Excitatory Actions of GABA Increase BDNF Expression via a MAPK-CREB–Dependent Mechanism—A Positive Feedback Circuit in Developing Neurons

KARL OBRIETAN,¹ XIAO-BING GAO,² AND ANTHONY N. VAN DEN POL²
¹Department of Neuroscience, The Ohio State University, Columbus, Ohio 43210; and ²Department of Neurosurgery, Yale University Medical School, New Haven, Connecticut 06520-8082

Obrietan, Karl, Xiao-Bing Gao, and Anthony N. van den Pol. Excitatory actions of GABA increase BDNF expression via a MAPK-CREB–dependent mechanism—a positive feedback circuit in developing hypothalamic neurons. J Neurophysiol 88: 1005–1015, 2002; 10.1152/jn.00910.2001. During early neuronal development, GABA functions as an excitatory neurotransmitter, triggering membrane depolarization, action potentials, and the opening of plasma membrane Ca²⁺ channels. These excitatory actions of GABA lead to a number of changes in neuronal structure and function. Although the effects of GABA on membrane biophysics during early development have been well documented, little work has been done to examine the possible mechanisms underlying GABA-regulated plastic changes in the developing brain. This study focuses on GABA-regulated kinase activity and transcriptional control. We utilized a combination of Western blotting and immunocytochemical techniques to examine two potential downstream pathways regulated by GABA excitation: the p42/44 mitogen-activated protein kinase (MAPK) cascade and the transcription factor cyclic AMP response element binding protein (CREB). During early development of cultured hypothalamic neurons (5 days in vitro), stimulation with GABA triggered activation of the MAPK cascade and phosphorylation of CREB at Ser 133. These effects were mediated by the GABAA receptor, since administration of the GABAA receptor-specific agonist muscimol (50 μM) triggered pathway activation, and pretreatment with the GABAA receptor-specific antagonist bicuculline (20 μM) blocked pathway activation. Immunocytochemistry revealed a spatial and temporal correlation between activation of the MAPK cascade and CREB phosphorylation. Pretreatment with the MAPK/ERK kinase (MEK) inhibitor U0126 (10 μM) attenuated CREB phosphorylation, indicating that the MAPK pathway regulates that activation state of CREB. In contrast to the excitatory effects observed during early development, in more mature neurons, GABA functions as an inhibitory transmitter. Consistent with this observation, GABAA receptor activation did not stimulate MAPK cascade activation or CREB phosphorylation in mature cultures (18 days in vitro). To determine whether GABAA receptor activation during early development stimulates gene expression, we examined the inducible expression of the neurotrophin brain-derived neurotrophic factor (BDNF). Both GABA and muscimol stimulated BDNF expression, and pretreatment with U0126 attenuated GABA-induced BDNF expression. Whole cell electrophysiological recording was used to assess the effects of BDNF on GABA release. BDNF (100 ng/ml) dramatically increased the frequency of excitatory GABAergic spontaneous post-synaptic currents. Together, these data suggest a positive excitatory feedback loop between GABA and BDNF expression during early development, where GABA facilitates BDNF expression, and BDNF facilitates the synaptic release of GABA. Signaling via the MAPK cascade and the transcription factor CREB appear to play a substantial role in this process.

INTRODUCTION

In contrast to its inhibitory role in the mature nervous system, GABA functions as a fast excitatory neurotransmitter during development. The principal mechanism by which GABA exerts its excitatory actions in hypothalamic neurons is via GABAA receptor-mediated membrane depolarization. This excitatory action of GABA results from a Cl⁻ reversal potential that is positive to the resting membrane potential (Chen et al. 1996). Thus the opening of GABAA receptors allows Cl⁻ to leave the cells, resulting in membrane depolarization. A secondary effect of GABA-mediated membrane depolarization is the activation of voltage-sensitive Ca²⁺ channels. Indeed, Ca²⁺-imaging experiments demonstrate that Ca²⁺ transients can be elicited by both exogenous and synaptically released GABA during early development of hypothalamic (Obrietan and van den Pol 1995) and spinal cord neurons (Reichling et al. 1994). GABA-mediated Ca²⁺ transients are observed in both the cell body and in distal neurites (Obrietan and van den Pol 1997).

Importantly, GABA is expressed during the early stages of neuronal development; it is found in axonal growth cones (van den Pol 1997), released from the growing axonal growth cone (Gao and van den Pol 2000), and secreted from synaptic terminals within days of the final mitosis (Obrietan and van den Pol 1995, 1996). Moreover, functional GABAA receptors are expressed at the earliest developmental time studied, embryonic day 15 in rats (Chen et al. 1995; van den Pol et al. 1995), and mature before receptors for the other excitatory transmitter, glutamate (Chen et al. 1995). Additionally, spike frequency in early hypothalamic development appears to be driven more by excitatory actions of GABA than by glutamate (Gao and van den Pol 2001).

