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Activating Transcription Factor 4 (ATF4) regulates neuronal activity by controlling $GABA_BR$ trafficking

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Title: Activating Transcription Factor 4 (ATF4) regulates neuronal activity by controlling $GABA_BR$ trafficking

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ABSTRACT

Activating Transcription Factor 4 (ATF4) has been postulated as a key regulator of learning and memory. We previously reported that specific hippocampal ATF4 down-regulation causes deficits in synaptic plasticity and memory and reduction of glutamatergic functionality. Here we extend our studies to address ATF4's role in neuronal excitability. We find that long-term ATF4 knockdown in cultured rat hippocampal neurons significantly increases the frequency of spontaneous action potentials. This effect is associated with decreased functionality of metabotropic $GABA_B$ receptors ($GABA_BRs$). Knocking down ATF4 results in significant reduction of $GABA_BR$-induced GIRK-currents and increased mIPSCs frequency. Furthermore, reducing ATF4 significantly decreases expression of membrane-exposed, but not total, $GABA_BR$ 1a and 1b subunits, indicating that ATF4 regulates $GABA_BR$ trafficking. In contrast, ATF4 knockdown has no effect on surface expression of $GABA_BR2s$, several $GABA_BR$-coupled ion channels or β2 and ɣ2 $GABA_ARs$. Pharmacologic manipulations confirmed the relationship between $GABA_BR$ functionality and action potential frequency in our cultures. Specifically, the effects of ATF4 down-regulation cited-above are fully rescued by transcriptionally active, but not by transcriptionally-inactive, shRNA-resistant, ATF4. We previously reported that ATF4 promotes stabilization of the

actin-regulatory protein Cdc42 by a transcription-dependent mechanism. To test the hypothesis that this action underlies the mechanism by which ATF4 loss affects neuronal firing rates and $GABA_BR$ trafficking, we down-regulated Cdc42 and found that this phenocopies the effects of ATF4 knockdown on these properties. In conclusion, our data favor a model in which ATF4, by regulating Cdc42 expression, affects trafficking of $GABA_BRs$, which in turn modulates the excitability properties of neurons.

Significance statement: $GABA_B$ receptors ($GABA_BRs$), the metabotropic receptors for the inhibitory neurotransmitter GABA, have crucial roles in controlling the firing rate of neurons. Deficits in trafficking/functionality of $GABA_BRs$ have been linked to a variety of neurological and psychiatric conditions, including epilepsy, anxiety, depression, schizophrenia, addiction, and pain. Here we show that $GABA_BRs$ trafficking is influenced by Activating Transcription Factor 4 (ATF4), a protein that has a pivotal role in hippocampal memory processes. We found that ATF4 down-regulation in hippocampal neurons reduces membrane-bound $GABA_BR$ levels and thereby increases intrinsic excitability. These effects are mediated by loss of the small GTPase Cdc42 following ATF4 down-regulation. These findings reveal a critical role for ATF4 in regulating the modulation of neuronal excitability by $GABA_BRs$.

Introduction

Normal cognitive functions rely on the balance of neuronal excitability properties throughout the brain as well as on synaptic plasticity (Beck and Yaari, 2008). Among the many proteins reported to influence cognition, mounting evidence suggests a pivotal role for Activating Transcription Factor 4 (ATF4), an ubiquitously expressed member of the ATF/CREB transcription factor family of basic leucine zipper proteins. In addition to its well-known functions as a stress-induced protein (Ameri and Harris, 2008), a number of studies have implicated ATF4 in synaptic plasticity and in learning and memory. Depending on cellular context, ATF4 has been characterized as either an inhibitor or promoter of synaptic plasticity (Pasini et al., 2015). Similarly divergent suggestions about ATF4's functions in learning and memory have been advanced, but these are largely based on indirect and non-selective manipulation of ATF4 activity or expression (Chen et al., 2003; Costa-Mattioli et al., 2007). To probe directly ATF4's role in normal brain function, we have monitored the consequences of its knockdown or knockout in neuronal culture and in animals. This has led to observations that ATF4 plays a role in regulation of mushroom dendritic spine density as well as in synaptic glutamatergic function (Liu et al., 2014). These effects appeared to be due to ATF4's direct transcriptional regulation of RhoGDIα (product of the *Arhgdia* gene), which in turn affects stability of the Rho family member Cdc42 that is involved in regulation of the actin cytoskeleton (Pasini et al., 2016). At the physiological level, loss of ATF4 manifested in reduced frequency and amplitude of mEPSCs, followed by defective LTP and LTD as well as in memory deficits (Pasini et al., 2015). Of relevance, similar deficiencies in plasticity and memory have been observed after conditional Cdc42 knockout in brain (Kim et al., 2014). In the context of ATF4's role in neuronal functionality, one area of interest is in its relation to $GABA_B$ receptors ($GABA_BRs$), the G-protein-linked metabotropic receptors for the inhibitory neurotransmitter GABA. Several

studies have shown direct association of $GABA_BRs$ with ATF4 (Nehring et al., 2000; White et al., 2000; Vernon et al., 2001; Ritter et al., 2004) while another study reported that ATF4 differentially regulates activity of promoters for the $GABA_BRs$ subunits 1a and 1b (Steiger et al., 2004). $GABA_BRs$ are widely expressed in brain and regulate neuronal excitability by modulating activity of G protein-gated inwardly rectifying K^+ channels (GIRKs), voltage-gated Ca^{2+} channels and adenylyl cyclase (Gassmann and Bettler, 2012). Activation of $GABA_BRs$ has been reported to hyperpolarize and decrease the threshold, while deactivation of the receptors increases the threshold required to generate an action potential (Ladera et al., 2008). Thus, alterations of $GABA_BR$ trafficking/functionality have the potential to significantly alter intrinsic neuronal excitability and brain function. In this work, we have investigated the role of ATF4 in neuronal excitability. We find that ATF4 knockdown in cultured hippocampal neurons significantly increases their firing rate and that this appears to be due to reduced trafficking of $GABA_BR$ to the cell surface. These effects in turn appear to be a consequence of ATF4's regulation of Cdc42 stability.

Methods

Cell cultures

Primary hippocampal cultures were prepared as previously described (Liu et al., 2015). Briefly, hippocampi were dissected from E18 rat embryos of either sex and, after dissociation, neurons were plated on poly-D-lysine-coated 12-well-plates at the density of 3×10^5 cells/well. Neurons were maintained in Neurobasal medium (Invitrogen) supplemented with 2% B-27 (Invitrogen) and 0.5 mM glutamine (Invitrogen). Elisa measurements of both cell media and cell lysates revealed the presence of both GABA and glutamate in the culture (glutamate=32.2 µg, GABA=13.9 µg).

