Brain-derived Neurotrophic Factor (BDNF)-induced Synthesis of Early Growth Response Factor 3 (Egr3) Controls the Levels of Type A GABA Receptor α4 Subunits in Hippocampal Neurons

Altered function of γ-aminobutyric acid type A receptors (GABARα4Rs) in dentate granule cells of the hippocampus has been associated with temporal lobe epilepsy (TLE) in humans and in animal models of TLE. Such altered receptor function (including increased inhibition by benzodiazepines and lack of modulation by benzodiazepines) is related, in part, to changes in the mRNA levels of certain GABAAR subunits, including α4 and may play a role in epileptogenesis. The majority of GABAARs that contain α4 subunits are extra-synaptic due to lack of the γ2 subunit and presence of δ. However, it has been hypothesized that seizure activity may result in expression of synaptic receptors with altered properties driven by an increased pool of α4 subunits. Results of our previous work suggests that signaling via protein kinase C (PKC) and early growth response factor 3 (Egr3) is the plasticity trigger for aberrant α4 subunit gene (GABRA4) expression after status epilepticus. We now report that brain derived neurotrophic factor (BDNF) is the endogenous signal that induces Egr3 expression via a PKC/MAPK-dependent pathway. Taken together with the fact that blockade of tyrosine kinase (Trk) neurotrophin receptors reduces basal GABRA4 promoter activity by 50%, our findings support a role for BDNF as the mediator of Egr3-induced GABRA4 regulation in developing neurons and epilepsy and, moreover, suggest that BDNF may alter inhibitory processing in the brain by regulating the balance between phasic and tonic inhibition.

The type A γ-aminobutyric acid (GABA) receptor (GABAAR) is an integral ligand gated ion channel that mediates the majority of inhibition in the central nervous system. Being a hetero-oligomeric complex, it is composed of five membrane spanning subunits that are chosen from the products of 19 different genes (α1–6, β1–3, γ1–5, δ, ε, π1–3, and τ). These genes are differentially transcribed during development and in various regions of the adult brain and spinal cord (1–5). Alteration in the function of GABAARs has been associated with a variety of diseases whose etiology leads to an imbalance between inhibition and excitation in specific populations of neurons (6–8).

For instance, changes in certain GABAAR subunit levels occur in dentate granule cells (DGCs) of both humans with temporal lobe epilepsy (TLE) and in animal models of TLE (6, 9). These molecular responses have been hypothesized to underlie persistent changes in GABAAR function associated with epileptogenesis. Most notably, individual DGCs display an elevation of α4 subunit mRNAs and a decrease in α1 (6). Receptors that contain α4 subunits have unique pharmacological properties that include heightened blockade of receptor function by zinc (11–13) and decreased benzodiazepine modulation (14). In addition, the majority of GABAARs that contain α4 subunits (co-assembled with a β and δ) are located extrasynaptically and mediate tonic GABA currents, while those containing α(1, 2, 3, or 5) without δ and with γ2 are targeted to the synapse (1, 15). Although the majority of synaptic receptors do not contain α4 subunits, α4 containing receptors are precipitated using γ2 subunit antibodies in thalamic ventrobasal neurons (14, 16) suggesting that they are found in a select group of synaptic receptors in neuronal subtypes where α4 is abundant. Assembly of an α, β, and γ2 subunit is also necessary for the pharmacological response to classical benzodiazepines but such a response is absent when α4 is present.

Recent data from our laboratory shows that seizure-induced transcriptional up-regulation of the α4 subunit gene (GABRA4) is regulated through the protein kinase C (PKC) pathway and specifically through binding of the inducible early growth response factor 3 (Egr3) (10). A possible endogenous mediator of Egr3 signaling in TLE has yet to be identified. Because brain-derived neurotrophic factor (BDNF) mRNAs and protein are elevated during seizures in TLE patients (17, 18) and in several animal models of TLE (19–21), we used cultured primary hippocampal neurons to determine whether BDNF could be the seizure-induced signal that up-regulates GABRA4 transcription and down-regulates GABRA1.

EXPERIMENTAL PROCEDURES

Primary Neuronal Cell Culture—Primary hippocampal neurons were cultured from prenatal day 18 Sprague-Dawley rats (E18) (Charles River Laboratories) as described previously (22, 23).

Drug Treatments—Drug treatments were performed for Western blotting, promoter analysis, and real-time PCR experi-
ments. Drugs were dissolved in dimethyl sulfoxide (Me₂SO) or water. Final vehicle concentration was 0.5% or less for all experiments. Drugs were diluted to 20 μl in warm conditioned media and added to each dish.

