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

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

Activation of the mesolimbic dopamine (DA) system, which originates in the ventral tegmental area (VTA), mediates rewarding effects of drugs of abuse[1]. Interestingly, different GABA$_B$R agonists are reported to have opposing effects on this system. While 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 to increase DA release from the VTA varies with individual drugs 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 DA-neurons. Beyond mediating rewarding effects, activation of the mesolimbic DA system is also thought to play a crucial role for the induction of repetitive drug use and may even trigger relapse[7]. Putative anti-craving compounds, including baclofen, may therefore be effective by modulating the mesolimbic system such that they interfere with DA release normally caused by drugs of abuse. The cellular mechanisms of this interference however remain elusive. The fact that GABA$_B$Rs are expressed on both DA 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$_B$Rs in the mesolimbic DA system. To resolve these apparent contradictions we undertook a systematic study of the effects of GABA$_B$ receptor activation in the VTA.

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 and b, lower traces) and a concomitant decrease of the membrane resistance (upper traces) in both DA- and GABA-neurons, indicating the opening of ionic conductances. In DA-neurons, however, currents were significantly larger and desensitized substantially during 15 minutes of continuous agonist application (Fig. 1c). Remarkably, the EC_{50} for baclofen activation of outward current in GABA-neurons was an order of magnitude smaller than in DA-neurons (Fig. 1d). For comparison, baclofen also inhibited evoked inhibitory postsynaptic currents (ipscs, measured in DA neurons) with an EC_{50} close to the one found in GABA-neurons for the postsynaptic inhibition (Fig 1e and f) as well as inhibition of ipsc of afferents onto GABA neurons (EC50=1.5±0.1, n=5, 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 EC50) was surprisingly low in DA-neurons.

Molecular identity of baclofen-evoked currents

In order to reveal the molecular identity of the baclofen-evoked current we took advantage of 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 was confirmed by the observation that in mice containing a null mutation in GIRK2 (*GIRK2* -/-) the baclofen-activated currents were dramatically reduced, revealing small, Ba-insensitive currents in both DA and GABA

neurons (Fig. 2d,e). 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 DA neurons, we applied brief voltage ramps ranging from –30mV to –130mV in extracellular solution of different K concentration. The i-v relationship showed a weak, outwardly rectifying current that reversed near the predicted K equilibrium potential, consistent with a K current (Fig. 3a). This current was insensitive to the two other K channel blockers TEA and 4AP but not the PKA inhibitors H7 or H8, (Fig. 3b). The Ba-insensitive current was inhibited by the cAMP analogue 8Br-cAMP, suggesting that the underlying channel may be a member of the two-pore K channel family [8]. Importantly, the EC_{50} of this current was 0.9±0.3μM (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$_B$R 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$_B$R to GIRK channels may vary in different neurons. We therefore hypothesized that DA-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[9-12], which form functional hetero- or homomeric channels by the assembly of four subunits[13]. GIRK channels in the brain are typically composed of GIRK1 and either GIRK2 or GIRK3[14]. GIRK2 also forms homomeric channels[15,16] (but see[17]) or co-assembles with GIRK3[18]. We next investigated whether DA 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[19,20], we found that GABA-neurons expressed mRNA for GIRK1, 2c and 3, whereas DA neurons contained mRNA for only GIRK2c and 3 (Fig. 4a-c). 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 hypothesized 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$_B$Rs 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 (Fig. 5). The change in EC_{50}, however, was not as large as that observed between DA and GABA neurons (see Discussion).

