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Roles of $GABA_B$ receptor subtypes in presynaptic auto- and heteroreceptor function regulating GABA and glutamate release

Peter C. Waldmeier · Klemens Kaupmann · Stephan Urwyler

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Abstract Γ-Aminobutyric acid B ($GABA_B$) receptors are heterodimers composed of two subunits $GABA_{B(1)}$ and $GABA_{B(2)}$, the former existing in two isoforms $GABA_{B(1a)}$ and $GABA_{B(1b)}$. The contributions of individual receptor subunits and isoforms to $GABA_B$ auto- and heteroreceptor functions were investigated, using release experiments in cortical slice preparations from corresponding knockout mice. Presynaptic $GABA_B$ autoreceptors are located on GABAergic terminals and inhibit GABA release, whereas presynaptic $GABA_B$ heteroreceptors control the release of other neurotransmitters (e.g. glutamate). Neither baclofen nor the selective antagonist CGP55845 at maximally active concentrations affected [^{3}H]GABA release in slices from $GABA_{B(1)}-/-$ mice. The amount of [^{3}H]GABA released per pulse was unaffected by the stimulation frequency in slices from $GABA_{B(1)}-/-$ and $GABA_{B(2)}-/-$ demonstrating a loss of $GABA_B$ autoreceptor function in these knockout animals. The $GABA_B$ receptor agonist baclofen was ineffective in modulating glutamate release in cortical slices from $GABA_{B(2)}-/-$ mice, showing that heteroreceptor function was abolished as well. Next we investigated knockout mice for the two predominant $GABA_{B(1)}$ isoforms expressed in brain, $GABA_{B(1a)}$ and $GABA_{B(1b)}$. In cortical, hippocampal and striatal slices from both $GABA_{B(1a)}-/-$ and $GABA_{B(1b)}-/-$ mice, the frequency dependence of [^{3}H]GABA released per pulse was maintained, suggesting that both isoforms participate or can substitute for each other in $GABA_B$ autoreceptor function. By contrast, the efficacy of baclofen to inhibit glutamate release was substantially reduced in $GABA_{B(1a)}-/-$, but essentially unaltered in $GABA_{B(1b)}-/-$ mice. Our data suggest that functional $GABA_B$ heteroreceptors regulating glutamate release are predominantly, but not exclusively composed of $GABA_{B(1a)}$ and $GABA_{B(2)}$ subunits.

Keywords $GABA_B$ receptor isoforms · Autoreceptors · Heteroreceptors · Knockout animals · [^{3}H]GABA release · Glutamate release

Abbreviations

AOAA	Aminooxyacetic acid
GDH	Glutamate dehydrogenase
Ko	Knockout
L-trans-PDC	L-trans-pyrrolidine-2,4-dicarboxylic acid
Wt	Wildtype

Introduction

$GABA_B$ receptors comprise the metabotropic, G protein-coupled receptor system for GABA and modulate inhibitory and excitatory neurotransmission. Activation of presynaptic $GABA_B$ receptors, via inhibition of Ca^{2+} channels, inhibits the release of several neurotransmitters and neuropeptides. Presynaptic $GABA_B$ autoreceptors are located on GABAergic terminals and inhibit GABA release, whereas presynaptic $GABA_B$ heteroreceptors control the release of other neurotransmitters (e.g. glutamate). Postsynaptic $GABA_B$ receptors activate inwardly rectifying potassium

P. C. Waldmeier · K. Kaupmann · S. Urwyler
Novartis Institutes for BioMedical Research, Neuroscience, Basel, Switzerland

S. Urwyler (✉)
Department of Chemistry and Biochemistry, University of Berne, p.Adr. Weissensteinweg 3, 3303 Jegenstorf, Switzerland
e-mail: stephan.urwyler@ioc.unibe.ch

channels (GIRKs) and induce the slow, long-lasting component of inhibitory postsynaptic potentials (IPSPs), the fast component of which is mediated through $GABA_A$ receptors. Based on the effectiveness of some $GABA_B$ receptor ligands in preferentially modulating the release of one versus another neurotransmitter, the existence of different auto- and heteroreceptor subtypes has been proposed (Bonanno and Raiteri 1993; Cunningham and Enna 1996), although this is controversial (Waldmeier et al. 1994).

