Inhibiting proBDNF to mature BDNF conversion leads to autism-like phenotypes *in vivo*

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Keywords:
BDNF, proBDNF, spines, synaptic plasticity, Autism spectrum disorders

Abstract

Autism spectrum disorders (ASD) comprise a range of early age-onset neurodevelopment disorders with genetic heterogeneity. Most ASD related genes are involved in synaptic function, which is oppositely regulated by brain-derived neurotrophic factor (BDNF): the precursor proBDNF inhibits while mature BDNF (mBDNF) potentiates synapses. Here we generated a knock-in mouse line (BDNF$^{met/leu}$) in which the conversion of proBDNF to mBDNF is inhibited. Biochemical experiments revealed residual mBDNF but excessive proBDNF in the brain. Similar to other ASD mouse models, the BDNF$^{met/leu}$ mice showed decreased brain volumes, reduced dendritic arborization, altered spines, and impaired synaptic transmission and plasticity. They also exhibited ASD-like phenotypes, including stereotypical behaviors, deficits in social interaction, hyperactivity, and elevated stress response. Interestingly, the plasma level of proBDNF, but not mBDNF, was significantly elevated in ASD patients. These results suggest that proBDNF level, but not *Bdnf* gene, is associated with autism-spectrum behaviors, and identify a potential blood marker and therapeutic target for ASD.

Introduction

Autism spectrum disorders (ASD) are early age-onset neurodevelopment disorders with genetic and clinical heterogeneity (Folstein & Rutter, 1977). There are three distinctive behavioral characteristics: social interaction deficits, communication disorders, and repetitive behaviors (De La Torre-Ubieta, Won, Stein, & Geschwind, 2016). A number of genes related to synaptic function have been genetically linked to ASD (De Rubeis et al., 2014), including *fmr1* in Fragile X syndrome (Bassell & Warren, 2008), *mecp2* in Rett syndrome (Amir et al., 1999), *scna1* in Dravet's syndrome (Mahoney et al., 2009; Oakley, Kalume, & Catterall, 2011), and *cacna1* in Timothy syndrome (Splawski et al., 2004). Animal models with mutations of these genes display repetitive stereotypes, and deficits in social interaction similar to ASD patients (De La Torre-Ubieta et al., 2016). Interestingly, nearly all ASD animal models exhibit synaptic deficits, such as alterations in synaptic density, synaptic protein synthesis, and synaptic plasticity (De La Torre-Ubieta et al., 2016; Mullins, Fishell, & Tsien, 2016).

A key regulator for synaptic function is brain-derived neurotrophic factor (BDNF) (Barde, 1994; Ceni, Unsain, Zeinieh, & Barker, 2014; Greenberg, Xu, Lu, & Hempstead, 2009; Kaplan & Miller, 2007), a secretory protein best known for its roles in neuronal survival and differentiation during development, and synaptic function in the adult (Bibel & Barde, 2000; Park & Poo, 2013; Reichardt, 2006). Most of the ASD-related synaptic genes are up- or downstream of BDNF (Lu, Nagappan, Guan, Nathan, & Wren, 2013), although genetic studies have so far not provided a direct link between BDNF and ASD (De La Torre-Ubieta et al., 2016; De Rubeis et al., 2014; Miles, 2011). BDNF is initially synthesized as a precursor protein (proBDNF), which is proteolytic cleaved either intracellularly by furin and pro-protein convertases or extracellularly by plasmin and matrix metalloproteases (MMPs) to form mature BDNF (mBDNF) (Chao & Bothwell, 2002; Lee, Kermani, Teng, & Hempstead, 2001; Lu, Pang, & Woo, 2005b; Mowla et al., 2001; P. T. Pang et al., 2004; Woo et al., 2005).

A number of cell biological and physiological studies implicated that proBDNF is a secreted protein (Keifer, Sabirzhanov, Zheng, Li, & Clark, 2009; Nagappan et al., 2009; Woo et al., 2005; J. Yang et al., 2009). More importantly, proBDNF is not simply an inactive precursor, but an active protein that elicits biological functions through its own receptor (Lu et al., 2005b). There is good evidence that extracellular cleavage of proBDNF plays an important role in late phase long-term potentiation (LTP) and activity-dependent synaptic competition (Je et al., 2012; Je et al., 2013; Petti T. Pang, Nagappan, Guo, & Lu, 2016; P. T. Pang et al., 2004; F. Yang et al., 2009). Previous *in vitro* studies showed that proBDNF promotes the apoptosis of sympathetic neurons and cerebellar granule neurons, reduces the neurite outgrowth of basal forebrain cholinergic neurons (Teng et al., 2005; Volosin et al., 2006) and induces the retraction of dendritic spines of hippocampal neurons (H. Koshimizu et al., 2009). It has also been shown that proBDNF enhances long-term depression (LTD) in hippocampal slices (Liu et al., 2004; Massey et al., 2004; Rosch, Schweigreiter, Bonhoeffer,

87   Barde, & Korte, 2005; Woo et al., 2005) These studies suggest that proBDNF, when secreted

88   extracellularly, could elicit biological effects very different from and even opposite to

89   mBDNF. Moreover, these apparently inhibitory effects are mediated by the pan-neurotrophin

90   receptor p75^{NTR}, but not by the high affinity BDNF receptor TrkB (H. Koshimizu et al., 2009;

91   Teng et al., 2005; Volosin et al., 2006). Binding of proBDNF to the complex comprising

92   p75^{NTR} and sortilin-related VPS10 domain containing receptor 2 (SorCS2) was shown to

93   induce growth cone retraction by initiating the dissociation of the guanine nucleotide

94   exchange factor Trio from the receptor complex (Deinhardt et al., 2011). Taken together,

95   these *in vitro* studies have led to the "Yin-Yang" hypothesis that (i) BDNF and proBDNF

96   elicit opposing biological functions and (ii) the conversion of proBDNF to mBDNF, or the

97   ratio of proBDNF and mBDNF may determine the functional outcomes (Lu et al., 2005b).

98

99   A number of key questions remain: what is the physiological role of proBDNF? How

100   important is the cleavage of proBDNF? A cleavage-resistant proBDNF knock-in mouse line

101   was developed by mutating the two key arginine residues (Figure 1A, 127R and 128R) at the

102   cleavage site of proBDNF (Yang et al., 2014). The homozygous mice were not viable,

103   possibly due to a complete lack of mBDNF, in addition to the strong apoptotic effects of

104   dramatically elevated levels of un-cleavable proBDNF in the brain. The heterozygous

105   (*probdnf-HA/+*) mice, which have one copy of un-cleavable proBDNF, displayed a reduced

106   dendritic complexity and impaired LTP similar to BDNF heterozygous mutant (BDNF+/-)

107   (Yang et al., 2014). In addition, the *probdnf-HA/+* showed a reduced spine density, a

108   decreased basal synaptic transmission, and an elevated LTD, which were not observed in

109   BDNF+/- mice (Woo et al., 2005). Because the *probdnf-HA/+* mice expressed a high level of

110   un-cleavable proBDNF but its mBDNF level was reduced by half, it is unclear whether the

111   phenotypes observed were due to an increase in proBDNF or a decrease in mBDNF, or both.

112   Moreover, the behaviors of the the *probdnf-HA/+* were not examined.

113

114   In the present study, we generated a proBDNF knock-in mouse line named BDNF$^{met/leu}$, in

115   which the arginine residues R125 and R127 at the cleavage site were converted to methionine

116   (R125M) and leucine (R127L) (rs1048220 and rs1048221, respectively), based on previously

117   reported human SNPs (H. Koshimizu et al., 2009). *In vitro* biochemical experiments

118   demonstrated that these two mutations, either alone or together, markedly inhibit the cleavage

119   of proBDNF (M. Koshimizu et al., 2015). The BDNF$^{met/leu}$ mice expressed high levels of

120   proBDNF but low levels of mBDNF, compared with their wild type (WT) littermates (H.

121   Koshimizu et al., 2009). Unlike the homozygotes of proBDNF knock-in mice (*probdnf-*

122   *HA/HA*) which died at birth, the homozygous BDNF$^{met/leu}$ mice survived until adulthood

123   (Kojima et al., 2020). This allowed us to perform detailed characterization of the adult

124   BDNF$^{met/leu}$ mice. Unexpectedly, this new line of mutant mice exhibited many morphological,

125   physiological, and behavioral phenotypes often seen in ASD patients. Further, preliminary

126   characterization of ASD patients revealed a significant increase in the plasma level of

127   proBDNF. Our results point to a possible non-genetic mechanism of ASD and suggest a

128   blood marker potentially useful in the clinic.

Results

Generation of BDNF[met/leu] mice predominantly expressing proBDNF

We generated a knock-in mouse line in which the endogenous *Bdnf* allele was replaced with proBDNF containing human SNPs that changed two arginines proximal to the cleavage site to methionine (R125M) and leucine (R127L), respectively (Figure 1A). We previously reported that these amino acid changes led to inefficient conversion of proBDNF to mature BDNF in cultured neurons (H. Koshimizu et al., 2009). Immunoblot analyses using a polyclonal BDNF antibody (N-20, Santa Cruz) demonstrated that hippocampal lysates from 8-week-old homozygous mutant (BDNF[met/leu]) mice contained an excess amount of proBDNF and only a residual amount of mBDNF, whereas those from wild-type (WT) littermates (or BDNF^{+/+}) contained more mBDNF than proBDNF (Figure 1B). This opposite ratio of mBDNF and proBDNF was quantitatively confirmed by Western blot analysis using a monoclonal anti-pan-BDNF antibody (clone; 3C11, Icosagen) (Kojima et al., 2020) (Figure 1C). A band with the same size as proBDNF was also observed via immunoblotting using another previously-generated proBDNF-specific antibody (Nagappan et al., 2009), further validating that this was indeed proBDNF (Figure 1D). Taken together, we generated a proBDNF knock-in mouse line that expresses a high level of proBDNF with a residual amount of mBDNF.

Despite insufficiency in the proBDNF cleavage, the BDNF[met/leu] homozygous mice were born in a Mendelian manner, and approximately 95% of the homozygous mutant mice survived to adulthood (Kojima et al., 2020). We therefore performed all subsequent experiments using BDNF[met/leu] homozygous, but not heterozygous, mice. The survival of the BDNF[met/leu] mice to adulthood was unexpected because the homozygous proBDNF knock-in mice with different mutations at the proBDNF cleavage site (RVRR→RVAA) died shortly after birth, as reported previously (Yang et al., 2014). Unlike the proBDNF knock-in mice, the morphology and TrkB activation of the hearts of BDNF[met/leu] mice were normal (Kojima et al., 2020). These morphological and immunohistochemical results suggest that the inefficient conversion of proBDNF into mBDNF due to R125M/ R127L mutation may not influence the survival of animals and/or development of the heart.

Brain volumes and dendritic complexity in BDNF[met/leu] mice

The brains of the BDNF[met/leu] mice were smaller than their WT counterparts (Figure 2A). The mean wet weight of the BDNF[met/leu] brains was $15.1 \pm 1.3\%$ lower than that of the BDNF^{+/+} brains (Figure 2B). Cavalieri analysis of Nissl-stained sections revealed that the whole brain volume of the BDNF[met/leu] group was $17.7 \pm 1.56\%$ lower than that of the BDNF^{+/+} group (Figure 2C). Similarly, the volume of the BDNF[met/leu] hippocampus and cortex was reduced by $14.0 \pm 2.3\%$ and $15.8 \pm 1.5\%$, whereas the reduction in olfactory bulb was not significant, compared with the BDNF^{+/+} group (Figure 2C). Thus, inefficient cleavage of proBDNF affects the volume of the adult brain, particularly in the hippocampus and cerebral cortex, two areas known to express high levels of BDNF mRNA (Timmusk et al., 1993).

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171   Since proBDNF is highly expressed in dentate gyrus (DG) (Zhou et al., 2004) the dendritic
172   complexity of the DG neurons was analyzed using Golgi staining. The BDNF$^{met/leu}$
173   homozygous mice had a marked decrease in dendritic arbor complexity compared with their
174   BDNF$^{+/+}$ littermates (Figure 2D, left panel). The difference became evident at 70 μm from
175   cell body (Figure 2D, right panel). Nevertheless, there was no significant difference between
176   the diameters of the cell bodies in the BDNF$^{met/leu}$ and WT animals (data not shown). No
177   apparent apoptotic cells were detected in the DG regions of brains from each group (Figure
178   2E). Thus, inhibition of proBDNF cleavage may lead to impairment of dendritic complexity
179   but not neuronal survival at least in hippocampal DG region in animals.

180

181   The reduction in brain volume was also reported in mouse models with autism-like
182   phenotypes (De La Torre-Ubieta et al., 2016), such as Nlgn3 KO (Baudouin et al., 2012),
183   Nlgn4 KO (Jamain et al., 2008) and ARX cKO mice (Fulp et al., 2008). Furthermore, reduced
184   dendritic complexity was observed in models with changes in the expression of ASD related
185   proteins, such as decreased TAO2 (de Anda et al., 2012) or elevated UBE3A (Khatri et al.,
186   2018). Thus, the BDNF$^{met/leu}$ mice manifest morphological changes similar to other ASD
187   models.

188

189   **Dendritic spine changes in BDNF$^{met/leu}$ mice**
190   Next, we performed a detailed analysis of the dendritic spine morphologies and density in
191   BDNF$^{met/leu}$ hippocampus. The maximal length, maximum width, and density of spine
192   protrusions in the secondary dendritic segments of hippocampal pyramidal neurons in the
193   stratum radiatum of the CA1 area were determined (Figure 3A). We found no significant
194   difference in the mean protrusion lengths between the BDNF$^{met/leu}$ and WT groups ($P = 0.25$)
195   (Figure 3B). However, the mean protrusion width of the BDNF$^{met/leu}$ mice was significantly
196   smaller than that of the WT mice (Figure 3C).

