Surface Expression of GABA_A Receptors Is Transcriptionally Controlled by the Interplay of cAMP-response Element-binding Protein and Its Binding Partner Inducible cAMP Early Repressor

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The regulated expression of type A γ-aminobutyric acid (GABA) receptor (GABA_A) subunit genes plays a critical role in neuronal maturation and synaptogenesis. It is also associated with a variety of neurological diseases. Changes in GABA_A receptor α1 subunit gene (GABA_A1) expression have been reported in animal models of epilepsy, alcohol abuse, withdrawal, and stress. Understanding the genetic mechanism behind such changes in α subunit expression will lead to a better understanding of the role that signal transduction plays in control over GABA_A-R function and brings with it the promise of providing new therapeutic tools for the prevention or cure of a variety of neurological disorders. Here we show that activation of protein kinase C increases α1 subunit levels via phosphorylation of CREB (pCREB) that is bound to the GABA_A1 promoter (GABA_A1p). In contrast, activation of protein kinase A decreases levels of α1 even in the presence of pCREB. Decrease of α1 is dependent upon the inducible cAMP early repressor (ICER) as directly demonstrated by ICER-induced down-regulation of endogenous α1-containing GABA_A-Rs at the cell surface of cortical neurons. Taken together with the fact that there are less α1γ2-containing GABA_A-Rs in neurons after protein kinase A stimulation and that activation of endogenous dopamine receptors down-regulates α1 subunit mRNA levels subsequent to induction of ICER, our studies identify a transcriptional mechanism for regulating the cell surface expression of α1-containing GABA_A-Rs that is dependent upon the formation of CREB heterodimers.

γ-Aminobutyric acid (GABA) type A receptors (GABA_A-Rs) are ligand-gated chloride ion channels that mediate the major aspect of fast synaptic inhibition in the mammalian brain (1). They are pentameric in structure and are composed of multiple subunit isoforms coming from eight distinct classes: α, β, γ, δ, θ, ε, π, and ρ. Diversity in receptor subtypes is controlled by the regulated expression of 19 different subunit genes and the alternative splicing of individual subunit transcripts (2–4). Most importantly, differential receptor subunit composition produces functionally and pharmacologically distinct GABA_A-Rs at certain times during development and in certain regions of the brain (5–8).

The transcription of different GABA_A-R subunit genes (GABRs) is likely to involve a complex system of regulatory controls that remain to be identified. Several lines of evidence suggest that α1 subunit expression is activity-dependent (9–11). Treatment with N-methyl-D-aspartate, a selective activator of an important class of excitatory ligand-gated ion channels, stimulates α1 subunit expression in cultured cerebellar granule cells (12, 13). In contrast, chronic treatment of cortical neurons with GABA decreases levels of GABA_A-R α1 subunit mRNAs (14–16), which is dependent on voltage-gated calcium channel activity and most likely an alteration in transcription (15). In addition, prolonged benzodiazepine (BZ) treatment decreases α1 mRNA levels in the rat hippocampal CA1 region. This decrease is associated with reduced GABA-mediated inhibition and a decrease in BZ potency to inhibit CA1 pyramidal cell-evoked responses (17).

Changes in the expression of α1 subunits have been observed in several animal models, including temporal lobe epilepsy, alcohol dependence and withdrawal, swim stress, and neonatal handling with maternal separation (18–21). Decreased levels of α1 subunit mRNAs in individual dentate granule cells have been observed in both patients and animals with temporal lobe epilepsy and are associated with functionally altered GABA_A-Rs.
that display decreased zolpidem potentiation and increased sensitivity to zinc blockade (18, 22–26).

As a foundation for understanding changes in GABA<sub>A</sub>R function that occur in vivo as a response to seizure activity, we determined whether the human and rat GABRA1 promoters contain consensus regulatory sites for activity-dependent transcription factors such as the cAMP-response element-binding protein (CREB). Phosphorylation-induced activation of CREB is regulated by multiple signal transduction pathways, including cAMP-dependent protein kinase (PKA), Ca<sup>2+</sup>-calmodulin dependent kinases, and mitogen-activated protein kinases (MAPks), that are critical for the transcription of important neural specific genes (41, 45). Once phosphorylated on serine 133, CREB bound to its DNA recognition site (CRE) can interact with the coactivator protein CBP (27, 28) leading to the recruitment of additional histone acetyltransferases to the promoter region (29, 30). The formation of a multiprotein complex containing CREB, CBP, and the basal transcriptional machinery initiates gene transcription (31, 32).

In addition to these proteins, there are bZIP repressors, including a class of truncated isoforms that can bind to CRE and CRE-like elements (33). One well characterized repressor isoform of the CREM family is inducible cAMP early repressor (ICER) (34, 35). ICER is expressed as a family of four isoforms that are produced from an internal promoter, p2, located in an intron of the CREM gene (34). Alternative splicing of the γ-exon and the ICER DNA-binding domains (DBDs I and II) generates ICERI, IIγ, II, and IIγ. The ICER proteins contain DNA binding/leucine zipper domains that make them endogenous inhibitors of transcription driven by CREB and its cognates, CREM and ATF1. ICER expression is inducible in the brain and in neuronal culture by a variety of stimuli. As an antagonist of CREB transcriptional activation, ICER appears to be of pivotal importance in gene regulation of the nervous system (36).

We now extend the importance of ICER to include the gene regulation of an important α subunit that is found in the majority of synaptic GABA<sub>A</sub>Rs. In addition, we establish a potential link between the number of α1γ2-containing GABA<sub>A</sub>Rs and the number of α1-containing GABA<sub>A</sub>Rs at the cell surface. Finally, we show that like forskolin-stimulated PKA activity, activation of D<sub>1</sub>-like dopamine receptors also induces ICER expression and subsequent down-regulation of α1 subunit levels. These studies lay the foundation for identifying the genomic programs that link the CREB/CREM family of transcription factors to long term processes of synaptic inhibition and diseases of the nervous system.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfections**—Primary rat neocortical cultures were prepared from E18 embryos (Charles River Breeding Laboratories) as described previously (37). Cultures were transfected 6–8 days after dissociation using a modified calcium phosphate precipitation method (38).

**Drug Treatments**—Drugs were dissolved in dimethyl sulfoxide (Me<sub>2</sub>SO). Final vehicle concentration was 0.5% or less for all experiments. Drugs were diluted in 20 μl of 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 15 min to 24 h after treatment. For treatments with the MAPK inhibitor (MEK1/MEK2), the following final concentration was utilized: U0126 (Calbiochem, 20 μM) and PD98059 (Calbiochem, 50 μM). The general PKC inhibitor staurosporine (Calbiochem, 500 nM), and a conventional PKC isomorph inhibitor Go6976 (Calbiochem, 1 μM) were used. The PKA inhibitor H-89 was used at a final concentration of 10 μM. Treatment with the signaling activators PMA (Sigma, 1 μM) and FSK (Sigma, 20 μM) was also utilized. Sister control dishes received vehicle (Me<sub>2</sub>SO) during the pretreatment and treatment phases. Cells were also treated for 6 h with 100 μM dopamine (DA) in the presence and absence of the D<sub>1</sub>-like antagonist SCH23390 (10 μM). Cells were pretreated with the antagonist for 1 h.