Given the early expression of both receptor and transmitter, one may realistically envision a pivotal role for excitatory
GABAergic neurotransmission during CNS development. Indeed, as an excitatory transmitter, GABA has been implicated in a wide array of developmentally related processes, including altering neurite length (Barbin et al. 1993), regulating DNA synthesis (LoTurco et al. 1995), regulating neuronal survival (Obata 1997), and affecting neuronal migration (Behar et al. 2000). Additionally, by stimulating BDNF expression during development, GABA has been shown to influence the phenotype of hippocampal interneurons (Marty et al. 1996). Importantly, these effects of GABA are only observed during development; concomitant with its transition to an inhibitory neurotransmitter, GABA’s trophic actions cease.

Because Ca²⁺ is a key regulator of numerous cellular signaling processes, it is likely that GABA’s ability to increase cytosolic Ca²⁺ levels during the early stages of development is linked to its trophic actions. In support of this possibility, Behar et al. (1996) found that uncoupling GABA_A receptor activation from Ca²⁺ influx disrupted GABA-induced motility during development. Thus, given the likelihood that many of the trophic actions elicited by GABA are mediated by Ca²⁺-dependent gene expression, we sought to identify Ca²⁺ responsive signaling pathways activated by GABA during early development. Toward this end we examined whether GABA activates a signaling cascade formed by the p42/44 mitogen activated protein kinase (MAPK) pathway and the transcription factor cyclic AMP response element binding protein (CREB). We focused on an examination of the MAPK cascade because of its well-characterized role as 1) a Ca²⁺ responsive kinase pathway, 2) a pathway that regulates cell morphology and synaptic plasticity, and 3) a pathway that influences the transcription of a diverse set of developmentally regulated genes (reviewed by Grewal et al. 1999; Impney et al. 1999; Sweatt 2001). CREB was examined because it is recognized as a downstream target of the MAPK pathway and because of its well-characterized role as a regulator of plasticity-associated gene expression in the CNS (reviewed by Lamprecht 1999; Martin and Kandel 1996).

Data presented here identifies the MAPK pathway and the transcription factor CREB as downstream effectors of GABA_A receptor activation during early development. Additionally, we provide evidence that GABA_A receptor activation leads to brain-derived neurotrophic factor (BDNF) expression and that BDNF functions presynaptically to facilitate GABA release, thus forming a positive feedback loop during development.

**METHODS**

**Tissue culture**

Hypothalamic neurons were cultured from rat embryos as described previously (Gao and van den Pol 1999). Briefly, dorsomedial hypothalami were dissected out of the brains of E18 Sprague-Dawley rat embryos and cut into small pieces (smaller than 1 mm³). The tissue was incubated at 37°C for 15–20 min in an enzyme solution containing 20 units/ml papain, 0.5 mM EDTA, 1.5 mM CaCl₂, and 0.2 mg/ml 1-cysteine. After enzymatic digestion and mechanical trituration in culture medium to obtain dissociated cells, cells were washed with culture medium containing 10% fetal calf serum. For Western blotting and electrophysiological analysis, cells were plated in 35-mm culture dishes at 200,000 cells per dish. For immunocytochemical examination, cells were plated on 22-mm coverslips. Cultures were maintained in an incubator at 37°C and 5% CO₂. Serum-containing medium was replaced by serum-free medium 1–2 h after plating. The serum-free culture medium contained Neurobasal medium (Gibco), 5% B27 supplement (Gibco), 0.5 mM l-glutamine, 100 units/ml penicillin-streptomycin, and 6 g/ml glucose. Neurons were fed twice a week.

**Calcium digital imaging**

Ratiometric fluorescent digital microscopy was performed as previously described (Obrietan and van den Pol 1998). Fura-AM ester was loaded into the neurons for 25 min, washed off, and then the glass coverslips on which hypothalamic neurons were growing were loaded into a microscope chamber and studied with ratiometric imaging using 340 and 380 nm excitation provided by a Sutter filter wheel.

