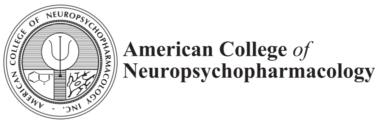

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ARTICLE

BDNF controls $GABA_AR$ trafficking and related cognitive processes via autophagic regulation of p62

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Reduced brain-derived neurotrophic factor (BDNF) and gamma-aminobutyric acid (GABA) neurotransmission co-occur in brain conditions (depression, schizophrenia and age-related disorders) and are associated with symptomatology. Rodent studies show they are causally linked, suggesting the presence of biological pathways mediating this link. Here we first show that reduced BDNF and GABA also co-occur with attenuated autophagy in human depression. Using mice, we then show that reducing Bdnf levels (*Bdnf*$^{+/-}$) leads to upregulated sequestosome-1/p62, a key autophagy-associated adaptor protein, whose levels are inversely correlated with autophagic activity. Reduced Bdnf levels also caused reduced surface presentation of α5 subunit-containing $GABA_A$ receptor (α5-$GABA_AR$) in prefrontal cortex (PFC) pyramidal neurons. Reducing *p62* gene dosage restored α5-$GABA_AR$ surface expression and rescued PFC-relevant behavioral deficits of *Bdnf*$^{+/-}$ mice, including cognitive inflexibility and reduced sensorimotor gating. Increasing p62 levels was sufficient to recreate the molecular and behavioral profiles of *Bdnf*$^{+/-}$ mice. Collectively, the data reveal a novel mechanism by which deficient BDNF leads to targeted reduced GABAergic signaling through autophagic dysregulation of p62, potentially underlying cognitive impairment across brain conditions.

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INTRODUCTION

Translational molecular studies consistently report lower expression of BDNF in postmortem brains in depression [1–3], bipolar disorder [4, 5], schizophrenia [6–8], Alzheimer's disease [9] and other age-related disorders [10, 11]. BDNF plays critical roles in the nervous system, including synaptogenesis, neurotransmission and cognition [12–14]. Animal models with reduced BDNF expression or activity show disturbances of neurotransmission or neuroplasticity, and associated cognitive deficits [14–16], consistent with a dimensional contribution of this pathway to cognitive symptoms across brain conditions and disorders.

BDNF primarily signals through binding to the TrkB receptor and its co-receptor, p75/NTR, leading to phosphorylation and activation of downstream signaling molecules [12]. BDNF signaling is regulated through ubiquitination of TrkB and p75/NTR by TRAF6, an E3 ligase, recruiting the ubiquitin-binding adaptor protein, sequestosome-1/p62, to form a protein complex (TrkB/p75/TRAF6/Ubi/p62), which is then trafficked to appropriate cellular compartments (e.g., the proteasome or autophagosome/lysosome for degradation, the endosome for internalization or recycling) to regulate BDNF signaling [17]. Consistently, recent studies reported that TrkB is located to the autophagosome and mediates retrograde transport of this organelle in neurons [18], and that BDNF modulates neuronal autophagy [19–21].

Autophagy is a specialized membrane trafficking machinery responsible for degrading damaged proteins and organelles in the lysosome [22]. Recent evidence shows new roles for neuronal autophagy in higher-order brain functions [23], particularly in cognitive processes, via regulation of synaptic components, including GABA-A receptors ($GABA_AR$) [24]. Importantly, the cellular autophagic activity determines p62 protein levels [25], and p62 interacts with $GABA_AR$-associated protein (GABARAP) [26], an adaptor protein implicated in endocytic trafficking of $GABA_AR$ [27]. Together the evidence suggests the possibility that BDNF may control $GABA_AR$ trafficking via autophagic regulation of p62.

While some studies showed pro-autophagic role of BDNF [18, 19], others reported inhibitory effects of BDNF on autophagy under nutrient-starved conditions [20, 21], suggesting that BDNF may affect neuronal autophagy in a physiological context-dependent manner. In streptozotocin-induced diabetes model rats, BDNF-TrkB signaling promotes autophagy in hippocampus [19]. In the absence of essential nutrients, BDNF promotes autophagosome formation but limits autophagic degradation through activation of the mammalian target of rapamycin pathway in cultured neurons [20]. In mice, prolonged fasting (~48h) upregulates BDNF expression, causing autophagy induction in several brain regions, including cortex, hippocampus and hypothalamus up to three months of age [21]. However, in older mice (>3 months of age), the effect of fasting-induced BDNF

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upregulation gradually shifts toward the opposite direction in the cortex and hippocampus, starting to cause autophagy suppression [21]. By contrast, the role of BDNF in autophagy regulation under normal physiological conditions remains to be studied.

Dysfunctions of GABAergic inhibitory neurotransmission are also consistently demonstrated in depression [1, 28, 29], schizophrenia [30–32] and age-related cognitive decline [11, 33], suggesting a contribution of this inhibitory pathway to the pathophysiology of mental illnesses. Mouse–human translational studies show that GABAergic changes occur downstream of reduced BDNF signaling, specifically affecting dendritic-targeting GABAergic interneurons [1, 11]. Using human postmortem samples, we demonstrated a positive correlation between *BDNF* and GABAergic synaptic gene expression, and showed in mouse models that blockade of global or dendritic BDNF signaling in PFC causes reduced expression of GABAergic genes mediating dendritic inhibition [11, 34], providing a causal link between reduced BDNF and deregulated GABAergic neurotransmission. GABA elicits its inhibitory neurotransmission through pentameric $GABA_ARs$ containing multiple subunits with diverse cellular and functional properties, including somatically/perisomatically-targeted $GABA_ARs$ (e.g., α1-, α2-$GABA_AR$). Notably, the dendritically-targeted α5-subunit-containing $GABA_AR$ (α5-$GABA_AR$) uniquely contributes to cognitive functions [35].

Summing up the evidence: (1) BDNF signaling is reduced in brain disorders; (2) markers of GABAergic function are significantly decreased in brain disorders and in mice with reduced BDNF signaling; (3) dendritic BDNF transcripts and dendritically-localized α5-$GABA_AR$ are specifically affected in these conditions; (4) α5-$GABA_ARs$ contribute to cognitive processes; and (5) autophagy-related protein (p62) regulates BDNF/TrkB signaling and $GABA_AR$ trafficking. Accordingly, we tested the hypothesis that autophagy-related mechanisms operate downstream of BDNF, regulating GABAergic functions preferentially through α5-$GABA_AR$ to affect cognitive processes. We first investigated the co-occurrence of autophagy-related gene changes with BDNF and GABAergic markers in human depression, using postmortem gene expression profiles. We next investigated in cell-based, genetic and behavioral animal models a putative causal mechanism by which BDNF controls cell surface presentation of α5-$GABA_AR$ via autophagic regulation of p62 expression levels, in turn affecting cognitive endophenotypes observed in $Bdnf^{+/-}$ mice [36, 37] and α5-$GABA_AR$-deficient mice [38, 39], namely, deficits in cognitive flexibility and sensorimotor gating.

