Cellular/Molecular

cAMP Response Element-Binding Protein, Activating Transcription Factor-4, and Upstream Stimulatory Factor Differentially Control Hippocampal $GABA_BR1a$ and $GABA_BR1b$ Subunit Gene Expression through Alternative Promoters

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Expression of metabotropic $GABA_B$ receptors is essential for slow inhibitory synaptic transmission in the CNS, and disruption of $GABA_B$ receptor-mediated responses has been associated with several disorders, including neuropathic pain and epilepsy. The location of $GABA_B$ receptors in neurons determines their specific role in synaptic transmission, and it is believed that sorting of subunit isoforms, $GABA_BR1a$ and $GABA_BR1b$, to presynaptic or postsynaptic membranes helps to determine this role. $GABA_BR1a$ and $GABA_BR1b$ are thought to arise by alternative splicing of heteronuclear RNA. We now demonstrate that alternative promoters, rather than alternative splicing, produce $GABA_BR1a$ and $GABA_BR1b$ isoforms. Our data further show that subunit gene expression in hippocampal neurons is mediated by the cAMP response element-binding protein (CREB) by binding to unique cAMP response elements in the alternative promoter regions. Double-stranded oligonucleotide decoys selectively alter levels of endogenous $GABA_BR1a$ and $GABA_BR1b$ in primary hippocampal neurons, and CREB knock-out mice show changes in levels of $GABA_BR1a$ and $GABA_BR1b$ transcripts, consistent with decoy competition experiments. These results demonstrate a critical role of CREB in transcriptional mechanisms that control $GABA_BR1$ subunit levels *in vivo*. In addition, the CREB-related factor activating transcription factor-4 (ATF4) has been shown to interact directly with $GABA_BR1$ in neurons, and we show that ATF4 differentially regulates $GABA_BR1a$ and $GABA_BR1b$ promoter activity. These results, together with our finding that the depolarization-sensitive upstream stimulatory factor (USF) binds to a composite CREB/ATF4/USF regulatory element only in the absence of CREB binding, indicate that selective control of alternative $GABA_BR1$ promoters by CREB, ATF4, and USF may dynamically regulate expression of their gene products in the nervous system.

Key words: **$GABA_B$ receptor; transcription; CREB; ATF4; CREB2; USF**

Introduction

Metabotropic $GABA_B$ receptors mediate slow inhibitory synaptic neurotransmission and play a critical role in forming neuronal circuitry and long-term synaptic plasticity (Davies et al., 1991; Mott and Lewis, 1991). Disruption of $GABA_B$ receptor-mediated synaptic pathways has been implicated in many diseases, including neuropathic pain, spasticity, drug addiction, schizophrenia, and epilepsy (Bowery et al., 2002; Calver et al., 2002). Formation of fully functional $GABA_B$ receptors requires the coassembly of $GABA_BR1$ and $GABA_BR2$ subunits (Kaupmann et al., 1997, 1998a; Jones et al., 1998; White et al., 1998; Kuner et al., 1999; Martin et al., 1999). Multiple isoforms of human $GABA_BR1$ ($GABA_BR1a$, $GABA_BR1b$, $GABA_BR1c$, and $GABA_BR1e$) have been described, but only one $GABA_BR2$ has been identified (Martin et al., 2001).

The $GABA_BR1a$ and $GABA_BR1b$ variants differ in their N-terminal amino acid sequences and were hypothesized to result from alternative splicing (Kaupmann et al., 1997). The human $GABA_BR1a$ contains 23 exons, the first five of which contain the $GABA_BR1a$ 5′-untranslated region (UTR) (exon 1), a signal peptide, and two Sushi domains (see Fig. 1*C*). The alternative N terminus of $GABA_BR1b$ is produced from the fifth intron of $GABA_BR1a$. However, it was not known whether consensus 5′- and 3′-splice sites were present at the appropriate locations to permit alternative splicing of $GABA_BR1b$ from the heteronuclear $GABA_BR1$ transcript. Splice junctions are consistent with the for-

Received March 31, 2004; revised May 19, 2004; accepted May 21, 2004.

This work was supported by a grant from the National Institute of Child Health and Human Development (HD22539). We are grateful to Dr. J. Blendy's laboratory (University of Pennsylvania, Philadelphia, PA) for generation of the CREB $^{\alpha\Delta}$ mice and to Dr. A. Brooks-Kayal's laboratory (University of Pennsylvania School of Medicine) for isolation of total RNA from the mutant mice. We thank Dr. M. E. Greenberg (Harvard Medical School, Boston, MA) for M1-CREB and Dr. T. Hai (Ohio State University, Columbus, OH) for the ATF4 expression vector. We also thank Drs. G. C. Lau and S. C. Martin for helpful contributions.

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DOI:10.1523/JNEUROSCI.1200-04.2004

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mation of $GABA_BR1c$ and $GABA_BR1e$ variants from a parent $GABA_BR1a$ transcript by exon skipping of exon 4 encoding the second Sushi domain ($GABA_BR1c$) or exon 11 producing a frameshift stop codon in the extracellular domain ($GABA_BR1e$).

Human temporal lobe epilepsy produces a significant increase in the levels of $GABA_BR1a$ and $GABA_BR1b$ mRNAs within individual neurons and increases in $GABA_B$ receptor binding parallel upregulation of $GABA_BR1$ mRNAs (Princivalle et al., 2002, 2003). Homozygous $GABA_BR1$ knock-out mice lack functional presynaptic and postsynaptic $GABA_B$ receptors and exhibit generalized epilepsy (Prosser et al., 2001; Schuler et al., 2001), as would be expected for the loss of slow synaptic inhibition.

In the hippocampus, $GABA_B$ receptor-mediated postsynaptic, but not presynaptic, responses are developmentally regulated (Lei and McBain, 2003). Moreover, responses mediated by postsynaptic $GABA_B$ receptors desensitize more rapidly than those mediated by presynaptic receptors (Wetherington and Lambert, 2002). Given the fact that the majority of $GABA_B$ receptors at the postsynaptic membrane contain $GABA_BR1a$ subunits and those at presynaptic membranes contain $GABA_BR1b$ (Benke et al., 1999), differential regulation of $GABA_BR1a$ and $GABA_BR1b$ gene expression may define the particular function of $GABA_B$ receptors in a cell.

$GABA_BR1$ interacts directly with the transcription factor cAMP response element-binding protein-2 (CREB2), also termed activating transcription factor-4 (ATF4) (Nehring et al., 2000; White et al., 2000; Vernon et al., 2001). ATF4 is a member of the CREB/ATF family of transcription factors that stimulates and represses the transcription of a variety of genes involved in neuronal survival and long-term memory (Bartsch et al., 1998; Mayr and Montminy, 2001). Baclofen-stimulated activation of $GABA_B$ receptors in hippocampal neurons causes a dramatic translocation of ATF4 out of the nucleus, which is presumably dependent on cAMP concentration (Vernon et al., 2001). Taken together with the fact that $GABA_BR1$ and $GABA_BR2$ are colocalized in the nuclei of neurons (Gonchar et al., 2001), interaction of $GABA_B$ receptors with transcription factors may provide a dynamic way for neurotransmitter receptors to control gene transcription.

Here, we provide the first demonstration that $GABA_BR1a$ and $GABA_BR1b$ are produced by distinct promoters and show that CREB-mediated activation of alternative $GABA_BR1$ promoters has the potential to regulate the differential expression of $GABA_B$ receptor subtypes. These findings identify the first functional regulatory elements in the $GABA_BR1a$ and $GABA_BR1b$ promoters and point to a novel regulatory pathway that may control $GABA_BR1$ gene expression in neurons.

Materials and Methods

RNase protection assay. RNase protection assays were performed using 50 μg of human adult and fetal brain total RNA (BD Biosciences Clontech, Palo Alto, CA) as described (McLean et al., 2000). Primer sequences for the $GABA_BR1a$ and $GABA_BR1b$ start site probes were: $GABA_BR1a$, 5′-GCAGCCGTCTTTCTCCAC-3′ and 5′-GGCCCTGGCTCTTACCTC-3′; $GABA_BR1b$, 5′-CCTGGTTCCTCCGTGCTTCAG-3′ and 5′-CCGCCATCACAAGAAGC-3′.

Methylation analysis. Human blood genomic DNA (10 μg; BD Biosciences Clontech) was digested with one of the methylation-sensitive enzymes, *Bsa*HI and *Hae*II (New England Biolabs, Beverly, MA). *Bsa*HI-digested DNAs also were cut with the methylation-insensitive enzyme *Eco*RI (New England Biolabs). Southern blots of the genomic fragments were prepared and hybridized with a $GABA_BR1$-specific DNA probe corresponding to the $GABA_BR1a$ cytosine-phospho-guanine (CpG) island or to the $GABA_BR1b$ CpG island (see Fig. 2). The combination of primers used to generate the subunit-specific DNA fragments were: $GABA_BR1a$, 5′-GTTGTTTGGCCCGCAGGTC-3′ and 5′-GGGAAGTGGAGCGAAGGA-3′; $GABA_BR1b$, 5′-CTCCCACTTCAGACCTCAG-3′and 5′-GAGCTCATAGTCCGGCAGG-3′.

Constructs and mutagenesis. To generate the $GABA_BR1a$ and $GABA_BR1b$ promoter constructs, we amplified 2.2 and 2.8 kb fragments of the $GABA_BR1a$ and $GABA_BR1b$ promoters by PCR with a human $GABA_BR1$ genomic clone (dJ271M21.2) and Pfu Turbo polymerase (Stratagene, La Jolla, CA). The combination of primers used to generate the PCR products were: $GABA_BR1a$, 5′-CCCAGGACATTCACGTAGTG-3′ and 5′-GGCCCTGGCTCTTACCTC-3′; $GABA_BR1b$, 5′-GAGCATCTGTAGTCAGGGCC-3′ and 5′-CCGCCATCACAACCAGAAGC-3′. Amplified DNA fragments were cloned into the pGL2-Basic vector (Promega, Madison, WI) upstream of the reporter gene, firefly luciferase. To generate the cAMP response element (CRE) substitution mutations in the context of the promoter constructs, the AC dinucleotide in the CRE consensus site was replaced with a TG dinucleotide. Substitution mutations were confirmed by sequence analysis.

