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$GABA_B$ Receptors Couple Directly to the Transcription Factor ATF4

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The inhibitory neurotransmitter γ-aminobutyric acid (GABA), acts at ionotropic ($GABA_A$ and $GABA_C$) and metabotropic ($GABA_B$) receptors. Functional $GABA_B$ receptors are heterodimers of $GABA_{B(1)}$ and $GABA_{B(2)}$ subunits. Here we show a robust, direct, and specific interaction between the coiled-coil domain present in the C-terminus of the $GABA_{B(1)}$ subunit and the transcription factor ATF4 (also known as CREB2). ATF4 and $GABA_{B(2)}$ binding to the $GABA_{B(1)}$ subunit were mutually exclusive. In rat hippocampal neurons native $GABA_{B(1)}$ showed surprisingly little similarity to $GABA_{B(2)}$ in its subcellular distribution. $GABA_{B(1)}$ and ATF4, however, were highly colocalized throughout the cell and displayed a punctate distribution within the dendrites. Activation of $GABA_B$ receptors in hippocampal neurons caused a dramatic translocation of ATF4 out of the nucleus into the cytoplasm. These data suggest a novel neuronal signaling pathway that could regulate the functional expression of $GABA_B$ receptors and/or modulate gene transcription.

INTRODUCTION

$GABA_B$ receptors have both pre- and postsynaptic distributions in the mammalian brain and play a fundamental role in regulating neurotransmission. In addition, they are involved in synaptic plasticity and nociception (Bowery and Enna, 2000). Presynaptic $GABA_B$ receptors inhibit neuronal activity through second-messenger systems that regulate the activity of Ca^{2+} channels and transmitter release (Kerr and Ong, 1995). Activation of postsynaptic $GABA_B$ receptors leads to an increased outward potassium current, causing hyperpolarization, which results in the slow late component of the inhibitory postsynaptic potential (IPSP) important in signal processing (Dutar and Nicoll, 1988). $GABA_B$ receptor activation also inhibits adenylyl cyclase activity, can have long-term effects on protein synthesis and has been reported to negatively regulate CREB-mediated transcription in the CNS (Barthel *et al.,* 1996; Suzuki *et al.,* 1998).

$GABA_B$ receptors are the first 7-transmembrane G-protein coupled receptors that have been shown to require two separate subunits for functional surface expression (Jones *et al.,* 1998; Kaupmann *et al.,* 1998; White *et al.,* 1998; Kuner *et al.,* 1999). These subunits, termed $GABA_{B(1)}$ and $GABA_{B(2)}$, have been shown to heterodimerize via a coiled-coil domain located in their intracellular C-terminal domains (for reviews see Marshall *et al.,* 1999; Bowery and Enna, 2000). Interestingly, however, it has been reported recently that a truncated splice variant of $GABA_{B(1)}$, $GABA_{B(1e)}$ that encodes only the extracellular ligand-binding domain of the subunit, can form heterodimers with $GABA_{B(2)}$ (Schwarz *et al.,* 2000). These results suggest that additional dimerisation domains exist in the N-terminal region of the $GABA_B$ receptor subunits.

ATF4, also called CREB2, is a ubiquitously expressed member of the CREB family of transcription factors that bind to and regulate, either positively or negatively, transcription from the cAMP response element (CRE; Karpinski *et al.,* 1992; Liang and Hai, 1997). These proteins share highly conserved C-terminal leucine zipper and basic DNA binding domains but the N-terminus varies between family members (Karpinski *et al.,* 1992). ATF4 can form dimers with several other transcription factors but, unlike CREB, it does not contain consensus phosphorylation sites for protein kinase A (PKA) or protein kinase C (PKC) (Karpinski *et al.,* 1992). It has been proposed that ATF4 can negatively regulate CREB (Yin and Tully, 1996; Abel and Kandel, 1998; Abel *et al.,*