MATERIALS AND METHODS

Human transcriptome analysis

Data from prior meta-analysis of altered gene expression in depression [40] were analyzed through differential expression summary statistics to rank the 10,621 genes from the upregulated gene with the lowest *p* value to the downregulated gene with the lowest *p* value. The area under the receiver operating curve statistics was used to test enrichment of the autophagy-related gene sets. The same datasets were also analyzed by a permutation-based ranking of gene changes approach (gene set enrichment analysis, GSEA) [41].

Animals

Animals were used in accordance with the NIH guidelines, and approved by Kyoto University and CAMH.

Additional details on transcriptome analysis, mouse lines and their use in molecular, cellular and behavioral characterization, and statistical analysis are described in Supplementary Information.

RESULTS

Human postmortem gene expression profiles suggest altered autophagy in depression

We first assessed the putative co-occurrence of deregulated autophagy with reduced BDNF and altered GABA signaling frequently observed in neuropsychiatric conditions [1, 11]. For this we re-analyzed genome-wide differential expression statistics from a meta-analysis of eight transcriptome datasets from corticolimbic areas in 51 depressed patients and 50 controls [40]. This study reported downregulation of BDNF, somatostatin (SST), a marker of dendritic targeting GABAergic neurons (Fig. 1A), and pre-synaptic GABAergic genes [40]. Autophagy-related genes were defined by gene ontology (GO) [42] and combined into "autophagy-enhancing" and "autophagy-attenuating" gene lists (Details in Tables S1, S2). Of the 95 genes included in the "autophagy-enhancing" gene list, one gene was significantly upregulated ($p < 0.05$), and eight were significantly downregulated ($p < 0.05$) in depression compared to controls (Table S1). By contrast, of the 38 "autophagy-attenuating" genes, the expression of 3 and 2 genes showed a significant increase and decrease, respectively, in depression compared to controls (Table S2). Notably, at the group level, an area-under-the-curve (AUC) analysis revealed a significant over-representation of upregulated autophagy-attenuating genes ($p < 0.01$; AUC = 0.60; 0.5 meaning no change) and a slight non-significant under-representation of downregulated autophagy-enhancing genes (AUC = 0.44) in depression (Fig. 1A). This result was confirmed using a complementary and permutation-based ranking of gene changes approach (gene-set enrichment analysis, GSEA) [41], showing no significant enrichment for autophagy-attenuating genes (Fig. 1B, red line), but a significant enrichment in downregulation for autophagy-enhancing genes compared to controls (Fig. 1B, green line, $p = 0.008$). Together these results suggest reduced autophagy at the transcriptome level in depression, co-occurring with reduced expression of BDNF and of GABAergic dendritic markers.

BDNF regulates autophagy in cortical neurons

To address the role of BDNF in autophagy under physiological conditions, we prepared cortical neurons from transgenic mice expressing GFP-LC3, a fluorescent marker for autophagosomes [43], and cultured them under normal nutrient conditions. BDNF treatment (30 min) caused a significant increase in fluorescence intensity and size of GFP-LC3-positive punctate structures (Fig. 2A), likely due to translocation or increased local concentration of the fluorescent proteins, because overall LC3 levels in culture are not changed during this assay period (Fig. 2B). This suggests either de novo autophagosome formation due to autophagy induction, or the accumulation of LC3 due to attenuated autophagic degradation.

To distinguish between these two possibilities, we performed autophagy flux assays [44] by treating the culture with BDNF in the presence or absence of bafilomycin A1 (BafA1), a vacuolar H^+-ATPase inhibitor that inhibits autophagic degradation. Previous autophagy flux assays were done either using a high dose of BafA1 (400 nM) causing neuronal cell death during the assay period [20] or by treating neurons with a low dose of BafA1 (1.5 nM) for the last 6 h of culture period while treating them with BDNF for 24 h [21], leaving the possibility for underestimating the degree of autophagic degradation by BDNF. To obviate this problem, the autophagy assay guideline recommends using BafA1 throughout assay periods [45]. We titrated the dose and duration of BafA1 treatment and found that cortical neurons remained viable (>90% survival as determined by trypan blue staining) after treatment with 20 nM BafA1 for 6 h; however, massive cell death (>70%) occurred by 8 h or even with a lower dose of BafA1 (1.5 nM) for 24 h due to BafA1 toxicity. We concluded that treating cortical neurons with 20 nM BafA1 for 6 h is the optimal condition for autophagy flux assays. During the 6 h assay period, we observed a BDNF-induced time-dependent increase in autophagy flux, i.e., the difference between the amount of membrane-bound LC3 (i.e., LC3-II) seen in the presence versus the absence of BafA1, which reflects the amount of LC3 degraded through an autophagy-dependent process within the lysosome (Fig. 2B).

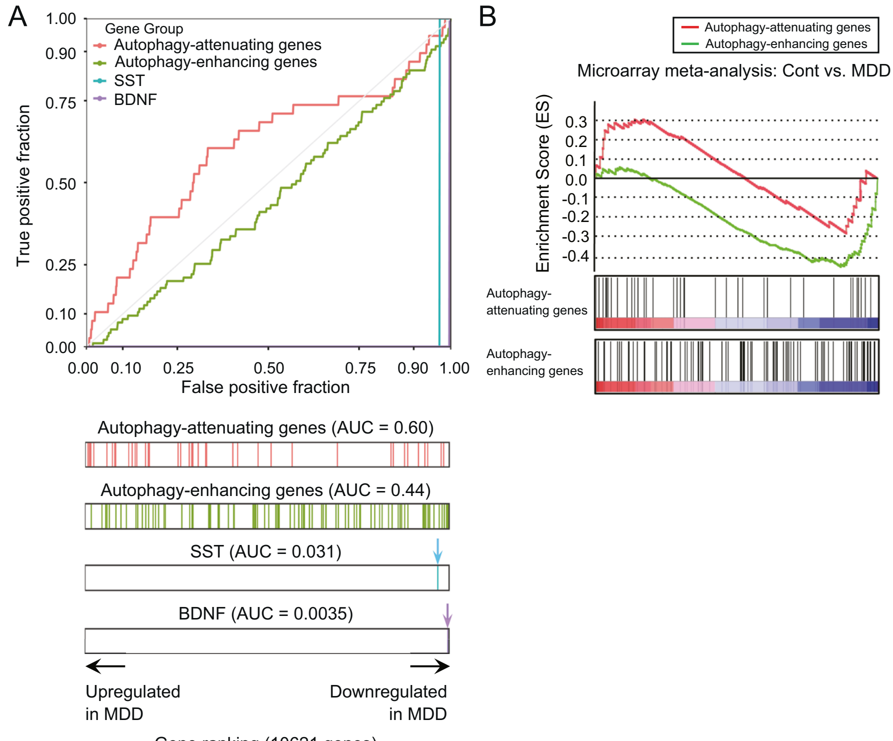


Fig. 1 Gene expression profiles in human postmortem brains suggest reduced autophagy in depression. A Upper panel: Expression levels of the autophagy-enhancing (95 genes, table S1) versus the autophagy-attenuating (38 genes, table S2) gene sets in corticolimbic areas from postmortem brains of depression were compared with those of the control cohorts. Overall expression levels of the autophagy-attenuating genes in depression, as represented by the area under the curve (AUC = 0.60), were significantly different from those of the autophagy-enhancing genes (AUC = 0.44) ($p < 0.01$). Lower panel: The autophagy-attenuating genes in depression clustered toward upregulated, whereas the autophagy-enhancing genes in depression clustered toward downregulated expression. As internal reference, the ranking of the *BDNF* and *Somatostatin* (*SST*) genes were plotted, both of which have been reported to be significantly downregulated in corticolimbic areas of depression subjects [40], as represented by the AUC values of 0.0035 and 0.031, respectively. **B** GSEA on gene expression profiles in depression versus control subjects obtained from meta-analysis of microarray datasets shows significant enrichment in downregulation for autophagy-enhancing genes (green line) ($p = 0.008$) but no significant enrichment for autophagy-attenuating genes (red line).