Cell culture and transfections. Primary rat hippocampal, neocortical, and fibroblast cultures were prepared from embryonic day (E) 18 embryos as described (McLean et al., 2000). Cultures were transfected 1 week after dissociation, using a Ca^{2+} phosphate precipitation method (Xia et al., 1996). To control for differences in transfectional efficiency, promoter activity was compared with background activity (as measured by the pGL2-Basic promoterless vector; Promega). For coexpression studies, CREB, M1-CREB, or ATF4 expression constructs were applied in the presence of $GABA_BR1a$- and $GABA_BR1b$-luciferase. As controls, pRC (Invitrogen, Carlsbad, CA) or pC-neo empty vectors under the control of the Rous sarcoma virus (RSV) or cytomegalovirus (CMV) promoter, respectively, were added with the reporter constructs. Cotransfection of RSV- or CMV-containing plasmids (in the absence of a transgene) markedly reduce $GABA_BR1$ promoter activity. To prevent competition of transcription factors between the $GABA_BR1$ promoter and a heterologous promoter, a 1:8 ratio of expression plasmid to reporter plasmid was used. The M1-CREB and ATF4 expression constructs were kindly provided by Dr. M. E. Greenberg (Harvard Medical School, Boston, MA) and Dr. T. Hai (Ohio State University, Columbus, OH), respectively. The CREB expression vector was constructed by site-directed mutagenesis of M1-CREB (Ala133Ser).

Electrophoretic mobility shift assay. Hippocampal nuclear extracts (10 μg/reaction) were used for electrophoretic mobility shift assay (EMSA) (Russek et al., 2000). The sequences of [^{32}P]-labeled probes and unlabeled competitors (where lowercase letters represent mutations) were: R1a(CRE), 5′-TCCCCTTTACGTTACAGAAA-3′; R1a(CRE$^-$), 5′-TCCCTTTtgGTTACAGAAA-3′; R1b(CRE), 5′-GCCGCCCGTGACGTCAGAGC-3′; R1b(CRE$^-$), 5′-GCCGCCCGTGtgGTCAGAGC-3′; Ebox(x2), 5′-TGTGGTCATGTGGTCATGTGGTCA-3′; R1bMUC, 5′-CCCCGCTGCCGCGCGCCGCCCGTGACGTCAGAGCCCCCTCC-3′; R1bMUC(MycMax$^-$), 5′- CCCCGCTGCCGCGCaCCGCCCGTGACGTCAGAGCCCCCTCC-3′; R1bMUC(USF$^-$), 5′- CCCCGCTGCCGCGCGCCGCCatTGACGTCAGAGCCCCCTCC-3′; R1bMUC(CRE$^-$), 5′-CCCCGCTGCCGCGCGCCGCCCGTGtgGTCAGAGCCCCCTCC-3′. Wild-type and mutant consensus CRE and Ebox oligonucleotides were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). For supershift experiments, 2–4 μl of polyclonal antibodies to CREB (Upstate Biotechnology, Lake Placid, NY), acute myeloid leukemia-1 (AML1) (sc-8563), MAX (sc-197), c-Myc (sc-764), USF1 (upstream stimulatory factor 1; sc-229), or USF2 (sc-861) (Santa Cruz Biotechnology) was added to the binding mixture.

Decoy oligonucleotide transfection. Treatment with decoy oligonucleotides was performed with modifications (Park et al., 1999; Mabuchi et al., 2001). The sequences of the double-stranded phosphorothioate oligonucleotides were: CRE-D, 5′-TGACGTCATGACGTCATGACGTCA-3′; mCRE-D (also termed USF-D), 5′-TGTGGTCATGTGGTCATGTGGTCA-3′. Using the cationic lipid DOTAP (*N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate; Roche Applied Science, Indianapolis, IN), the decoys (200 μM) were applied to cultured hippocampal neurons (7 d *in vitro*). After 5 hr, the media was replaced with untreated, conditioned media. Cells were harvested 48 hr after decoy transfection, and

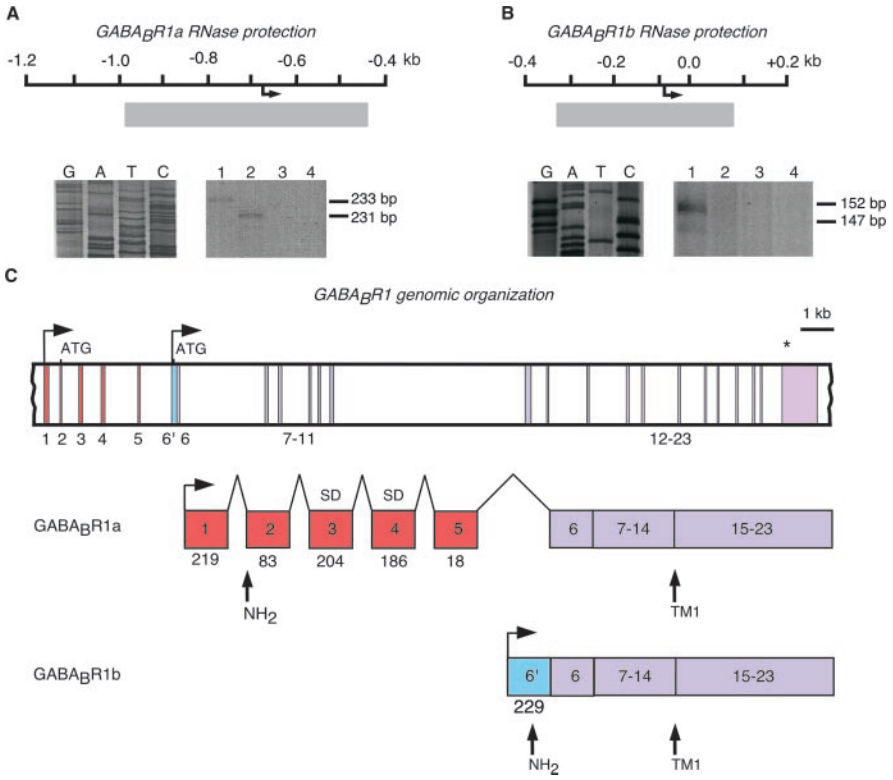

Figure 1. Identification of transcriptional start sites for GABA$_B$R1a and GABA$_B$R1b. *A*, *B*, Top, Schematic of the human GABA$_B$R1a (*A*) and GABA$_B$R1b (*B*) 5′-flanking regions. Base positions are indicated relative to the ATG start codon, and the major transcription start site is indicated by an arrow. The approximate location of the cRNA probe is shown (gray bar). Bottom, RNase protection was performed using 50 μg of human adult brain RNA (lane 1), human fetal brain RNA (lane 2), tRNA (lane 3), and a no RNA control (lane 4). Protected bands are indicated on the right. The ladder shown on the left was generated by chain termination sequencing. *C*, Top, Genomic organization of the human GABA$_B$R1 gene. The GABA$_B$R1a-specific exons are shown in red, the GABA$_B$R1b-specific exons are shown in blue, and the common exons, encoding both GABA$_B$R1a and GABA$_B$R1b, are shown in purple. The transcription start sites (arrows), ATG translation initiation codons, and translational stop codon (asterisk) are labeled. The scale is indicated above the diagram. Bottom, Patterns of promoter use that lead to generation of GABA$_B$R1a and GABA$_B$R1b mRNAs. Exons are indicated as boxes (not drawn to scale), and the size of the exons (base pairs) is indicated below. The GABA$_B$R1a mRNA contains five exons from the 5′-end of the gene that are not present in the GABA$_B$R1b transcript. Exon 1 contains the GABA$_B$R1a 5′-UTR. Exon 6′ is the alternative first exon for GABA$_B$R1b and contains the GABA$_B$R1b 5′-UTR and the GABA$_B$R1b-specific coding region. The N terminus (NH$_2$) and first transmembrane domain (TM1) are labeled (arrows).

GABA$_B$R1a and GABA$_B$R1b protein levels were measured by quantitative Western analysis.

Western analysis. Total cellular proteins were extracted from primary neuronal and fibroblast cultures. Western blot analysis was performed using a polyclonal GABA$_B$R1 antibody (Chemicon, Temecula, CA) and a monoclonal β-actin antibody (Sigma-Aldrich, St. Louis, MO). Quantitation of enhanced chemiluminescent signals was analyzed by densitometry (Amersham Biosciences, Piscataway, NJ) and normalized to β-actin expression.