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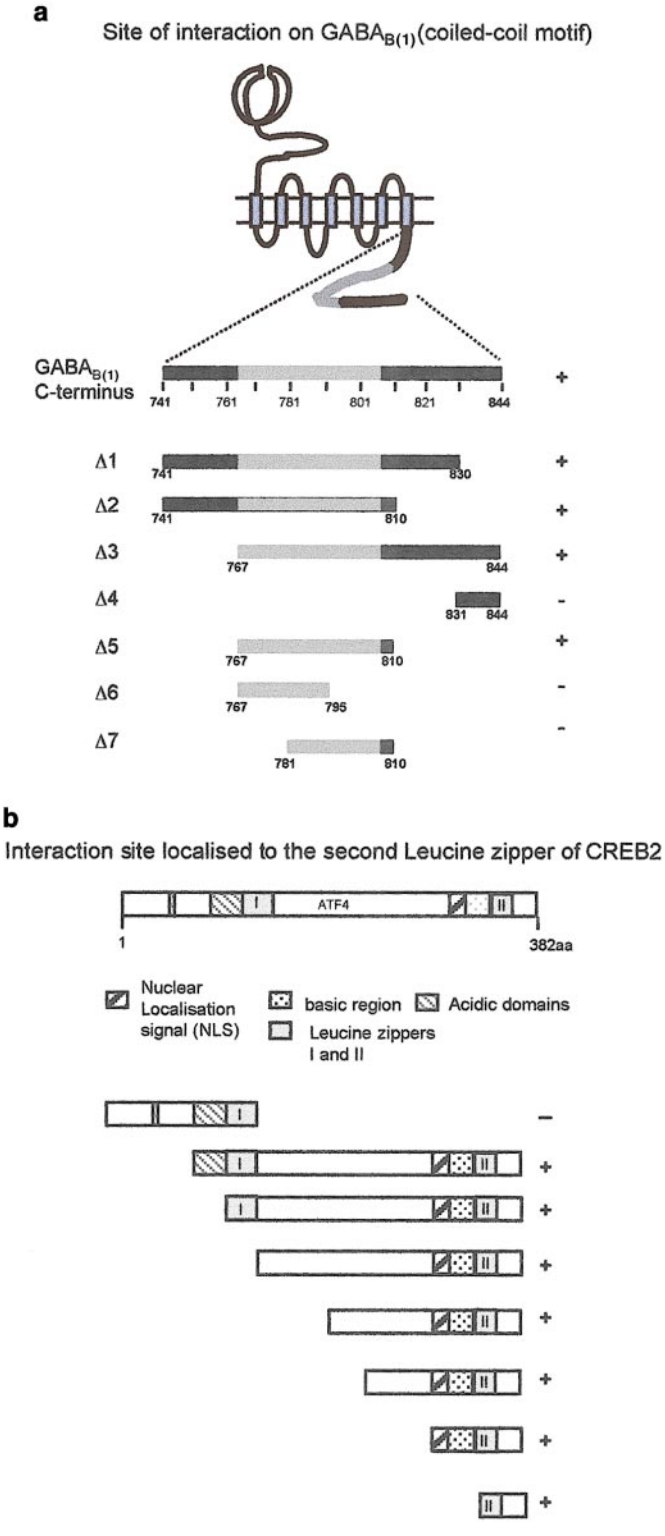

FIG. 1. GABA$_{B(1)}$ binds to ATF4. Identification of binding sites on GABA$_{B(1)}$ and ATF4. (a) Mapping of the ATF4 interaction site on ct-GABA$_{B(1)}$. The smallest region that gave a positive interaction corresponded to the coiled-coil domain (shaded grey) present in GABA$_{B(1)}$. (b) Mapping of the GABA$_{B(1)}$ interaction site on ATF4. The smallest truncation mutant that interacted corresponded to the second leucine zipper domain. Positive two-hybrid interactions are denoted by +.

1998). One reason that this may be of potential importance is because CREB is activated during long-term potentiation and other forms of synaptic plasticity (Silva and Giese, 1994; Abel *et al.*, 1998).

Here we show that the C-terminal domain of the GABA$_{B(1)}$ subunit binds directly to ATF4 via the coiled-coil domains present in both proteins. Coexpression of GABA$_{B(1)}$ with ATF4 in HEK293 cells resulted in a change in distribution of ATF4 compared to cells expressing ATF4 alone. There was a marked colocalization of GABA$_{B(1)}$, but not GABA$_{B(2)}$, with ATF4 in dendrites of cultured hippocampal neurons. Furthermore, baclofen activation of GABA$_B$ receptors caused nuclear export of ATF4 in neurons. These data raise the possibilities that there is a novel signaling pathway from the synapse to the nucleus and that ATF4 has cellular actions in addition to being a transcription factor.

RESULTS AND DISCUSSION

Detection of the Interaction between GABA$_{B(1)}$ and ATF4

We used the C-terminal domain (residues R^{741}–K^{844}) of the GABA$_{B(1b)}$ subunit (Kaupmann *et al.*, 1997) as a bait in a yeast two hybrid screen of a rat brain cDNA library. The strongest interacting clone we isolated, L14, comprised a 1.1-kb cDNA, that showed near identity to mouse activating transcription factor-4 (ATF4; Hai *et al.*, 1989; Liang and Hai, 1997) and human cyclic AMP response element binding protein-2 (CREB2; Karpinski *et al.*, 1992). L14 (hereafter called ATF4) covered 84% of the coding sequence of the corresponding ATF4 and CREB2 clones.

Specificity of the GABA$_{B(1)}$ and ATF4 Interaction

More than 120 separate positive interactors were obtained from two yeast two-hybrid screens of the rat brain library with the C-terminal GABA$_{B(1b)}$ bait. Of these in excess of 100 were copies representing at least 6 independent clones of ATF4. ATF4 did not interact with lamin or with the C-terminal domains of AMPA, kainate, or NMDA ionotropic glutamate receptor sub-