Molecular cloning and pharmacological characterization has revealed that native $GABA_B$ receptors are heterodimers composed of two subunits, $GABA_{B(1)}$ (GB1) and $GABA_{B(2)}$ (GB2; reviewed in Bettler et al. 2004). $GABA_B$ receptors are exceptional among G protein coupled receptor heterodimers in that only one subunit, GB1, contains the ligand binding domain whereas effector coupling is entirely mediated through the second subunit, GB2. Genetic inactivation of either the GB1 or the GB2 subunits abolishes physiological $GABA_B$ receptor responses (GB1−/− and GB2−/− mice; Prosser et al. 2001; Schuler et al. 2001; Quéva et al. 2003; Gassmann et al. 2004; Thuault et al. 2004). The lack of physiological pre- and postsynaptic $GABA_B$ receptor functions in respective knockout (ko) mice demonstrates that GB1 and GB2 are essential subunits of all brain $GABA_B$ receptors (Prosser et al. 2001; Schuler et al. 2001; Gassmann et al. 2004).

In the brain two predominant, differentially expressed isoforms are transcribed from the *Gabbr1* gene, $GABA_{B(1a)}$ (GB1a) and $GABA_{B(1b)}$ (GB1b), which are conserved in different species including humans (Kaupmann et al. 1997; Bischoff et al. 1999; Fritschy et al. 1999). Both isoforms heterodimerize with GB2 to form functional receptors. The GB1a isoform differs from GB1b by the presence of a pair of "sushi repeats" at the very N terminal protein sequence (Hawrot et al. 1998). Using established ligands the pharmacological profiles of both isoforms are however indistinguishable (Kaupmann et al. 1997; Kaupmann et al. 1998a, b). Sushi repeats were originally identified in proteins of the complement system and are likely involved in protein–protein interactions (Lehtinen et al. 2004). In the rat brain GB1a is the prevalent isoform present at birth whereas in adult brain tissue GB1b is more abundant (Malitschek et al. 1998). Transcription of the two isoforms is regulated by different promoters (Steiger et al. 2004). Additional isoforms (splice variants) transcribed from the *Gabbr1* gene have been described, however their physiological significance is unclear to date (reviewed in Bettler et al. 2004).

The goal of the present study was to assess the contributions of individual $GABA_B$ receptor isoforms and subtypes to $GABA_B$ autoreceptor and heteroreceptor functions, using release studies in respective ko mice. Using brain slice preparations from the recently generated GB1a and GB1b isoform selective ko mice (Vigot et al. 2006) we measured GABA and glutamate release in comparison with previously characterized GB1 and GB2 subunit ko mice (Schuler et al. 2001; Gassmann et al. 2004). Our data suggest that the GB1a isoform is mainly, although not exclusively, involved in heteroreceptor function regulating glutamate release, whereas both GB1a and GB1b contribute to autoreceptor function.

Materials and methods

Chemicals

The $GABA_B$ antagonist CGP55845 ([3-[[1-(*S*)-(3,4-dichlorophenyl)-ethyl]-amino]-2-(S)-hydroxypropyl]-phenylmethylphosphinic acid), the agonist *R*(–)-baclofen (*p*-chlorophenyl-GABA) and the GABA uptake inhibitor SK&F 89976 (1-(4,4-diphenyl-3-butenyl)-3-piperidine carboxylic acid) have been synthesized at Novartis. The GABA transaminase inhibitor, aminooxyacetic acid (AOAA), and the glutamate uptake inhibitor, L-trans-pyrrolidine-2,4-dicarboxylic acid (L-trans-PDC), were obtained from FLUKA AG (Buchs, Switzerland) and Tocris (Bristol, UK), respectively. Luciferase (from Photobacterium fischeri, E.C. 1.14.14.3) and NAD(P)H:FMN oxidoreductase (E.C. 1.6.8.1) were from Roche Diagnostics (Mannheim, Germany). FMN, NAD, glutamate dehydrogenase (E.C. 1.4.1.3, GDH) and decanal were purchased from Sigma Aldrich (Buchs, Switzerland), and ADP was from ICN Biomedicals Inc. (Aurora, OH, USA).

$GABA_B$ receptor knockout mice

Ko mice of the different $GABA_B$ receptor subunits and isoforms were generated in the Balb/c inbred strain using a newly established Balb/c embryonic stem (ES) cell line. The generation of GB1 and GB2 subunit ko mice (GB1−/− and GB2−/−) has been described (Schuler et al. 2001; Gassmann et al. 2004). To generate GB1 isoform selective ko mice (GB1a−/− and GB1b−/−) the respective start codons of GB1a and GB1b isoforms (Steiger et al. 2004) were converted into stop codons (Vigot et al. 2006).

Release experiments

In each experiment in which the electrically stimulated release of [^{3}H]GABA or endogenous glutamate was measured, usually the slices obtained from two or more mice per genotype were pooled and distributed into the number of superfusion chambers indicated in the figure legends (depending on the number of different conditions, the superfusion apparatus containing 12 chambers). The

data from the number of experiments given in the figure legends were pooled for statistical analysis to give a total of *N* data points, each data point reflecting a sample obtained from one superfusion chamber.