**Reporter Assays**—GABRA1 promoter fragments (−894/+70) were cloned upstream of luciferase gene in pGL2 vector (Promega) and were a generous gift of the Farb laboratory. This promoter fragment confers full promoter activity in primary neocortical neurons (Farb laboratory; +70 corresponds to exonic sequence 70 bp downstream from the first nucleotide of the first exon in the human gene).<sup>5</sup> The GABRA1 promoter/reporter (GABRA1p/reporter) containing the 2-bp mutation at the CRE site (mCRE–GABRA1) was made by site-directed mutagenesis of the wild type–GABRA1p/reporter construct. The ICER expression vector was generated from the CREM cDNA. ICER Iγ cDNA was amplified from an IMAGE human cDNA clone (GenBank<sup>®</sup> accession number BC090051, Open Biosystems, Huntsville, AL) using PCR. The sequences of the primers were as follows: ICER IIγ forward 5′-CGGGATCCATGGCTGTAACCTGGAGATGACACACAGCTGCCACTGGTG-3′; ICER IIγ reverse 5′-GCTCTAGACTGTAATCTGTTTTGGGAGAACAAATG-3′. The amplified fragments were cloned into the BamHI (5′ end) and XbaI (3′ end) sites of the expression vector pcDNA3 (Invitrogen). The construct was sequenced to confirm identity. A Western blot analysis was performed after overexpression of the ICER IIγ cDNA construct in HEK293 cells to confirm expression levels and identity. The dominant-negative M-CREB, K-CRE, or 250 ng of ICER or same amount of control vector (pRC or pcDNA3 empty vectors) were utilized for each well. 24 h after transfection, cells were assayed for luciferase (Promega) using the Victor 1420 detection system (E. G. Wallace). Luciferase counts were normalized to protein within each dish.

<sup>5</sup> M. Leach, Ph.D. thesis, unpublished data.
Regulation of GABRA1 by CREB Homo- and Heterodimers

A.

Real Time RT-PCR—PCR primers were designed using primer express software (PE Biosystems). Primer sets for α1 subunit and ICER were as follows: α1 subunit forward 5'-CCCGGGCTTGGCAACTA-3'; α1 subunit reverse 5'-CGGTTTTGTCTCAGGCTTGAC-3'; α1 subunit forward 5'-TGCTAAAAATGGACACCATAAGCCAAAGA-3'. CRE site in the GABRA1 gene. Primer sets for the CRE site were the following sequences: forward 5'-TGGTACCCTTCCCTTTTCTTTGAGAAAATTTAGGAGATGATC-3'; reverse 5'-ATACGCTCGAGCCGCGCACAG-3'. Western Blot Analysis—Total cellular proteins were extracted from primary neuronal cultures after drug treatments with standard procedures and RIPA lysis buffer (Tris, pH 7.4, 10 mM; Nonidet P-40 1%; NaCl 150 mM; SDS 0.1%; protease inhibitor mixture (Roche Applied Science) 1×; EDTA 1 mM; sodium orthovanadate 1 mM; sodium deoxycholate 0.1%; phenylmethylsulfonyl fluoride 1 mM). ~30 μg of whole cell extracts were separated by SDS-PAGE under reducing conditions on a 10 or 4–20% Tris-glycine gel and transferred to nitrocellulose membrane. Western blot analysis was performed using antibodies to CREB, pCREB (Cell Signaling), ERK, pERK, CREM1 (Santa Cruz Biotechnology), α1 GABAR subunit (Upstate), γ2 subunit (Alpha Diagnostics), and β-actin (Sigma) antibodies. The membranes were developed after incubation with peroxidase-conjugated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology) by ECL enhanced chemiluminescence.

Chromatin Immunoprecipitation (ChIP) Assay—ChIP was performed as described previously (39). Genomic DNA and protein complex were collected from primary cultured neurons after drug treatment. The DNA-protein complex was immunoprecipitated with an anti-CREB antibody or anti-pCREB antibody. Immunoprecipitated GABRA1 genomic DNA fragments were detected by real time PCR using specific primers that flank the CRE site in the GABRA1 gene. Primer sets for the CRE site were the following sequences: forward 5'-TGGTACCCTTCTCTTTTCTTTGAGAAAATTTAGGAGATGATC-3'; reverse 5'-ATACGCTCGAGCCGCGCACAG-3'. Western Blot Analysis—Total cellular proteins were extracted from primary neuronal cultures after drug treatments with standard procedures and RIPA lysis buffer (Tris, pH 7.4, 10 mM; Nonidet P-40 1%; NaCl 150 mM; SDS 0.1%; protease inhibitor mixture (Roche Applied Science) 1×; EDTA 1 mM; sodium orthovanadate 1 mM; sodium deoxycholate 0.1%; phenylmethylsulfonyl fluoride 1 mM). ~30 μg of whole cell extracts were separated by SDS-PAGE under reducing conditions on a 10 or 4–20% Tris-glycine gel and transferred to nitrocellulose membrane. Western blot analysis was performed using antibodies to CREB, pCREB (Cell Signaling), ERK, pERK, CREM1 (Santa Cruz Biotechnology), α1 GABAR subunit (Upstate), γ2 subunit (Alpha Diagnostics), and β-actin (Sigma) antibodies. The membranes were developed after incubation with peroxidase-conjugated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology) by ECL enhanced chemiluminescence.

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Regulation of GABA<sub>R</sub>1 by CREB Homo- and Heterodimers

A. | PD98059 |
PMA (min) | 0 | 5 | 15 | 30 | 60 | 15 | P-CREB | P-Erk1/2 |
--- | --- | --- | --- | --- | --- | --- | --- | --- |
control | - | - | - | - | - | - | - | - |
PMA | - | - | - | - | - | - | - | - |
Staur | - | - | - | - | - | - | - | - |

FIGURE 2. PMA activates CREB phosphorylation through a PKC-dependent pathway as measured by Western blot. A, PMA leads to time-dependent CREB activation through a MAPK-independent pathway. B, PKC inhibitor staurosporine (Staur) blocks PAM-induced CREB activation. Primary neocortical neurons were pretreated with the MEK inhibitors PD98059 (50 μM), PKC inhibitors staurosporine (500 nM), or vehicle (MeSO) for 1 h. Cultures were then treated with either vehicle (MeSO) or PMA (1 μM) for the indicated time. Total cellular proteins were analyzed using Western blot with polyclonal CREB and pCREB antibodies. Proteins were visualized using ECL following incubation with an anti-rabbit horseradish peroxidase-conjugated antibody. Normalized data (P-CREB/CREB) are presented as means ± S.E. and expressed as percent change with respect to vehicle-treated cultures (defined as 100%). Levels of total CREB did not vary with PMA treatment and were used as an internal control. Significant changes are as indicated (***, p < 0.01; mean ± S.E.; n = 3).

