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# RGS2 modulates coupling between $GABA_B$ receptors and GIRK channels in dopamine neurons of the ventral tegmental area

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**Agonists of $GABA_B$ receptors exert a bi-directional effect on the activity of dopamine (DA) neurons of the ventral tegmental area, which can be explained by the fact that coupling between $GABA_B$ receptors and G protein-gated inwardly rectifying potassium (GIRK) channels is significantly weaker in DA neurons than in GABA neurons. Thus, low concentrations of agonists preferentially inhibit GABA neurons and thereby disinhibit DA neurons. This disinhibition might confer reinforcing properties on addictive $GABA_B$ receptor agonists such as γ-hydroxybutyrate (GHB) and its derivatives. Here we show that, in DA neurons of mice, the low coupling efficiency reflects the selective expression of heteromeric GIRK2/3 channels and is dynamically modulated by a member of the regulator of G protein signaling (RGS) protein family. Moreover, repetitive exposure to GHB increases the $GABA_B$ receptor-GIRK channel coupling efficiency through downregulation of RGS2. Finally, oral self-administration of GHB at a concentration that is normally rewarding becomes aversive after chronic exposure. On the basis of these results, we propose a mechanism that might underlie tolerance to GHB.**

GIRK channels (also known as Kir3 channels) are the effectors of $G_{i/o}$-coupled receptors, including the $GABA_B$ receptor[1]. The main function of neuronal GIRK channels is to mediate the postsynaptic inhibitory effects of $G_{i/o}$-coupled receptors. Four mammalian GIRK subunits have been identified (GIRK1–4; also referred to as Kir3.1–Kir3.4)[2–5], which assemble as either homo- or hetero-tetrameric channels. For example, immunoprecipitation studies indicate that heteromeric GIRK1/2 (ref. 6) and GIRK2/3 (ref. 7) channels, and homo-tetrameric GIRK2 (ref. 8) channels, exist in the brain. In heterologous expression systems, GIRK1 requires co-assembly with GIRK2/3/4 subunits to form functional channels, whereas GIRK2 and GIRK4 seem to form functional homo-tetramers[9,10]. GIRK4 is expressed only sparsely in the brain, which effectively reduces its contribution to neuronal GIRK channel formation[11,12].

$GABA_B$ receptors are activated by endogenous GABA and in response to $GABA_B$ receptor agonists such as baclofen and GHB. GHB is of particular interest because it is an addictive and increasingly popular club drug[13,14]. Like all addictive drugs, GHB targets the meso-corticolimbic dopamine system[15,16], which originates in the VTA. With the opioids and cannabinoids, GHB forms a group of addictive drugs that mediate their effects on the VTA through $G_{i/o}$-coupled receptors[16]. The release of DA from mesocorticolimbic projections is thought to be crucial in the induction of compulsive addictive behavior. GHB also fulfills the criteria of an addictive drug in animal models, according to reports of self-administration and conditioned place preference[17,18]. Conversely, the canonical high-affinity $GABA_B$ receptor agonist baclofen reduces self-administration and re-instatement of several drugs in rodents[19], indicating that it might be useful as an anti-craving compound in human addicts[20].

Unlike baclofen, GHB has two binding sites in the brain. One is an orphan G protein-coupled receptor (GPCR)[21] and the other is the $GABA_B$ receptor. However, all tested pharmacological effects of GHB are abolished in $GABA_B$ receptor knockout mice[22], which identifies the $GABA_B$ receptor as the responsible target. Many of the basic pharmacological properties of GHB and its derivatives can therefore be studied using various concentrations of baclofen. Nevertheless, it remains unclear why the brain responds so differently to two $GABA_B$ receptor agonists (GHB and baclofen).

Previously, we investigated the coupling of $GABA_B$ receptors to GIRK channels in DA and GABA neurons of the VTA and discovered

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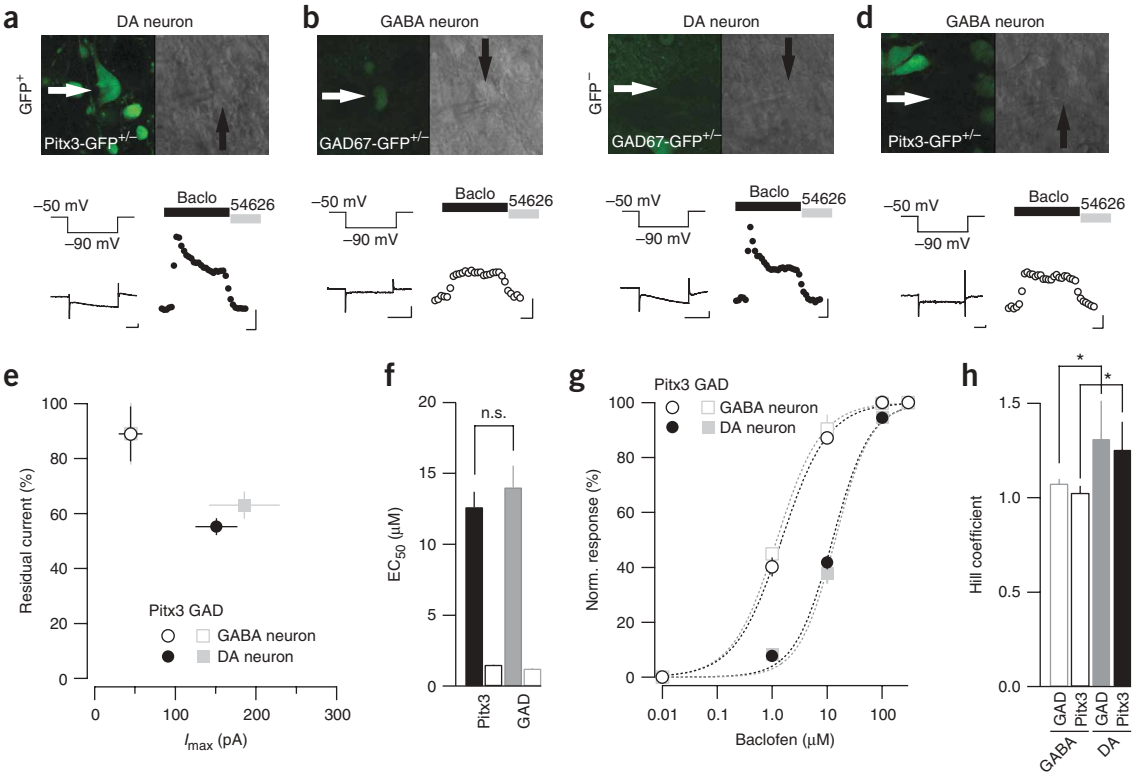