Difference in EC50 imparts bi-directional effects of GABA$_B$R agonists

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 DA-neurons. Given the 10-fold difference in coupling efficiency of the GABA$_B$Rs 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 DA-neurons. Conversely, DA-neurons would be directly inhibited via GABA$_B$Rs with high agonist concentrations. To test this, we selected for DA-neurons with preserved synaptic connectivity to GABA neurons (see methods) and monitored action potentials in the 'cell-attached' patch technique while

stepwisely increasing agonist concentrations. Importantly, concentrations of baclofen below 1μM did not induce desensitization of GIRK currents (Suppl. Fig 1). Low doses of baclofen (0.1μM) caused a threefold increase of the firing frequency (Fig. 6a-c). At a moderate dose (0.5μM) of baclofen, the frequency returned to basal firing rates whereas action potentials were completely abolished at a high concentration (100μM) of balcofen. These effects were fully reversible with the GABA$_B$ receptor antagonist GCP 54 626. Thus, depending on the concentration of agonist, GABA$_B$ agonists can either enhance or suppress the DA 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$_B$R 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 appeared to fully activate GIRK channels (Fig. 7A). By contrast, GHB (10 mM) activated only 30% of the baclofen-induced current (Fig. 7b), suggesting that GHB had the same effects on GIRK channels as a more canonical GABA$_B$R agonist, but with a much lower affinity (Fig. 7a,b). These effects of GHB were completely blocked with GCP54 626, 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 full, but low-affinity agonist properties at the GABA$_B$R (concentrations of >100 mM GHB would be required to determine the maximal stimulation with GHB). Consistent with this conclusion, previous observations with reconstituted GABA$_B$ receptors[21], native receptors in midbrain slices[22] and in vivo brain recordings[23] have suggested that GHB is a full, low affinity agonist. Importantly, the difference of the EC50 between GABA-neurons and DA-neurons was preserved for

GHB (Fig. 7c). Taken together, GHB showed a similar pattern of GABA$_B$R activation in VTA neurons as baclofen, albeit at higher concentrations due to its low receptor affinity.

DISCUSSION

In this study, we discovered that EC$_{50}$ for GABA$_B$R coupling to GIRK channels in DA neurons is an order of magnitude larger than in GABA neurons of the VTA. We provide a molecular explanation for this difference and discuss the ramifications for GABA$_B$R agonists on the mesolimbic DA system.

The measurement of the EC$_{50}$ in our experiments reflects 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$_B$R or G protein seems unlikely to explain the shift in EC$_{50}$. The GABA$_B$R is an obligatory heterodimer, formed by the co-assembly of the R1 and R2 subunits[24,25]. Consequently, animals deficient for the R1 subunit lack any behavioral and electrophysiological responses to baclofen as well as ^{35}S-GTPγS binding. Receptor heterogeneity may therefore only be associated with splice variants of the R1 subunit[25]. It has been suggested that R1a may be preferentially expressed presynaptically whereas R1b has a predilection for somatodendritic expression[26]. In our study, the EC$_{50}$'s for coupling GABA$_B$Rs to presynaptic voltage-gated Ca channels, postsynaptic Ba-insensitive current and GABA$_B$-R 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$_B$Rs couple to pertussis toxin sensitive G proteins and have similar agonist affinities and coupling efficacy to a number

of effectors including GIRK channels[27,28]. Taken together, these studies suggest the difference in EC_{50} between DA and GABA neurons is not due to a change in GABA$_B$R or the type of G proteins.

RT-PCR experiments, however, demonstrated that cell-specific expression of GIRK subunits in VTA neurons, with GIRK1/2c/3 expressed in GABA and GIRK2c/3 expressed in DA neurons, correlated with the difference in EC50. We reasoned that GIRK channels composed of different subunits may contribute to the shift in EC50. 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[14,29-31]. However exceptions to this rule are adrenergic neurons of the locus coeruleus where GIRK currents have been observed in GIRK2-/- animals[32] and DA neurons of the substantia nigra, where RT-PCR and immunoprecipitation experiments suggest GIRK2a/2c heteromultimers exist[17]. Here, we show that DA neurons of the VTA also lack GIRK1, but express GIRK2c and GIRK3, which suggest that functional channels in these neurons may be composed of GIRK2c homomeric and/or GIRK2c/GIRK3 heteromeric assemblies. Results obtained by co-expressing GIRK2c and GIRK3 subunits with GABA$_B$R1 and R2 in HEK-293T cells argue for the latter as they yielded a significantly higher EC50 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 co-expressed with GIRK3. Alternatively, the cytosolic environment in DA-neurons may further increase the EC_{50}, for example by differential expression of RGS proteins[33] and PIP2 levels[34]. However, it has been shown that GIRK2/3 channels are less sensitive than GIRK1/3 channels to the G protein $G_{B\gamma}$ dimer[18],