Cultures were treated with signaling inhibitors and returned to the incubator for 1 h. Cultures were then treated with signaling activators. Following activation, cultures were harvested at variable time intervals ranging from 45 min to 24 h after treatment. For treatments with the MAPK inhibitor (MEK1/MEK2), the following final concentration was utilized: U0126 (Calbiochem, catalog number 662006, 20 μM). The general PKC inhibitor calphostin C (Calbiochem, catalog number 208725) was used at a final concentration of 1 μM. The TrK signaling inhibitor (K252a, Calbiochem, catalog number 420298) was used as a final concentration of 200 nM. Treatment with the signaling activators phorbol 12-myristate 13-acetate (PMA) (Sigma, 1 mM NaCl, pH 7.6, 0.1% Tween) for 1 h or 24 h was also utilized. Sister control dishes received vehicle (Me₂SO or H₂O) during the pretreatment and treatment phases.

**Transient Transfection Studies**—Primary cultures in 6-well dishes (Nunc) were transfected using a modified calcium phosphate precipitation method (22, 23). Eight micrograms of total DNA was transfected into each well of a 6-well dish for studies that accessed reporter activity (GABRA4) after drug treatment. Vectors containing 500 bases of the 5′-flanking sequence specific to GABRA4 were cloned upstream of the luciferase reporter gene in the pGL2 vector (Promega) (10). Promoter fragments confer full promotor activity in primary hippocampal or neocortical neurons, respectively.

Luciferase reporter activity was monitored using the cell culture lysis Reagent and luciferase substrate (Promega) and a Victor 1420 detection system (PerkinElmer Life Sciences). Luciferase counts were normalized to protein within each dish.

**Western Blot Analysis**—Whole cell lysates (10 μg) were electrophoresed on 10% Tris-glycine gels (Novex) and transferred to nitrocellulose membranes. Membranes were blocked in 2% nonfat dry milk (Carnation)/40 ml of TBS-T (20 mM Tris base, 137 mM NaCl, pH 7.6, 0.1% Tween) and 50 ng/ml were also utilized. Sister control dishes received vehicle (Me₂SO or H₂O) during the pretreatment and treatment phases.
was extracted and real-time PCR was performed. Stimulation with BDNF and PMA increases Egr3 mRNA levels at both 45 min and 2 h, with levels peaking at 45 min (Fig. 1B). Levels return to baseline by 6 h after stimulation and remain at baseline for up to 24 h (data not shown). Maximal stimulation by either BDNF or PMA is blocked by the MEK inhibitor U0126 or the PKC inhibitor calphostin C (Fig. 1C).

Changes in Egr3 mRNA levels are paralleled by changes in Egr3 protein after BDNF treatment. Similar to Egr3 mRNA levels, Egr3 protein is elevated by BDNF (Fig. 2). Egr3 levels increase by almost 3-fold 1 h after treatment. Levels are maximally elevated after 2 h (5-fold) and remain elevated for at least 6 h.

BDNF can stimulate MAPK/PKC pathways (25, 26), and our previous work (10) has shown that GABA<sub>4</sub>α subunit synthesis is increased in the pilocarpine model. To investigate whether BDNF can produce similar changes in GABA<sub>4</sub>α subunit levels that occur after PKC stimulation and whether such changes are accompanied by down-regulation of GABA<sub>4</sub>α as seen in the pilocarpine model (6), hippocampal cultures were treated with BDNF or vehicle for 6 or 24 h. Following BDNF treatment, cultures were then harvested and
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A.

** FIGURE 3.** BDNF regulates levels of α subunit and GABRA4 promoter activity in primary hippocampal cells. Rat hippocampal cultures, 7–9 days in vitro, were treated for 6 or 24 h with BDNF (50 ng/ml) or vehicle (H<sub>2</sub>O). Whole cell extracts were prepared as described under “Experimental Procedures” and resolved by SDS-PAGE under reducing conditions. Proteins were visualized using ECL following incubation with an anti-rabbit HRP-conjugated antibody. A, α4 (or α1 subunit) and β-actin levels were quantified by densitometry. Normalized data (α subunit/β-actin) are presented as mean ± S.E. and expressed as percent change with respect to vehicle-treated cultures (defined as 100%). Levels of α-actin did not vary with BDNF treatment and were used as an internal control (∗ = significantly different from control as determined by 95% confidence interval, n = 5; ** = significantly different from control as determined by 95% confidence interval, n = 5). B, representative Western blot shows effect of a 6 and 24 h treatment with BDNF on α4, α1, and β-actin protein levels in hippocampal neurons. C, BDNF up-regulates GABRA4 promoter activity, and activation is dependent upon TrkB receptor signaling. Cultures were transfected with the GABRA4 promoter luciferase reporter construct (~471 to +71). Eighteen hours after transfection, cells were pretreated with vehicle (Me<sub>2</sub>SO) or the Trk signaling inhibitor K252a (200 nM) followed by a 1-, 6-, or 12-h treatment with BDNF (50 ng/ml) or vehicle. Data are presented as mean ± S.E. and are expressed as percent activity from control (% control) (∗ = significantly different from control as determined by 95% confidence interval).