**Figure 1** Baclofen-evoked currents in DA and GABA neurons of the VTA. (**a**) Top, visualization of GFP-positive VTA neurons in a slice from a Pitx3-GFP$^{+/-}$ mouse. Images were obtained in the fluorescent (left) and transmitted (right) light channels of a two-photon laser-scanning microscope. White and black arrows point to the recorded neuron. Bottom, representative whole-cell traces obtained in this neuron. A hyperpolarizing pulse evoked an $I_h$ current (left); the right panel shows a typical response to baclofen (baclo; 100 μM). Currents were reversed by the selective antagonist CGP54626 (2 μM). (**b**) Visualization (top) and whole-cell recordings (bottom) obtained in a GABA neuron identified by GFP-fluorescence in the VTA of a GAD67-GFP$^{+/-}$ mouse. (**c**,**d**) Recordings obtained in GFP$^-$ neurons from a GAD67-GFP$^{+/-}$ and a Pitx3-GFP$^{+/-}$ mouse, respectively. Currents strongly resemble the recordings in **a**,**b** so the cells are probably DA and GABA neurons, respectively. Scale bars for bottom left panels, 300 ms/200 pA; for right panels, 2 min/50 pA (DA neurons) 25 pA (GABA neurons). (**e**) Normalized residual current against initial maximal amplitude of current evoked by 10 min baclofen (100 μM). In both mouse lines, currents in DA neurons ($n = 8$) were significantly larger and showed stronger desensitization than in GABA neurons ($n = 9$). (**f**) Bar graph representing $EC_{50}$ values from DA neurons (filled bars) and GABA neurons (empty bars) in GAD67-GFP and Pitx3-GFP mice. (**g**) The $EC_{50}$ values were derived from concentration-response curves for baclofen-evoked currents in GABA neurons ($n = 7$) and DA neurons ($n = 8$) in both genotypes. (**h**) Hill coefficient derived from the dose-response curves in GABA and DA neurons obtained in slices from both mouse lines.



that $GABA_B$ receptors couple less efficiently to GIRK channels in DA neurons than in GABA neurons. GHB, which has a very low receptor affinity, might preferentially inhibit GABA neurons at concentrations typically obtained with recreational use, thus producing disinhibition and consequent activation of DA neurons. The coupling efficiency for $GABA_B$ receptor evoked GIRK channels differed by almost an order of magnitude between GABA and DA neurons in rats, which we proposed was due to differences in the subunit compositions of GIRK channels in these neurons[23].

Here, by measuring the $EC_{50}$ (the concentration of agonist that is needed to obtain 50% of the maximal effect) we tested whether the coupling of $GABA_B$ receptors to GIRK channels in DA neurons is subject to modulation. Such modulation could occur through several mechanisms, targeting the receptors, the G proteins or the GIRK channel itself. We focused on GTPase-accelerating proteins (GAP) that promote GTP hydrolysis, thereby accelerating the termination of the βγ dimer signaling and affecting the $EC_{50}$ for receptor-dependent channel activation[24]. RGS proteins constitute a large family of multi-functional GAPs that can modulate the coupling of GIRK channels to their respective GPCRs[25]. If such a modulation of the $EC_{50}$ occurred in DA neurons of the VTA, it could profoundly alter the pharmacological effect of $GABA_B$ receptor agonists. For example, we would predict that GHB, at recreationally relevant doses, could under certain circumstances inhibit rather than excite DA neurons. Baclofen, on the other hand, would exert stronger inhibition on DA neurons, which would enhance its anti-craving properties.

In the present study, we found that RGS2 uniquely modulates the coupling efficiency of heteromeric GIRK2/3 channels. Furthermore, we show that chronic exposure to GHB can increase the coupling efficiency in DA neurons by inhibiting RGS2 transcription.

