nature neuroscience

Bi-directional effects of $GABA_B$ receptor agonists on the mesolimbic dopamine system

Hans G Cruz[1], Tatiana Ivanova[1], Marie-Louise Lunn[3], Markus Stoffel[4], Paul A Slesinger[3] & Christian Lüscher[1,2]

The rewarding effect of drugs of abuse is mediated by activation of the mesolimbic dopamine system, which is inhibited by putative anti-craving compounds. Interestingly, different $GABA_B$ receptor agonists can exert similarly opposing effects on the reward pathway, but the cellular mechanisms involved are unknown. Here we found that the coupling efficacy (EC_{50}) of G-protein-gated inwardly rectifying potassium (GIRK, Kir3) channels to $GABA_B$ receptor was much lower in dopamine neurons than in GABA neurons of the ventral tegmental area (VTA), depending on the differential expression of GIRK subunits. Consequently, in rodent VTA slices, a low concentration of the canonical agonist baclofen caused increased activity, whereas higher doses eventually inhibited dopamine neurons. At behaviorally relevant dosages, baclofen activated GIRK channels in both cell types, but the drug of abuse γ-hydroxy-butyric acid (GHB) activated GIRK channels only in GABAergic neurons. Thus $GABA_B$ receptor agonists exert parallel cellular and behavioral effects due to the cell-specific expression of GIRK subunits.

Activation of the mesolimbic dopamine system, which originates in the VTA, mediates rewarding effects of drugs of abuse[1]. Different $GABA_B$ receptor agonists are reported to have opposing effects on this system. Whereas the prototypical agonist baclofen reduces self-administration and re-instatement of a number of drugs in rodents[2] and is considered a putative anti-craving compound in humans[3], γ-hydroxy-butyric acid (GHB) is readily self-administered[4] and has been recognized as a drug with a strong abuse potential[5]. The cellular mechanism involved in the increase in dopamine release from the VTA varies with the specific drug of abuse. Opioids and cannabinoids, for example, bind to $G_{i/o}$-coupled GPCRs that are selectively expressed on inhibitory interneurons (GABA neurons) of the VTA[6]. This leads to the suppression of their spontaneous activity through concomitant hyperpolarization (postsynaptic inhibition) and decreased transmitter release (presynaptic inhibition), causing a disinhibition of the dopamine neurons. Beyond mediating rewarding effects, activation of the mesolimbic dopamine system is also thought to be centrally involved in the induction of repetitive drug use and may even trigger relapse[7]. Putative anti-craving compounds, including baclofen, may therefore work by modulating the mesolimbic system such that they interfere with dopamine release normally caused by drugs of abuse. The cellular mechanisms of this interference, however, remain elusive. The fact that $GABA_B$ receptors are expressed on both dopamine neurons and GABA neurons adds to the complexity. Moreover, an explanation is lacking for why GHB and baclofen have different behavioral effects in spite of their similar actions on $GABA_B$ receptors in the mesolimbic dopamine system. Here we provide a model that reconciles these apparent contradictions based on the differential coupling efficacy (EC_{50}) of GIRK channels in VTA neurons leading to bi-directional effects of $GABA_B$ receptor agonists on the mesolimbic dopamine system. We provide evidence that the EC_{50} is determined by cell-specific expression of GIRK subunits.

RESULTS

Effects of baclofen on neurons of the VTA

In acute horizontal slices of VTA, whole-cell patch-clamp recordings revealed that bath application of baclofen led to outward currents (**Fig. 1a**,**b** lower traces) and a concomitant decrease of the membrane resistance (upper traces) in both dopamine and GABA neurons, indicating the opening of ionic conductances. In dopamine neurons, however, currents were significantly larger and desensitized substantially during 15 min of continuous agonist application (**Fig. 1c**). Remarkably, the EC_{50} for baclofen activation of outward current in dopamine neurons was an order of magnitude larger than in GABA neurons (**Fig. 1d**). For comparison, baclofen also inhibited evoked inhibitory postsynaptic currents (IPSCs, measured in dopamine neurons) with an EC_{50} close to the one found in GABA neurons for the postsynaptic inhibition (**Fig. 1e**,**f**) as well as inhibition of IPSC of afferents onto GABA neurons (EC_{50} = 1.5 ± 0.1, n = 5, data not shown). Taken together, these results confirm the presynaptic expression of $GABA_B$ receptors on GABA neurons and the postsynaptic somatodendritic expression on both cell types; however, the efficiency of receptor–G-protein–effector coupling (high EC_{50}) was surprisingly low in dopamine neurons.

Molecular identity of baclofen-evoked currents

To determine the molecular identity of the baclofen-evoked current, we used pharmacological tools and knockout animals. In both cell types, a large component was sensitive to 300 μM barium (Ba, **Fig. 2a–c**), suggesting an underlying inwardly rectifying K^+ channel. This

[1]Department of Basic Neurosciences and [2]Clinic of Neurology, University of Geneva, 1 Michel Servet, CH-1211 Geneva 4, Switzerland. [3]Peptide Biology Laboratory, The Salk Institute for Biological Studies, Box 85800, San Diego, California 92186, USA. [4]Laboratory of Metabolic Diseases, The Rockefeller University, 1230 York Avenue, New York, New York 10021, USA. Correspondence should be addressed to C.L. (Christian.Luscher@medecine.unige.ch) or P.A.S. (Slesinger@salk.edu).