**Immunocytochemistry**

Cells cultured for 5 days in vitro (DIV) were transferred from culture medium to a HEPESS-based buffer containing (in mM) 137 NaCl, 25 glucose, 10 HEPESS, 5 KCl, 1 MgCl₂, and 3 CaCl₂ (pH 7.4) against the Ser 133 phosphorylated form of CREB (P-CREB) and phosphorylated extracellular signal-regulated kinase (P-ERK) 30 min prior to stimulation. For immunostaining, cells were stimulated for 15 min and rapidly fixed in ice-cold 90% methanol and 10% acetic acid for 5 min; for BDNF immunostaining, cells were stimulated for 9 h and fixed with 5% formaldehyde for 20 min. Next, cells were washed in phosphate-buffered saline (PBS; 3 × 5 min per wash) and blocked with 1% normal goat serum and 10% bovine serum albumin in PBS with 0.1% Triton X-100 (PBST) and 0.02% azide for 2 h at room temperature. For experiments examining regulation of ERK and CREB, cultures were immunolabeled with an affinity-purified rabbit polyclonal antibody raised against the stimulated form of CREB [(phosphorylated at Ser 133, 1:500 final dilution, New England Biolabs (NEB))] and a mouse monoclonal antibody directed against the activated forms of erk-1 and erk-2 (phosphorylated at Thr-202 and Tyr-204; 1:500 final dilution, NEB). For experiments analyzing BDNF expression, cells were incubated with rabbit polyclonal BDNF antibody (2 μg/ml final dilution; Chemicon International). Primary antibody incubations were performed overnight at 4°C. For visualization of P-CREB and BDNF expression, cells were incubated (4 h at room temperature) with an Alexa 488-conjugated secondary antibody (2 μg/ml final dilution; Molecular Probes) raised against rabbit immunoglobulin G (IgG). For visualization of activated ERK expression, an Alexa 594 conjugated secondary antibody (2 μg/ml final dilution; Molecular Probes) was used. After washing, the immunolabeled cells were mounted using Gelmount (Biomedia). For double-labeling experiments, immunofluorescence was examined using an MRC-600 scanning laser confocal microscope. For single-labeling experiments, an inverted epifluorescent microscope (Leica DMRB) connected to a Micromax 1300 YHS camera was used. Signal quantitation was performed using Metamorph software (Universal Imaging). To minimize spurious variability, all groups within each experiment were processed in parallel using the same antibodies dilution stocks.

**Western blotting**

Cultured cells were transferred from culture medium to a HEPESS-based buffer 30 min prior to stimulation. Cells were stimulated for 15 min and lysed in hot (85°C) sample buffer. Lysates were stored at −80°C until ready to use. Before loading, lysates were heated to 85°C for 10 min, vortexed (10 s), and centrifuged for 7 min at 15,000 g. Extracts (25 μl/lane) were electrophoresed into a 10% sodium dodecyl sulfate polyacrylamide gel and transblotted onto polyvinylidene fluoride (Immobilon-P; Millipore). Next, the membrane was washed with 10% (wt/vol) powdered milk dissolved in PBST, followed by incubation (4°C overnight) with affinity-purified rabbit polyclonal antibody raised against the Ser 133 form of CREB (1:1000 final dilution, NEB). Membranes were treated with a goat anti-rabbit IgG.
alkaline horseradish peroxidase (HRP) conjugated secondary antibody (1:2,000; NEN). The signal was visualized using Renaissance chemiluminescent HRP substrate (NEN). Membranes were probed for expression of activated ERK using a mouse monoclonal antibody against the activated forms of erk-1 and erk-2 (1:5,000 final dilution; Sigma). Membranes were reacted with a rabbit anti-mouse IgG alkaline phosphatase-conjugated secondary antibody (1:2,000 final dilution; NEN); immunoreactivity was revealed using the CDP star alkaline phosphatase chemiluminescent detection system (NEN). As a control for equal loading of protein across the gel, membranes were stripped and probed for expression of total ERK using a goat polyclonal antibody against erk-1 and erk-2 (1 µg/ml final dilution; Santa Cruz Biochemicals). ERK expression was revealed using a donkey, anti-goat IgG antibody (1:2,000 dilution; NEN) conjugated to horseradish peroxidase, followed by visualization with Renaissance anti-goat IgG antibody (1:2,000 dilution; NEN). ERK expression was revealed using a donkey, anti-goat IgG antibody (1:2,000 dilution; NEN) conjugated to horseradish peroxidase, followed by visualization with Renaissance chemiluminescent HRP substrate. Blots were washed a minimum of 6 times (5 min per wash) in PBST with 5% milk after each antibody treatment. Scion Image analysis software was used to quantitate band intensity. Band intensity for phosphorylated erk-2 and phosphorylated CREB was normalized to total erk-2 for the corresponding lane. Each experiment was repeated a minimum of 3 times.

**Image analysis**

Quantitative immunofluorescent analysis was performed using Metamorph image analysis software (Universal Imaging). For each condition, the fluorescent signal from a minimum of four coverslips (4 regions per coverslip) were captured. After thresholding the image, the fluorescent intensity of each neuron was recorded. Data for phosphorylated CREB immunoreactivity were collected from the nucleus; data for activated ERK, and BDNF immunoreactivity was collected from the cell body. All cells with a neuronal morphology were used for quantitation. Data were collected and analyzed by a person “blinded” to the experimental conditions. Data are represented as the mean ± SE fluorescent intensity on a 0–4,095 scale. Significance was determined via a two-tailed Student’s t-test.