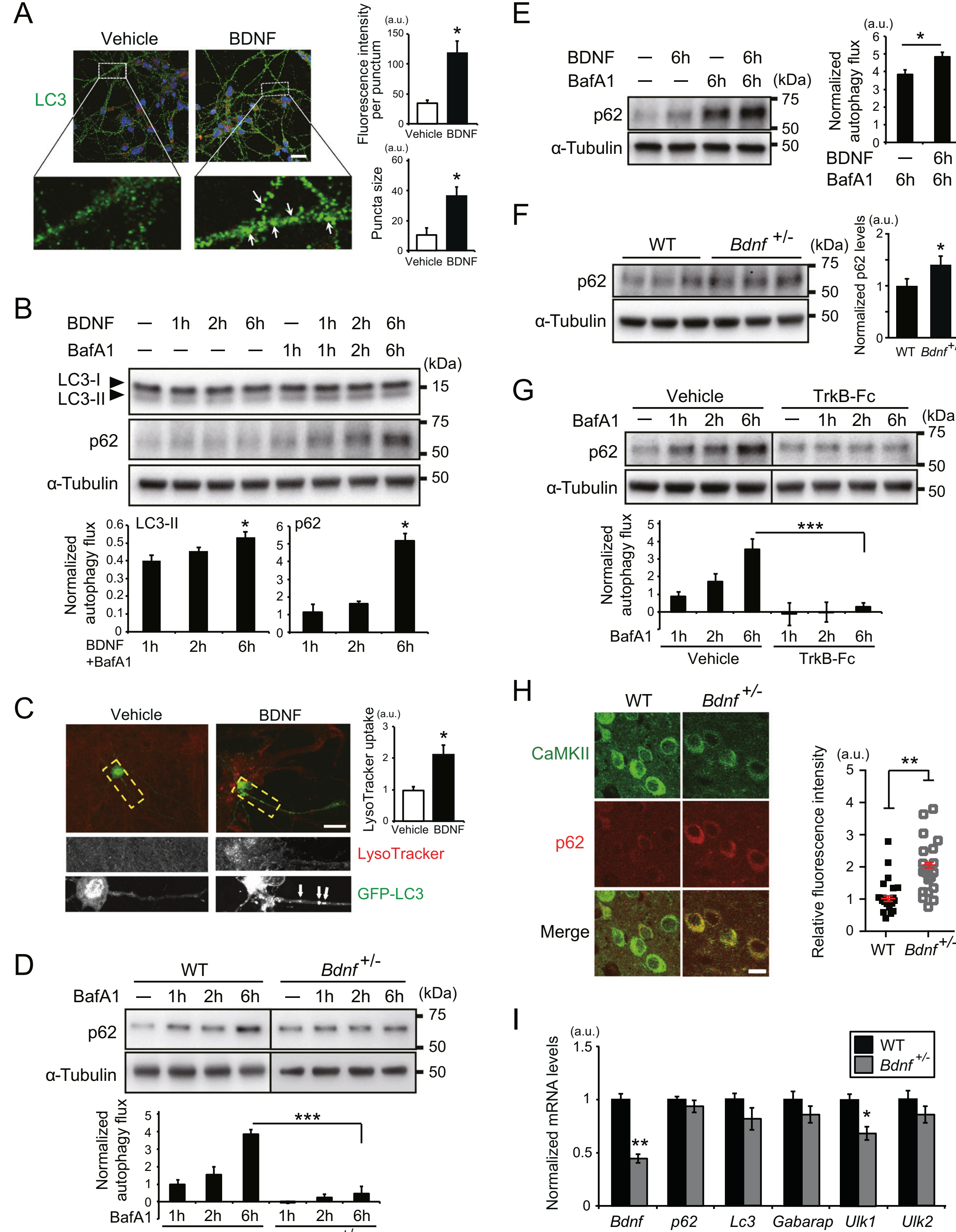
BDNF-induced autophagy flux evaluated by p62, the obligatory adaptor protein targeted for autophagic degradation [25], was similarly and significantly increased, reaching the autophagy flux index of 5.18 ± 0.37 at 6 h time point (Fig. 2B). The observed increase in autophagy flux concurrent to BDNF exposure is not attributable to transcriptional changes in *Lc3* or *p62* expression, as BDNF did not upregulate expression of these genes during our assay period (Fig. S1). This implies that the observed increase in LC3 or p62 protein levels was caused by BafA1-dependent inhibition of lysosomal degradation of these proteins and that continuous degradation of these proteins had occurred in the absence of BafA1 during the assay period.

We next tested whether BDNF could affect the later maturation stage of autophagy, where proteins and organelles are degraded in the acidophilic lysosome. This can be assessed by evaluating the acidity of the autophagosome/lysosome using LysoTracker [45]. To facilitate simultaneous observation of autophagosome formation and maturation, cortical neurons were transfected with GFP-LC3 and then labeled with LysoTracker after BDNF treatment. BDNF markedly increased the extent of LysoTracker uptake by the soma (Fig. 2C), indicating autophagosome maturation in neurons. Consistently, neurons with greater levels of LysoTracker uptake also exhibited an increase in number and size of autophagosomes located in neurites (Fig. 2C, arrows), suggesting a sequence of events elicited by BDNF, from autophagosome formation to maturation.

Together these data show that BDNF has an autophagy-enhancing activity in cultured cortical neurons under normal physiological conditions.

Reduced BDNF expression leads to elevated p62 levels in cortical pyramidal neurons

To address the endogenous BDNF activity in autophagy regulation, we performed the autophagy flux assay in cortical neurons of WT mice, compared to mice with reduced *Bdnf* levels (*Bdnf*$^{+/-}$ mice). In time-course experiments with BafA1, WT neurons showed a consistent increase in autophagy flux (i.e., the difference between the amount of p62 seen in the presence vs. the absence of BafA1), reaching the autophagy flux index of 3.98 ± 0.19 at 6 h time point, whereas *Bdnf*$^{+/-}$ neurons showed significantly lower levels of the flux (0.48 ± 0.41 at 6h, $p < 0.001$ vs. WT, 6 h) (Fig. 2D), suggesting that higher endogenous BDNF expression contributed to greater levels of p62 degradation. Notably, the autophagy flux in the presence of exogenous BDNF (4.81 ± 0.20) was significantly higher than the flux without exogenous BDNF (3.90 ± 0.25) ($p = 0.031$), implying that exogenous BDNF has the ability to augment autophagy and contributes to greater levels of p62 degradation in the absence of BafA1 in WT cortical neurons (Fig. 2E). In agreement with higher autophagy flux in WT, the steady-state levels of p62 normalized to α-Tubulin were significantly lower in WT than in *Bdnf*$^{+/-}$ neurons (Fig. 2F). In addition, perturbation of BDNF signaling using TrkB-Fc, the TrkB ectodomain fused with the constant region of IgG, confirmed the role of endogenous BDNF in upregulating autophagy flux in culture (flux index: 3.45 ± 0.47 vs. 0.15 ± 0.11 for control vs. TrkB-Fc at 6h, respectively, $p < 0.001$, Fig. 2G). Furthermore, TrkB-Fc-treated neurons showed significantly lower levels of lysosomal activity than control neurons, as measured by LysoTracker uptake assays (Fig. S2),



demonstrating that endogenous BDNF promotes autophagic maturation in neurons.