which directly activates GIRK channels[35,36], 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 of 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$_B$ R and GIRKs along with the intrinsic circuitry of the VTA[37] provides a cellular mechanism for establishing bi-directional modulation of the mesolimbic DA system (Figure 8). Accordingly, GABA neurons, due to 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 DA neurons. At higher doses of baclofen, GIRK channels in DA neurons are now directly activated, which leads to a hyperpolarization of these projection neurons and therefore a decrease DA output. This bi-directional control of DA output was monitored directly in VTA slices (Figure 6). 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$_B$R, while local interneurons give rise to GABA$_A$R mediated responses[38]. Consistent with this segregation, we observed disinhibition with picrotoxin but not with GCP 54 626 (Fig 6). In summary, our model predicts that the instrinsic circuitry of the VTA is sufficient 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[39]. Interestingly GABA neurons are also more sensitive to GABA$_A$R agonists than DA neurons (reviewed in 40). This may explain why modulators of GABA$_A$R such as benzodiazepines have rewarding effects and suggest that 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$_B$ R 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$_B$R would at typical therapeutic levels inhibit both GABA- and DA-neurons and decrease DA release in the target structures, which is confirmed by *in vivo* studies[41]. Conversely, GHB, through its low affinity binding-site for the GABA$_B$ receptor (~100μM), would preferentially inhibit interneurons at concentrations associated with recreational use (<1mM)[5]. At higher concentrations, however, GHB could also inhibit DA release, which may explain the long-standing controversy on whether GHB inhibits or stimulates the DA system[42] and reconcile the paradoxical finding that GHB, although a drug of abuse, in some conditions also has anti-craving effects[43]. Based on the observation of a high affinity binding site [44] and recent cloning data[45] 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 since GABA$_B$R antagonists fully block self-administration of GHB in rats[23]. Moreover, behavioral changes as well as hypothermia induced by GHB are absent in GABA$_B$R1 -/- animals[46,47], suggesting that the GABA$_B$R mediates most, if not all of the pharmacological effects of GHB.

In conclusion, our findings, extended to other GABA$_B$R 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$_B$R.

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 ACSF containing (in mM) NaCl 119, KCl 2.5, $MgCl_2$ 1.3, $CaCl_2$ 2.5, NaH_2PO_4 1.0, $NaHCO_3$ 26.2 and glucose 11 and continuously bubbled with 95% O_2 and 5% CO_2. Slices were progressively warmed up to 32-34°C, and transferred after 1h 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 medially to the MT (medial terminal nucleus of the accessory optical tract). DA cells were identified by a large I_h current while GABA neurons showed no I_h, but an outward current in response to the μ-opioid selective agonist DAMGO (1μM). Whether a short pulse (<2min) of DAMGO was applied in the beginning or the end of the experiment did not have any influence on the amplitude of baclofen (100μM) evoked currents (Imax 90±20pA versus 100±15pA) Kynurenic acid (2mM) 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 (in mM): K-Gluconate 140, NaCl 4, $MgCl_2$ 2, EGTA 1.1, HEPES 5, Na_2ATP 2, Na_2-Creatine-phosphate 5, and Na_3GTP 0.6, pH adjusted to 7.3 with KOH. In some experiments, 8-Bromo-cAMP (10 mM) was added to the internal solution. Currents were amplified (Visual patch 500, Bio-logic, France), filtered at 1 kHz and digitized at 5 kHz (National Instruments Board PCI-MIO-16E4, NI-DAQ Igor Software, Wave Metrics, Lake Oswego, OR) 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, DA 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 ± sem. For statistical comparisons the non parametric 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, Bristol, GB; 8Br-cAMP, TEA and 4-AP and kynurenic acid from Sigma/RBI and GHB from Unavera, Mittenwald, FRG.