**Whole cell electrophysiological recording**

All experiments were performed at room temperature on developing hypothalamic neurons cultured 3–7 days in vitro. The recording chamber was continuously perfused at a rate of 1.5–2 ml/min with a bath solution containing (in mM) 150 NaCl, 2.5 KCl, 2 CaCl₂, 10 HEPES, and 10 glucose, pH 7.3 with NaOH. Whole cell voltage-clamp recording was used to observe spontaneous postsynaptic currents at ~60 mV with a L/M EPC-7 amplifier. The patch pipette was made of borosilicate glass (World Precision Instruments) with a Narashige puller (PP-85). The tip resistance of the recording pipettes was 4–6 MΩ after filling with a pipette solution containing (in mM) 116 KMeSO₄, 27 KCl, 1 MgCl₂, 10 HEPES, 1.1 EGTA, 2 Mg-ATP, and 0.5 Na₂-GTP, pH 7.3 with KOH. In some experiments with perforated patch recording, gramicidin (50–100 µg/ml) was added to the pipette solution to verify inward currents and depolarizing actions of GABA transmission in developing cells, with undisturbed Cl⁻ levels of the same age and culture conditions used in the present set of experiments. The preparation of gramicidin was described previously (Gao et al. 1998). After a gigahm seal was formed, the series resistance was between 20 and 40 megaohms and partially compensated by the amplifier. Both input resistance and series resistance were monitored throughout the experiments. Only those recordings with input resistance higher than 0.8 gigaohms and a stable series resistance were used. GABA (50 μM) was briefly (10 ms) ejected by air pressure (4 psi) through micropipettes (with 2–3 µm tip diam and 2–4 µm away from the soma) with a picospritzer (General Valves) to study the GABA-evoked postsynaptic current. All data were sampled at 3–10 kHz and filtered at 1–3 kHz with an Apple Macintosh computer using AxoData 1.2.2 (Axon Instruments). Data were analyzed with Axograph 3.5 (Axon Instruments) and plotted with Igor Pro software (WaveMetrics). Spontaneous postsynaptic currents were detected and measured with an algorithm in Axograph 3.5 as described elsewhere (Gao and van den Pol 1999). To eliminate electronic noise, we only used signals >5 pA. All data were reported as mean ± SE. Student’s t-test was used to compare two groups of data.

Recombinant BDNF was obtained from PeproTech. The lyophilized protein was reconstituted in water to a concentration of 100 µg/ml and stored in −20°C as instructed by the vendor. The stock solution was diluted to the working concentration of 100 ng/ml just prior to use. 2-amino-5-phosphono-pentanoic acid (AP5), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) were obtained from RBI-Sigma.

**RESULTS**

Contrary to its inhibitory role in the mature nervous system, during early development, the amino acid neurotransmitter GABA is excitatory, triggering membrane depolarization, action potentials, and a rise in intracellular Ca²⁺. In our preparation, GABA (50 μM) elicited a Ca²⁺ rise ≥20 nM in approximately 40% of neurons, and the GABAreceptor-specific agonist muscimol (50 μM) elicited a robust Ca²⁺ rise in 42% of the neurons (n = 402), indicating that the effects of GABA were mediated by GABAA receptor activation (Fig. 1). Stimulation of GABAB receptors does not alter basal Ca²⁺ levels (Obrietan and van den Pol 1998). Given that changes in cytosolic Ca²⁺ trigger an array of developmentally regulated processes, we tested the hypothesis that the excitatory effects of GABA lead to changes in the activation state of signaling pathways and transcription factors. Toward this end we focused on GABA-mediated activation of the p42/44 MAPK pathway and the transcription factor CREB.

After 5 days in vitro, hypothalamic neurons cultured from embryonic day 18 rat pups were stimulated with GABA (50 μM) for 15 min, and then lysed and examined via Western analysis for the expression of the Ser 133 phosphorylated form of CREB (P-CREB) and the activated (i.e., dually phosphorylated) CREB (P-ERK). Ser 133 phosphorylation is a measure of CREB (P-CREB) and the activated (i.e., dually phosphorylated) CREB (P-ERK).