Published online 25 January 2004; doi:10.1038/nn1181

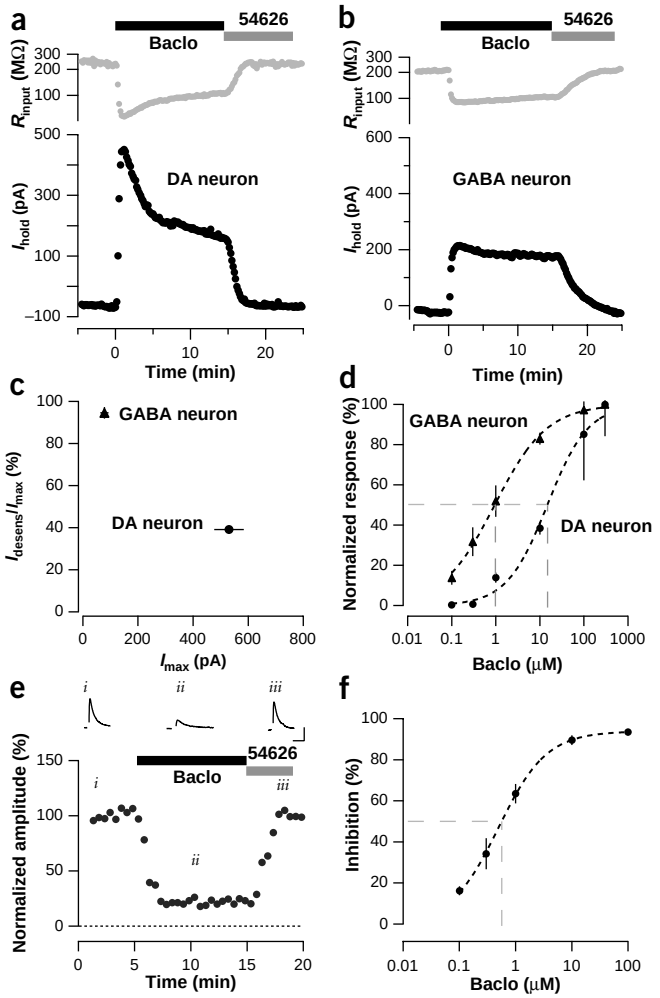

Figure 1 Pre- and postsynaptic $GABA_B$ receptor–mediated inhibition in VTA neurons. (**a**) Bath application of baclofen (100 μM) in a dopamine (DA) neuron. (**b**) A GABA interneuron elicits outward currents (black trace) in whole cell voltage clamp (V_h = –63 mV) recordings in rats slices, which were reversed by CGP 54626 (2 μM). Notice the concomitant drop of the membrane resistance (gray trace). (**c**) Desensitization plotted against the maximal amplitude of the outward current for dopamine (n = 34) and GABA neurons (n = 18). (**d**) Concentration-response curves are shown for dopamine neurons (circle; EC_{50} = 14.8 ± 2.5 μM, h = 0.9 ± 0.1, n = 5) and GABA neurons (triangle; EC_{50} = 0.9 ± 0.1 μM, h = 0.7 ± 0.1, n = 5). (**e**) Baclofen (100 μM)-induced presynaptic inhibition of IPSCs recorded in a dopamine neuron. Insets are averaged traces at corresponding time points. Scale bar, 100 pA, 20 ms. (**f**) Concentration-response curve of IPSC inhibition (EC_{50} = 0.5 ± 0.2 μM, h = 1.0 ± 0.1, n = 5).

was confirmed by the observation that in mice containing a null mutation in GIRK2 ($GIRK2^{-/-}$)[8], the baclofen-activated currents were markedly reduced, revealing small, Ba-insensitive currents in both dopamine and GABA neurons (**Fig. 2d–f**). We conclude that the majority of the baclofen-evoked current is carried by GIRK channels containing the GIRK2 subunit in both cell types.

To characterize the Ba-insensitive, baclofen-induced current in dopamine neurons, we applied brief voltage ramps ranging from –30 mV to –130 mV in extracellular solutions containing potassium concentrations. The current-voltage (I-V) relationship showed a weak, outwardly rectifying current that reversed near the predicted potassium equilibrium potential, consistent with a K^+ current (**Fig. 3a**). This current was insensitive to the two other K^+ channel blockers TEA and 4-AP, and the protein kinase A (PKA) inhibitors H7 and H8 (**Fig. 3b**). The Ba-insensitive current was inhibited by the cAMP analog 8Br-cAMP, suggesting that the underlying channel may be a member of the two-pore K^+ channel family[9]. Importantly, the EC_{50} of this current was 0.9 ± 0.3 μM (**Fig. 3c**), which is substantially less than the EC_{50} of the total current, mediated predominantly by GIRK channels. This difference in EC_{50} for GIRK channels and Ba-insensitive K^+ channels suggests that GIRK channels may have a particularly low affinity for G proteins. The efficiency of $GABA_B$ receptor coupling may in fact vary depending on the type of effector (*i.e.*, two-pore versus GIRK channel). In addition, the coupling efficiency of $GABA_B$ receptors to GIRK channels may vary in different neurons. We therefore hypothesized that dopaminergic neurons express a subset of GIRK channels with a particularly low affinity for G proteins.

Cell-specific GIRK subunit composition

To date, four different mammalian GIRK subunits (GIRK1–4) have been cloned[10–13], which form functional hetero- or homomeric channels by the assembly of four subunits[14]. GIRK channels in the brain are typically composed of GIRK1 and either GIRK2 (ref. 15) or GIRK3 (ref. 16). GIRK2 also forms homomeric channels[17,18] (but see ref. 19) or co-assembles with GIRK3 (ref. 20). We next investigated whether dopamine and GABA neurons in the VTA expressed different combinations of GIRK subunits. Using single-cell RT-PCR for all four GIRK subunits, including three splice variants for GIRK2 (refs. 21,22), we found that GABA neurons expressed mRNA for GIRK1, 2c and 3, whereas dopamine neurons contained mRNA for only GIRK2c and 3 (**Fig. 4**). This difference raised the possibility that the presence of the GIRK1 subunit, by forming GIRK1/3 and GIRK 1/2c heteromeric channels, ensures highly efficacious coupling of the channel to G proteins. We reasoned that GIRK2c/3 heteromeric channels may couple less efficiently and therefore have a lower EC_{50}. When we expressed various combinations of GIRK subunits with $GABA_B$ receptors in HEK-293T cells, the EC_{50} values were indistinguishable for all combinations containing GIRK1 and the GIRK2c homomer. Importantly, the EC_{50} was significantly higher in HEK-293T cells co-expressing GIRK2c and GIRK3 without affecting the Hill coefficient (h; **Fig. 5**). The change in EC_{50}, however, was not as large as that observed between dopamine and GABA neurons (see Discussion).

Difference in EC_{50} imparts bi-directional effects

Taken together, our data suggest that the high EC_{50} for GIRK current activation is due to the unique combination of GIRK2c/3 heteromeric channels in dopamine neurons. Given the ten-fold difference in coupling efficiency of the $GABA_B$ receptors in the two different types of neurons in the VTA, we predicted that a $GABA_B$ agonist would exert a bi-directional effect on the VTA output. With a low agonist concentration, GABA neurons would be electrically silenced, leading to disinhibition of the dopamine neurons. Conversely, dopamine neurons would be directly inhibited via $GABA_B$ receptors with high agonist concentrations. To test this, we selected for dopamine neurons with preserved synaptic connectivity to GABA neurons (see methods) and monitored action potentials in the 'cell-attached' patch technique while increasing agonist concentrations in a stepwise manner. Importantly, concentrations of baclofen below 1 μM did not induce desensitization of GIRK currents (**Supplementary Fig. 1** online). Low doses of baclofen (0.1 μM) caused a three-fold increase in firing frequency (**Fig. 6**). At a moderate dose (0.5 μM) of baclofen, the frequency returned to basal firing rates, whereas action potentials were completely abolished at a high concentration (100 μM) of baclofen.

These effects were fully reversible with the $GABA_B$ receptor antagonist CGP 54626. Thus, depending on the concentration of agonist, $GABA_B$ agonists can either enhance or suppress the dopamine output of the VTA.

