Gephyrin plays a key role in BDNF-dependent regulation of amygdala surface GABA$_A$Rs

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Abstract

Brain–derived neurotrophic factor (BDNF) is critically involved in synaptic plasticity and neurotransmission. Our lab has previously found that BDNF activation of TrkB is required for fear memory formation and that GABA$_A$ receptor subunits and the GABA$_A$ clustering protein gephyrin are dynamically regulated during fear memory consolidation. We hypothesize that TrkB-dependent internalization of GABA$_A$ receptors may partially underlie a transient period of amygdala hyperactivation during fear memory consolidation. We have previously reported that BDNF modulates GABA$_A$ receptor α1 subunit sequestration in cultured hippocampal and amygdala neurons by differential phosphorylation pathways. At present, no studies have investigated the regulation of gephyrin and GABA$_A$ receptor α1 subunits following BDNF activation in amygdala. In this study, we confirm the association of GABA$_A$ receptor α1 and γ2 subunits with gephyrin on mouse amygdala neurons by coimmunoprecipitation and immunocytochemistry. We then demonstrate that rapid BDNF treatment, as well as suppression of gephyrin protein levels on amygdala neurons, induced sequestration of surface α1 subunits. Further, we find that rapid exposure of BDNF to primary amygdala cultures produced decreases in gephyrin levels, whereas longer exposure resulted in an eventual increase. While total α1 subunit levels remained unchanged, gephyrin was downregulated in whole cell homogenates, but enhanced in complexes with GABA$_A$ receptors. Our data with anisomycin suggest that BDNF may rapidly induce gephyrin protein degradation, with subsequent gephyrin synthesis occurring. Together, these findings suggest that gephyrin may be a key factor in BDNF-dependent GABA$_A$ receptor regulation in amygdala. This work may inform future studies aimed at elucidating the pathways connecting BDNF, GABA$_A$ systems, gephyrin, and their role in underlying amygdala-dependent learning.

Keywords

Amygdala; Fear; GABA; Memory; Consolidation; Gephyrin

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Introduction

The activation of GABA_A receptors (GABA_ARs) mediates the majority of fast inhibitory neurotransmission in the CNS. These receptors are pentameric structures predominantly comprised of alpha (α) and beta (β) subunits, but must also contain either gamma (γ) or delta (δ) subunits. Among these combinations, at least 16 GABA_AR subtypes have been identified; the most abundant subtype in brain is composed of $\alpha 1 \beta 2 \gamma 2$ subunits, representing over half of all GABA_ARs (McKernan and Whiting, 1996; Gao and Fritschy, 1994; Sperk et al., 1997; Olsen and Sieghart, 2009). In some brain regions, including the amygdala, $\alpha 1$-containing subtypes (GABA_AR$\alpha 1$) are present on both pyramidal cells and parvalbumin-positive interneurons (Freund and Gulyas, 1997; McDonald and Mascagni, 2004; Muller et al., 2007). Such receptors play a role in both reinforcing and negative feedback as well as tonic inhibition, in addition to mediating the synchronized rhythmic activity of pyramidal cells important for proper functioning (Mann et al., 2005; Wu et al., 2012).

GABA_ARs undergo dynamic changes on the neuronal cell surface. Their trafficking to and from the synapse is regulated by activation of several cell-signaling pathways, which have profound effects on both GABA_AR function and the efficacy of GABA_AR-mediated synaptic inhibition. Past studies have demonstrated that intracellular signaling pathways activated by brain-derived neurotrophic factor (BDNF) influence GABAergic transmission. For example, Brunig et al. (2001) found a decrease in miniature inhibitory postsynaptic current (mIPSC) amplitude after a 5-minute application of BDNF in hippocampal neurons. In cerebellar granule cells, BDNF application induces the internalization of GABA_AR $\beta 2/3$ subunits and a depression of GABA-induced currents (Cheng and Yeh, 2003). Additionally, we have previously reported that BDNF application to cultured hippocampus and amygdala neurons induced the rapid internalization of GABA_AR $\alpha 1$ subunits (Mou et al., 2010). However, the mechanism by which GABA_ARs are regulated by BDNF signaling is unknown. The current literature suggests that BDNF-induced changes in GABAergic transmission may differ across brain regions and cell types (Jovanovic et al., 2004; Cheng and Yeh, 2005; Palma et al., 2005). Several factors could be underlying the variability reported across studies, including the type of neurons studied (Cheng and Yeh, 2005), duration of BDNF application (Henneberger et al., 2005), and the maturation of cells (Baldelli et al., 2002; Mizoguchi et al., 2003; Yamada et al., 2002). Yet the steps between BDNF induced TrkB activation and changes in GABA_AR function remain unclear.

Previous work in our lab has demonstrated that gephyrin, a clustering protein of GABA_AR, is dynamically regulated along with GABA_AR following emotional learning. For example, we have demonstrated that gephyrin protein levels and GABA_AR surface expression in the amygdala were decreased in parallel after fear acquisition (Chhatwal et al., 2005), and that fear conditioning is both BDNF- and TrkB-dependent (Rattiner et al., 2004a,b, 2005; Choi et al., 2010). In another study, we reported that gephyrin gene expression was significantly downregulated in the amygdala during consolidation, after fear acquisition (Ressler et al., 2002). We further showed that fear acquisition induced a down-regulation of mRNA markers related to GABAergic function within the amygdala, whereas fear extinction upregulated gephyrin (Heldt and Ressler, 2007). However, the mechanism of learning-dependent, rapid GABA_AR downregulation and alteration of gephyrin levels is unknown.

A growing literature suggests a role for gephyrin in the formation and/or stabilization of GABA_AR clusters. Gephyrin anti-sense oligonucleotides have been shown to destabilize postsynaptic GABA_AR clusters in treated neuronal cultures (Essrich et al., 1998). Cultured hippocampal neurons from gephyrin knockout mice failed to express clusters of GABA_ARs containing $\gamma 2$ and $\alpha 2$ subunits in one study (Kneussel et al., 1999). However, another study reported that GABA_AR $\alpha 2$ and $\gamma 2$ subunits did cluster at synapses in hippocampal cultures

from gephyrin knockout mice, indicating gephyrin-independent GABAergic synapses (Levi, et al., 2004). Additionally, the removal of gephyrin by gene targeting or RNA expression interference dramatically alters $GABA_A R$ clustering (Yu et al., 2007). More recently, $GABA_AR$ $\alpha1$ subunits have been shown to be directly associated with gephyrin at inhibitory synapses in cultured rat hippocampal neurons (Mukherjee et al., 2011). Importantly, inhibiting gephyrin expression significantly decreases the number of $GABA_AR$ clusters at the cell surface, while having no effect on the total number of surface $GABA_ARs$ expressed (Jacob, et al., 2005). However, to date there have been few studies examining the interactions between surface $GABA_ARs$ and gephyrin on amygdala neurons, particularly the $\alpha1$-containing $GABA_ARs$. In the present study, we investigated the role of gephyrin in the BDNF-mediated decrease of surface $GABA_AR\alpha1$ in cultured amygdala neurons.