Release of [^{3}H]GABA

Electrically stimulated release of [^{3}H]GABA from preloaded mouse brain slices was measured as previously described for slices from rat brain (Baumann et al. 1990). Briefly, cross-chopped slices (1,000 × 350 × 350 µm) were incubated for 5 min with 100 nM [2,3-^{3}H(*N*)] GABA (1.25 TBq/mmol, Perkin-Elmer Lifesciences, Boston, MA, USA) in physiological buffer (composition in mM: NaCl, 118; KCl, 4.8; $CaCl_2$, 2.6; $MgSO_4$, 1.2; KH_2PO_4, 1.2; (+)-glucose, 10; $NaHCO_3$, 25; AOAA as an inhibitor of GABA transaminase, 0.05). 25 µl of slice suspension (≈1 mg protein) were transferred to stimulation chambers and superfused at 0.4 ml/min with physiological buffer gassed with 5% CO_2 in O_2, containing 10 µM of the GABA uptake inhibitor, SK&F 89976. Fractions of 6 min were collected, beginning 50 min after starting the superfusion. Radioactivity in the fractions and the slices [collected at the end of the experiments and solubilized in Soluene 350 (Packard, Meriden, CT, USA)] was counted after addition of Irgasafe Plus scintillator (Zinsser Analytics, Maidenhead, UK). Data are means of the stimulated fractional release ± SEM.

For the comparison of the effect of the $GABA_B$ antagonist, CGP55845, on [^{3}H]GABA release from cortical slices of wildtype (wt) and GB1−/− mice, four groups of three slice preparations (wt control; wt + 1 µM CGP55845; GB1−/− control; GB1−/− + 1 µM CGP55845) were stimulated three times (S_1–S_3): at 2 Hz for 2 min (240 monophasic pulses, 2 ms, 25 mA) during fraction 3, and at 2 Hz for 0.5 min (60 pulses) during fractions 9 and 15; 17 fractions were collected in total. The compound was added between S_2 and S_3, in fraction 12. Two identical experiments were carried out and the results pooled. A similar procedure was used for assessing the effect of the $GABA_B$ agonist, *R*(–)-baclofen (10 µM), except that S_2 and S_3 were at 0.125 Hz (8 min, 60 pulses).

In the experiments in which the dependence of [^{3}H]GABA release on the stimulation frequency was assessed in cortical slices, six chambers each containing slice preparations from wt and ko mice (for gender and age see figure legends) were stimulated five times (S_1–S_5; 0.125 Hz for 8 min, during fractions 3 and 4, 0.25 Hz for 4 min (fraction 7), 0.5 Hz for 2 min (fraction 10), 1 Hz for 1 min (fraction 13), and 2 Hz for 30 s (fraction 16), delivering a constant number of 60 monophasic pulses (2 ms, 25 mA) at each frequency. A total of 18 fractions were collected. Each experiment was then repeated, inversing the frequency sequence (i.e. beginning with 2 Hz, ending with 0.125 Hz). The frequency dependence experiments with striatal and hippocampal slices from wt, GB1a−/− and GB1b−/− mice were only done with increasing frequencies, because no difference between the two procedures had been seen in the previous experiments.

Data analysis The significancies of the effects of baclofen and CGP55845 on GABA release (Fig. 1), as well as those of the differences in GABA release levels between wt and ko mice (Figs. 2, 3) were assessed by Student's *t* test. On the other hand, the significance of the dependence of GABA release on the stimulation frequency was calculated by one-way analysis of variance (ANOVA) followed by Dunnett's test, using the values obtained at the lowest stimulation frequency (0.125 Hz) as the control group. GraphPad Prism software was used for these calculations.

Release of endogenous glutamate

Mouse cortical slices were prepared as above and superfused at 0.4 ml/min with physiological buffer as described above for [^{3}H]GABA release experiments, but containing 100 µM of the glutamate uptake inhibitor L-trans-PDC instead of SK&F 89976, as previously described (Waldmeier et al. 1993b). Collection of twelve 6 min fractions was begun 50 min after starting the superfusion. Four groups of slice preparations pooled from at least two animals each (wt control; wt + *R*(–)-baclofen 10 µM; ko control; ko + *R*(–)-baclofen 10 µM) were electrically stimulated (1 Hz for 4 min, 2 ms, 25 mA) twice, during fractions 3 and 10; *R*(–)-baclofen was added before S_2, in fraction 6.