Real-time reverse transcription-PCR. Wild-type and CREB$^{\alpha\Delta}$ mutant mice were kindly provided by Dr. J. Blendy's laboratory (University of Pennsylvania, Philadelphia, PA), and total RNA from the whole brain was gratefully prepared by Dr. A. Brooks-Kayal's laboratory (University of Pennsylvania School of Medicine). In the CREB$^{\alpha\Delta}$ mutant mice, expression of CREBα and CREBΔ isoforms is disrupted (Walters and Blendy, 2001). Real-time reverse transcription (RT)-PCRs were performed using the ABI PRISM 7900HT instrument (Applied Biosystems, Foster City, CA). Primers were designed using Primer Express version 1.5a software (Applied Biosystems). Cyclophilin was used as an endogenous control to normalize mRNA levels. The forward and reverse primers for mouse GABA$_B$R1a were 5′-CACACCCAGTCGCTGTG-3′ and 5′-GAGGTCCCCACCCGTCA-3′. Primers for mouse GABA$_B$R1b were 5′-GGGACCCTGTACCCCGGTG-3′ and 5′-GGAGTGAGAGGCCCACACCCC-3′ and 5′-CCCAAGGGCTCGTCA-3′. Taqman probes were purchased from Applied Biosystems; each was synthesized with the fluorescent reporter FAM (6-carboxy-fluorescein) attached to the 5′-end and the quencher dye TAMRA (6-hyroxy-tetramethyl-rhodamine) attached to the 3′-end. The sequence of gene-specific Taqman probes was: GABA$_B$R1a, 5′-CCGAATCTGCTCCAAGTCTTATTTGACCC-3′; GABA$_B$R1b, 5′-CCGCTGCCTCTTCTGCTGGTGATG-3′; cyclophilin, 5′-CCGTGTTCTTCGACATCACGGCCG-3′. Thermocycling was done in a final volume of 10 μl containing 4 ng of total RNA, GABA$_B$R1a- or GABA$_B$R1b-specific primers (900 nM), and cyclophilin primers (200 nM) as required for the QuantiTect Probe RT-PCR kit (Qiagen, Valencia, CA). PCR parameters were 50°C for 30 min, 95°C for 10 min, 50 cycles of 95°C for 15 sec, and 60°C for 1 min. A semiquantitative measurement of relative levels of gene expression in knock-out samples, compared with the wild-type samples, was performed for GABA$_B$R1a and GABA$_B$R1b using cyclophilin as a control.

Results

Transcription of GABA$_B$R1a and GABA$_B$R1b initiates upstream of the exons encoding the alternative 5′-UTRs

To identify the most 5′-end of GABA$_B$R1a and GABA$_B$R1b 5′-UTRs and determine whether they are located at splice junctions, we performed RNase protection analyses with riboprobes specific to the alternative 5′-UTRs. Using an antisense RNA probe complementary to GABA$_B$R1a (−988/−450 bp, in which +1 is relative to the GABA$_B$R1a ATG start codon), we detected products of 233 bp and 231 bp in human adult and fetal brain RNAs (Fig. 1*A*). The intensity of the band observed in fetal brain RNA was greater than that observed in adult brain RNA.

RNase protection analysis using a GABA$_B$R1b probe (−330/+63, in which +1 is relative to the GABA$_B$R1b ATG start codon) produced multiple products, indicative of more than one transcription start site for GABA$_B$R1b in adult human brain RNA (Fig. 1*B*). The greater intensity of the 152 bp band, when compared with that of the 147 bp band, indicates the prevalence of the longer transcript and therefore has been assigned as the major transcriptional start site for GABA$_B$R1b. In contrast to GABA$_B$R1a, protected fragments specific to GABA$_B$R1b were not detected using RNA from the fetal human brain. These results support the hypothesis that transcripts specific to GABA$_B$R1a and GABA$_B$R1b are differentially regulated during development, possibly through the use of alternative promoters.

To determine whether the 5′-ends of the protected transcripts reflect alternative splicing of heteronuclear RNA, we examined the genomic sequence for the presence or absence of 5′-donor and 3′-acceptor splice sites. Because of the fact that no such sites were observed, and the fact that there would have to be a common first exon to which exon 6′ could splice to generate GABA$_B$R1b, the 5′-end of the GABA$_B$R1b transcript is most likely a site of transcription initiation, rather than an internal

exon. Results support a genomic structure in which exon 1 (219 bp) encodes the GABA$_B$R1a 5′-UTR and exon 6′ encodes the first exon for GABA$_B$R1b that contains the GABA$_B$R1b 5′-UTR (88 bp) as well as a coding sequence (141 bp) (Fig. 1*C*). The first nucleotide corresponding to the adult GABA$_B$R1a transcript has been designated as +1. Additionally, the first nucleotide of GABA$_B$R1b is designated as +1. The structure for all luciferase reporter constructs used in evaluating GABA$_B$R1a and GABA$_B$R1b promoter activity is defined relative to these sites.

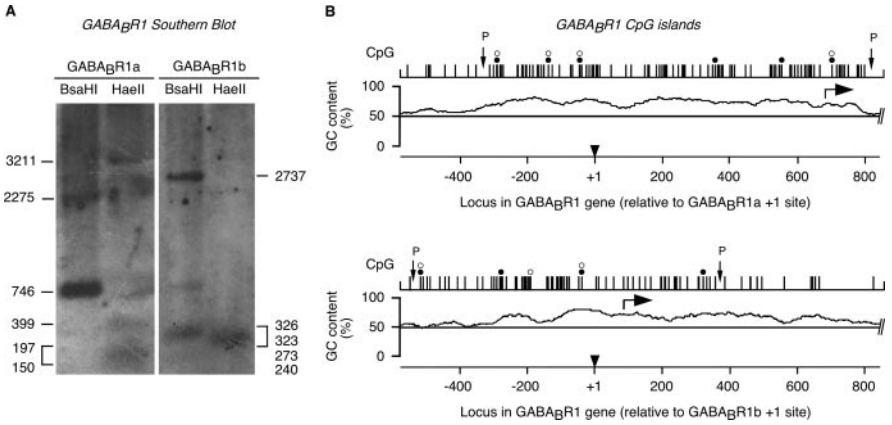

Figure 2. DNA methylation state of the human GABA$_B$R1 gene. *A*, Methylation-sensitive Southern blot for GABA$_B$R1a (left) and GABA$_B$R1b (right). The methylation state of CpG dinucleotides was evaluated by sensitivity to *Bsa*HI and *Hae*II restriction digestion in human blood genomic DNA. GABA$_B$R1a- and GABA$_B$R1b-specific fragments (1135 and 908 bp) were generated by PCR and radiolabeled as probes. The sizes (base pairs) of the GABA$_B$R1a and GABA$_B$R1b hybridization products are shown. *B*, GC content analysis of the GABA$_B$R1a (top) and GABA$_B$R1b (bottom) 5′-flanking regions. Locations of CpG dinucleotides are shown above the GC plot; each vertical black line represents the presence a CpG dinucleotide. The inverted triangle represents the transcription start site, and the arrow above the GC plot indicates the translation initiation site. The open and solid circles refer to CpG dinucleotides that are not methylated, as identified, respectively, by a *Hae*II- and *Bsa*HI-restricted Southern blot. Fragments that correspond to the GABA$_B$R1a and GABA$_B$R1b probes are indicated (P).

Analysis of genomic sequence: CpG islands

The genomic sequences flanking the GABA$_B$R1a and GABA$_B$R1b transcription start sites are GC-rich (75 and 72%, respectively) (Fig. 2*B*). Two regions of highest GC content span 1113 bp (−310/+803, GABA$_B$R1a) and 867 bp (−518/+349 bp, GABA$_B$R1b). Their sequence composition, based on computational predictions developed by the Wellcome Trust Sanger Institute, suggests that the GABA$_B$R1a and GABA$_B$R1b transcriptional start sites are embedded within CpG islands, genomic structures (~1 kb) distinguished by an abundance of unmethylated CpG dinucleotides (Gardiner-Garden and Frommer, 1987; Cross et al., 1994). Alterations in CpG dinucleotide methylation are believed to regulate promoter activity by remodeling the DNA–chromatin superstructure (Cross and Bird, 1995).

To determine whether methylation could account for the tissue-specific regulation of GABA$_B$R1a and GABA$_B$R1b, we used Southern blot hybridization with the methylation-sensitive restriction enzyme *Bsa*HI to analyze the methylation status of the GABA$_B$R1a and GABA$_B$R1b CpG islands (Fig. 2*A*). The ability of *Bsa*HI to cleave mammalian genomic DNA is blocked by CpG methylation. The GABA$_B$R1a CpG island contains four *Bsa*HI restriction sites, whereas the GABA$_B$R1b CpG island contains three sites. In human blood genomic DNA, *Bsa*HI digestion produced two GABA$_B$R1a-specific fragments and two GABA$_B$R1b fragments. These findings indicate that the CpG dinucleotides found in the *Bsa*HI recognition sites were not methylated. Similar results also were obtained with the methylation-sensitive enzyme *Hae*II (Fig. 2*A*). The presence of unmethylated CpG dinucleotides in GABA$_B$R1a and GABA$_B$R1b promoters suggests that global DNA methylation of the promoters does not account for the absence of GABA$_B$R1 expression in blood. We cannot rule out, however, the possibility that selective methylation may regulate expression of GABA$_B$R1 in the brain.

Functional analysis of the GABA$_B$R1a and GABA$_B$R1b promoters

GABA$_B$R1a and GABA$_B$R1b mRNA and protein are present in most brain structures and peripheral tissues. Within the brain, GABA$_B$R1 mRNA is first detected at E11.5, and expression in the hippocampus and neocortex is found at E12.5 (Kim et al., 2003). Moreover, GABA$_B$R1 proteins are present in the neocortex at E14 (Lopez-Bendito et al., 2002). The concentration of GABA$_B$R1a protein is highest during early postnatal CNS development, whereas the GABA$_B$R1b isoform predominates in the adult (Malitschek et al., 1998; Fritschy et al., 1999). Although GABA$_B$R1a and GABA$_B$R1b mRNAs also are present in the periphery of the adult rat (Castelli et al., 1999; Calver et al., 2000), it is unknown whether GABA$_B$R1a and GABA$_B$R1b mRNAs are expressed in peripheral tissues during embryonic development. To this end, we characterized GABA$_B$R1 gene expression in cultures of E18 hippocampal, neocortical, and fibroblast cells using quantitative Western analysis. GABA$_B$R1a and GABA$_B$R1b proteins were present in hippocampal and neocortical neurons with concentrations of GABA$_B$R1a that were threefold to fourfold higher than GABA$_B$R1b (GABA$_B$R1a/GABA$_B$R1b: hippocampus, 3.7 ± 0.26; neocortex, 4.5 ± 0.68). Additional evidence indicates that GABA$_B$R1a and GABA$_B$R1b also are found in fibroblasts in a 1:1 ratio (GABA$_B$R1a/GABA$_B$R1b: 1.65 ± 0.48), indicating that GABA$_B$R1a and GABA$_B$R1b promoters are not neural specific.

