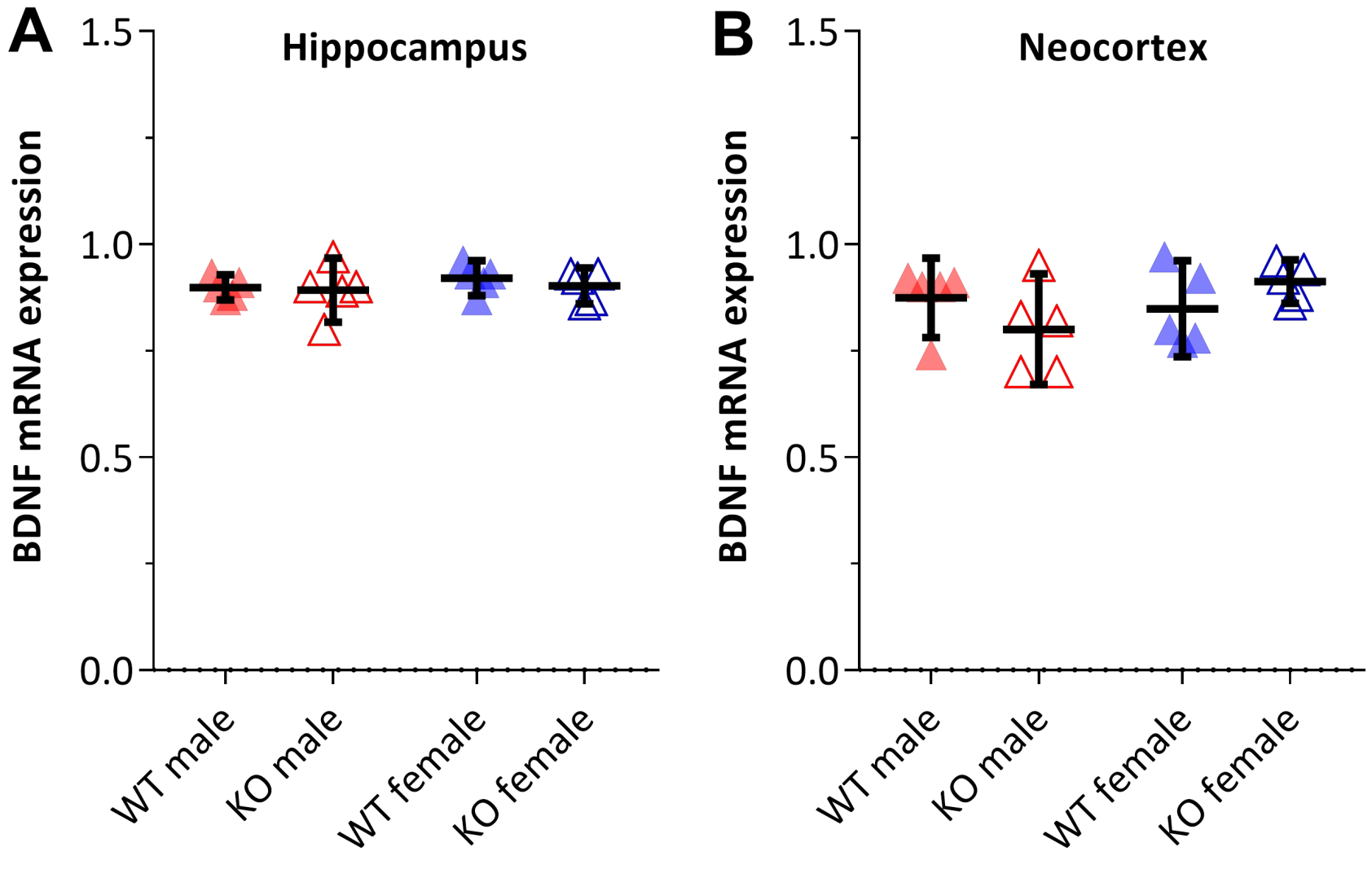
2.0








A
Hippocampus
BDNF mRNA expression
1.5
1.0
0.5
0.0
WT male
KO male
WT female
KO female
B
Neocortex
BDNF mRNA expression
1.5
1.0
0.5
0.0
WT male
KO male
WT female
KO female

Supplemental Table 1. Primer sequences and melting temperatures for qRT-PCR conducted to assess mRNA expression BDNF and two housekeeping genes.

Gene symbol	Gene name	Primer sequence	Tm (°C)
Bdnf	Brain-derived neurotrophic factor	(+)ccataaggacgcggacttgt (-)gaggctccaaaggcacttga	60.0
CycA	Cyclophilin A	(+)agcactggggagaaaggatt (-)agccactcagtcttggcagt	58.0
Ywhaz	Tyrosine 3-monooxygenase/tryptophan, 5-monooxygenase activation protein, zeta	(+)ttgagcagaagacggaaggt (-)gaagcattggggatcaagaa	56.1

Supplemental Table 2. ANOVA results (effects of age, sex, and genotype).