DNA Pulldown Assay—DNA pulldown assay was performed as described previously (40). Oligonucleotide duplexes corresponding to the sequence of the CRE site in the α1 promoter and its flanking region were covalently linked to a biotin moiety using the Pro-found mammalian coimmunoprecipitation kit form Pierce. Affinity-purified rabbit polyclonal antibodies (50 mg), raised against GABA<sub>R</sub>α2 subunit (Alpha Diagnostics), were coupled to AminoLink Plus gels specified by the manufacturer. Whole cell proteins were extracted with M-PER (mammalian protein extraction reagent) plus protease inhibitor mixture (Roche Applied Science) one time and 1 mM phenylmethylsulfonyl fluoride. Whole cell extracts were centrifuged at 10,000 × g for 30 min to remove cellular debris. Supernatants were incubated with the antibody-coupled gel overnight at 4 °C with gentle rocking. Gels were washed five times with buffer, and complexes were recovered with 100 μl of the elution buffer provided with the kit. Controls were performed by quenching the same gel before coupling antibodies to test for proteins that may bind nonspecifically to the gel. Approximately 35 μl of the eluates were analyzed by Western blot as described above with anti-α1 and γ2 polyclonal antibodies.
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A. CRE: TGAGTCATGACCTATCAGCTCA
mCRE: TGGTCATGACGCTATGGTACT
A1–CRE: CTCGGCCATGAATCTCAGGCTAT
A1–mCRE: CTCGGCCATGAACACGCGCTAT

B. Nuclear

| Treatment | CREB | A1–CRE | A1–mCRE | CRE | mCRE |
|-----------|------|--------|---------|----|------|
| control   | 250  | 250    | 250     | 250| 250  |
| PMA       | 250  | 250    | 250     | 250| 250  |
| Staurosp. | 250  | 250    | 250     | 250| 250  |

C. Binding of pCREB to GABRA1

D. GABRA1p activity

FIGURE 3. CREB-mediated regulation of GABRA1. A. Identity of oligonucleotide sequences (sense strand) used in this study. All 5'-nucleotides were biotinylated. The CRE site in the α1 promoter is highlighted in blue; and mutations with red. B, CREB binds directly to the CRE site in the GABRA1 promoter as measured by DNA pulldown assay and Western blot. Nuclear extract was obtained from primary cultured neocortical neurons with or without PMA treatment. 600 μg of nuclear extract was incubated with 1 mg of Dynabeads Streptavidin-bound to the appropriate oligonucleotides. After 15 min of incubation, beads were washed three times. Eluted proteins were then analyzed by SDS-PAGE and followed by Western blot with anti-CREB antibodies. C, PMA treatment increases association of pCREB with endogenous GABRA1 as measured by ChIP. Primary neocortical neurons were preincubated with the PKC inhibitor staurosporine (500 nM) or vehicle (Me2SO) for 1 h. Cultures were then treated with either vehicle (Me2SO) or PMA (1 μM) for 30 min. Cross-linked genomic DNA and protein complex were collected from primary cultured neurons after drug treatment. CREB-immunoprecipitated GABRA1 genomic DNA fragments were detected by real time PCR using specific primers that flank the CRE site in the GABRA1 gene. (**, p < 0.001; mean ± S.E.; n = 3). D, up-regulation of WT-CREB increases GABRA1 promoter activity in transfected primary cultured neurons. WT-CREB and dominant-negative M-CREB and K-CREB expression vectors were cotransfected with GABRA1 promoter/reporter constructs. 24 h after transfection, cultures were harvested for luciferase assays. Results are expressed as a percentage of activity measured from control (cotransfected control Prc vector) without (transgene) and GABAAR α1p/reporter construct, defined as 100%. (***, p < 0.001; mean ± S.E.)

Fluorescence Microscopy—Primary neocortical neurons were cotransfected with pDsRed2-Monomer vector (BD Biosciences) and CMV-ICER construct or control vector pcDNA3. Both ICER or control vector and pDsRed2-Monomer vector were used at a 1:1 ratio (2 μg per 35-mm dish). At 48 h after transfection, cells were washed and fixed by using a standard protocol. Immunocytochemistry was performed by using an α1-specific antibody (Upstate) without permeabilizing cells at a 1:200 dilution in 1% bovine serum albumin for 12 h at 4 °C, and secondary antibody was conjugated to fluorescein isothiocyanate at a dilution of 1:50 (Jackson ImmunoResearch). DsRed transfected cells were viewed by using an Olympus IX 71 fluorescence microscope equipped with an UPlanapo 60X/0.9 water objective lens. Fluorescence was quantified by using IPLab software (Scanalytics, Inc.) and normalized to the total area of a selected cell. Changes in subunit expression were monitored by double-blind cell-selection procedure with 28 transfected neurons per condition (n = cultures from three different animals).

RESULTS

α1 Subunit Expression Is Increased after PMA Treatment via PKC Activation—As a first step toward understanding how GABRA1 transcription is regulated by extracellular signaling, primary cultured neurons from E18 embryonic cortex were exposed to PMA, a stimulator of both PKC and MAPK. A 6-h treatment with PMA (1 μM) increases α1 subunit mRNA levels by 65% as compared with vehicle control when measured by real time RT-PCR (Fig. 1A). Such an increase was completely blocked by the broad spectrum PKC inhibitor, staurosporine (500 nM, 78% of control), and also by the conventional PKC isoform inhibitor Go6976 (1 μM, 114% of control). Increased levels were not blocked by the MEK (MAPK kinase) inhibitors, PD98059 and U0126 (Fig. 1A). To determine whether changes in α1 mRNA levels might be because of a transcriptional mechanism, we monitored the activity of a −894/+70-bp fragment of the human GABRA1 promoter (GABRA1p) in transfected cortical neurons. GABRA1p promoter activity, as measured by the luciferase reporter assay, is up-regulated in response to PMA. 1 μM PMA produces a 40% increase in GABRA1p/reporter activity that is dependent upon PKC activation (as determined by specific blockade with the PKC inhibitor, staurosporine) (Fig. 1B). Consistent with changes in α1 mRNA levels, levels of α1 subunit protein are increased by almost 2-fold 24 h after treatment with PMA (Fig. 1C). Taken together, these observations suggest that neurons possess a mechanism to up-regulate α1 subunit levels via activation of PKC and not MAPK.