DNA constructs, lentivirus preparation and infection

All shRNAs were cloned in the pLVTHM vector (Addgene), which contains an EF-1α promoter for target gene expression, using the following oligo DNA pairs as previously described (Liu et al., 2014).

Lenti-shRNA control:

5′-CGCGTCACAGCCCTTCCACCTCCATTCAAGAGATGGAGGTGGAAGGGCTGTGTTTTTT

A-3′ and 5′-CGCGTAAAAAACACAGCCCTTCCACCTCCATCTCTTGAATGGAGGTGGA AGGGCTGTGA-3′.

Lenti-shATF4:

5′ -CGCGTGCCTGACTCTGCTGCTTATTTCAAGAGAATAAGCAGCAGAGTCAGGC

TTTTTTA-3′ and5′ -CGCGTAAAAAAGCCTGACTCTGCTGCTTATTCTCTTGAA

ATAAGCAGCAGAGTCAGGCA-3′

Lenti-shATF4 addback was generated using the QuickChange Site-directed Mutagenesis kit (Stratagene). Point mutations were introduced into the Lenti-ATF4 at the recognition site for shATF4 (CCTGACTCTGCTGCTTAT to CCAGAGTCAGCTGCTTAC).

Lenti-shATF4 mut/addback was generated from shATF4addback by introducing point mutations at the DNA binding site (292RYRQKKR298 to 292GYLEAAA298).

Lenti-shCdc42 was generated according to a published siRNA sequence 5′-GAUAACUCACCACUGUCCATT-3′ (Deroanne et al., 2005). A scrambled shRNA (lenti-shCdc42scr) was generated by using the following oligo DNA pair: 5′-CGCGTGTCCAACGTCCATATACCATTCAAGAGATGGTATATGGACGTTGGACTTTTTTA-3′ and 5′-CGCGTAAAAAAGTCCAACGTCCATATACCATCTCTTGAATGGTATATGGACGTTG G ACA-3′. Lentiviral particles were produced using the 2nd generation packaging system. Briefly, HEK293T cells were transfected with the respective lentiviral constructs for shRNA together with the packaging vectors psPAX2 and pMD2.G (Addgene) using calcium phosphate. Two and three days after transfection, cell supernatants were collected and lentiviral particles were concentrated 20–30x by centrifugation in Amicon Ultra centrifugal filters (100KD) (Millipore). Viral titer ranged between 1–5×10^6 virions/µl. Primary neuronal cultures were infected with viral particles on *Day In Vitro 7* (*DIV7*) and RNA and protein extraction were performed at the indicated time points.

RNA Extraction and Quantitative RT-PCR

Total RNA was extracted from rat primary hippocampal cultures 4, 8, and 12 days after lentiviral infection according to the RNeasy Mini Protocol (Quiagen kit). RNA concentration and purity were determined using a NanoDrop 8000 (Thermo Scientific, Wilmington, DE). mRNA was then reverse-transcribed into cDNA using the 1st Strand cDNA Synthesis System for quantitative RT-PCR (Origene) following the manufacturer's instructions. Reaction mixtures were diluted 5-fold and subjected to qRT-PCR amplification (Eppendorf) using FastStart SYBR Green Master mix (Roche). The following primers were used: ATF4: F 5′-ATGCCAGATGAGCTCTTGACCAC-3′ and R 5′ -GTCATTGTCAGAGGGAGTGTCTTC-3′; αTubulin: F 5′-TACACCATTGGCAAGGAGAT-3′ and R 5′-GGCTGGGTAAAT GGAGAACT-3; $GABA_BR$ 1a: F 5'- CACACCCAGTCGCTGTG-3' and R 5'-GAGGTCCCCACCCGTCA-3'; $GABA_BR$ 1b 5′-GGGACCCTGTACCCCGGTG-3' and R 5'-GGAGTGAGAGGCCCACACC-3'. Relative product quantities for each transcript were performed in triplicate, normalized to αTubulin mRNA as an endogenous control, and determined using the comparative CT method.

Electrophysiology

Primary hippocampal neurons (19-21 *DIV*, 2 weeks after lentiviral infection) were used for tight seal conventional whole-cell patch clamp. All the currents were recorded from pyramidal-like neurons, based on the their large (~15µm) triangular shaped somas. Coverslips were placed in a recording chamber with bath solution containing (in mM): 119 NaCl, 5 KCl, 20 Hepes, 30 glucose, 2 $CaCl_2$, 2 $MgCl_2$. The pH and osmolarity of the bath solution were adjusted to 7.3 and 330

mOsm/L, respectively. For spontaneous action potential recordings, glass pipettes were filled with intracellular electrode solution (pH 7.3, 285 mOsm/L) containing (in mM): 130 K-gluconate, 10 KCl, 10 HEPES, 1 $MgCl_2$, 0.06 $CaCl_2$, 0.1 EGTA, 3 MgATP, 0.3 Na_2GTP, and typically registered 4–8 MΩ pipette resistances. Following acquisition of electrical access, cells were held in current-clamp mode at I=0. For mIPSCs experiments, glass pipettes were filled with intracellular electrode solution (pH 7.3, 285 mOsm/L) containing (in mM): 130 KCl, 10 Hepes, 0.5 $CaCl_2$, 1 EGTA, 3 MgATP, 0.3 Na_2GTP. Furthermore, 1 µM TTX, 10 µM CNQX, and 50 µM D-APV were continuously perfused during the experiment. All the cells were recorded at −70 mV for 10 min and a 5 mV hyperpolarizing test pulse was applied periodically during recordings to ensure that the access resistance did not change significantly and was less than 25 MΩ. If not, the recordings were discarded. Signals were filtered at 2 kHz, digitized at 10 kHz, stored and analyzed offline using MiniAnalysis Software (Synaptosoft, Version 6.0.7). The threshold for event detection was set at 5 pA. Recordings were performed at room temperature under constant perfusion (2 mL/min) and acquired using Clampex software and a microamplifier (MultiClamp 700B, Molecular Devices). For Baclofen-induced GIRK currents, hippocampal neurons were bathed initially with a solution containing (in mM) 119 NaCl, 5 KCl, 20 Hepes, 30 glucose, 2 $CaCl_2$, 2 $MgCl_2$ (pH 7.3, 330 mOsm/L) and then switched to a high potassium solution (hK) containing (in mM) 85 NaCl, 60 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 Hepes, 10 Glucose (pH 7.3) to determine the amplitude of the basal potassium current. When the basal current reached equilibrium, Baclofen diluted in hK was applied. The hK induced current was subtracted from the total current to obtain the Baclofen-induced GIRK current. Membrane potential was held at -70 mV throughout the duration of the experiment.

