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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 ($GABA_A$R) subunits and the $GABA_A$ clustering protein gephyrin are dynamically regulated during fear memory consolidation. We hypothesize that TrkB-dependent internalization of $GABA_A$Rs may partially underlie a transient period of amygdala hyperactivation during fear memory consolidation. We have previously reported that BDNF modulates $GABA_A$R α1 subunit sequestration in cultured hippocampal and amygdala neurons by differential phosphorylation pathways. At present, no studies have investigated the regulation of gephyrin and $GABA_A$R α1 subunits following BDNF activation in the amygdala. In this study, we confirm the association of $GABA_A$R 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 $GABA_A$Rs. 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 $GABA_A$R regulation in the 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.

Key words: amygdala, fear, GABA, memory, consolidation, gephyrin.

INTRODUCTION

The activation of $GABA_A$ receptors ($GABA_A$Rs) 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 $GABA_A$R subtypes have been identified; the most abundant subtype in brain is composed of α1β2γ2 subunits, representing over half of all $GABA_A$Rs (Gao and Fritschy, 1994; McKernan and Whiting, 1996; Sperk et al., 1997; Olsen and Sieghart, 2009). In some brain regions, including the amygdala, α1-containing subtypes ($GABA_A$R α1) 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).

$GABA_A$Rs undergo dynamic changes on the neuronal cell surface. Their trafficking to and from the synapse is regulated by the activation of several cell-signaling pathways, which have profound effects on both $GABA_A$R function and the efficacy of $GABA_A$R-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-min application of BDNF in hippocampal neurons. In cerebellar granule cells, BDNF application induces the internalization of $GABA_A$R β2/3 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 $GABA_A$R α1 subunits (Mou et al., 2010). However, the mechanism by which $GABA_A$Rs 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

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Abbreviations: BDNF, brain-derived neurotrophic factor; $GABA_A$Rs, $GABA_A$ receptors; GFP, green fluorescent protein; ICC, immunocytochemistry; IP, immunoprecipitation; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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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; Yamada et al., 2002; Mizoguchi et al., 2003). 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 antisense 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 $\gamma2$ and $\alpha2$ subunits in one study (Kneussel et al., 1999). However, another study reported that $GABA_AR$ $\alpha2$ and $\gamma2$ subunits did cluster at synapses in hippocampal cultures from gephyrin knockout mice, indicating gephyrin-independent GABAergic synapses (Lévi 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.

EXPERIMENTAL PROCEDURES

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/cm^2 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 phosphate-buffered saline (PBS) as 100 mg/ml stock. The aliquots of stock were stored at −30 °C and final concentration of application on neurons was 100 ng/ml.

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

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. 1 μl of