Activation of CREB after PMA Treatment Is PKC- and Not MAPK-dependent—Given that α1 subunit up-regulation is dependent upon PKC and not MAPK, and that activity of GABRA1p is also increased in response to PKC stimulation, we sought to identify the critical transcription factors that mediate this process. Based on the presence of an atypical CRE site in GABRA1 and the fact that CREB is known to be activated by PKC (41–43), we asked whether CREB might play a role in PMA-induced α1 subunit up-regulation. Stimulation of cortical neurons with PMA leads to a time-dependent activation of CREB that reaches a peak level of ~3-fold increase when compared with control at 15 min (Fig. 2A). Like α1 subunit mRNA levels, pretreatment with the MEK inhibitor PD98059 fails to block PMA-induced CREB activation (Fig. 2A, upper panel). To test for the activity of MAPK inhibitors, we also measured MAPK activation of ERK as a control in our studies. As expected, PMA treatment increases MAPK activation in a time-dependent manner, and PD98059 inhibits the increase (Fig. 2A, lower panel).
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Figure 4. FSK stimulation of primary cultured neocortical neurons decreases α1 subunit expression and promoter activity. A, alteration of α1 subunit mRNA levels by FSK treatment over time. Cultures were treated with either vehicle (Me2SO) or FSK (20 μM) as indicated. Total RNA was extracted, and real-time PCR was performed using PCR primers and probes specific for α1 subunit and cyclophilin mRNAs. Experimental data were normalized to levels of cyclophilin. mRNA abundance in each treatment group is expressed relative to the vehicle (Me2SO) (*, p < 0.05; **, p < 0.01; mean ± S.E.; n = 3). B, FSK produces a decrease in GABRA1p activity consistent with changes in endogenous α1 mRNAs. Rat neocortical neurons were transfected with GABA1α mutant constructs (−894/+70). 18 h after transfection, cultures were treated with FSK (20 μM) or Me2SO. Cultures were harvested, and luciferase assays were performed. Results are expressed as percentage of activity measured from control (Me2SO). (**, p < 0.001; mean ± S.E.; n = 5). C, FSK decreases α1 subunit levels. Cultures were treated with FSK (20 μM) for 4, 6, or 24 h. Whole cell extracts were resolved by SDS-PAGE and visualized using ECL. α1 subunit and β-actin levels were quantified by densitometry. Normalized data (α1/β-actin) are presented as means ± S.E. and expressed as percent change with respect to vehicle-treated cultures (defined as 100%). Levels of β-actin did not vary with PMA treatment and were used as an internal control. (*, p < 0.05; mean ± S.E.; n = 3).

lower panel). These results confirm that PMA-induced activation of CREB occurs through a MAPK-independent pathway.

To confirm PKC-dependent activation of CREB, we used the PKC inhibitor staurosporine to block PMA-induced CREB phosphorylation (Fig. 2B). Staurosporine also blocks PMA-induced MAPK activation supporting previous observations in the literature that PKC activates ERK as well as its immediate upstream activator MEK (44). Taken together, these data indicate that PKA activates both CREB and MAPK through a PKC-dependent pathway. Most importantly, the activation of CREB by PKA is independent of the PKC activation of MAPK, paralleling our PKA-specific change in GABRA1 expression as reported in Fig. 1.

CREB Regulates GABRA1 Gene Expression through PKC Activation—Because there is a CRE in the proximal GABRA1 promoter region (Fig. 3A), we asked whether PMA-induced increase in GABRA1 expression is dependent upon CREB recognition of the proximal promoter region. DNA pulldown assays were used to detect whether PMA treatment enriches binding of CREB to the GABRA1p CRE-binding site. Oligonucleotide duplexes corresponding to the sequence of the CRE site in GABRA1p and its flanking region, or three copies of a consensus CRE site (Fig. 3A), were covalently linked to a biotin moieties at their 5′ ends, and such oligonucleotides and their nuclear binding proteins were recovered using magnetic streptavidin Dynabeads. Nuclear binding proteins were prepared from cultured neocortical neurons before and after PMA treatment. Beads were collected and washed, and the bound proteins were eluted, separated by SDS-PAGE, and then analyzed by Western blot. As shown in Fig. 3B, 2nd and 3rd lanes, CREB binds to the CRE site in GABRA1p with or without PMA treatment. Moreover, binding is sequence-specific because the presence of a 2-bp mutation in the CRE site removes CREB recognition (Fig. 3B, 4th, 5th, and 7th lanes). CREB binding also occurs at the control consensus oligonucleotides that contain three copies of a classical CRE site.

To determine whether activated CREB is bound to the endogenous GABRA1 in primary cortical neurons, ChIPs were performed. The endogenous GABRA1p DNA-protein complex was immunoprecipitated with either anti-CREB or anti-phospho-CREB (Ser-133) antibodies after a 30-min 1 μM PMA treatment, and precipitated GABRA1 genomic fragments were detected by real-time PCR. Although binding of CREB to the endogenous GABRA1p is detected by ChiP, levels of total CREB do not change with PMA stimulation (data not shown). However, when DNA is precipitated with the phospho-CREB (pCREB) antibody, there is an ~2-fold increase of pCREB association with the promoter suggesting that PMA increases CREB phosphorylation while bound to GABRA1p, consistent with the classical model of CREB-mediated gene regulation. Increase in pCREB association at GABRA1p is also blocked by the PKC inhibitor staurosporine (Fig. 3C).

To further investigate if increased association of pCREB is necessary for GABRA1 up-regulation, wild type CREB (WT-CREB) and serine 133 mutated dominant-negative CREB (M-CREB) were cotransfected with the human GABRA1p reporter construct (894/+70, see “Experimental Procedures”) into neocortical neurons in culture, and promoter activity was measured 24 h after transfection. Overexpression of WT-CREB increases GABRA1p activity ~2-fold in transfected primary cultured neurons; however, overexpression of M-CREB is without effect (Fig. 3D). These data strongly suggest that although both CREB and activated CREB bind to the GABRA1 promoter, only the activated CREB mediates PMA-induced GABRA1 expression.

FSK Stimulation Decreases α1 Subunit Expression—It is well known that multiple pathways can lead to CREB activation and the regulation of gene expression (41, 42, 45). Evidence has shown that CREB phosphorylation occurs through the activation of distinct signaling pathways that differentially regulate the expression of multiple target genes (42). To investigate whether CREB phosphorylation via an additional signaling pathway also regulates GABRA1 expression, forskolin (FSK) was used to activate PKA in primary cortical neurons. Although FSK treatment leads to robust and long-lasting CREB activation (data not shown), unlike PMA, a 20 μM FSK treatment decreases α1 subunit mRNA levels in a time-dependent manner (Fig. 4A). 1 h of FSK treatment decreases α1 mRNA levels by ~30%. This decrease peaks at 2–4 h of treatment (~50%). To
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A.

B.

C.

D.