Surface and total protein isolation

Membrane-bound and total protein isolation was conducted using the EZ-Link NHS-PEO4-Biotinylation Kit (Pierce), following manufacturer's instructions. Briefly, cells were gently washed three times with ice-cold PBS containing 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$ (PBS/CM) and then incubated with 500 µg/ml of EZ-link NHS-PEO4-biotin dissolved in ice-cold PBS at 4ºC for 1 hour. Cells were then washed once with ice-cold PBS and the reaction quenched by adding 500 µl of 100 mM glycine for 10 minutes, followed by 3 washes in ice-cold-PBS. Cells were then harvested in RIPA buffer supplemented with protease/phosphatase inhibitor and centrifuged at 14000 rpm for 15 minutes at 4ºC. 30 µl of the resulting supernatant were collected for total protein input and the rest incubated with 50 µg of streptavidin beads, rotating overnight at 4ºC. Beads were washed 5 times with RIPA buffer and bound proteins eluted with 1x sample buffer by boiling for 5 minutes.

Immunoblotting

After adding NuPAGE LDS Sample Buffer (Invitrogen) and 5% β-mercaptoethanol, samples were boiled for 15 min and proteins were separated by electrophoresis on 4-12% BisTris SDS-acrylamide gels (Invitrogen). After transfer, the membranes were blocked for 1 hour at room temperature with 5% milk and then incubated overnight with primary antibody. The following primary antibodies were used: rabbit anti-$GABA_B$ R1 (1:1000, Abcam, #55051 (Zapata et al., 2017)), $GABA_B$ R2 (1:1000, Cell Signaling, #3839), rabbit anti-$GABA_A$R β2 (1:1000, Synaptic Systems, #224803), rabbit anti-$GABA_A$R ɣ2 (1:1000, Synaptic Systems, #224003), rabbit anti-

ATF4 (1:1000, Cell Signaling, #11815), rabbit anti-Cdc42 (1:1000, Cell Signaling, #2462S), rabbit anti-GIRK1 (1:500, Abcam, #129182), rabbit anti-GIRK2 (1:200, Sigma-Aldrich, #P8122), rabbit anti-GIRK3 (1:400, Sigma-Aldrich, #P8247), mouse anti-GAPDH (1:10000, Invitrogen, #MA1-16757), rabbit anti-K_V1.1 (1:400, Sigma-Aldrich, #P8247), mouse anti-K_V2.1 (1:500, Abcam, #ab192761), rabbit anti-K_V4.2 (1:200, Sigma-Millipore, #07-491), rabbit anti-Ca_V2.1 (1:500, abcam, #ab32642). Densitometric quantification of the bands was performed using ImageJ software (NIH). Total level of proteins (input) was normalized to GAPDH, while membrane-bound samples were normalized with the ratio input/GAPDH. GAPDH was undetectable in the membrane-bound fraction, therefore excluding the possibility that the membrane was leaky or compromised.

Statistical analysis

Data are shown as means ± SEM. Comparison between two groups was performed with a two-tailed unpaired Student's t test. Comparison between multiple groups was performed using two-way ANOVA, followed by a Bonferroni *post hoc* test when applicable. Statistical significance was set at $p < 0.05$.

Results

ATF4 knockdown increases neuronal excitability

We previously described a key role for ATF4 in modulating glutamatergic neurotransmission both *in vitro* and *in vivo* and in regulating dendritic spines (Liu et al., 2014; Pasini et al., 2015). Given the pivotal roles of these two aspects in controlling the excitability properties of neurons, we next set out to directly investigate the role of ATF4 in intrinsic neuronal excitability. For this purpose, we used lentivirally delivered shRNAs to specifically down-regulate ATF4 expression in 7 *DIV* cultured rat hippocampal neurons and performed whole-cell patch-clamp two weeks after the infection to record the frequency of spontaneously-occurring action potentials (sAPs). At this time in culture (3 weeks total), the neurons have formed extensive synaptic connections (Liu et al., 2014). As shown in Figure 1A,B, knockdown of ATF4 resulted in an approximately 3-fold increase in the frequency of sAPs compared to neurons infected with a control shRNA (shCtrl=0.35±0.07Hz, shATF4=1.11±0.26Hz; *post hoc* Bonferroni, shCtrl vs shATF4 $p<0.01$) To confirm that this result was not due to off-target effects, we performed a rescue experiment in which the neurons were co-infected with lentiviruses expressing shATF4 and an ATF4 construct (ATF4add) conservatively mutated to make it unresponsive to shATF4. This resulted in knockdown of endogenous and overexpression of exogenous ATF4, respectively (Liu et al., 2014). Our results indicate that ATF4add restored the firing rate to the control level (Fig. 1A,B; shATF4+ATF4add=0.46±0.07Hz; *post hoc* Bonferroni, shCtrl vs shATF4+ATF4add, $p>0.05$). However, while ATF4 over-expression rescued the firing rate, it did not reduce it below that seen in control cultures. Next, to test whether the effects of ATF4 on sAP frequency requires its transcriptional activity, we co-infected cultured hippocampal neurons with shATF4 and a mutant

ATF4 construct, ATF4add/mut, that is not recognized by shATF4 and that encodes a mutant ATF4 that does not bind DNA and thus is transcriptionally inactive. This results in knockdown of endogenous ATF4 and overexpression of inactive exogenous ATF4 (Liu et al., 2014). In contrast to ATF4add, ATF4add/mut failed to rescue the effect of ATF4 knockdown (Fig. 1A,B; shATF4+ATF4add/mut=1.17±0.21Hz; *post hoc* Bonferroni, shCtrl vs shATF4+ATF4add/mut, $p<0.01$), suggesting that ATF4 must retain its transcriptional capability to regulate the frequency of neuronal firing. Because APs are generated by voltage-gated sodium (Na_V) and potassium (K_V) channels (Bean, 2007), we investigated whether ATF4 down-regulation could affect the neuronal firing rate by influencing these major AP constituents. However, our results show no differences in either Na_V or K_V I-V curves obtained from shCtrl- or shATF4-infected hippocampal cultures (Fig. 1C-E), suggesting that ATF4 regulates neuronal excitability by a mechanism independent of effects on Nav or Kv.

