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The Metabotropic $GABA_B$ Receptor Directly Interacts with the Activating Transcription Factor 4*

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G protein-coupled receptors regulate gene expression by cellular signaling cascades that target transcription factors and their recognition by specific DNA sequences. In the central nervous system, heteromeric metabotropic γ-aminobutyric acid type B ($GABA_B$) receptors through adenylyl cyclase regulate cAMP levels, which may control transcription factor binding to the cAMP response element. Using yeast-two hybrid screens of rat brain libraries, we now demonstrate that $GABA_B$ receptors are engaged in a direct and specific interaction with the activating transcription factor 4 (ATF-4), a member of the cAMP response element-binding protein /ATF family. As confirmed by pull-down assays, ATF-4 associates via its conserved basic leucine zipper domain with the C termini of both $GABA_B$ receptor ($GABA_BR$) 1 and $GABA_BR2$ at a site which serves to assemble these receptor subunits in heterodimeric complexes. Confocal fluorescence microscopy shows that $GABA_BR$ and ATF-4 are strongly coclustered in the soma and at the dendritic membrane surface of both cultured hippocampal neurons as well as retinal amacrine cells *in vivo*. In oocyte coexpression assays short term signaling of $GABA_BRs$ via G proteins was only marginally affected by the presence of the transcription factor, but ATF-4 was moderately stimulated in response to receptor activation in *in vivo* reporter assays. Thus, inhibitory metabotropic $GABA_BRs$ may regulate activity-dependent gene expression via a direct interaction with ATF-4.

Many stimulatory neurotransmitters and hormones in the mammalian central nervous system have been found to cause long term changes in neuronal function, such as differentiation, plasticity, and learning (1–4). These changes generally require agonist-driven activation of cellular signaling cascades, followed by the induction of transcriptional regulators that recognize cis-acting promoter and enhancer elements (5). Among the best studied examples of DNA target motifs in many neuronal genes is the octanucleotide cAMP response element (CRE)[1] that is bound by transcription factors of the ATF/CREB family when phosphorylated by protein kinase A upon an increase in cellular cAMP levels (6, 7). Inhibitory neurotransmitters that lower cytoplasmic cAMP levels are expected to negatively regulate neuronal transcription through CREB-dependent mechanisms. Indeed, previous reports on the main inhibitory neurotransmitter in the central nervous system, γ-aminobutyric acid (GABA), have shown that in cerebellar granule neurons the specific agonist baclofen inhibits forskolin-initiated CREB-transcriptional programs by lowering cytosolic cAMP or Ca^{2+} levels (8).

In the central nervous system, GABA targets to two distinct types of receptors, ligand-gated ionotropic $GABA_A$ receptors (including $GABA_C$ receptors) and G protein-linked, metabotropic $GABA_B$ receptors ($GABA_BR$; Refs. 9–11), thus mediating both fast and slow inhibition of excitability at central synapses. In short term signaling, presynaptically located $GABA_BRs$ suppress neurotransmitter release by inhibiting voltage-sensitive P, N, and L-type Ca^{2+} channels (11–14). Postsynaptically, $GABA_BR$ stimulation generally causes inhibition of adenylate cyclase via $G\alpha_i$ subunits (15), as well as activation of Kir3 type potassium channels by liberated $G\beta\gamma$ subunits, thereby hyperpolarizing the postsynaptic membrane (16, 17). Molecularly, two major isoforms of the metabotropic receptor, $GABA_BR1$ and $GABA_BR2$, and various splice variants thereof, have been recently described (18–25). Their primary amino acid sequences indicate heptahelical membrane topology and are most closely related to the family 3 of G-protein coupled receptors: metabotropic glutamate receptors (mGluR; Refs. 26 and 27), the Ca^{2+} sensing receptor (28), and the vomeronasal receptors (29, 30). In central neurons $GABA_BR1$ and $GABA_BR2$ are widely coexpressed and, a novelty for heptahelical receptors, were found to generate fully functional receptors only when linked by their C-terminal tails in a heterodimeric assembly (19–23). Although the precise functional consequences of this association have not yet been deciphered in detail, it is thought

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[1] The abbreviations used are: CRE, cAMP response element; CREB, cAMP response element-binding protein; bZIP, basic leucine zipper domain; GABA, γ-aminobutyric acid; $GABA_BR$, metabotropic B-type GABA receptor; Kir, inwardly rectifying potassium channel; NLS, nuclear localization signal; SFV, Semliki forest virus; Y2H, yeast-two hybrid; MEKK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase; ATF, activating transcription factor; GST, glutathione *S*-transferase; MBP, maltose-binding protein; PBS, phosphate-buffered saline; EGFP, enhanced green fluorescent protein.

that subunit dimerization promotes proper posttranslational processing, membrane targeting, and assembly into specific signaling matrices in subcellular neuronal specializations (31).