FIGURE 5. Induction of ICER is PKA-dependent and regulated by the D1 dopamine receptor. A, ICER induction is specific to the cAMP pathway. Cultures were treated with either vehicle (Veh; Me2SO), PMA (1 μM), or FSK (20 μM) as indicated. Total RNA was extracted, and ICER mRNA abundance was measured by real time RT-PCR. Data were normalized to cyclophilin (**, p < 0.01; ***, p < 0.001; mean ± S.E.; n = 3). B, ICER proteins are induced by FSK. Cultures were treated with either vehicle (Me2SO) or PMA (1 μM) or FSK (20 μM) as indicated. Total cellular proteins were extracted and Western blot was performed using polyclonal CREM antibodies. Proteins were visualized using ECL. Levels of β-actin protein did not vary with FSK treatment and were used as an internal control. C, dopamine (DA) induces ICER synthesis via the D1-like receptor and the PKA signaling pathway in neocortical neurons. Cultures were pretreated with the D1 antagonist SCH23390 (SCH, 10 μM), PKA inhibitor H-89 (10 μM), or vehicle (water, Me2SO) for 1 h. Subsequently, cultures were treated with either DA (100 μM), vehicle (water), antagonist (SCH or H-89), or DA and antagonist for 4 h. Total RNA was extracted, and ICER mRNA abundance was measured by real time RT-PCR. Data were normalized to cyclophilin (**, p < 0.01; *, p < 0.05; mean ± S.E.; n = 4). D, DA decreases α1 subunit mRNA levels. Cultures were treated with either vehicle (water) or DA (100 μM) for 6 h. Total RNA was extracted, and α1 subunit mRNA abundance was measured by real time RT-PCR. Data were normalized to cyclophilin (**, p < 0.01; mean ± S.E.; C, n = 5; D, n = 4).

determine whether decreased mRNA levels reflect a change in GABRA1 transcription, functional promoter/reporter assays were used to confirm that 4 h of FSK (20 μM) decreases GABRA1p activity by ~40% (Fig. 4B). The decrease of α1 mRNA levels and GABRA1p activity are paralleled by changes in α1 protein levels. As shown in Fig. 4C, α1 subunit levels show no change after 4 h of FSK but decrease by 32% at 6 h and further decrease by 51% after 24 h. A representative Western blot is shown in Fig. 4C, upper panel.

Activation of cAMP Pathway Induces ICER Expression—As shown in Figs. 2 and 4, PMA and FSK both activate CREB, but α1 mRNA levels differ in polarity (PMA increases levels although FSK decreases levels). One possible explanation is that phosphorylated CREB recruits different binding partners via activation of distinct signaling pathways. Like all bZIP transcription factors, CREB family members contain a C-terminal bZIP domain that mediates DNA binding and a leucine zipper domain that facilitates dimerization. One feature common to all bZIP transcription factors is the high degree of homology in the DNA binding domain (DBD) of these proteins. Different members of the family can form homo- and heterodimers that bind to similar DNA-binding sites, CRE or CRE-like elements (46, 47). In particular, CREB/ICER heterodimers form in a cell- and signal-specific manner (34, 35). To determine whether induction of ICER might occur in response to FSK treatment and contribute to GABRA1 repression, RT-PCR was used to monitor the presence of ICER mRNAs after selective drug treatment. As shown in Fig. 5A, ICER mRNA is not induced by PMA, but after a 1-h exposure to FSK, ICER mRNA levels rise reaching a peak at ~4 h and then decreasing at 6 h.

ICER gene products are remarkably small proteins of either 108 or 120 amino acids with the predicted sizes of 12 and 13.5 kDa, excluding or including the γ domain, respectively (ICER γ and ICER). To analyze the presence of ICER proteins, Western blot analysis was performed to analyze extracts of FSK-treated primary cortical neurons. FSK induces ICER proteins with kinetics similar to ICER transcripts, rapid and transient, a characteristic of immediate early genes (Fig. 5B). In contrast, ICER is not induced after 4 h of PMA treatment of neocortical neurons (Fig. 5B, 8th lane). The time course for ICER induction in response to FSK is similar to the time course for α1 mRNA down-regulation, suggesting that ICER may be a repressor of GABRA1 transcription.

To investigate whether a physiological signal that activates the cAMP/PKA pathway would also regulate α1 gene expression, neocortical neurons were treated with dopamine (DA, 100 μM), and ICER and α1 mRNA levels were detected using real time RT-PCR. Four-hour DA treatment dramatically increases ICER mRNA levels (4.7-fold; Fig. 5C). This increase precedes the decrease in the levels of α1 mRNAs and is attenuated by cotreatment with the D1-like receptor antagonist SCH23390 and the PKA inhibitor H-89, suggesting that D1 receptor-mediated activation of the cAMP/PKA pathway is responsible for induction of ICER synthesis. As shown in Fig. 5D, 6-h DA treatment decreases α1 mRNA levels by 33%.

PKA-induced ICER Synthesis Is Responsible for GABRA1 Transcriptional Repression—As shown in Fig. 3, B and C, CREB can bind to the CRE site in GABRA1. Activation of the PKC pathway increases pCREB association with GABRA1 through
the CRE site. To investigate whether ICER and CREB can both bind to the CRE site in GABRA1 after FSK treatment, DNA pulldown assays were performed as described for Fig. 5. Nuclear extracts prepared from cultured neocortical neurons, either treated with or without FSK for 4 h, are incubated with beads to which α1 CRE-containing oligonucleotide duplexes have been attached. After incubation with lysates, bead-purified CRE-binding proteins were separated by SDS-PAGE and then analyzed by Western blot using both CREB and ICER antibodies. Consistent with the results of Fig. 3B, CREB binds to the GABRA1-CRE site before FSK treatment (Fig. 6A, 1st lane, upper panel). Presence of CREB at the CRE site is not altered after a 4-h FSK treatment (Fig. 6A, 2nd lane, upper panel). As ICER is absent after 4-h FSK treatment (Fig. 6A, 1st lane, lower panel), in contrast, after 4 h of FSK stimulation, there is robust binding of ICER to the GABRA1-CRE (Fig. 6A, 2nd lane, lower panel).

Whether ICER induction is responsible for the decrease of α1 mRNA levels after FSK treatment still remains to be identified. To begin to address this possibility, we performed promoter assays in neocortical neurons in culture in the presence and absence of overexpressed CREB and ICER along with GABRA1p/reporter constructs. Similar molar amounts of CREB and ICER were transfected in each promoter study. As shown in Fig. 6B, CREB overexpression alone increases GABRA1 activity, whereas overexpression of ICER alone or in combination with CREB reduces GABRA1p activity.

We next determined whether ICER induction is PKA-dependent consistent with FSK-induced down-regulation of endogenous α1 subunit expression. The PKA inhibitor H-89 (10 μM) partially blocks FSK-induced ICER expression (53% blockade, Fig. 6C) as well as CREB phosphorylation (Fig. 6D) by activation of the PKA pathway in primary neocortical neurons. Because it has been reported that FSK can also activate the MAPK pathway (48), the MEK inhibitor U0126 was also used to determine whether an additional signaling pathway contributes to FSK-induced pCREB and ICER synthesis. In contrast to H-89, pretreatment with U0126 (10 μM) has no effect on FSK-induced ICER expression, suggesting that the induction of ICER under these conditions is MAPK-independent (Fig. 6D, 4th lane). The MAPK pathway may, however, be involved in FSK-induced CREB phosphorylation. As shown in Fig. 6D, 3rd and 4th lanes, U0126 alone does not block FSK-induced pCREB; 10 μM H-89 alone partially inhibits pCREB; and Fig. 6D, 5th lane, U0126 with 10 μM H-89 completely blocks pCREB. The same degree of blockade is seen with both 10 μM H-89 and U0126 by using double the concentration of U0126 (20 μM). These results suggest that PKA and MAPK pathways synergistically contribute to FSK-induced pCREB levels. Such an effect was not observed in FSK-induced ICER synthesis (Fig. 6D, 3rd and 5th lanes) further supporting the conclusion that FSK induction of ICER is MAPK-independent.