## RESULTS

### Baclofen-evoked currents in DA and GABA neurons of the VTA

To measure the $EC_{50}$ for GIRK currents elicited by baclofen in DA neurons and GABA neurons of mice, we took advantage of transgenic lines that express green fluorescent protein (GFP) in defined types of VTA neurons. This approach is more reliable than the identification of DA and GABA neurons previously used in rat slices, which was based on the presence of $I_h$ currents and their response to μ-opioid and D2 receptor agonists, criteria that have been challenged recently[26,27]. In the first mouse line, GFP was selectively expressed in cells that express Pitx3, a transcription factor that is required for the development of

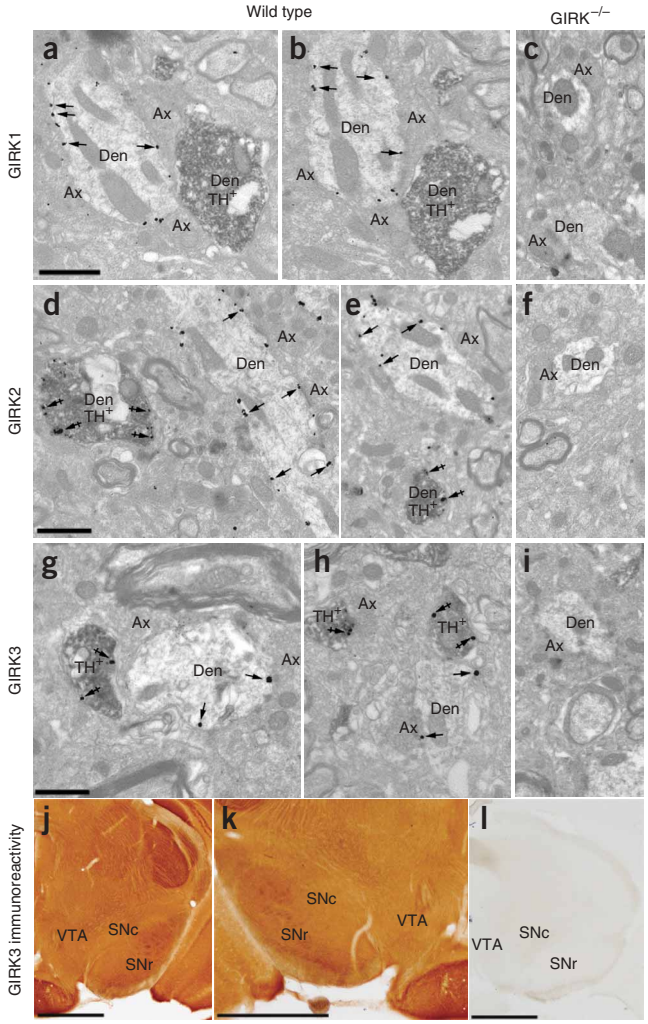


**Figure 2** Cell-type-specific subcellular localization of GIRK subunits. Pre-embedding electron micrographs combining immunoperoxidase (HRP) staining for TH and immunogold (gold) labeling for GIRK1, GIRK2 or GIRK3 in the VTA. (**a**) Immunoparticles for GIRK1 were observed along the extrasynaptic plasma membrane (arrows) of dendritic shafts that were immunonegative for TH (Den). Peroxidase reaction product (immunoreactivity for TH) filled dendritic shafts (Den, TH$^+$), which were devoid of immunoreactivity for GIRK1. (**b**) The selective presence of GIRK1 in TH$^-$ neurons is consistent throughout serial sections. (**c**) GIRK1 immunogold labeling is abolished in GIRK1$^{-/-}$ mice. (**d**,**e**,**g**,**h**) Similar images obtained with immunolabeling for GIRK2 and GIRK3, respectively, reveal that these GIRK subunits are found along the extrasynaptic plasma membrane (arrows) of dendritic shafts of TH$^-$ and TH$^+$ cells (crossed arrows). (**f**,**i**) Absence of GIRK2 and GIRK3 labeling in GIRK2$^{-/-}$ and GIRK3$^{-/-}$ mice, respectively. Ax, axon terminals. Scale bars, 0.2 μm. (**j**–**l**) Light-microscopic immunoreactivity in slices from wild-type mice (two examples at different magnification) and example in GIRK3$^{-/-}$ mouse. Scale bars, 1 mm.

DA neurons of the midbrain[28] and therefore is selectively expressed in these neurons, as confirmed with immunohistochemistry for tyrosine hydroxylase (TH)[29]. The second strain was a glutamate decarboxylase 67-GFP (Δneo) mouse, here called GAD67-GFP mouse for simplicity[30], in which GFP is selectively expressed in GABA neurons. Therefore, in heterozygous mice of either line, specific types of neuron are fluorescent and can be easily identified (**Fig. 1a**,**b**). Patch-clamp recordings in Pitx3-GFP–positive neurons revealed large $I_h$ currents and desensitizing baclofen-evoked currents (**Fig. 1a**). In most cases, the GAD67-GFP phenotype did not show an $I_h$ current and baclofen-evoked currents did not desensitize (**Fig. 1b**). GFP-negative cells in GAD67-GFP mice behaved like DA neurons, whereas GFP-negative neurons in Pitx3-GFP mice showed currents that resembled those of GABA neurons (**Fig. 1c**,**d**). In fact, in GFP-negative cells the maximal baclofen-evoked current (GABA neurons 45 ± 15 pA for GFP-negative cells versus 44 ± 15 pA for GFP-positive cells; DA neurons 186 ± 44 pA versus 151 ± 26 pA; **Fig. 1e**) and its desensitization, (GABA neurons 89 ± 11% versus 89 ± 10%; DA neurons: 55 ± 3% versus 63 ± 5%; **Fig. 1e**) did not differ from those of corresponding GFP-positive cells. Successive brief applications of increasing concentrations of baclofen elicited responses that were normalized to the maximal response obtained with a supramaximal concentration. These responses were used to construct dose-response curves and to calculate the $EC_{50}$ in both mouse lines (GABA neurons 1.5 ± 0.1 μM for GFP-negative cells versus 1.2 ± 0.1 μM for GFP-positive cells; DA neurons: 12.6 ± 1.1 μM versus 14 ± 1.6 μM; **Fig. 1f**,**g**). Thus, we confirm that in mice, just as in rats[23], the $EC_{50}$ for GIRK channel activation by $GABA_B$ receptors is about an order of magnitude higher in DA neurons than in GABA neurons in the VTA.