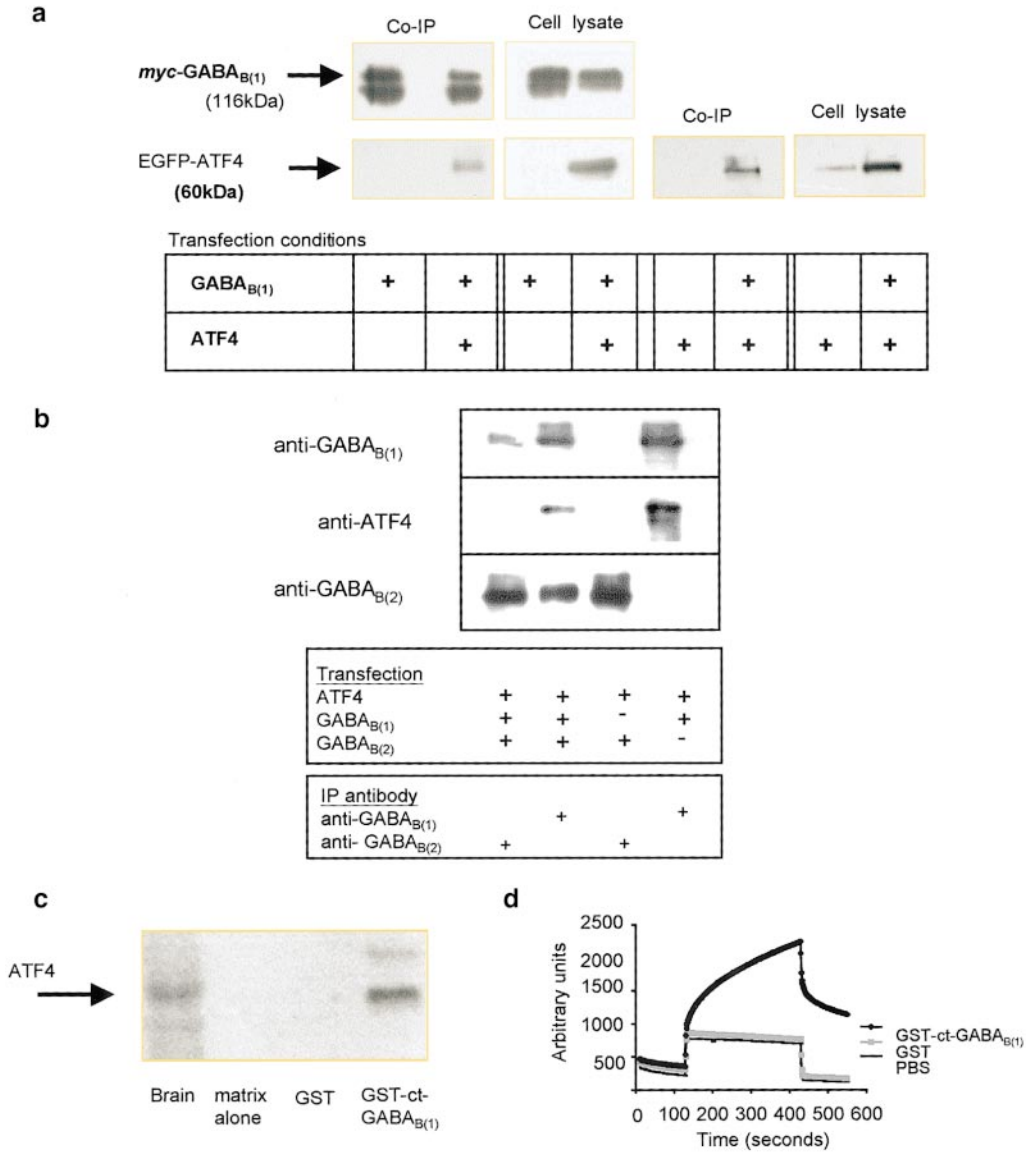

FIG. 2. Biochemical assays confirming the interaction between ATF4 and GABA$_{B(1)}$. (a) Western blots showing total HEK293 cell lysates (input) and coimmunoprecipitation of GFP-ATF4 with *myc*-GABA$_{B(1)}$. The anti-*myc* antibody 9E10 was used for immunoprecipitation. HEK293 cells were transfected with either GFP-ATF4 or *myc*-GABA$_{B(1)}$ alone or cotransfected with GFP-ATF4 and *myc*-GABA$_{B(1)}$. (b) Western blots showing coimmunoprecipitation of untagged ATF4 with an anti-GABA$_{B(1)}$ antibody but not with an anti-GABA$_{B(2)}$ antibody. (c) Pull-down showing native rat brain ATF4, prepared from the P2 fraction excluding the cell nucleus, was retained by GST-ct-GABA$_{B(1)}$ but not by uncoupled or GST-coupled glutathione sepharose matrix. (d) Interaction between HIS6-ATF4 and GST-ct-GABA$_{B(1)}$ analyzed by surface plasmon resonance. GST-ct-GABA$_{B(1)}$, but not GST alone, was retained by the immobilized HIS6-ATF4.

units (GluR1-4, GluR5-6, KA1, NR1, NR2a) or the metabotropic glutamate receptors (mGluR1-5, 7; data not shown) in yeast. To identify the binding site for ATF4 we constructed a series of truncation mutants of the GABA$_{B(1b)}$ C-terminal domain in the bait vector and tested them for interaction in the yeast (Fig. 1a). The smallest truncation that interacted comprised residues E^{767} to H^{810}. This region of GABA$_{B(1b)}$ is a predicted coiled-coil domain (Lupas, 1996) that has also been proposed as a site of dimerisation with the GABA$_{B(2)}$ subunit (for review see Marshall *et al.*, 1999). A similar truncation strategy defined the site of GABA$_{B(1)}$ interac-

tion on ATF4 as the coiled-coil region corresponding to the C-terminal leucine zipper domain (Fig. 1b).