Single-cell RT-PCR

Recordings were preformed as above. After 20 min, the cell contents (including a nucleus in most of case) 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 of Rnase-inhibitor and 20 U of 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 hr at 37 °C in a total reaction volume 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 200U 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 (accession number L22651), sense TTCTCAACCTGCTCTTCTCCC (position 612), antisense ACCTTGTCCTCTCTGGCACTG (position 838); GAD67 (accession number M76177) sense AGCCTGGAAGAGAAGAGTCGT (position 347), antisense CAACTGGTGTGGGTGGTGGAA (position 602); GIRK1 (accession number U09243) sense, TTTCCGACCTCTTCACTACCC (position 389) antisense TCGGTGATGTAGCGGTAGCCA (position 624); GIRK2 sense for every splice variants, GGAACTGGAGATTGTGGTCAT (position 1117); GIRK2a (accession number AB073754) antisense, CAAGCAGTTAGGGAGGAAAAT (position 1533); GIRK2b (accession number AB073756) antisense TTCCCTTTTGGCTTGATAACA, GIRK2a,c (accession number AB073753) antisense CATCACCATTCCTCTCTGTCA (position 1437); GIRK3 (accession number L77929) sense CGAGACCTACCGCTACCTGAC (position 368), antisense GAAGCCGTTGAGGTTGTTGAC (position 552); GIRK4 (accession number L35771), 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 transcription, 20 pM of each primers 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 T3Thermocycler (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 performed followed by a final elongation period of 10 min at 72 °C. The second PCR were performed in individual reactions for every pair of primers, in each case with 3 µl of the multipex 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 (base pairs) of PCR fragments were: 226bp (TH), 255bp (GAD67), 235 bp (GIRK1), 416 bp (GIRK2a), 608 bp (GIRK2b), 320 bp (GIRK2a,c), 184 bp (GIRK3), 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$_B$R cDNA were subcloned in pcDNA3. GIRK2c was constructed by adding the 11 amino acid C-terminal sequence of GIRK2c to that of GIRK2a using PCR[19]. HEK-293T cells were cultured in DMEM supplemented with fetal bovine serum (10%), glutamine (2mM), 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 12mm glass cover slips (Warner Instruments) coated with poly-D-lysine (20µg/ml; Sigma) and collagen (100µg/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 ~1N NaOH)) and added (50µl) to each well and incubated for 16-32 hrs. Whole-cell patch-clamp currents were recorded with an Axopatch 200B (Axon Inst.) amplifier, adjusted electronically for cell capacitance and series resistance (80-100%), filtered at 2 kHz with an 8-pole Bessel filter, digitized at 5 kHz with a Digidata 1320 interface (Axon Inst.) and stored on a laboratory computer. Intracellular pipet solution contained (in mM) 130 KCl, 20 NaCl, 5 EGTA, 2.56 K_2ATP, 5.46 $MgCl_2$ and 10 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 (20K) contained (in mM) 140 NaCl, 20 KCl, 0.5 $CaCl_2$, 2 $MgCl_2$, and 10 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) and averaged together to calculate the mean and SEM. The EC50s 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 [48,49].

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Figures

Figure 1 Pre- and postsynaptic GABA$_B$R mediated inhibition in VTA neurons. (a) Bath application of baclofen (100 µM) in DA neuron and **(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 CGP54626 (2µM). Notice the concomitant drop of the membrane resistance (grey trace). **(c)** Desensitization plotted against the maximal amplitude of the outward current for DA- (n=34) and GABA-neurons, (n=18). **(d)** Concentration-response curves are shown for DA-neurons (circle; EC_{50} = 14.8 ± 2.5 µM, Hill coefficient = 0.9 ± 0.1 , n= 5) and GABA-neurons (triangle; EC_{50} = 0.9 ± 0.1 µM, Hill coefficient = 0.7 ± 0.1, n= 5). **(e)** Baclofen (100 µM)-induced presynaptic inhibition of IPSCs recorded in a DA-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, Hill coefficient = 1.0 ± 0.1, n= 5).