Methods

Amygdala neuronal cell culture

All procedures involving animal use were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Primary cultures of postnatal amygdala neurons were performed as described previously (Mou et al., 2011). Briefly, C57BL/6J mice (postnatal 14 days) were decapitated, and the amygdala were punched from brain slides and immersed in ice-cold dissection buffer consisting of Hibernate-A medium (BrainBits, Springfield, IL, USA), B27 supplement (Invitrogen, Carlsbad, CA, USA), 2 mM Glutamax (Invitrogen), and gentamycin (Invitrogen) (12 μg/ml). Then the amygdala tissues were sliced and enzymatically digested with papain (Worthington, Lakewood, NJ, USA) in Hibernate-A medium at 32°C for 30 min. Cells were dissociated by triturating with pasteur pipettes. Neurons were purified in the density gradient media including Hibernate-A and OptiPrep (Sigma, St. Louis, MO, USA) by centrifugation. Neurons were then transferred into a new tube. After being washed with dissection buffer, neuronal cells were plated onto Poly-D-Lysine (Sigma) coated plates at the density of 2.5×10^5 cells/cm2 in culture media consisting of Neurobasal A medium (Invitrogen) with 2% B27 supplement, 2 mM glutamax and gentamycin (5 μg/ml). Thereafter, the cultures were kept in a humidified incubator at 37°C and 5% CO_2, and media were changed every 5 days until used for experiments. Cells were used for the experiments in this study after 2 weeks *in vitro*. The neuronal culture viability was tested by adding 4% Trypan Blue solution (Mediatech Inc., Herndon, VA, USA) onto cultures and >99% viability was assured before experiments.

BDNF peptide and antibodies

Recombinant human BDNF was purchased from Cell Sciences (Canton, MA, USA) and reconstituted in sterile PBS as 100mg/ml stock. The aliquots of stock were stored at −30°C and final concentration of application on neurons was 100ng/ml.

The solution of anisomycin (Sigma) was made in sterile water as a stock of 10mM and stored at −30°C in small aliquots. The final working concentration on neurons is 10μM.

The following antibodies were used in the described experiments: polyclonal rabbit antisera against $\alpha1$-$GABA_AR$ subunits (epitope region: N-terminus, Millipore, Temecula, CA, USA); polyclonal rabbit antisera against $\gamma2$-$GABA_AR$ subunits (Affinity BioReagents, Rockford, IL, USA); monoclonal mouse antibody against gephyrin (BD Transduction Laboratories, San Jose, CA, USA; Synaptic Systems, Goettingen, Germany); goat anti-rabbit IgG conjugated with Alexa Fluor 488 (Invitrogen); goat anti-rabbit IgG conjugated with Alexa Fluor 568 (Invitrogen); Donkey anti-mouse IgG conjugated with Alexa Fluor 568 (Invitrogen); peroxidase-conjugated horse anti-mouse secondary (Vector Laboratories,

Burlingame, CA, USA); peroxidase-conjugated goat anti-rabbit secondary (Vector Laboratories).

Neuronal Transfection

Before starting transfection, the culture media in cell chambers were changed with half fresh. 1μl of Lipofectamine2000 (Invitrogen) was diluted with 50μl culture media, and 0.5μg DNA (either pLV-siGephyrin, a gephyrin siRNA containing GFP, or pLV-siRNA control) diluted in another 50μl culture media. After 5 minutes of incubation at room temperature, the Lipofectamine and DNA mixtures were combined into one vial. Following 20 minutes of incubation at room temperature, the DNA-lipofectamine mixture in 100μl culture media was added into the cell chamber (area 1.8cm^2). After 3 hours in the cell incubator, the media in the cell chamber was changed to regular. The cells were used for experiments 48 hours post-transfection. The cells that received lipofectamine without DNA also served as controls for transfection and ICC experiments.

Immunocytochemistry and analysis of Immunofluorescence

Antibody feeding protocol—The surface GABA$_A$R α1 subunits were tagged in living cultured amygdala neurons with the primary antibody against α1-GABA$_A$R subunits. The tagged α1 subunits were allowed to undergo endocytosis at 37 °C before fixation of cells. First, half of the culture media was changed with fresh media, and the cultures were incubated with rabbit antibody against α1-GABA$_A$R subunits (diluted 1:200) for 30 min at 37°C. After washing three times with dissection buffer, culture media was returned to cells with half fresh media. Cells were then treated with BDNF for 10 minutes, 20 minutes, or 1 hour. Treatments were stopped by removing media and rinsing cells three times with dissection buffer. To label the surface α1 subunits, cells were then incubated with goat anti-rabbit IgG conjugated with Alexa Fluor 568 or Alexa Fluor 488 (1:2000) diluted in culture media for 20 min in incubator. Cells were then rinsed with ice-cold PBS on ice and fixed with methanol at −20°C for 20 min. After washing with PBS, cells were ready for microscopy or for further gephyrin staining.

Immunocytochemistry (ICC) of total GABA$_A$R α1 subunits and gephyrin or of gephyrin following surface α1 staining—Following removal of the culture media, the cells were fixed in 4% paraformedehyde (Sigma) in 1X PBS at room temperature for 20 minutes. After being washed five times with 1X PBS, cells were incubated with blocking buffer (1% BSA, 3% normal goat serum and 0.01% Triton X-100 (Sigma) in 1X PBS) at room temperature for 1 hour with gentle shaking. All subsequent antibodies were diluted in the blocking buffer. Rabbit anti- GABA$_A$R α1 (1:500), mouse anti-gephyrin (1:500), or a combination, was added onto cells and incubated overnight at 4°C. Following washing with 1X PBS, the cells were incubated with either goat anti-rabbit IgG conjugated with Alexa Fluor 488 (1:2000) or donkey anti-mouse conjugated with Alexa Fluor 568 (1:2000), or a combination, at room temperature for 2 hours. After final washing with 1X PBS, the cells were ready for microscopy. Cells without primary antibodies or secondary antibodies added were used as controls.

Analysis of immunofluorescence—Immunofluorescence images were visualized and captured using Nikon eclipse TE300 microscope with a high resolution digital camera (Nikon, Melville, NY, USA) and using Olympus confocal microscope FV1000. The relative immnofluorescence intensity was analyzed by using software of NIS-Elements BR2.30 (Nikon). Measured immunofluorescence intensity levels were subtracted from non-fluorescence background. The maximum intensities were normalized to controls. For quantitative analysis in each experiment, imaged cells were chosen based on the presence of fluorescence signals, and randomly selected neurites including ones close or remote to soma

or soma from 20 neurons were measured. Student's t-test was applied at p <0.05 to determine significance. ImageJ was used for quantitative analysis of confocal microscopy images. The ICC experiment was done independently at least three times.

Surface biotinylation and isolation of surface proteins

BDNF or vehicle was added into indicated wells for 20 minutes, then the media was aspirated and the cells were washed with ice-cold 1X PBS on ice. The cell surface protein isolation kit (Thermo scientific, Rockford, IL, USA) was used for the cell surface biotinylation process. Briefly, 1 mg biotin was applied onto cells in each well of 6-well plates. The cell lysates were obtained by sonicating cells in lysis buffer containing proteinase inhibitors (Roche diagnostics, Indianapolis, IN, USA) and supernatants were collected. The biotin-labeled protein was isolated and the surface protein was eluted from the biotin in SDS-PAGE sample buffer.