197

198   A previous report using heterozygous *probdnf-HA/+* mice showed a marked decrease in total
199   spine density, without detailed classification of different types of spines (Yang et al., 2014). It
200   was also unclear whether the decrease in spine density was due to an increase in proBDNF or
201   a decrease in mBDNF. In contrast, we observed a significant increase in total spine density
202   (Figure 3D). According to previous studies, the spine protrusions could be classified as thin (a
203   protrusion with length to width ratio >2) or mushroom-like ones (Bourne & Harris, 2008;
204   Harris, Jensen, & Tsao, 1992; Matsuzaki et al., 2001). Detailed quantitative analyses of CA1
205   neuron dendrites revealed a small but significant decrease in mushroom-like spines (Figure
206   3E), but a more than 2-fold increase in thin spines (Figure 3F), in the BDNF$^{met/leu}$ mice. These
207   results reveal that our BDNF$^{met/leu}$ mice are distinct from the previously reported *probdnf-*
208   *HA/+* mice and suggest that the prevention of proBDNF cleavage results in instability of
209   dendritic spines in hippocampus. Selective increase in the density of thin spines (or filopodia)
210   has been observed in Fmr1 KO mice (de Vrij et al., 2008), a mouse model for Fragile X
211   Syndrome (Bernardet & Crusio, 2006; Gross et al., 2015; Huber, Gallagher, Warren, & Bear,

212   2002; Lim et al., 2014). Selective decrease in the density of mushroom-like dendritic spines
213   has been reported in Tg1 mice that over-express human MeCP2, a mouse model for Rett
214   syndrome.
215
216   **Altered postsynaptic scaffold**
217   Recent studies suggest that some of the biological effects of proBDNF require its interaction
218   with p75^{NTR} and the sortilin family proteins such as SorCS2 and SorCS3 (Glerup et al.,
219   2016). SorCS3 is expressed at a high level in the hippocampal CA1 region and is localized to
220   the postsynaptic density (PSD), and the loss of SorCS3 in mice leads to the impairment of
221   hippocampal LTD (Breiderhoff et al., 2013). We therefore investigated whether the proBDNF
222   signaling machinery is intact in the BDNF$^{met/leu}$ hippocampus. We found that the expression
223   levels of p75^{NTR}, SorCS2, and SorCS3 in the hippocampus were not affected by the
224   BDNF$^{met/leu}$ mutation (Figure 3-supplement 1). Thus, proBDNF signaling through the p75^{NTR}-
225   SorCS2 and/or SorCS3 receptor complex occurs normally in the BDNF$^{met/leu}$ mice.
226
227   The deficits in postsynaptic scaffold proteins have been reported in several ASD mouse
228   models (Mullins et al., 2016; Peça et al., 2011). Two proteins have been implicated in spine
229   structure/function: PSD-95, a postsynaptic scaffold protein abundant in spines, and SynGAP,
230   a Ras GTPase-activating protein known to regulate spine morphology (Kim, Liao, Lau, &
231   Huganir, 1998; Vazquez, Chen, Sokolova, Knuesel, & Kennedy, 2004). The expression levels
232   of PSD-95 and SynGAP were not affected by the BDNF$^{met/leu}$ mutation (Figure 3G).
233   However, immunoprecipitation studies revealed that the interaction of PSD-95 and SynGAP
234   was attenuated markedly in the lysates prepared from 9-week-old BDNF$^{met/leu}$ mice,
235   compared with the WT control (Figure 3H).
236
237   proBDNF has been shown to promote hippocampal LTD by regulating the expression of the
238   GluN2B subunit of the N-methyl-D-aspartate receptor (NMDAR)(Woo et al., 2005). The
239   expression of hippocampal GluN2B were comparable between WT and BDNF$^{met/leu}$ animals
240   (Figure 3I). Interestingly, the level of GluN2B was significantly higher in the crude
241   synaptosomal fraction (P2) prepared from BDNF$^{met/leu}$ hippocampi (Figure 3J and K). In
242   contrast, its interaction with PSD-95 was slightly decreased (Figure 3L). However, the levels
243   of PSD-95, synapsin I, or beta-actin in the same P2 fraction were not altered (Figure 3J and
244   K). Overall, these data suggest that proBDNF signaling *in vivo* leads to a down-regulation of
245   the molecular interaction of scaffolding proteins in dendritic spines but an up-regulation of
246   GluN2B expression at extrasynaptic sites in the hippocampal neurons. These findings are
247   reminiscent to the impairments in NMDA receptor function observed in the Shank3 mutant
248   (Duffney et al., 2015) and Shank2 exons 6-7 KO mice (Won et al., 2012), which are also used
249   as ASD models..
250
251   **Impaired synaptic transmission and plasticity**
252   We further examined whether deficits in dendritic spines would alter synaptic function in
253   Schaffer collateral-CA1 pyramidal neurons (Mizui et al., 2015). The slope of the input/output

254 (I/O) curve was significantly reduced, suggesting an impairment in basal synaptic function in
255 BDNF$^{met/leu}$ synapses (Figure 4A). However, paired-pulse facilitation (PPF) was not altered in
256 CA1 synapses of the BDNF$^{met/leu}$ mice, indicating normal presynaptic function (Figure 4B).
257 The decrease in mushroom spines but normal PPF suggest that the reduction in basal synaptic
258 transmission could be mediated by post-synaptic rather than pre-synaptic mechanisms.
259
260 We next examined long-term synaptic plasticity in BDNF$^{met/leu}$ mice. Previous studies have
261 shown that the application of exogenous proBDNF enhanced LTD in juvenile (5-week-old)
262 hippocampal slices (Woo et al., 2005). LTD was elevated in 3-week-old hippocampal slices
263 from the previously reported in the heterozygous *probdnf-HA/+* mice, which have half of its
264 mBDNF but elevated proBDNF (Yang et al., 2014). We tested LTD in hippocampal slices
265 from 3-week-old BDNF$^{met/leu}$ mice which predominantly express proBDNF with residual
266 mBDNF (Figure 1C). The Low-frequency stimulation (LFS; 1 Hz, 900 pulses, 15 min) was
267 applied to Schaffer collaterals of hippocampal slices. In marked contrast to what was
268 observed in *probdnf-HA/+* mice, both induction and expression of LTD was severely
269 impaired in BDNF$^{met/leu}$ mice (Figure 4D). We further examined whether hippocampal LTP
270 was altered in BDNF$^{met/leu}$ mice. Two-month-old hippocampal slices were used, field EPSP
271 slopes at CA1 synapses were recorded, and high-frequency stimulation (HFS, 100Hz, 1
272 second) was applied. Although control mice expressed normal LTP (Figure 4C), the
273 BDNF$^{met/leu}$ mice failed to express LTP (Figure 4C). Taken together, it appears that
274 BDNF$^{met/leu}$ mice, similar to most ASD models (De La Torre-Ubieta et al., 2016), exhibit
275 deficits in all aspects of synaptic functions, including synaptic transmission and plasticity. For
276 example, the Shank2 exons 6–7 KO mice showed impairment in both LTD and LTP (Won et
277 al., 2012).
278
279 **Stereotypes and deficits in social interactions**
280 Reduced brain volume, decreased dendritic complexity, increased thin spines, as well as
281 deficits in postsynaptic scaffold proteins and synaptic functions are all characteristics of
282 mouse models of ASD. In humans, ASD is a group of developmental brain disorders with
283 several distinctive features, including reduced social interactions, language problems, and
284 repetitive and restrictive behaviors (De La Torre-Ubieta et al., 2016; Mullins et al., 2016).
285 While it is difficult to investigate language problems in animals, several mouse ASD models
286 display common autistic behaviors such as hyperactivity, repetitive and stereotyped
287 behaviors, and anxiety (De La Torre-Ubieta et al., 2016). We therefore investigate whether
288 BDNF$^{met/leu}$ mice have some of the ASD-related behavioral phenotypes.
289
290 An obvious repetitive and stereotypical behavior seen in the BDNF$^{met/leu}$ mice was
291 "stargazing." In home cages, BDNF$^{met/leu}$ mice displayed repeated head-tossing very similar
292 to that seen in the classic "stargazer" mouse (Figure 5A, Movie#1) (L. Chen et al., 2000).
293 Quantitative analyses showed that the mutant mice underwent stargazing over 50 times per
294 min (Figure 5B). Such behavior was hardly seen in WT mice.
295

296   Next, we performed two tests to assess social interaction by BDNF$^{met/leu}$ mice. First, a three-
297   chamber test was performed to examine whether a BDNF$^{met/leu}$ mouse placed in the middle
298   chamber prefers a stranger mouse (in one side chamber) to a novel object (in the opposite
299   side chamber). The WT mice spent twice more time with a mouse than with an object (Figure
300   5B). In contrast, the BDNF$^{met/leu}$ mice showed no preference to social partner, spending equal
301   amount of time interacting with the mouse partner and the object (Figure 5B). Second, we
302   measured the interaction of a BDNF$^{met/leu}$ mouse with their littermates in the home cage. A
303   mouse generally interacts less with an aggressive littermate than with a non-aggressive one.
304   We therefore measured the interactions with the two types of littermates separately.
305   BDNF$^{met/leu}$ mice displayed dramatically reduced interaction time with both aggressive and
306   non-aggressive littermates, compared with the WT mice (Figure 5C). These results indicated
307   that social interaction was impaired in BDNF$^{met/leu}$ mice.
308
309   **Hyperactivity and elevated stress response**
310   As most ASD mice models are reportedly hyperactive (De La Torre-Ubieta et al., 2016), we
311   tested locomotion and motor ability of BDNF$^{met/leu}$ mice. In an open field test, the BDNF$^{met/leu}$
312   mice traveled much longer distances, approximately 1000cm/min, than their WT littermates
313   (Figure 5D, left). They also traveled mostly in the center of the field, spending approximately
314   40sec/min there (Figure 5D, center). There was no difference in their vertical activities
315   between the BDNF$^{met/leu}$ mice and their WT littermates (Figure 5D, right). Interestingly, the
316   hyperactivity phenotype appeared to be more apparent during the nighttime when the mice
317   are mostly awake (Figure 5-supplement 1).
318
319   While hyperactive, ASD patients often become restrictive and immobile when challenged or
320   stressed (De La Torre-Ubieta et al., 2016). Tail-suspension test (TST) is often used to test
321   stress-induced immobile behavior in mice. The BDNF$^{met/leu}$ mice (10-12-week old) showed
322   significantly longer immobility time in TST than their WT littermates (Figure 6A). However,
323   unlike in WTs, the immobility time was not shortened by fluoxetine (20 mg/kg, 30 min, i.p.),
324   a selective serotonin reuptake inhibitor commonly used as an antidepressant (Crowley,
325   Blendy, & Lucki, 2005)(Figure 6A). To determine whether prolonged immobility in TST is
326   associated with stress, we determined blood concentrations of corticosterone, an adrenal
327   steroid known to increase in response to stress. Blood samples were collected from the tail
328   vein of BDNF$^{met/leu}$ and WT littermates reared in the same cage for 2 weeks. As expected, the
329   blood corticosterone concentration in resting time in WT mice was comparable to that
330   reported previously (Dugovic, Maccari, Weibel, Turek, & Van Reeth, 1999). In contrast, the
331   BDNF$^{met/leu}$ mice demonstrated a 3-fold higher blood corticosterone concentration (Figure
332   6B, Before stress), suggesting that BDNF$^{met/leu}$ animals are severely stressed compared with
333   WT littermates reared in the same cage. However, 60 min after the exposure to
334   immobilization stress, both BDNF$^{met/leu}$ and WT mice exhibited similar, elevated levels of
335   corticosterone (Dugovic et al., 1999), (Figure 6B, right, After stress). Consistent with these
336   results, in an unstressed home cage environment, the net weight of the adrenal glands, which
337   secrete corticosterone (Jankord & Herman, 2008), was significantly higher in BDNF$^{met/leu}$

338   than that in WT mice (Figure 6C, Adrenal gland/Body). As a negative control, the weight of

339   the kidney did not differ between the BDNF$^{met/leu}$ and WT animals ((Figure 6C,

340   Kidney/Body). These results suggest that mice deficient in proBDNF-processing are sensitive

341   to stress and exhibit activation of the HPA axis under normal conditions.

342

343

344   **Plasma proBDNF and BDNF levels in ASD patients and controls**

345   A number of previous studies have suggested that blood (serum) level of BDNF is

346   proportional to its level in the brain (Klein et al., 2011). Given that the BDNF $^{met/leu}$ mice

347   expressed excessive proBDNF in the brain, we determined whether blood proBDNF

348   concentration was also elevated. However, blood platelets contain a large quantity of BDNF

349   (Fujimura et al., 2002; Yamamoto & Gurney, 1990), which could interfere the accurate

350   measurement of BDNF in the blood. Furthermore, the majority of ELISAs used in previous

351   literature could not distinguish between proBDNF and mBDNF. We therefore measured

352   proBDNF specifically, using plasma, which do not contain platelets or its content instead of

353   serum. We used a sandwich ELISA, which included a capture antibody against the C-terminal

354   BDNF, a detection antibody against the pro-domain of proBDNF, and another detection

355   antibody against the N-terminal of mBDNF. This allowed us to measure both mBDNF and

356   proBDNF levels simultaneously in the same sample. Unfortunately, our highly sensitive

357   ELISA assay (down to 2 pg/ml) could not detect any trace amount of mBDNF nor proBDNF

358   with the 10 ul of mouse blood. Consistent with this, several previous reports indicated that,

359   unlike human and rat blood, mouse blood does not have BDNF (Klein et al., 2011; Radka,

360   Holst, Fritsche, & Altar, 1996).

361

362   We therefore turned to examine the levels of mBDNF and proBDNF in the plasma of human

363   ASD patients and healthy controls. We obtained 1 ml of blood from human volunteers

364   (controls), 100 times the amount from mice. In the initial, pilot experiments, 9 ASD patients

365   and 10 healthy volunteers were enrolled, with well-documented consent forms and the

366   approval of the Institutional Ethics Board. The age of participants ranged from 3~13 years: 7

367   boys and 3 girls in the ASD group; 1 boy and 10 girls in the control group (Table 1). Among

368   those 10 ASD patients, all had delayed development of speech, and most showed repetitive

369   behaviors, a common feature in ASD diagnosis. We also noticed that the intellectual abilities

370   of at least 8 out of 10 ASD children were also impaired, suggesting that these ASD patients

371   likely belonged to the traditional ASD, but not some special groups of ASD with normal

372   intellectual ability, such as Asperger's syndrome.