To further address the endogenous BDNF activity in autophagy regulation in vivo, we quantitated p62 protein levels in the medial prefrontal cortex (mPFC) of $Bdnf^{+/-}$ and WT mice. In mPFC, BDNF is predominantly produced by CaMKII-positive pyramidal neurons and functions as an autocrine and paracrine factor to modulate the activity of neighboring excitatory and inhibitory neurons [46]. The results show a significant increase in p62 protein levels in CaMKII-positive neurons of $Bdnf^{+/-}$ compared to WT mice (Fig. 2H), consistent with findings in culture (Fig. 2F). This increase in protein level was not paralleled by an increase in *p62* transcriptional

Fig. 2 BDNF regulates autophagy and p62 protein levels in cortical pyramidal neurons. A Primary cortical neurons prepared from GFP-LC3 mice and cultured for 16 days in vitro (DIV) were treated with BDNF (100 ng/ml) or vehicle for 30 min, and the fluorescence intensities of GFP$^+$ autophagosomes and their sizes were scored from >50 GFP$^+$ puncta per condition (a.u., arbitrary unit). Scale bar, 20 µm. *$p < 0.05$ (Student's *t* test). **B** Autophagy flux assay: wild-type (WT) primary cortical neurons (14–16DIV) were treated with BDNF (100 ng/ml) for the indicated times in the presence or absence of bafilomycin A1 (BafA1, 20 nM), and the cell lysates were analyzed by Western blot using LC3 and p62 antibodies. The autophagy flux (the difference of LC3-II (or p62) in the presence vs. the absence of BafA1 at a given time point) was normalized to the steady-state level of LC3-II (or p62) measured without BDNF and BafA1 (lane 1) to allow cross-experimental comparisons. All autophagy flux assays were done in triplicate. *$p < 0.05$ (vs. 1h, Kruskal–Wallis test). **C** WT primary cortical neurons were transfected with GFP-LC3 at 14DIV and treated with BDNF (100 ng/ml) for 30 min at 16DIV. LysoTracker was included in culture media for the last 5 min of culture period immediately before microscopic observation of epifluorescence in culture. LysoTracker fluorescence intensities per some were scored. Scale bar, 20 µm. The dotted line areas were magnified with separate LysoTracker and LC3 signals in the two lower panels. Arrows indicate GFP$^+$ autophagosomes located in the neurite. *$p < 0.05$ (Student's *t* test). **D** WT or *Bdnf*$^{+/-}$ primary cortical neurons (16DIV) were incubated in the presence or absence of BafA1 for the indicated times and the autophagy flux was analyzed by Western blot using p62 antibody. ***$p < 0.001$ (Kruskal–Wallis test). **E** WT primary cortical neurons (14–16DIV) were treated with or without BDNF (100 ng/ml) or BafA1 for 6h, and the autophagy flux assays were done ($N = 6$) using p62 antibody. *$p < 0.05$ (Mann–Whitney *U* test). **F** WT or *Bdnf*$^{+/-}$ primary cortical neurons (16DIV) were analyzed by Western blot using p62 antibody. *$p < 0.05$ (Student's *t* test). **G** WT primary cortical neurons (16DIV) were treated with TrkB-Fc (1 µg/ml) in the presence or absence of BafA1 for the indicated times and the autophagy flux was analyzed by Western blot using p62 antibody. ***$p < 0.001$ (Kruskal–Wallis test). **H** The medial PFC (layer 2/3) of *Bdnf*$^{+/-}$ mice and their WT littermates (2–2.5 months of age, $N = 4$ per genotype) were immunostained with p62 and CaMKII antibodies, and p62 fluorescence intensities in CaMKII$^+$ neurons were scored and plotted in the graph. Scale bar, 10 µm. **$p < 0.01$ (Kruskal–Wallis test). **I** Quantitative PCR analysis of autophagy-related genes expressed in PFC of WT and *Bdnf*$^{+/-}$ mice (2 months of age, $N = 3$ per genotype). *$p < 0.05$, **$p < 0.01$ (Student's *t* test).

activity (Fig. 2I), implying it may result from reduced protein degradation rates. To obtain further evidence for reduced autophagy in *Bdnf*$^{+/-}$ mice, we quantitated expression levels of additional genes in this pathway. Expression of several autophagy genes (e.g., *Lc3*, *Gabarap*, *Ulk2*) remained unchanged, whereas expression of *Ulk1*, a gene critical to autophagy induction [22], was significantly downregulated by ~25% (Fig. 2I). Together the data demonstrate a constitutive role for BDNF in enhancing autophagy in PFC. Unlike previous reports showing negative impacts of BDNF on autophagy under nutrient-starved conditions, where elevated BDNF and subsequent reduction in core autophagy machinery genes were observed [21], our current results are in line with other studies [18, 19] showing pro-autophagic roles of BDNF under conditions relevant to neuropsychiatric conditions (i.e., reduced BDNF, nearly intact levels of core autophagy machinery genes; Fig. 2I; Fig. S1).

Elevated p62 expression in *Bdnf*$^{+/-}$ cortical neurons causes downregulated surface presentation of α5-GABA$_A$R