To determine whether the 5′-flanking regions of GABA$_B$R1a and GABA$_B$R1b contain independent promoters that are active in neuronal and non-neuronal cells, we measured the transcriptional activity of GABA$_B$R1a and GABA$_B$R1b genomic fragments using a transient transfection system with luciferase as a reporter gene. A 2.2 kb fragment from the GABA$_B$R1a 5′-flanking region and a 2.8 kb fragment from the GABA$_B$R1b 5′-flanking region were cloned upstream of the firefly luciferase gene in the pGL2-Basic vector (Fig. 3).

In primary cultures of rat hippocampal neurons, reporter constructs containing the GABA$_B$R1a 5′-flanking region were 165 times more active than the promoterless control (Fig. 3*A*), whereas those containing the GABA$_B$R1b 5′-flanking region were weaker but significantly greater (fivefold) than background (Fig. 3*B*). Promoter activity of GABA$_B$R1a and GABA$_B$R1b 5′-flanking regions also was examined in primary cultures of rat neocortical neurons and fibroblasts (Fig. 3). Although the 5′-flanking region of GABA$_B$R1a had strong activity in neocortical and fibroblast cells, activity in fibroblasts was significantly lower (−42%; $p < 0.05$) than in hippocampal neurons (Fig. 3*A*). In contrast, there was no significant difference in GABA$_B$R1b promoter activity between brain and fibroblast cultures (Fig. 3*B*). Taken together with the observation that GABA$_B$R1 promoters do not contain

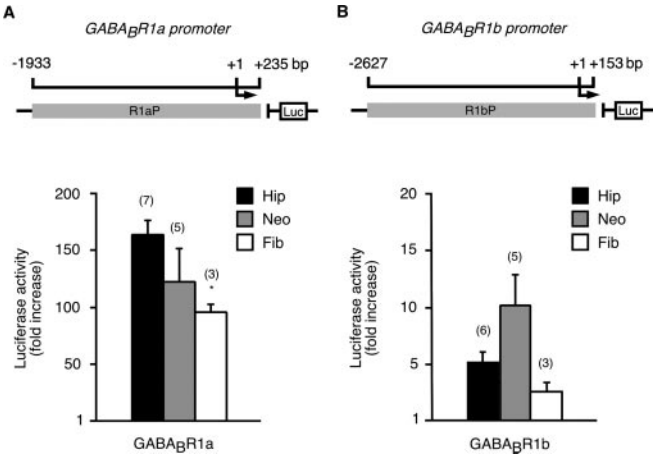

Figure 3. GABA$_B$R1a and GABA$_B$R1b promoter activity in neuronal and non-neuronal cell types. *A*, *B*, Top, Schematic of the human GABA$_B$R1a (*A*) and GABA$_B$R1b (*B*) luciferase (Luc) reporter constructs R1aP and R1bP. Base positions of the alternative GABA$_B$R1 5′-flanking regions are indicated relative to the GABA$_B$R1a and GABA$_B$R1b transcription start sites. The GABA$_B$R1 5′-flanking regions (gray bars) were cloned upstream of the luciferase gene (open bars). *A*, *B*, Bottom, GABA$_B$R1a (*A*) and GABA$_B$R1b (*B*) promoter activity was monitored in primary cultures of rat hippocampal neurons (Hip), neocortical neurons (Neo), and fibroblasts (Fib). Data shown are mean ± SEM. $*p < 0.05$, hippocampus compared with fibroblasts (one-way ANOVA with Games-Howell *post hoc* comparison).

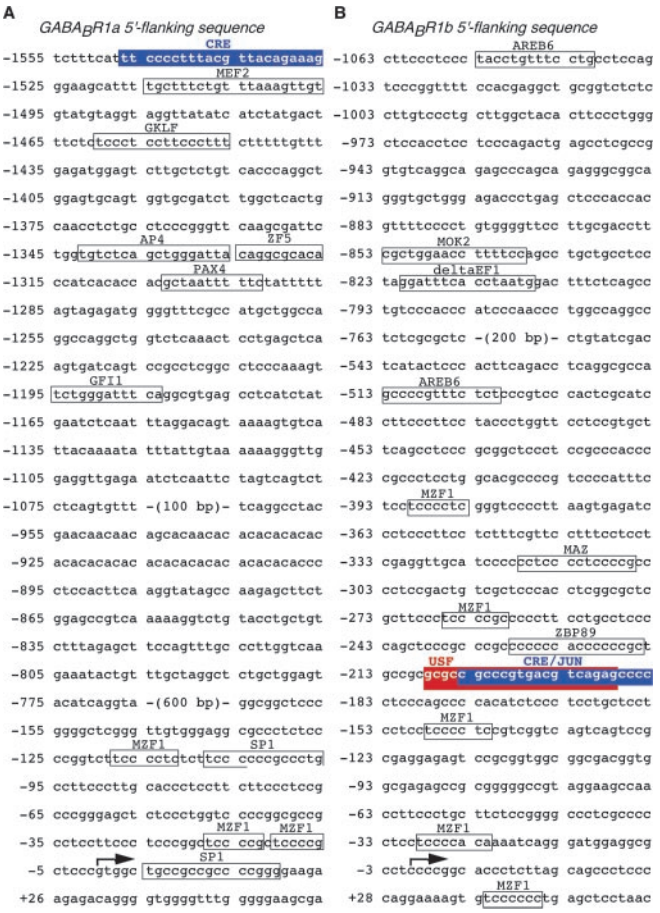

Figure 4. Features of the GABA$_B$R1a and GABA$_B$R1b 5′-flanking regions. *A*, *B*, The nucleotide sequence of the human GABA$_B$R1a (*A*) and GABA$_B$R1b (*B*) 5′-flanking regions are shown. Arrows indicate the major transcription start site based on RNase protection. Consensus sites for known transcription factors are identified by name above the corresponding boxed sequence. The numbering is relative to the transcription start site for GABA$_B$R1a and GABA$_B$R1b. AP4, Activator protein-4; AREB6, Atp1a1 regulatory element binding factor-6; deltaEF1, delta-crystallin/E2-box factor-1; GFI1, growth factor independence-transcriptional repressor factor-1; GKLF, gut-enriched Krueppel-like binding factor; MAZ, Myc-associated zinc finger protein; MEF2, myocyte-specific enhancer-2; MOK2, ribonucleoprotein-associated zinc finger protein; MZF1, myeloid zinc finger protein-1; PAX4, paired box factor-4; SP1, stimulatory protein-1; USF, upstream stimulatory factor; ZBP89, zinc finger transcription factor-89; ZF5, zinc finger-5.

the neuron-restrictive silencer element, these results demonstrate that the GABA$_B$R1a and GABA$_B$R1b promoters are not neural-specific but may be differentially regulated in neurons.

CREB functions as a transcriptional activator at GABA$_B$R1a(CRE)

The GABA$_B$R1a and GABA$_B$R1b promoter regions lack a canonical TATA-box but contain other putative *cis*-acting elements (Fig. 4*A*, *B*). In particular, one CRE is located in the GABA$_B$R1a promoter at −1540/−1533 [R1a(CRE)] and in the GABA$_B$R1b promoter at −202/−188 [R1b(CRE)]. Consensus CRE elements are bound by members of the basic leucine zipper (bZip) family of transcription factors, including CREB and ATF proteins.

To determine the importance of the CRE in the GABA$_B$R1a 5′-flanking region, a substitution mutation was introduced into the R1a(CRE) site. Activity of the mutant reporter construct was assayed by transient transfection in primary hippocampal neurons. A 2 bp substitution mutation in R1a(CRE) effectively abolished GABA$_B$R1a promoter activity (Fig. 5*A*), because all endogenous CREB binding should be eliminated. Consistent with this, overexpression of CREB significantly increased GABA$_B$R1a promoter activity (258 ± 77%) (Fig. 5*A*).

The effect of a CREB dominant-negative mutant was determined on the magnitude of GABA$_B$R1a promoter activity. The M1-CREB dominant-negative mutant contains a nucleotide substitution at Ser133Ala. Previous studies have demonstrated that phosphorylation of CREB at Ser-133 is a critical event that mediates initiation of CRE-dependent activity (Gonzalez and Montminy, 1989). M1-CREB cannot be phosphorylated but it is able to bind CRE sites. Overexpression of M1-CREB competes for the binding of endogenous CREB family members that transactivate. Cotransfection of M1-CREB with the GABA$_B$R1a reporter construct decreased GABA$_B$R1a promoter activity (−33 ± 16%) in cultured hippocampal neurons (Fig. 5*A*).

There is, therefore, general agreement between the effect of the CRE mutation on GABA$_B$R1a promoter activity and M1-CREB overexpression. Whereas there is a quantitative difference in the amount of inhibition of promoter activity using these two methods, there is no reason to expect quantitatively identical outcomes. In the case of CRE mutations, all endogenous CREB binding should be eliminated, consistent with the elimination of promoter activity. In the case of the M1-CREB overexpression experiment, however, there are additional variables that would tend to explain the results. First, the level of M1-CREB overexpression is not known and thus its ability to compete with endogenous CREB cannot be predicted. Second, cotransfection of a RSV-containing plasmid (in the absence of a transgene) markedly reduces GABA$_B$R1 promoter activity.

To test whether CREB interacts with the R1a(CRE) element, we prepared nuclear extracts from cultured hippocampal neurons and incubated these extracts with a radiolabeled probe encompassing the human R1a(CRE) sequence (Fig. 5*B*). The R1a(CRE) probe formed one DNA–protein complex that disappeared with the addition of a 100-fold excess of cold probe. Excess consensus CRE oligonucleotides also competed for specific binding, but mutant consensus CRE oligonucleotides did not. The

addition of a CREB antibody to the incubation supershifted the complex, indicating that CREB is bound either directly or indirectly through protein–protein interactions.