Immunoprecipitation and immunoblot analysis

The above surface proteins were applied to further immunoprecipitation (IP) experiments. 100μg protein per sample was used after being adjusted to equal with buffer. In parallel experiments, cells were treated with BDNF for 20 minutes in indicated wells. Then the cells were harvested with RIPA buffer (50mM Tris–HCl, pH 8, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing protease inhibitors (protease inhibitor cocktail tablet, Roche diagnostics). The protein solutions were collected after cell lysis and centrifugation. 260μg protein per sample was used for IP. Then, each sample was divided into two equal parts. Mouse anti-gephyrin (1:100) was added into first half, and rabbit anti-$GABA_A R$ α1 (1:100) or γ2 (1:100) into the second. The reactions were incubated overnight at 4°C. The antigen-antibody complex was added into Protein A/G agarose (Thermo Scientific) and incubated for 2 hours at room temperature with gentle mixing. The immune complex was eluted with electrophoresis loading buffer and evaluated by SDS-PAGE. After SDS-PAGE, the blot of IP with anti-gephyrin was incubated with rabbit anti-$GABA_A R$ α1 or γ2, and vice versa. The dilution of antibodies was 1:500. Finally, immunocomplexes were visualized by using a peroxidase-conjugated goat anti-rabbit or horse anti-mouse secondary (1:2000). Super Signal west pico chemiluminescent substrate (Thermo Scientific) was used for detecting horse radish peroxidase. Bands were quantified by Fluorchem Sp (Alpha Innotech, San Leandro, CA, USA). For each IP experiment, 20μl of protein solution was always analyzed along with IP samples on SDS-PAGE as a control of IP. The experiment was performed three times independently.

Construct of LV-siGephyrin

Viral vectors containing siGephyrin were derived from the HIV-based lentivirus backbone pLV-CMV–GFP–U3Nhe, and were designed, tested, and produced as recently described for siGAD67 (Heldt et al., 2012). Each small interfering RNA-expressing viral vector (LV-siRNA) was created by subcloning a H1-siRNA coding cassette into the *Nhe*1 restriction enzyme site of the pLV-CMV–GFP–U3Nhe backbone as described (Tiscornia et al., 2003). The sense and antisense sequences targeting Gephyrin were 5′-ACAUGGCGACCGAGGGAAU and 5′-AUUCCCUCGGUCGCCAUGU, respectively. Active viral particles were produced by co-transfecting these lentiviral packaging constructs with plasmids coding for delta8.9 and VSV-G into HEK-293 T cells following standard methods (Tiscornia et al., 2006; Jasnow et al., 2009; Heldt et al., 2012), using a titer of approaximately 1×10^9 infectious particles per ml. The pLV-siRNA control construct was designed to have no complementarity to gephyrin, with the following sequence: 5′-UGGCAGUGACUAGCUGGUUGU.

Results

GABA$_A$R α1 and γ2 subunits and gephyrin are associated in cultured mouse amygdala neurons

In this study, we first wanted to confirm that GABA$_A$Rs are co-localized with gephyrin in cultured mouse amygdala neurons. By using co-IP, protein samples from primary amygdala neuronal cultures were precipitated with antibodies either against GABA$_A$R α1 or γ2, and then immunoblotted with anti-gephyrin antibody. Our data clearly indicated that GABA$_A$R α1 or γ2 subunits were associated with gephyrin (Figure 1A1). The protein samples were also immunoprecipitated with the antibody against gephyrin and then blotted with either anti-α1 or γ2 on western blot. The bands of α1 and γ2 subunits were clearly observed as expected (Figure 1A2 and 1A3). We next performed ICC with amygdala neurons by staining for the presence of GABA$_A$R α1 subunits (green fluorescent secondary) and gephyrin (red fluorescent secondary), and observed their co-localization on neuronal soma and dendrites (Figure 1B).

Decreased gephyrin expression with siRNA decreases surface GABA$_A$R α1 on cultured amygdala neurons

Previously, we have shown that rapid BDNF treatment on amygdala neurons induced the sequestration of surface GABA$_A$R α1 subunits (Mou et al. 2010). Here, we first replicated this effect using ICC. We observed that 10 or 20 minutes, but not 1 hour, of BDNF treatment on neurons resulted in a decrease of surface GABA$_A$R α1 subunits (Figure 2A). This reduction was more than 80%, when compared with the control (Figure 2C).

Published studies demonstrated that GABA$_A$R α1 subunits binding directly to gephyrin could facilitate the receptor clustering at inhibitory synapses (Tretter et al., 2008, Mukherjee et al., 2011; Tretter et al., 2011). We have previously found that activation of TrkB by BDNF was required for fear memory formation and, separately, that GABA$_A$R subunits and gephyrin were dynamically regulated during fear memory consolidation (Chhatwal et al. 2005; Heldt and Ressler 2007). Therefore, we hypothesized that the BDNF-induced surface GABA$_A$Rs changes could be mediated by a change in gephyrin expression. To test this, we created a lentivirus construct harboring siGephyrin (LV-siGephyrin). To validate the construct, we applied it to our primary amygdala neuron cultures. The cells were infected for 3 days, then harvested and protein homogenates were probed for gephyrin via Western blot. The LV-siGephyrin was verified to be able to inhibit the gephyrin expression (Figure 3A1) up to 40% comparing with the control (Figure 3A2). We also tested LV-siGephyrin action by ICC. Gephyrin expression was significantly inhibited (Figure 3B1) in infected neurons up to 90%, comparing to non-infected control neurons (Figure 3B2). Next, we used this construct to further assess whether blocking gephyrin expression had any effect on surface GABA$_A$R α1 subunits by ICC (Figure 2B). Infection with LV-siGephyrin is indicated by GFP expression (Top panels of Figure 2B1, 2B2, 2B3 and 2B4), and surface α1 is labeled with red-fluorescent secondary antibody. In the no-BDNF treatment group that received LV-siGephryin (Figure 2B1), surface α1 subunits were also greatly decreased in infected neurons more than 90% compared to control (non-infected, no-BDNF) neurons (Figure 2C). The effect of blocking gephyrin expression on the surface α1 subunits is same as the effect of 10 or 20 minutes BDNF treatment on neurons (Figure 2A). Application of BDNF for 10 minutes (Figure 2B2) or 20 minutes (Figure 2B3) in LV-siGephyrin-infected cells decreased surface α1 levels not only on infected neurons but also on non-infected ones by more than 95% (Figure 2C). In LV-siGephyrin infected neurons treated with BDNF for 1 hour (Figure 2B4), LV-siGephyrin only lowered surface α1 subunit levels in infected neurons that had GFP expression (Figure 2C). Moreover, neurons infected with LV-siGephyrin that were treated with BDNF for 10 minutes (Figure 2B2) showed a further, significant reduction in

surface α1 subunit levels, compared to BDNF-treated neurons lacking siGephyrin (Figure 2A and 2C). Overall, suppression of gephyrin expression had similar depressive effects as rapid BDNF treatment on the surface level of GABA$_A$R α1 subunits.

We performed several additional control experiments to confirm the above results. First, when similar experiments were performed with a control (scrambled) siRNA that has no complementarity to gephyrin (Figure 4), no effects were seen, demonstrating that it was the specificity of the siGephryin, and not the constructs in general, providing these findings. Additionally, co-localization studies were performed using confocal microscopy, to confirm that the gephyrin and GABA$_A$R α1 signals were located on synaptic puncta (Figure 5).