By means of yeast-two-hybrid (Y2H) interaction cloning, biochemical, and functional reporter assays, as well as immunocytochemistry, we now provide evidence that metabotropic $GABA_BRs$ are also capable of directly interacting with transcription factors and thus may utilize a mechanism for transcriptional regulation unique to membrane receptors. Our mutational analysis indicates that $GABA_BR$ bind to ATF-4, a transcription factor of the leucine zipper ATF/CREB family, via their C-terminal leucine zipper motifs, which *in vivo* may result in the regulation of gene transcription upon stimulation.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screening—Two independent Y2H assays were used in parallel. Both the MATCHMAKER II (CLONTECH) and the LexA (OriGene Technologies) systems were used to screen rat brain cDNA libraries constructed with the activation domain vectors pAD-GAL4 and pJG4–5, respectively, using amino acids 848–960 of the C-terminal coding region of $GABA_BR1a$ (18) as bait. C-terminal baits were amplified by polymerase chain reaction from a rat brain library and inserted into the DNA-binding domain vector pGBT9 and the galactose-inducible vector pGilda, respectively. Screening with the pGBT9 and pGilda bait yielded colonies that grew on the corresponding selection plates complemented with 10 mM 3-aminotriazole for the MATCHMAKER system and were positive in the β-galactosidase assay. Isolated plasmids were sequenced on both strands using the ABI PRISM sequenase dye terminator kit on an automatic sequencer. For analysis of the interaction site, $GABA_BR1$ and ATF-4 deletions were generated by polymerase chain reaction with specific oligonucleotides and subcloned into pGBT9 and pAD-GAL4. Yeast strains HF7c and EGY 48 were cotransformed with 100 ng each of bait and prey vector, streaked out on agar plates lacking tryptophan, leucine, and histidine (MATCHMAKER), and also lacking uracil (LexA). Colony growth/activation of the *HIS3* and *LEU* reporter genes, respectively, as well as β-galactosidase activity were controlled after 4 days.

Preparation of Brain Homogenates —Rat cerebral cortices were homogenized in a Teflon glass Potter homogenizer with 12 strokes at 900 rpm in 20 ml of ice-cold 0.32 M sucrose, 4 mM HEPES/NaOH, pH 7.3, containing Complete and a protease inhibitor mixture (Roche Diagnostics). The homogenate was centrifuged for 10 min at $800 \times g$ in a Sorvall SS-34 rotor (DuPont). The resulting pellet was used as crude nuclear fraction (P1). The supernatant was recovered and spun at $27{,}000 \times g$ for another 30 min. The resulting pellet (P2) was resuspended in 3 ml of homogenization buffer and frozen until needed.

Bacterial Recombinant Fusion Proteins —Glutathione *S*-transferase (GST) fusion proteins of $GABA_BR$ (GST-GBR1 and GST-GBR2) and synaptoporin tail regions (amino acids 198–265) were constructed in pGEX-5X-1 (Amersham Pharmacia Biotech) using specific *Eco*RI- and *Sal*I-flanked oligonucleotides. Full-length ATF-4 was fused to the C terminus of GST in pGEX-5X-1, and maltose-binding protein (MBP-ATF-4) in pMAL-c2 (New England Biolabs) using *Eco*RI and *Xho*I sites, and electroporated into Epicurian *Escherichia coli* BL21 (Stratagene). Expression of fusion protein was induced by 1 mM isopropyl-1-thio-β-D-galactopyranoside for 3–5 h. Cells were broken in a French press in PBS, and soluble protein fractions were recovered in the supernatant after centrifugation at $100{,}000 \times g$ for 1 h and kept frozen until needed.

MBP-ATF-4 Binding to GST Fusion Proteins—Glutathione beads (30 μl) were loaded by incubation for 1 h at 4 °C in PBS with 150 μg of GST and the fusion proteins between GST and the C termini of synaptoporin, GBR1 and GBR2. An extract (100 μl) of bacterially expressed MBP-ATF-4 was then added to preloaded beads in binding buffer (PBS, 0.2% Triton X-100, and Complete) and rotated at 4 °C for 3 h. Pelleted beads were washed three times with binding buffer and once with PBS. Bound proteins were eluted using SDS sample buffer and separated on a 10% SDS-polyacrylamide gel. To control for MBP-ATF-4 binding, samples were subjected to Western blotting with rabbit anti-MBP antibodies (1:10,000; New England Biolabs).

Pull-down Assays—ATF-4 pull-downs from P1 brain extract were achieved by mixing 100 μl of GST or GST-GBR1 bacterial extracts with 10 mg of brain P1 Triton extract in 10 ml of 10 mM HEPES/NaOH, pH 7.3, containing 0.5% (w/v) Triton X-100 for 2 h at 4 °C. After incubation, 50 μl of glutathione beads were added and the mixture was incubated for an additional 2 h. Glutathione beads were then recovered by centrifugation and washed three times vigorously with PBST (PBS with 0.5% (w/v) Triton X-100). Proteins bound to the beads were eluted by SDS sample buffer and analyzed by Western blotting using anti-CREB2/ATF-4 antibodies (1:1000; Santa Cruz Biotechnology). Affinity purification of native $GABA_BR1$ from the solubilized P2 fraction was performed using an aliquot of the P2 fraction. The P2 (8 mg of protein) fraction was solubilized for 1 h with 1.5% Triton X-100 in a 3-ml final volume of Tris-buffered saline (25 mM Tris, pH 7.4, 150 mM NaCl, and protease inhibitors). After ultracentrifugation at $100{,}000 \times g$ for 1 h, the supernatant was incubated for 5 h with 30 μl of glutathione beads preloaded with either GST or GST-ATF-4 (150 μg). Bound material was recovered from glutathione beads after washing four times with Tris-buffered saline containing 0.2% (w/v) Triton X-100 and one wash with Tris-buffered saline alone. Bound proteins were then eluted with SDS sample buffer. Proteins were analyzed by Western blotting using a polyclonal goat anti-$GABA_BR1$ antibody (Santa Cruz Biotechnology).

Miscellaneous Methods—SDS-polyacrylamide gel electrophoresis was performed on 6%, 10%, or 12% polyacrylamide gels. For Western blot analysis, proteins were transferred to nitrocellulose membranes (Schleicher & Schuell). First antibodies were overlaid with goat anti-rabbit or anti-mouse horseradish peroxidase-coupled secondary antibodies, and chemiluminescence was detected using the Pico detection kit (Pierce).