To further address the importance of CREB in regulating endogenous $GABA_BR1a$ gene expression, we used decoy oligonucleotides containing a canonical CRE sequence to compete for binding of endogenous CREB/ATF family members. It has been demonstrated previously that CRE decoys can specifically interfere with endogenous CRE-directed transcription in cell lines (Park et al., 1999) and neurons (Mabuchi et al., 2001). Transfection of cultured hippocampal neurons with a CRE decoy oligonucleotide reduced $GABA_BR1a$ expression ($-29 \pm 6\%$), as measured by Western analysis (Fig. 5*C*). A control decoy containing a 2 bp pair mismatch (Fig. 5*C*) had no effect on levels of $GABA_BR1a$ protein.

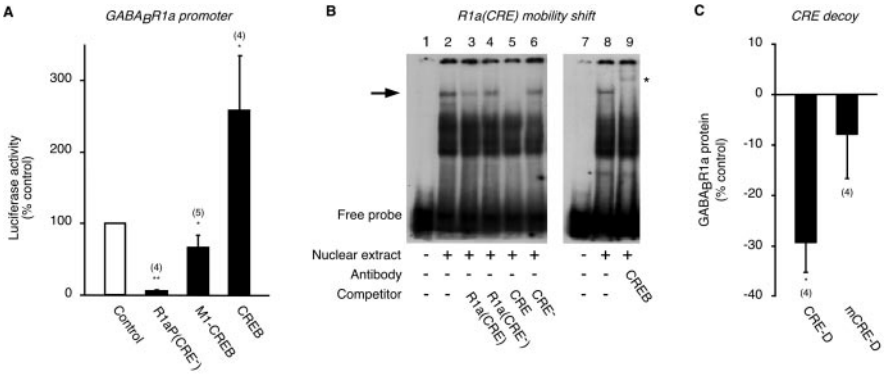

Figure 5. CREB controls $GABA_BR1a$ gene expression through $GABA_BR1a$CRE (R1aCRE). *A*, CREB family proteins activate $GABA_BR1a$ promoter activity through R1aCRE. A 2 bp substitution mutation (AC to TG) was introduced into R1aCRE of the $GABA_BR1a$ promoter–luciferase construct (R1aP), producing R1aP(CRE$^-$). Activity of the mutant reporter construct was assayed by transient transfection in primary hippocampal cultures. Dominant-negative M1-CREB or wild-type CREB expression vectors were cotransfected with R1aP. Data are expressed as a percentage of R1aP, defined as 100% (open bar). *B*, Sequence-specific binding of CREB to R1aCRE in the $GABA_BR1a$ promoter. EMSA competition and supershift analyses were performed. Nuclear extracts (10 μg/lane) were prepared from cultured hippocampal neurons and incubated with a [^{32}P] end-labeled double-stranded oligonucleotide probe, termed R1a(CRE) (lanes 2 and 8). Unlabeled competitors for the wild-type or mutant R1aCRE sequences [R1a(CRE) and R1a(CRE$^-$)] and the wild-type and mutant consensus CRE sequences (CRE and CRE$^-$) were added at a 100-fold excess (lanes 3–6). The position of the major DNA–protein complex is indicated (arrow). Hippocampal nuclear extracts were preincubated with a CREB antibody (lane 9). The asterisk indicates the position of the supershifted complex. *C*, Application of wild-type CRE-decoys (CRE-D) inhibits endogenous $GABA_BR1a$ protein expression. Primary cultures of rat hippocampal neurons were treated with DOTAP (*N*-[1-(2,3-dioleoyloxy)propyl]- *N*,*N*,*N*-trimethylammonium methylsulfate) in the presence or absence of CRE-D and mutant CRE decoy (mCRE-D) oligonucleotides. Total cellular proteins were harvested after 48 hr, and Western analysis was performed with a $GABA_BR1$ antibody. Data were quantified using densitometry, normalized to β-actin protein levels, and expressed as a percentage of vehicle control (DOTAP treatment only). Data shown are mean $\pm$ SEM. $^*p < 0.05$; $^{**}p < 0.01$ (confidence limits of the means).

CREB functions as a transcriptional activator at $GABA_BR1b$(CRE)

To determine whether CREB family members contribute to $GABA_BR1b$ transcription, we performed cotransfection experiments using dominant-negative M1-CREB in the presence of the $GABA_BR1b$ promoter construct (Fig. 6*A*). Overexpression of M1-CREB caused specific downregulation ($-38 \pm 14\%$) in $GABA_BR1b$ promoter activity, indicating that CREB/ATF proteins are transcriptional activators of the $GABA_BR1b$ promoter. This effect most likely is mediated through the CRE in the $GABA_BR1b$ 5′-flanking region [R1b(CRE)]. To characterize the nuclear proteins interacting with R1b(CRE), we performed EMSA, using hippocampal nuclear extracts and radiolabeled oligonucleotides complementary to the human R1b(CRE) site (Fig. 6*B*). The addition of a 100-fold excess of unlabeled R1b(CRE) or consensus CRE oligonucleotides inhibited formation of the DNA–protein interaction, whereas the addition of a 100-fold excess of mutant R1b(CRE) and mutant consensus CRE oligonucleotides failed to prevent complex formation. Incubation with a CREB antibody supershifted the complex, indicating that CREB is bound to R1b(CRE). These results suggest that $GABA_BR1b$ gene transcription, like $GABA_BR1a$ (Fig. 5), is positively regulated by CREB binding to a distinct CRE site.

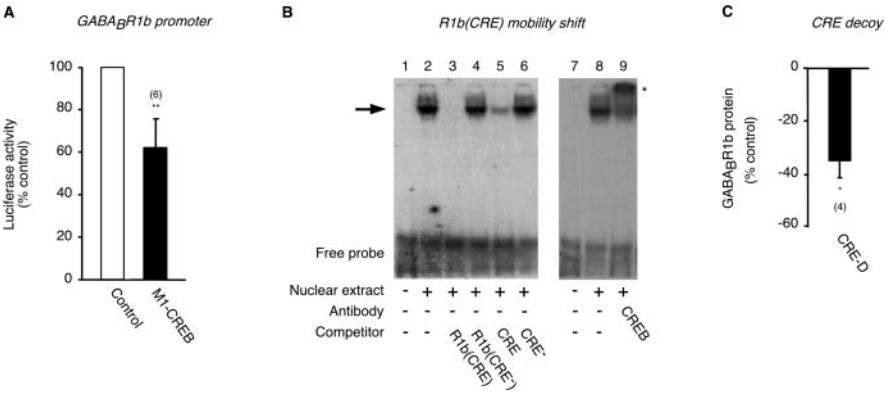

Figure 6. CREB controls $GABA_BR1b$ gene expression through $GABA_BR1b$CRE (R1bCRE). *A*, CREB family proteins activate $GABA_BR1b$ promoter activity. A dominant-negative M1-CREB expression vector was cotransfected with the $GABA_BR1b$–luciferase promoter construct (R1bP). Data are expressed as a percentage of R1bP in the presence of an empty control, defined as 100% (open bar). *B*, Sequence-specific binding of CREB to R1bCRE. EMSA competition and supershift analyses were performed. Nuclear extracts (10 μg/lane) were prepared and incubated with a [^{32}P] end-labeled double-stranded oligonucleotide probe, R1b(CRE) (lanes 2 and 8). Unlabeled competitors for the wild-type or mutant R1bCRE [R1b(CRE) and R1b(CRE$^-$)] and consensus CRE sequences [CRE and CRE$^-$] were added at a 100-fold excess (lanes 3–6). Hippocampal nuclear extracts were preincubated with a CREB antibody (lane 9). The position of the major DNA–protein complex is indicated (arrow). The asterisk indicates the position of the supershifted complex. *C*, The wild-type CRE decoy (CRE-D) inhibits endogenous $GABA_BR1b$ expression. Hippocampal neurons were treated with DOTAP (*N*-[1-(2,3-dioleoyloxy)propyl]- *N*,*N*,*N*-trimethylammonium methylsulfate) in the presence or absence of CRE-D; total cellular proteins were harvested after 48 hr, and Western analysis was performed with a $GABA_BR1$ antibody. Data were quantified using densitometry, normalized to β-actin protein levels, and expressed as a percentage of vehicle control (DOTAP treatment only). Data shown are mean $\pm$ SEM. $^*p < 0.05$; $^{**}p < 0.01$ (confidence limits of the means).

To explore the possibility that CREB regulates endogenous $GABA_BR1b$ gene expression, we monitored levels of a $GABA_BR1b$ subunit protein after CRE decoy treatment. Consistent with results of transient transfection, the triple-repeat CRE decoy oligonucleotide reduced $GABA_BR1b$ expression ($-35 \pm 6\%$) (Fig. 6*C*). Surprisingly, the 2 bp mismatch decoy, which did not affect $GABA_BR1a$ gene expression, upregulated $GABA_BR1b$ protein levels by $58 \pm 16\%$ (see Fig. 8*D*), suggesting that the mutant sequence contains a negative regula-

Table 1. $GABA_BR1a$ and $GABA_BR1b$ mRNAs are differentially expressed in $CREB^{\alpha\Delta}$ mutant mice

Subunit	$CREB^{+/+}$	$CREB^{\alpha\Delta}$	Change (%)	*n*
$GABA_BR1a$	0.64 ± 0.02	0.79 ± 0.02	+24.6 ± 1.9*	3
$GABA_BR1b$	1.29 ± 0.10	0.85 ± 0.08	−33.9 ± 1.3**	3

Whole-brain RNA was isolated from wild-type (+/+) and $CREB^{\alpha\Delta}$ mutant mice. Relative mRNA levels for $GABA_BR1a$ and $GABA_BR1b$ were determined by real-time RT-PCR and normalized to cyclophilin mRNA levels (means ± SEM).