The glutamate content of the superfusate fractions from release experiments was determined with a bioluminescence assay, as described by Fosse et al. (1987). The principle of the assay is based on an enzymatic cascade, in which the NADH generated by glutamate dehydrogenase is reoxidized by NADH:FMN oxidoreductase, the reduced FMNH then being accepted as a co-factor in the oxidation of a long-chain aldehyde (decanal) by a light-producing bacterial luciferase. The measurements were performed in opaque, white 96 well microtiter plates on a WALLAC Micro Beta 1450 Jet instrument. To sample or standard solutions (25 µl), 45 µl of a luminescence reaction mixture (25 mM potassium phosphate buffer pH 7.0, 100 µM dithiothreitol, 5 µg/ml luciferase, 200 mU/ml NAD(P)H:FMN oxidoreductase, 2.5 µM FMN, 2 mM NAD, 250 µM ADP, 16% glycerol, 44 U/ml GDH, 20 µM decanal) were injected using a six channel—injector module. After allowing an initial peak occurring upon reagent mixing to decay for 12 s, the light production was measured during 45 s, at room temperature.

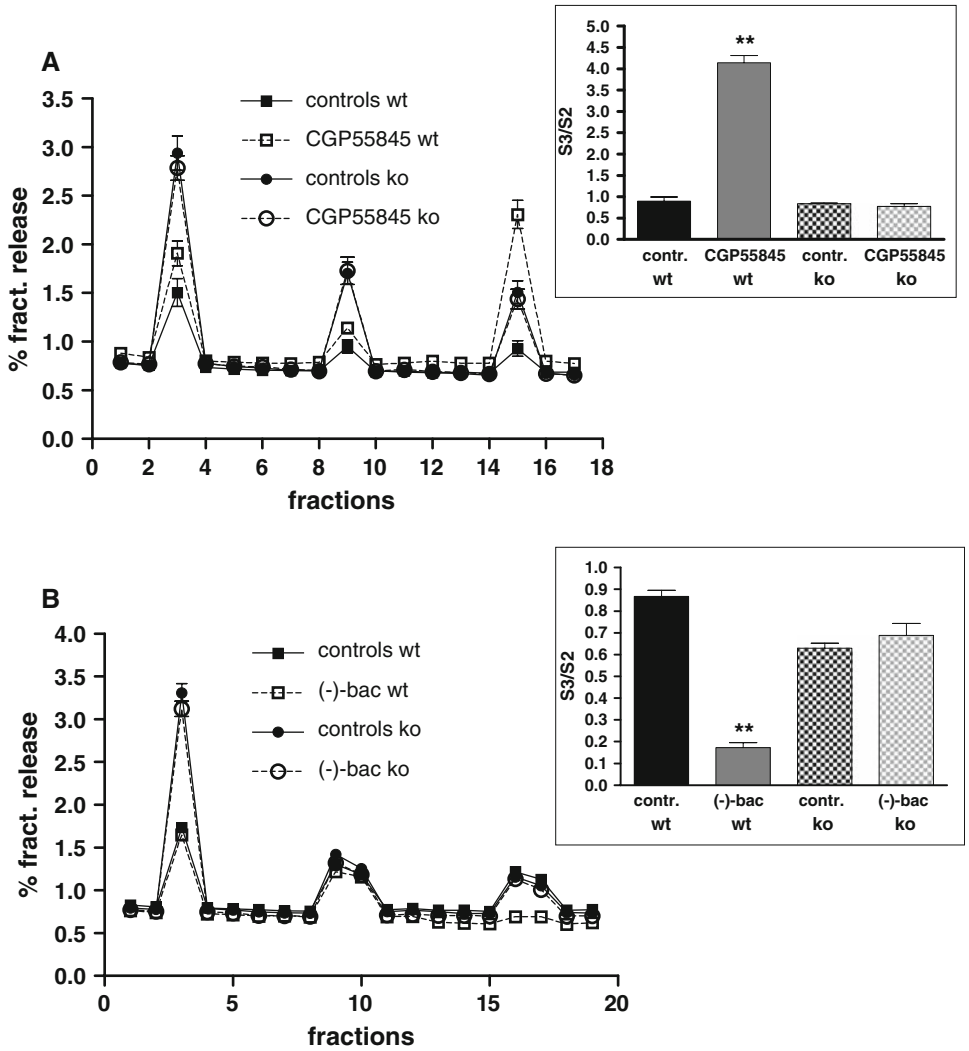

Fig. 1 Effects of a $GABA_B$ antagonist and an agonist on [^{3}H]GABA release in cortical slices from wt and GB1−/− mice. **a** In each experiment, slices were prepared from 3 wt or 3 ko mice (males, age 10–13 weeks) and distributed into 3 different superfusion chambers for each condition. The slices were stimulated 3 times (S_1: 2 Hz, 2 min; S_2 and S_3: 2 Hz 0.5 min); to three chambers each containing slices from wt or GB1−/− mice, 1 μM CGP55845 was added after S_2, from fraction 12 onwards. S_1 only served to ensure comparability with the results depicted in (**b**). Two identical experiments were performed and the results pooled. Data show means ± SEM ($n = 6$) of the fractional release per fraction. The S_3/S_2 ratios are given in the *inset*. $^{**}P < 0.01$ versus control wt (two-tailed t test). **b** In each experiment, slices were prepared from 3 wt or 3 ko mice (males, age 11–15 weeks) and distributed into three different superfusion chambers for each condition. The slices were stimulated three times (S_1: 2 Hz, 2 min; S_2 and S_3: 0.125 Hz, 8 min); to three chambers each containing slices from wt or GB1−/− mice, 10 μM R(–)-baclofen was added after S_2, from fraction 12 onwards. Two identical experiments were performed and the results pooled. Data show means ± SEM ($n = 6$) of the fractional release per fraction. The S_3/S_2 ratios are given in the *inset*. $^{**}P < 0.01$ versus control wt (two-tailed t test)