Effects of GHB on VTA neurons

Although baclofen is considered a putative anti-craving compound in humans[3], GHB, another putative $GABA_B$ receptor agonist, has been recognized as a drug with a strong abuse potential[5]. We therefore investigated the coupling of GHB to GIRK channels in VTA slices. In GABA neurons, both GHB and baclofen seemed to fully activate GIRK channels (**Fig. 7a**). By contrast, GHB (10 mM) activated only 30% of the baclofen-induced current in dopamine neurons (**Fig. 7b**), suggesting that GHB had the same effects on GIRK channels as a more canonical $GABA_B$ receptor agonist, but with a much lower affinity (**Fig. 7a,b**). These effects of GHB were completely blocked with CGP 54626, and a saturating dose of GHB occluded subsequent saturating doses of baclofen (**Fig. 7a**). We concluded that the effects of GHB in the VTA are mediated by low-affinity agonist properties at the $GABA_B$ receptor (concentrations >100 mM GHB would be required to determine the maximal stimulation with GHB). Consistent with this conclusion, previous observations with reconstituted $GABA_B$ receptors[23], native receptors in midbrain slices[24] and *in vivo* brain recordings[25] have suggested that GHB is a full, low-affinity agonist. Importantly, the difference in the EC_{50} between GABAergic neurons and dopaminergic neurons was preserved for GHB (**Fig. 7c**). In summary, GHB showed a similar pattern of $GABA_B$ receptor activation in VTA neurons as baclofen, albeit at higher concentrations because of its low receptor affinity.

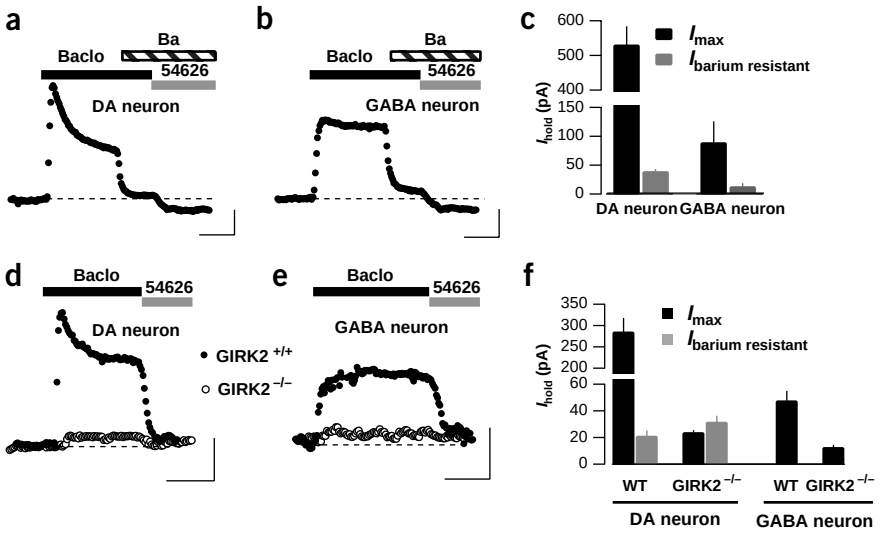

Figure 2 $GABA_B$ receptor agonist–evoked currents in VTA neurons are mediated by two distinct conductances. (**a**,**b**) A large fraction of the current elicited by baclofen (100 μM) was inhibited by $BaCl_2$ (300 μM) in dopaminergic (DA) neurons (**a**) as well as in GABAergic neurons (**b**). Scale bars: 50 pA, 5 min. (**c**) Mean amplitude of current ($n > 5$). (**d**,**e**) Overlay of a baclofen-elicited currents (100 μM) obtained in a DA neuron (**d**) and GABA neuron (**e**) of a GIRK2$^{-/-}$ and a wild-type mouse (open and closed symbols, respectively). Scale bar: 50 pA, 5 min. (**f**) Average data indicate that in dopamine neurons the amplitude of Ba-resistant current is not different in wild-type and GIRK2$^{-/-}$ mice, and is indistinguishable from the residual current in GIRK2$^{-/-}$ mice without Ba. Similarly, in GABA neurons of GIRK2$^{-/-}$, only a small residual current was elicited.

DISCUSSION

In this study, we found that the EC_{50} for $GABA_B$ receptor coupling to GIRK channels in dopamine neurons is an order of magnitude larger than in GABA neurons of the VTA. We provide a tenable molecular explanation for this difference and discuss the ramifications for $GABA_B$ receptor agonists on the mesolimbic dopamine system.

The measurement of the EC_{50} in our experiments reflects the outcome of several cellular events, including agonist binding, GPCR activation, G protein turnover and $G_{\beta\gamma}$ affinity of the GIRK channels. Therefore, a shift in the EC_{50} could in principle arise from a change in any one of these steps. A difference in the type of $GABA_B$ receptor or G protein seems unlikely to explain the shift in EC_{50}. The $GABA_B$ receptor is an obligatory heterodimer, formed by the co-assembly of the R1 and R2 subunits[26,27]. Consequently, animals deficient in the R1 subunit lack any behavioral and electrophysiological responses to baclofen as well as ^{35}S-GTPγS binding[28]. Receptor heterogeneity may therefore only be associated with splice variants of the R1 subunit[27]. It has been suggested that R1a may be preferentially expressed presynaptically, whereas R1b has a predilection for somatodendritic expression[29]. In our study, the EC_{50} for coupling $GABA_B$ receptors to presynaptic voltage-gated calcium channels, postsynaptic Ba-insensitive current and $GABA_B$ receptor–activated GIRK currents in GABA neurons were all surprisingly similar despite their coupling to different types of effectors and membrane localization. Moreover, heterologous expression studies have shown that $GABA_B$ receptors couple to pertussis toxin–sensitive G proteins and have similar agonist affinities and coupling efficacy to a number of effectors including GIRK channels[30,31]. Taken together, these studies suggest

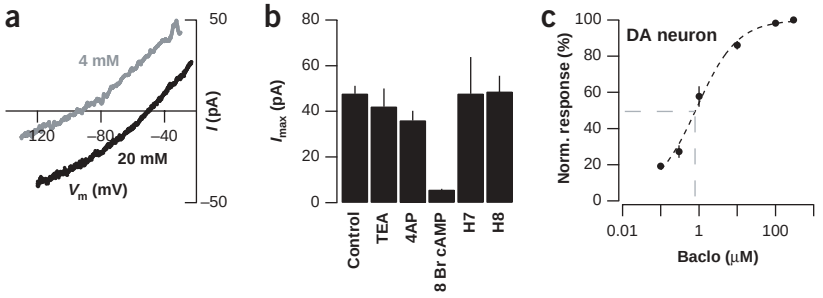

Figure 3 The Ba-resistant component is an outward rectifying potassium conductance that has a low EC_{50}. (**a**) Current-voltage relationship obtained in the presence of 300 μM Ba and 4 mM (dark trace) or 20 mM external K^+ (light trace) reveals an underlying outward rectifying potassium conductance. Zero current potentials match calculated E_K. (**b**) The barium resistant current was not sensitive to the K^+ channel blockers TEA (2 mM) and 4-AP (500 μM) or to H7 or H8 hydrochloride (20 μM) intracellularly pre-applied for 15 min but was almost completely blocked by 8Br-cAMP (10 mM). ($V_h = -53$ mV, $n = 6$). (**c**) Concentration-response curve of barium resistant current ($EC_{50} = 0.9 \pm 0.3$ μM, $h = 0.8 \pm 0.2$ μM, $n = 5$).