ATF4 regulates trafficking of $GABA_BRs$ to the membrane

Among the many proteins reported to modulate the excitability of neurons, we focused on $GABA_B$ receptors ($GABA_BRs$), the metabotropic (G-protein coupled) receptors for GABA. Postsynaptic $GABA_BRs$ induce a slow inhibitory postsynaptic current (sIPSC) by gating Kir-type K^+-channels. This in turn hyperpolarizes the membrane and shunts excitatory currents, thereby inhibiting generation of action potentials (Leung and Peloquin, 2006). In this light, we confirmed that blocking $GABA_BR$ activity in our cultures increases the neuronal firing rate. As shown in Figure 2A, application of the specific $GABA_BR$ antagonist CGP55845 (10 μM) produced a rapid 8-fold rise (from about 0.4 to 3.2 Hz) of sAP frequency. Of potential relevance, ATF4 has been reported to directly bind $GABA_BRs$ as well as to differentially regulate promoter activity of the subunits 1a and 1b of $GABA_BRs$ (Steiger et al., 2004). We therefore first investigated whether ATF4 down-regulation affects expression of $GABA_BRs$ at the transcriptional level. To achieve this, we infected cultured hippocampal neurons with either shATF4 or shCtrl for 4-15 days and then carried out qRT PCR. As shown in Figure 2B, knockdown of ATF4 did not significantly affect the transcript levels of either $GABA_BR$ 1a or 1b subunits (ATF4, 4 days: shCtrl=100±7.1%, shATF4=19.1±2.4%, t test $p<0.01$; ATF4, 8 days: shCtrl=100±3%, shATF4=11.6±2.8%, t test $p<0.01$; ATF4, 12 days: shCtrl=100±4.19%, shATF4=9.47±1.63%, t test $p<0.01$).

Next we determined whether knocking down ATF4 would alter total or membrane- bound protein expression of $GABA_BRs$. To achieve this, we performed biotinylation of plasma membrane proteins on cultured hippocampal neurons with or without ATF4 knockdown (infected at 7 *DIV* for 2 weeks), isolated the biotinylated proteins on streptavidin-bound beads and carried out western immunobloting analysis for $GABA_BR$ subunits 1a and 1b on both the input (total cell lysate) and membrane fractions. Densitometric quantification from multiple experiments showed that ATF4 down-regulation did not affect total $GABA_BR$ 1a and 1b protein levels (Fig. 2C), but significantly decreased the levels of $GABA_BR$ subunits 1a and 1b in the biotinylated membrane fraction (Fig. 2C; $GABA_BR$ 1a: shCtrl=100±11.8%, shATF4=46.7±10.7%; *post hoc* Bonferroni test: shCtrl vs shATF4 $p<0.01$; $GABA_BR$ 1b: shCtrl=100±7.2%, shATF4=48.2±7.9%; *post hoc* Bonferroni test: shCtrl vs shATF4 $p<0.01$) thus indicating a role for ATF4 in regulating $GABA_BR$ trafficking, but not overall expression.

To address the question of whether the effect of ATF4 on membrane trafficking of $GABA_BRs$ involves ATF4's transcriptional activity, we performed a rescue experiment as above and found that ATF4add/mut, in contrast to ATF4add, failed to reverse the effects of ATF4 knockdown (Fig. 2C; $GABA_BR$ 1a, shATF4+ATF4add=95.1±10.3%, shATF4+ATF4add/mut=39.8±8.2%; *post hoc* Bonferroni test: shCtrl vs shATF4+ATF4add $p>0.05$, shATF4 vs shATF4+ATF4add $p<0.05$, shCtrl vs shATF4+ATF4add/mut $p<0.01$. $GABA_BR$ 1b, shATF4+ATF4add=110.5±12.7%, shATF4+ATF4add/mut=55.2±10%; *post hoc* Bonferroni test: shCtrl vs shATF4+ATF4add $p>0.05$, shATF4 vs shATF4+ATF4add $p<0.001$, shCtrl vs shATF4+ATF4add/mut $p<0.05$). This indicates, as with neuronal excitability, that ATF4 has a transcriptional role in regulating trafficking of $GABA_BRs$. The data also show, as with excitability, that ATF4 over-expression is not sufficient to drive surface expression of $GABA_BRs$ beyond that seen with basal endogenous expression.

We next asked whether knocking down ATF4 might produce a more general or non-specific effect on membrane proteins. Interestingly, both the total and membrane-bound levels of $GABA_BR$ 2 (biotin labeled as above) were unaffected by ATF4 down-regulation (Fig. 2D). We also examined the effect of shATF4 on membrane-exposed β2 and ɣ2 subunits of $GABA_A$ receptors and found no effects on either the total or membrane-exposed protein levels (Fig. 3A). In addition to $GABA_BRs$, a wide variety of voltage-sensitive K and Ca channels has been described to regulate excitability properties of neurons (Chen et al., 2006; Hsiao et al., 2009; Rossignol et al., 2013; Speca et al., 2014). We therefore queried whether the effect of ATF4 on neuronal excitability was in part due to its capability to regulate the expression or localization of $K_v1.1$, $K_v2.1$, $K_v4.2$, and $Ca_v2.1$. As shown in Fig. 3B-E, neither the total nor the membrane-bound levels of these proteins was affected by ATF4 down-regulation. These findings thus indicate that ATF4 has a selective role in regulation of membrane-bound proteins involved in neuronal excitability and that this includes $GABA_BRs$.