*$p \leq 0.01$; **$p \leq 0.005$ (confidence limits of the means).

tory element that may normally function in the endogenous $GABA_BR1b$ gene. Bioinformatic analysis revealed a consensus site for USF in the mismatch decoy and a corresponding USF site near the CRE in the $GABA_BR1b$ promoter.

CREB and ATF4 can regulate $GABA_BR1a$ and $GABA_BR1b$ gene transcription

Functional promoter analysis using transient transfection and EMSA indicate that CREB acts via distinct CRE elements in the human $GABA_BR1a$ and $GABA_BR1b$ promoters (Figs. 5, 6). To determine whether CREB proteins mediate endogenous $GABA_BR1a$ and $GABA_BR1b$ gene transcription, we monitored $GABA_BR1$ mRNA levels in wild-type and $CREB^{\alpha\Delta}$ mutant mice. These mice are deficient in the major CREB isoforms, CREBα and CREBΔ (Walters and Blendy, 2001).

Using total RNA extracted from the whole brain of $CREB^{\alpha\Delta}$ mutant mice and quantitative real-time RT-PCR, we observed decreased levels of $GABA_BR1b$ transcripts in CREB-deficient mice (Table 1), consistent with the hypothesis that CREB is important in activating $GABA_BR1b$ gene transcription. Increased levels of $GABA_BR1a$ mRNA in the $CREB^{\alpha\Delta}$ mutant mice (Table 1) suggest that CREB functions as a transcriptional repressor at the $GABA_BR1a$ promoter or other CREB/ATF family members compensate for the loss of CREB expression. However, because of the fact that transfection studies were performed in rat hippocampal neurons and *in vivo* expression studies were performed in $CREB^{\alpha\Delta}$ mutant mouse brain, we cannot rule out the possibility that CREB functions as a regulator of $GABA_BR1$ gene expression in the brain as a whole differs from its specific function in the hippocampus.

Given the fact that promoter analysis supports the notion of an active CRE site in the $GABA_BR1a$ promoter that may also bind other CREB/ATF proteins, we monitored $GABA_BR1a$ and $GABA_BR1b$ promoter activity in transfected hippocampal neurons after cotransfection with an ATF4 expression vector. Although our *in vitro* transcription studies (Fig. 5) have suggested that R1a(CRE) preferentially recognizes CREB, cotransfection of ATF4 with the $GABA_BR1a$ reporter construct increased $GABA_BR1a$ promoter activity in transfected neurons (Fig. 7) to the same extent as seen for induction of $GABA_BR1a$ mRNA in $CREB^{\alpha\Delta}$ mutant mice (Table 1). Moreover, overexpression of ATF4 caused a specific downregulation in $GABA_BR1b$ promoter activity (Fig. 7). These results establish the first link between ATF4 and regulation of the $GABA_BR1a$ and $GABA_BR1b$ promoters. Together with the observation that canonical CREs are recognized by several CREB family member proteins (Habener, 1990; Meyer and Habener, 1993), and that CREM and CREBβ are overexpressed in $CREB^{\alpha\Delta}$ mutant mice (Hummler et al., 1994; Blendy et al., 1996; Walters and Blendy, 2001), evidence from ATF4 cotransfection studies suggests that CREB family members can regulate $GABA_BR1$ gene expression in the presence of CREB *in vivo*.

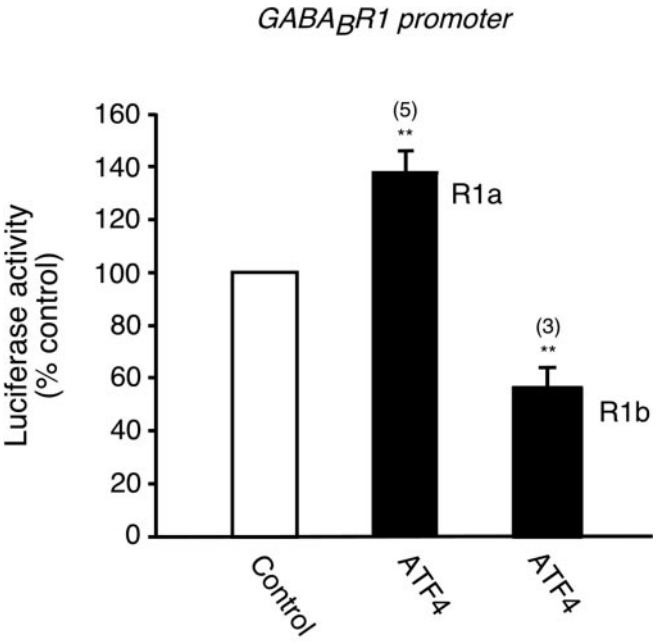

Figure 7. ATF4 is a bifunctional regulator of transcription from the $GABA_BR1$ gene. A wild-type ATF4 expression vector was cotransfected with either the $GABA_BR1a$ (R1a) or $GABA_BR1b$ (R1b) promoter construct into primary hippocampal neurons. Promoter activity is normalized to the activity of the $GABA_BR1$–luciferase promoter construct in the presence of an empty control vector, defined as 100% (open bar). Data shown are mean ± SEM. **$p \leq 0.001$ (confidence limits of the means).

USF family proteins inhibit $GABA_BR1b$ gene expression

Sites for USF proteins (USF1 and USF2) are localized with CRE elements in several promoters (Cvekl et al., 1994; Scholtz et al., 1996; Durham et al., 1997; Kingsley-Kallesen et al., 1999; Rourke et al., 1999; Pan et al., 2001; Wu and Wiltbank, 2001; Tabuchi et al., 2002; Chen et al., 2003). More importantly, recent evidence suggests that the USF–CRE composite regulatory region plays a critical role in mediating activity-dependent gene expression in neurons (Tabuchi et al., 2002; Chen et al., 2003). The USF isoforms (USF1 and USF2) bind to Ebox elements (CANNTG) as homodimers or heterodimers (Blackwell et al., 1990; Gregor et al., 1990; Blackwood and Eisenman, 1991), as do Myc and Max (MycMax) (Blackwood et al., 1992). Bioinformatic analysis of the $GABA_BR1b$ promoter revealed the presence of MycMax and USF consensus elements that overlap the R1b(CRE) site in the human, mouse, and rat genome (Fig. 8*A*). This potential composite regulatory site is defined as the $GABA_BR1b$ promoter MycMax/USF/CRE (MUC) region.

Introduction of a 2 bp substitution mutation in the $GABA_BR1b$ promoter eliminated USF- and CRE-binding sites. Activity of the mutant $GABA_BR1b$ promoter–luciferase construct markedly enhanced promoter activity in transfected hippocampal neurons when compared with wild type (Fig. 8*B*). Because CREB activator proteins recognize R1b(CRE), this finding suggests that USF proteins may function as transcriptional repressors at the $GABA_BR1b$ promoter MUC regulatory region.

To examine whether a consensus Ebox element binds USF or MycMax proteins derived from hippocampal nuclear extracts, EMSA analysis was performed using a radiolabeled probe containing two copies of a consensus Ebox sequence. The probe formed one DNA–protein complex that disappeared with the addition of a 100-fold excess of unlabeled consensus oligonucleotides containing a single copy of the Ebox sequence (Fig. 8*C*).

The addition of mutant Ebox oligonucleotides failed to prevent complex formation (Fig. 8*C*). The addition of a USF1 antibody inhibited complex formation, and a USF2 antibody supershifted the complex, identifying USF1 and USF2 as part of the protein complex recruited by the consensus Ebox probe (Fig. 8*C*). In contrast, the addition of antibodies that recognize Myc (Fig. 8*C*), as well as Max and CREB (data not shown), had no effect on the appearance or migration of the DNA–protein complex. Although two copies of the Ebox sequence in tandem creates an AML1-binding site that is identical to the AML1 consensus sequence (TGTGGT) (Wang and Speck, 1992), there was no alteration in DNA–protein binding with addition of an AML1 antibody (Fig. 8*C*).

In agreement with the results of transient transfection and EMSA analysis, USF proteins also were found to repress endogenous GABA$_B$R1b gene expression in hippocampal neurons (Fig. 8*D*). Transfection of a USF decoy oligonucleotide increased GABA$_B$R1b protein levels by 58 ± 16% (Fig. 8*D*). These data suggest that endogenous USF1, USF2, or both, are part of the protein complex that binds to the GABA$_B$R1b MUC regulatory region.