Although H-89 partially inhibits ICER expression, it fails to reverse the down-regulation of α1 subunit mRNA levels that is induced by FSK (Fig. 7A). This result might occur if activation of PKA is also necessary for maintaining basal levels of α1 subunit consistent with FSK-induced down-regulation of α1 CRE-containing oligonucleotide duplexes have been attached. After incubation with lysates, bead-purified CRE-binding proteins were separated by SDS-PAGE and then analyzed by Western blot using both CREB and ICER antibodies. Consistent with the results of Fig. 3B, CREB binds to the GABRA1-CRE site before FSK treatment (Fig. 6A, 1st lane, upper panel). Presence of CREB at the CRE site is not altered after a 4-h FSK treatment (Fig. 6A, 2nd lane, upper panel). As ICER is absent after 4-h FSK treatment (Fig. 6A, 1st lane, lower panel), in contrast, after 4 h of FSK stimulation, there is robust binding of ICER to the GABRA1-CRE (Fig. 6A, 2nd lane, lower panel).

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Although H-89 partially inhibits ICER expression, it fails to reverse the down-regulation of α1 subunit mRNA levels that is induced by FSK (Fig. 7A). This result might occur if activation of PKA is also necessary for maintaining basal levels of α1 sub-
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**FIGURE 7. Down-regulation of α1 subunit mRNA levels is reversed after treatment with ICER-specific siRNAs.** A, inhibition of PKA activation fails to reverse FSK-induced down-regulation of α1 subunit mRNA levels. Primary neocortical neurons were pretreated with the PKA inhibitor H-89 (10 μM) or Me2SO for 1 h. Cultures were then treated with either Me2SO, FSK (20 μM), or FSK and H-89 for the following 4 h. α1 subunit mRNA abundance was measured by real-time RT-PCR. Data were normalized to cyclophilin mRNA levels. Levels of RNA are expressed relative to the vehicle (VER), defined as 100%. (**, p < 0.01; mean ± S.E.; n = 3.) B, ICER siRNA treatment attenuates ICER induction after FSK (*, p < 0.05; **, p < 0.01; mean ± S.E.; n = 6.) C, ICER siRNA treatment attenuates FSK-induced decreases in α1 mRNA levels (**, p < 0.01; *** p < 0.001; mean ± S.E.; n = 6.) ICER siRNAs (ICER1 or ICER2) and scramble siRNAs (control) were transfected into primary cultured neocortical neurons prior to FSK treatment. After 4 h of FSK, RNA was isolated and mRNA levels of ICER (B) or α1 (C) were determined by real-time RT-PCR. Data were normalized to cyclophilin mRNA levels. Bars and C, inset histograms display the relationship between % reduction in ICER and % recovery of α1 mRNA levels in the presence of ICER siRNAs after treatment with FSK. Note that % recovery of α1 mRNA levels is consistent with % reduction of ICER mRNA levels after siRNA treatment.

units. In this scenario, inhibiting PKA may inhibit the function of a yet to be identified critical activator. To further determine the importance of ICER for FSK-induced down-regulation of GABRA1 transcription, two pairs of ICER siRNAs were transfected into primary cultured neurons prior to FSK treatment. Transfection of ICER siRNAs was found to successfully inhibit ICER induction (~50%) after a 4-h FSK treatment (Fig. 7B). The percentage of inhibition is consistent with the transfectional efficiency of siRNAs in the assay. Most importantly, specific inhibition of ICER induction by siRNAs attenuates the decrease of α1 mRNA levels induced by FSK. α1 subunit mRNA levels decrease by 48% in scramble siRNA-transfected neurons after 4 h of FSK treatment, similar to the decrease in 4-h FSK-treated cultures in the absence of siRNA transfection (Fig. 5A). In ICER siRNA-transfected neurons, 4-h FSK treatment causes an increase in α1 mRNA levels to 69 and 71% of control levels, again consistent with transfectional efficiency of siRNAs in these studies (30–50%) (Fig. 7, B and C, see insets). Taken together, our results strongly suggest that induction of ICER is responsible for PKA-induced down-regulation of GABRA1 transcription.

The CRE Site in GABRA1 Is Responsible for Bi-directional Regulation of α1 Subunit Expression—To determine whether CREB and ICER recognition of the GABRA1-CRE site is necessary for their regulation of α1 promoter activity in cultured cortical neurons, a mutant form of CREB (K-CREB), which does not bind DNA but can dimerize to endogenous CREB, was cotransfected with GABRA1p/reporter constructs. Unlike wild type CREB, overexpression of K-CREB fails to increase GABRA1p activity (Fig. 3D, 4th column). Interestingly, a 2-bp mutation in the GABRA1-CRE site (Fig. 8A) does not change promoter activity in transfected neurons suggesting that CREB is not responsible for basal levels of α1 gene expression (Fig. 8B). However, the PMA-induced increase in GABRA1 promoter activity is abolished in promoter constructs that contain the CRE mutation. Similar to the PMA dependence on an intact CRE site, FSK down-regulation of α1 promoter activity is also dependent on the CRE site (Fig. 8B). Finally, the role of the CRE site in GABRA1p was determined using overexpression of CREB/ICER constructs to show loss of CRE-mediated bidirectional regulation (Fig. 8C) and determined directly using a DNA pulldown purification assay with nuclear extracts from treated and untreated primary cultured neocortical neurons. CREB binding to the GABRA1-CRE site is lost when the oligonucleotides contain a 2-bp mutation (Fig. 8B). Taking together, these results show that CREB regulates GABRA1 gene expression through the CRE site of the GABRA1p and that binding of CREB and/or CREB and ICER is necessary for bi-directional changes in GABRA1 transcription.

ICER Induction Is Associated with Decrease in α1γ2-Containing GABAARs and Decrease in α1 Subunits Detected at the Cell Surface—The majority of GABAAR subtypes that contain α1 subunits are composed of α1β2γ2 (49). To determine whether the decreased expression of GABRA1 after FSK treatment may result in an alteration in the subunit composition of GABAARs, we performed immunoprecipitation of GABAARs. Neocortical neurons were treated with FSK or vehicle for 24 h, and whole cell protein extracts were prepared and then immunoprecipitated with anti-γ2 subunit antibodies that were chemically linked to AminoLink Plus gel. After several stringent washes, the precipitate was eluted and separated by SDS-PAGE. Western blot was performed using anti-α1 or -γ2 antibodies. The ratio of α1 to γ2 subunits was used to reflect the abundance of α1 subunits in γ2 containing GABAARs. 24 h of FSK treatment decreases ~40% of the ratio of α1 to γ2 subunits compared with vehicle-treated cultures (Fig. 9A). This decrease is consistent with
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A.

GABRA1 promoter CRE region

Wildtype Mutation

CTTCGGCATAAGGTCAACGCCTAT
CTTCGGCATGACACAACGCCTAT

B.