Our data also reveal that the Hill coefficient is slightly larger in DA neurons than in GABA neurons (1.3 ± 0.2 versus 1.0 ± 0.03, average of both genotypes, $P < 0.05$; **Fig. 1h**). This difference is probably due to the desensitization of the GIRK currents, which is larger in DA neurons than in GABA neurons. Even with short baclofen applications, we cannot exclude the possibility that a small underestimation of the current amplitude in DA neurons, when normalized to the maximal response, would make the concentration-response curve look steeper. In fact, throughout our study, we observed similar differences for the Hill coefficients in experiments where desensitization was affected by the manipulation. Importantly, any underestimation of the amplitude measurements due to desensitization would lead to a slightly smaller $EC_{50}$ value in the control condition, and therefore to a small underestimation of the difference (see Methods).

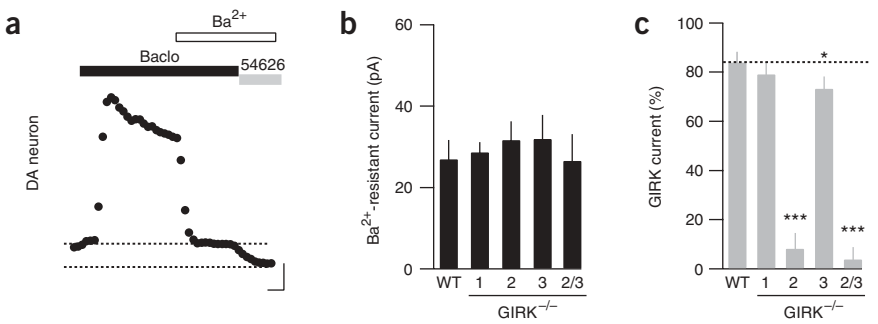


**Figure 3** Contribution of GIRK channel subunits to total outward current in DA neurons of the VTA. (**a**) A large fraction of the baclofen-evoked current (100 μM) is inhibited by $BaCl_2$ (1 mM) in wild-type (WT) mice. Subsequent reversal with CGP54626 reveals the $Ba^{2+}$-resistant component of the current. Scale bars, 50 pA/2 min. (**b**) The $Ba^{2+}$-resistant component was of similar magnitude in all the GIRK$^{-/-}$ mice tested. (**c**) Fraction of GIRK currents obtained by normalization to the $Ba^{2+}$-resistant component in DA neurons in all the genotypes.

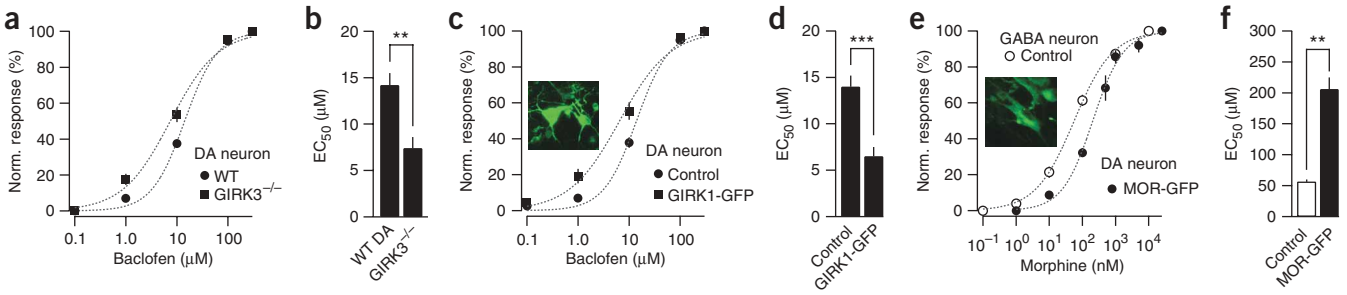


**Figure 4** $GABA_B$-GIRK coupling efficiency in DA neurons is determined by GIRK subunit expression. (**a**) Concentration-response curve to baclofen in DA neurons of GIRK3$^{-/-}$ mice ($n = 8$) compared to DA neurons of wild-type mice ($n = 8$). (**b**) Mean of the $EC_{50}$ value calculated for each cell. (**c**) Concentration-response curve of baclofen-evoked currents in GIRK1-GFP transfected DA neurons (green cells, inset; $n = 9$) and neighboring untransfected DA neurons (as determined by the presence of $I_h$ and response to D2 receptor agonist) in wild-type mice ($n = 8$). (**d**) $EC_{50}$ values for neurons in **c**. (**e**) Concentration-response curve for morphine in MOR-GFP transfected DA neurons (green neurons, inset; $n = 6$) and untransfected GABA neurons (absence of $I_h$ and response to MOR agonist) in wild-type mice ($n = 6$). (**f**) Mean $EC_{50}$ values from **e**.