**FIG. 1.** GABAreceptor activation triggers a Ca²⁺ rise during early development. Hypothalamic neurons from embryonic day 18 rat pups were cultured for 5 days in vitro (DIV) and then were loaded with the Ca²⁺-sensitive fluorophore fura-2. Administration of GABA (50 μM, arrow) elicited a robust Ca²⁺ rise. On GABA washout, intracellular Ca²⁺ returned to basal levels. The GABAreceptor-specific agonist muscimol (50 μM, arrow) also elicited a Ca²⁺ transient. Administration of N-methyl-D-aspartate (NMDA; 50 μM, arrow) to the perfusion media triggered a robust Ca²⁺ rise. To inhibit action potential–mediated transmitter release, all bath solutions used in this study contained tetrodotoxin (500 nM). Horizontal bar below Ca²⁺ trace denotes time in minutes (min); vertical bar to the left of the trace denotes the calibrated cytosolic Ca²⁺ values.
Relative to mock-treated cultures, GABA administration increased the expression phosphorylated CREB (Fig. 2A). As a positive control and to assess the relative magnitude of CREB phosphorylation by GABA, cultures were also stimulated with high potassium (40 mM), which triggers membrane depolarization, and with forskolin, which stimulates adenyl cyclase activity. Both potassium and forskolin triggered robust increases in CREB phosphorylation. Potassium administration elicited a marked \( \text{Ca}^{2+} \) rise in \( \sim 94\% \) of the cells examined \((n = 206)\); forskolin did not significantly affect basal \( \text{Ca}^{2+} \) levels (data not shown). The same blot was then probed for expression of P-ERK. Relative to mock treatment, GABA elicited ERK phosphorylation, indicating that GABA receptor activation during early development triggers activation of the MAPK cascade. Both high potassium and forskolin also triggered ERK activation.

GABA acts on both GABA\(_A\) and GABA\(_B\) receptors in the CNS. To test whether CREB and MAPK pathway activation was triggered by GABA\(_A\) receptor stimulation, cells were challenged with the GABA\(_A\) receptor-specific agonist muscimol (50 \( \mu \text{M}\); Fig. 2B). As was seen with GABA administration, muscimol triggered both CREB and ERK phosphorylation, indicating that GABA\(_A\) receptor activation is the mechanism by which GABA signals to kinase pathway and transcription factor stimulation. Furthermore, pretreatment with the GABA\(_A\) receptor antagonist bicuculline (20 \( \mu \text{M}\)) inhibited GABA\(_A\) receptor-mediated phosphorylation of CREB and ERK, while not inhibiting high potassium- or cAMP-dependent stimulation of these pathways (Fig. 3B).

These experiments were designed to obviate the issue of synaptic activity (both GABAergic and non-GABAergic) contributing to kinase and transcription factor activation. To this end, tetrodotoxin (500 nM) was added to the cell-stimulation media to inhibit action potential–mediated transmitter release. Furthermore, we cultured at relatively low density (200,000 cells/per 35-mm dish), approximately 10-fold lower than what we typically use for experiments that examine \( \text{Ca}^{2+} \) influx driven by excitatory GABAergic neurotransmission (Obrietan and van den Pol 1997).

Immunocytochemical techniques in combination with confocal microscopy were also used to examine GABA-mediated CREB and ERK phosphorylation in immature hypothalamic neurons. In these experiments, cultures were either mock stimulated (buffer change), or stimulated (15 min) with GABA (50 \( \mu \text{M}\)), muscimol (50 \( \mu \text{M}\)), or a depolarizing concentration of potassium (40 mM), and then fixed and processed for P-ERK and P-CREB. Double-labeling experiments revealed that GABA receptor stimulation and potassium-induced depolarization triggered the phosphorylation and the colocalized expression ERK and CREB (Fig. 4). Given the known role of the MAPK pathway as an upstream regulator of CREB phosphorylation at Ser-133 (Impey et al. 1998), we tested the hypothesis that GABA-mediated CREB phosphorylation in early development is mediated by activation of the MAPK pathway. Five DIV cultures were pretreated (30 min) with the MEK inhibitor U0126 (10 \( \mu \text{M}\)) and stimulated with GABA or muscimol. As a positive control, cultures were also stimulated with high potassium (40 mM). For all three stimuli, disruption of the MAPK pathway activity significantly attenuated CREB phosphorylation (Fig. 5, A and B). PD 98059, another MEK antagonist, also attenuated GABA-mediated CREB phosphorylation (data not shown). Together, these data reveal the MAPK pathway as a signaling intermediate that couples GABA\(_A\) receptor activation to CREB phosphorylation during early development of hypothalamic neurons.