Figure 2 GABA$_B$R 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 $BaCl_2$ (300µM) in DA-neurons (**a**) as well as in GABA-neurons in rats (**b**). Scale bars; 50 pA, 5 minutes **(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) . Scale bar; 50 pA, 5 min. **(f)** Average data indicate that in DA neurons the amplitude of Ba resistant current is not different in WT and GIRK2 -/- mice, and is indistinguishable form the residual current in GIRK2-/-

mice without Ba. Similarly, in GABA neurons of GIRK2-/- again, only a small residual current was elicited.

Figure 3 Ba-resistant component is a outward rectifying K conductance that has a low EC50. (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 (2mM) and 4-AP (500µM) or H7/H8 hydrochloride (20µM) intracellularly pre-applied for 15 minutes but was almost completely blocked by 8 Br-cAMP (10mM). (V_h=-53mV, n= 6). **(c)** Concentration-response curve of barium resistant current (EC_{50} = 0.9 ± 0.3 µM, Hill coefficient = 0.8 ± 0.2 µM , n= 5).

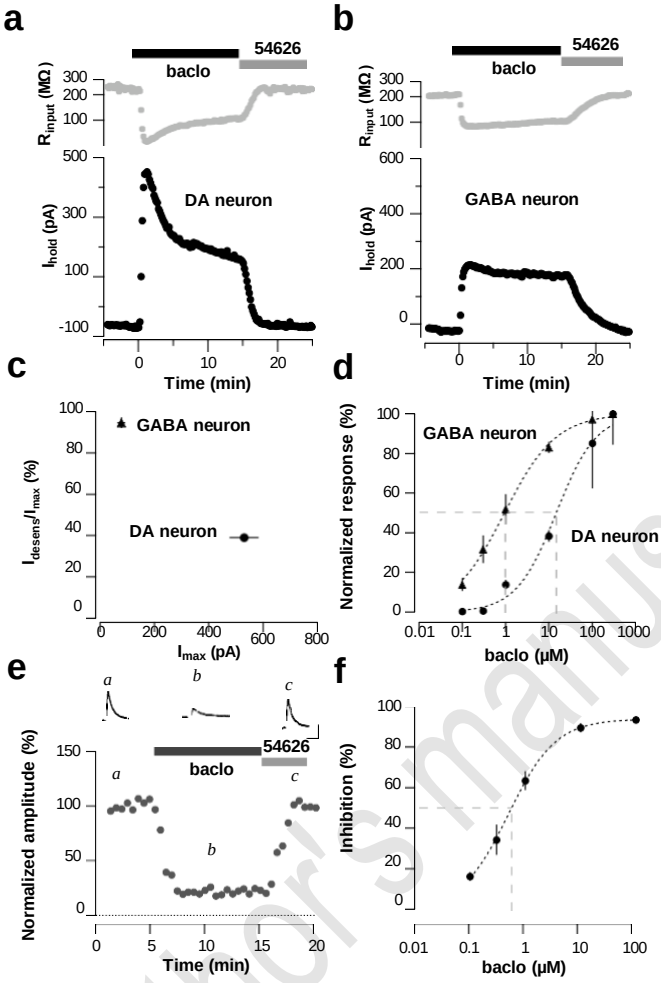
Figure 4 Low efficacy of GABA$_B$Rs in DA-neurons correlates with specific combination of GIRK channel subunits. (a) Example of single cell multiplexed RT-PCR in DA-neuron; a baclofen-induced current was recorded first and then RT-PCR revealed mRNA for tyrosine hydroxylase (TH) and GIRK2c and GIRK3 (top). **(b)** In a GABA-neuron, RT-PCR showed mRNA for glutamic acid decarboxylase (GAD) along with GIRK1, GIRK2c and GIRK3. Scale 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).

Figure 5 Coupling efficiency of GABA$_B$Rs to different combinations of GIRK subunits expressed heterologously in HEK-293T cells. HEK-293 cells were transfected with cDNA for GABABR1 and GABABR2 along with the indicated GIRK subunits. (**a)** whole-cell patch-clamp recording from HEK-293T cell expressing GIRK1/2c or GIRK2c/3. Broken line indicates 50% activation. Holding potential was -80 mV**.** Scale bars: 10s /0.5 and 0.2nA respectively. **(b-c)**. The average EC_{50} (**a**) and Hill coefficient (**b**) 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).