GABRA1p activity (% control)

Vehicle

160

140

120

100

80

60

40

20

0

wildtype mutated

**

**

C.

GABRA1p activity (% control)

CREB ICER CREB ICER

Vehicle

150

140

130

120

110

100

90

80

70

60

50

40

30

20

10

0

wildtype mutated

**

**

FIGURE 8. The CRE site of GABRA1p directs bidirectional regulation of GABRA1 in neocortical neurons. A, identity of the 2-bp mutation in the CRE site of GABRA1p is indicated. CRE site (blue); mutation (red). B, loss of PMA and FSK stimulation in transfected primary cultured neurons that contain the CRE-mutated GABRA1p (mCRE-promoter). Rat neocortical neurons were transfected with GABRA1p/reporter or mCRE-promoter/reporter constructs. 18 h after transfection, cultures were treated with PMA (1 μM), FSK (20 μM), or vehicle for 6 or 4 h. Cultures were harvested for luciferase assays. Results are expressed as percentage of activity measured from wild type GABRA1p. Data are shown as mean ± S.E., n = 5. **, p < 0.01. C, cotransfection of WT-CREB or ICER with mCRE-promoter has no effect on promoter activity. WT-CREB and/or ICER were cotransfected with wild type or CRE-mutated GABRA1p/reporter constructs. 24 h after transfection, cultures were harvested, and luciferase assays were performed. Results are expressed as percentage of activity measured from control (cotransfection of empty Prc and Pcneo vectors and mCRE-promoter/reporter constructs), defined as 100%. (Data are shown as mean ± S.E., n = 5.) **, p < 0.01.

the decrease in expression of α1 subunits after FSK stimulation. ICER induction as mimicked by overexpression in individual neurons was also shown to regulate the levels of endogenous α1 subunits to the same degree as FSK-induced down-regulation when assayed using nonpermeabilized transfected neurons (Fig. 9B). This result strongly suggests that ICER can regulate the number of α1-containing GABA_1Rs at the plasma membrane.

DISCUSSION

We have shown that two distinct signal transduction pathways regulate GABRA1 through CREB binding and activa-

tion. It is known that CREB can be phosphorylated through a PKC-dependent pathway (41). Results of our studies show that PMA up-regulates GABRA1 expression by activating CREB via the PKC pathway. Although PKC can directly phosphorylate CREB in vitro at multiple sites, including Ser-133 (43), most of the results in the literature suggest that CREB phosphorylation occurs downstream of PKC after activation of the ERK pathway (41, 44, 48). Because PMA, in addition to PKC stimulation, also activates the MAPK pathway, we used MEK (MAPK kinase) inhibitors in our studies to determine whether PMA-induced CREB phosphorylation and GABRA1 up-regulation are MAPK-dependent, as suggested by studies in the literature. Results of our studies suggest that activation of PKC up-regulates GABRA1 expression in a MAPK/ERK-independent manner that is mediated by CREB phosphorylation. G66976, an inhibitor of the conventional PKC isoforms, blocks both CREB phosphorylation and PMA-induced up-regulation of GABRA1, whereas MAPK inhibitors do not. These results when taken together suggest that PMA may directly activate the conventional form of PKC to phosphorylate CREB. Recent findings that PKCa, one isoform of conventional PKCs, can translocate to the nucleus and directly phosphorylate CREB support our observations of a direct mechanism (50).

Increased GABA_1R α1 subunit mRNA levels in response to PMA treatment are accompanied by increased binding of pCREB to the endogenous GABRA1 promoter in cultured neocortical neurons as measured by chromatin precipitation. Overexpression of WT-CREB increases GABRA1 promoter activity, whereas a Ser-133 mutated dominant-negative form of CREB (M-CREB) is without effect. Taken together, these findings suggest that CREB is a stimulus-inducible transcriptional activator controlled by phosphorylation that up-regulates endogenous GABRA1 transcription.

In addition to PKC activation, we show that activation of the cAMP pathway, using FSK, decreases α1 subunit mRNA levels in neocortical cultures in the presence of pCREB. One feature common to bZIP transcription factors is the high degree of homology in their DBDs. Different members of this family form homo- and/or heterodimers binding to similar DNA recognition sites. It is known that when a CRE is occupied by a homodimer or heterodimer specific to the activator isoforms, transcription of a target gene is augmented. However, when the same DNA site is occupied by a heterodimer containing an activator and a repressor isoform or by repressor homodimers, transcription is repressed, presumably because of impaired interaction with CBP or TFIID (46, 47). Control over the occupancy of the CRE site by activating or repressing dimers is believed dependent upon the relative abundance and affinities of the three classes of dimers for the target CRE.

We show that ICER, a powerful repressor among the CREB family of transcription factors, is induced in primary cultured neocortical neurons through the activation of the cAMP/PKA pathway by forskolin and, most importantly, by the neurotransmitter DA (Fig. 5, C and D). The fact that DA induces ICER strongly suggests that the activity of endogenous neurotransmitter receptor systems use the CREB signaling pathway to regulate inhibition in the brain. Given the important role of DA in
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A.

\[
\begin{align*}
\alpha_1: & \quad \gamma_2 \text{ IP} & \text{ control} \\
\gamma_2: & \quad \text{ Western blot with anti-} \alpha_1 & \text{ and } \gamma_2 \text{ subunit antibodies. Controls were performed by quenching the gel before coupling antibodies to test for proteins that may bind nonspecifically to the gel matrix. } \\
\end{align*}
\]

B.

FIGURE 9. Effects of ICER induction on receptor levels and cell surface expression. A. FSK stimulation of primary cultured neocortical neurons decreases the abundance of \( \alpha_1 \gamma_2 \) containing GABARs. Cultures were treated with either vehicle (MeSO) or FSK (20 \( \mu \)M) for 24 h. Total cellular proteins were extracted and applied to \( \gamma_2 \) subunit antibody-coupled AminoLink plus gel. After overnight incubation, the \( \gamma_2 \) subunit antibody-coupled protein complexes were eluted and separated by SDS-PAGE and then followed by Western blot with anti-\( \alpha_1 \) and \( \gamma_2 \) subunit antibodies. Controls were performed by quenching the gel before coupling antibodies to test for proteins that may bind nonspecifically to the gel matrix. \( \alpha_1 \) and \( \gamma_2 \) subunit levels were quantified by densitometry. Normalized data (\( \alpha_1 \) subunit/\( \gamma_2 \) subunit) as presented as means \( \pm \) S.E. and expressed as percent change with respect to vehicle (VEH)-treated cultures (defined as 100%). A representative Western blot shows decreases in \( \alpha_1 \) subunit abundance after \( \gamma_2 \) subunit immunoprecipitation in FSK-treated cultures (upper); quantification of data is shown in the lower histogram. (*, \( p < 0.05; \) mean \( \pm \) S.E.; \( n = 3 \)). B. Overexpression of ICER decreases the endogenous levels of \( \alpha_1 \) subunit detected at the cell membrane. Primary cultured neocortical neurons were cotransfected with pDsRed2-Monomer and ICER expression (CMV-ICER) or control vectors (CMV-empty). At 48 h after transfection, unpermeabilized cells were fixed and stained with an \( \alpha_1 \)-specific antibody using a standard protocol. The DsRed transfected cells were viewed by using a Olympus IX71 fluorescence microscope, and the images were analyzed by using IPlab software (see “Experimental Procedures”). Representative images are shown (empty vector, upper panel; ICER construct, lower panel). Quantitation data are presented in the histogram (***, \( p < 0.01; \) mean \( \pm \) S.E.; \( n = 3 \)).