Biochemical Verification That $GABA_{B(1)}$ Binds to ATF4

To confirm the interaction in mammalian cells we coexpressed *myc*-$GABA_{B(1)}$ and green fluorescent protein-tagged ATF4 (GFP-ATF4) in HEK293 cells. GFP-ATF4 was detected on Western blots with a commercial anti-ATF4 antibody; (Santa Cruz; Nilsson *et al.*, 1995; Ferrer *et al.*, 1996; Reddy *et al.*, 1997) that detected a single predominant band on Western blots of whole rat brain (data not shown). GFP-ATF4 was coimmunoprecipitated with *myc*-$GABA_{B(1)}$ by the anti-*myc* antibody 9E10 (Fig. 2a). We also performed coimmunoprecipitation from HEK293 cells expressing GFP-ATF4 with untagged $GABA_{B(1)}$ and/or $GABA_{B(2)}$ subunits. An anti-$GABA_{B(1)}$ antibody coimmunoprecipitated both $GABA_{B(2)}$ and ATF4. In contrast, an anti-$GABA_{B(2)}$ antibody precipitated $GABA_{B(1)}$ but not ATF4 (Fig. 2b). These results indicate that $GABA_{B(1)}$ can bind to either $GABA_{B(2)}$ or ATF4, but not both proteins at the same time. In addition, they show that ATF4 does not bind to $GABA_{B(2)}$.

For technical reasons we were unable to visualise untagged ATF4 following coimmunoprecipitation experiments. Specifically, this was because cross-reactivity between the secondary antibody used for ECL detection in Western blots and the light chain of the precipitating antibody resulted in a strong artifactual (IgG) signal exactly in the location of the ATF4 immunoreactive band. This occurred with all of the combinations of antibodies available to us (data not shown). The nonspecific IgG band was not present in no antibody controls but was present in controls containing no tissue input but primary and secondary antibodies. Furthermore, it was not possible to use anti-ATF4 antibodies for the precipitation step since the epitope for the antibody overlaps with the interaction domain on ATF4, thereby precluding the possibility of coimmunoprecipitation of $GABA_{B(1)}$ with ATF4. Therefore, to determine whether native rat brain ATF4 interacts with $GABA_{B(1)}$ a glutathione-S-transferase-(GST)-ct-$GABA_{B(1)}$ fusion protein was used for pull-down assays. As shown in Fig. 2c, native ATF4 from the P2 fraction (excluding nuclei) was retained by GST-ct-$GABA_{B(1)}$ but not by GST alone. We also tested the interaction between GST-ct-$GABA_{B(1)}$ and ATF4 expressed as a hexahistidine fusion (HIS6-ATF4) using plasmon resonance with a BIAcore 1000. This system allows monitoring of binding events between molecules, in real time, without the use of labels. In these experiments HIS6-ATF4 was immobilized on the BIAcore chip and GST-ct-$GABA_{B(1)}$ or GST alone perfused over it. GST-ct- $GABA_{B(1)}$, but not GST alone or PBS, showed a robust interaction with HIS6-ATF4 (Fig. 2d).

Coexpression of ATF4, $GABA_{B(1)}$, and $GABA_{B(2)}$ in Transiently Transfected Cells

In HEK293 cells expressing $GABA_{B(1)}$ alone there was a perinuclear localisation with no intranuclear staining (Fig. 3). In cells expressing GFP-ATF4 alone the fluorescence was distributed diffusely in the cell nucleus with no evidence of cytoplasmic GFP-ATF4. There was, however, a dramatic redistribution of GFP-ATF4 to the region of the nuclear membrane (Fig. 3e) on coexpression with $GABA_{B(1)}$. The $GABA_{B(1)}$ immunoreactivity appeared unchanged from the distribution in cells expressing $GABA_{B(1)}$ alone (Fig. 3c). In HEK293 cells transfected with $GABA_{B(2)}$ alone nearly all immunoreactivity appeared to be plasma membrane associated (Fig. 3d). In cells cotransfected with GFP-ATF4 and $GABA_{B(2)}$ no change in the distribution of either protein was observed (Fig. 3f), consistent with the coimmunoprecipitation data, suggesting that $GABA_{B(2)}$ does not interact with GFP-ATF4. Finally, in cells expressing GFP-ATF4, $GABA_{B(1)}$, and $GABA_{B(2)}$ the GFP-ATF4 fluorescence redistributed to the nuclear membrane but to a lesser extent that that observed with $GABA_{B(1)}$ in the absence of $GABA_{B(2)}$ (Fig. 3g). We attribute the nuclear redistribution of GFP-ATF4 to an interaction with $GABA_{B(1)}$ close to the nuclear membrane.

Compartmentalization of ATF4, $GABA_{B(1)}$, and $GABA_{B(2)}$ in Hippocampal Neurons

To determine the distribution and possible interactions of native proteins in neurons we used postnatal hippocampal neuronal cultures (Noel *et al.*, 1999). $GABA_{B(1)}$ immunoreactivity was widely distributed throughout the neuron with the exception of the nucleus. While some $GABA_{B(1)}$ puncta co-localized with the synaptic marker synaptophysin, the majority of staining was somatodendritic (Fig. 4a) consistent with a recent electron microscopy study showing that over 80% of $GABA_{B(1)}$ is extrasynaptic (Fritschy *et al.*, 1999). In contrast, nearly all $GABA_{B(2)}$ immunoreactivity colocalized with synaptophysin (Fig. 4b). Since >90% of the synapses on our hippocampal neurons are glutamatergic (Noel *et al.*, 1999) this suggests that $GABA_{B(2)}$ is predominantly presynaptic. Nonetheless, electrophysiological analysis of our cultured hippocampal neurones

showed baclofen-induced K^+ currents demonstrating the presence of functional postsynaptic $GABA_B$ receptors (data not shown).