In Vivo Reporting Systems —The trans PathDetect® reporting system (Stratagene) was used to test if ATF-4 was involved in activating transcriptional expression upon receptor stimulation. For all experiments HEK293-GBR cells were used that had been stably tranfected with $GABA_BR1$ and $GABA_BR2$ subunits. A fusion transactivator protein between ATF-4 (or constitutively MEKK-activated Jun) and the GAL4 DNA binding domain was constructed and transfected (pFA-ATF-4; 50 ng) into HEK293-GBR cells together with vectors carrying the luciferase reporter gene under the control of the GAL4 promoter (pFR-Luc; 1 μg) and a vector expressing β-galactosidase (50 ng). 24 h after transfection, cells were treated with serum-free medium, and 18 h later lysed with 500 μl of lysis buffer and the supernatant centrifuged to remove cell debris. Luciferase activity was measured with a luminometer (Luciferase Reporter Gene Assay, Roche Diagnostics).

Electrophysiology—For expression in *Xenopus laevis* oocytes, capped run-off poly(A^+) cRNA transcripts were synthesized from $GABA_BR1a$, $GABA_BR2$, ATF-4, and Kir3.1/3.2 concatemers (32) and ~3 ng of each injected in defolliculated oocytes. Oocytes were incubated at 19 °C in ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM HEPES, pH 7.4 -7.5), supplemented with 100 μg/ml gentamicin and 2.5 mM sodium pyruvate, and assayed 72 h after injection. Two-electrode voltage-clamp measurements were performed with a Turbo Tec-10 C amplifier (npi) and sampled through an EPC9 (Heka Electronics) interface using Pulse/Pulsefit software (Heka). Oocytes were placed in a small volume perfusion chamber and bathed with ND96 or "high K^+" solution (96 mM KCl, 2 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM HEPES, pH 7.4–7.5).

Neuronal Expression via Semliki Forest Viruses (SFV) and Immunocytochemistry —Recombinant SFV harboring ATF-4 were engineered and processed as described previously (33). In brief, the cDNA of the N-terminally EGFP-tagged fusion protein ATF-4-EGFP was subcloned into pSFV1 (Life Technologies, Inc.). After linearization with *Spe*I, cDNA was *in vitro* transcribed using SP6 RNA polymerase (Roche Molecular Biochemicals). BHK21 cells were transfected by electroporation (400 V, 975 microfarads) with a mixture of 10 μg of pSFV/ATF-4-EGFP and pSFV-helper2, respectively. After 24 h supernatant was collected and stored in 450-μl aliquots at −80 °C. Prior to treatment of neuronal cultures, aliquots of virus were activated by 100 μl of chymotrypsin (2 mg/ml). Primary cultures of hippocampal neurons were prepared from 1-day-old rats as described previously (34). Hippocampal neurons were cultured in Neurobasal A medium supplemented with B27 (Life Technologies, Inc.) in 12-well plates (2 ml in each well) for 14 days. Half of the medium was removed from each well and stored at 37 °C. For infection, 20–50 μl of activated virus was added per well. After incubation for 2 h at 37 °C, the virus-containing medium was replaced with stored aliquots. Expression of ATF-4-EGFP was observed 12–14 h after infection.

For immunostaining, hippocampal neurons were fixed with 2% (w/v) paraformaldehyde, 0.1% (w/v) Triton X-100 and blocked with 2% (v/v) normal goat serum. Subsequently, neurons were incubated overnight with guinea pig anti-$GABA_BR1$ (1:1000; PharMingen) and rat anti-synaptophysin (1:1000; a gift of R. Jahn, Göttingen) antibodies, respectively. After washing with PBS, cells were incubated 1 h with Cy3-conjugated IgG (1:1000; Jackson Immunoresearch Laboratories). Coverslips were washed again with PBS, mounted on slides, and analyzed on a confocal LSM410 microscope (Zeiss).

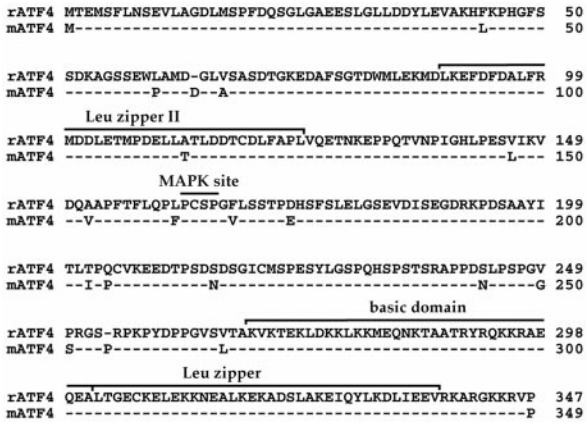

FIG. 1. **Amino acid sequence alignment of rat and mouse ATF-4.** Shown are the leucine zipper I and II motifs, the putative mitogen-activated protein kinase phosphorylation site, and the basic DNA binding domain. The rATF-4 sequence has been deposited to GenBank™ under accession no. AF252627.

Retina Preparation and Immunocytochemistry—Adult albino rats were anesthetized deeply with halothane and decapitated. Eyes were enucleated and opened along the ora serrata, and the posterior eyecups with the retinae attached were immersion-fixed for 15–30 min in 4% (w/v) paraformaldehyde in 0.1 M PB, pH 7.4. After dissection retinae were cryoprotected in 10% (w/v), 20% (w/v) sucrose in PB for 1 h each and in 30% (w/v) sucrose in PB overnight at 4 °C. Pieces of retinae were mounted in freezing medium (Reichert-Jung, Bensheim, Germany), sectioned vertically at 12-μm thickness on a cryostat, and collected on slides. For double-labeling experiments, guinea pig anti-GABA$_B$R1 (1:1000; PharMingen) and rabbit anti-CREB2/ATF-4 antibodies (1:1000; Santa Cruz Biotechnology) were used and visualized by red and green fluorescence secondary antibodies, goat anti-rabbit IgG, and goat anti-guinea-pig IgG (Alexa™ 594, Alexa™ 488; 1:500; Molecular Probes). Sections were examined by confocal laser-scanning microscopy (Leica DM IRBE; Leica Microsystems, Heidelberg, Germany) using a ×63 objective and special filter settings (Leica TCS SP).