	D.F.	Age	Sex	Genotype
Neocortex				
BDNF (Western blot)	1, 32	***F* = 11.56, *p* = .002**	*F* = 2.85, *p* = .101	*F* = 0.92, *p* = .345
BDNF (ELISA)	1, 32	*F* = 1.54, *p* = .224	*F* = 1.92, *p* = .176	*F* = 0.35, *p* = .557
BDNF (ELISA with acid treatment)	1, 15	N/A	*F* = 0.01, *p* = .937	*F* = 0.08, *p* = .781
TrkB (Western blot)	1, 32	*F* = 0.89, *p* = .353	***F* = 5.13, *p* = .030**	*F* = 1.28, *p* = .267
TrkB.T (Western blot)	1, 32	*F* = 0.08, *p* = .777	*F* = 0.10, *p* = .757	*F* = 1.29, *p* = .265
Actin (Western blot)	1, 32	*F* = 1.75, *p* = .195	*F* = 2.00, *p* = .167	*F* < 0.01, *p* = .980
BDNF mRNA	1, 16	N/A	*F* = 1.43, *p* = .250	*F* = 0.01, *p* = .932
Hippocampus				
BDNF (Western blot)	1, 32	*F* = 0.68, *p* = .416	*F* = 0.01, *p* = .924	*F* = 2.69, *p* = .111
BDNF (ELISA)	1, 32	*F* = 0.55, *p* = .362	*F* = 0.15, *p* = .697	*F* = 0.36, *p* = .552
TrkB (Western blot)	1, 32	*F* = 0.34, *p* = .566	*F* = 0.47, *p* = .496	*F* = 1.60, *p* = .215
TrkB.T (Western blot)	1, 32	*F* < 0.01, *p* = .959	*F* < 0.01, *p* = .958	*F* = 1.49, *p* = .231
Actin (Western blot)	1, 32	*F* = 0.17, *p* = .680	*F* = 0.03, *p* = .869	*F* = 0.03, *p* = .860
BDNF mRNA	1, 16	N/A	*F* = 0.65, *p* = .432	*F* = 0.52, *p* = .481

Supplemental Table 2 (Cont.). ANOVA results (effects of age, sex, and genotype).

	Age × sex	Sex × genotype	Age × genotype	Age × sex × genotype
Neocortex				
BDNF (Western blot)	$F = 0.99$, $p = .327$	$F = 2.07$, $p = .160$	$F = 0.03$, $p = .858$	$F = 1.04$, $p = .316$
BDNF (ELISA)	***F* = 6.60, *p* = .015**	$F = 0.09$, $p = .761$	$F = 0.80$, $p = .377$	$F = 0.42$, $p = .523$
BDNF (ELISA with acid treatment)	N/A	$F = 0.04$, $p = .854$	N/A	N/A
TrkB (Western blot)	$F = 0.60$, $p = .446$	$F = 0.21$, $p = .649$	$F = 0.54$, $p = .470$	$F = 1.16$, $p = .290$
TrkB.T (Western blot)	$F = 0.52$, $p = .477$	$F = 1.09$, $p = .304$	$F = 0.06$, $p = .816$	$F = 0.02$, $p = .904$
Actin (Western blot)	$F = 0.08$, $p = .776$	$F = 0.04$, $p = .840$	$F = 0.25$, $p = .619$	$F = 0.08$, $p = .777$
BDNF mRNA	N/A	$F = 3.67$, $p = .073$	N/A	N/A
Hippocampus				
BDNF (Western blot)	$F = 1.27$, $p = .268$	$F = 0.01$, $p = .917$	$F = 0.73$, $p = .401$	$F < 0.01$, $p = .958$
BDNF (ELISA)	***F* = 7.95, *p* = .008**	$F = 1.26$, $p = .270$	$F = 0.62$, $p = .438$	$F = 0.45$, $p = .508$
TrkB (Western blot)	$F = 1.01$, $p = .322$	$F = 0.46$, $p = .504$	$F = 0.07$, $p = .795$	$F = 1.51$, $p = .228$
Truncated TrkB (Western blot)	$F = 1.07$, $p = .309$	$F = 0.97$, $p = .332$	$F = 0.06$, $p = .814$	$F = 1.22$, $p = .278$
Actin (Western blot)	$F = 1.10$, $p = .302$	$F = 0.15$, $p = .705$	$F = 0.01$, $p = .921$	$F = 1.35$, $p = .254$
BDNF mRNA	N/A	$F = 0.16$, $p = .694$	N/A	N/A