As expected for a transcription factor, high levels of ATF4 immunoreactivity were present in the nucleus. There was also a widespread distribution throughout the neurons with well-defined puncta in the dendrites. The somatodendritic staining was specific since it was blocked by preabsorbing the anti-ATF4 antibody with recombinant ATF4. The majority of the ATF4 immunoreactivity was not associated with synapses (Fig. 4c) but showed a high degree of colocalization with $GABA_{B(1)}$ (Fig. 4d). We quantified colocalization by counting puncta for $GABA_{B(1)}$ and ATF4 in a series of randomly selected visual fields of neurons stained with antibodies against both proteins. $GABA_{B(1)}$ (81 ± 7%) immunoreactivity (six fields analyzed, 248 $GABA_{B(1)}$ puncta counted) was coincident with ATF4 and, excluding the nucleus, which shows only ATF4 staining, 60 ± 6% of ATF4 puncta were colocalized with $GABA_{B(1)}$ (six fields analyzed, 315 ATF4 puncta counted).

$GABA_B$ Receptor Activation Causes Translocation of ATF4 in Hippocampal Neurons

We investigated the effects of the $GABA_B$ receptor agonist baclofen on the distribution of ATF4 in cultured hippocampal neurons. Qualitatively, the confocal images of the ATF4 immunostaining showed little or no change in nuclear staining 30 min after 10 or 100 μM baclofen addition. However, from 1 h after addition of either concentration of baclofen there was a dramatic decrease in nuclear ATF4 immunostaining in all neurons investigated (Fig. 4, Panel 2). Inclusion of the $GABA_B$ receptor selective antagonist CGP55845A (10 μM) completely prevented the 100 μM baclofen-evoked decrease in ATF4 in the nucleus (Fig. 4, Panel 2). Neither 100 nM TTX nor a cocktail of ionotropic receptor inhibitors (picrotoxin, CNQX, AP5) in addition to TTX blocked the baclofen-evoked translocation of ATF4 (data not shown). These results suggest that the effect of baclofen on ATF4 redistribution is not mediated by the presynaptic depression of neurotransmitter release.

A cell ELISA assay of neurons permeabilized with either digitonin (0.002%) or Triton-X100 (1%) (Adam *et al.*, 1992) was used to quantify the baclofen-evoked redistribution of ATF4 on coverslips of cultured hippocampal cells. Overall there was a lower level of ATF4 translocation measured by cell ELISA in populations of cells compared the near complete emptying of nuclei seen by confocal microscopy of individual neurons. We attribute this to a high background signal in the cell ELISA, for example, from glial cells. In control cells 46 ± 6% of the total ATF4 was present in the nuclei. In contrast, 4 h after 10 μM baclofen addition nuclear ATF4 accounted for 27 ± 3% of the total ATF4 immunoreactivity. In other words there was a 42% decrease in the proportion of ATF4 present in the nucleus 4 h after baclofen addition. Importantly, the total amount of ATF4 immunoreactivity in neurons 4 h after baclofen addition decreased by only 11% compared to controls. Therefore, the change in nuclear ATF4 signal is mainly or exclusively the result of translocation out of the nucleus and not selective degradation or down-regulation in the nuclear compartment.

These experiments show that $GABA_B$ receptor activation can result in the export of ATF4 out of the nucleus to the dendrites of hippocampal neurons. They do not, however, address the issue of the intermediates in the signaling cascade that is involved in this process. It has been reported previously that increased levels of cAMP in hippocampal cultures leads to increased levels of ATF4 protein in the nucleus without changing ATF4 mRNA levels (Yukawa *et al.*, 1999). It is possible, therefore, that a $GABA_B$ receptor-evoked reduction in cAMP levels could be involved in the nuclear export of ATF4. This proposal does not exclude the possibility that activation of other receptors that reduce cAMP levels may also lead to ATF4 translocation out of the nucleus. Since ATF4 contains a nuclear localization signal the default cellular compartment is the nucleus. Therefore, extranuclear ATF4 must be retained outside the nucleus by anchoring protein(s). Our results suggest that $GABA_{B(1)}$ can act as one such somatodendritic anchoring protein. Within the time periods we have investigated following a 10-min baclofen stimulation (up to 4 h) there was no return of the translocated ATF4 back to the nucleus. It is likely that another signal, possibly a rise in cAMP levels may be required to cause ATF4 to move back to the nucleus.

One possible role for the $GABA_{B(1)}$-ATF4 interaction is in gene regulation (Yukawa *et al.*, 1999). ATF4 is a member of a family of cAMP response element binding proteins that has been shown to negatively regulate CREB (Yin and Tully, 1996; Abel and Kandel, 1998; Abel *et al.*, 1998). CREB itself has been widely implicated in memory formation and, interestingly, CREB is activated during long-term potentiation and other forms of synaptic plasticity (Silva and Giese, 1994; Abel and Kandel, 1998). Therefore, docking of ATF4 to somatodendritic $GABA_{B(1)}$ could prevent its nuclear function and thereby effect the transcriptional regulation of proteins. In this way $GABA_B$ receptors may influence the expression of proteins, including those important for synaptic plas-