RESULTS

Y2H Assay—Using the Y2H system (35), we sought to isolate candidate proteins that directly interact with and modulate the signaling function of GABA$_B$ receptors. Therefore, the complete C-terminal intracellular region of GABA$_B$R1a was initially used as bait to screen rat brain cDNA libraries. Two independent screenings of $\sim 2 \times 10^7$ recombinants resulted in the isolation of 27 and 180, respectively, positive clones, the open reading frames of which all encoded regions of the same polypeptide. Data base analysis indicated very high similarity to the mouse transcription factor mATF-4 (36), also known as C/ATF (37) or mTR67 (38). The complete open reading frame of the rat orthologue (rATF-4) is shown in Fig. 1. rATF-4 is 347 amino acids in length and shares 94% and 86% amino acid identity with mouse ATF-4 and the human ATF-4 (hCREB2/TAXREB67; Refs. 39 and 40), respectively. At the C terminus, rATF-4 harbors a conserved basic leucine zipper (bZIP) dimerization motif that binds CRE and is present in all CREB/ATF proteins. In addition, rATF-4 contains a second heptad repeat of leucines between amino acids 89 and 124 near the N terminus, and a potential phosphorylation site for mitogen-activated protein kinase (amino acid position 164).

We performed control experiments (i) with bait or prey vectors missing, (ii) using empty vectors, (iii) using vectors that expressed unrelated proteins such as pRHFM1 encoding the *Drosophila* bicoid protein homeodomain, and (iv) using vectors that expressed other proteins harboring a bZIP domain, *e.g.* CREB. All these controls were negative, excluding autoactivation and corroborating the specificity of the interaction between GABA$_B$R1 and rATF-4. For a detailed mapping of the interaction domains, deletion mutants of both GABA$_B$R1 and ATF-4

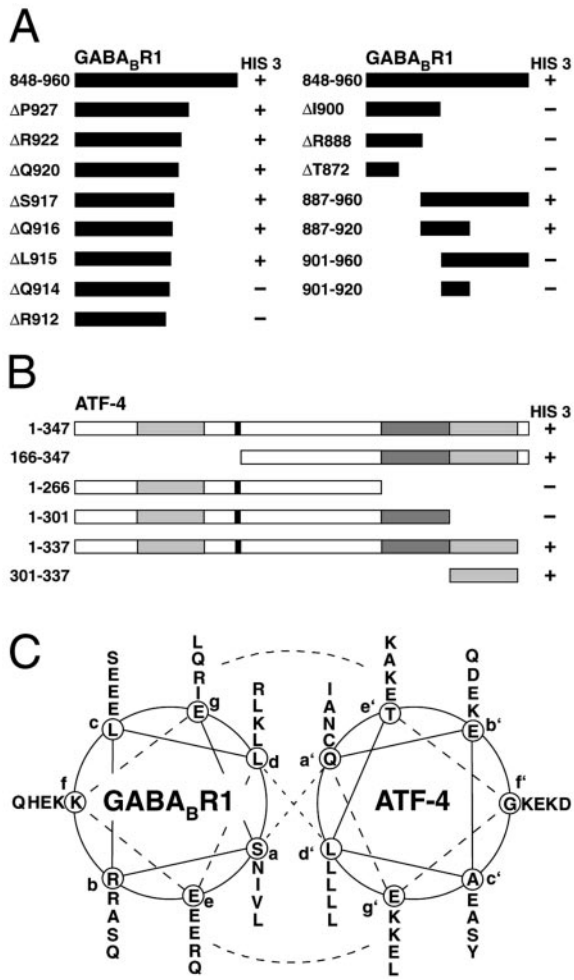

FIG. 2. **GAL-4-based Y2H analysis of the interaction between GABA$_B$R1 and ATF-4.** *A*, deletion constructs of GABA$_B$R1 (amino acid region on the *left*) were used as baits for binding ATF-4 as prey. The HIS3 marker was used as reporter in the Y2H assay. *B*, the equivalent analysis is shown for deletion constructs of rATF-4 used as prey for binding the C terminus of GABA$_B$R1. Functional sites depicted in Fig. 1 are *boxed* in this schematic representation of ATF-4. *C*, helical wheel diagrams of the putative leucine zipper-based interface between GABA$_B$R1 and rATF-4. The view is from the N termini starting from S887 (position *a* in GABA$_B$R1a) and Q299 (*a′* in rATF-4). Heptad positions are labeled *a*–*g* (*a′*–*g′*).

were constructed and tested for complementation in the Y2H assay. These experiments showed that deletion constructs in the GABA$_B$R1 bait, removing partial sequences from the C terminus, allow binding of rATF-4 (Fig. 2*A*) until a leucine at amino acid position 915 (Leu-915) is removed (ΔQ914) or exchanged by a glycine (L915G) or serine (L915S) residue (data not shown in the illustration). In contrast, replacement of Gln-916 by an alanine (Q916A) did not disturb the interaction. Further restriction analysis on the 5′ end of GABA$_B$R1a assigns the region of interaction to amino acids 887–915. Interestingly, this domain has been recently mapped to likely participate in the obligate assembly of GABA$_B$R1 and GABA$_B$R2 subunits into heteromeric receptor complexes (23, 41), suggesting a bifunctional role of this site.