373

374   Next, peripheral venous blood, from patients and controls. was collected in EDTA-coated

375   tubes and centrifuged within 5 min to obtain plasma (see Methods). Using the sandwich

376   ELISA specific for proBDNF and mBDNF, respectively, we found a selective increase in

377   proBDNF, but not mBDNF, in the ASD patients (Figure 7, and Table 6). The concentration of

378   proBDNF was 1316.17 pg/ml, whereas that of mBDNF was 946.27 pg/ml in ASD patients. In

379   healthy controls, the concentration of proBDNF was 688.39 pg/ml, nearly half of that in the

380   ASD group. However, the concentration of mBDNF is 713.12 pg/ml, which was comparable
381   to that in the ASD group. Thus, the increase in plasma proBDNF, but not mBDNF, is
382   associated with ASD.
383

384   Furthermore, we observed, evaluated, and documented all related symptoms of these ASD
385   patients (Table 2~5). 8 out of 10 patients were firmly diagnosed with ASD, while in the
386   remaining two, autistic features were clearly found, but these features did not match the
387   criteria of ASD diagnosis (Table 2). All patients exhibited conventional autistic features,
388   including the delay of speech development (Table 2), intellectual disabilities (Table 2),
389   repetitive behavior (Table 4), and ADHD (Table 4). However, in some of these ASD patients,
390   we also observed unconventional symptoms, including delays in motor development (Table
391   2), sleep disturbances (Table 3), anxiety (Table 4), self-injury (Table 4), obsessive behavior
392   (Table 4), and aggressive behavior (Table 4). Interestingly, we found that the proBDNF data
393   of ASD patients was associated with a few of these unconventional symptoms. Several ASD
394   children with aggression, eye abnormalities, sleep disorders, and self-injurious behavior
395   tended to express lower proBDNF levels (patient HN0544.p1, HN0555.p1, HN0557.p1, and
396   HN0560.p1). This observation may imply that proBDNF is more likely a biomarker only for
397   ASD with common features, rather than all the ASD subtypes. Further studies with a larger
398   patient number are necessary to validate this point.
399

Discussion

401   Most of the previous models for ASD were based on SNPs or gene mutations found in ASD
402   patients. However, emerging studies have shown that most of ASD patients are not familial
403   but sporadic, and genetic association studies have identified numerous risk variations but few
404   causal genes (De La Torre-Ubieta et al., 2016). Interestingly, nearly all well-founded ASD
405   related genes, such as *fmr1*, *mecp2*, *scna1*, and *cacnal1*, are associated with synaptic function
406   (Amir et al., 1999; Bassell & Warren, 2008; De Rubeis et al., 2014; Mahoney et al., 2009;
407   Oakley et al., 2011; Splawski et al., 2004). Furthermore, most ASD animal models exhibit
408   reductions in synaptic density, synaptic protein synthesis, or synaptic plasticity. In this study,
409   we took an unconventional approach, focusing not on genetic mutations but on the processing
410   of BDNF, a neurotrophic protein known to play a critical role in synaptic function. We have
411   developed an ASD-like mouse model in which synaptic function is decreased, through
412   elevating the proBDNF level but reducing mBDNF level. To the best of our knowledge, this
413   is the first ASD mouse model not based on genetic mutations, but on protein processing.
414

415   Due to the lack of tools to non-invasively detect cellular changes in human brain, we do not
416   know the neuronal phenotypes in the brains of ASD patients. However, studies using
417   postmortem ASD brain tissues have revealed increased spine density in many brain regions,
418   such as the frontal, parietal and temporal lobes (Hutsler & Zhang, 2010; Tang et al., 2014).
419   Efforts have been made to study neuronal phenotypes using ASD animal models. Compared
420   with some of previous models, our BDNF$^{met/leu}$ mice exhibit more comprehensive neuronal

421   phenotypes relevant to ASD. Specifically, the BDNF$^{met/leu}$ mice showed reduced brain volume

422   and dendritic complexity, increased thin spines but decreased mushroom spines, altered

423   synaptic proteins, decreased basal synaptic transmission, and impairments in both LTP and

424   LTD. While previously published ASD models display some synaptic deficits, there is no

425   model that express all of these phenotypes (De La Torre-Ubieta et al., 2016). The similarity in

426   the synaptic phenotypes between our BDNF$^{met/leu}$ mice and previous ASD models suggests

427   that processing of BDNF protein, rather than genetic alteration of *Bdnf* gene, may be an

428   important factor contributing to ASD etiology. However, animal and human studies have so

429   far not implicated BDNF in ASD. Thus, our study provides the first link between ASD and

430   BDNF. Given that BDNF is a key regulator of synaptic development and function (Lu, Pang,

431   & Woo, 2005a), the present work also opens up a new direction for ASD research.

432

433   Another important finding is that the BDNF$^{met/leu}$ mice exhibit comprehensive behavior

434   deficits resembling humans with ASD. We showed the BDNF$^{met/leu}$ mice have impaired social

435   interaction and repetitive behaviors, two key components in the diagnostic criteria for ASD as

436   defined by DSM-5. Compared with all previously established ASD models, the repetitive

437   behaviors of the BDNF$^{met/leu}$ mice are much stronger and more like the symptoms of human

438   ASD patients. A unique aspect of the BDNF$^{met/leu}$ mice, not seen in other ASD models, is the

439   strong and spontaneous 'star-gazing' stereotype (Movie #1), in the absence of any external

440   stimuli ('self-stimulating'). In many children with ASD, especially for those diagnosed with

441   Tourette's syndrome, the 'self-stimulating' behaviors are extremely frequent and apparent,

442   such as flapping their arms or wiggling their toes repeatedly(Kurlan, 2010; Leckman, 2002),

443   which are categorically similar to the 'star-gazing' stereotype in the BDNF$^{met/leu}$ mice. It

444   should be noted that the BDNF$^{met/leu}$ mice do not show over-grooming, a form of repetitive

445   behavior observed in some ASD models but not in human ASD. Bead-burying, a repetitive

446   behavior seen in some ASD models but not in ASD children, is also absent in our BDNF$^{met/leu}$

447   mice. Thus, the BDNF$^{met/leu}$ mice could be a different and perhaps better ASD model, with

448   regards to 'self-stimulating' behaviors. Regardless, the robust behavioral phenotype of the

449   BDNF$^{met/leu}$ mice provides a unique opportunity for mechanistic studies and drug testing.

450

451   Finally, our preliminary data indicates that the plasma level of proBDNF could be used as a

452   potential ASD biomarker that may help in monitoring disease progression and drug efficacy.

453   Specifically, in the pilot experiment with 9 ASD patients and 10 healthy volunteers, we found

454   that an increase in plasma proBDNF level, but not mBDNF level, is associated with ASD.

455   However, a biomarker for clinical use requires both good sensitivity and specificity (Brower,

456   2011). Our data (Figure 7 and Table 6) indicates that the concentration of proBDNF in the

457   ASD group is twice of that in healthy controls, suggesting that the sensitivity of the current

458   ELISA might be in the right range. Technologies exist to further improve the sensitivity

459   greatly. Given the small sample size, we cannot claim the specific association of elevated

460   proBDNF with ASD. Further studies with much larger sample sizes are necessary to validate

461   the specificity of this potential biomarker and to investigate whether it is associated with a

462   distinct ASD subtype. Previous studies have suggested that the plasma level of BDNF could

463 reflect BDNF concentration in the brain (Klein et al., 2011). It is unclear whether the plasma
464 level of proBDNF also correlates with proBDNF concentration in the brain. The source and
465 the functional implication of plasma proBDNF should also be investigated in the future.
466
467 Although most of the phenotypes found in the BDNF$^{met/leu}$ mice coincide with the symptoms
468 of ASD patients or neuronal features of the other ASD models, the brain volume changes may
469 not be the same as those found in human studies. In contrast to the decrease of brain volume
470 in the BDNF$^{met/leu}$ mice, previous studies with large cohorts have shown brain overgrowth in
471 ASD children (De La Torre-Ubieta et al., 2016). However, no such brain overgrowth was
472 reliably detected in the adult ASD patients. In addition, neuroimaging analysis of ASD brains
473 also showed decreased volume of the corpus callosum, cerebellum and brainstem (Penn,
474 2006). These clinical observations suggest that brain volume may not be used as a general
475 pathological criterion for ASD. Interestingly, mutations of the X-linked genes encoding
476 neuroligins, which are known for their association with ASD, have been shown to lead to
477 decreased brain volume (Jamain et al., 2003). Moreover, both neuroligins-3 and -4 knock-out
478 mice, which could display all ASD-like phenotypes, have reduced brain volume (Baudouin et
479 al., 2012; Jamain et al., 2008). Thus, our BDNF$^{met/leu}$ mice could be an ASD-like model
480 similar to fragile X syndrome.
481
482 Another phenotype which shows a potential inconsistency with a previous finding is the
483 reduced LTD of the BDNF$^{met/leu}$ mice. A previous study found an increase, not decrease, in
484 hippocampal LTD in the heterozygotes of cleavage-resistant *probndfHA* knock-in mice (Yang
485 et al., 2014). The design of *probndfHA* knockin line was such that the last two arginines on
486 the proBDNF cleavage site was replaced by two alanines (RR➔AA), and therefore, the
487 cleavage of proBDNF was completely blocked. Since the homozygous *probndfHA* die at
488 birth, only the heterozygous mice were used for the LTD experiment. The heterozygous
489 *probndfHA* knock-in line contains one allele with uncleavable proBDNF and another WT
490 allele. Thus, the proBDNF/mBDNF ratio should be approximately 1:1. The increased LTD
491 seen in *probndfHA* heterozygotes is consistent with the observation that application of
492 exogenous proBDNF to WT hippocampal slices facilitates LTD (Woo et al, 2015). In
493 contrast, our BDNF$^{met/leu}$ mice are homozygous and the mBDNF level in this line was
494 decreased by nearly 90% of that in the WT mice. The extremely low level of mBDNF in the
495 BDNF$^{met/leu}$ mice may not maintain normal function and morphology of hippocampal spines
496 and thus results in a decrease rather than an increase in LTD. This may explain the difference
497 in LTD phenotype between our BDNF$^{met/leu}$ mice and the *probndfHA* heterozygous mice.
498
499 In summary, we have developed a new ASD model, not based on genetic mutations, but on
500 protein processing, and provided a link between ASD and BDNF, a key factor for synaptic
501 regulation. With its robust phenotypes, the BDNF$^{met/leu}$ mouse line may serve as a new ASD
502 model with more comprehensive behavioral deficits resembling human autism patients.
503 Finally, our preliminary study using human blood samples raises the possibility of using
504 plasma proBDNF level as an ASD biomarker.

Materials and Methods

Animals

All animal experiments were performed in strict accordance with protocols that were approved by the Institutional Animal Care and Use Committee of AIST. All efforts were made to minimize animal suffering during the experiments. Animals

All animal experiments were performed in strict accordance with protocols that were approved by the Institutional Animal Care and Use Committee of AIST (number.2019-084, approval date 6 June 2015). All efforts were made to minimize animal suffering during the experiments. Mice were housed with access to food and water ad libitum at constant room temperature ($24 \pm 2°C$) and were exposed to a 12 h light/dark cycle (lights on between 7 a.m. and 7 p.m.). All mice were housed in groups of 3–4 per cage.

Generation of proBDNF knock-in mouse line

Generation of proBDNF knock-in mouse line was described in the report of Kojima et al. (2020) (Kojima et al., 2020). Briefly, the proBDNF knock-in mouse line was generated by replacing the endogenous Bdnf allele with a cDNA encoding cleavage-resistant form of BDNF that contained two amino acid substitutions proximal to the cleavage site of proBDNF (Figure 1A, RVRR to MVLR). A 6.0 kb long arm fragment (NCBI accession number: AY057907; 49015–54460) and a 3.4 kb short arm fragment (55207–58754) flanking the 5' and 3' ends of mouse Bdnf exon 5, respectively, were amplified from 129SV mouse genomic DNA using PCR. The long and short arm fragments were introduced into the ClaI-NotI and PacI-AscI sites of the pMulti-ND 1.0 vector, respectively. A DNA fragment encoding mutant proBDNF was then inserted into the ClaI-NotI site of the targeting vector. A pGK-thymidine kinase gene was used as a negative selectable marker (Figure 1A, Vector). Linearized targeting vector was electroporated into embryonic stem (ES) cells of the D3 line (strain 129 SV). DNA derived from G418-resistant ES clones was screened using PCR and positive ES clones were injected into blastocysts obtained from C57BL/6 mice. The injected blastocysts were then introduced into the uteri of pseudo-pregnant females. Chimeric mice were mated with C57BL/6 mice to produce heterozygotes, and these mice were subsequently crossed with mice expressing Cre recombinase in germ cells to excise the neo cassette. A genetic backcross to the 129/SvEv background were performed over 10 generations before the animals were used in behavioral analyses and all other analyses.