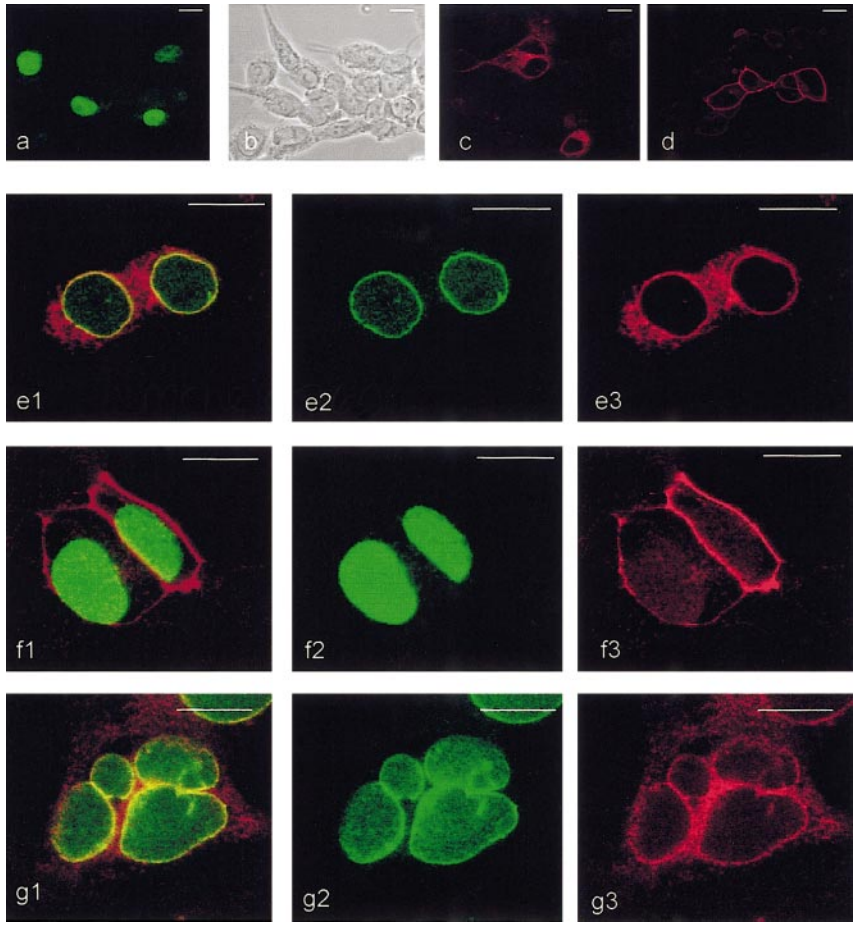

FIG. 3. Redistribution of ATF4 in the nucleus of HEK293 cells coexpressing $GABA_{B(1)}$. (a) Nuclear distribution of ATF4 in cells transfected with GFP-ATF4 alone. (b) Transmission image of transfected HEK293. (c) Localization of $GABA_{B(1)}$ in cells transfected with $GABA_{B(1)}$ alone. (d) Plasma membrane-associated localisation of $GABA_{B(2)}$ in cells transfected with $GABA_{B(2)}$ alone. (e1–3) Redistribution of GFP-ATF4 within the nucleus on coexpression with $GABA_{B(1)}$; (e1) combined image of both GFP-ATF4 and $GABA_{B(1)}$ channels. Areas of colocalization are shown yellow; (e2) GFP-ATF4, green channel only; (e3) $GABA_{B(1)}$, red channel only. (f) GFP-ATF4 did not redistribute within the nucleus on coexpression with $GABA_{B(2)}$; (f1) combined image of GFP-ATF4 and $GABA_{B(2)}$ channels; (f2) GFP-ATF4 green channel only; (f3) $GABA_{B(2)}$ red channel only. (g) Redistribution of GFP-ATF4 on coexpression with $GABA_{B(1)}$ and $GABA_{B(2)}$. (g1) combined image of GFP-ATF4 and $GABA_{B(1)}$ channels; (g2) GFP-ATF4 green channel only; (g3) $GABA_{B(1)}$ red channel only. In all panels the scale bar denotes 5 μm.

ticity, by both modulating the levels of cAMP (Barthel *et al.*, 1996) and by a direct interaction between $GABA_{B(1)}$ and ATF4.

Our results suggest that $GABA_{B(1)}$ can bind to either $GABA_{B(2)}$ or ATF4, but not both simultaneously. One possibility, therefore, is that ATF4 is involved in the regulation of $GABA_B$ subunit heterodimerisation and thus functional receptors. This hypothesis is consistent with the synaptic localization of $GABA_{B(2)}$ compared to the predominantly nonsynaptic, but dendritic localization of $GABA_{B(1)}$ and ATF4. The excess of nonsynaptic $GABA_{B(1)}$ that has also been identified by other workers (Fritschy *et al.*, 1999) may be bound to ATF4 so preventing the formation of functional heterodimeric $GABA_B$ receptors.