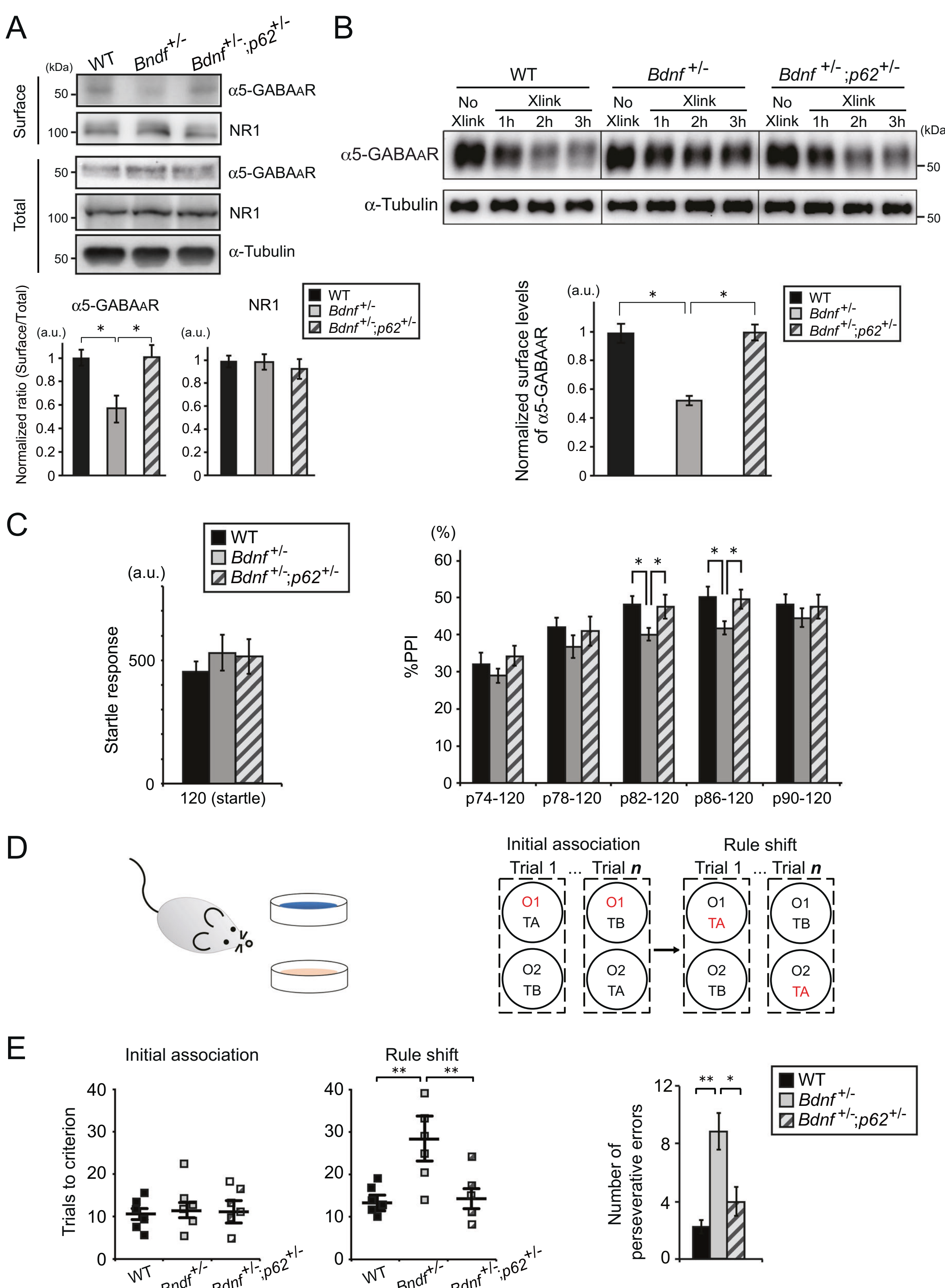
We recently reported that attenuated autophagy causes downregulation of surface expression of GABA$_A$R through sequestration of GABARAP by elevated p62 protein levels [24]. Besides, we previously showed that reduced BDNF signaling in PFC causes decreased expression of GABA synaptic genes, most notably α5-GABA$_A$R [11], a GABA$_A$R subtype predominantly expressed in pyramidal neuron dendrites [47]. These results suggest that BDNF affects GABA neurotransmission through regulation of α5-GABA$_A$R levels in pyramidal neuron dendrites, consistent with *Bdnf*$^{+/-}$ mice exhibiting reduced amplitude and frequency of inhibitory miniature currents (mIPSC) in cortical and thalamic neurons [48]. We therefore reasoned that elevated p62 protein levels in *Bdnf*$^{+/-}$ neurons may influence the surface presentation of α5-GABA$_A$R.

Surface biotinylation of cultured cortical neurons showed reduced cell surface α5-GABA$_A$R protein levels in *Bdnf*$^{+/-}$ neurons, as compared with WT neurons, with no significant changes in total levels of α5-GABA$_A$R, or in both total and surface levels of the glutamate receptor NR1 (Fig. 3A). Notably, reducing the *p62* gene dosage in *Bdnf*$^{+/-}$ neurons, using cortical neurons from *Bdnf*$^{+/-}$;*p62*$^{+/-}$ mice, restored α5-GABA$_A$R surface levels to WT levels (Fig. 3A), suggesting that p62 is a critical adaptor mediating BDNF-induced altered surface presentation (i.e., trafficking) of α5-GABA$_A$R. Gene expression analysis in animal models used (WT, *Bdnf*$^{+/-}$, *p62*$^{+/-}$, *Bdnf*$^{+/-}$;*p62*$^{+/-}$) showed expected changes in *Bdnf* and *p62* gene expression, confirming the validity of the models for use in subsequent analysis (Fig. S3).

To validate this finding in vivo, we performed receptor cross-linking assays using bis (sulfosuccinimidyl) suberate (BS3), a membrane-impermeable chemical cross-linker [49]. As the cross-linking reaction proceeds in the presence of BS3, only the fraction of receptors expressed on the plasma membrane surface are covalently cross-linked with anonymous cell surface proteins, thereby transforming into higher molecular weight species, while the rest of the receptors associated with the endomembrane remains intact, maintaining their original molecular weight. The PFC from WT and *Bdnf*$^{+/-}$ mice were subjected to the cross-linking reaction for 3 h and the levels of a series of GABA$_A$R subunits (α1, α2, α5) were measured as a function of time. The cross-linking reaction reached a plateau in 3 h, allowing us to calculate the surface receptor levels by subtracting the intact, non-crosslinked receptor levels measured in the presence of BS3 at 3 h time point from the total receptor levels measured without BS3. The results showed equivalent surface levels of α1- or α2-GABA$_A$R between WT and *Bdnf*$^{+/-}$ mice (Fig. S4), but a significant decrease in α5-GABA$_A$R surface levels in *Bdnf*$^{+/-}$ mice; specifically ~53% of α5-GABA$_A$R were estimated to be expressed on the cell surface in WT PFC (Fig. 3B, left), whereas a lesser extent (~25%) of α5-GABA$_A$R were presented on the cell surface under conditions of reduced *Bdnf* levels (Fig. 3B, middle). In contrast, ~56% of α5-GABA$_A$R were observed on the cell surface in the PFC of *Bdnf*$^{+/-}$ in which p62 levels were genetically reduced (*Bdnf*$^{+/-}$; *p62*$^{+/-}$) (Fig. 3B, right), demonstrating that reducing the *p62* gene dosage restored the surface expression of α5-GABA$_A$R to a level equivalent to WT mice. Collectively, the data suggest that decreased BDNF expression results in specific reduction in surface presentation of α5-GABA$_A$R through elevated p62 expression in PFC.

Elevated p62 expression in *Bdnf*$^{+/-}$ mice mediates behavioral deficits relevant to PFC dysfunction

We then investigated the potential role of elevated p62 expression in PFC-relevant brain functions of *Bdnf*$^{+/-}$ mice, such as information processing and cognition. Previous studies reported that *Bdnf*$^{+/-}$ mice have reduced prepulse inhibition (PPI) of acoustic startle response [36], demonstrating a role of BDNF in sensorimotor gating function. This mechanism of filtering sensory information to render appropriate motor responses is partly regulated by the cortical circuitry involving PFC [50]. In agreement with previous studies, we confirmed normal startle response and reduced PPI levels in *Bdnf*$^{+/-}$ mice (Fig. 3C). We next show that reducing the *p62* gene dosage in *Bdnf*$^{+/-}$ mice (i.e., using *Bdnf*$^{+/-}$; *p62*$^{+/-}$ mice) rescued the PPI deficits back to levels observed in control WT mice (Fig. 3C).