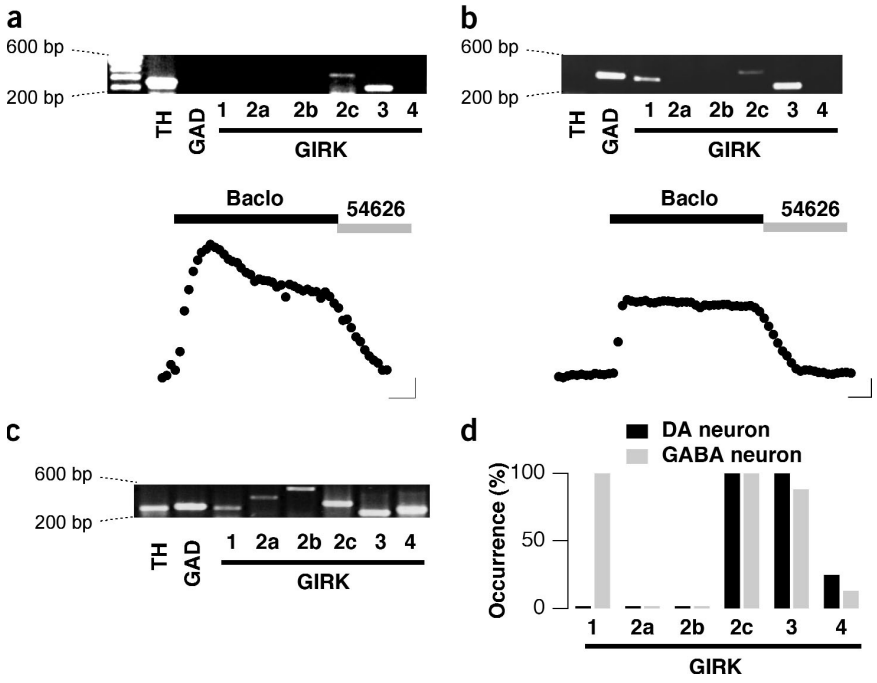

Figure 4 Low efficacy of $GABA_B$ receptors in dopaminergic neurons correlates with specific combination of GIRK channel subunits. (**a**) Example of single cell multiplexed RT-PCR in dopamine neuron; a baclofen-induced current was recorded first and then RT-PCR revealed mRNA for tyrosine hydroxylase (TH) and GIRK2c and GIRK3 (top). (**b**) In a GABA neuron, single-cell RT-PCR showed mRNA for glutamic acid decarboxylase (GAD) along with GIRK1, GIRK2c and GIRK3. Scale bar: 25 pA, 1 min. (**c**) Multiplexed RT-PCR of the whole mesencephalon as positive control. (**d**) Relative occurrence of GIRK subunits with single-cell multiplexed RT-PCR ($n = 8$ for each cell type).

the difference in EC_{50} between dopamine and GABA neurons is not due to a change in $GABA_B$ receptor or the type of G proteins.

RT-PCR experiments, however, showed that cell-specific expression of GIRK subunits in VTA neurons, with GIRK1/2c/3 expressed in GABA neurons and GIRK2c/3 expressed in dopamine neurons, correlated with the difference in EC_{50}. We reasoned that GIRK channels composed of different subunits may contribute to the shift in EC_{50}. Data from mice carrying targeted disruptions of various GIRK subunits and immunohistochemical studies suggest that GIRK1/2 heteromeric channels carry GIRK currents in most neurons[8,16,32,33]. However, exceptions to this rule are adrenergic neurons of the locus coeruleus, where GIRK currents have been observed in $GIRK2^{-/-}$ animals[34], and dopamine neurons of the substantia nigra, where RT-PCR and immunoprecipitation experiments suggest GIRK2a/2c heteromultimers exist[19]. Here, we show that dopamine neurons of the VTA also lack GIRK1, but express GIRK2c and GIRK3, which suggests that functional channels in these neurons may be composed of GIRK2c homomeric and/or GIRK2c/GIRK3 heteromeric assemblies (or a homomeric channel modulated by GIRK3)[35]. Results obtained by coexpressing GIRK2c and GIRK3 subunits with $GABA_BR1$ and R2 in HEK-293T cells argue for the latter, as they yielded a significantly higher EC_{50} than GIRK1/2c, GIRK1/3 or GIRK2c alone. Since GIRK2 is also an obligatory subunit in GABA neurons, these cells mostly express GIRK1/2 heteromultimeric channels at the surface. The subunit-dependent increase in EC_{50} observed in heterologous cells, however, was smaller than that observed in the VTA. This could be due to preferential assembly of GIRK2c homomeric channels in HEK-293T cells even when coexpressed with GIRK3. Alternatively, the cytosolic environment in dopaminergic neurons may further increase the EC_{50}, for example by differential expression of RGS proteins[36] and PIP2 levels[37]. However, it has been shown that GIRK2/3 channels are less sensitive than GIRK1/3 channels to the G protein $G_{\beta\gamma}$ dimer[20], which directly activates GIRK channels[38,39], supporting the conclusion that the increase in EC_{50} involves the GIRK2c/3 heteromer. If found in other neurons, our data suggest a general mechanism for altering the coupling efficiency by regulating the expression of specific GIRK subunits.

What is the physiological significance of a higher EC_{50} for coupling to GIRK channels? The differential coupling efficiency of $GABA_B$ receptors and GIRKs along with the intrinsic circuitry of the VTA[40] provide a cellular mechanism for establishing bi-directional modulation of the mesolimbic dopamine system (**Supplementary Fig. 2** online). Accordingly, GABA neurons, because of their high coupling efficacy (low EC_{50}) would be targeted preferentially at low agonist doses. Thus, activation of GIRK channels in GABA neurons would suppress the spontaneous activity of these interneurons and lead to disinhibition of the dopamine neurons. At higher doses of baclofen, GIRK channels in dopamine neurons would be directly activated, leading to a hyperpolarization of these projection neurons and therefore less dopamine output. This bi-directional control of dopamine output was monitored directly in VTA slices (**Fig. 6**).

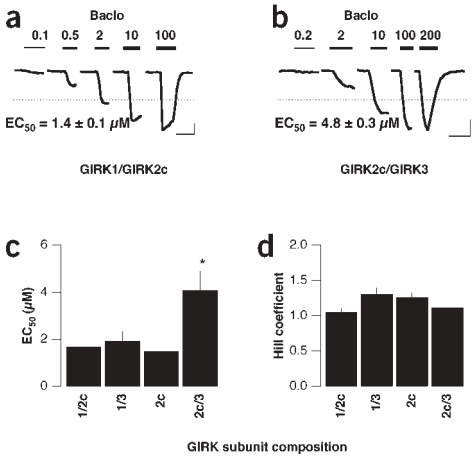

Figure 5 Coupling efficiency of $GABA_B$ receptors to different combinations of GIRK subunits expressed heterologously in HEK-293T cells. HEK-293 cells were transfected with cDNA for $GABA_BR1$ and $GABA_BR2$ along with the indicated GIRK subunits. (**a**,**b**) Whole-cell patch-clamp recording from HEK-293T cell expressing GIRK1/2c or GIRK2c/3. Broken line indicates 50% activation. EC_{50} is indicated for each cell. Holding potential was –80 mV. Scale bars: 10 s/0.5 nA (**a**) and 10 s/0.2 nA (**b**). (**c**,**d**) The average EC_{50} (**c**) and Hill coefficient (**d**) were measured for baclofen activation. Only coexpression of GIRK2c and GIRK3 led to a significantly higher value (*$P < 0.05$ using one-way ANOVA followed by *post hoc* Fisher LSD test, $n = 8$–18).