In our studies, although both CREB and ICER bind to the CRE site of GABRA1 as monitored using DNA pulldown assay, it is difficult to determine whether ICER homodimers, or CREB and ICER heterodimers, or both, drive down-regulation of \( \alpha_1 \) expression in vivo. Based on the literature, when CREB and ICER are coexpressed, both ICER homodimers and ICER/CREB heterodimers have been detected at various CRE sites (35, 51, 53). The precise sequence of the CRE site has been shown to affect the affinity of CREB and its family members for its DNA element (54, 55). Given that the \( \alpha_1 \) CRE site is a noncanonical CRE, it is reasonable to expect that one form of the repressing dimers might have a higher affinity for the asymmetric CRE site. Further studies will be needed to determine whether there is, in fact, a preferential ICER dimer form that recognizes the GABRA1 in response to FSK. In contrast, PMA stimulation fails to induce ICER; therefore, activated CREB most likely forms homodimers to up-regulate GABRA1 transcription.

The GABA\(_{\text{A}}\)R \( \alpha_1 \) subunit is the most abundant \( \alpha \) subunit variant in the brain. At least half of the GABA\(_{\text{A}}\)Rs are believed to contain the \( \alpha_1 \) subunit that is highly expressed throughout most brain regions (56–58). The majority of GABA\(_{\text{A}}\)R subtypes containing the \( \alpha_1 \) subunit are found with a \( \beta_2 \) and \( \gamma_2 \) subunit, \( \alpha_1 \beta_2 \gamma_2 \) (49), at the synapse. In vitro studies have suggested that \( \alpha_1 \) subunit expression confers specific pharmacological properties to the GABA\(_{\text{A}}\)R. Recombinant expression studies indicate that the presence of the \( \alpha_1 \) subunit contributes to the efficacy of GABA agonists (59) and the maximal response to BZs (60). Knockdown of \( \alpha_1 \) subunit expression using antisense deoxyoligonucleotides results in a decrease in GABA-mediated chloride flux (61). Patch clamp recordings show a reduction in the amplitude of evoked inhibitory postsynaptic currents in slices from visual cortex after treatment with an antisense deoxyoligonucleotides (62). In addition, a decrease in \( \alpha_1 \) subunit expression has been associated with a decrease in the \( E_{\text{max}} \) of muscimol-stimulated chloride flux after chronic ethanol administration (19), although there is no change in total receptor number (63).

Recently, two mouse lines in which the \( \alpha_1 \) subunit has been genetically deleted have been developed and characterized
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α1-containing GABAA Rs that can be detected at the cell surface membrane (Fig. 9B).

Taken together, our results are summarized as a working model (Fig. 10); CREB activation via the PKC pathway leads to the formation of CREB homodimers that bind to the GABRA1p-CRE site to increase transcription. In contrast, CREB activation via the PKA pathway, along with PKA-induced ICER synthesis, leads to the formation of ICER homodimers or CREB/ICER heterodimers to repress transcription. The discovery that α1 subunit levels can be differentially regulated by CREB/CREM family members uncovers a new mechanism of GABRA1 gene expression that may be operative in neurological diseases (such as temporal lobe epilepsy, alcohol dependence/withdrawal, and stress) and may provide novel opportunities for therapeutic intervention.

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REFERENCES

1. MacDonald, R. L., and Olsen, R. W. (1994) Annu Rev. Neurosci. 17, 569–602
2. Olsen, R. W., and Tobin, A. J. (1990) FASEB J. 4, 1469–1480
3. Burt, D. R., and Kamatchi, G. L. (1991) FASEB J. 5, 2916–2923
4. Sieghart, W. (2000) Trends Pharmacol. Sci. 21, 411–413
5. Beattie, C. E., and Siegel, R. E. (1993) J. Neurosci. 13, 1794–1792
6. Laurie, D. J., Wisden, W., and Seeburg, P. H. (1992) J. Neurosci. 12, 4151–4172
7. Wisden, W., Laurie, D. J., Monyer, H., and Seeburg, P. H. (1992) J. Neurosci. 12, 1040–1062
8. Reynolds, D. S., O’Meara, G. F., Newman, R. J., Bromidge, F. A., Atack, J. R., Whiting, P. J., Rosahl, T. W., and Dawson, G. R. (2003) Neuropharmacology 44, 190–198
9. Huntsman, M. M., Isackson, P. J., and Jones, E. G. (1994) J. Neurosci. 14, 2236–2259
10. Meinecke, D. L., and Rakic, P. (1990) Brain Res. Rev. Brain Res. 55, 73–86
11. Zheng, T., Santi, M. R., Bovolin, P., Marlier, L. N., and Grayson, D. R. (1993) Brain Res. Dev. Brain Res. 75, 91–103
12. Harris, B. T., Charlton, M. E., Costa, E., and Grayson, D. R. (1994) Mol. Pharmacol. 45, 637–648
13. Zhu, W. J., Vicini, S., Harris, B. T., and Grayson, D. R. (1995) J. Neurosci. 15, 7692–7701
14. Baumgartner, B. J., Harvey, R. J., Darlison, M. G., and Barnes, E. M., Jr. (1994) Brain Res. Mol. Brain Res. 26, 9–17
15. Lyons, H. R., Gibbs, T. T., and Farb, D. H. (2000) J. Neurochem. 74, 1041–1048
16. Montpied, P., Ginnis, E. J., Martin, B. M., Roca, D., Farb, D. H., and Paul, S. M. (1991) J. Biol. Chem. 266, 6011–6014
17. Tietz, E. L., Huang, X., Weng, X., Rosenberg, H. C., and Chiu, T. H. (1993) J. Mol. Neurosci. 4, 277–292
18. Brooks-Kayal, A. R., Shumate, M. D., Jin, H., Rikhter, T. Y., and Coulter, D. A. (1998) Nat. Med. 4, 1166–1172
19. Devaud, L. L., Fritschy, J. M., Sieghart, W., and Morrow, A. L. (1997) J. Neurochem. 69, 126–130
20. Montpied, P., Weizman, A., Weizman, R., Kook, K. A., Morrow, A. L., and Paul, S. M. (1993) Brain Res. Mol. Brain Res. 18, 267–272
21. Hsu, F. C., Zhang, G. J., Raol, Y. S., Valentino, R. J., Coulter, D. A., and Brooks-Kayal, A. R. (2003) Proc. Natl. Acad. Sci. U. S. A. 100,
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