These results suggest that decreased BDNF expression leads to PPI deficits through elevated p62 expression.

Bdnf$^{+/-}$ mice also exhibit reduced cognitive flexibility in a visual discrimination task [37]. Here we assessed cognitive flexibility in *Bdnf*$^{+/-}$ mice using a rule-shifting paradigm [51, 52], in which mice were initially trained to associate food reward with a specific stimulus (i.e., either an odor or a digging medium) and subsequently evaluated for cognitive flexibility by changing the type of stimulus that predicts the reward (Fig. 3D). *Bdnf*$^{+/-}$ and WT mice learned the association rule in a similar number of trials

Fig. 3 Decreased surface presentation of α5-GABA$_A$R and behavioral deficits in *Bdnf*$^{+/-}$ mice are rescued by reducing *p62* gene dosage. A Surface biotinylation of primary cortical neurons (16DIV) prepared from WT, *Bdnf*$^{+/-}$ or *Bdnf*$^{+/-}$; *p62*$^{+/-}$ mice, analyzed by Western blot using the indicated antibodies. Surface levels of expression were normalized by the total levels of expression for each genotype. Surface levels of α5-GABA$_A$R were significantly reduced in *Bdnf*$^{+/-}$ neurons compared to the other genotypes, whereas those of NR1 subunit of glutamate receptors were equivalent across the three genotypes. The assays were performed in triplicate. *$p < 0.05$ (Kruskal−Wallis test). **B** BS3 cross-linking assays using the PFC extracts from WT, *Bdnf*$^{+/-}$ or *Bdnf*$^{+/-}$;*p62*$^{+/-}$ mice (2–2.5 months of age, $N = 4$ per genotype). The cross-linking reaction reached a plateau in 2–3 h in our assay conditions. Levels of non-crosslinked α5-GABA$_A$R (~50 kDa) were normalized to levels of α-Tubulin at each time point. Differences in the levels of non-crosslinked α5-GABA$_A$R at a given time point versus those of the control sample (No Xlink: no cross-linker added) represent the amount of surface α5-GABA$_A$R that underwent mobility shift toward a higher molecular weight range due to covalent cross-linking with anonymous cell surface proteins. The calculated surface α5-GABA$_A$R levels (= levels at No Xlink – levels at 3 h Xlink) in *Bdnf*$^{+/-}$ mice were significantly reduced compared to the other genotypes. *$p < 0.05$ (Kruskal−Wallis test). See Fig. S4 for cross-linking assays for α1- and α2-GABA$_A$Rs. **C** The amplitude of startle response and the percentage of PPI were evaluated for WT, *Bdnf*$^{+/-}$ and *Bdnf*$^{+/-}$; *p62*$^{+/-}$ mice (2 months of age, $N = 10$ per genotype). No significant difference in startle response (left panel; $F_{2,27} = 0.378$, $P = 0.9243$, one-way ANOVA). PPI was reduced in *Bdnf*$^{+/-}$ mice compared to WT mice, and rescued to control levels in *Bdnf*$^{+/-}$; *p62*$^{+/-}$ mice for the prepulse–pulse pair of trials (p82–120 and p86–120 dB) (right panel; $F_{2,27} = 9.775$, $P < 0.001$, two-way ANOVA with repeated measures; *$p < 0.05$, Bonferroni post-hoc test). **D** Schematic diagram of the rule shift assay: Mice were habituated to food, feeding apparatus, different odor cues (O1, O2, etc.; e.g., coriander vs. garlic powder) and texture cues (TA, TB, etc.; e.g., fine versus coarse digging media) prior to testing, and then food-deprived a day before the assays. Mice were initially trained in a sequence of trials to associate a food reward with a specific stimulus (i.e., either an odor or a digging medium; a stimulus associated with food reward is shown in red). A varying combination of stimulus and food reward was presented to mice per trial. Eight consecutive correct responses to the food reward were considered reaching criterion (i.e., successful establishment of association between the stimulus and the food reward), and the number of trials to reach criterion were scored for each mouse tested, before and after rule shifting (e.g., from an odor cue to a different texture cue to predict reward). **E** Numbers of trials to criterion were scored for WT, *Bdnf*$^{+/-}$ and *Bdnf*$^{+/-}$; *p62*$^{+/-}$ mice (2–2.5 months of age, $N = 6$ per genotype) during the initial association phase, as well as the rule shift phase of the assays. No significant difference during the initial association phase ($F_{2,15} = 1.25$, $P = 0.934$). Statistical significance during the rule shift phase: $F_{2,15} = 9.93$, $P < 0.001$ (one-way ANOVA); **$p < 0.01$ (Bonferroni post-hoc test). During the rule shift phase, *Bdnf*$^{+/-}$ mice made a greater number of perseverative errors than the other genotypes. *$p < 0.05$, **$p < 0.01$ (Kruskal–Wallis test).

during the initial association phase of trials; however, *Bdnf*$^{+/-}$ mice required significantly higher numbers of trials to shift their behavior during the rule-shifting phase of trials (Fig. 3E). Reducing the *p62* gene dosage in *Bdnf*$^{+/-}$ mice (i.e., in *Bdnf*$^{+/-}$; *p62*$^{+/-}$) restored cognitive performance to control levels (Fig. 3E), together suggesting that decreased BDNF expression results in cognitive deficits through elevated p62 expression.

Elevated p62 expression is sufficient to cause downregulation of surface α5-GABA$_A$R expression and behavioral deficits

To further address the causal role of elevated p62 expression in the regulation of α5-GABA$_A$R surface expression and the associated behavioral changes, we generated p62-transgenic (Tg) mice, in which p62 transgene expression was driven by the CaMKII promoter. Among three Tg lines established, two lines (#1, #3) showed ~60% increase in p62 protein expression in PFC, while one line (#2) failed to overexpress p62, as evaluated by Western blot (Fig. 4A). BS3 cross-linking assays demonstrated reduced surface α5-GABA$_A$R levels in the PFC of p62-overexpressing Tg line (#1) compared to WT, whereas the non-overexpressing line #2 displayed surface α5-GABA$_A$R expression equivalent to WT levels (Fig. 4B).

We next evaluated sensorimotor gating in the p62-Tg mice. p62-Tg lines (#1, #3) exhibited reduced PPI levels, whereas the non-overexpressing line #2 had PPI levels equivalent to WT levels (Fig. 4C). Similarly, in the cognitive flexibility test, mice from the overexpressing p62-Tg line (#1, #3) learned the association rule during the initial association phase of trials in a similar manner as WT, but were impaired during the rule-shifting phase (Fig. 4D).

Together these data demonstrated that elevated p62 levels in CaMKII$^+$ pyramidal neurons in the corticolimbic areas are sufficient to replicate the molecular (reduced surface α5-GABA$_A$R levels) and behavioral phenotypes (deficits in PPI and cognitive flexibility) of *Bdnf*$^{+/-}$ mice.