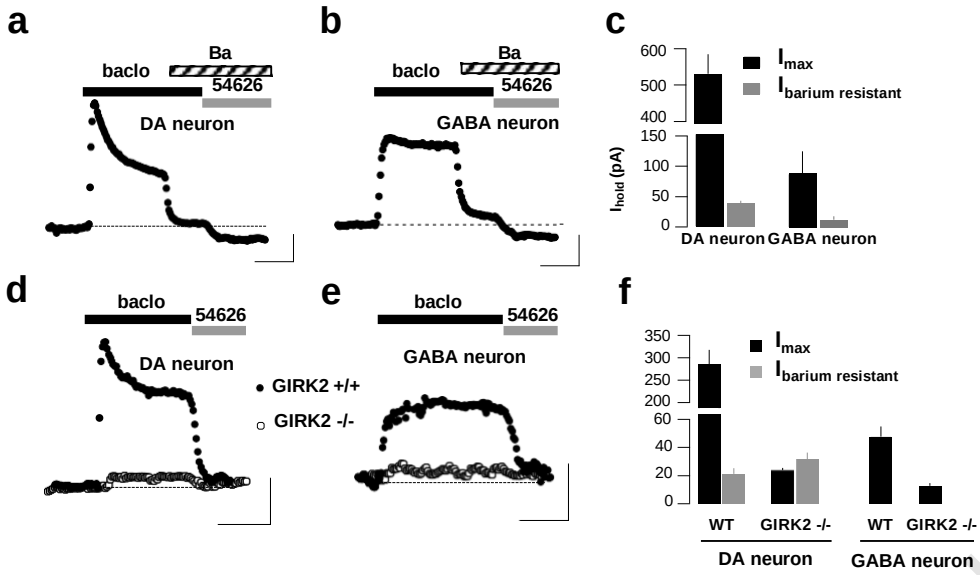
Figure 6 Bi-directional effects of GABA$_B$ receptor agonists on the firing rate of DA neurons in the VTA. **(a)** Single-spike activity recorded in cell-attached configuration (10 s duration) from a DA neuron at corresponding time-points of (**b**) showing the spiking frequency as a function of different baclofen doses. Low concentrations led to a substantial increase of the firing frequency, while high doses eventually suppressed all action potentials. Effects were reversed by CGP54626 (2 µM). **(c)** Average change in firing frequency, n=4. In all experiments excitatory inputs were blocked with kynurenic acid (2mM).

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

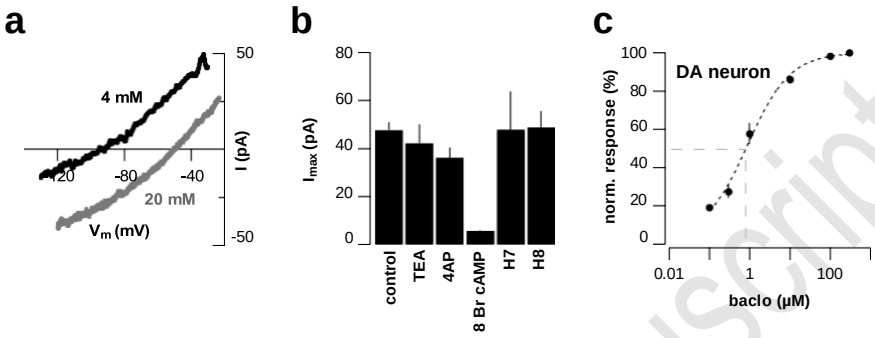
Figure 8 Differential coupling efficacy (EC_{50}) of GIRK channels in VTA neurons is sufficient to explain bi-directional effects of GABA$_B$R agonists on the mesolimbic DA system. The VTA contains DA neurons that are under inhibitory control by local GABA neurons. Both cell types receive excitatory (mainly from the prefrontal cortex and the pedunculo pontine tegmental nucleus; not drawn) and inhibitory inputs (from the pallidum (GP) and nucleus accumbens, NAc)[40]. In the acute slice preparation all these distant afferents are cut, leaving local GABA neurons to exert an inhibitory control of DA neurons. Since DA neurons are less sensitive to GABA$_B$R agonists than GABA neurons (note significantly higher EC_{50} for baclofen compared to the EC_{50} of pre- as well as postsynaptic inhibition in GABA neurons) low agonist concentrations will lead to disinhibition while at higher concentrations both cell types will stop firing.