There is increasing interest in the roles of $GABA_B$ receptors, but little is known about their trafficking, targeting, desensitization or internalization. It is also unclear precisely how $GABA_B$ receptor activation influences gene transcription. Since these are the parameters most likely to be influenced by ATF4 binding to $GABA_{B(1)}$ a great deal of future work is required before it will be possible to determine the exact physiological role(s) of this interaction. Nonetheless, the demonstra-

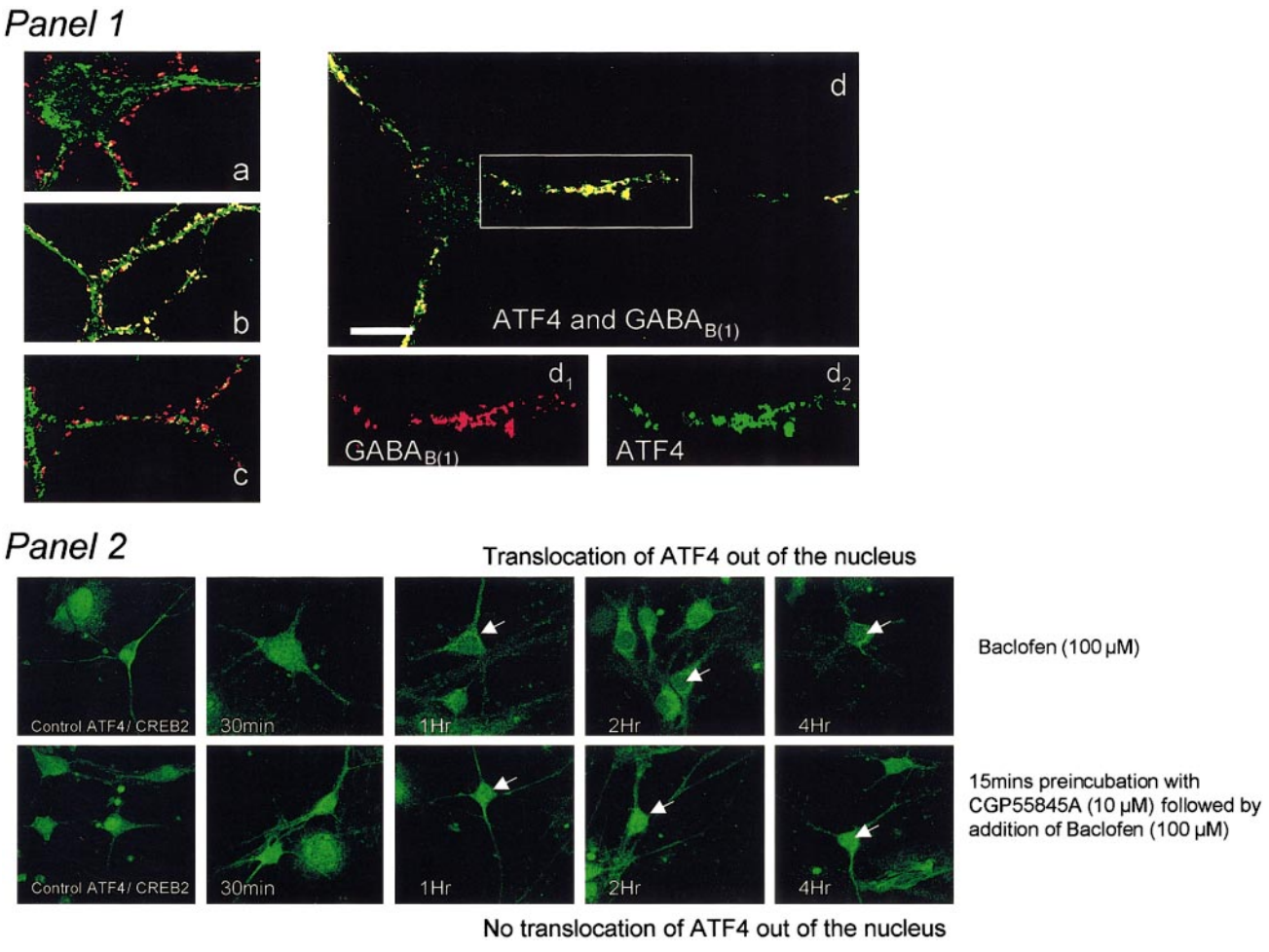

FIG. 4. Localization of ATF4 and $GABA_{B(1)}$ in hippocampal neurons. (Panel 1) Dual immunolabeling of (a) $GABA_{B(1)}$ (green) and synaptophysin (red) in hippocampal neurons after 17 days in culture. Areas of colocalization are shown as yellow; (b) $GABA_{B(2)}$ (green) and synaptophysin (red); (c) ATF4 (green) and synaptophysin (red); (d) ATF4 (green) and $GABA_{B(1)}$ (red). Note the high degree of colocalization (yellow) between $GABA_{B(2)}$, but not $GABA_{B(1)}$, and synaptophysin and between $GABA_{B(1)}$, but not $GABA_{B(2)}$, with ATF4. Because the nuclei in the cell body are typically found in different confocal planes, no cell body is present in the images presented in b or c. The pictures show dendritic processes that highlight the presence of ATF4 at synapses. The scale bar denotes 5 μm. (Panel 2) Baclofen caused a redistribution of ATF4. (a) Time-course of the decrease in nuclear ATF4 following addition of baclofen. Each panel shows representative neurons. Similar results were obtained with 10 and 100 μM baclofen. In these examples baclofen (100 μM) was present for the entire period but similar results were obtained when baclofen was applied for 10 min and then washed out. The lower set of panels shows that no baclofen (100 μM)-evoked translocation of ATF4 out of the nucleus occurred in the presence of the $GABA_B$ receptor antagonist CGP55845A (10 μM).

tion that there is a direct and dynamic association between ATF4 and $GABA_{B(1)}$ suggests a novel neuronal signaling pathway that could modulate gene transcription and/or regulate the functional expression of $GABA_B$ receptors.