DISCUSSION

The current study demonstrates a novel mechanism by which (1) BDNF regulates autophagy in PFC pyramidal neurons under normal and neuropsychiatric-related conditions, and (2) reduced BDNF signaling negatively impacts GABA functions via autophagy-related control of GABA$_A$R trafficking, and show that (3) these changes underlie behavioral manifestations relevant to PFC-mediated symptoms of psychiatric disorders. Specifically, controlling levels of p62, a molecule implicated in autophagic regulation of cellular function, serves as a key molecular event linking BDNF signaling, GABAergic neurotransmission, and specific behavioral manifestations related to PFC functions.

Reduced BDNF expression and deregulated GABA transmission frequently co-occur with psychiatric disorders, including depression, schizophrenia and during aging [1, 11, 30], suggesting a shared biological mechanism across these conditions. We previously demonstrated that reduced activity of BDNF, predominantly produced by pyramidal neurons, leads to reduced expression of presynaptic genes (e.g., *Gad1*, *SLC32A1*) and neuropeptide genes (e.g., *SST*, *neuropeptide Y*, *cortistatin*) expressed in neighboring GABAergic inhibitory neurons targeting pyramidal neuron dendrites [11], suggesting a paracrine mode of BDNF action responsible for attenuated GABA signaling (Fig. 5, right). Moreover, expression levels of *Gabra5*, a gene encoding α5-GABA$_A$R that is predominantly localized to the pyramidal neuron dendrites, were among the most significantly downregulated, suggesting an autocrine mode of BDNF action responsible for attenuated GABA signaling. Both modes of transcriptional mechanisms, coupled with the attenuated GABA$_A$R trafficking through the autophagy regulator (this study, Fig. 5, left), are expected to synergistically downregulate GABA neurotransmission across pre- and post-synaptic compartments, leading to altered neuroplasticity or excitation-inhibition balance in PFC. Mechanistically, reduced BDNF causes decreased autophagy, leading to p62 protein accumulation and reduced surface expression of α5-GABA$_A$R, at least in part through p62-mediated sequestration of GABARAP, a molecule responsible for trafficking and surface presentation of GABA$_A$R [24].

In the current study, we demonstrated a role for BDNF in enhancing autophagy under normal nutrient conditions in culture or in vivo using mice with reduced BDNF function (*Bdnf*$^{+/-}$ mice) to model the widespread lower BDNF pathobiological signature of neuropsychiatric conditions [1–11]. We focused on earlier stages (2–3 months of age) for analysis in mice, in order to be consistent