Data analysis The light produced in the luminometric assay was converted into the corresponding quantities of glutamate with the aid of calibration curves obtained with glutamate standard solutions in the range from 0.5 to 10 picomol per sample. The amounts of glutamate released in response to the depolarization of the slices were calculated by subtracting baseline values (means of fractions 2/4 and 9/11, respectively) from the values of the stimulated fractions 3 and 10. These values were used to calculate S2/S1 ratios. The statistical significances of baclofen effects and of possible differences between wt versus ko controls were assessed by analysis of variance (one-way ANOVA) followed by Tukey's test for post hoc comparisons. Prism 3.03 (GraphPad Software Inc., San Diego, CA, USA) was used for all the calculations.

Results

Absence of autoreceptor-mediated regulation of [^{3}H]GABA release in cortical slices of GB1−/− and GB2−/− mice

In rat brain slice preparations, $GABA_B$ antagonists increase electrically stimulated [^{3}H]GABA release and agonists decrease it via the release-regulating $GABA_B$ autoreceptor (Waldmeier and Baumann 1990). Therefore, we compared the effects of the potent $GABA_B$ antagonist, CGP55845 (Waldmeier et al. 1993a), and of *R*(–)-baclofen, the active enantiomer of the prototypical $GABA_B$ agonist, baclofen (Baumann et al. 1990), on [^{3}H]GABA release from cortical slices of GB1−/− and corresponding wt mice. Based on our previous work (Waldmeier et al. 1993a), we chose stimulation frequencies of 2 Hz and 0.125 Hz for testing antagonist and agonist effects, respectively. The concentrations of the agents in the superfusion medium (1 μM CGP55845; 10 μM *R*(–)-baclofen) were previously shown to be near-maximally to maximally effective in rat brain slices (Waldmeier et al. 1993a).

Stimulation at 2 Hz for 2 min (S_1 in Fig. 1a, b), as well as for 0.5 min (S_2 in Fig. 1a) caused a clearly more marked release of [^{3}H]GABA from slice preparations of GB1−/− mice than from wt mice in the absence of any agent. CGP55845, added after S_2, caused an about fourfold increase of [^{3}H]GABA release at 2 Hz (0.5 min) in slices from wt mice, similar to what was found in rat cortical slices (Waldmeier et al. 1993a), but no change at all in those from GB1−/− mice (Fig. 1a; for a comparison of S_3/S_2 ratios see inset). Conversely, *R*(–)-baclofen reduced [^{3}H]GABA release from wt slices about fivefold, similar to its effect on rat brain slices (Waldmeier et al. 1993a). However, *R*(–)-baclofen was entirely ineffective in slices from GB1−/− mice (Fig. 1b, see inset for S_3/S_2 values).

Cortical slice preparations from wt, GB1−/− and GB2−/− mice were then stimulated consecutively with progressively increasing frequencies to generate a frequency dependence curve, in which the fractional release of [^{3}H]GABA per pulse (or rather, per 60 pulses in our case) is plotted against the stimulation frequency. In a second experiment, the same frequencies were applied in a decreasing sequence, to eliminate any potential bias to repetitive stimulation. It finally turned out that there was no such bias, and the data were pooled with those of the first experiment.