## Cell type–specific GIRK subunit expression

We next combined immunohistochemistry for TH with immunogold labeling for GIRK subunits in ultrastructural experiments that were designed to visualize the subcellular distribution of GIRK subunits in the VTA (**Fig. 2**). This high-resolution technique revealed that GIRK1 is present only at extrasynaptic sites in TH-negative dendrites (**Fig. 2a,b**), whereas GIRK2 and GIRK3 were found at extrasynaptic sites in both TH-positive and TH-negative cells (**Fig. 2d,e,g,h**). Immunogold labeling for GIRK1, GIRK2 and GIRK3 was absent in GIRK1$^{-/-}$, GIRK2$^{-/-}$ and GIRK3$^{-/-}$ mice, respectively, confirming the specificity of the antibodies used for electron microscopic staining (**Fig. 2c,f,i**) and of the GIRK3 antibody that was used in this study for the first time for light microscopy (**Fig. 2j,k,l**). These observations extend our previous analysis of cell type–specific GIRK subunit expression in the VTA, which was carried out using single-cell PCR with reverse transcriptase (RT-PCR)[23], and confirm that GIRK1 is selectively expressed in TH-negative neurons while GIRK2 and GIRK3 are found in both TH-positive and TH-negative cells (see **Supplementary Table 1** online).

## Baclofen-evoked currents in mice lacking GIRK subunits

We next tested the involvement of GIRK1–3 subunits in baclofen-evoked currents in GIRK1$^{-/-}$, GIRK2$^{-/-}$, GIRK3$^{-/-}$ and GIRK2$^{-/-}$ GIRK3$^{-/-}$ double-knockout mice in DA neurons of the VTA. Previous studies have shown that baclofen activates, in addition to GIRK channels, a $Ba^{2+}$-resistant conductance whose molecular identity remains unknown, but that shows some of the pharmacological properties of two-pore $K^+$ channels[23,31]. In subsequent experiments, we used the $Ba^{2+}$-insensitive conductance as a reference, which had the advantage of reducing the variability caused by the size of the cell. We measured the amplitude of the $Ba^{2+}$-insensitive current component by applying $Ba^{2+}$ (1 mM) before antagonizing $GABA_B$ receptors (**Fig. 3a**). The $Ba^{2+}$-insensitive component did not differ across all knockout mice tested (**Fig. 3b**), confirming that it is not carried by GIRK channels. For each cell, the amplitude of the $Ba^{2+}$-insensitive current was divided by the maximal current. This ratio was then subtracted from 100% to calculate the relative GIRK component. Currents were unaltered in GIRK1$^{-/-}$ mice (79 ± 5% versus wild type 84 ± 4%), virtually abolished in GIRK2$^{-/-}$ mice (8 ± 6%, $P < 0.001$) and GIRK2$^{-/-}$ GIRK3$^{-/-}$ mice (4 ± 5%, $P < 0.001$), and slightly but significantly reduced in GIRK3$^{-/-}$ mice (73 ± 5%, $P < 0.05$; **Fig. 3c**). Together, these results indicate that GIRK2 and GIRK3 contribute to endogenous currents in DA neurons, indicating that these neurons contain GIRK2/3 heteromers. Although the lack of GIRK3 can be compensated partially (presumably through the formation of GIRK2 homomers), this is not possible after knockout of GIRK2.

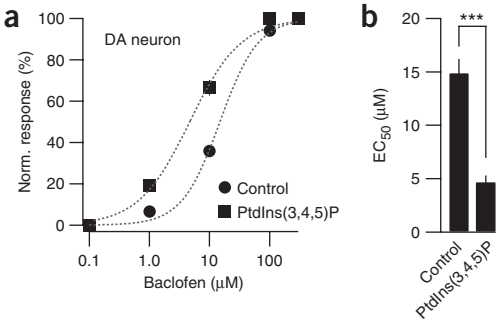


**Figure 5** Inhibition of RGS proteins increases the $GABA_B$-GIRK coupling efficiency in DA neurons. (**a**) Concentration-response curve of baclofen-evoked currents (100 μM) in presence (square, $n = 8$) or in absence (circle, $n = 10$) of $PtdIns(3,4,5)P_3$. (**b**) Corresponding $EC_{50}$ values.

## $EC_{50}$ is determined by GIRK subunits in DA neurons

As GIRK2/3 heteromeric channels show a lower affinity for $G_{\beta\gamma}$-dimers than do other GIRK subunit combinations[7], and because GIRK2c/3 heteromers expressed in HEK293 cells show a higher $EC_{50}$ for activation by baclofen[23], we predicted that the $EC_{50}$ for baclofen-evoked currents in DA neurons of GIRK3$^{-/-}$ mice would be lower than in wild-type mice. In fact, we found a value of 7.4 ± 1.2 μM, which was significantly lower than in neurons from wild-type mice (14.2 ± 1.3 μM, $P < 0.01$; **Fig. 4a,b**). Because DA neurons lack GIRK1, we postulated that ectopic expression of GIRK1 might shift the $EC_{50}$ to lower concentrations. We used *in vivo* stereotaxic delivery of a viral vector harboring GIRK1 and GFP into the VTA (see Methods). In transfected DA neurons, baclofen yielded currents with a significantly lower $EC_{50}$ than in neighboring untransfected cells (6.5 ± 1 μM versus 14 ± 1.2 μM, $P < 0.001$; **Fig. 4c,d**).