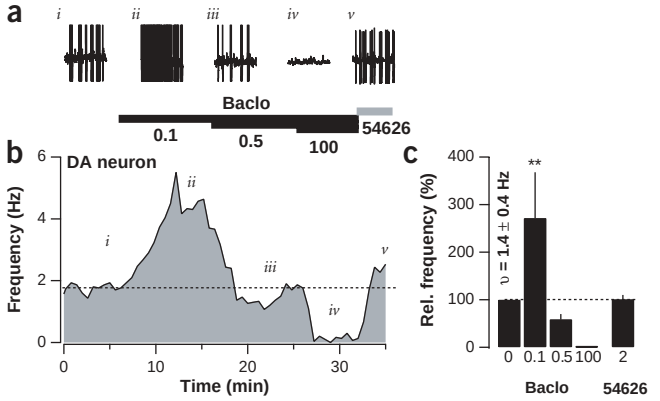

Figure 6 Bi-directional effects of $GABA_B$ receptor agonists on the firing rate of dopamine neurons in the VTA. (**a**,**b**) Single-spike activity (**a**) recorded in cell-attached configuration (10 s duration) from a dopamine neuron at corresponding time points of **b** showing the spiking frequency as a function of different baclofen doses (in μM). Low concentrations led to a substantial increase of the firing frequency, whereas high doses eventually suppressed all action potentials. Effects were reversed by CGP 54626 (2 μM). (**c**) Average change in firing frequency (υ), $n = 4$. In all experiments excitatory inputs were blocked with kynurenic acid (2 mM).

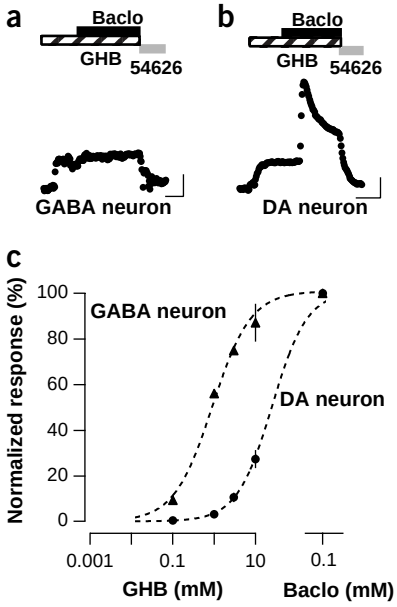

Figure 7 GHB-evoked currents in VTA neurons. (**a**,**b**) Representative responses to GHB (10 mM) followed by baclofen (100 μM) in a GABAergic neuron (**a**) and a dopaminergic (DA) neuron (**b**). Scale bars: 50 pA, 5 min (**a**); 100 pA, 5 min (**b**). Note that in DA neurons, 10 mM GHB is not saturating. (**c**) Concentration-response curve normalized to the response of a saturating dose of baclofen (100 μM) in dopamine neurons (circle; estimated $EC_{50} = 26.8 \pm 1.2$ mM, $h = 0.9 \pm 0.1$, $n = 4$) and GABA neurons (triangle; $EC_{50} = 0.9 \pm 0.1$ mM, $h = 1 \pm 0.1$, $n = 3$).

The involvement of excitatory afferents can be excluded, as experiments were conducted in the presence of kynurenic acid blocking all excitatory glutamtergic transmission. In addition, distant inhibitory afferents from the pallidum and nucleus accumbens were cut by the slicing procedure and therefore did not interfere. Moreover, afferents from pallidum and accumbens preferentially activate $GABA_B$ receptors, while local interneurons give rise to $GABA_A$ receptor–mediated responses[41]. Consistent with this segregation, we observed disinhibition with picrotoxin but not with CGP 54626 (**Fig. 6**). In summary, our model predicts that the intrinsic circuitry of the VTA is sufficient such that for a given agonist, with increasing concentrations, the mesolimbic system would first become activated, followed by a subsequent inhibition. Such bi-directional modulation could explain the fact that, in addition to the suppression of self-administration[2], euphoria is a well-established side effect of baclofen[42]. Interestingly, GABA neurons are also more sensitive to $GABA_A$ receptor agonists than dopamine neurons (for review, see ref. 43). This may explain why modulators of $GABA_A$ receptors such as benzodiazepines have rewarding effects. The integration of 'natural rewards' in the VTA may depend on GABA afferents acting synergistically through ionotropic as well as metabotropic GABA receptor systems.

The differential coupling of $GABA_B$ receptors to GIRK channels may also offer an explanation for the difference in abuse liability of GHB and baclofen. Baclofen, due to its high affinity for the $GABA_B$ receptor, would at typical therapeutic levels inhibit both GABAergic and dopaminergic neurons and decrease dopamine release in the target structures, which is confirmed by *in vivo* studies[44]. Conversely, GHB, through its low-affinity binding site for the $GABA_B$ receptor (~100 μM), would preferentially inhibit interneurons at concentrations typically seen in recreational use (< 1 mM)[5]. At higher concentrations, however, GHB could also inhibit dopamine release, which may explain the long-standing controversy over whether GHB inhibits or stimulates the dopamine system[45] and reconcile the paradoxical finding that GHB, although a drug of abuse, in some conditions also has anti-craving effects[46]. Based on the observation of a high-affinity binding site[47] and recent cloning data[48], it has been proposed that some GHB actions are mediated by a specific GHB receptor. This possibility seems unlikely for the acute effects on the reward system, as $GABA_B$ receptor antagonists fully block self-administration of GHB in rats[25]. Moreover, behavioral changes as well as hypothermia induced by GHB are absent in $GABA_BR1^{-/-}$ animals[49,50], suggesting that the $GABA_B$ receptor mediates most, if not all, of the pharmacological effects of GHB.

In conclusion, our findings, extended to other $GABA_B$ receptor agonists, suggest that low-affinity compounds are more likely to cause activation of the mesolimbic dopamine system than high-affinity agonists, which at typical concentrations will inhibit the system. For developing improved anti-craving drugs, future drug development should therefore focus on creating compounds with a high affinity for the $GABA_B$ receptor.