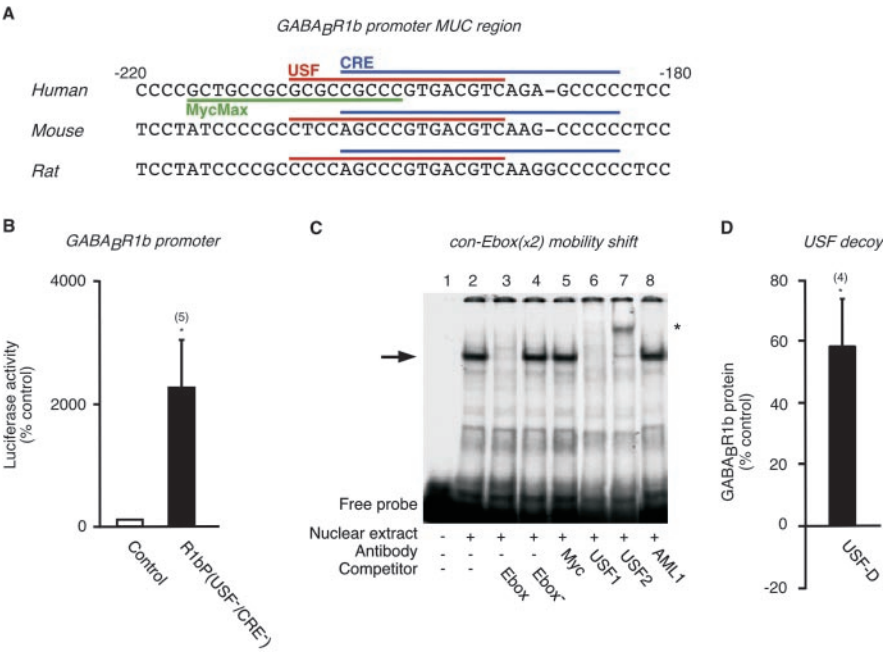

Figure 8. USF transcription factors inhibit GABA$_B$R1b gene expression. *A*, Characterization of the GABA$_B$R1b promoter Myc-Max/USF/CRE (MUC) region. Potential MycMax and USF regulatory elements overlap the CREB-binding site in the GABA$_B$R1b promoter. The homology of the MycMax (green), USF (red), and CRE (blue) sites are shown for human, mouse, and rat. *B*, Functional analysis of the USF–CRE site on GABA$_B$R1b promoter activity. A 2 bp substitution mutation (AC to TG) was introduced into the GABA$_B$R1b promoter–luciferase construct (R1bP), producing R1bP(USF$^-$/CRE$^-$). Activity of the mutant reporter construct was assayed by transient transfection in primary hippocampal cultures. Data are expressed as a percentage of R1bP, defined as 100% (open bar). *C*, USF1 and USF2 proteins bind the consensus Ebox sequence. Nuclear extracts (10 μg/lane) were prepared from cultured hippocampal neurons and incubated with a [^{32}P] end-labeled double-stranded oligonucleotide probe [Ebox(x2)] (lane 2). Unlabeled competitors for wild-type and mutant consensus Ebox sequences (Ebox and Ebox$^-$) were added at a 100-fold excess (lanes 3–4). The position of the major DNA–protein complex is indicated (arrow). Hippocampal nuclear extracts were preincubated with Myc, USF1, USF2, and AML1 antibodies (lanes 5–8). The asterisk indicates the position of the supershifted complex. *D*, Application of a wild-type USF decoy (USF-D) upregulates endogenous GABA$_B$R1b protein expression. Primary cultures of rat hippocampal neurons were treated with DOTAP (*N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate) in the presence or absence of USF-D oligonucleotides. Total cellular proteins were harvested after 48 hr, and Western analysis was performed with a GABA$_B$R1 antibody. Data were quantified using densitometry, normalized to β-actin protein levels, and expressed as a percentage of vehicle control (DOTAP treatment only). Results are shown as mean ± SEM. *$p < 0.05$ (confidence limits of the means).

CREB and USF proteins may compete for binding to an overlapping CREB–USF site in the GABA$_B$R1b promoter

To isolate the binding activity of USF proteins from that of CREB on the GABA$_B$R1b promoter, EMSA was performed with a composite probe (R1bMUC) with and without mutations. Nuclear extracts from embryonic hippocampal neurons held in culture specifically recognized the R1bMUC probe (Fig. 9*A*). Moreover, binding shows specificity toward the CRE site, and not the MycMax and USF sites, as determined by competition with an excess of unlabeled R1bMUC oligonucleotides containing a substitution mutation in one of the individual elements of the composite sequence (i.e., a mutated MycMax site [R1bMUC(MycMax$^-$)], a mutated USF site [R1bMUC(USF$^-$)], or a mutated CRE [R1bMUC(CRE$^-$)]). Both R1bMUC(MycMax$^-$) and R1bMUC(USF$^-$) competed for protein binding to the probe, indicating that the binding activity was not specific to either of these consensus sites. Only R1bMUC(CRE$^-$) was ineffective in the competition assay, indicating that factors in the nuclear extract recognized the CRE site of the composite GABA$_B$R1b regulatory sequence. This was confirmed by the fact that the addition of a CREB antibody to the reaction mixture caused a supershift of the DNA–protein complex (Fig. 9*A*). In contrast, the addition of an antibody that recognized USF1 and USF2 had no effect on complex formation or mobility.

Despite the preferential binding of CREB to the GABA$_B$R1b MUC regulatory region *in vitro*, results of decoy analyses and transient transfection assays (Fig. 8) raise the possibility that USF proteins regulate endogenous GABA$_B$R1b promoter activity in neurons. To examine whether USF proteins have a potential role in GABA$_B$R1b gene regulation *in vivo*, we first tested the ability of USF to bind to the composite site in the absence of the CRE site, using a mutated radiolabeled probe R1bMUC(CRE$^-$). Like the double-stranded oligonucleotides used for competition assays (Fig. 9*A*), the R1bMUC(CRE$^-$) probe contained wild-type MycMax and USF elements and a mutant CRE site. Nuclear extracts prepared from hippocampal neurons formed two DNA–protein complexes when incubated with the R1bMUC(CRE$^-$) probe (Fig. 9*B*). The addition of a USF antibody that recognizes USF1 and USF2 markedly inhibited formation of the faster-migrating DNA–protein complex, whereas a CREB antibody had no effect (Fig. 9*B*).

Discussion

Significance of multiple GABA$_B$R1 promoters

GABA$_B$R1a and GABA$_B$R1b are generally thought to arise from alternative splicing of a parent heteronuclear RNA. Here, we report that expression of GABA$_B$R1a and GABA$_B$R1b transcripts is under differential control of alternative promoters in the GABA$_B$R1 gene and not alternative splicing. Using RNase protection analyses, we have demonstrated that the 5′-ends of the GABA$_B$R1a and GABA$_B$R1b transcripts are found upstream of exon

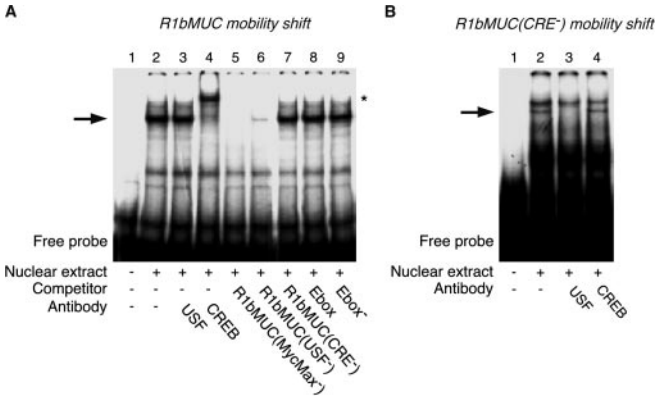

Figure 9. USF proteins bind the $GABA_BR1b$ MycMax/USF/CRE (MUC) region in the absence of CREB binding. *A*, CREB preferentially binds the composite MycMax/USF/CRE site. Nuclear extracts (10 μg/lane) were prepared from cultured hippocampal neurons and incubated with a [^{32}P] end-labeled double-stranded oligonucleotide probe containing the human $GABA_BR1b$ MycMax/USF/CRE site (R1bMUC) (lane 2). Hippocampal nuclear extracts were preincubated with USF and CREB antibodies (lanes 3 and 4). Unlabeled competitors containing substitution mutations for the individual MycMax, USF, or CRE site [R1bMUC(MycMax$^-$), R1bMUC(USF$^-$), and R1bMUC(CRE$^-$)] were added at a 100-fold excess (lanes 5–7). Unlabeled competitors for wild-type and mutant consensus Ebox sequences (Ebox and Ebox$^-$, respectively) also were added at a 100-fold excess (lanes 8 and 9). The position of the major DNA–protein complex is indicated (arrow). The asterisk indicates the position of the supershifted complex. *B*, USF proteins are recruited to the MycMax/USF/CRE site in the absence of CREB binding. Hippocampal nuclear extracts were incubated with a [^{32}P] end-labeled double-stranded oligonucleotide probe that contained the wild-type MycMax/USF site and a mutated CRE site [R1bMUC(CRE$^-$)] (lane 2). Antibodies against USF and CREB were added to the reaction mixture before the DNA-protein binding reaction was started. The position of the major DNA–protein complex is indicated (arrow).

1 and exon 6′. This, along with the fact that the 5′-flanking regions of $GABA_BR1a$ and $GABA_BR1b$ exhibit significant promoter activity, indicates that differential expression of $GABA_BR1a$ and $GABA_BR1b$ reflect differential use of alternative promoters.

In eukaryotic genes, alternative promoters are known to mediate developmental or tissue-specific gene expression (Schibler and Sierra, 1987; Ayoubi and Van De Ven, 1996). Therefore, alternative promoters in the $GABA_BR1$ gene could provide an explanation for the differential developmental and tissue-specific regulation of $GABA_BR1a$ and $GABA_BR1b$ isoforms. The relative use of $GABA_BR1a$ and $GABA_BR1b$ transcription initiation sites in human adult brain are different from those in human fetal brain (Fig. 1*A*,*B*). Whereas both $GABA_BR1$ transcripts are present in the adult brain, $GABA_BR1a$, but not $GABA_BR1b$, mRNA is detected in the fetal brain. Consistent with this observation, the $GABA_BR1a$ promoter is 33 times stronger than $GABA_BR1b$ in cultures of embryonic hippocampal neurons. Differential use of $GABA_BR1$ alternative promoters in different developmental stages may be related to binding of regulatory factors to unique transcriptional elements in the $GABA_BR1a$ and $GABA_BR1b$ flanking regions. When considered with the observation that $GABA_BR1a$ may be the preferred postsynaptic receptor, whereas the majority of presynaptic receptors may contain $GABA_BR1b$ (Benke et al., 1999), our results suggest that genetic control over the number of $GABA_B$ receptors targeted to a particular location in the neuron may control the functional phenotype of that neuron and provide a dynamic way to respond to synaptic input.