We next investigated whether the type of GPCR helped to determine the low coupling efficiency with GIRK channels. We expressed another pertussis toxin (PTX)-sensitive $G_{i/o}$-coupled GPCR, the μ-opioid receptor (MOR), in DA neurons using a viral construct. Normally MORs are selectively expressed on GABA neurons of the VTA. The recombinant MORs expressed in DA neurons reliably coupled to GIRK channels, producing an $EC_{50}$ for morphine-induced GIRK currents

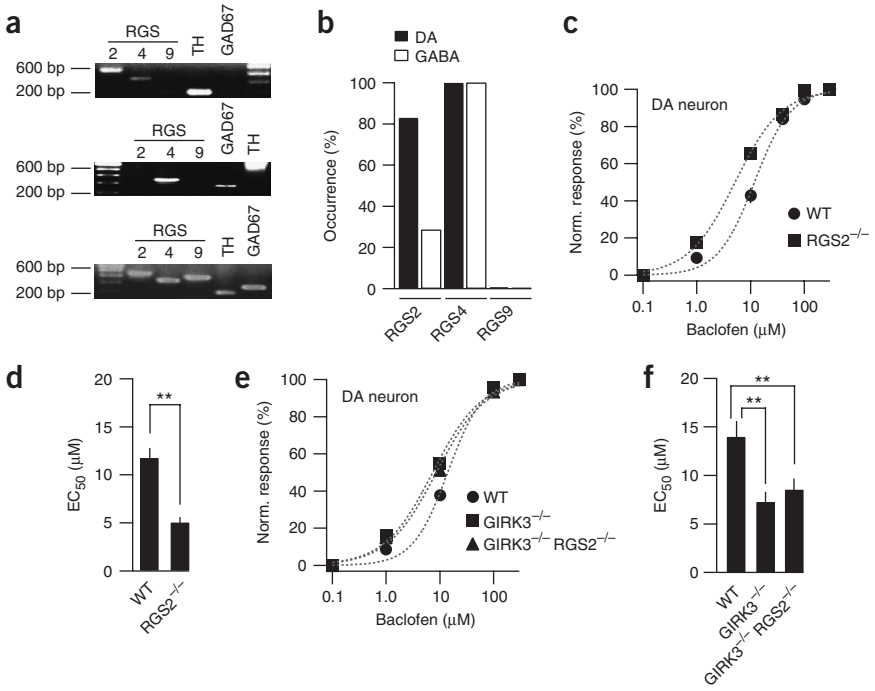


**Figure 6** RGS2 modulates $GABA_B$-GIRK coupling in DA neurons. (**a**) Example of single cell multiplexed RT-PCR in DA neuron (top) revealing mRNA for tyrosine hydroxylase (TH), RGS2 and RGS4. In a GABA neuron (middle), single-cell RT-PCR showed mRNA for glutamic acid decarboxylase (GAD67) and RGS4. Multiplexed RT-PCR of the whole mesencephalon as positive control (bottom). (**b**) Relative occurrence of RGS proteins with single-cell multiplexed RT-PCR ($n = 6$–7 for each condition). (**c**) Concentration-response curve of baclofen-evoked currents in DA neurons of $RGS2^{-/-}$ and wild-type control mice ($n = 8$ for each genotype); (**d**) corresponding $EC_{50}$ values. (**e**) Concentration-response curve of baclofen-evoked current in DA neurons of $GIRK3^{-/-}$ ($n = 9$), $GIRK3^{-/-}$ $RGS2^{-/-}$ ($n = 10$) or wild-type control mice ($n = 7$). (**f**) $EC_{50}$ values in the three genotypes.

that was significantly higher than in GABA neurons (206 ± 19 nM versus 56 ± 4 nM, $P < 0.01$; **Fig. 4e,f**). These results show that the $EC_{50}$ for GPCR activation of neuronal GIRK channels is determined by the subunit composition of the GIRK channel and does not depend on the identity of the GPCR.

### Inhibition of RGS proteins increases coupling efficiency

We next tested whether RGS proteins were involved in modulating the $EC_{50}$ for coupling between $GABA_B$ receptors and GIRK channels in DA neurons of the VTA. We loaded DA neurons through a patch pipette with the non-specific RGS inhibitor, phosphatidylinositol (3,4,5)-trisphosphate ($PtdIns(3,4,5)P_3$)[32] and found that $GABA_B$ receptor-evoked currents had a significantly lower $EC_{50}$ than in untreated neurons (4.7 ± 0.6 μM versus 14.9 ± 1.3 μM, $P < 0.001$; **Fig. 5a,b**) while the maximal amplitude remained unchanged (159 ± 33 pA versus 207 ± 35 pA, n.s.; data not shown).

### Cell-specific expression of RGS proteins in the VTA

We next investigated whether there was cell type–specific expression of RGS proteins in neurons of the VTA. We performed single-cell RT-PCR from DA and GABA neurons, for three members of the RGS family that have previously been implicated in the action of addictive drugs[33]. We found that RGS2 was preferentially expressed in DA neurons, whereas RGS4 was always found in both DA and GABA neurons; RGS9 was not found in either cell type (**Fig. 6a,b**). The absence of RGS9 in both cell types of the VTA is in line with other studies that found that this RGS protein was enriched almost exclusively in striatal regions[34,35].

### Coupling efficiency in RGS2 knockout mice

Reasoning that the selective expression of RGS2 in DA neurons could contribute to the low $GABA_B$-GIRK coupling efficiency, we established concentration-response curves in $RGS2^{-/-}$ mice. The $EC_{50}$ was significantly lower in DA neurons from $RGS2^{-/-}$ mice (5.1 ± 0.5 μM) than in neurons from littermate controls (11.8 ± 1 μM, $P < 0.01$; **Fig. 6c,d**). We next asked whether RGS2 works selectively on GIRK3-containing channels or whether it also affects other subunits. We crossbred the knockout lines to generate mice lacking both GIRK3 and RGS2 ($GIRK3^{-/-}$ $RGS2^{-/-}$). As in the $RGS2^{-/-}$ mice, baclofen-evoked currents in the $GIRK3^{-/-}$ $RGS2^{-/-}$ mice showed a significantly smaller $EC_{50}$ (8.6 ± 1.1 μM) than in wild-type littermate controls (14 ± 1.6 μM; $P < 0.01$; **Fig. 6e,f**). Interestingly, this reduced value was similar to the $EC_{50}$ in $GIRK3^{-/-}$ mice (7.3 ± 1, n.s.). These experiments indicate that the effect of RGS2 on $GABA_B$ receptor coupling uniquely requires channels with the GIRK3 subunit.