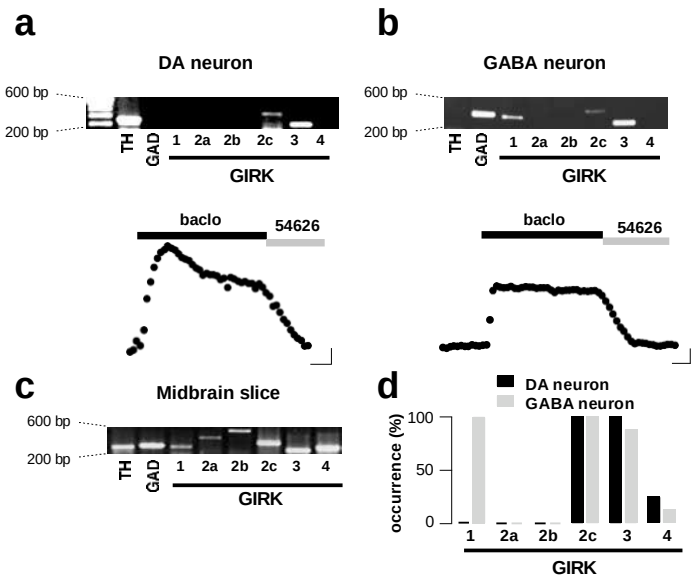
Cruz et al. **Figure 1**



Cruz et al. **Figure 2**



Cruz et al. **Figure 3**



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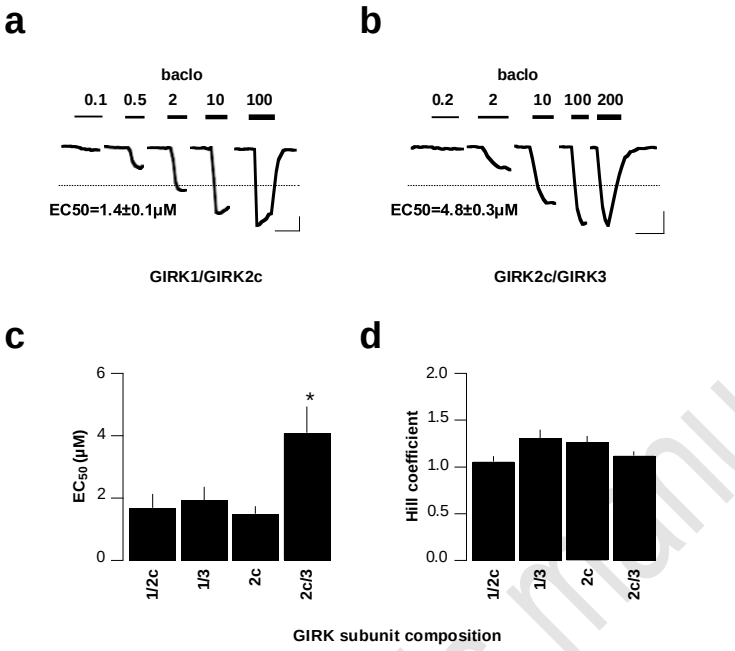
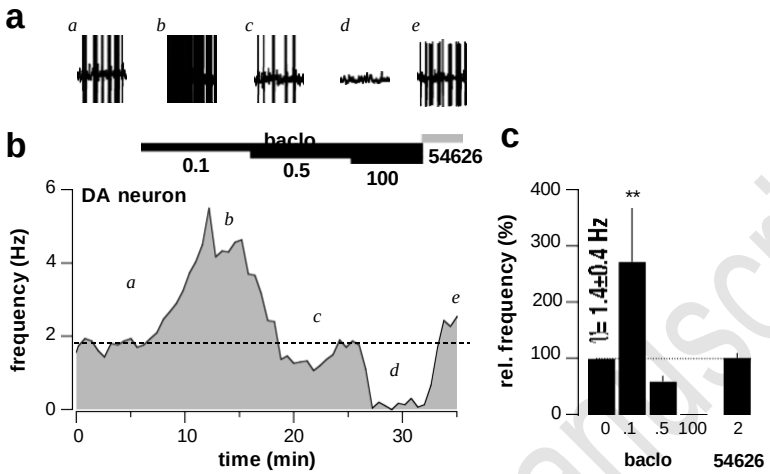
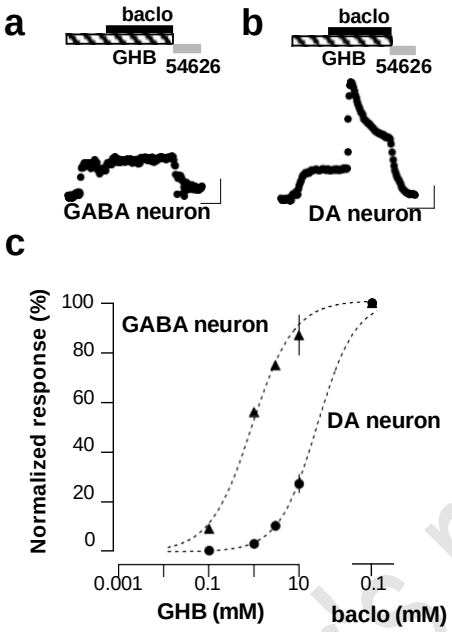