Conversely, deletion constructs in rATF-4 demonstrated that the first leucine zipper an the putative mitogen-activated protein kinase site of rATF-4 were dispensable for binding, whereas the C-terminal leucine zipper (amino acids 301–337) was required for association with GABA$_B$R1 (Fig. 2*B*). When described in terms of the heptad patterns seen in a helical wheel diagram, the interaction sites between the C termini of

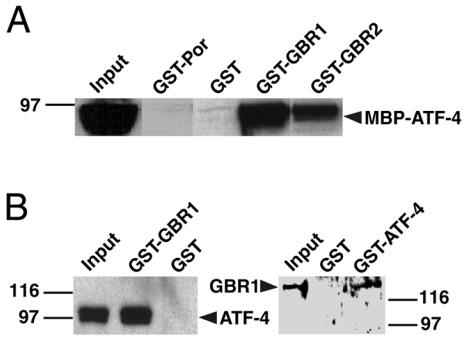

FIG. 3. **Interaction of GABA$_B$Rs and ATF-4 *in vitro*.** *A*, bacterially expressed GST and GST fusion proteins (*GST-Por*, fusion with the C-terminal tail of the synaptic vesicle protein synaptoporin; *GST-GBR1* and *GST-GBR2*, fusion proteins of the C-terminal tails of GABA$_B$R1a and R2) were immobilized on glutathione-Sepharose and then incubated with recombinant MBP-ATF-4. *B*, GST-GBR1 or GST-ATF-4 were used to test the binding of ATF-4 (*left panel*) and native GBR1 (*right panel*) present in a Triton X-100 extract from rat brain. Bound material was eluted using SDS sample buffer, separated by SDS-PAGE, and immunoblotted with anti-MBP (*A*), anti-ATF-4 (*B*, *left*), or anti-GBR1 (*B*, *right*).

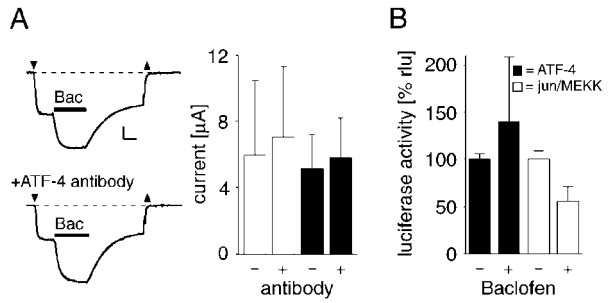

FIG. 4. **Expression in *Xenopus* oocytes and *in vivo* luciferase reporter assay.** *A*, expression of GABA$_B$R1, GABA$_B$R2, ATF-4, and Kir3.1/3.2 concatemeric channels in *Xenopus* oocytes elicits basal and agonist-dependent (as indicated by *black bars*) K^+ currents in the absence (*top left trace*) and presence of injected ATF-4 antibodies (*bottom left trace*). High K^+ (96 mM, *arrowheads*) and 10 μM baclofen (*black bars*) were applied to the bath and oocytes voltage-clamped at -80 mV. *Dashed lines* indicate zero-current levels, *scale bar* represents 2 μA and 10 s. The *right panel* shows a bar graph summary of Kir3.1/3.2 inward currents at -80 mV in the presence (*black bars*) and absence (*white bars*) of overexpressed ATF-4 and injected anti-ATF-4 antibodies. *B*, *in vivo* luciferase trans-reporter assay performed on HEK293 cells stably transformed with GABA$_B$R1 and GABA$_B$R2. Fusion transactivator proteins between the GAL4 DNA binding domain and ATF-4 and Jun (activated with MEKK), respectively, were transfected together with the luciferase reporter gene under the control of the GAL4 promoter. Luciferase activity was measured in the presence or absence of 10 μM baclofen.

ATF-4 and GABA$_B$R1 conform with good approximation to classic coiled-coil structures (Fig. 2*C*). In the bZIP domain of rATF-4, the periodic array of leucines at every seventh position likely interdigitates with that of a matching helix formed by the GABA$_B$R1 C terminus to form a zipper-like structure. It has been suggested earlier that bZIP domains not only mediate association between transcription factors prior to DNA binding (42), but also form coiled-coil structures from up to four helices with various other proteins (43). Together with the array of leucines at position d in GABA$_B$R1 and d′ in ATF-4, several features are consistent with such an interaction: (i) the β-branched amino acids valine, isoleucine (and alanine) occur at the alternate hydrophobic positions a and a′; (ii) there are highly conserved breaks at these positions caused by polar asparagines; and (iii) the amino acids preceding the alternate juxtaposed hydrophobic residue and following the leucine of the next heptad are often oppositely charged in both proteins to allow electrostatic interactions (38). Consistent with our pull-down experiments (see below), helical wheel representation of GABA$_B$R2, but not the C-terminal splice variant GABA$_B$R1d (24), which misses the leucine zipper, does also reveal complementarity to ATF-4 as required for stable dimerization.

Biochemical Assays—To verify the interaction between ATF-4 and GABA$_B$R1a by an independent assay, we evaluated binding of the respective bacterially expressed fusion proteins. In addition, we have tested the binding of C-terminal regions of GABA$_B$R2 and the unrelated synaptic protein synaptoporin (Por). GST and the GST-fusion proteins GST-GBR1, GST-GBR2, and GST-Por were immobilized on glutathione beads and incubated with either MBP or MBP-ATF-4 bacterial lysates. After washing the resin, bound material was eluted and analyzed by Western blotting using anti-MBP antibody. MBP-ATF-4, but not MBP (data not shown), specifically interacted with both immobilized GST-GBR1 and GST-GBR2, but not with GST or GST-Por (Fig. 3*A*).