Autoreceptor-mediated regulation of transmitter release results in a progressive reduction of the amount of transmitter released per pulse with increasing stimulation frequency (Waldmeier et al. 1993a), a pattern which is evident in all our results with slices from wt mice (Figs. 2a, b, 3a, b, 4a). In contrast, stimulation frequency did not affect the amount of transmitter release per 60 pulses from slices of GB1−/− (Fig. 2a) and GB2−/− mice (Fig. 2b), indicating absence of autoreceptor-mediated regulation of transmitter release. This becomes particularly clear in the insets of Fig. 2a and b, where the values are expressed in percent of those obtained at the lowest frequency (0.125 Hz) for each mouse type. It should be noted that, more so in the GB2−/− than in the GB1−/− mice, as becomes visible at the lowest stimulation frequency, the [^{3}H]GABA release per (60) pulse(s) was lower than what one might expect to obtain by just eliminating autoreceptor regulation in wt mice, suggesting the occurrence of compensatory adaptation.

Both GB1a and the GB1b subunit isoforms are involved in autoreceptor function

Experiments identical to those above were carried out with slice preparations from mice in which the $GABA_{B(1)}$ isoforms GB1a and GB1b were inactivated separately (i.e. GB1a−/− and GB1b−/− mice, respectively). The results show that in either of these mouse lines, autoreceptor-mediated regulation of [^{3}H]GABA release is maintained (Figs. 3a, b). The average release per 60 pulses at each frequency was lower in ko compared to wt mice, but the shapes of the curves were identical, as shown in the insets of Figs. 3a and b, where the values are expressed in percent of those obtained at the lowest frequency (0.125 Hz) for each mouse type.

Since the density of GB1a is higher than that of GB1b in the striatum (Malitschek et al. 1998), one might expect the frequency dependence curve in this area to be flatter in GB1a−/− than in GB1b−/− mice and the reverse in hippocampus, where GB1b prevail. To examine this, frequency dependence curves were generated using striatal and hippocampal slice preparations from corresponding ko mice and wt controls. In striatum, the fractional release per 60 pulses was similar in all three types of mice at all frequencies, but lower than in hippocampus. In the latter area, fractional release per 60 pulses in GB1a−/− and GB1b−/− was lower than in wt. The shapes of the frequency dependence curves (insets), however, were not different between mouse types nor areas (Fig. 4a–c).

Absence of $GABA_B$-receptor-mediated inhibition of the release of endogenous glutamate in cortical slices from GB2−/−, but not from GB1a−/− or GB1b−/− mice

The pooled data from *all* experiments with cortical slices from wt mice indicate that 10 μM *R*(–)-baclofen inhibited the electrically stimulated release of endogenous glutamate by approximately 40% (S_2/S_1 in the control group:

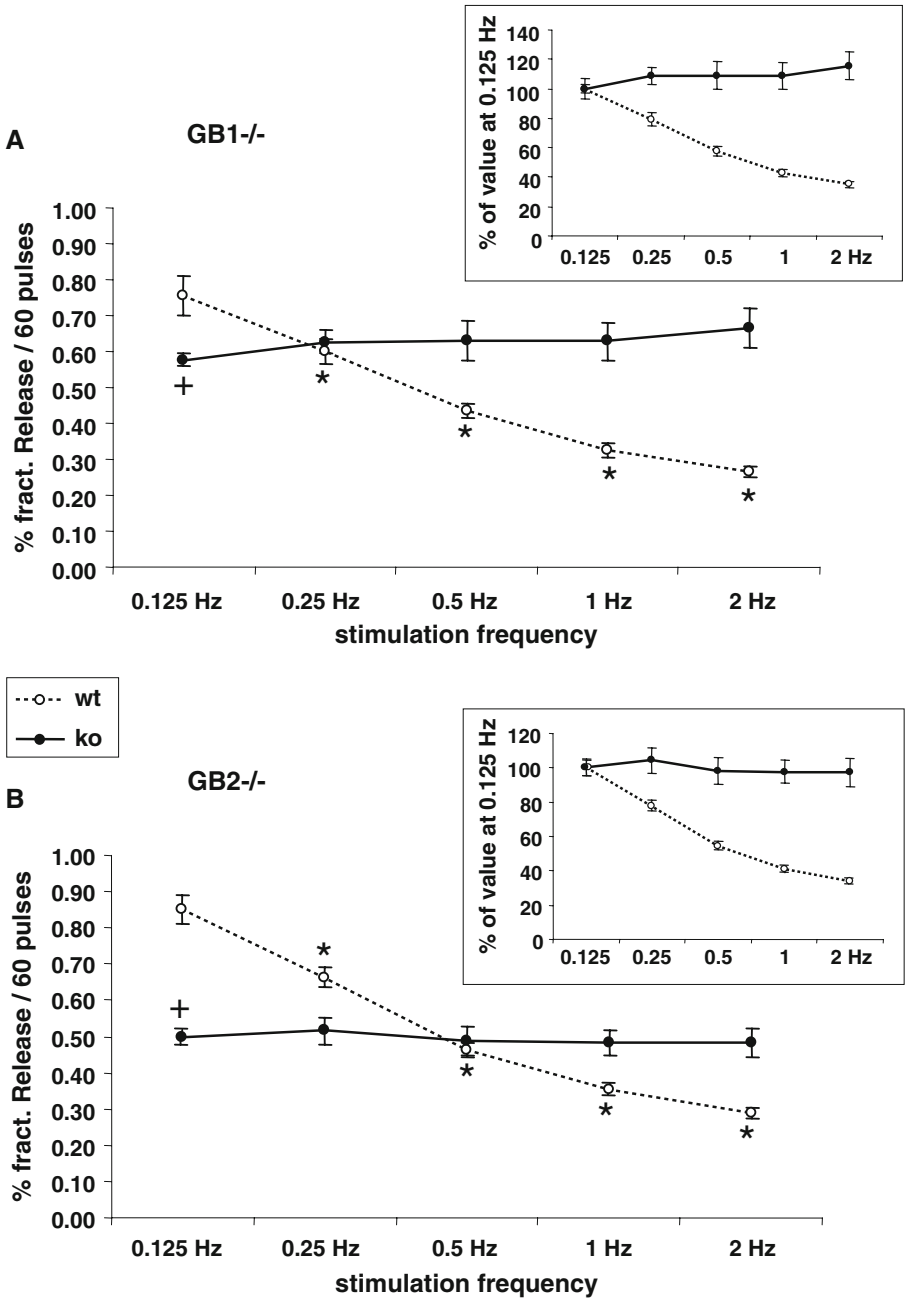

Fig. 2 Comparison of frequency dependence of [^{3}H]GABA release in cortical slices from wt, GB1−/− and GB2−/− mice. **a** Slices were prepared from two male wt or two male GB1−/− (age 13 weeks) mice and distributed into six different superfusion chambers for each group. The slices were stimulated five times with the same number of pulses (60), but progressively increasing frequency. A second experiment with slice preparations from two male wt mice and two male GB1−/− mice (age 15 weeks) was performed in which the frequency sequence was reversed (degressive). The results were pooled. Data represent the means ± SEM ($n = 12$) of the stimulated fractional release at the different frequencies ($S_{1[0.125\ \mathrm{Hz}]} - S_{5[2\ \mathrm{Hz}]}$). The inset shows the same data expressed as percent of S_1 of the respective groups. **b** Slices were prepared from a male and a female wt mouse (age 11 weeks) or a male and a female GB2−/− mouse (age 10 weeks) and distributed into six different superfusion chambers for each group (wt or ko). The slices were stimulated five times with the same number of pulses (60), but progressively increasing frequency. A second experiment with slice preparations from a male and a female wt mouse (age 16 weeks) or a male and a female GB2−/− mouse (age 16 weeks) was performed in which the frequency sequence was reversed (degressive). The results were pooled. Data represent the means ± SEM ($n = 12$) of the stimulated fractional release at the different frequencies ($S_{1[0.125\ \mathrm{Hz}]} - S_{5[2\ \mathrm{Hz}]}$). The *inset* shows the same data expressed as percent of S_1 of the respective groups. + significantly lower ($P < 0.01$) than the corresponding wt group; * significantly different from the wt value at the lowest stimulation frequency (S_1) ($P < 0.01$, ANOVA/Dunnett's test)

1.19 ± 0.04 [$n = 29$], with baclofen: 0.73 ± 0.03, $n = 28$, $P < 0.001$). The *individual* results obtained with slices from wt mice used as a reference for comparison with slices from ko animals are shown on the respective graphs in Fig. 5. The inhibitory effect of baclofen on glutamate release was completely abolished in cortical slices from ko mice lacking the GB2 receptor subunit (S_2/S_1 without baclofen: 1.13 ± 0.1, with baclofen: 1.28 ± 0.13, $n = 5$ per group) (Fig. 5a). On the other hand, in slices from GB1a−/− mice, the inhibitory effect of 10 μM *R*(–)-baclofen was reduced, but not abolished, when compared to wt controls (Fig. 5b). In fact, the S_2/S_1 ratios in the slices from the ko animals were 1.05 ± 0.04 ($n = 12$) in the absence, and 0.9 ± 0.04 ($n = 10$) in the presence of 10 μM *R*(–)-baclofen, corresponding to an inhibition of 14%. A different situation was found in tissue from GB1b−/− mice. In these cortical slices, *R*(–)-baclofen was still able to produce essentially the same degree of inhibition of glutamate release as in tissue from wt animals ($S_2/S_1 = 1.09 \pm 0.05$, $n = 12$, in the control group; $S_2/S_1 = 0.74 \pm 0.05$, $n = 9$, with baclofen, 32% inhibition, $P < 0.001$).