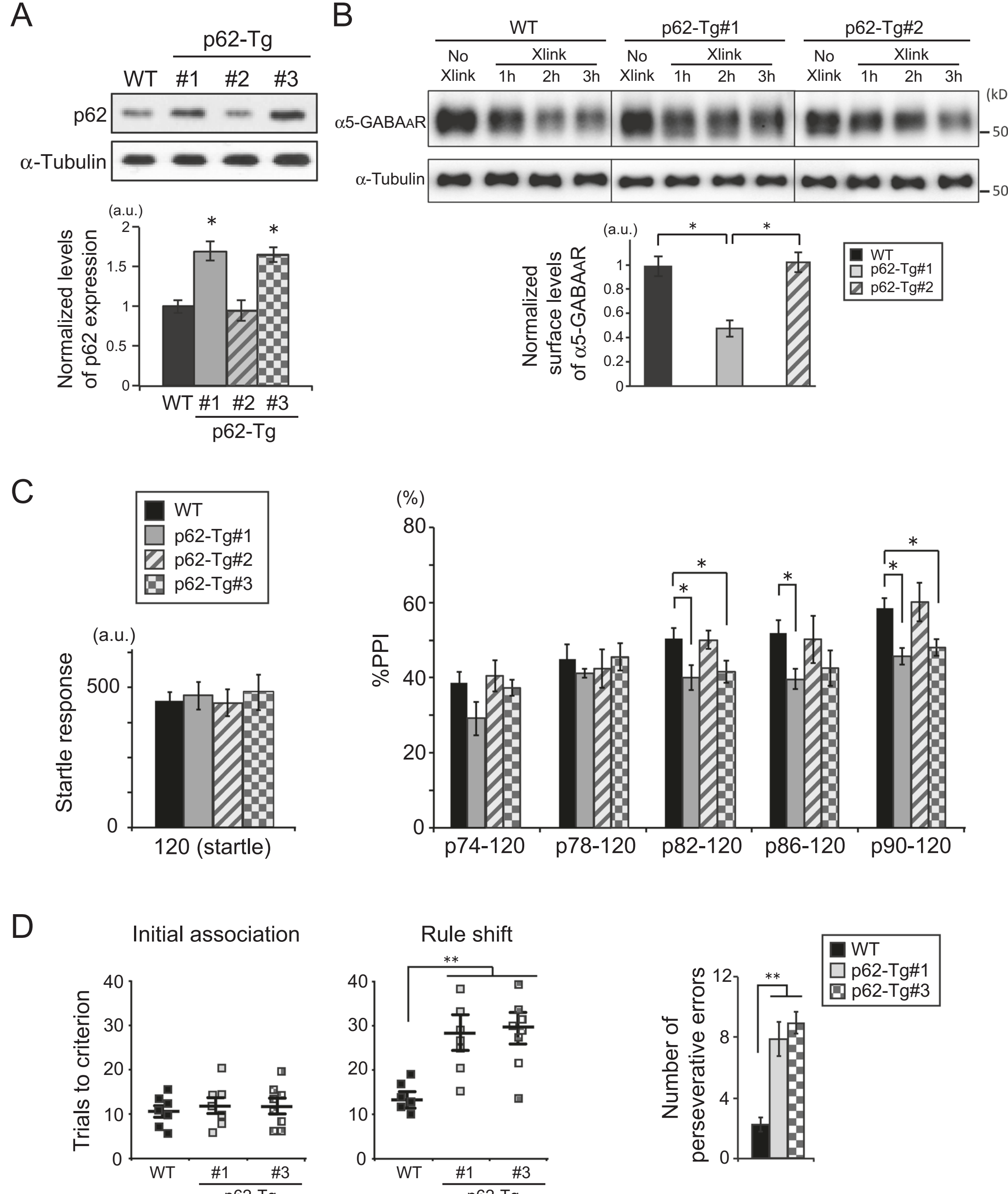


Fig. 4 Elevated p62 expression is sufficient to cause downregulation of surface α5-GABA$_A$R expression and behavioral deficits. **A** Generation of CaMKII-p62-transgenic mouse lines. Among three lines established, two lines (#1, #3) showed ~60% higher levels of p62 protein expression in thePFC when compared with WT, whereas the line #2 showed no apparent increase in p62 expression. $*p < 0.05$ (Kruskal–Wallis test). **B** BS3 cross-linking assays using the PFC extracts from WT, p62-Tg#1 and Tg#2 mice (2–2.5 months of age, $N = 4$ per genotype). Levels of non-crosslinked α5-GABA$_A$R (~50 kDa) were normalized to levels of α-Tubulin at each time point. Calculated surface α5-GABA$_A$R levels (= levels at No Xlink – levels at 3 h Xlink) in p62-Tg#1 mice were significantly reduced compared to the other genotypes. $*p < 0.05$ (Kruskal–Wallis test). **C** The amplitude of startle response and the percentage of PPI were evaluated for WT, p62-Tg#1, p62-Tg#2 and p62-Tg#3 mice (2 months of age, $N = 10$ per genotype). No significant difference in startle response ($F_{3,36} = 0.726$, $P = 0.7121$, one-way ANOVA). Statistical significance for %PPI; $F_{3,36} = 12.474$, $P < 0.001$ (two-way ANOVA with repeated measures); $*p < 0.05$ (Bonferroni post-hoc test). **D** Numbers of trials to criterion were scored for WT ($N = 7$), p62-Tg#1 ($N = 7$) and p62-Tg#3 ($N = 8$) mice (2–2.5 months of age) during the initial association phase, as well as the rule shift phase of the assays. No significant difference during the initial association phase ($F_{2,19} = 1.968$, $P = 0.936$). Statistical significance during the rule shift phase: $F_{2,19} = 12.56$, $P < 0.001$ (one-way ANOVA); $**p < 0.01$ (Bonferroni post-hoc test). During the rule shift phase, mice from p62-Tg lines #1 and 3 made a greater number of perseverative errors than WT. $**p < 0.01$ (Kruskal–Wallis test).

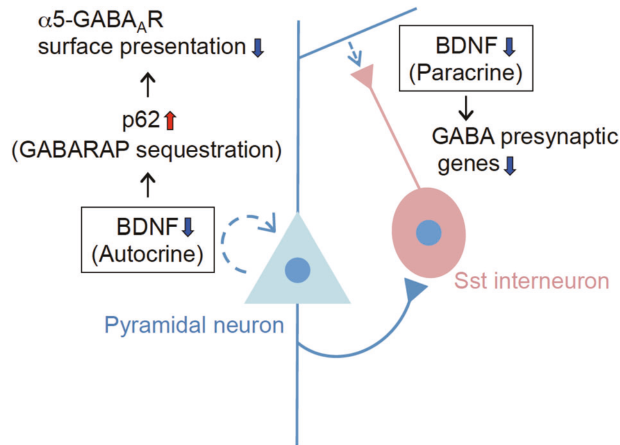


Fig. 5 Two modes of mechanisms underlying GABAergic dysfunction following reduced BDNF signaling. Reduced BDNF expression or signaling in cortical pyramidal neurons, due to chronic stress or other neuropsychiatric insults, leads to GABA dysfunction through transcriptional suppression of GABA synapse genes in neighboring inhibitory neurons (paracrine mode) [11, 34] and also via reduced surface presentation of α5-GABA$_A$R in pyramidal neurons (autocrine mode), as demonstrated in this study, together contributing to cognitive and other behavioral deficits relevant to neuropsychiatric disorders, including depression and schizophrenia.

with previous literature [36, 37], which used this age range of BDNF mutant mice to establish the psychopathological phenotypes that we also assessed in our study (PPI deficits, cognitive inflexibility), allowing direct comparisons. While our findings are in line with previous reports showing pro-autophagic roles of BDNF [18, 19], several previous studies showed negative impacts of BDNF on autophagy flux in hippocampal neurons under nutrient-starved conditions [20], or in older mice (>3 months) when the effect of fasting-induced BDNF on autophagy converts from activation to suppression [21]. Curiously, expression of the core autophagy machinery proteins (e.g., LC3) were significantly downregulated by ~90% in hippocampal neurons after 24 h of BDNF treatment or in cortex after 24 h of fasting, resulting in diminished autophagy flux [21]. These changes in physiological condition (i.e., elevated BDNF, loss of core autophagy machinery proteins) appear to be uniquely associated with nutrient starvation, and are not relevant to neuropsychiatric conditions in our models (i.e., reduced BDNF in *Bdnf*$^{+/-}$ mice; nearly intact core autophagy machinery protein levels in cortical neurons during 24 h treatment with BDNF). Together these data support the need for evaluating the role of BDNF in autophagy in a context-dependent and experimental condition-specific manner.

There are several limitations to this study. First, given the multiple roles and binding partners of p62 adaptor protein, it is unlikely that α5-GABA$_A$R is the only receptor system or cellular target affected by elevated p62 levels. Additional cellular machineries may further contribute to the behavioral deficits in *Bdnf*$^{+/-}$ mice, despite that we found here and in our prior transcriptomic studies [11] that α5-GABA$_A$Rs are preferentially affected, as opposed to (peri)somatically-localized α1-/α2-GABA$_A$Rs. Second, although we show that increasing p62 levels in pyramidal neurons was sufficient to cause reduced surface α5-GABA$_A$R levels and others previously showed that reduced surface α5-GABA$_A$R or defective α5-GABA$_A$R signaling caused deficits in cognitive flexibility and sensorimotor gating [38, 39], a direct causal link of reduced GABAergic neurotransmission or GABA$_A$R trafficking to the observed behavioral deficits remains to be tested in our model. In addition, we do not rule out the possibility of altered levels of p62 or relevant signaling components (e.g., TrkB) in other cell types in *Bdnf*$^{+/-}$ mice, which may indirectly affect α5-GABA$_A$R system in pyramidal neurons. Cell type-specific manipulation of these components will further address the causal link of p62 and GABA$_A$R functions. Third, multiple adaptor proteins, including gephyrin, are reported to regulate GABA$_A$R trafficking downstream of BDNF [53, 54]. Future studies will need to integrate these regulatory mechanisms into the p62-dependent mechanism shown here for more complete understanding of GABA$_A$R trafficking, as well as functional outcomes at the electrophysiological levels. Finally, the current studies were performed in male mice and comparative analyses in female mice are warranted.

Given the critical role of p62 in regulating α5-GABA$_A$R trafficking and behavioral outcomes in *Bdnf*$^{+/-}$ mice, we propose that p62, BDNF and α5-GABA$_A$R function in concert to regulate cognitive processes under normal and pathophysiological conditions. p62 protein levels typically increase with age, reflecting a gradual decrease in cellular autophagic activity [55], and elevated p62 levels or increased p62$^+$ inclusions are cardinal features of age-related neurological disorders [56, 57]. Furthermore, we recently reported upregulated p62 protein expression in cultured neurons isolated from subjects with schizophrenia and bipolar disorder [24], and in brains of a mouse model of schizophrenia [58]. Hence, controlling the p62 protein levels may provide a potential target for therapeutic intervention against symptoms shared across these disorders, such as cognitive impairment, through augmenting inhibitory neurotransmission. Notably, cognitive impairment is among symptoms most difficult to treat and frequently persists during remission in depression [59]. Because surface availability of GABA$_A$R represents a rate-limiting step for GABAergic neurotransmission, either regulating this step through p62 dosage control or augmenting GABA$_A$R activity (e.g., via α5-GABA$_A$R positive allosteric modulation [35]) may provide an alternative therapeutic approach, for instance for depression subjects who do not respond to current antidepressant treatment, or for targeting cognitive deficits during remission of depression, across brain disorders and during aging.

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AUTHOR CONTRIBUTIONS

TT and ES conceived the studies; TT, AS and YH-T carried out experiments; HM generated CaMKII-p62 transgenic mice; HO provided analytical tools; RS and LF analyzed gene expression profiles; TT and ES wrote and edited the paper.

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COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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