BDNF treatment leads to biphasic changes in gephyrin levels in cultured amygdala neurons

Next, we sought to determine whether BDNF application on amygdala neurons directly induced any changes on gephyrin. Cultured amygdala neurons were treated with BDNF for 20 minutes, 30 minutes, 1 hour, 2 hours, 4 hours and 17 hours, respectively. Then cells were harvested and an antibody against gephyrin was added into samples of protein homogenates for western blot. We observed that the level of gephyrin protein was significantly decreased after 20 minutes of BDNF treatment, but was similar to baseline levels at the 30-minute timepoint. Then, we saw an increase in gephyrin expression after 1 hour of BDNF exposure, which was maintained at 4 hours exposure to BDNF. However, gephyrin levels returned back to control levels after overnight BDNF exposure (Figure 6A1 and 6A2). In a parallel set of ICC experiments, gephyrin expression was decreased more than 60% after 20 minutes BDNF treatment while greatly increased on 4 hours BDNF treatment (Figure 6B1 and 6B2). The agreement of these data supports the hypothesis that BDNF treatment can directly alter gephyrin levels in neurons.

Decreased GABA$_A$R α1 subunits on cell membranes were associated with gephyrin changes induced by rapid BDNF treatment

After we confirmed that gephyrin was also decreased by rapid BDNF treatment on neurons, we wanted to test whether GABA$_A$R α1 subunits previously located on the surface (identified with surface biotinylation) were associated with gephyrin after BDNF treatment, which presumably leads to receptor internalization. To answer the question, we performed a co-immunoprecipitation on protein samples obtained from cultured amygdala neurons treated with BDNF. After treatment, cells were incubated with biotin, and the biotinynated proteins were isolated. After releasing the proteins from the biotin, samples were immunoprecipitated with the anti-gephyrin antibody, and then immunoblotted with an anti-GABA$_A$R α1 antibody. We found that the surface α1 subunits internalized after a 20-minute BDNF treatment were indeed associated with gephyrin on cell membranes (Figure 7A1 and 7A2).

We also performed a separate set of Co-IP experiments with whole cell homogenates to determine whether gephyrin levels were altered in complex with GABA$_A$Rs. We found that gephyrin was significantly enhanced by 20 minutes of BDNF treatment on neurons compared to control, while GABA$_A$R α1 subunits remained unchanged (Figure 7B1 and 7B2). Also, we did western blot experiments on gephyrin and GABA$_A$R α1 subunits on whole cell homogenates. We observed that gephyrin was significantly decreased following 20 minutes of BDNF treatment, while there was no change in total GABA$_A$R α1 subunit levels (Figure 7C1 and 7C2). Thus, we conclude that BDNF treatment induced rapid sequestration of surface GABA$_A$Rs through dynamic regulation of gephyrin levels in amygdala neurons. This said, it is also possible that in some cases GABA$_A$R regulation is

the initial target of BDNF treatment, which then results in secondary alterations in gephyrin levels.

Protein synthesis is not involved in the initial gephyrin decrease but is required for its later rebound

We wished to determine if protein synthesis was involved in BDNF-dependent gephryin modulation. Our data (Figure 8) suggest that the processes of protein synthesis and degradation activated by BDNF may be involved in this modulation. At 20 minutes after BDNF treatment, the application of a protein synthesis inhibitor, anisomycin, further decreased gephyrin (or doesn't prevent its decrease) (Figure 8A1 and 8A2). However, the longer BDNF treatment does enhance gephyrin synthesis as inhibition of protein synthesis by anisomycin blocks the increased gephyrin levels at 1 hour (Figure 8B1 and 8B2). These data suggest that BDNF may rapidly induce gephyrin protein degradation, with subsequent gephyrin synthesis occuring, perhaps in a homeostatic fashion.

Discussion

Work from our lab has begun to investigate the molecular mechanism behind the neuronal adaptation of GABAergic inhibition influenced by signal pathways initiated by BDNF. We reported previously that on cultured hippocampal and amygdala neurons, rapid BDNF application (minutes) induces sequestration of surface GABA$_A$Rs (Mou et al., 2011). In earlier studies, our lab demonstrated that after fear acquisition, gephyrin gene expression was greatly downregulated (Ressler et al., 2002) and surface GABA$_A$Rs and gephyrin protein levels were decreased in amygdala in parallel (Chhatwal et al., 2005). Building on this work, we conducted the current study and found that BDNF application on cultured amygdala neurons induces a change in gephyrin expression which leads to a decrease of surface GABA$_A$R α1.

The association of gephyrin and GABA$_A$Rs in the cultured amygdala neurons

In the present study, we first confirmed that GABA$_A$Rs and gephyrin are associated on cultured amygdala neurons. There has been some disagreement in the literature as to whether the association of these two proteins takes place. Kneussel *et al*. (1999) reported that in brain sections and cultured hippocampal neurons derived from gephrin knock-out mice, synaptic GABA$_A$R clusters containing either the γ2 or the α2 subunit were absent, suggesting gephyrin may be directly involved with the localization of these receptors. However, Levi *et al*. (2004) reported that gephyrin does not contribute to insertion or stabilization of α2- or γ2-containing GABA$_A$Rs in cell membranes, and found GABA$_A$R clusters at synapses in hippocampal neurons to be gephyrin-independent. Our data by co-IP and ICC suggests that gephyrin and GABA$_A$Rs are indeed colocalized with each other in cultured amygdala neurons, in agreement with the Kneussel study.

Surface GABA$_A$R α1 subunits are decreased by reduced gephyrin which is a result of rapid BDNF application on amygdala neurons

Further, this study demonstrates that the association of gephyrin and GABA$_A$Rs has functional consequences. Our data suggest that knocking down gephyrin expression by siRNA decreases the population of surface GABA$_A$Rs. Small interfering RNA of gephyrin has long been used to study GABA$_A$R clustering and stability (Yu et al., 2007). We applied this technique to the present study and found that lowered gephyrin expression via siRNA decreases surface levels of GABA$_A$R α1 on cultured amygdala neurons, the same effect we observed previously with rapid BDNF treatment on neurons (Mou et al., 2011). Our co-IP of surface GABA$_A$Rα1 likewise indicates that the reduction of surface α1 is gephyrin-related. Postsynaptic GABA$_A$Rs are critical for effective synaptic inhibition. Their trafficking to and

from the cell surface is targeted for regulation at multiple steps. These include receptor localization, stability, insertion and diffusion into membranes, receptor removal, and degradation (Jacob et al., 2008). At least 40% of the total cell surface population of GABA$_A$Rs is capable of being dynamically internalized (Kittler et al., 2004). To date, the number of published studies on surface GABA$_A$Rs is limited, and studies specifically on surface GABA$_A$Rα1 on cultured amygdala neurons are few. To our knowledge, the present study is the first to demonstrate a loss of surface GABA$_A$R α1 subunits induced by gephyrin decrease in response to rapid BDNF application on cultured amygdala neurons. These changes are accompanied by no change in total GABA$_A$R α1 and a decrease of total gephyrin. Our data suggest that BDNF-dependent gephyrin downregulation has a consequential effect on the presentation and localization of surface GABA$_A$Rs.