METHODS

Electrophysiology in acute slices. Care of experimental animals was in accordance with institutional guidelines and carried out with the permission of the Cantonal Veterinary Office of Geneva, Switzerland. Horizontal slices (300 μm thick, VT1000 vibratome, Leica) of the midbrain were prepared from P10–P21 Sprague-Dawley rats and, where stated, C57BL6 mice in cooled artificial cerebrospinal fluid (ACSF) containing NaCl (119 mM), KCl (2.5 mM), $MgCl_2$ (1.3 mM), $CaCl_2$ (2.5 mM), NaH_2PO_4 (1.0 mM), $NaHCO_3$ (26.2 mM) and glucose (11 mM) and continuously bubbled with 95% O_2 and 5% CO_2. Slices were progressively warmed up to 32–34 °C, and transferred after 1 h to the recording chamber superfused (2 ml/min) with ACSF. Visualized whole-cell voltage-clamp recording techniques were used to measure holding currents and synaptic responses of neurons of the VTA. The VTA was identified as the region medial to the medial terminal nucleus of the accessory optical tract. Dopamine cells were identified by a large I_h current, whereas GABA neurons showed no I_h but did show an outward current in response to the μ-opioid-selective agonist DAMGO (1 μM). Whether a short pulse (<2 min) of DAMGO was applied in

the beginning or at the end of the experiment did not have any influence on the amplitude of baclofen (100 μM) evoked currents (I_{max} 90 ± 20 pA versus 100 ± 15 pA). Kynurenic acid (2 mM) was applied while monitoring action potentials and to isolate stable inhibitory synaptic currents, which were obtained by stimuli (0.1 ms duration) delivered at 0.1 Hz through bipolar stainless steel electrodes positioned just rostral to the VTA. The internal solution contained potassium gluconate (140 mM), NaCl (4 mM), $MgCl_2$ (2 mM), EGTA (1.1 mM), HEPES (5 mM), Na_2ATP (2 mM), sodium creatine phosphate (5 mM) and Na_3GTP (0.6 mM); pH adjusted to 7.3 with KOH. In some experiments, 8Br-cAMP (10 mM) was added to the internal solution. Currents were amplified (Visual patch 500, Bio-logic), filtered at 1 kHz and digitized at 5 kHz (National Instruments Board PCI-MIO-16E4, NI-DAQ Igor Software, WaveMetrics) and stored on a hard disk. Cells were clamped at either –53 or –63 mV. With each sweep, a 10 mV hyperpolarizing step (200 ms duration) was imposed to measure cell membrane resistance and approximate access resistance. Voltage errors due to liquid junction potential (–13 mV) were corrected. For the experiments in **Figure 6**, dopamine neurons were selected by discarding cells that have become disconnected by the slicing procedure, which was revealed by the lack of an increase the firing frequency when the $GABA_A$ receptor blocker picrotoxin (100 μM) was applied.

Compiled data are expressed as means ± s.e.m. For statistical comparisons, the nonparametric Mann-Whitney or Wilcoxon matched tests were used and the level of significance was taken at $P = 0.05$.

Baclofen, CGP 54626, H7, H8 and picrotoxin were from Tocris; 8Br-cAMP, TEA, 4-AP and kynurenic acid from Sigma/RBI and GHB from Unavera.

Single-cell RT-PCR. Recordings were preformed as above. After 20 min, the cell contents (including a nucleus in most cases) were aspirated as completely as possible into patch pipette under visual control. Pipettes were then quickly removed from the cell, washed through the solution interface, and the pipette contents were immediately expelled into 0.2 ml tubes containing 20 U RNAse inhibitor and 20 U DNAase for digestion of genomic DNA. After 30 min at 37 °C, DNAase was inactivated by incubation at 75 °C for 5 min. Strand cDNA was then synthesized for 1 h at 37 °C in a total reaction volume of 15 μl containing random hexamer primers (Roche; 5 μM), the four deoxyribonucleotide triphosphates (Invitrogen; 2.5 mM of each), 20 U of ribonuclease inhibitors (Promega) and 200 U of reverse transcriptase (Superscript II, Invitrogen).

After reverse transcription, the cDNAs for GIRK1, GIRK2a/b/c, GIRK3, GIRK4, TH and GAD 67 were simultaneously amplified in a multiplex PCR using the following set of primers (from 5′ to 3′): TH, sense TTCTCAACCTGCTCTTCTCCC (position 612), antisense ACCTTGTCCTCTCTGGCACTG (position 838); GAD67 sense AGCCTGGAAGAGAAGAGTCGT (position 357), antisense CAACTGGTGTGGGTGGTGGAA (position 602); GIRK1 sense, TTTCCGACCTCTTCACTACCC (position 389) antisense TCGGTGATGTAGCGGTAGCCA (position 624); GIRK2 sense for every splice variant, GGAACTGGAGATTGTGGTCAT (position 1117); GIRK2a antisense, CAAGCAGTTAGGGAGGAAAAT (position 1533); GIRK2b antisense TTCCCTTTTGGCTTGATAACA (position 1725), GIRK2a,c antisense CATCACCATTCCTCTCTGTCA (position 1437); GIRK3 sense CGAGACCTACCG CTACCTGAC (position 368), antisense GAAGCCGTTGAGGTTGTTGAC (position 552); GIRK4, sense CCAAACAGGCTCGGGATTACA (position 258), antisense ATGAGCCACCAGATGAAGCCA (position 493). Multiplex PCR was performed as hot start in a final volume of 50 μl containing the 15 μl reverse transcriptase, 20 pM of each primer, 2.5 mM of each dNTP (Invitrogen), 1.5 mM $MgCl_2$, 50 mM KCl, 20 mM Tris-HCl, pH 8.4 and 1 U hot start polymerase (Stratagen) in a T3 Thermocycler (Biometra) with the following cycling protocol: after 15 min at 94 °C, 10 cycles (94 °C, 1 min; 60 °C 1 min; 72 °C 3 min) of PCR were followed by a final elongation period of 10 min at 72 °C. The second PCR reaction was done in individual reactions for every pair of primers, in each case with 3 μl of the multiplex PCR reaction product under similar conditions for 35 cycles. To investigate the presence and size of the amplified fragments, 10 μl aliquots of PCR products were separated and visualized in ethidium bromide–stained agarose gels (1.5%) by electrophoresis. Predicted sizes (in base pairs, bp) of PCR fragments were 226 bp (TH), 255 bp (GAD67), 235 bp (GIRK1), 416 bp (GIRK2a), 608 bp (GIRK2b), 320 bp (GIRK2a,c), 184 bp (GIRK3) and 235 bp (GIRK4). Because primers annealing in the specific C-terminal tail of GIRK2c were unreliable in single-cell conditions, we determined the presence of GIRK2c indirectly by the absence of the specific GIRK2a product in the presence of a band common to GIRK2a and 2c. The PCR products were verified several times ($n > 3$) by direct sequencing.