For demonstrating that GABA$_B$R1 is capable to interact with rATF-4 present in rat brain, we performed pull-down experiments with immobilized GST or GST-GBR1 using crude P1 rat brain extracts. Triton X-100 extracts from a crude rat brain nuclear fraction was incubated with GST or GST-GBR1, and protein binding to the fusion proteins was recovered by adding glutathione beads. Bound material was eluted and analyzed by Western blotting using anti-rATF-4 antibodies. A doublet of 95–100-kDa proteins, corresponding most probably to a dimer of rATF-4, was detected exclusively in the GST-GBR1, but not in the GST eluates (Fig. 3*B*, *left*). In parallel, we showed that ATF-4 can bind to native GABA$_B$R1 by performing similar pull-down experiments using immobilized GST or GST-ATF-4 and Triton X-100 extract from crude P2 rat brain fraction. Bound material was eluted and analyzed by Western blotting using anti-GABA$_B$R1 antibodies. A 130-kDa protein was detected exclusively in the GST-ATF-4, but not in the GST eluates (Fig. 3*C*).

Electrophysiology and in Vivo Reporting System—In a further series of experiments with heterologously expressed proteins, we sought to reveal a functional consequence of the interaction between GABA$_B$R and ATF-4. First, we investigated whether cytosolic ATF-4, when binding to the dimeric receptor, might provide a negative regulator of receptor function in that it interferes with G protein binding and thus classic short term signaling. This putative role of ATF-4 on G protein signaling of the receptors was studied in *Xenopus* oocytes under two-electrode voltage-clamp. Coinjection at a 1:1:1 ratio of cRNAs for GABA$_B$R1, GABA$_B$R2, and Kir3.1/3.2 concatemers as target channels for receptor signaling resulted in the expression of large basal and baclofen-induced inwardly rectifying K^+ currents as described previously (17). Two days after injection, Kir3 current amplitudes activated by baclofen (10 μM) averaged -6.0 ± 4.5 μA ($n = 7$) at -80 mV in the presence of 96 mM external K^+ (Fig. 4*A*). To test for the role of endogenous ATF-4, anti-ATF-4 antibodies were injected 1 h prior to the experiment at a dilution of 1:100. In the presence of antibody, the activation kinetics of baclofen-induced Kir currents (as determined from responses to hyperpolarizing voltage steps) was unchanged, but mean amplitudes were slightly increased by statistically significant 18% ($p < 0.05$). Similarly, when ATF-4 was overexpressed under otherwise unchanged recording conditions, ligand-activated currents in the presence of antibody (-5.8 ± 2.4 μA) were elevated by 12% compared with control conditions (-5.1 ± 2.1 μA, $n = 7$), indicating that the presence of intrinsic or exogenously expressed ATF-4 plays only a marginal role in receptor-stimulated G protein activation.

Next, we employed the PathDetect® luciferase reporting system to investigate the *in vivo* consequences of the GABA$_B$R/

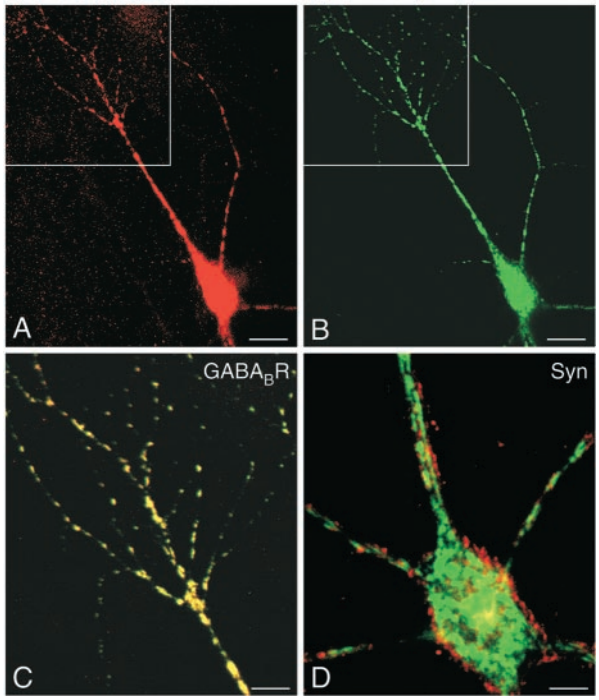

FIG. 5. **Colocalization of ATF-4 and GABA$_B$R1a in hippocampal neurons.** Confocal fluorescence micrographs of primary hippocampal cells immunostained with anti-GABA$_B$R1 antibodies and detected with Cy3-labeled secondary antibody (*A*). *B*, EGFP-tagged ATF-4 expressed through a recombinant Semliki forest virus. *C*, fluorescence overlay in the detail figure of regions *boxed* in *A* and *B* shows complete coclustering of ATF-4 and GABA$_B$R1 in the dendritic membrane. *D*, fluorescence overlay of ATF-4-EGFP (*green*) and antibody-labeled synaptophysin (*Syn*, Cy3 signal in *red*). *Scale bars*, 20 μm (*A* and *B*), 10 μm (*C*), and 7 μm (*D*).

ATF-4 interaction on transcriptional activation in HEK293 cells that were engineered to stably express GABA$_B$R1 and GABA$_B$R2. In the trans-reporting assay, the GAL4 DNA binding domain was joined to ATF-4 and the construct cotransfected with a GAL4-driven luciferase reporter plasmid. Cells were assayed after 18 h in serum-free medium. Although results varied considerably between different wells, we found that stimulation with 50 μM baclofen for 1 h increased luciferase levels to 144 $\pm$ 72% (n = 10) compared with unstimulated cells (Fig. 4*B*), which is a statistically significant increase (Student's t test; $p < 0.05$). In another experiment, the GAL4 DNA binding domain was fused to c-Jun and stimulated with constitutively activate MEKK. Under these conditions stimulation with baclofen significantly decreased luciferase levels to 56 $\pm$ 16% (Fig. 4*B*). The precise mechanisms underlying this differential receptor signaling are presently not understood, but are in accordance with previous findings, if we assume that endogenous ATF-4 is also present in HEK293 cells. ATF-4 has been found to suppress the transcriptional action of Jun family members (44), but is a transcriptional activator by itself (Ref. 45; see "Discussion").