BDNF application induces biphasic expression changes of gephyrin

We found that BDNF dynamically regulates gephyrin protein levels over time in cultured amygala neurons. While 20 minutes of BDNF exposure leads to a decrease in gephyrin levels, 1–4 hours of BDNF leads to an increase in gephyrin. Focusing on the more rapid BDNF effect, we found that while total gephyrin is decreased, a greater proportion of gephyrin is present in the complexes with GABA$_A$Rs than at baseline. This may serve as a compensatory or homeostatic mechanism to recruit receptors back to the cell membrane. Indeed, it was recently reported that gephyrin plays a major role as a cytoskeletal structure in transporting GABA$_A$Rs to and from the synapses (Tretter et al., 2012). However, the mechanism underlying how BDNF regulates gephyrin is unclear. Our data with anisomycin suggest that BDNF may rapidly induce gephyrin protein degradation, with subsequent gephyrin synthesis occuring, perhaps in a homeostatic fashion. We are continuing to study signaling pathways and the dynamic changes in gephyrin induced by BDNF activation, with the goal of identifying underlying mechanisms.

BDNF is a critical mediator of short-term neuronal plasticity (Lessmann et al., 1994; Berninger and Poo., 1996) and plays a diverse role in GABAergic inhibition (Brunig et al. 2001; Cheng and Yeh, 2003). Our lab and others have shown that BDNF is required for amygdala-related fear learning (Rattiner et al., 2004; Mahan et al., 2012). The BDNF-TrkB signaling cascade activates multiple intracellular responses, including the Ras/ERK pathway, the PI3-K pathway, and phospholipase C-gamma (Carvalho et al., 2008). The rapid effects of BDNF on neuronal plasticity are partly mediated by protein phosphorylation downstream of TrkB receptor activation (Santos et al., 2010). Currently, there are no published studies showing the effect of activation of BDNF-TrkB on gephyrin. Elucidating the signaling pathways between BDNF-TrkB and gephyrin, and determining the role of these pathways in fear memory consolidation, are goals for future study.

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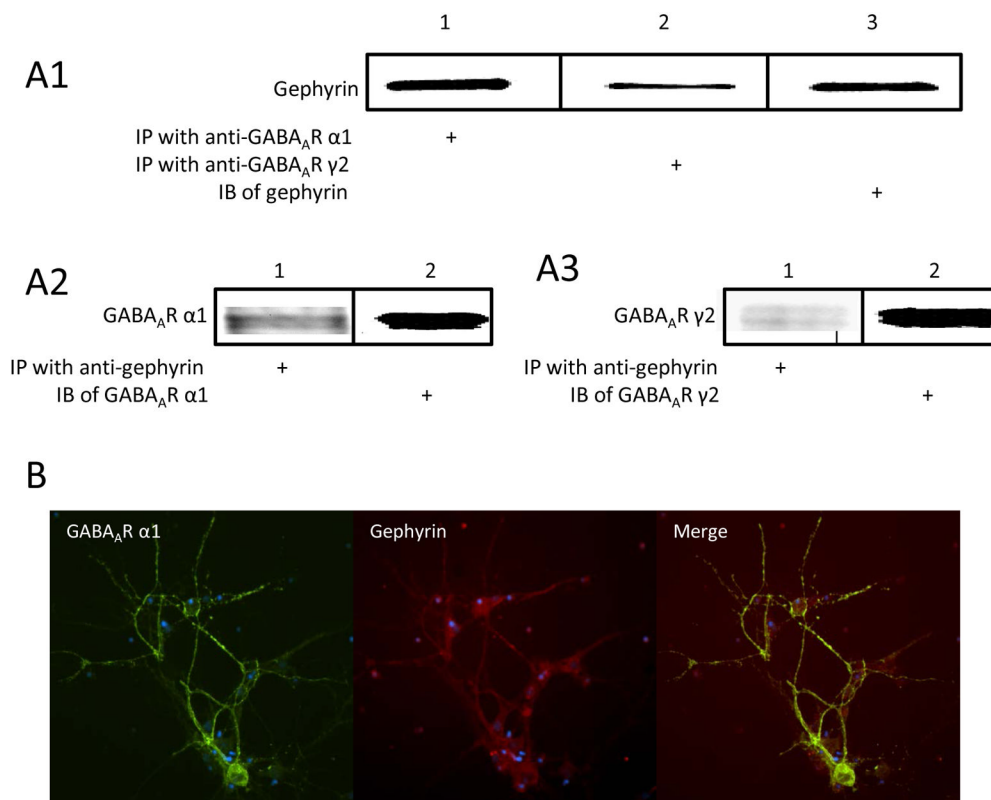
Figure 1. GABA$_A$R α1 and γ2 subunits were associated with gephyrin on cultured mouse amygdala neurons

Panel A shows results of co-IP of gephyrin and GABA$_A$R α1 and γ2 subunits. A1 shows gephyrin protein from samples immunoprecipitated with either anti-α1 or anti-γ2. A2 shows α1 subunits from samples immunoprecipitated with gephyrin antibody. A3 represents γ2 subunits from samples immunoprecipitated with anti-gephyrin. Panel B illustrates the green-fluorescent labeled GABA$_A$R α1 subunits and red fluorescent labeled gephyrin and the colocalization of both.
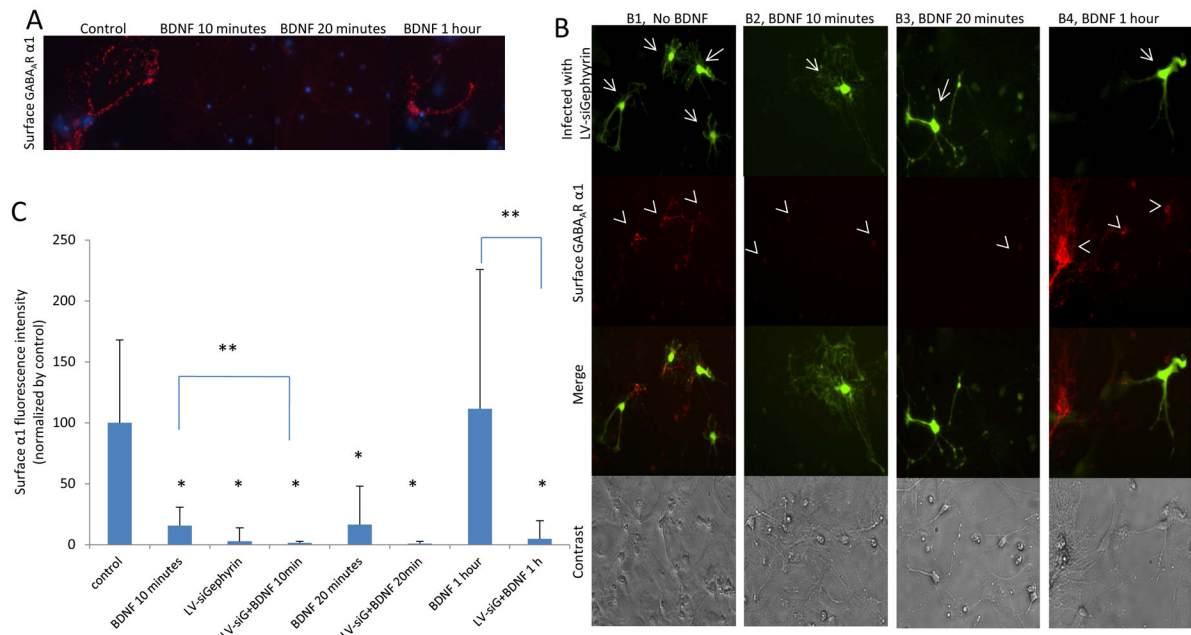
Figure 2. Decreased gephyrin expression with siRNA decreases surface GABA$_A$R α1 on cultured amygdala neurons