Transfection and electrophysiology in HEK-293T cells. GIRKs and $GABA_B$ receptor cDNAs were subcloned in pcDNA3. GIRK2c was constructed by adding the 11 amino acid C-terminal sequence of GIRK2c to that of GIRK2a using PCR[21]. HEK-293T cells were cultured in DMEM supplemented with fetal bovine serum (10%), glutamine (2 mM), penicillin (50 units/ml) and streptomycin (50 μg/ml; Gibco) in a humidified 37 °C. incubator with 95% air/5% CO_2. HEK cells were plated onto 12 mm glass cover slips (Warner Instruments) coated with poly-D-lysine (20 μg/ml; Sigma) and collagen (100 μg/ml; BD Biosciences) in 24-well plates. HEK-293T cells were transiently transfected with cDNA using the calcium phosphate method. Briefly, cDNA (0.08 μg/ml) was mixed in sterile de-ionized water with 0.25 M $CaCl_2$, then combined 1:1 with HEPES buffered saline (280 mM NaCl, 10 mM KCl, 1.5 mM Na_2HPO_4, 12 mM glucose, 50 mM HEPES (pH 6.9 with ~1 N NaOH)) and added (50 μl) to each well and incubated for 16–32 h. Whole-cell patch-clamp currents were recorded with an Axopatch 200B (Axon Instruments) amplifier, adjusted electronically for cell capacitance and series resistance (80–100%), filtered at 2 kHz with an eight-pole Bessel filter, digitized at 5 kHz with a Digidata 1320 interface (Axon Instruments) and stored on a laboratory computer. Intracellular pipet solution contained 130 mM KCl, 20 mM NaCl, 5 mM EGTA, 2.56 mM K_2ATP, 5.46 mM $MgCl_2$ and 10 mM HEPES (pH 7.2 with ~14 mM KOH). 300 μM Li_3-GTP (RBI) was added fresh to the intracellular pipet solution to sustain activation of GIRK channels. The external bath solution contained 140 mM NaCl, 20 mM KCl, 0.5 mM $CaCl_2$, 2 mM $MgCl_2$ and 10 mM HEPES (pH 7.2). The osmolarity was 310–330 mosm. A baclofen dose-response curve for each cell was fit with the Hill equation ($y = 1/(1 + ([EC_{50}]/[x])^h)$, where EC_{50} is the half-maximal concentration and h is the Hill coefficient. The EC_{50}s were calculated by normalization to a response to saturating doses, to account for possible differences in the efficiency of expression of GIRK channels composed of different GIRK subunits in heterologous systems[35].

GenBank accession numbers. TH, L22651; GAD67, M76177; GIRK1, U09243; GIRK2a, AB073754; GIRK2b, AB073756; GIRK2c, AB073753; GIRK3, L77929; GIRK4, L35771.

Note: Supplementary information is available on the Nature Neuroscience website.

ACKNOWLEDGMENTS

We thank the members of the Lüscher lab for many helpful discussions, L. Jan for the $GABA_BR1$ and $GABA_BR2$ cDNA, D. Clapham for GIRK3 cDNA, M. Lazdunski for GIRK2a cDNA, and A. Gittis for some of the dose-response data. We also thank C. Fiorillo, M. Frerking, and M. Mühlethaler for comments on an earlier version of the manuscript, and F. Loctin for technical assistance. M.L is supported, in part, by the Danish Natural Science Research Council. C.L. is supported by grants of the Swiss National Science Foundation. C.L. and P.A.S. share a Human Science Frontier Project Young Investigator's grant.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Received 1 December; accepted 24 December 2003
Published online at http://www.nature.com/natureneuroscience/

1. Robbins, T.W. & Everitt, B.J. Drug addiction: bad habits add up. *Nature* **398**, 567–570 (1999).
2. Brebner, K., Childress, A.R. & Roberts, D.C. A potential role for $GABA_B$ agonists in the treatment of psychostimulant addiction. *Alcohol Alcohol* **37**, 478–484 (2002).
3. Cousins, M.S., Roberts, D.C. & de Wit, H. $GABA_B$ receptor agonists for the treatment of drug addiction: a review of recent findings. *Drug Alcohol Depend.* **65**, 209–220 (2002).
4. Martellotta, M.C., Cossu, G., Fattore, L., Gessa, G.L. & Fratta, W. Intravenous self-administration of gamma-hydroxybutyric acid in drug-naive mice. *Eur. Neuropsychopharmacol.* **8**, 293–296 (1998).
5. Nicholson, K.L. & Balster, R.L. GHB: a new and novel drug of abuse. *Drug Alcohol Depend.* **63**, 1–22 (2001).
6. Johnson, S.W. & North, R.A. Opioids excite dopamine neurons by hyperpolarization of local interneurons. *J. Neurosci.* **12**, 483–488 (1992).