CREB and ATF4 differentially regulate the alternative $GABA_BR1$ promoters

The results show that CREB activates transcription through distinct CREs in the alternative $GABA_BR1a$ and $GABA_BR1b$ promoters. First, EMSA experiments reveal that endogenous CREB from hippocampal nuclear extracts binds specific $GABA_BR1a$ and $GABA_BR1b$ CRE sequences *in vitro*. Second, specific and selective CRE decoy oligonucleotides that compete for binding of endogenous CREB proteins inhibit endogenous $GABA_BR1$ gene expression in neurons. Third, transient transfection experiments show that overexpression of CREB increases promoter activity, whereas overexpression of M1-CREB decreases promoter activity. Finally, inactivation of the $GABA_BR1a$ CRE by mutation reduces promoter activity. These data strongly support a critical role for CREB in the activation of $GABA_BR1$ transcription from alternative $GABA_BR1a$ and $GABA_BR1b$ promoters.

Additional CREB/ATF family members may also regulate the expression of $GABA_BR1$ isoforms in the presence of endogenous CREB. ATF4 can bind to $GABA_BR1$ in the nucleus and cytoplasm of neurons (Nehring et al., 2000; White et al., 2000; Vernon et al., 2001). Overexpression of ATF4 stimulates $GABA_BR1a$ and inhibits $GABA_BR1b$ promoter activity in transfected primary hippocampal neurons that contain endogenous CREB.

$CREB^{\alpha\Delta}$ mutant mice are characterized by a partial loss of hippocampal-dependent memory (Graves et al., 2002). Although $CREB^{\alpha\Delta}$ mutant mice display normal spatial learning, they have impaired short- and long-term cued and contextual fear conditioning. In the absence of CREB binding, compensation within the CREB/ATF family of proteins may prevent a total loss of hippocampal-mediated behaviors (Blendy et al., 1996; Graves et al., 2002; Balschun et al., 2003).

The activity of the $GABA_BR1a$ promoter is increased rather than decreased, as initially expected, in the CREB knock-out. The difference between the results of $GABA_BR1a$ promoter analysis with a CRE mutation and M1-CREB overexpression versus the effect of the knock-out on the expression of the $GABA_BR1a$ gene can be understood from our results using primary hippocampal neurons. First, we have shown that the CRE site in $GABA_BR1a$ and $GABA_BR1b$ promoters is occupied by CREB. Second, overexpression of ATF4 stimulates $GABA_BR1a$ and inhibits $GABA_BR1b$ promoter activity. Finally, unlike adult rat tissue, primary hippocampal neurons exhibit barely detectable levels of ATF4, so it follows that CREB binding to the CRE site would predominate. Thus, because CREB knock-out animals show high levels of ATF4, the observed increase in $GABA_BR1a$ and decrease in $GABA_BR1b$ gene expression directly parallel the changes in promoter activity observed when ATF4 is overexpressed in hippocampal neurons.

Results from studies on *Aplysia* provide insight into how CREB and ATF4 might produce different effects on $GABA_BR1b$ gene transcription. Bidirectional modification of chromatin by CREB and ATF4 can lead to gene activation or repression through recruitment of CREB-binding protein and induction of histone acetylation or recruitment of histone deacetylase 5 and histone deacetylation (Guan et al., 2002). Transcriptional activation by CREB stimulates long-term facilitation, whereas ATF4-directed transcriptional repression mediates long-term depression.

Interaction of $GABA_B$ receptor subunits with ATF4 in neurons (Nehring et al., 2000; White et al., 2000; Vernon et al., 2001) and the genomic regulation of $GABA_BR1$ isoforms by such factors points to an additional potential role of $GABA_B$ receptor subunits. $GABA_BR1$ can bind to $GABA_BR2$ or ATF4, but not to both, simultaneously (White et al., 2000; Vernon et al., 2001). ATF4 may, therefore, prevent $GABA_B$ receptor subunit dimerization and inhibit formation of functional heterodimeric $GABA_B$ receptors. Conversely, the C terminus of $GABA_BR1$ may mask the ATF4 nuclear localization signal to control its concentration in

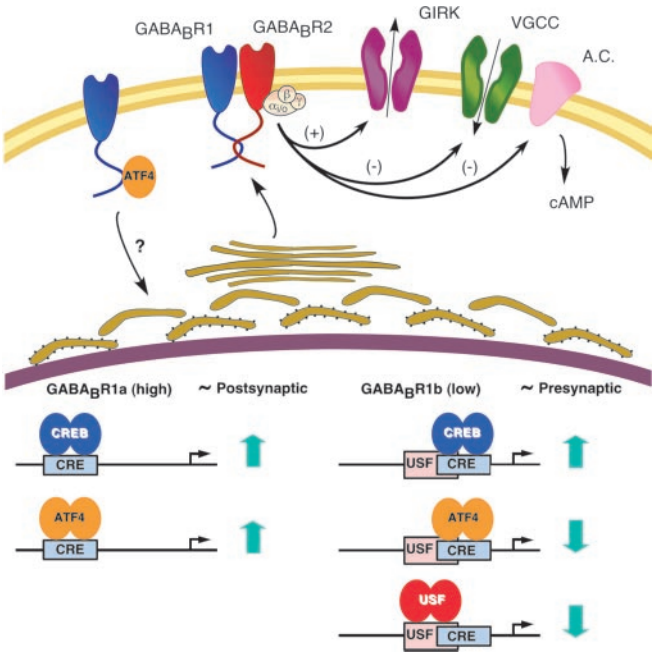

Figure 10. A model for combinatorial regulation of the alterative $GABA_BR1$ promoters. Whereas it is known that $GABA_BR1$ interacts directly with the transcription factor ATF4 in the cytoplasm of neurons, $GABA_B$ receptor activation may stimulate translocation of ATF4 from one cellular compartment to another (Nehring et al., 2000; White et al., 2000; Vernon et al., 2001). Previous reports suggest a presynaptic and postsynaptic localization for both $GABA_BR1a$ and $GABA_BR1b$ subunits (Kaupmann et al., 1998b; Benke et al., 1999; Bischoff et al., 1999; Princivalle et al., 2000, 2001; Towers et al., 2000). However, $GABA_BR1a$ appears to be located predominately in postsynaptic sites, whereas $GABA_BR1b$ is in presynaptic terminals (Benke et al., 1999; Princivalle et al., 2001). We propose a model in which activator and repressor proteins selectively recognize DNA regulatory elements specific to the $GABA_BR1a$ and $GABA_BR1b$ promoters, independently controlling expression of these receptor subunit subtypes. Binding of CREB and ATF4 activator proteins may mediate $GABA_BR1a$ expression at postsynaptic sites. In contrast, binding of the CREB activator as well as ATF4 and USF repressor proteins may mediate $GABA_BR1b$ expression at presynaptic sites. The precise cellular and subcellular localization of $GABA_BR1a$ and $GABA_BR1b$ subunits in the hippocampal formation awaits further characterization. GIRK, G-protein-gated inwardly rectifying potassium channel; VGCC, voltage-gated calcium channel; A.C., adenylate cyclase.

the nucleus (Nehring et al., 2000; Vernon et al., 2001). Taken together with the fact that $GABA_BR1$ has been found in the nucleus of neurons (Gonchar et al., 2001), the interaction of a $GABA_B$ receptor subunit with a transcription factor suggests a novel feedback mechanism linking receptor number to gene regulation.

Overlapping USF- and CRE-binding sites define a novel $GABA_BR1b$ regulatory region (R1bMUC)

Activation of CREB is required for activity-dependent transcription of genes that are important for neural development and synaptic plasticity (Sheng and Greenberg, 1990; Shieh et al., 1998; Tao et al., 1998). In addition, members of the USF family, USF1 and USF2, contribute to the Ca^{2+} signal-mediated transcription of BDNF (Tabuchi et al., 2002; Chen et al., 2003), possibly through recognition of an overlapping CREB–USF site in alternative BDNF promoters. Mice devoid of CREB or USF transcription factors show a marked disruption of brain function (Sirito et al., 1998; Graves et al., 2002). Our research has shown that, like BDNF, the $GABA_BR1b$ promoter is specifically controlled by the dynamic interaction of CREB and USF factors at an overlapping CREB–USF site.

Using transient transfection, we have shown that the USF-binding site mediates transcriptional repression from the $GABA_BR1b$ promoter. Whereas CREB is the dominant transcription factor at this composite regulatory region, we find that USFs bind the R1bMUC sequence in the absence of CREB binding. Moreover, USF decoy oligonucleotides markedly increase levels of $GABA_BR1b$ protein in embryonic hippocampal cells that normally contain very low levels of $GABA_BR1b$, suggesting that USF transcription factors may be a nexus point for regulation over isoform-specific transcription. Relief of USF-mediated repression has been implicated in the induction of other genes (McMurray and McCance, 2003). Whether de-repression of $GABA_BR1b$ gene expression during CNS development is regulated by USF transcription factors remains to be determined.

In summary, we have defined novel *cis*-regulatory elements in the alternative $GABA_BR1$ promoters and have begun to reveal how multiple transcription factors (CREB, ATF4, and USF) cooperate to regulate $GABA_BR1a$ and $GABA_BR1b$ transcription in neurons (Fig. 10). $GABA_BR1a$ and $GABA_BR1b$ alternative promoters are regulated by positive- and negative-acting CREs. Whereas CREB stimulates transcription of both $GABA_B$ receptor isoforms, ATF4 produces differential effects. The observations that $GABA_BR1b$-containing receptors may be presynaptic and regulate neurotransmitter release, that USF proteins have been shown to activate with depolarization, and that activity of multiple transmitter-gated receptors, including $GABA_B$, regulate CREB phosphorylation (Ito et al., 1995; Ishige et al., 1999), when taken together with an association of $GABA_BR1$ subunits with ATF4 in the cytoplasm, suggest that a novel regulatory pathway from the synapse to the nucleus may exist to control inhibitory neurotransmission in the CNS.

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