Cruz et al. **Figure 5**



Cruz et al. **Figure 6**

b



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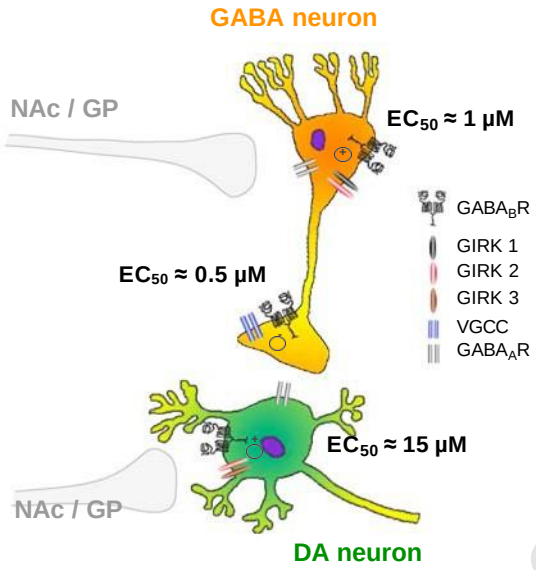
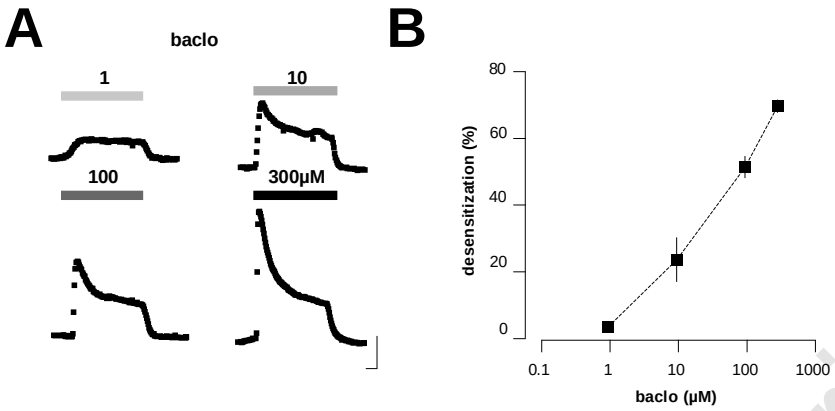


Cruz et al. **Figure 8**



Cruz et al. **Suppl. Figure 1**