Panel A shows red-fluorescent surface GABA$_A$R α1 subunits. The surface α1 subunits are decreased in neurons treated with BDNF for 10 or 20 minutes. Panel B shows single- and double-label ICC. GFP expression indicates neurons infected by LV-siGephyrin (arrows) and the surface GABA$_A$R α1 is labeled with red fluorescence (arrowheads). B1 represents neurons infected with LV-siGephyrin and without BDNF treatment. The surface GABA$_A$R α1 subunits were decreased only in infected neurons. B2 and B3 are neurons treated with BDNF for 10 or 20 minutes, respectively, and also infected with LV-siGephyrin. The surface α1 was decreased in infected neurons and non-infected neurons. B4 presents neurons treated with BDNF for 1 hour and infected with LV-siGephyrin. The surface α1 decreases only on infected neurons. Panel C illustrates the quantified surface α1 fluorescence intensity of Panel A and B. (*LV-siG* stands for LV-siGephyrin). (* P   0.01 comparing with the control. ** p   0.05 comparing between groups).
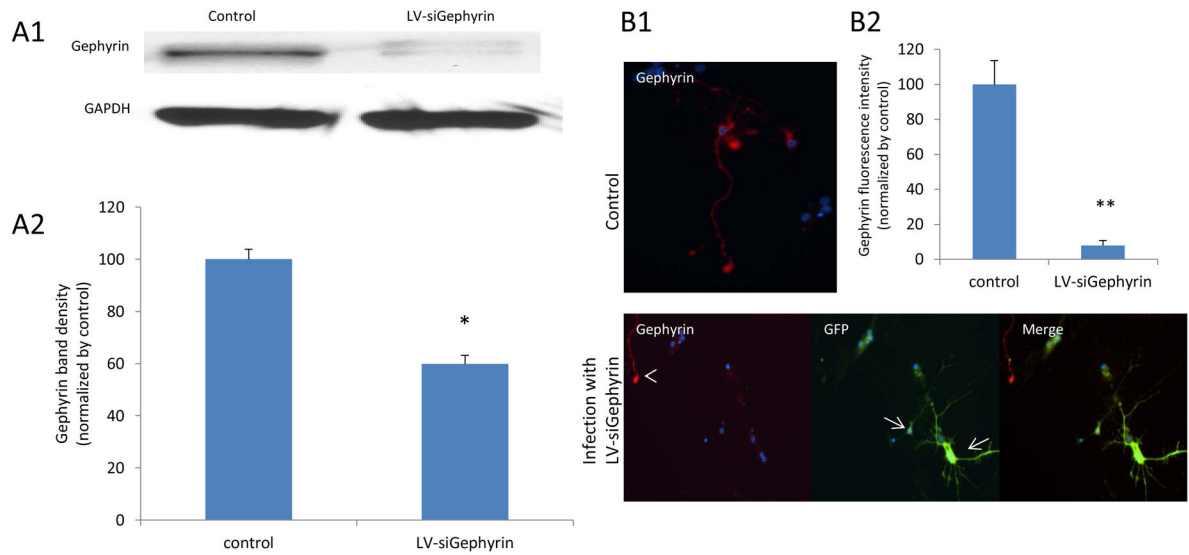
Figure 3. Infection by LV-siGephyrin inhibits gephyrin expression on cultured mouse amygdala neurons

Panel A1 is a western blot result showing gephyrin expression on neurons infected with LV-siGephyrin compared to non-infected control neurons. The gephyrin expression in infected cells was significantly decreased, compared to the control, * $p<0.002$ (A2). Panel B is an ICC result, demonstrating decreased gephyrin on infected, GFP expressing neurons, (arrows in B1) compared to non-infected neurons (arrowhead). B2 represents the quantified fluorescence intensity of gephyrin from B1. (** *p = 0.001,* compared to control).
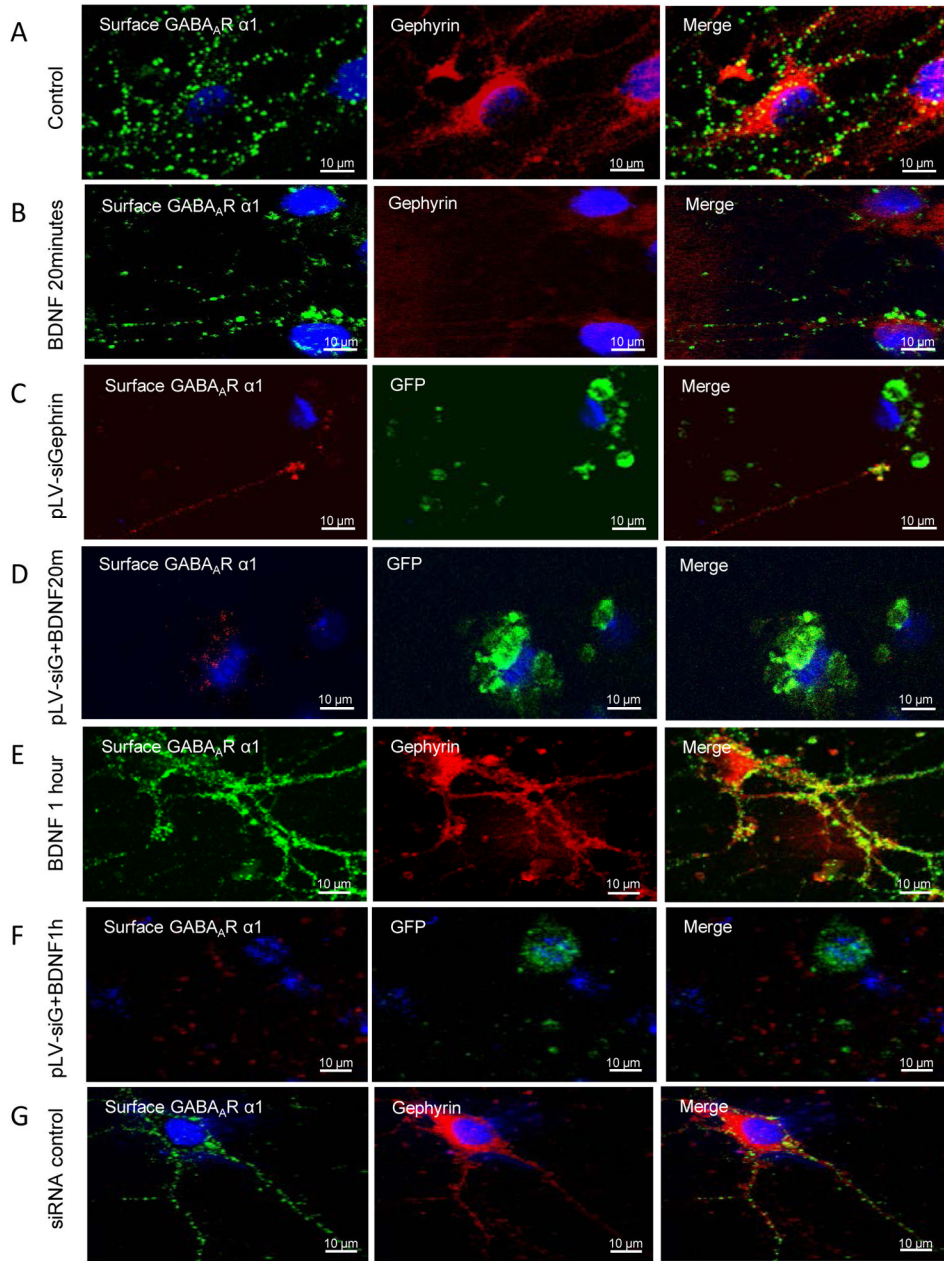
Figure 4. Confocal microscopy verifies that surface GABA$_A$R α1 subunits are decreased by gephyrin siRNA on amygdala neurons