As neurons mature, GABA’s role as an excitatory neuron-
The CREB/cAMP response element (CRE) pathway regulates an array of activity-inducible genes in the CNS (reviewed by Montminy 1997). One of these gene products, BDNF, plays an important role in many developmentally regulated processes, including neuronal differentiation, synapse formation, and cell survival (reviewed by Lu and Figurov 1997). GABA receptor stimulation during early development triggers BDNF mRNA expression in hippocampal neurons (Marty et al. 1996). Similar to the observations in hippocampal neurons, we found that GABA receptor activation triggered a significant increase in BDNF protein expression in immature hypothalamic neurons (Fig. 7). Given that the expression of BDNF is regulated by the CRE/CRE transcriptional pathway (Shieh et al. 1998; Tao et al. 1998) and that disruption of MAPK signaling attenuates CREB phosphorylation, we tested the possibility that disruption of MAPK-dependent signaling would uncouple GABA_A receptor activation from BDNF expression. For these experiments, cells were pretreated (30 min) with the MEK inhibitor U0126 (10 μM), stimulated with GABA or muscimol for 9 h, and immunocytochemically processed for BDNF expression. Figure 7D shows that GABA receptor-mediated BDNF expression was significantly attenuated by disruption of MAPK pathway activation. Together, these data suggest that a signaling cassette formed by the MAPK cascade and the

transmitter diminishes and is replaced by its well-characterized role as an inhibitory neurotransmitter. To assess whether GABA_A receptor-mediated CREB and ERK phosphorylation is lost as a function of maturation, hypothalamic neurons were maintained in culture for 18 days (a time point at which GABA_A receptors are inhibitory; Obrietan and van den Pol 1995) and stimulated (15 min) with GABA (G: 50 μM), high potassium (K: 40 mM), or forskolin (F: 5 μM). Under this condition, GABA administration did not increase P-CREB expression nor did it trigger expression of the catalytically active form of ERK. In the presence of bicuculline, both potassium and forskolin stimulated CREB and ERK phosphorylation. For each lane, the level of p-erk-2 and CREB phosphorylation was quantitated relative to the total erk-2 level.

**FIG. 3.** Inhibition of GABA_A receptor activity depressed GABA-mediated ERK and CREB phosphorylation. Hypothalamic neurons cultured for 5 DIV were incubated (15 min) with bicuculline (20 μM) and then stimulated (15 min) with GABA (G: 50 μM), high potassium (K: 40 mM), or forskolin (F: 5 μM). Under this condition, GABA administration did not increase P-CREB expression nor did it trigger expression of the catalytically active form of ERK. In the presence of bicuculline, both potassium and forskolin stimulated CREB and ERK phosphorylation. For each lane, the level of p-erk-2 and CREB phosphorylation was quantitated relative to the total erk-2 level.

**FIG. 4.** During development, GABA_A receptor activation triggers colocalized activation of CREB and ERK. Hypothalamic neurons cultured for 5 DIV were stimulated (15 min) with GABA (50 μM), muscimol (50 μM), or potassium (K: 30 mM) and then immunocytochemically processed for P-ERK and P-CREB. Relative to mock-treated cells (Control), the administration of potassium triggered a robust increase in ERK (green hue) and CREB (red hue) phosphorylation. An orange/yellow hue denotes a high level of colocalized signal. Activated ERK was found in the cell body and processes, as well as in the nucleus; P-CREB was found in the nucleus. Both GABA and muscimol triggered a robust, colocalized increase in ERK and CREB phosphorylation in a subset of neurons. To effectively reveal subcellular colocalized expression of the 2 antigens, images were captured using a scanning laser confocal microscope.
CREB/CRE transcriptional pathway link GABA excitation to BDNF expression during early development.

Despite the potential importance of BDNF in developing CNS neurons, previous electrophysiological studies have focused primarily on more mature neurons (Kang and Schuman 1995). Since BDNF may participate in mechanisms related to synaptic development, we focused on the effects of BDNF on GABA transmission at a developmental time period when GABA actions are depolarizing. Initially, to demonstrate that GABA generated excitatory actions from hypothalamic cells, four cells were recorded with gramicidin-perforated whole cell recording. Gramicidin-based recording has the advantage over conventional whole cell recording in that the gramicidin perforation leaves the internal Cl– concentration at physiological levels, critical for a rigorous assessment of the polarity of GABA actions. Application of GABA evoked an inward current in all four developing hypothalamic cells (Fig. 8), consistent with our previous observations that GABA was excitatory at this stage of development (Chen et al. 1996, Gao and van den Pol 2001).

Next we assessed the modulatory effects of BDNF on spontaneous GABAergic postsynaptic currents (PSC). Toward this end, 10 to 15 min after whole cell access was achieved and a stable baseline was recorded, 100 ng/ml human recombinant BDNF was bath-applied to the recorded neuron. Within 8 min of the application of BDNF, an enhancement of spontaneous PSC (sPSC) frequency >20% was observed in 8 of 9 cells. The enhancement of GABAergic sPSC frequency reached its peak within 8 min after the initiation of BDNF application, shown in Fig. 9B. Figure 9A depicts the traces recorded from a typical

![Images and graphs related to the text content.](http://jn.physiology.org/...)