Neuronal Expression via SFV—The cellular expression of GABA$_B$R1 and ATF-4 *in vivo* was first analyzed in low density rat hippocampal cultures by immunofluorescence. Primary cultures of rat hippocampal neurons were infected with an SFV vector containing EGFP-tagged ATF-4 (ATF-4-EGFP) and expression evaluated 12–18 h after infection. In confocal images ATF-4 was found in the nucleus and diffusely in the cytoplasm, but primarily clustered at specific sites in the outer membranes of hippocampal cell somata and along dendrites (Fig. 5*B*). Under whole cell patch-clamp conditions, all hippocampal neurons cultured from this age displayed GABA$_B$Rs as revealed by

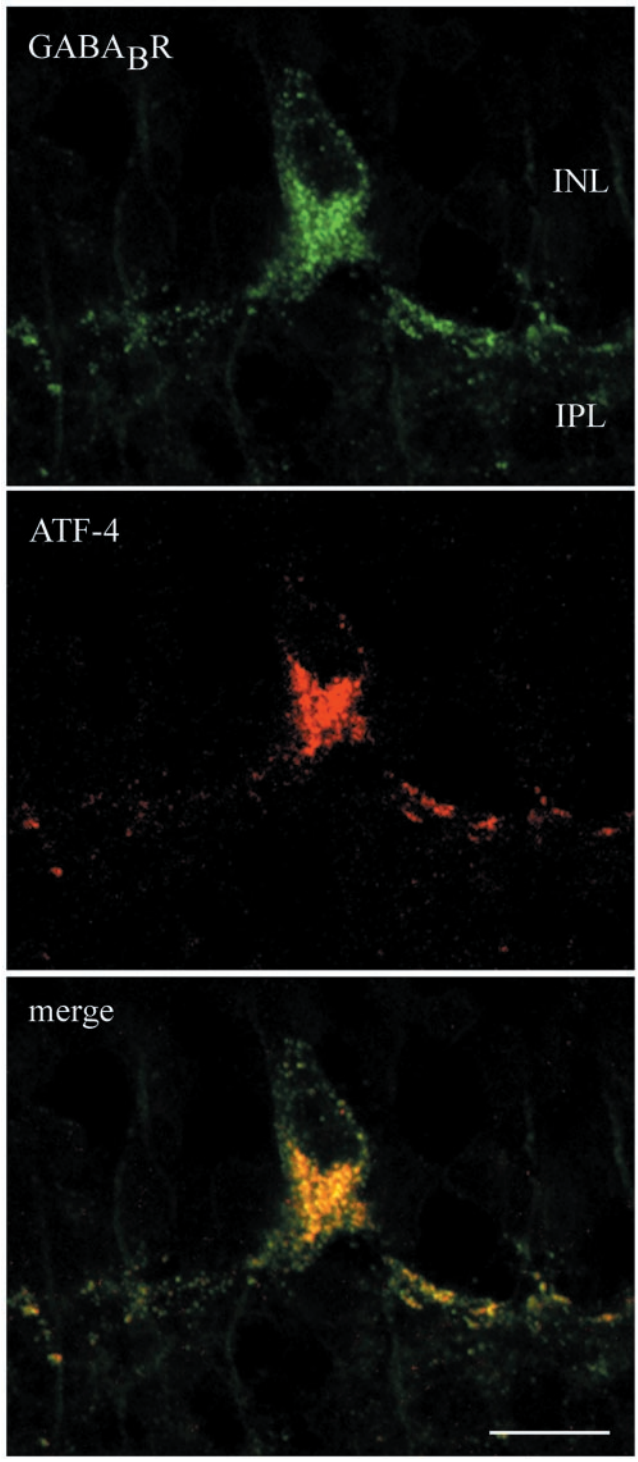

FIG. 6. **Colocalization of GABA$_B$R1 and ATF-4 in an amacrine cell of the rat retina.** Confocal laser-scanning micrographs of a transverse section of rat retina showing an amacrine cell in the inner nuclear layer (*INL*) coimmunostained for GABA$_B$R1 (*green*) and ATF-4 (red). In the merged panel below, coexpression of the two proteins is clearly visible in the cell's cytoplasm and along its processes in the inner plexiform layer (*IPL*; *yellow-orange*). *Scale bar*, 10 μm.

prominent baclofen-induced inwardly rectifying K^+ currents (data not shown). After staining with Cy3-labeled secondary antibodies, GABA$_B$R1 antibodies gave rise to a punctate membrane pattern of dendritic immunoreactivity that exactly colocalized with coexpressed ATF-4 (Fig. 5, *A* and *C*). A similar pattern of GABA$_B$R immunoreactivity was obtained in hippocampal cells that were not infected with ATF-4 SFV, ensur-

ing that overexpression of ATF-4 did not affect receptor distribution.

In many cases these puncta were not congruent with synaptic regions, as indicated by the differential distribution of the presynaptic terminal marker synaptophysin (Fig. 5*D*) or glutamic acid decarboxylase that shows inhibitory GABAergic terminals (data not shown). Our findings suggest that $GABA_BR1$ and ATF-4 are clustered predominantly at extrasynaptic sites in neuronal cells.