Panel A is an example of control group, showing green-fluorescent α1 and red-fluorescent gephyrin, and merged picture. Panel B represents neurons treated with BDNF for 20 minutes. Both α1 and gephyrin puncta are decreased compared with Panel A control. Panel C shows cells transfected with pLV-siGephyrin, a gephyrin siRNA with GFP expression. The red-fluorescent α1 puncta are decreased. Panel D shows cells transfected with pLV-siGephyrin and treated with BDNF for 20 minutes. As in Panel C, the red-fluorescent α1puncta are greatly lowered. Panel E shows cells treated with BDNF for 1 hour, a time at which both gephyrin and α1puncta are markedly increased. Panel F shows cells transfected with pLV-siGephyrin and treated with BDNF for 1 hour. The red-fluorescent α1 subunits are largely decreased comparing with α1 in Panel E. Panel G is an example of cells

transfected with pLV-siRNA control. The α1 and gephyrin expression is same as the control Panel A. (pLV-siG stands for pLV-siGephyrin).
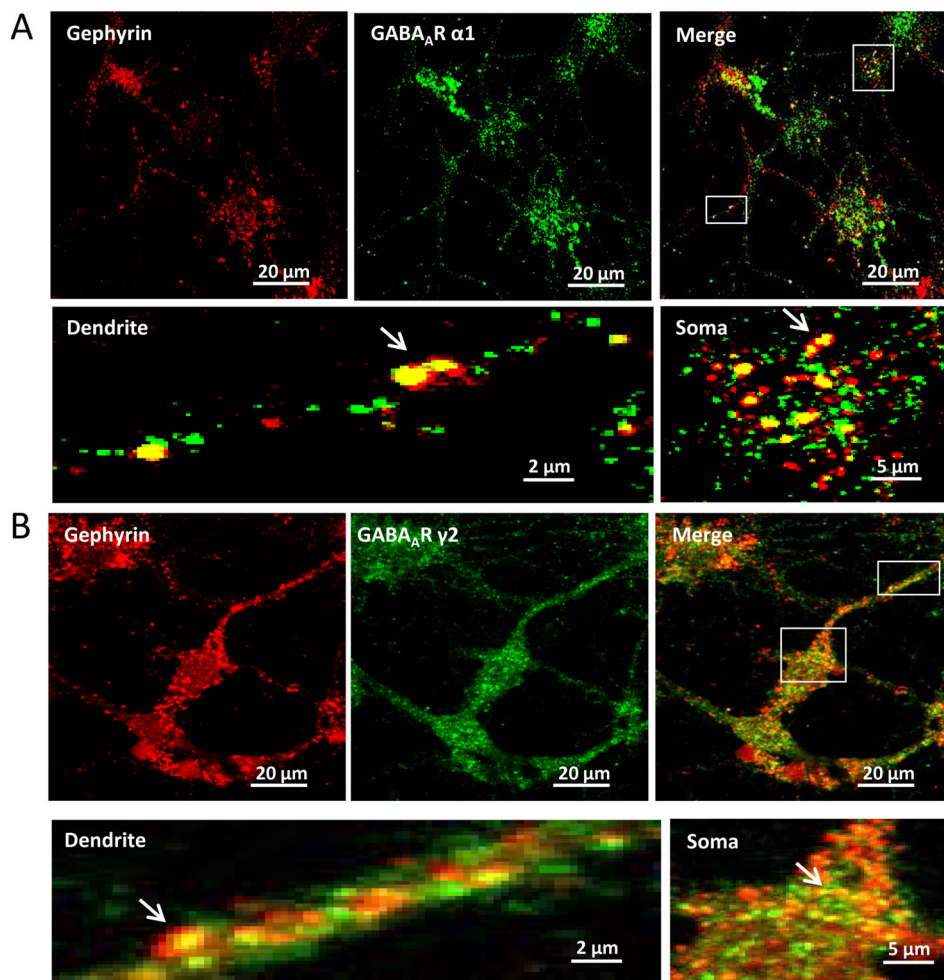
Figure 5. Confocal microscopy shows co-localization of gephyrin and GABA$_A$R α1 or γ2 subunits on cultured mouse amygdala neurons

Panel A shows red-fluorescent gephyrin and green-fluorescent total α1 subunits on dendrites and somata. The merged picture emphasizes the co-localization of both. The pictures of dendrite and soma correspond to the areas in the merged picture, showing details of co-localization of puncta pointed by arrows. Panal B represents red-fluorescent gephyrin and green-fluorescent total γ2 subunits. The pictures of dendrite and soma are the framed areas in the merged picture. The arrows point to the co-localized puncta.
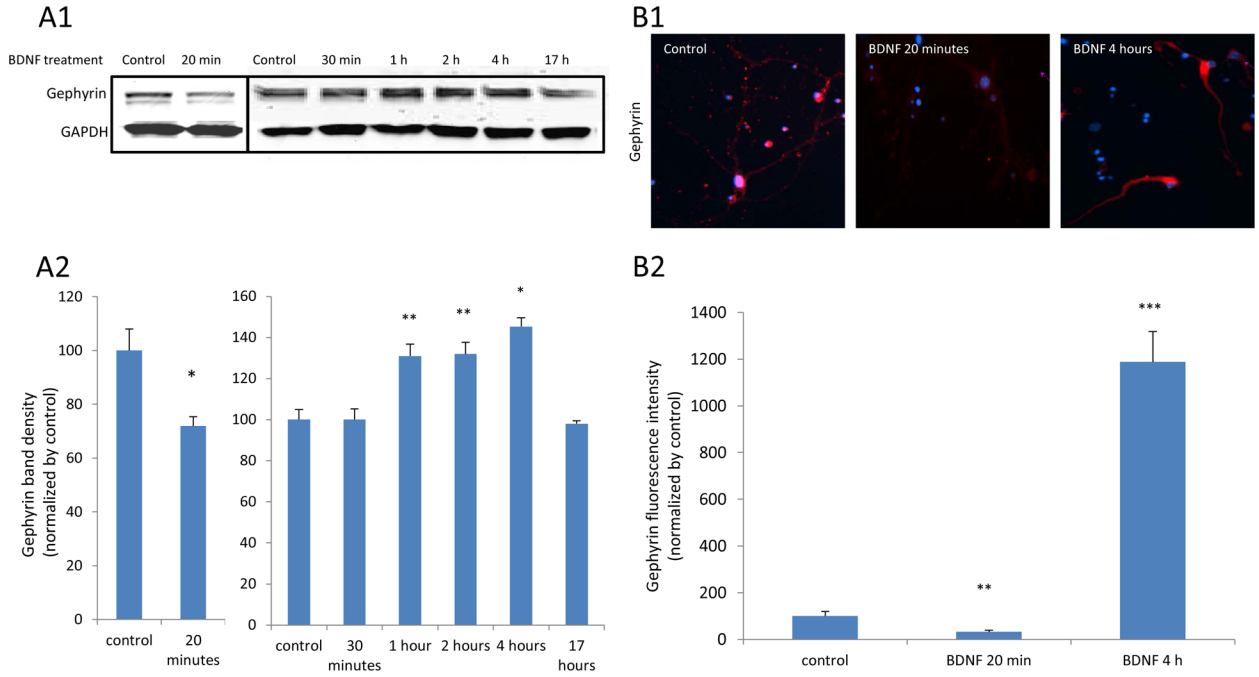
Figure 6. BDNF treatment leads to biphasic changes in gephyrin levels on cultured amygdala neurons