EXPERIMENTAL METHODS

Strain L40 yeast expressing the DNA binding domain of GAL4 fused to the residues R^{741}–K^{845} of the C-terminal $GABA_{B(1)}$ (pBTM116-ct-$GABA_{B(1)}$; bait vector) were used to screen an adult rat brain cDNA library cloned into the GAL4 activation domain carrying vector pGAD10 (fish vector; Clontech) as previously described (Nishimune *et al.*, 1998). Positive $GABA_{B(1)}$ interactors were selected by growth on triple dropout media (-Trp/-Leu/-His) and by β-galactosidase filter assays. To map the interaction sites truncation mutants of the $GABA_{B(1)}$ C-terminal and ATF4 were made by PCR and, after sequence verification, subcloned into pBTM116

and tested for interaction with full-length ATF4 or $GABA_{B(1)}$ C-terminal, respectively.

The ct-$GABA_{B(1)}$ was subcloned into pGEX-3X (Pharmacia) and expressed in *Escherichia coli* strain TOP10 (Invitrogen). Hexahistidine-tagged ATF4 (HIS6-ATF4) was constructed by subcloning the *Bgl*II fragment of pGAD10-ATF4 (amino acids 60–382) into the prokaryotic expression vector pRSET C (Invitrogen) and expression in *E. coli* strain BLRDE3 (Novagen). A BIAcore 1000 biosensor was used for surface plasmon resonance analyses. Purified HIS6-ATF4 fusion protein was immobilized onto the flow-cell surface according to the manufacturer's instructions, and GST-tagged proteins (50 $\mu g \cdot ml^{-1}$ in phosphate-buffered saline) were flowed over at a constant rate of 10 $\mu l \cdot min^{-1}$ for 5 min. Data were analyzed using BIAcore software.

The *Bgl*II fragment from pGAD10-ATF4 was subcloned into pEGFP-C2 (Clontech) to make the mammalian expression construct pEGFP-ATF4. HEK293 cells were transfected with either pEGFP-ATF4 alone or in conjunction with myc-$GABA_{B(1)}$ using calcium phosphate precipitation. Cells were harvested and homogenized in 4 ml of 1× PBS supplemented with "complete" protease inhibitors (Boehringer). The homogenate was incubated on ice with 1% (v/v) Triton X-100 and 0.5 M NaCl for 1 h and then precleared with 100 μl protein G-agarose (Sigma) for 2 h. The protein G-agarose was pelleted and the supernatant incubated with monoclonal anti-*myc* 9E10 antibody (2 μg total) overnight at 4°C then for a further 2 h after addition of 100 μl of protein G-agarose. Immune complexes run on SDS–PAGE gels and immunoblotted with anti-ATF4 antibody (Santa Cruz). The P2 fraction of rat brain homogenates (excluding nuclei) was used for pull down assays of native rat brain ATF4 with GST-ct-$GABA_{B(1)}$ using a previously published protocol (Nishimune *et al.*, 1998).

HEK293 cells were transfected with various combinations of pEGFP-ATF4, *myc*-$GABA_{B(1)}$, and pC1neo-$GABA_{B(2)}$ using Superfect (Qiagen). Forty-eight hours after transfection cells were fixed with ice-cold methanol (−20°C for 5 min). GFP-ATF4 fluorescence was imaged directly by confocal microscopy. $GABA_B$ receptors were visualised using anti-$GABA_{B(1)}$ (Chemicon), anti-*myc* (9E10; Calbiochem), or anti-$GABA_{B(2)}$ (Kaupmann *et al.*, 1998) antibodies.

CA3-CA1 postnatal hippocampal cultures were prepared as previously described (Noel *et al.*, 1999). Neurons were used for experiments 10–21 days after plating. Confocal microscopy of rabbit anti-ATF4 (2 $\mu g \cdot ml^{-1}$; Santa Cruz), guinea pig anti-$GABA_{B(1)}$ (200 $\mu g \cdot ml^{-1}$; Chemicon), rabbit anti-$GABA_{B(2)}$ (Kaupmann *et al.*, 1998), and mouse anti-synaptophysin antibody (10 $\mu g \cdot ml^{-1}$; Boehringer-Mannheim) staining was carried out in methanol fixed and permeabilized cells as described previously using a Leica TCS-NT (Noel *et al.*, 1999). All matched control and experimental images were obtained using the same confocal settings and were subject to identical image processing procedures. For ATF4 translocation experiments cells were treated with 10 or 100 μM (R)-baclofen for times ranging from 10 min to 4 h. At appropriate time points the cells were washed in Hepes-buffered saline (HBS containing TTX), fixed with methanol (−20°C), washed in HBS and the ATF4 visualized as described. In some experiments TTX (500 nM) or 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 20 μM), D-AP5 (100 μM), and picrotoxin (100 μM) plus TTX were included with the 10 μM baclofen challenge. For the cell ELISA assays hippocampal neurons after 17 days in culture were treated with 10 μM baclofen then washed in phosphate-buffered saline (PBS). The cells were fixed with 4% paraformaldehyde in PBS for 30 min on ice then washed with ice-cold HBS and 100 mM glycine in HBS. Cells were treated with either 0.002% digitonin to permeabilize the plasma membrane only (Adam *et al.*, 1992) or 1% Triton X-100 to permeabilize all cellular membrane compartments. Selective permeabilization was confirmed by imaging ATF4 staining. Cell ELISA was performed as described previously (Noel *et al.*, 1999). The percentage of ATF4 present in the nucleus was calculated by subtraction of the extranuclear from the total ATF4 signal.

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