To test for a preferential interaction between RGS2 and GIRK3 channels, we tested for fluorescence resonance energy transfer (FRET) between CFP and YFP-tagged proteins expressed heterologously in HEK293 cells. We focused on the possible association in the plasma membrane using total internal reflection fluorescence (TIRF) microscopy[36] and the acceptor photobleaching method[37] to determine the FRET efficiency (%FRET)[38]. Similar to CFP-GIRK4 and GIRK1-YFP (9.12 ± 0.54%), which are known to associate in the plasma membrane[38], we measured significant %FRET between RGS2-YFP and CFP-GIRK3 in the presence of untagged GIRK2 (6.92 ± 0.27%, $P < 0.05$), when compared to cells expressing CFP-GIRK3, untagged RGS2 and GIRK2 (2.39 ± 0.29%, n.s.; **Fig. 7a–e**). Furthermore, the association of GIRK3 and RGS2 did not appear to originate from non-specific association, since CFP-GIRK2 and RGS2-YFP did not produce significant %FRET (2.51 ± 0.55%, n.s.; **Fig. 7e**). The detection of significant %FRET indicates that RGS2 proteins and GIRK3 channels move within the 100 Å needed to produce a FRET signal[37].

Together, these findings support the conclusion that RGS2 can directly interact with GIRK3 but not with GIRK2.

### Modulation of RGS2 expression

If RGS2 has a modulatory role, any condition that alters RGS2 expression could exert a profound impact on coupling between $GABA_B$ receptors and GIRK channels. In the context of drug abuse, it has been reported that the expression of RGS proteins can change in response to chronic drug exposure[33]. We therefore injected the mice twice daily with GHB (250 mg $kg^{-1}$) for 5 days and prepared slices 1 h after the last injection on the morning of the 6th day. In these conditions, the $EC_{50}$ was significantly lower than in slices from untreated mice (6.1 ± 0.2 μM versus 12.3 ± 0.2 μM, $P < 0.01$; **Fig. 8a**). To test whether changes in RGS expression could also be elicited by chronic exposure to other addictive drugs, we injected increasing doses of morphine (20–100 mg $kg^{-1}$ twice daily) over 6 days.

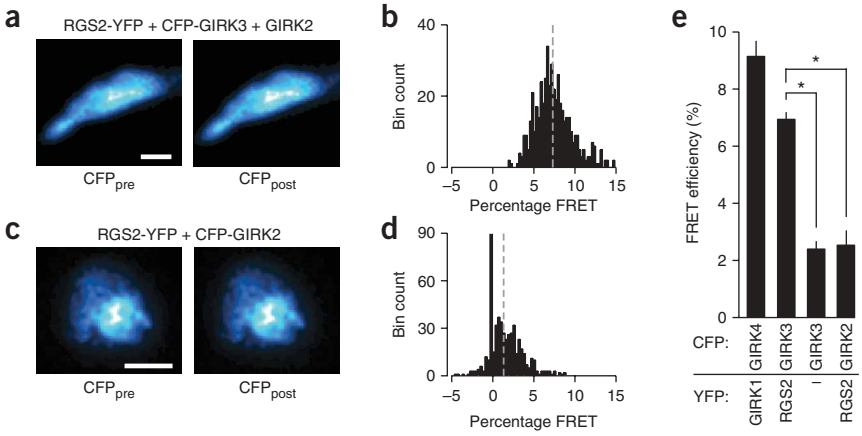


**Figure 7** Close association between RGS2 proteins and GIRK3 channels. HEK293 cells were transfected with the following cDNAs: CFP-GIRK4 and GIRK1-YFP; CFP-GIRK3, RGS2-YFP and untagged GIRK2; CFP-GIRK3, untagged RGS2 and untagged GIRK2; CFP-GIRK2 and RGS2-YFP. (**a**,**c**) CFP fluorescence before and after photobleaching YFP (left and right, respectively). Note increase in CFP fluorescence for CFP-GIRK3, RGS2-YFP and untagged GIRK2, but not for CFP-GIRK2 and RGS2-YFP. Scale bars represent 10 μm. (**b**,**d**) Pixel-by-pixel distribution of %FRET, with the mean indicated by the dashed line. (**e**) Average %FRET (± s.e.m., $n = 10$–18) for the indicated conditions. In addition, possible FRET was examined between CFP-GIRK3, PSD95-YFP and GIRK2 as controls (not shown, n.s., $n = 7$).

In slices prepared from these mice 2 h after the last morphine injection (without naloxone challenge), the $EC_{50}$ for baclofen-evoked currents in DA neurons was significantly lower than in slices from saline-treated mice (8.8 ± 1.2 μM versus 13.2 ± 0.7 μM, $P < 0.01$; **Fig. 8b**). Quantifying the level of RGS2 messenger RNA with real-time PCR revealed a significant reduction after both chronic GHB and chronic morphine exposure when the VTA was surgically isolated from midbrain slices (34 ± 0.3% and 45 ± 8% reduction, respectively, compared with saline-injected control mice, $P < 0.01$; **Fig. 8c**).