Panel A1 is a result of a western blot for gephyrin from neurons treated with BDNF for 20 and 30 minutes and 1, 2, 4 or 17 Hours, respectively. A2 shows quantified western blot data demonstrating that gephyrin levels are decreased with 20 minutes of BDNF treatment. In a separate experiment, we showed that 1, 2 and 4 hours of BDNF treatment increased the gephyrin. (*P < 0.05 and ** p < 0.01, compared to control). Panel B1 shows ICC results of red fluorescent-labeled gephyrin on neurons exposed to BDNF for 20 minutes or 4 hours. B2 represents the quantified Gephyrin fluorescence intensities from ICC. (** p < 0.01 and *** p < 0.004, compared to control).

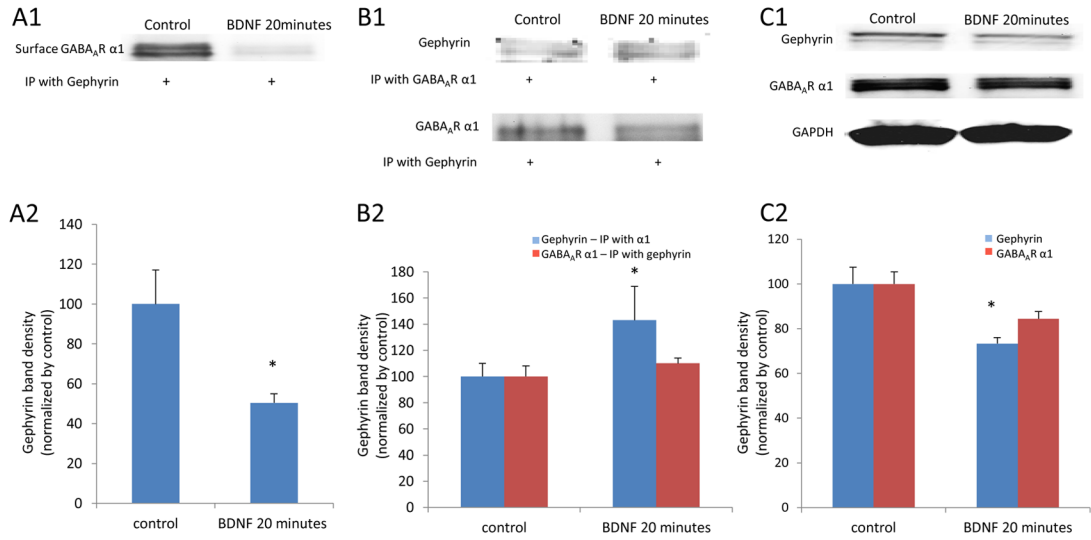**Figure 7. Decreased GABA$_A$R α1 subunits on cell membranes were associated with gephyrin changes induced by rapid BDNF treatment**

Panel A1 is a western blot result of the surface GABA$_A$R α1 subunits from biotinylated protein samples and then co-immunoprecipitated with an antibody against gephyrin. The surface GABA$_A$R α1 specifically bound to gephyrin was significantly decreased by 20 minutes of BDNF treatment. (* $p < 0.05$, compared to control) (A2). Panel B1 shows co-IP results of gephyrin and GABA$_A$R α1 from neurons treated with BDNF for 20 minutes and compared to untreated control neurons. B2 shows that gephyrin bound to GABA$_A$R α1 was increased with BDNF treatment (* $p < 0.05$, compared to control). Panel C1 is a western blot result of gephyrin and total GABA$_A$R α1 subunits. There is no change in total GABA$_A$R α1 while gephyrin was significantly decreased (C2). (* $p < 0.05$, compared to control).
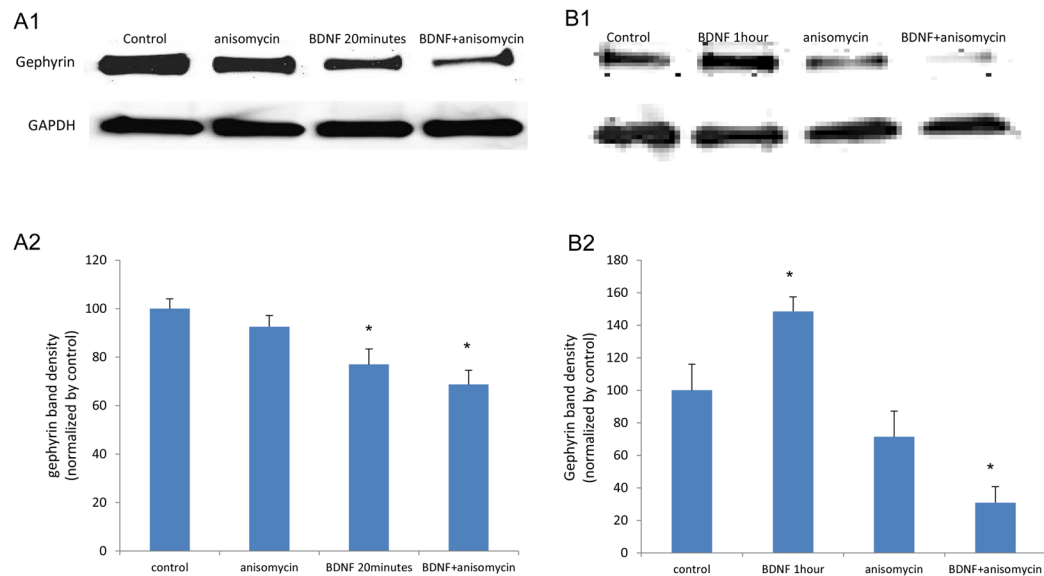
Figure 8. In cultured mouse amygdala neurons, anisomycin further decreases gephyrin after 20 minutes of BDNF treatment and blocks the enhanced gephyrin induced by 1 hour of BDNF treatment
Panel A1 represents the western blot result on gephyrin from neurons treated with 20 minutes of BDNF either with or without application of anisomycin (a protein synthesis inhibitor). GAPDH is the protein loading control. Anisomycin stimulation lowers the dereased gephyrin (Panel A2) (* $p < 0.05$ compared to control). Panel B is a result of gephyrin western blot data on neurons in 1 hour BDNF with or without anisomycin. Anisomycin application blocks the increased gephyrin. (* $p < 0.05$ compared to control).