Colocalization in the Retina—For the mammalian retina, it has been shown that $GABA_BRs$ are strongly expressed in amacrine cells both pre- and postsynaptically (46). We therefore investigated whether $GABA_BRs$ and ATF-4 would indeed cocluster at distinct subcellular sites in a native central nervous system neuron. Fig. 6 depicts a wide field amacrine cell with the cell body located in the inner nuclear layer and its processes stratifying in the inner plexiform layer close to the inner nuclear layer. Immunocytochemical double-labeling experiments with anti-$GABA_BR$ and anti-ATF-4 antibodies revealed the striking overlap of $GABA_BR$ immunoreactivity (*green*) and ATF-4 immunoreactivity (*red*) both in the cytoplasmic compartment of the amacrine cell and also along its processes and terminal arborizations, suggesting a strong functional relationship between these two proteins in selected neurons.

DISCUSSION

Here we demonstrate for the first time a tight and direct interaction between a heptahelical neurotransmitter receptor and a soluble transcription factor (ATF-4). We show that the bZIP domain of ATF-4 associates with the $GABA_BR$ C termini in a coiled coil-confirmation, which is common, *e.g.*, among structural proteins and contains between two and four helices. Given that the site at which ATF-4 interacts with both $GABA_BR1$ and likely $GABA_BR2$ overlaps with the putative interaction site between the two subunits (23, 41), it may be hypothesized that, at least temporarily, triple-helix structures exist. It is currently thought that individually neither of the two receptor subtypes is expressed and transported with high efficiency to the outer membrane, because of homodimer instability (41). Instead, the majority of native $GABA_BRs$ are likely to exist as heterodimers between $GABA_BR1$ and $GABA_BR2$ (19–23) with specific electrostatic interactions in their C termini giving rise to parallel coiled-coil α-helices. Our assays convincingly showed that ATF-4 is tightly linked to $GABA_BRs$ expressed in neuronal membranes. Similarly, overlapping edge fluorescence of ATF-4 and $GABA_BR1$ immunoreactivity was observed by confocal microscopy after cotransfection into COS-7 cells (data not shown). In COS-7 cells that lack $GABA_BR1$ and $GABA_BR2$, ATF-4 was not seen at the plasma membrane, but only diffuse cytoplasmic staining was found, suggesting that $GABA_BRs$ play a role in recruiting ATF-4 to the outer plasma membrane. The notion that in neurons transcription factors are localized in dendrites and may be retrogradely transported to the nucleus has emerged only recently (47). One of the possible cellular consequences of the documented $GABA_BR$/ATF-4 interaction would be that agonist stimulation of the receptor at the outer surface membrane releases ATF-4, which then translocates into the nucleus to increase the nuclear pool of this transactivating protein. Like other transcription factors, ATF-4 is likely imported across the nuclear membrane by shuttling proteins that recognize nuclear localization signals (NLS; Refs. 48 and 49). A putative bipartite NLS sequence of clustered basic residues is present in the basic region of ATF-4 at amino acid position 279–296. In analogy to the processes that mask and expose NLS in other transcription factors (50, 51), we speculate that the NLS of cytosolic ATF-4 is masked upon binding to the $GABA_BR$ C-terminal region and available to shuttle carriers only after agonist-driven release. So far, the functional assays performed in our study do raise several unsolved questions on the stimuli and targets of cytosolic ATF-4. Although transfected COS cells and primary neurons in our experiments were depleted overnight of serum and other extracellular stimuli, prominent ATF-4 signals often remained in the nuclei. This may have masked a pronounced, visually detectable translocation signal of ATF-4 upon receptor stimulation with baclofen. As an alternative explanation, ligand binding to the receptor may be insufficient to fully activate ATF-4 as a transcriptional protein, but require a coincident stimulus through another signaling protein of the receptor matrix.

When using the trans luciferase reporter assay, a moderate rise in transcriptional activity via ATF-4 was seen upon receptor stimulation. This trend was supported by a cis reporting assay in HEK-GBR1 stable cells, in which CRE-driven luciferase expression was enhanced by ~20% by baclofen in the presence of 10 ng/ml pertussis toxin (data not shown). In this assay gene expression was reduced by >30% in the absence of pertussis toxin which disrupts receptor stimulation of G_i proteins. This indicates that, even under experimental suppression of G protein-mediated $GABA_BR$ signaling, ATF-4 may be involved in CRE-mediated stimulation of gene expression independent of G proteins. Yet, this action is far from being understood in detail. Similar to other proteins of the CREB/ATF family, ATF-4/CREB2 is known to bind to the transcriptional enhancer motif CRE as homo- or heterodimers in conjunction with c-Jun, but also together with TATA-binding protein, TFIIB, RAP30 subunit of TFIIF, or the coactivator CREB-binding protein CBP/p300 (38, 45, 52). It has been reported that ATF-4, like CREB, can act as a transcriptional activator (45), but under a variety of experimental conditions significantly represses CRE-dependent transcription (39, 44). This bifunctional role may be explained by the displacement of other CREB activator/coactivator proteins from the CRE promoter site by high amounts of ATF-4 (squelching), resulting in inhibition of transcriptional activation. A repressive action of ATF-4 orthologues on CREB1-mediated transcription in *Aplysia*, *Drosophila*, and rodents (53) has been interpreted to impede synaptic plasticity and affect spatial and social learning (54, 55). Based on its repressive character in the nervous system and in analogy to the function of tumor suppressor genes, CREB2/ATF-4 has been suggested to act as a "memory suppressor gene" that decreases synaptic strength or removes inhibitory constraints of long term memory storage (56, 57).

Heptahelical receptors are commonly thought to signal primarily through coupling to G proteins. Only recently, however, they have been found to interact with a growing number of membrane and cytosolic proteins, including proteins that function in signal termination (58), synaptic targeting (59), mitogenic signaling (60, 61), and translational regulation (62, 63). With the direct interaction of metabotropic $GABA_BR$ and the ubiquitously expressed transcription factor ATF-4 (39) demonstrated here, a novel alternative mechanism by which transcriptional regulation important for long term memory formation (64) may be initiated at inhibitory synapses of the mammalian central nervous system, is emerging.

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