Genotyping

Genotyping of the proBDNF knock-in mouse line was performed according to our recent report (Kojima et al., 2020). using the following primers: 5'-TGCACCACCAACTGCTTAG-3' and 5'-GGATGCAGGGATGATGTTC-3'. PCR analyses using these primers generated 550 bp and 320 bp DNA fragments from wild-type and mutant alleles, respectively. Genotyping of the p75NTR knock-out mice (Ngfrtm1Jae) from the Jackson Laboratory (Bar Harbor, ME) was performed using the following primers: 5'-GCTCAGGACTCGTGTTCTCC-3', 5'-CCAAAGAAGGAATTGGTGGA-3', and 5'-TGGATGTGGAATGTGTGCGAG-3'. PCR

546 analyses using these primers generated 386 bp and 193 bp DNA fragments from wild-type
547 and mutant alleles, respectively. Genotyping of the BDNF knock-out mice (kindly provided
548 by Prof. Nakamura, Tokyo University of Agriculture and Technology, Tokyo) was performed
549 using the following primers: 5'-ATGAAAGAAGTAAACGTCCAC-3', 5'-
550 CCAGCAGAAAGAGTAGAGGAG-3', and 5'-GGGAACTTCCTGACTAGGGG-3'. PCR
551 analyses using these primers generated 275 bp and 340 bp DNA fragments from wild-type
552 and mutant alleles, respectively. Wild-type mice with the background of 129S6/SvEv and
553 C57BL6/J were obtained from Taconic Biosciences (Hudson, NY) and Crea Japan (Shizuoka,
554 Japan), respectively.
555
556 **Golgi impregnation of granule neurons in the hippocampal dentate gyrus**
557 Golgi impregnation of mouse brains was performed using the FD Rapid GolgiStain Kit.
558 Dentate gyrus (DG) neurons were examined in the dorsal hippocampus. For Sholl analyses of
559 dendritic arborization (Z. Y. Chen et al., 2006), Golgi-impregnated DG granule cells that met
560 the following criteria were used: (i) isolated cell body with a clear relationship between the
561 primary dendrite and the soma, (ii) presence of untruncated dendrites, (iii) consistent and
562 dark impregnation along the extent of all of the dendrites, and (iv) relative isolation from
563 neighboring impregnated cells that could interfere with the analysis. For quantitative
564 analyses, 50 neurons from the hippocampal DG area were selected. The cells were traced
565 under 40× magnification using Neurolucida software (MicroBrightField Inc., Colchester, VT)
566 and the morphological traits were analyzed using the NeuroExplorer analysis package. The
567 data were processed and statistical analyses were performed using Graph Pad Prism 4.0
568 (Graph Pad Software Inc., San Diego, CA).
569
570 **Cavalieri analysis of Nissl-stained sections**
571 Cavalieri analysis of Nissl-stained sections were used for hippocampal volume estimation.
572 Steroinvestigator software was applied to measure the entire volume of the hippocampus at
573 4X objective magnification. The external capsule, alveus of hippocampus, and white matter
574 were used as boundary landmarks. All sections throughout each hippocampus were traced
575 and reconstructed. The Cavalieri estimator function was used to calculate the volume of each
576 hippocampus. Following total hippocampal measurements, the cellular layer of each
577 subregion of the hippocampus (DG, CA1, CA2/3) was traced separately and analyzed in the
578 same manner.
579
580 **Immunohistochemistry**
581 Adult mice were anesthetized and transcardially perfused with phosphate buffered saline
582 (PBS) followed by 4% paraformaldehyde in PBS. The brains were postfixed overnight at
583 4°C, cryoprotected, and then cut into 30 μm sections. The sections were washed three times
584 in PBS for 10 min, permeabilized with 0.2% Triton X-100 in PBS, washed a further three
585 times in PBS for 10 min, incubated in 3% bovine serum albumin (BSA) in PBS for 2 h, and
586 then incubated at 4°C for 48 h with anti-proBDNF rabbit antiserum(H. Koshimizu et al.,
587 2009)diluted 1:500 in PBS containing 3% BSA. Subsequently, the sections were washed

588 three times in PBS for 5 min and then incubated with Alexa555 goat anti rabbit IgG (1:1000,
589 Life Technologies, Carlsbad, CA) and 4',6'-diamidino-2-phenylindole dihydrochloride (1
590 μg/ml, Life Technologies) for 2 hr. Fluorescent images were obtained using a Nikon C1
591 confocal or Nikon Ti E inverted microscope (Nikon, Japan) and a 20× Plan Apo, NA 0.75
592 numerical aperture objective lens (Nikon). Immunocytochemical staining using a rat
593 monoclonal anti-mouse CD31 antibody (1:100; clone MEC 13.3; BD Pharmingen, San Jose,
594 CA) was used to detect endothelial cells in the mouse heart. Purified anti-phospho-TrkB
595 (pTrkB) antiserum, which was generously provided by Dr. Chao(Arevalo et al., 2006), was
596 used to detect the activated TrkB receptor. Mouse heart sections were treated with 0.1%
597 hydrogen peroxide and then incubated with the primary antibody. Signal amplification was
598 performed using the avidin:biotinylated horseradish peroxidase complex method (Vectastain
599 ABC; Vector Labs, Burlingame, CA). An anti-serotonin (5-HT) antibody (1:15,000; Incstar,
600 Stillwater, MN) was used for immunocytochemical analyses of 5-HT in brain tissues and the
601 fiber density was quantified as described by Lyons et al(Lyons et al., 1999).
602
603 **In situ hybridization**
604 Brains from 8-week-old mice were fixed by transcardial perfusion with PBS followed by 4%
605 paraformaldehyde in PBS at 4°C. The brains were immersed in the fixative overnight at 4°C,
606 treated with 20% sucrose in PBS, embedded in Tissue-Tek Optimal Cutting Temperature
607 Compound (Sakura Finetek, Tokyo, Japan), and then frozen on a block of dry-ice. In situ
608 hybridization was performed on 20 μm coronal sections. The hybridization and post-
609 hybridization wash steps were performed at 65°C to avoid nonspecific binding. Stained
610 sections were photographed under the bright field system of a BioRevo microscope
611 (Keyence, Osaka, Japan).
612
613 **Immunoblotting**
614 Immunoblotting was performed according to our previous report(Suzuki et al., 2007). Briefly,
615 hippocampal tissues were homogenized in cold lysis buffer comprising 50 mM Tris-HCl (pH
616 7.4), 1 mM EDTA, 150 mM NaCl, 10 mM NaF, 1 mM Na_3VO_4, 1% Triton X-100, 10 mM
617 $Na_2P_2O_7$, 100 μM phenylarsine oxide, and 1% protease inhibitor cocktail (Complete Mini,
618 Roche Diagnostics, Hertforshire, UK). The lysed tissues were incubated at 4°C for 20 min
619 and then centrifuged at 15,000 rpm for 15 min. The protein levels in the supernatants were
620 determined using the BCA Protein Assay Kit (Pierce/Thermo Scientific, Rockford, IL). To
621 detect BDNF, the lysates were boiled for 5 min at 100°C, separated on SDS-polyacrylamide
622 gels, and then transferred to polyvinylidene fluoride membranes (Immobilon P; Millipore,
623 Billerica, MA). The membranes were blocked in Tris-buffered saline containing 0.2% Tween-
624 20 (TBST) and 5% BSA or Block Ace (Dainippon Pharmaceutical, Osaka, Japan), incubated
625 with a rabbit polyclonal anti-BDNF antibody (N-20, Santa Cruz Biotechnology, San Diego,
626 CA) in TBST containing 0.5% BSA or 1% Block Ace at room temperature for 90 min, and
627 then washed three times with TBST. Subsequently, the membranes were incubated at room
628 temperature for 30 min with peroxidase-conjugated secondary antibodies in TBST containing
629 5% BSA and washed three times with TBST. The signal was detected using ImmunoStar

630   Reagents (Wako, Tokyo, Japan) or SuperSignal WestFemto Maximum Sensitivity Substrate

631   (Pierce). The exposure time was adjusted such that the intensity of the bands was within the

632   linear range. To quantify the amount of proBDNF relative to the amount of total BDNF, the

633   blots were scanned and the images were converted to TIFF files and quantified using the NIH

634   ImageJ software (version 1.37v). After subtracting the background in the same lane, the

635   signals for proBDNF and mature BDNF were summed to obtain the total amount of BDNF.

636

637   To detect other molecules, the lysates were separated on SDS-polyacrylamide gels and

638   transferred to polyvinylidene fluoride membranes (Immobilon P membrane, Millipore). The

639   membranes were blocked at room temperature for 30 min in TBST containing 5% skimmed

640   milk (Nacalai Tesque, Kyoto, Japan), and then incubated with the primary antibodies in

641   TBST containing 3% BSA at room temperature overnight. The following primary antibodies

642   were used: anti-p75NTR (1:1000; clone D4B3; Cell Signaling Technology, Danvers, MA),

643   anti-SorCS2 (1:500; R&D Systems, Minneapolis, MN), anti-SorCS3 (1:500; R&D Systems),

644   anti-PSD-95 (1:1000; clone D27E11; Cell Signaling Technology), anti-SynGAP (1:1000;

645   clone D88G1; Cell Signaling Technology), anti-GluN2B (1:1000; Alomone Labs, Jerusalem,

646   Israel), anti-synapsin I (1:1000; Millipore), and anti-β-actin (1:10000; clone AC-15; Sigma,

647   St. Louis, MO). After washing, the membranes were incubated with TBST containing

648   horseradish peroxidase-conjugated secondary antibody (1:1000; GE Healthcare, Pittsburgh,

649   PA) for 1 h at room temperature. The blots were developed using Luminata Forte Western

650   HRP substrate (Millipore) and images of the immunoblots were captured using an

651   ImageQuant LAS500 (GE Healthcare) imaging system. Quantitative analyses of the band

652   intensities were performed using ImageQuant software (Image Analysis Software v8.1, GE

653   Healthcare). Hippocampal tissues were isolated from 9-week-old mice and rapidly

654   homogenized in lysis buffer comprising 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 150 mM

655   NaCl, 10 mM NaF, 1 mM Na_3VO_4, 1% Triton X-100, 10 mM $Na_2P_2O_7$, 100 μM phenylarsine

656   oxide, 1% protease inhibitor cocktail, and 1% protein phosphatase inhibitor cocktail (Sigma).

657   Lysed tissues were incubated at 4°C for 20 min and centrifuged at 15,000 rpm for 15 min.

658   The protein levels in the supernatants were determined using the BCA Protein Assay kit

659   (Pierce). Protein G Sepharose (50 μl, GE Healthcare) was added to the hippocampal lysates

660   and the mixture was rotated at 4°C for 60 min. After removing the Protein G Sepharose, 1 μg

661   of a rabbit monoclonal anti-p75NTR antibody (clone D4B3; Cell Signaling Technology) or

662   mouse monoclonal anti-PSD-95 antibody (clone 7E3-1B8; Thermo Scientific, Pittsburgh, PA)

663   was added to the supernatant (containing 2.5 mg of total protein). The lysates were incubated

664   with the beads (50 μl) at 4°C overnight. The immune complexes were then pelleted and

665   immunoblotted with mouse monoclonal anti-PSD-95 (1:1000, Thermo Scientific), rabbit

666   monoclonal anti-PSD-95 (1:1000; clone D27E11; Cell Signaling Technology), or rabbit

667   monoclonal anti-SynGAP (1:1000; clone D88G1; Cell Signaling Technology).