Extracts were subjected to Western blot analysis. As shown in Fig. 3A, α4 subunit levels increase by 57% after 6 h of treatment and 120% after 24 h, while α1 subunit levels show no change at 6 h and decrease by 42% after 24 h. A representative Western blot is shown in Fig. 3B.

To test the hypothesis that BDNF regulates α4 subunits through the up-regulation of GABRA4 transcription, primary hippocampal neurons were transfected with the GABRA4 promoter luciferase reporter construct. Eighteen hours after transfection, neurons were pretreated with a Trk receptor signaling inhibitor (K252a) for 1 h followed by a 6- or 12-h treatment with BDNF. Treatment with BDNF increases promoter activity by more than 50% after 12 h and activation is fully reversible by K252a (Fig. 3C). K252a alone reduces promoter activity to about 50% of base line.

** DISCUSSION **

Changes in GABA<sub>R</sub> subunit gene expression may play an important role in the etiology of TLE (27). In both animal models of TLE or in TLE patients, DGCs have elevated levels of GABA<sub>R</sub>Rs with distinct pharmacological properties (6, 28, 29). These receptors are associated with a marked increase in α4 and decrease in α1 subunit gene expression (6). We previously reported that elevated levels of α4 mRNAs and protein are due to binding of Egr3 to GABRA4 in cultured hippocampal neurons and that there are increased levels and increased association of Egr3 and GABRA4 in dentate gyrus of animals exposed to SE (10). Additionally, our data showed that activation of PKC signaling elevates α4 mRNA levels most likely through transcriptional regulation of endogenous GABRA4 by Egr3. We now report that BDNF is a likely mediator of GABRA4 expression in TLE through its stimulation of Egr3 mRNA and protein synthesis.

Previous evidence has suggested a relationship between BDNF and the modulation of GABA<sub>R</sub> receptor function. In primary hippocampal neurons, BDNF reduces GABAergic miniature inhibitory postsynaptic currents and causes a reduction in GABA<sub>R</sub> subunit α2, β(2,3), and γ2 immunoreactivity (31). A role for BDNF has not been established in the regulation of GABRA4. We now show that BDNF increases α4 while decreasing α1 subunit levels in hippocampal neurons suggesting that the neurotrophin has the potential to differentially regulate the expression of extrasynaptic and synaptic GABA<sub>R</sub>Rs.

Egr3 is a member of the early growth response (Egr) transcription factor family that includes four members (Egr1–4). Our data suggest that Egr3 mRNAs and protein are elevated in primary hippocampal neurons after BDNF treatment through PKC/MAPK activation. These changes are also seen after treatment with PMA, a drug known to activate both PKC and MAPK pathways. Our observations are consistent with those in non-neuronal systems where both Egr3 mRNA levels (32) and Egr3 promoter activity (33) are activated upon treatment with PMA. Egr3 mRNAs and protein levels are also elevated in several animal models of TLE (34–36). Taken together with our data, where the elevation of BDNF mRNA levels occurs before or at a time when Egr3 levels are also elevated, evidence suggests that BDNF is a likely endogenous regulator of Egr3 in response to SE.

Elevated Egr3 levels after BDNF stimulation are also the likely mediator of increased α4 subunit levels in both cultured neurons and in DGCs of TLE animals. In addition to the observation that Egr3 knock-out mice lack muscle spindles, display sensory ataxia, resting tremor, and scoliosis (37), these animals also have around 50% less GABRA4 mRNAs in the hippocampus (9). Interestingly, regulation of GABRA4 by Egr3 appears to be context independent because overexpression of Egr3 increases GABRA4 mRNA levels by over 7-fold in non-neuro-
nal primary myotubes (38) suggesting that Egr3 may override the neuron-specific expression of GABRA4.

BDNF has received attention as a putative regulator of epileptogenesis (39). Intrahippocampal infusion of BDNF can cause spontaneous limbic seizures (40), and one controversial hypothesis is that BDNF may contribute to epileptogenesis through mossy fiber sprouting, a process whereby DGCs send glutamatergic projections to one another to form hyperexcitatory circuits. With data both supporting (41) and refuting this hypothesis (42, 43), it will be important to parcel out the relationship between BDNF, Egr3, and GABAergic neurotransmission in the central nervous system. Equally important is the study of how BDNF selectively decreases the levels of α1 subunits, by an Egr-independent mechanism (5), while increasing those of α4. Future studies will be aimed at determining the balance between these two interrelated yet distinct BDNF-induced regulatory pathways that control GABA receptor function in the nervous system.

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