ATF4 knockdown reduces GIRK currents

We next queried whether the reduction we observed in the number of membrane-inserted $GABA_BRs$ after ATF4 down-regulation reflected a change in the functionality of the receptors themselves. Post-synaptic $GABA_BRs$ are associated with, and mediate part of their functions through G protein-coupled inwardly-rectifying potassium (GIRK) channels (Gassmann and Bettler, 2012). We therefore studied the function of $GABA_BRs$ by whole-cell patch-clamp recording of $GABA_BR$-induced K^+ GIRK currents in 7 *DIV* hippocampal cultured neurons infected for 2 weeks with lentiviruses carrying either shCtrl or shATF4. As shown in Fig. 4A, we first calibrated our recordings by applying the $GABA_BR$ agonist Baclofen (100 μM), which elicited sustained K^+ currents in control neurons that were prevented by pre-treating the cells with a specific $GABA_BR$ antagonist (SCH50911, 100 μM). Consistent with our finding that ATF4 down-regulation reduces cell surface $GABA_BR$ levels, when we recorded Baclofen-induced GIRK currents in ATF4 knockdown neurons, we found them to be significantly reduced when compared to those in ShCtrl infected neurons (Fig. 4B; shCrl 725.9±58.4pA, shATF4 435.1±39.2pA; *post hoc* Bonferroni: $p<0.01$). This effect did not appear to be mediated by effects on GIRK channels in that shATF4 did not affect total or membrane-bound GIRK1, GIRK2, or GIRK3 protein levels (Fig. 4C). Finally, ATF4add, but not ATF4add/mut, completely restored the currents to the control level (Fig. 4B; shATF4+ATF4add=786.9±69.2pA, shATF4+ATF4add/mut=545.9±43.8pA; *post hoc* Bonferroni:

shATF4 vs shATF4+ATF4add, p<0.001; shATF4add vs shATF4add/mut, p<0.01), further confirming the idea that ATF4 needs to retain its transcriptional capability to regulate the trafficking of $GABA_BRs$. As with $GABA_BR$ membrane trafficking, ATF4 over-expression did not raise GIRK current amplitude beyond that in control cultures.

ATF4 knockdown increases the frequency, but not amplitude of mIPSCs

Given that $GABA_BR$ manipulations have been reported to affect the frequency, but not the amplitude of spontaneous miniature inhibitory postsynaptic currents (mIPSCs; (Ulrich and Huguenard, 1996; Kubota et al., 2003; Kirmse and Kirischuk, 2006)), we assessed mIPSCs (confirmed by picrotoxin blockade) in cultured hippocampal neurons infected with either shCtrl or shATF4 as a further readout of $GABA_BR$ functionality. As shown in Figure 5A, we found that shAT4 significantly increases (by about 2-fold) the frequency, but not the amplitude of mIPSCs (mIPSCs frequency: shCtrl=1.65±0.21Hz, shATF4=3.43±0.41Hz; *post hoc* Bonferroni: p<0.05). In addition, adding back ATF4 completely restored the frequency of mIPSCs to control values (Fig. 5A; shATF4+ATF4add=1.19±0.37Hz; *post hoc* Bonferroni: shATF4 vs shATF4+ATF4add, p<0.05). As with other properties described above, ATF4add overexpression did not increase mIPSC frequency beyond the level observed in control cultures. Furthermore, our whole-cell patch-clamp recordings showed that, unlike ATF4add, ATF4add/mut was unable to reverse the effect of ATF4 down-regulation on mIPSC frequency (Fig. 5A; shATF4+ATF4add/mut=3.83±0.76Hz; *post hoc* Bonferroni: shCtrl vs shATF4+ATF4add/mut, p<0.01), confirming that the transcriptional activity of ATF4 is required for this action. To further confirm the idea that membrane-bound $GABA_BRs$ are reduced by shATF4, we treated both shCtrl and shATF4-infected neurons with the specific $GABA_BR$ agonist Baclofen (20 μM) or $GABA_BR$ antagonists SCH50911 and CGP55845 (used at 100 and 10 μM, respectively). As shown in Fig. 5B, Baclofen application significantly reduced the frequency of mIPSCs both in shCtrl- and shATF4-infected hippocampal neurons (shCtrl+Baclofen=0.79±0.14Hz, shATF4+Baclofen=1.23±0.15Hz; *post hoc* Bonferroni: shCtrl vs shCtrl+Baclofen, p<0.05; shATF4 vs shATF4+Baclofen, p<0.01), thus confirming that the membrane-bound $GABA_BRs$ of shATF4-infected hippocampal neurons are properly responding to stimulation. Interestingly, the application of $GABA_BR$ antagonists CGP55845 and SCH50911 significantly elevated mIPSCs frequency in shCtrl but not in shATF4 neurons (Fig. 5B; shCtrl+SCH=3.19±0.30Hz, shCrl+CGP=3.67±0.46Hz, shATF4+SCH=3.38±0.44Hz, shATF4+CGP=3.59±0.15Hz; *post hoc* Bonferroni: shCtrl vs shCtrl+SCH, p<0.05; shCtrl vs shCtrl+CGP, p<0.05, shATF4 vs shATF4+SCH, shATF4 vs shATF4+CGP, p>0.05), which is consistent with the observation that shATF4 reduces membrane-bound levels of $GABA_BRs$. As expected, none of the treatments significantly affected the amplitude of mIPSCs (Fig. 5B).

The effects of ATF4 on excitability and $GABA_BRs$ appear to be mediated by changes in Cdc42 expression

We previously reported that ATF4's modulation of dendritic spine density and glutamatergic functionality is mediated, at least in part, by its capacity to regulate the stability and expression of the total and activated forms of the small Rho GTPase Cdc42 (Liu et al., 2014; Pasini et al., 2015). Of particular relevance here, Cdc42 has been shown to be involved in regulating receptor

trafficking (Hussain et al., 2015). We therefore next tested the hypothesis that the effects of ATF4 down-regulation on $GABA_BR$ trafficking and neuronal excitability could be mediated by loss of Cdc42. To achieve this, we used a previously characterized Cdc42 shRNA (Liu et al., 2014) to deplete Cdc42 in cultured hippocampal neurons and determined whether this phenocopies the effects of ATF4 knockdown. We first assessed whether specific Cdc42 down-regulation affects neuronal excitability. As in the case of ATF4 knockdown, silencing Cdc42 protein produced a 2-fold increase of AP frequency (Fig. 6A; shCtrl=0.43±0.13Hz, shCdc42=1.08±0.15Hz; t test, $p<0.01$). Next we queried whether Cdc42 down-regulation phenocopies the effect of ATF4 knockdown on $GABA_BR$ trafficking and found that this was sufficient to significantly decrease the levels of membrane-exposed, but not total $GABA_BRs$ (Fig. 6B; for $GABA_BR$ 1a shCtrl=100%±23.3%, shCdc42 44.2%±7.4%; t test, $p<0.05$; $GABA_BR$ 1b shCtrl=100%±20.3%, shCdc42 38.7%±4.7%; t test, $p<0.05$). In addition, we found that Baclofen-induced GIRK currents were significantly decreased in shCdc42-infected neurons, compared to controls (Fig. 6C; shCtrl=627.8±77.6pA, shCdc42=406.9±52.9pA; t test, $p<0.05$). Finally, Cdc42 knockdown also increased the frequency mIPSCs (Fig. 6D; shCtrl=1.55±0.16Hz, shCdc42=3.21±0.67Hz; t test, $p<0.05$) to a degree similar to that observed with ATF4 knockdown. However, with Cdc42 knockdown, there was also a small, but significant increase in mIPSCs amplitude (Fig. 6D; shCtrl=25.22±1.26pA, shCdc42=31±1.21pA; t test, $p<0.01$). Taken together, these findings support the idea that regulation of Cdc42 levels mediates the effects of ATF4 on neuronal $GABA_BR$ trafficking and excitability.