668

669   **Golgi staining and quantitative analyses of dendritic spine morphology**

670   Mice were anesthetized with isoflurane and the brains were removed and washed in ice-cold

671   PBS (pH 7.4). Rapid Golgi staining was performed using the FD Rapid GolgiStain Kit (FD

672 NeuroTechnologies, Ellicott City, MD), according to the manufacturer's instructions. Briefly,
673 whole brains were silver impregnated for 2 weeks, cryoprotected for 1 week, and then
674 sectioned (80 m) on a sliding microtome (REM-700, Yamato Kohki. Industrial Co., Ltd.,
675 Saitama, Japan). The sections were developed, clarified, and then coverslipped with resinous
676 medium. During staining, image acquisition, and image analysis, the operators were blinded
677 to the genotype of each animal. Images of secondary dendritic segments of hippocampal
678 pyramidal neurons in the stratum radiatum in the CA1 region were obtained using an
679 AxioObserver Z1 microscope (Zeiss, Oberkochen, Germany) with a 63×/0.75 numerical
680 aperture objective lens (LD PlanNeo; Zeiss) for high magnification imaging or a 20×/0.8
681 numerical aperture objective lens (PlanApo; Zeiss) for low magnification imaging, and a
682 cooled CCD camera (CoolSnap HQ2; Photometrics, Tucson, AZ). For morphological
683 analyses of dendritic spines, a z-series with 0.5 μm intervals was created to visualize the
684 entire extent of each dendritic process and to capture spine details in all focal planes. The
685 density, maximum length, and maximum width of the dendritic protrusions was quantified
686 using the Region Measurements tool of MetaMorph Software (Universal Imaging
687 Corporation, West Chester, PA, USA) (10). The form factor of each protrusion, defined as the
688 ratio of its maximum length to its maximum width, was calculated(Takahashi et al., 2003).
689 The age, genotype, and number of mice used for morphological analyses are described in the
690 figure legends. The morphologies of dendritic protrusions were quantified using 33–36
691 segments of the secondary dendrites. These segments represented a total dendritic length of
692 1329–1683 μm per group of mice.
693
694 **Electrophysiology**
695 Field excitatory synaptic potentials were recorded as follows. Transverse slice was placed on
696 custom-made recording chamber according to a previous report (Tominaga, Tominaga, &
697 Ichikawa, 2002)with some modifications. Briefly, both sides of slice were perfused with
698 carbogenize aCSF containing (in mM) 118 NaCl, 3 KCl, 2.5 CaCl$_2$, 1.2 MgCl$_2$, 11 D-glucose,
699 10 HEPES, and 25 NaHCO$_3$, at 2-3ml/min at 28 C. Field EPSPs were elicited by 10-100 μA
700 constant currents pulse (100 microsecond duration) with Pt bipolar electrode (FHC) onto the
701 Schaffer-collateral pathway and recored with glass electrode filled with aCSF (1-2 M ohm).
702 EPSPs were amplified by multiclamp700A (Axon instruments, Sunnydale CA, USA) and
703 digitized at 10Hz (National Instruments, Austin, Texas, USA) controlled by WinLTP
704 programs (Anderson, Fitzjohn, & Collingridge, 2012).
705
706 To set stimulus intensities, maximum responses were determined by increasing stimulus
707 intensity stepwise to saturating responses. For paired-pulse experiment and long-term
708 potentiation experiments, stimulus intensity which elite about 33% of maximum
709 response were employed.   Input-output relationship were studied by plotting fiber volley
710 response amplitude (mV) against fEPSP slope (mV/ms). Paired-pulse responses were also
711 recorded with various inter-pulse intervals from 500ms, 100ms, 50ms, 20ms and paired-pulse
712 ration (2nd pulse response to first response) were calculated.   LTP was induced by four theta
713 burst stimulation, which consist of 4 burst at 0.05Hz of theta burst stimuli (4 bursts at

714   10Hz of 5 pulse at 100Hz). On the other hands, LTD was induced by low frequency
715   simulation (1Hz 900 pulse). In case of LTD experiments, stimulus intensity which elicited
716   50 % of maximum response was employed.
717

718   **Blood sampling and corticosterone measurements**
719   Corticosterone measurements were performed as described previously(Schmidt et al., 2007).
720   To determine basal corticosterone levels, blood samples were collected between 10 and 12
721   a.m. The mice were moved to an adjacent room and placed individually in a transparent
722   restraint tube. Blood samples (approximately 100 µl) were collected into plastic tubes from
723   the tail vein without anesthesia, as described previously(Fluttert, Dalm, & Oitzl, 2000). In all
724   cases, the time between the first disturbance of the animals and the sampling was less than 1
725   min. Following blood collection, the mice were released and returned to their cages. Blood
726   samples were separated by centrifugation at 4°C for 15 min and the plasma was stored in the
727   freezer until use. To measure the change in blood corticosterone levels in response to acute
728   stress, sampling was performed 60 min before and after restraint stress was initiated.
729   Restraining was performed in plastic cylinders identical to those used for the prenatal stress
730   procedure. The AssayMax Corticosterone ELISA Kit (Assaypro, St. Charles, MO) was used
731   to measure corticosterone levels in the blood samples, according to the manufacturer's
732   instructions.
733

734   **Reverse transcription and quantitative PCR**
735   Hippocampal tissues from adult (8–15-week-old) mice (single mutants: $BDNF^{+/+}$, n = 9; and
736    $BDNF^{met/leu}$, n = 8; and double mutants: $BDNF^{+/+}$; $p75^{NGFR+/+}$, n = 6; $BDNF^{+/+}$; $p75^{NGFR+/-}$, n =
737   7, $BDNF^{met/leu}$; $p75^{NGFR+/+}$, n = 7; and $BDNF^{met/leu}$; $p75^{NGFR+/-}$, n = 6) were dissected and
738   stored in RNAlater (Applied Biosystems, Foster City, CA) at −30°C until use. Total RNA was
739   extracted using ISOGEN reagent (Nippon Gene, Tokyo, Japan), treated with RNase-free
740   DNase I (Qiagen, Valencia, CA) to remove contaminating genomic DNA, and then purified
741   using the RNeasy Mini Kit (Qiagen), according to the manufacturer's instructions. Total RNA
742   (0.5 µg) was reverse-transcribed into cDNA using SuperScript III (Invitrogen, Carlsbad, CA)
743   and an oligo $(dT)_{20}$ primer or BDNFrt-3 primer (for *Bdnf* antisense-specific PCR) in a total
744   volume of 5 µl. Quantitative real-time PCR was performed using the 7300 Real-Time PCR
745   system (Applied Biosystems). Duplicate standards were included in each plate and each
746   sample was measured in triplicate. PCR was performed in a total volume of 15 µl containing
747   1.5 µl of diluted cDNA (1/80 dilution), specific forward and reverse primers (50 nM), and
748   Power SYBR Green PCR Master Mix (Applied Biosystems). The PCR conditions for all
749   genes were as follows: 50°C for 2 min, 95°C for 10 min, and then 40 cycles of 95°C for 15 s
750   and 60°C for 1 min. All mRNA levels were normalized to those of Gapdh to adjust for small
751   differences in the amount of input RNA. The primer sequences were as follows: BDrt-1 (for
752   BDNF mRNA), 5'-TGTGTGACAAGTATTAGCGAGTGG-3'; BDrt-2 (for BDNF mRNA),
753   5'-ATGGGATTACACTTGGTCTCGT-3'; BDrt-3 (for BDNF antisense RNA), 5'-
754   ACTCTGGAGAGCGTGAATG-3'; BDrt-4 (for BDNF antisense RNA), 5'-
755   GGCTCCAAAGGCACTTGAC-3'; NGF-F, 5'-GATGGCATGCTGGACCCAAG-3'; NGF-R,

756 5'-CAACATGGACATTACGCTATGCAC-3'; NT3-F, 5'-GGATGATGACAAACACTGG-3';
757 NT3-R, 5'-ACAAGGCACACACACAGGAA-3'; GAPDH-F, 5'-
758 TGCACCACCAACTGCTTAGC-3'; and GAPDH-R, 5'-GGCATGGACTGTGGTCATGAG-
759 3'. The expression levels of genes in mutant tissues were normalized to those of the
760 corresponding genes in wild-type tissues.
761

762 **Tail-suspension test (TST) and antidepressant treatment**
763 For tail-suspension test (TST) (Figure 6), Male mice at 8-12-week-old with the indicated
764 genotype were used. TST was performed on an automated tail suspension device (Muromachi
765 Kikai, Tokyo, Japan). Animals were suspended from a strain gauge for 6 minutes. The
766 settings for the equipment were time constant= 0.25, gain=4, threshold 1=3, and
767 resolution=200 ms. Time spent immobile was recorded in seconds. To test the effect of
768 antidepressant treatment on depressive-behavior, animals were administered saline or
769 fluoxetine 30 min before the tail suspension test (10 mg/kg i.p.).
770

771 **Human plasma collection**
772 All human plasma collection protocols were approved by the Ethics Committee at Xiangya
773 Hospital Central South University. 11 Autism children and their 10 normal siblings as control
774 were recruited for blood sampling. Peripheral venous blood from patients and controls was
775 collected in EDTA-coated tubes and centrifuged at 1000g, 10 minutes to obtain the raw
776 plasma. Then, the raw plasma was centrifuged at 1000g, 10 minutes again to eliminate
777 residual blood cells. All plasma samples were stored at -80°C before the proBDNF and
778 BDNF ELISA experiment.
779

780 **BDNF and proBDNF ELISA**
781 BDNF and proBDNF protein levels were determined by a two-side ELISA (Genstar, China,
782 C643-02 and C546-02) as described by the manufacturer. Plasma were loaded directly into 96
783 well plates without dilution. Absorbance was recorded and analyzed using a Cytation BioTeK
784 plate reader (Winooski, USA). BDNF and proBDNF concentration (pg/ml) was normalized
785 to the volume of plasma in each sample.
786

787 **Statistics**
788 Typically, statistical significance was determined using Student's t-tests after confirming
789 normal distribution of the data. In multi-bar figures, statistical significance was determined
790 by analysis of variance followed by a post hoc test. Data are represented as the mean ± SEM.
791
792

793 # Table Legends

794 **Table 1 The general information of ASD patients and controls**
795 Subject ID, gender, birthday, recruit data, and age of all ASD patients and controls were
796 displayed.
797

798 **Table 2 Neurodevelopment problems in the ASD patients**

799 Neurodevelopment symptoms of the ASD patients, including developmental delay of speech

800 and motor ability, ASD or autisitc features, and intellectual disability were summarized. In

801 this and all the following tables, '+' represents a positive symptom in an ASD patient, '—'

802 represents a negative one, and the blank means the imformation is not reported. Please note

803 '±' means the patient exhibit autistic features but not severe enough to be disagoised as the

804 ASD.

805

806 **Table 3 Neurological problems in the ASD patients**

807 Neurological tests for epilepsy, EEG and MRI abnormities, macrocephaly and sleep disorders

808 in the ASD patients. Please note '±' in the column of epilepsy means seizure was found in

809 that patients, but not severe enough for epilepsy diagnosis.

810

811 **Table 4 Behavioral problems in the ASD patients**

812 Behavior problems, including, the repetitive behavior, ADHD, anxiety, obsessive behavior,

813 self-injury, and aggressive behavior, were displayed in the ASD patients.

814

815 **Table 5 No systemic problems observed in the ASD patients**

816 Systemic features were documented for these ASD patients. No systemic problems, such as

817 eye abnormalities, recurrent infections, hypotonia, hand deformity, and short stature were

818 observed in all the ASD patients.

819

820 **Table 6 The mBDNF and proBDNF level of plasma in the ASD patients**

821 The mBDNF and proBDNF level of plasma in each of ASD patients were displayed.

822

823

824 Table 1 The general information of ASD patients and controls

Subject ID	Gender	Birthday	RecruitDate	Age (year)
ASD				
HN0539.p1	M	2015/11/27	2018/12/9	3.04
HN0543.p1	M	2013/10/6	2018/12/9	5.18
HN0544.p1	M	2015/4/27	2018/12/9	3.62
HN0555.p1	F	2016/2/3	2018/12/16	2.87
HN0557.p1	F	2009/8/21	2018/12/16	9.33
HN0560.p1	F	2013/11/21	2019/1/4	5.12
HN0562.p1	M	2016/7/8	2019/3/20	2.70
HN0612.p1	M	2017/6/30	2019/7/21	2.06
HN0620.p1	M	2016/11/19	2019/7/21	2.67
HN0620.p2	M	2016/11/19	2019/7/21	2.67
Control				
HN0539.s1	F	2012/1/1	2018/12/9	6.94
HN0543.s1	F	2016/8/27	2018/12/9	2.28

HN0544.s1	F	2007/8/23	2018/12/16	11.32
HN0555.s1	F	2013/3/13	2018/12/16	5.76
HN0555.s2	F	2016/2/3	2018/12/16	2.87
HN0557.s1	F	2005/6/17	2018/12/16	13.51
HN0557.s2	F	2009/8/21	2018/12/16	9.33
HN0560.s1	F	2006/9/2	2019/1/4	12.35
HN0562.s1	F	2014/12/9	2019/3/20	4.28
HN0612.s1	M	2015/6/26	2019/7/21	4.07
HN0620.s1	F	2013/11/11	2019/7/21	5.69

825

826 Table 2 Neurodevelopment problems in the ASD patients.

Subject ID	developmental delay (speech)	developmental delay (motor)	ASD/autisitc features	Intellectual disability
HN0539.p1	+		±	+
HN0543.p1	+	+	+	+
HN0544.p1	+	−	+	+
HN0555.p1	+	+	+	+
HN0557.p1	+		+	
HN0560.p1	+		+	
HN0562.p1	+		+	+
HN0612.p1	+	−	±	+
HN0620.p1	+	−	+	−
HN0620.p2	+	−	+	+

827

828

829 Table 3 Neurological problems in the ASD patients.

Subject ID	Epilepsy /Seizure	EEG abnormalities	MRI Brain abnormalities	Macrocephaly	Sleep disturbances
HN0539.p1				−	−
HN0543.p1	−	−	−	−	−
HN0544.p1	−	−	−	−	−
HN0555.p1	−	−	−	−	−
HN0557.p1				−	+
HN0560.p1				−	+
HN0562.p1				−	−
HN0612.p1	−	−	−	−	−
HN0620.p1	−			−	−
HN0620.p2	−			−	−

830

831 Table 4 Behavioral problems in the ASD patients

Subject ID	Repetitive behavior	ADHD	Anxiety	Obsessive behavior	Self-injurious behavior	Aggressive behavior
HN0539.p1	+			−	−	−
HN0543.p1	+	+	−	+	−	−
HN0544.p1	+	+	+	−	−	−
HN0555.p1	+	+	−	−	−	−
HN0557.p1	+	+	+		+	−
HN0560.p1	+	+			−	+
HN0562.p1	+	+		+	+	−
HN0612.p1		−	−	−	−	−
HN0620.p1	+	−	−	−	−	−
HN0620.p2	+	−	−	−	−	−

832

833

834   Table 5 No systemic problems observed in the ASD patients

Subject ID	Eye abnormalities	Recurrent infections	Hypotonia	Hand deformity	Short stature
HN0539.p1	−			−	−
HN0543.p1	−	−	−	−	−
HN0544.p1	−	−	−	−	−
HN0555.p1	−	−	−	−	−
HN0557.p1				−	−
HN0560.p1			−	−	−
HN0562.p1	−		−	−	−
HN0612.p1	−	−	−	−	−
HN0620.p1	−	−	−	−	−
HN0620.p2	−	−	−	−	−

835

836   Table 6 The mBDNF and proBDNF level of plasma in the ASD patients.

Subject ID	mBDNF level (pg/ml)	proBDNF level (pg/ml)
HN0539.p1	1172.76	1877.297
HN0543.p1	132.7449	1495.534
HN0544.p1	1693.265	608.195
HN0555.p1	175.8991	824.8709
HN0557.p1	92.91032	633.9897
HN0560.p1	60.37873	740.608
HN0562.p1	1621.231	878.18
HN0612.p1	938.3997	950.4053
HN0620.p1	1206.951	2589.232
HN0620.p2	2368.13	2563.437

837

Acknowledgements

Author Contributions

T.M., H.K., T.H., and M.K. (AIST) performed the biochemical assays, analyzed the data; T.M. and K.K.[a] (AIST), performed the electrophysiological experiments and analyzed the data; K.T., T.M.[a,c], K.K.[a,c], M.O., and M.K. (AIST) engaged in the behavioral analysis of mice; T.M., K.T., M.O., M.K. performed the analysis of neuronal morphology and its quantitative analysis; T.B. and K.X. collected plasma samples of ASD patients; H.Y. conducted the ELISA analysis; H.Y., T.M., K.T., B.L., T.M., H.K., and M.K. wrote the paper; B.L., H.Y. and M.K. conceived and supervised the project.