**FIG. 5.** Disruption of signaling via the MAPK pathway attenuates GABA-induced CREB phosphorylation. Five DIV hypothalamic neurons were pretreated (30 min) with the MEK inhibitor U0126 (10 μM), stimulated (15 min), and then examined for P-CREB expression. A: relative to mock-treated cultures (Control), GABA (50 μM), muscimol (50 μM), and high potassium (K+; 40 mM) triggered CREB phosphorylation. Disruption of signaling via the MAPK cascade (+U0126) attenuated CREB phosphorylation elicited by all 3 stimuli. B: single-cell P-CREB immunofluorescence was plotted as a histogram in ascending order of signal intensity for each experimental group. C: quantitative examination of CREB phosphorylation revealed that muscimol, GABA, and K+ all elicited a significant increase in CREB phosphorylation relative to mock-treated cells. Pretreatment with U0126 significantly attenuated the capacity of the depolarizing stimuli to elicit CREB phosphorylation. *P < 0.001. Number above each bar indicates the number of cells examined per condition.
experiment. In Fig. 9C, data from all 9 neurons were analyzed and plotted. The range of this enhancement was from 124.6% above control level to 218.2% above control (defined as 100% with an average increase of 172.2 ± 19.3% of control level (61 ± 27/min, n = 9) from all nine tested neurons (P < 0.05, n = 9). Interestingly, the excitatory effects of BDNF were preserved after BDNF washout. Even 10 min after BDNF withdrawal, the frequency of spontaneous GABAergic activity was still increased by 161.1 ± 24.1% over controls (P > 0.05), suggesting a long-lasting action of BDNF. In contrast to the long-lasting effects of BDNF, control application of glutamate (100 μM) caused an increase in activity that returned to baseline within seconds after glutamate washout (data not shown).

DISCUSSION

We examined the intracellular signaling mechanisms elicited by the excitatory actions of GABA during early development. Results reveal that the MAPK pathway and the transcription factor CREB are downstream effectors of GABA receptor stimulation. In addition, we characterized a GABA-dependent increase in BDNF expression that is, in part, the result of signaling via the MAPK pathway. Finally, we observed that BDNF facilitates the synaptic release of GABA, thus a positive feedback loop is formed between GABA and BDNF, where GABA facilitates BDNF synthesis and BDNF facilitates GABA release.
GABA excitation, Ca\(^{2+}\), and the MAPK pathway

GABA’s diverse neurotrophic actions during early development are likely the result of its ability to increase intracellular Ca\(^{2+}\). We and others have reported extensively on GABA-mediated Ca\(^{2+}\) transients during early development. Ostensibly, an increase in intracellular Ca\(^{2+}\) couples to downstream kinase pathways that amplify the Ca\(^{2+}\) signal, both spatially and temporally, thereby leading to profound long-term alterations in developmentally regulated cellular processes. Indeed, numerous studies have examined how changes in cytosolic Ca\(^{2+}\) affect neuronal development. For example, the application of depolarizing levels of potassium to immature neurons increases cell survival and facilitates neurite outgrowth (Collins et al. 1991). These effects may be attributable to the opening of voltage-activated Ca\(^{2+}\) channels on the cell surface. Similar to the depolarizing actions of K\(^{+}\), GABA\(\lambda\) receptor activation triggers Ca\(^{2+}\) influx via depolarization induced opening of voltage-activated Ca\(^{2+}\) channels. Interestingly, in some models, the route of Ca\(^{2+}\) entry may determine the downstream physiological ramifications. For example, Ca\(^{2+}\) influx mediated by voltage-activated Ca\(^{2+}\) channels initiates a different set of intracellular signaling responses from NMDA receptor activation (Bading et al. 1993). Given that GABA\(\lambda\) receptors are expressed at the earliest development time studied, embryonic day 15 in rats (Chen et al. 1995; van den Pol et
al. 1995), and mature before glutamate receptors (Chen et al. 1995), GABA is well positioned to initiate a unique set of Ca^{2+}-dependent signaling events (via voltage-activated Ca^{2+} channel activation) during early neuronal development.

The trophic actions of GABA during early development result in part from changes in gene expression patterns. Given that GABA elicits a robust Ca^{2+} rise during this period, we endeavored to identify Ca^{2+}-responsive signaling pathways that were activated by GABA. Thus we focused on activation of the MAPK pathway. Brief treatment with GABA elicited activation of the MAPK pathway. Activation was likely to be the result of GABA_A receptor activation, since the effects of GABA were paralleled by the GABA_A receptor-specific agonist muscimol. Further support for a direct role for GABA_A receptors comes from the stimulus paradigm, where the cultures were pretreated with the voltage-activated Na^{+} channel antagonist tetrodotoxin, thereby blocking action potential-mediated release of transmitter. Under this condition, GABA would not be able to stimulate the action potential-dependent release of a secondary transmitter(s) that in turn could activate the MAPK cascade.