Discussion

In the present study we delineate a novel role for the transcription factor ATF4 in regulating $GABA_BR$ trafficking and neuronal excitability. Our data show that chronic ATF4 down-regulation in hippocampal neurons reduces membrane levels of $GABA_BRs$, diminishes their functionality, and consequently leads to a substantial increase of neuronal firing rate. In addition, we found that these effects are mediated by ATF4's transcriptional capability and reflect changes in expression of the small GTPase Cdc42. Studies on ATF4's role in the regulation of synaptic plasticity and memory have led to divergent views. ATF4 has been characterized to be either a negative or positive influence on plasticity. Such interpretations appear to be dependent on cellular context (Bartsch et al., 1995; Hu et al., 2015) or have been based on experimental manipulations that are not specific to ATF4 such use of a dominant-negative construct (Chen et al., 2003) or regulation of eIF2α phosphorylation (Costa-Mattioli et al., 2007). To more directly assess ATF4's function in unstressed brain, we have used the strategy of studying the effects of long-term ATF4 knockdown or deletion in hippocampal and cortical neurons both *in vitro* and *in vivo*. This has revealed required roles for ATF4 in maintaining mushroom spines and glutamatergic functionality as well as in long-term spatial memory and behavioral flexibility (Liu et al., 2014; Pasini et al., 2015). The present findings significantly extend the range of cognition-relevant neuronal properties that are dependent on the presence of ATF4.

In the present work, we found that ATF4 plays a novel role in regulating the proportion of $GABA_BRs$ that are exposed on the neuronal surface. Two subunit isoforms of $GABA_B$ 1 receptors have been described, $GABA_BR$ 1a and $GABA_BR$ 1b, which differ by the presence of two sushi domains near the N-terminus of $GABA_BR$ 1a (Hawrot et al., 1998). Our findings indicate that ATF4

knockdown leads to comparable reductions of both $GABA_BR$ 1a and $GABA_BR$ 1b on the cell surface. There is evidence that $GABA_BR$ 1a subunits are mainly located presynaptically, while $GABA_BR$ 1b subunits are predominantly expressed postsynaptically (Vigot et al., 2006). Consistent with this localization and with the reductions of both subunit types on the cell surface, we observed both an increase in the frequency of mIPSCs (which is mainly a presynaptic measurement) and a decrease of GIRK currents (mainly postsynaptic in origin) after ATF4 knockdown. Presynaptic $GABA_BRs$ act as presynaptic brakes on release of neurotransmitters (Sakaba and Neher, 2003; Laviv et al., 2010) and $GABA_BR$ modulation consequently affects the frequency, but not the amplitude, of mIPSCs (Ulrich and Huguenard, 1996; Kubota et al., 2003; Kirmse and Kirischuk, 2006). Our observation that ATF4 down-regulation increases the frequency, but not amplitude, of mIPSCs is thus consistent with a decrease of pre-synaptic surface-exposed $GABA_BRs$. Further supporting this view, we found that application of the specific $GABA_BR$ antagonists CGP55845 and SCH50911 significantly elevated mIPSC frequency in shCtrl- but not in shATF4-treated neurons, indicating that presynaptic $GABA_BR$ activity is compromised in the ATF4 knockdown neurons.

Pre- and post-synaptic $GABA_BR$ function has been widely reported to be associated with G-protein inward rectifying potassium channels (GIRKs), which hyperpolarize neurons in response to $GABA_BR$ activation (Ladera et al., 2008). We therefore measured $GABA_BR$-induced K^+ currents as a readout of $GABA_BR$ activity and found a 40% reduction of GIRK current in ATF4 down-regulated neurons. These observations are consistent with a decline in trafficking of post-synaptic $GABA_BRs$. Thus, our findings suggest effects of ATF4 on both pre- and post-synaptic $GABA_BRs$. Additional studies will be needed to further characterize these effects and to define their individual influences on synaptic transmission.

In our study, we observed that the total and surface levels of GIRK proteins were unaltered by ATF4 down-regulation; this finding suggests the idea that $GABA_BRs$ and GIRK can be independently trafficked to the cell surface. In addition, our finding that ATF4 down-regulation reduces GABABR functionality without affecting $GABA_BR$ 2 levels is in line with the evidence that the 2 subunits ($GABA_BR$ 1 and 2) need to heterodimerize in order to produce a functionally active $GABA_BR$ (Jones et al., 1998).

A major issue addressed in our study is the mechanism by which ATF4 regulates the surface expression of $GABA_BRs$. As a leucine zipper protein, ATF4 has the capacity to undergo direct protein-protein interactions, among which is binding to $GABA_BRs$. However, this interaction did not appear to be relevant to the observed regulation of $GABA_BR$ membrane trafficking since the deficits in this parameter were not rescued by a mutant ATF4 lacking DNA binding capacity, but possessing an intact leucine zipper. These observations support rather a transcriptional role for ATF4 in regulating $GABA_BR$ surface exposure. A prior study reported that ATF4 over-expression elevated expression of a $GABA_BR$ 1a promoter-reporter construct and decreased expression of a $GABA_BR$ 1b promoter-reporter construct (Steiger et al., 2004). However, we noted no changes either in $GABA_BR$ 1a or $GABA_BR$ 1b mRNA or total protein after ATF4 knockdown. Moreover, ATF4 over-expression also failed to affect surface or total $GABA_BR$ 1a or $GABA_BR$ 1b levels in our experiments. Our findings instead indicate that the role of ATF4 in controlling $GABA_BR$ trafficking stems from its transcription-dependent capacity to regulate Cdc42 expression. In

support of this idea, Cdc42 knockdown fully phenocopied the effects of ATF4 knockdown on surface expression of $GABA_BRs$. While the ATF4/Cdc42 pathway appears to be key player in $GABA_BR$ trafficking, this does not seem to be a universal mechanism for regulating receptor surface expression in neurons. Thus, ATF4 knockdown did not affect total or membrane expression of $GABA_BR$ 2, $GABA_A$ β2 and γ2 subunits, or $K_v1.1$, $K_v2.1$, $K_v4.2$, and $Ca_v2.1$ channels. It remains to be seen whether ATF4/Cdc42 pathway affects trafficking of other neurotransmitter receptors.