Competing Interests

The authors declare no conflict of interests.

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Figure 1
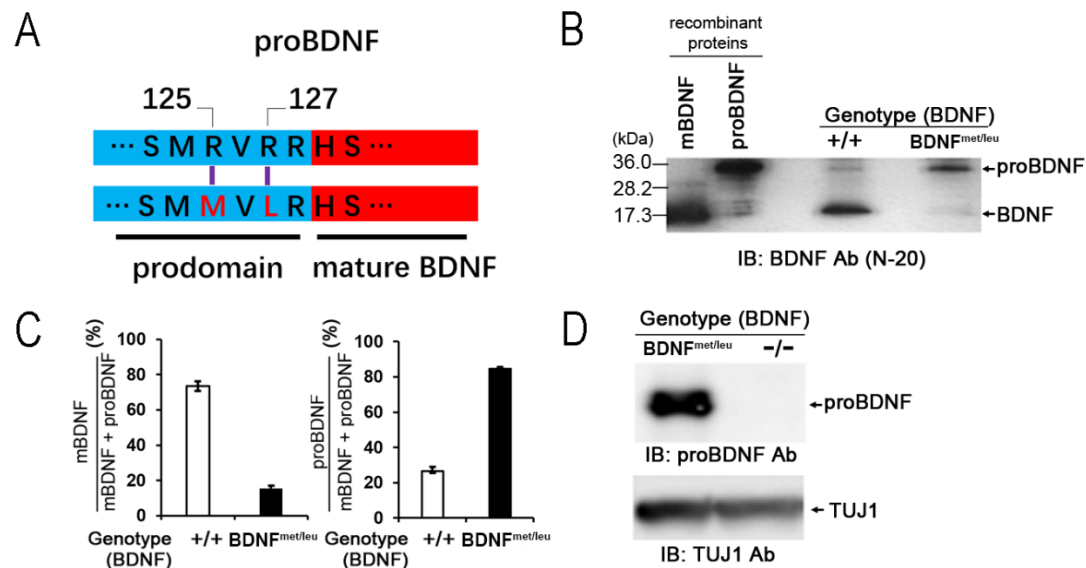
BDNF$^{met/leu}$ knock-in mice expressing excessive proBDNF but residual mBDNF.

(A) Schematic diagram showing the replacement of two arginine (R) with a methionine (M) and a leucine (L) in the conserved cleavage site of the *bdnf* gene to generate a cDNA encoding cleavage-resistant-type proBDNF. **(B)** Immunoblot analyses of BDNF protein expression in the hippocampus. Hippocampal lysates (30 μg of protein/lane) from 8-week-old littermates (wild type (+/+ or BDNF$^{+/+}$) and BDNF$^{met/leu}$ knock-in mice were processed for Western blot and probed with an antibody against the C-terminus of BDNF (N-20). Recombinant mature BDNF (mBDNF) and proBDNF proteins were used for comparison. **(C)** Quantification of the mBDNF or proBDNF bands on Western blot using Image J. N = 3 littermate mice per group. Note a dramatic increase in proBDNF and a dramatic decrease in mBDNF. In this and all other figures, bar graphs represented as the mean ± SEM. Student's t-test was performed unless indicated otherwise. *: p<0.05; **: p<0.001. **(D)** Detection of proBDNF in BDNF$^{met/leu}$ hippocampus, using a proBDNF-specific antibody. Hippocampal lysates from 10-day-old *Bdnf* homozygous knockout mice (-/-) were used as a negative control.

Figure 2
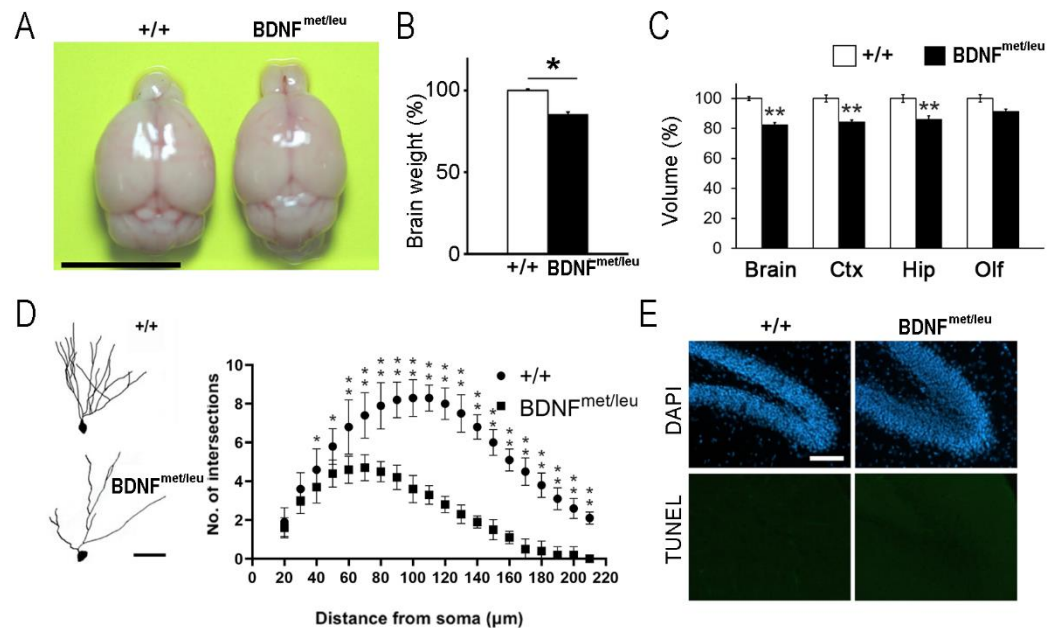
Decrease in brain volume and dendritic arborization in BDNF$^{met/leu}$ mutants.

(A) Gross view of representative brains of +/+ and BDNF$^{met/leu}$ littermates. Scale bars: 10 mm. **(B)** Quantification of brain weights of 2-month-old mice with the indicated genotype. N = 6 animals per group. **(C)** Cavalieri analyses of Nissl-stained brain sections of +/+ and BDNF$^{met/leu}$ littermates showing volumes of whole brain and its sub-regions. N = 6 animals per genotype. Ctx: cortex; Hip: hippocampus; Olf: olfactory bulb. **(D)** Sholl analyses of dentate gyrus (DG) neurons from 8-week-old mice N = 10 neurons per group. Left: an example of the Golgi-stained neurons. Scale bars: 50 μm. **(E)** Detection of apoptotic cells in DG granule cell layers. Coronal sections of hippocampi from 8-week-old mice were stained using a TUNEL assay. Scale bars: 100 μm.

Figure 3
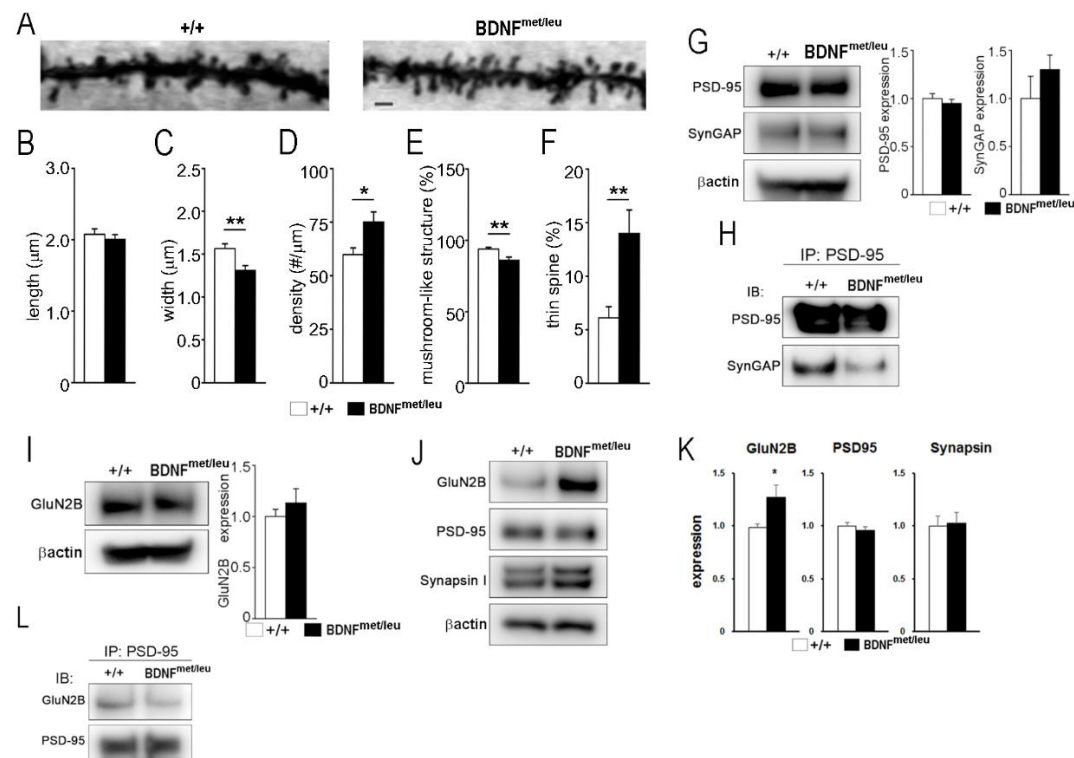
Alterations in dendritic spines and interactions of postsynaptic proteins.

(A) Representative high-magnification images of Golgi-stained CA1 pyramidal neurons isolated from 9-week-old +/+ and BDNF$^{met/leu}$ littermates. Scale bar, 2 μm. **(B–F)** Quantitative analyses of the morphologies of secondary dendritic segments of hippocampal pyramidal neurons in the stratum radiatum of the CA1 area. The length (B), width (C), and density (D) of the spine protrusions, as well as the percentages of mushroom-like (E) and thin (F) spines, were quantified. Data are represented as the mean ± SEM of N = 3 mice per genotype and n = 33 (+/+) or n = 36 (BDNF$^{met/leu}$) neurons per mouse. **(G)** Immunoblot analyses of PSD-95 and SynGAP expression levels in hippocampal lysates (25 μg protein) from +/+ and BDNF$^{met/leu}$ mice. The expression levels of PSD-95 and SynGAP were normalized to that of β-actin. The left panel shows a representative blot and the right panel shows quantification of the data (PSD-95, p = 0.19; and SynGAP, p = 0.73, by Student's t-tests). N = 3 mice per group. **(H)** The interaction of PSD-95 with SynGAP in +/+ and BDNF$^{met/leu}$ hippocampus. Hippocampal lysates were immunoprecipitated with an anti-PSD-95 antibody, followed by immunoblotting using anti-PSD-95 and anti-SynGap antibodies. Note a selective decrease in SynGap in BDNF$^{met/leu}$ hippocampus. **(I)** Immunoblot analyses of GluN2B expression in hippocampal lysates from +/+ and BDNF$^{met/leu}$ mice. The expression levels of GluN2B were normalized to that of β-actin. The left panel shows a representative blot and the right panel shows quantification of the data (N = 3 mice per group. p = 0.18 by Student's t-test). **(J-K)** The level of synaptic proteins in hippocampal synaptosomal preparations from +/+ and BDNF$^{met/leu}$ mice. Note a selective increase in GluN2B in BDNF$^{met/leu}$ hippocampus. **(L)** The interaction of GluN2B with PSD-95 in +/+ and BDNF$^{met/leu}$ hippocampi. Hippocampal lysates (2 mg protein) were immunoprecipitated (IP) with an anti-PSD-95 antibody, and equal aliquots of the

immunoprecipitants were subjected to immunoblotting (IB) with the indicated antibodies. Note that the interaction between PSD95 and GluN2B was decreased in BDNF$^{met/leu}$ hippocampus, compared with that in the +/+ hippocampus.