By comparison, potassium-induced depolarization elicited a more robust activation of the MAPK pathway than GABA receptor stimulation. Likely reasons for this may include the relative magnitude of evoked Ca^{2+} response (on average, K^{+} elicits a larger Ca^{2+} transient) or the percentage of responsive cells; GABA elicited a robust Ca^{2+} response in a subset of the neurons, whereas the vast majority of neurons responded to potassium with a robust Ca^{2+} rise.

Many of the trophic effects of GABA during early development are similar to the actions of the MAPK pathway. For example, GABA has been shown to affect the rate of neurite outgrowth (Barbin et al. 1993) and increase cell survival (Obata 1997). Paralleling these effects are the well-characterized roles of the MAPK cascade as a regulator of cell survival (reviewed by Cobb 1999) and a stimulator of neurite outgrowth (Barbin et al. 1993) and increase cell survival (Chen et al. 1995), and mature before glutamate receptors (Montminy 1997). The CRE consists of a palindromic 8-bp sequence (TGACGTCA) found within the enhancer regions of a variety of genes shown to play important roles in CNS physiology. Interestingly, a recent characterization of the BDNF gene identified a CRE within its exon III regulatory region (Tao et al. 1998). Robust Ca^{2+}-induced expression of BDNF requires a functional CRE, since mutation of the CRE or disruption of CRE-dependent signaling attenuates Ca^{2+}-dependent expression of BDNF (Shieh et al. 1998; Tao et al. 1998). Because of its inducibility by Ca^{2+} and its well-characterized neurotrophic effects during development, we examined whether BDNF expression is stimulated by GABA. GABA excitation led to a significant increase in BDNF expression during early development, and the disruption of signaling via the MAPK pathway attenuated BDNF expression. Given that Ca^{2+}-mediated expression of BDNF requires CREB-dependent signaling in postnatal neurons, coupled with our observation that the MAPK pathway couples to CREB phosphorylation at Ser 133, we postulate that a signaling cassette, forming the MAPK/CREB pathways, contributes to GABA-mediated BDNF expression. Our observation that GABA stimulates BDNF protein expression is in agreement with previous work showing that GABA_A receptor stimulation increases BDNF mRNA expression (Berninger et al. 1995). Interestingly, Marty et al. (1996) found that GABA-elicted BDNF expression regulates hippocampal neuronal phenotype during early development. Thus during early development, GABA may elicit many of its trophic actions by stimulating expression of genes that in turn act in autocrine and/or paracrine manner.

Similar to the effects at the level of CREB phosphorylation, disruption of MAPK signaling significantly attenuated, but did not block, GABA-evoked BDNF expression. This result indi-
cates that a signaling pathway(s) in addition to the MAPK cascade couple GABA receptor stimulation to BDNF expression. One possibility is that GABA also couples to BDNF expression via CaMKIV. Another possibility is that GABA-mediated increases in Ca$$^{2+}$$ facilitate cAMP accumulation (via type I or VIII adenyl cyclase), which in turn triggers PKA-dependent increases in BDNF expression. Either, or both, of these routes may augment the effects of MAPK pathway activation.

**BDNF facilitates synaptic GABA activity**

In the paragraphs above, the signaling pathways by which excitatory GABA activity results in an increase in BDNF expression are examined. It is of considerable interest that BDNF exerted a robust excitatory modulation of GABA activity in hypothalamic neurons. Furthermore, the excitatory actions of BDNF on excitatory GABA activity lasted long after BDNF was cleared from the bath. Long-term facilitation of GABA release raises the likelihood of a robust, amplifying, feed-forward loop formed between BABA and BDNF. The excitatory modulation of BDNF is consistent with our previous examination of another neurotrophic factor in the hypothalamus, neurotrophin 3 (NT-3). NT-3 also exerted an excitatory modulation of GABA activity, but only during the developmental period when GABA functions as an excitatory neurotransmitter (Gao and van den Pol 1999). Thus both neurotrophic factors BDNF and NT-3 can independently increase excitatory GABA activity.

The data presented here suggest a positive feedback loop between GABA and BDNF during early development, where BDNF stimulates BDNF expression, and BDNF facilitates the synaptic release of GABA. These events, which are likely to be coupled via a signaling cassette formed by the MAPK cascade and the transcription factor CREB, may be of critical importance to CNS development.

This work was supported by a National Science Foundation Grant IBN-0090974 to K. Obrietan, and National Institute of Neurological Disorders and Stroke Grants NS-34887 and NS-41454 to A. N. van den Pol.

**REFERENCES**