Cdc42-dependent signaling has been implicated in trafficking of GluA1 AMPA receptors (Hussain et al., 2015). One way in which the effect of Cdc42 knockdown differed from ATF4 knockdown was that the former, but not the latter caused a small, but significant increase in the amplitude of mIPSCs. One possible explanation for this discrepancy may be the different magnitude of Cdc42 protein silencing exerted by the two shRNA constructs. While shATF4 caused a ~40-50% reduction of Cdc42 protein, shCdc42 depleted 70-80% of total Cdc42 protein. It may be that the greater loss of Cdc42 with shCdc42 results in post-synaptic modifications that affect GABA sensitivity.

Our findings establish roles for transcriptionally active ATF4 and for Cdc42 in regulation of $GABA_BR$ trafficking. Our prior work has shown that ATF4 influences Cdc42 levels by promoting its stability and that this in turn reflects the Cdc42-stabilizing activity of RhoGDIα, a direct transcriptional target of ATF4 (Pasini et al., 2016). Fig. 7 shows a proposed mechanistic pathway by which ATF4 regulates $GABA_BR$ trafficking and neuronal excitability via Cdc42 and RhoGDIα. An important feature of this mechanism is that it appears to occur over a prolonged time course. Cdc42 is a relatively stable protein and knocking down ATF4 in neurons reduces Cdc42's apparent half-life from 31.5 to 18.5 hrs (Liu et al., 2014). This suggests that sustained changes in ATF4 protein levels/activity over many hours may be required to materially affect Cdc42 expression and thereby to affect synaptic activity. ATF4 itself is a rapidly turning-over protein and its expression is regulated both by translational and transcriptional mechanisms. Thus ATF4 may serve as a "sentinel" protein whose ambient expression levels in neurons influence $GABA_BR$ trafficking as well as other elements of neuronal plasticity.

Figure Legends:

Figure 1:

ATF4 knockdown increases neuronal excitability. A, The frequency of spontaneously-occurring action potentials (sAPs) is increased upon ATF4 down-regulation; this effect appears to be mediated by ATF4's transcriptional capability. Left panel, representative traces of sAPs recorded from cultured hippocampal neurons infected at *DIV7* for 2 weeks with lentivirus expressing either shCtrl (black trace), shATF4 (red trace), shATF4+ATF4add (purple trace), or shATF4+ATF4add/mut (cyan trace). Right panel, summary bar graph showing the mean frequencies (± SEM) of sAPs of hippocampal neurons infected with shCtrl (black bars, n=24), shATF4 (red bars, n=22), shATF4+ATF4add (purple bars, n=18), shATF4+ATF4add/mut (cyan bars, n=18) constructs. **C-E**, ATF4 down-regulation does not affect Na^{2+} and K^+ voltage-gated

channels in shATF4-infected neurons (red dots) when compared to shCtrl neurons (black dots). **C**, Voltage-gated Na^{2+} currents were evoked by 1s step depolarizations from −40 to +60 mV with 10-mV increments (shCtrl n=15; shATF4 n=16). **D**, A-type K^+ currents were evoked by 1s step depolarizations from −30 to +60 mV, with 10-mV increments (shCtrl n=29; shATF4 n=33), and successively isolated. **E**, Delayed-rectifying K^+ currents were evoked by 1s step depolarizations from −90 to +60 mV, with 10 mV increments (shCtrl n=30; shATF4 n=34), and successively isolated. Values are expressed as mean ± SEM. *p<0.05; **p<0.01.

Figure 2:

ATF4 regulates $GABA_BRs$ surface expression. A, $GABA_BR$ blockade increases the frequency of sAPs in control neurons. Representative trace of sAPs recorded from cultured hippocampal neurons infected with shCtrl-carrying lentivirus treated with the specific $GABA_BR$ antagonist CGP55845 (10 μM). **B**, Time-course experiment showing no changes in the mRNA levels for $GABA_BR$ subunits 1a and 1b upon ATF4 down-regulation. Bar graphs represent qPCR analysis of ATF4 (left), $GABA_BR$ 1a (center) and $GABA_BR$ 1b (right) mRNA levels extracted 4, 8, and 12 days after infection with lentivirus carrying either shCtrl (black bars) or shATF4 (red bars) constructs. Values are expressed as mean ± SEM from three independent experiments. **C**, ATF4 down-regulation decreases membrane levels of $GABA_BR$. *DIV7* hippocampal cultured neurons were infected with indicated lentiviral constructs (black for shCtrl, red for shATF4, purple for shATF4+ATF4add, and cyan for shATF4+ATF4add/mut) for 14 days before undergoing extraction of total and membrane proteins. Left side of the panel shows a representative western immunoblot. Bar graphs on the right portion of the panel show densitometric analysis of total (left) or membrane (right) $GABA_BR$ 1a and 1b protein normalized to GAPDH. **D**, ATF4 down-regulation does not affect total or surface levels of $GABA_BR$ 2. Left side of the panel shows a representative western immunoblot. Bar graphs on the right portion of the panel show densitometric analysis of total (left) or membrane (right) $GABA_BR$ 2 protein normalized to GAPDH. Values are expressed as mean ± SEM from three independent experiments. *p<0.05. **p<0.01; ***p<0.001.

Figure 3:

ATF4 down-regulation does not affect total and membrane levels of $GABA_ARs$ and voltage-sensitive K or Ca channels. *DIV7* cultured hippocampal neurons were infected with indicated lentiviral constructs (black for shCtrl, red for shATF4) for 14 days before undergoing extraction of total and membrane proteins. **A**, Left side of the panel shows a representative western immunoblot. Bar graphs show densitometric analysis of total (left) or membrane (right) $GABA_AR$ β2 and γ2 protein normalized to GAPDH. **B**, Upper panel shows a representative western immunoblot. Bar graphs show densitometric analysis of total (left) or membrane (right) $K_v1.1$ protein normalized to GAPDH. **C**, Upper panel shows a representative western immunoblot. Bar graphs show densitometric analysis of total (left) or membrane (right) $K_v2.1$ protein normalized to GAPDH. **D**, Upper panel shows a representative western immunoblot. Bar graphs show densitometric analysis of total (left) or membrane (right) $K_v4.2$ protein normalized to GAPDH. **E,**

Upper panel shows a representative western immunoblot. Bar graphs show densitometric analysis of total (left) or membrane (right) $Ca_V2.1$ protein normalized to GAPDH. Values are expressed as mean ± SEM from three independent experiments, each run in duplicate. The differences betwen shCtrl and shATF4 were not significant ($p>0.05$) in all cases.