7. McFarland, K. & Kalivas, P.W. The circuitry mediating cocaine-induced reinstatement of drug-seeking behavior. *J. Neurosci.* **21**, 8655–8663 (2001).
8. Lüscher, C., Jan, L.Y., Stoffel, M., Malenka, R.C. & Nicoll, R.A. G protein-coupled inwardly rectifying K^+ channels (GIRKs) mediate postsynaptic but not presynaptic transmitter actions in hippocampal neurons. *Neuron* **19**, 687–695 (1997).
9. Patel, A.J. & Honore, E. Properties and modulation of mammalian 2P domain K^+ channels. *Trends Neurosci.* **24**, 339–346 (2001).
10. Krapivinsky, G. *et al.* The G-protein-gated atrial K^+ channel IKACh is a heteromultimer of two inwardly rectifying K^+-channel proteins. *Nature* **374**, 135–141 (1995).
11. Lesage, F. *et al.* Cloning provides evidence for a family of inward rectifier and G-protein coupled K^+ channels in the brain. *FEBS Lett.* **353**, 37–42 (1994).
12. Dascal, N. *et al.* Atrial G protein-activated K^+ channel: expression cloning and molecular properties. *Proc. Natl. Acad. Sci. USA* **90**, 10235–10239 (1993).
13. Kubo, Y., Reuveny, E., Slesinger, P.A., Jan, Y.N. & Jan, L.Y. Primary structure and functional expression of a rat G-protein-coupled muscarinic potassium channel. *Nature* **364**, 802–806 (1993).
14. Wickman, K., Pu, W.T. & Clapham, D.E. Structural characterization of the mouse Girk genes. *Gene* **284**, 241–250 (2002).
15. Liao, Y.J., Jan, Y.N. & Jan, L.Y. Heteromultimerization of G-protein-gated inwardly rectifying K^+ channel proteins GIRK1 and GIRK2 and their altered expression in weaver brain. *J. Neurosci.* **16**, 7137–7150 (1996).
16. Jelacic, T.M., Sims, S.M. & Clapham, D.E. Functional expression and characterization of G-protein-gated inwardly rectifying K^+ channels containing GIRK3. *J. Membr. Biol.* **169**, 123–129 (1999).
17. Schoots, O. *et al.* Co-expression of human Kir3 subunits can yield channels with different functional properties. *Cell Signal* **11**, 871–883 (1999).
18. Slesinger, P.A. *et al.* Functional effects of the mouse weaver mutation on G protein-gated inwardly rectifying K^+ channels. *Neuron* **16**, 321–331 (1996).
19. Inanobe, A. *et al.* Characterization of G-protein-gated K^+ channels composed of Kir3.2 subunits in dopaminergic neurons of the substantia nigra. *J. Neurosci.* **19**, 1006–1017 (1999).
20. Jelacic, T.M., Kennedy, M.E., Wickman, K. & Clapham, D.E. Functional and biochemical evidence for G-protein-gated inwardly rectifying K^+ (GIRK) channels composed of GIRK2 and GIRK3. *J. Biol. Chem.* **275**, 36211–36216 (2000).
21. Lesage, F. *et al.* Molecular properties of neuronal G-protein-activated inwardly rectifying K^+ channels. *J. Biol. Chem.* **270**, 28660–28667 (1995).
22. Wei, J. *et al.* Characterization of murine Girk2 transcript isoforms: structure and differential expression. *Genomics* **51**, 379–390 (1998).
23. Lingenhoehl, K. *et al.* Gamma-hydroxybutyrate is a weak agonist at recombinant $GABA_B$ receptors. *Neuropharmacol.* **38**, 1667–1673 (1999).
24. Madden, T.E. & Johnson, S.W. Gamma-hydroxybutyrate is a $GABA_B$ receptor agonist that increases a potassium conductance in rat ventral tegmental dopamine neurons. *J. Pharm. Exp. Ther.* **287**, 261–265 (1998).
25. Carter, L.P. *et al.* The role of $GABA_B$ receptors in the discriminative stimulus effects of gamma-hydroxybutyrate in rats: time course and antagonism studies. *J. Pharm. Exp. Ther.* **305**, 668–674 (2003).
26. White, J.H. *et al.* Heterodimerization is required for the formation of a functional $GABA_B$ receptor. *Nature* **396**, 679–682 (1998).
27. Kaupmann, K. *et al.* $GABA_B$-receptor subtypes assemble into functional heteromeric complexes. *Nature* **396**, 683–687 (1998).
28. Schuler, V. *et al.* Epilepsy, hyperalgesia, impaired memory, and loss of pre- and postsynaptic $GABA_B$ responses in mice lacking $GABA_B1$. *Neuron* **31**, 47–58 (2001).
29. Billinton, A., Upton, N. & Bowery, N.G. $GABA_B$ receptor isoforms GBR1a and GBR1b, appear to be associated with pre- and post-synaptic elements respectively in rat and human cerebellum. *Br. J. Pharmacol.* **126**, 1387–1392 (1999).
30. Brauner-Osborne, H. & Krogsgaard-Larsen, P. Functional pharmacology of cloned heterodimeric $GABA_B$ receptors expressed in mammalian cells. *Br. J. Pharmacol.* **128**, 1370–1374 (1999).
31. Pfaff, T. *et al.* Alternative splicing generates a novel isoform of the rat metabotropic $GABA_BR1$ receptor. *Eur. J. Neurosci.* **11**, 2874–2882 (1999).
32. Karschin, C., Dissmann, E., Stuhmer, W. & Karschin, A. IRK(1–3) and GIRK(1–4) inwardly rectifying K^+ channel mRNAs are differentially expressed in the adult rat brain. *J. Neurosci.* **16**, 3559–3570 (1996).
33. Wickman, K., Karschin, C., Karschin, A., Picciotto, M.R. & Clapham, D.E. Brain localization and behavioral impact of the G-protein-gated K^+ channel subunit GIRK4. *J. Neurosci.* **20**, 5608–5615 (2000).
34. Torrecilla, M. *et al.* G-protein-gated potassium channels containing Kir3.2 and Kir3.3 subunits mediate the acute inhibitory effects of opioids on locus ceruleus neurons. *J. Neurosci.* **22**, 4328–4334 (2002).
35. Ma, D. *et al.* Diverse trafficking patterns due to multiple traffic motifs in G protein-activated inwardly rectifying potassium channels from brain and heart. *Neuron* **33**, 715–729 (2002).
36. Jeong, S.W. & Ikeda, S.R. Endogenous regulator of G-protein signaling proteins modify N-type calcium channel modulation in rat sympathetic neurons. *J. Neurosci.* **20**, 4489–4496 (2000).
37. Huang, C.L., Feng, S. & Hilgemann, D.W. Direct activation of inward rectifier potassium channels by PIP2 and its stabilization by Gbetagamma. *Nature* **391**, 803–806 (1998).
38. Logothetis, D.E., Kurachi, Y., Galper, J., Neer, E.J. & Clapham, D.E. The beta gamma subunits of GTP-binding proteins activate the muscarinic K^+ channel in heart. *Nature* **325**, 321–326 (1987).
39. Reuveny, E. *et al.* Activation of the cloned muscarinic potassium channel by G protein beta gamma subunits. *Nature* **370**, 143–146 (1994).
40. Johnson, S.W. & North, R.A. Two types of neurone in the rat ventral tegmental area and their synaptic inputs. *J. Physiol. (Lond.)* **450**, 455–468 (1992).
41. Sugita, S., Johnson, S.W. & North, R.A. Synaptic inputs to $GABA_A$ and $GABA_B$ receptors originate from discrete afferent neurons. *Neurosci. Lett.* **134**, 207–211 (1992).
42. *Matindale – The Complete Drug Reference* p. 2496 (The Pharmaceutical Press, 2002).
43. Kalivas, P.W. Neurotransmitter regulation of dopamine neurons in the ventral tegmental area. *Brain Res. Brain Res. Rev.* **18**, 75–113 (1993).
44. Kalivas, P.W., Duffy, P. & Eberhardt, H. Modulation of A10 dopamine neurons by gamma-aminobutyric acid agonists. *J. Pharm. Exp. Ther.* **253**, 858–866 (1990).
45. Feigenbaum, J.J. & Howard, S.G. Does gamma-hydroxybutyrate inhibit or stimulate central DA release? *Int. J. Neurosci.* **88**, 53–69 (1996).
46. Gallimberti, L. *et al.* Gamma-hydroxybutyric acid for treatment of alcohol withdrawal syndrome. *Lancet* **2**, 787–789 (1989).
47. Snead, O.C. III. Evidence for a G protein-coupled gamma-hydroxybutyric acid receptor. *J. Neurochem.* **75**, 1986–1996 (2000).
48. Andriamampandry, C. *et al.* Cloning and characterization of a rat brain receptor that binds the endogenous neuromodulator gamma-hydroxybutyrate (GHB). *FASEB J.* **17**, 1691–1693 (2003).
49. Quéva, C. *et al.* Effects of GABA agonists on body temperature regulation in $GABA_B(1)^{-/-}$ mice. *Brit. J. Pharmacol.* **140**, 315–322 (2003).
50. Kaupmann, K. *et al.* Specific γ-hydroxybutyrate (GHB) binding sites but loss of pharmacological effects of GHB in $GABA_B(1)$ deficient mice. *Eur. J. Neurosci.* **18**, 2722–2730 (2003).