Figure 3-figure supplement 1
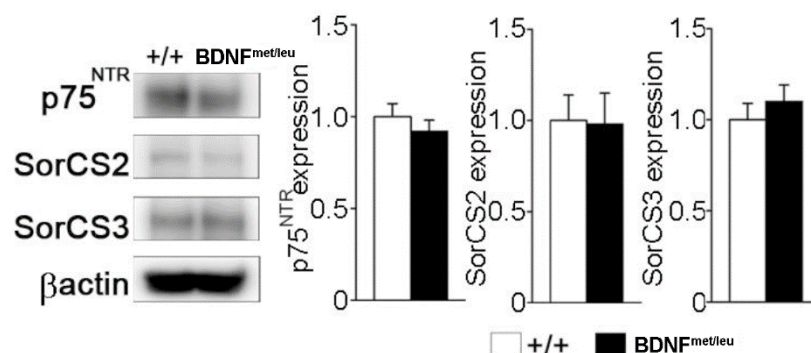
Immunoblot analyses of p75^{NTR}, SorCS2, and SorCS3 expression in hippocampal lysates (25 μg of protein) from BDNF$^{+/+}$ and BDNF$^{met/leu}$ mice. The expression level of indicated proteins was normalized to that of beta-actin. The left panel shows a representative blot and the right panel shows quantification of the data (p75NTR, p = 0.18; SorCS2, p = 0.46; SorCS3, p = 0.77, by Student's t-test.).   N = 3 mice per group.

Figure 4
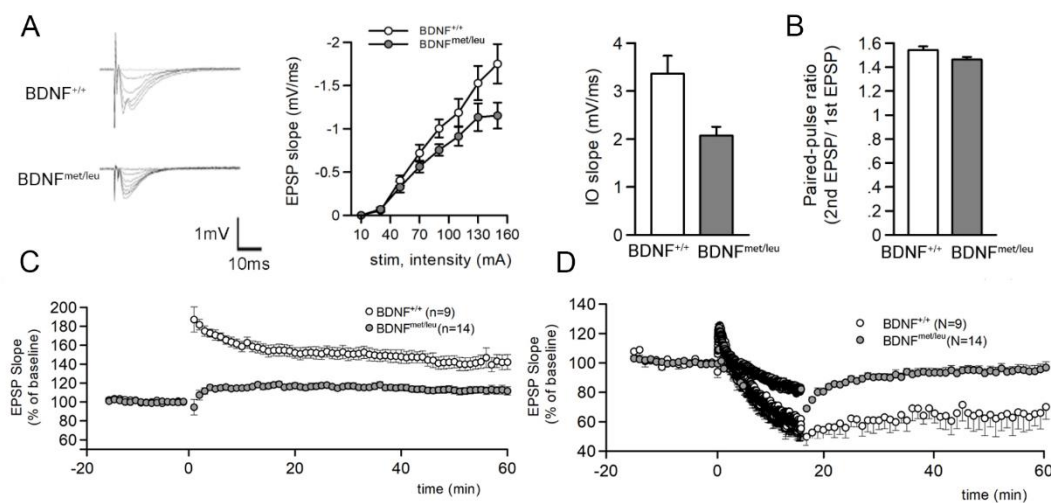
Impairments in synaptic transmission and plasticity.

(**A**) A decrease in basal synaptic transmission in BDNF[met/leu] mice. Representative traces (Left), quantitative analysis (Middle), and slope (right) of the input-output (I/O) curve of field excitatory postsynaptic potentials (fEPSPs) recorded in Schaffer collateral-CA1 pyramidal neurons in the hippocampus of +/+ and BDNF[met/leu] mice. The slopes of fEPSP were plotted against the fiber volley amplitude. +/+: N= 4 animals, n=9 slices; BDNF[met/leu]: N= 4 animals, n=9 slices. (**B**) Normal paired-pulse facilitation (PPF) in BDNF[met/leu] mice. Two stimuli were applied at various inter-pulse intervals (IPI, 10 to 100 msec). The ratios of the second and first fEPSP slopes were calculated, and mean values were plotted against different inter-pulse intervals (IPI, 10 to 100 msec). +/+: N= 4 animals, n=9 slices; BDNF[met/leu]: N= 4 animals, n=9 slices. (**C**) Impairments of long-term potentiation (LTP) in BNDF[met/leu] mice. The fEPSP slopes were plotted against time (in minutes) before and after theta-burst stimulation (TBS, 5 pulses at 100Hz per burst, 10 bursts with an interval of 200 msec). n in this and (D) indicates number of slices used. (**D**) Impairments of long-term depression (LTD) in BNDF[met/leu] mice. The experiments were carried out in the same way as above except low-frequency stimulation (1Hz 900 pulse) were applied to hippocampal slices to induce LTD.

Figure 5
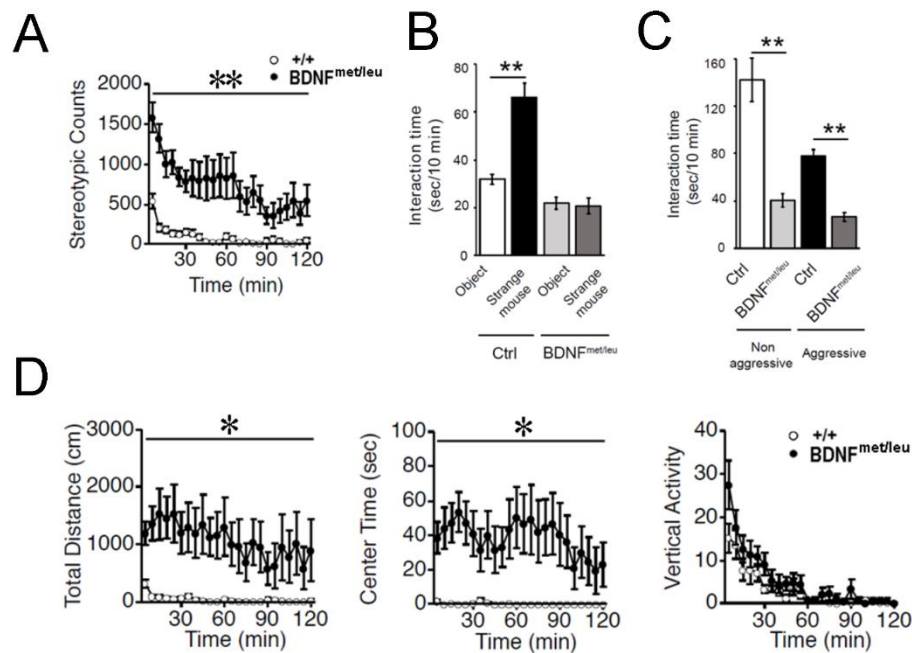
Autism spectrum disorders (ASD)-related behavioral phenotypes.

(**A**) Stereotype behavior. In the same open field, the number of "star-gazing" like behaviors were counted over a 120-min period. (**B**) Social-interaction test in the open field. Interaction time was measured as the time mice spent in the quadrant of target object or stranger mouse within in a 10-min period. When presented both an object and a stranger mouse simultaneously, +/+ mice spent more time in the quadrant to interact with the caged stranger mouse. However, BDNF$^{met/leu}$ mice had no preference for the stranger mouse. (**C**) Social interactions of freely moving BDNF$^{met/leu}$ and +/+ littermates with aggressive or non-aggressive test mice. A mouse generally interacts less with an aggressive littermate than with a non-aggressive one. In contrast, BDNF$^{met/leu}$ mice displayed a decreased time in both non-aggressive and aggressive interactions. N = 7 mice per genotype. **P < 0.01. (**D**) BDNF$^{met/leu}$ mice exhibit severe hyperactivity. Mice were placed in a standard open field, and total distance traveled (left), the amount of time spent in the center of the open field (center), and vertical activity (right) were counted in a 120-min period.

Figure 5-figure supplement 1
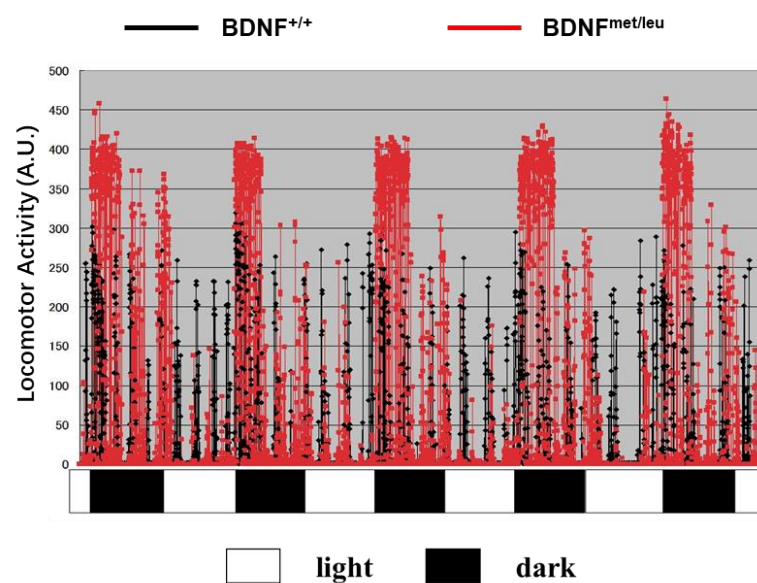
The recording of locomotor activity of BDNF$^{+/+}$ (N=4) and BDNF$^{met/leu}$ (N=4) mice during 4.5 days, with 12 h light and 12 h dark phases. Y axis indicates the horizontal activity for 5 minutes.

Figure 6
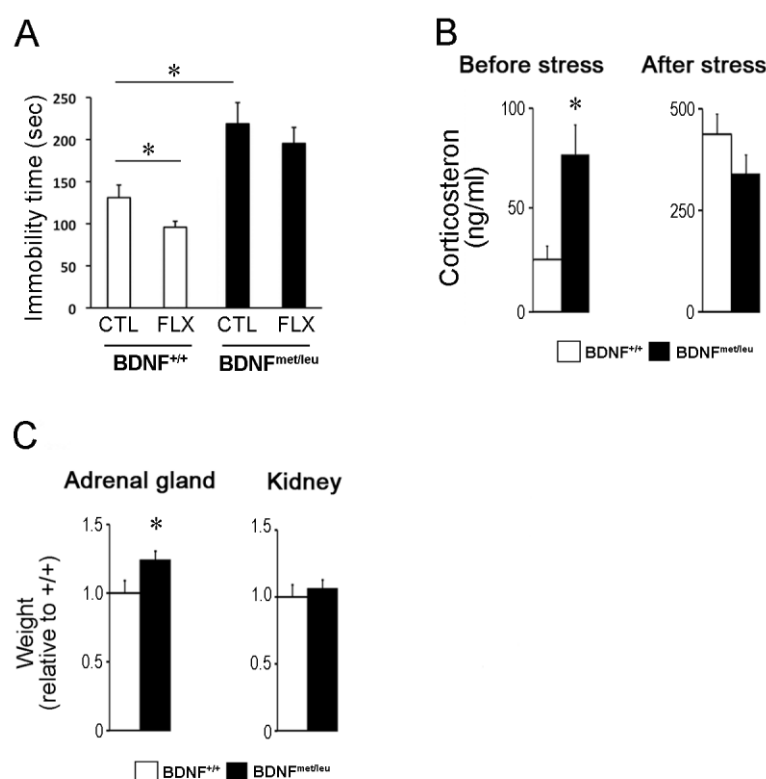
Elevated stress response in BDNF$^{met/leu}$ mice.