We next tested whether prolonged *in vitro* exposure of the slice to morphine was sufficient to alter RGS2 expression. We incubated slices for 5 h in morphine (5 μM) and found a similar reduction in the $EC_{50}$ (6.5 ± 0.3 μM versus 14 ± 1 μM; **Fig. 8d**,**e**), which was occluded when the same procedure was repeated in slices obtained from RGS2$^{-/-}$ mice (4.1 ± 0.5 μM versus 4.9 ± 0.6 μM, n.s; **Fig. 8f**,**g**). Together, these results indicate that chronic exposure to GHB and morphine downregulates RGS2, thereby causing a reduction in the $EC_{50}$ for activation by baclofen.

### Loss of disinhibition of DA firing in RGS2$^{-/-}$ mice

The magnitude of the shift in the dose-response curves between GABA neurons and DA neurons determines the concentration window in which $GABA_B$ receptor agonists can lead to disinhibition. In RGS2$^{-/-}$ mice, the shift in $EC_{50}$ predicts that there will be a small effect of disinhibition. While monitoring the firing frequency in connected DA neurons, we applied a low (0.1 μM) concentration of baclofen, which led to a frequency increase in slices from wild0-type mice, as shown previously[23]. By contrast, the same concentration of baclofen reduced the firing rate in DA neurons from RGS2$^{-/-}$ mice. Applying 10 μM baclofen completely suppressed firing (7 ± 2% versus 1 ± 0.3%; **Fig. 9a**) in both genotypes. The effect of baclofen was reversed by the $GABA_B$ receptor antagonist CGP54626 (105 ± 5% versus 96 ± 7%; **Fig. 9a**). On average, the firing frequency increased to 177 ± 9% in wild-type mice, while it was reduced to 53 ± 15% of baseline in the RGS2$^{-/-}$ mice with a baclofen concentration of 0.1 μM ($P < 0.01$; **Fig. 9b**). Importantly, there was no change in the spontaneous firing of DA neurons from the RGS2$^{-/-}$ mice (1.5 ± 0.4 Hz versus 1.9 ± 0.2 Hz, n.s.; inset **Fig. 9b**). Like the effects of baclofen, the disinhibition was not observed with a low concentration of GHB in the RGS2$^{-/-}$ mice (62 ± 13% versus –7 ± 4%, $P < 0.01$; **Fig. 9c**,**d**).

### Chronic GHB alters preference for self-administration

In DA neurons from the VTA, we have shown that chronic treatment with GHB increases the coupling efficiency of $GABA_B$ receptors to GIRK channels, through downregulation of RGS2. As a consequence of this lower $EC_{50}$, we predicted that GHB would lose its rewarding

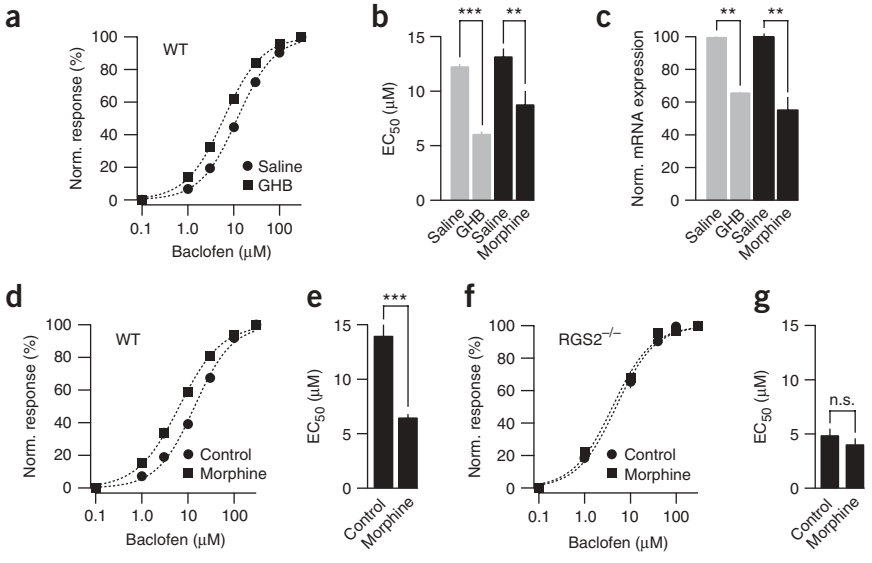


**Figure 8** Exposure to GHB and morphine modulates $GABA_B$-GIRK coupling efficiency by reducing the transcription level of RGS2. (**a**) Concentration-response curve of baclofen-evoked currents in DA neuron in mice treated with chronic GHB ($n = 7$) versus saline ($n = 6$). (**b**) Mean $EC_{50}$ values of baclofen-evoked currents in DA neurons of mice treated with GHB, morphine and saline ($n = 6$–7). (**c**) Quantitative real-time PCR measurement of RGS2 mRNA in the VTA of mice treated with chronic GHB, morphine and saline ($n = 6$ for each condition). (**d**) Concentration-response curve obtained in slices that were incubated in morphine (square, $n = 13$) or regular ACSF ($n = 8$) for 5 h. (**e**) $EC_{50}$ values obtained in the same conditions. (**f**) Concentration-response curve obtained in slices from RGS2$^{-/-}$ mice that were incubated in morphine ($n = 7$) or ACSF (circle, $n = 8$) for 5 h. (**g**) Mean $EC_{50}$ values showing that the effect of morphine is occluded in RGS2$^{-/-}$ mice.