Figure 4:

ATF4 knockdown reduces $GABA_BR$-induced GIRK currents. A, Specific $GABA_BR$ agonist Baclofen (100 µM) elicited a sustained GIRK current in cultured hippocampal neurons infected with shCtrl lentivirus (top panel shows a representative trace). This effect is abolished by pretreating the culture with the specific $GABA_BR$ antagonist CGP55845 (10 µM; bottom panel). **B**, shATF4 infection reduces Baclofen-induced GIRK currents; this effect is dependent on ATF4's transcriptional capability. Left panel shows representative traces of Baclofen-elicited currents recorded from *DIV7* cultured hippocampal neurons infected for two weeks with lentivirus expressing either shCtrl (black), shATF4 (red), shATF4+ATF4add (purple), or shATF4+ATF4add/mut (cyan). Bar graphs on the right indicate measurements of the currents (shCtrl n=37; shATF4 n=39; shATF4+ATF4add n=36; shATF4+ATF4add/mut n=51). Values are expressed as mean ± SEM. *p<0.05; **p<0.005. **C,** ATF4 down-regulation does not affect total and membrane levels of GIRK proteins**.** *DIV7* cultured hippocampal neurons were infected with indicated lentiviral constructs (black for shCtrl, red for shATF4) for 14 days before undergoing extraction of total and membrane proteins. Left side of the panel shows a representative western immunoblot. Bar graphs show densitometric analysis of total (left) or membrane (right) GIRK proteins normalized to GAPDH. Values are expressed as mean ± SEM from three independent experiments. *p<0.05. **p<0.01; ***p<0.001.

Figure 5:

ATF4 knockdown increases the frequency, but not amplitude of mIPSCs. A, Left portion of the panel shows representative mIPSC traces recorded from *DIV7* hippocampal neurons infected for two weeks with lentivirus expressing either shCtrl (black), shATF4 (red), shATF4+ATF4add (purple), or shATF4+ATF4add/mut (cyan). On the right portion of the panel, bar graphs quantify the frequency (left) and the amplitude (right) of mIPSCs shown in A. (shCtrl, n=46; shATF4, n=29; shATF4+ATF4add, n=16; shATF4+ATF4add/mut, n=25). Values are expressed as mean ± SEM. **B**, Effect of pharmacological manipulation of $GABA_BRs$ on mIPSCs recorded from *DIV7* hippocampal neurons infected for two weeks with lentivirus expressing either shCtrl,or shATF4. Top panel shows representative traces of mIPSCs recorded from shCtrl (black traces) or shATF4 (red traces) infected neurons alone (shCtrl, n=49; shATF4, n=29) or in the presence of either 20 µM Baclofen ($GABA_BR$ agonist; shCtrl, n=16; shATF4, n=15), 10 µM SCH50911 ($GABA_BR$ antagonist; shCtrl, n=36; shATF4, n=18), or 100 µM CGP55845 ($GABA_BR$ antagonist; shCtrl, n=21; shATF4, n=17). In the bottom portion of the panel, bar graphs quantify the frequency (left) and the amplitude (right) of the mIPSCs. Values are expressed as mean ± SEM. * indicates p<0.05; ** indicates p<0.01; *** indicates p<0.001.

Figure 6:

The effects of ATF4 on excitability and $GABA_BRs$ are driven by changes in Cdc42 expression. A, Cdc42 down-regulation increases intrinsic neuronal excitability. Top panel shows representative traces of sAPs recorded from cultured hippocampal neurons infected at *DIV7* for 2 weeks with lentivirus expressing either shCtrl (black trace) or shCdc42 (blue trace). Bottom panel shows summary bar graphs showing the frequency of sAPs of neurons infected with shCtrl (black bars, n=13) or shCdc42 (blue bars, n=13) constructs. **B**, Cdc42 down-regulation decreases membrane but not total $GABA_BR$ levels. *DIV7* cultured hippocampal neurons were infected with different lentiviral constructs (black for shCtrl, blue for shCdc42) for two weeks before undergoing extraction of total and membrane proteins. Left portion of the panel shows a representative Western immunoblot. Bar graphs on the right portion of the panel show densitometric analysis of immunoblots for total (left) or membrane (right) $GABA_BR$ 1a and 1b protein, normalized to GAPDH. **C**, Cdc42 down-regulation decreases Baclofen-elicited GIRK currents. Top side of the panel shows representative traces of Baclofen-elicited currents recorded from *DIV7* cultured hippocampal neurons infected for two weeks with lentivirus carrying either shCtrl (black) or shCdc42 (blue). Bar graph on the bottom side shows the measurements of the currents (shCtrl, n=22; shCdc42, n=26). **D**, Cdc42 down-regulation increases mIPSCs. Top portion of the panel shows representative mIPSC traces recorded from *DIV7* hippocampal neurons infected for two weeks with lentivirus carrying either shCtrl (black) or shCdc42 (blue) constructs. On the bottom portion of the panel, bar graphs quantify the frequency (left) and the amplitude (right) of mIPSCs shown in A. (shCtrl, n=34; shCdc42, n=29). Values are expressed as mean ± SEM. * indicates $p<0.05$.

Figure 7:

Proposed mechanism by which ATF4 regulates neuronal excitability. Left panel shows a control condition where basal levels of ATF4 protein ensure the appropriate amount of RhoGDIα expression to bind and stabilize cytoplasmic Rho GTPase family members, including Cdc42. Appropriate levels of Cdc42 result in basal levels of membrane-bound $GABA_BRs$ that contribute to control the pace of neuronal firing. The right side of the panel shows a condition of chronic ATF4 down regulation, the consequent decrease of RhoGDIα levels and augmented Cdc42 turnover. This in turn negatively affects the amount of membrane-bound $GABA_BRs$, altering neuronal intrinsic excitability properties, which results in increased sAP frequency.

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