(A) Tail suspension test of BDNF$^{+/+}$ and BDNF$^{met/leu}$ mice at the age of 10~12 weeks. Animals were administered saline or fluoxetine 30 min before the tail suspension test (10 mg/kg i.p.). *, p < 0.05; ANOVA with post-hoc test. N=7~8 littermate animals. **(B)** Corticosterone levels in the serum before and after acute stress exposure. The tail blood samples were collected in group-housed BDNF$^{+/+}$ and BDNF$^{met/leu}$ mice (10-week-old, N = 8 per genotype) (Left panel, before stress). After an additional 2 weeks of group-housing, the animals were subjected to immobilization stress (60 min) and the tail blood samples were collected immediately (Right panel, after stress). The serum corticosterone concentration was determined immediately after sampling. **(C)** The relative weights of the adrenal gland and kidney in 8-week-old littermate animals (N = 6 per genotype) with the indicated genotypes. The organ weights were normalized to the body weight of each animal.
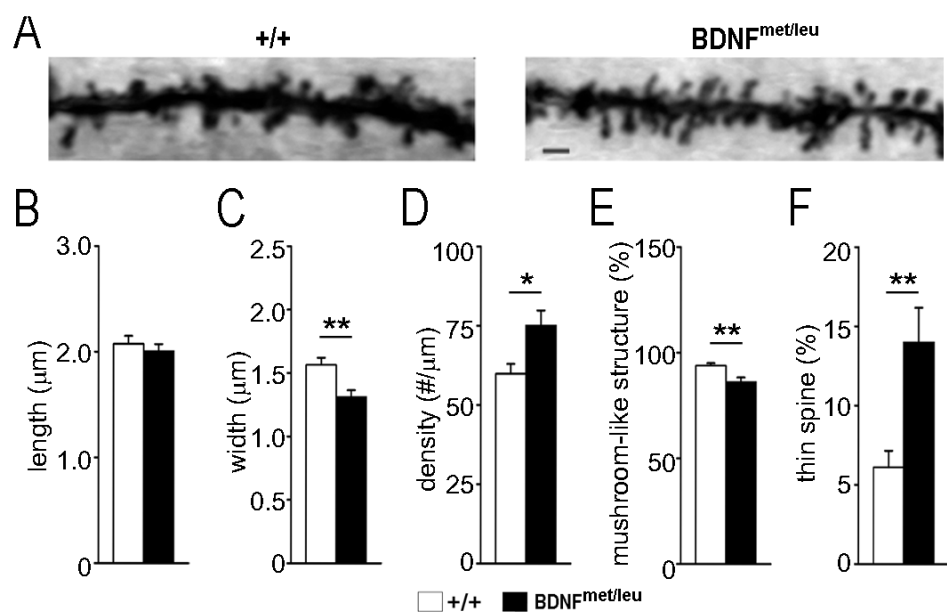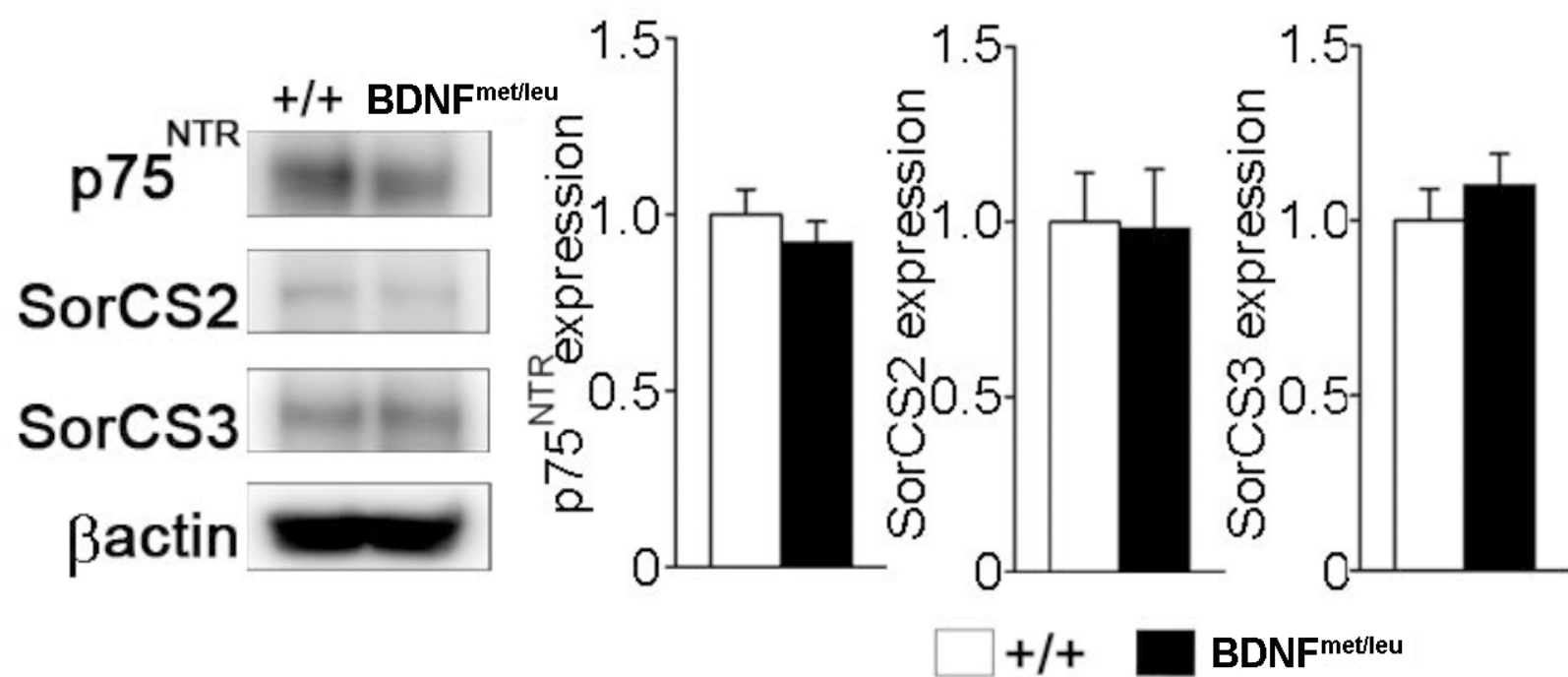
Figure 7
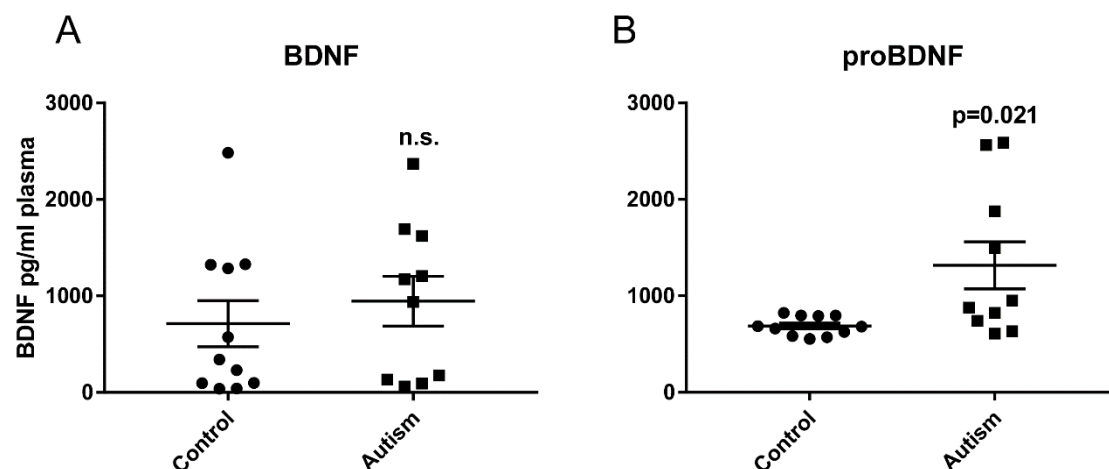
proBDNF and BDNF levels in human plasma.
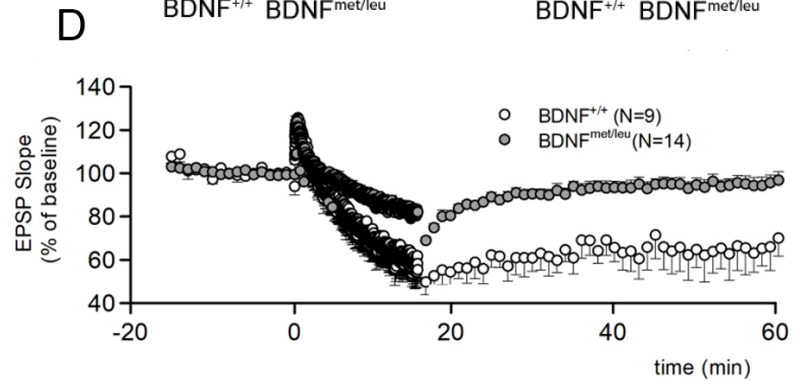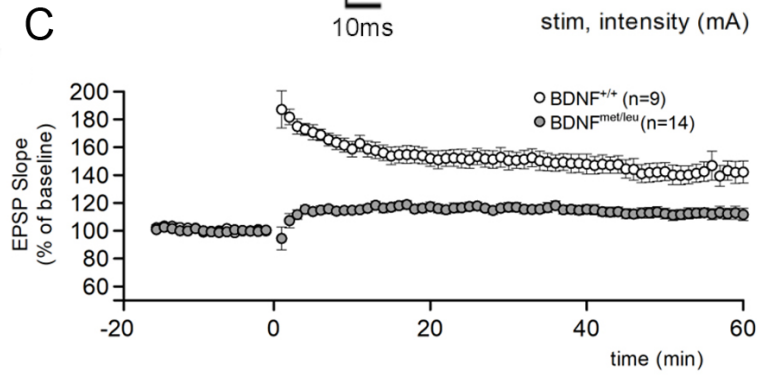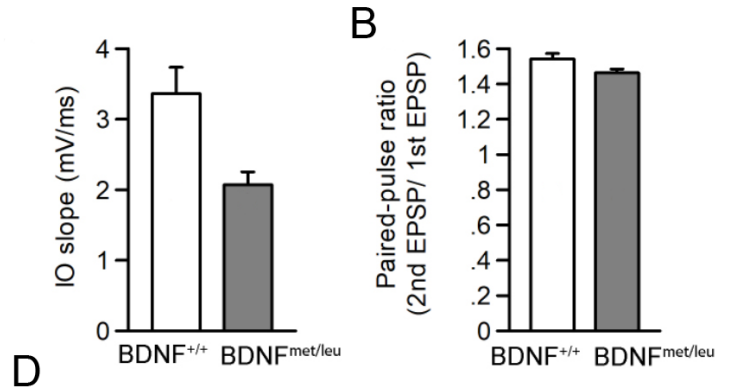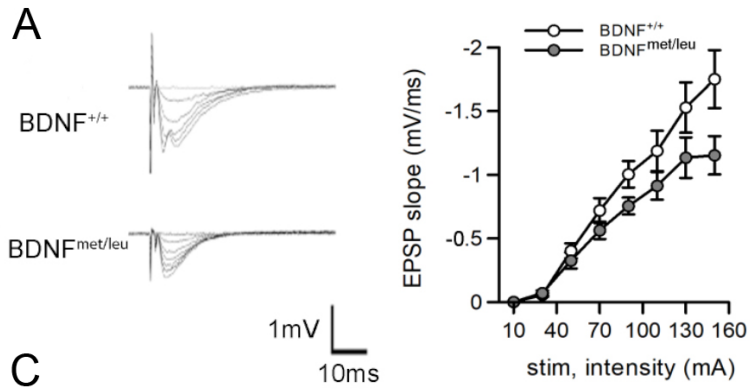
(**A, B**) ELISA analysis of mBDNF (**A**) and proBDNF (**B**) in human plasma of ASD (n=11) and control (n=10) children. Blood sampling and the separation of plasma was described in Material and Methods. A sandwich ELISA was employed to detect both mBDNF and proBDNF levels simultaneously. Data are shown as mean ± S.E.M.
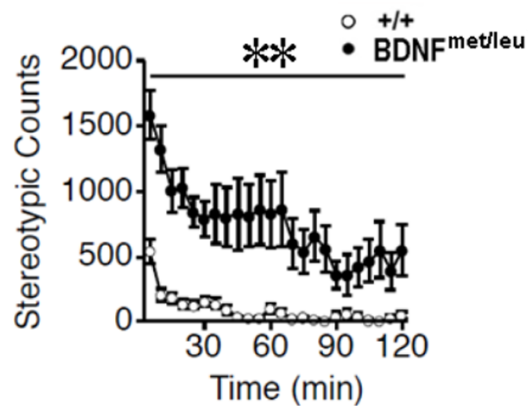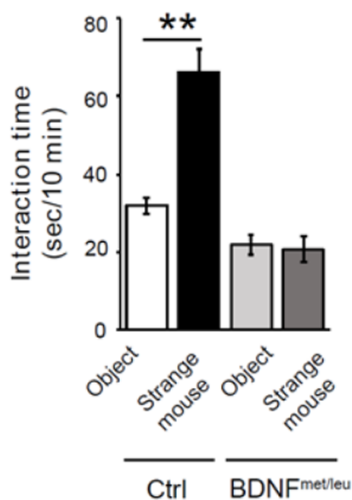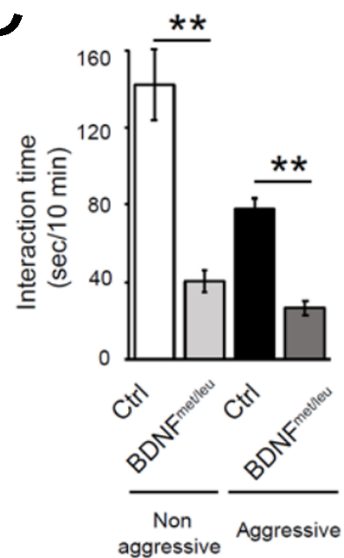
A proBDNF

125 ⎤ ⎡ 127

··· S M **R** V **R** R H S ···

··· S M **M** V **L** R H S ···

prodomain   mature BDNF

B

recombinant proteins

(kDa) mBDNF proBDNF

Genotype (BDNF)

+/+   BDNF^met/leu

36.0 —   ← proBDNF

28.2 —

17.3 —   ← BDNF

IB: BDNF Ab (N-20)

C

$\frac{mBDNF}{mBDNF + proBDNF}$ (%)

Genotype (BDNF)   +/+   BDNF^met/leu

$\frac{proBDNF}{mBDNF + proBDNF}$ (%)

Genotype (BDNF)   +/+   BDNF^met/leu

D Genotype (BDNF)

BDNF^met/leu   −/−

← proBDNF

IB: proBDNF Ab

← TUJ1

IB: TUJ1 Ab

A

+/+   BDNF^met/leu

B

Brain weight (%)

100

50

0

+/+   BDNF^met/leu

C

□ +/+   ■ BDNF^met/leu

Volume (%)

100
80
60
40
20
0

Brain   Ctx   Hip   Olf

D

+/+

BDNF^met/leu

No. of intersections

10
8
6
4
2
0

● +/+
■ BDNF^met/leu

0 20 40 60 80 100 120 140 160 180 200 220

Distance from soma (μm)

E

+/+   BDNF^met/leu

DAPI

TUNEL

A   +/+   BDNF^met/leu

B
length (μm)

C
width (μm)
**

D
density (#/μm)
*

E
mushroom-like structure (%)
**

F
thin spine (%)
**

□ +/+   ■ BDNF^met/leu

G   +/+   BDNF^met/leu
PSD-95
SynGAP
βactin

PSD-95 expression
SynGAP expression
□ +/+   ■ BDNF^met/leu

H   IP: PSD-95
IB: +/+   BDNF^met/leu
PSD-95
SynGAP

I   +/+   BDNF^met/leu
GluN2B
βactin

GluN2B expression
*
□ +/+   ■ BDNF^met/leu

J   +/+   BDNF^met/leu
GluN2B
PSD-95
Synapsin I
βactin

K   GluN2B   PSD95   Synapsin
expression
*
□ +/+   ■ BDNF^met/leu

L   IP: PSD-95
IB: +/+   BDNF^met/leu
GluN2B
PSD-95

BDNF$^{+/+}$ **BDNF$^{met/leu}$**

light   dark

A   BDNF

B   proBDNF