Discussion

The release of [^{3}H]GABA from GABAergic nerve terminals is normally under an inhibitory control by the synaptic concentration of GABA, mediated by autoreceptors of the $GABA_B$ type. This can be conveniently demonstrated by measuring [^{3}H]GABA overflow (in the following termed release) from preloaded brain slices, or the release of endogenous GABA, evoked by electrical field stimulation. The presence of a GABA uptake inhibitor facilitates the measurements, but does not qualitatively affect the results. Characteristically, the electrically evoked release of [^{3}H]GABA per impulse progressively decreases with increasing stimulation frequency in the range of approximately 0.05–2 Hz, reflecting activation of the autoreceptor by endogenously released GABA (Waldmeier et al. 1994). This can be used for assessing the effects of $GABA_B$ agonists, which reduce, and of antagonists, which enhance [^{3}H]GABA release at a given stimulation frequency. Most of the previous work on autoreceptor-mediated regulation of GABA release has been done using rat brain slices, but our control data shown here demonstrate that results obtained with brain slices from wt mice are very similar.

Thus, the prototypical $GABA_B$ antagonist CGP55845 increased and the prototypical $GABA_B$ agonist (–)-baclofen reduced the release of [^{3}H]GABA from cortical slices of wt Balb/c mice in much the same way as previously shown in rat cortical slices (Waldmeier et al. 1993a, 1994). The effects of both compounds were eliminated in mice lacking both subtypes of ligand-binding GB1 subunits (Fig. 1). Moreover, in the absence of any drug, [^{3}H]GABA release evoked by 2 Hz was much higher in GB1−/− mice (Fig. 1). Both this finding and the absence of drug effects are most likely due to absence of inhibitory autoreceptor control. This was confirmed by comparing frequency curves, i.e. the fractional release of [^{3}H]GABA per a given number of pulses plotted against the stimulation frequency: while wt mice showed frequency curves comparable to those reported in rats, no dependence of this parameter on the stimulation frequency was observed in GB1−/− mice. This result is an unmistakable sign of absence of autoreceptor regulation. Eliminating the GB2 subunit of the heterodimeric $GABA_B$ receptor complex, responsible for effector coupling, also causes a complete loss of frequency dependence of [^{3}H]GABA release, indicating that this subunit is just as essential for autoreceptor function as the GB1 subunits.

Corresponding experiments with cortical, hippocampal and striatal slices from mice in which expression of the GB1a or GB1b subunits, respectively, was disabled, suggested that both subtypes can convey autoreceptor function to their heterodimeric receptor complex with GB2. Fractional release per 60 pulses was lower in slices from both ko types than in wt mice at all stimulation frequencies examined, making the frequency curves look shallower. However, on normalization to the values at 0.125 Hz (insets in Figs. 3, 4), wt and ko frequency curves were identical, indicating little if any difference in autoreceptor function of the corresponding heterodimeric receptor complexes. This is also suggested by the fact that frequency curves obtained with striatal and hippocampal slices of GB1a−/− and GB1b−/− mice showed the same pattern, i.e. shallower curves in striatum. If there were a difference in autoreceptor function of the corresponding heterodimeric receptor complexes, one might have expected a change in this pattern, since GB1a subunits prevail in the striatum and GB1b subunits in the hippocampus (Malitschek et al. 1998).

Of note, fractional release per 60 pulses was also lower at 0.125 Hz in both GB1a−/− and GB1b−/− mice. A downregulation of GABA release by unknown compensatory mechanisms as a reaction to the absence of these subunits might be responsible for this phenomenon.

$GABA_B$ receptor-mediated inhibition of glutamate release, as measured by the ability of R(–)-baclofen to reduce electrically evoked release of endogenous glutamate from cortical slices, was also completely abolished in GB2−/− mice, whereas this agent caused a reduction in wt mice similar in extent as previously reported for rats (Waldmeier et al. 1993b). Interestingly, a difference in the ability of *R*(–)-baclofen to reduce glutamate release emerged in the experiments on GB1a−/− and GB1b−/−

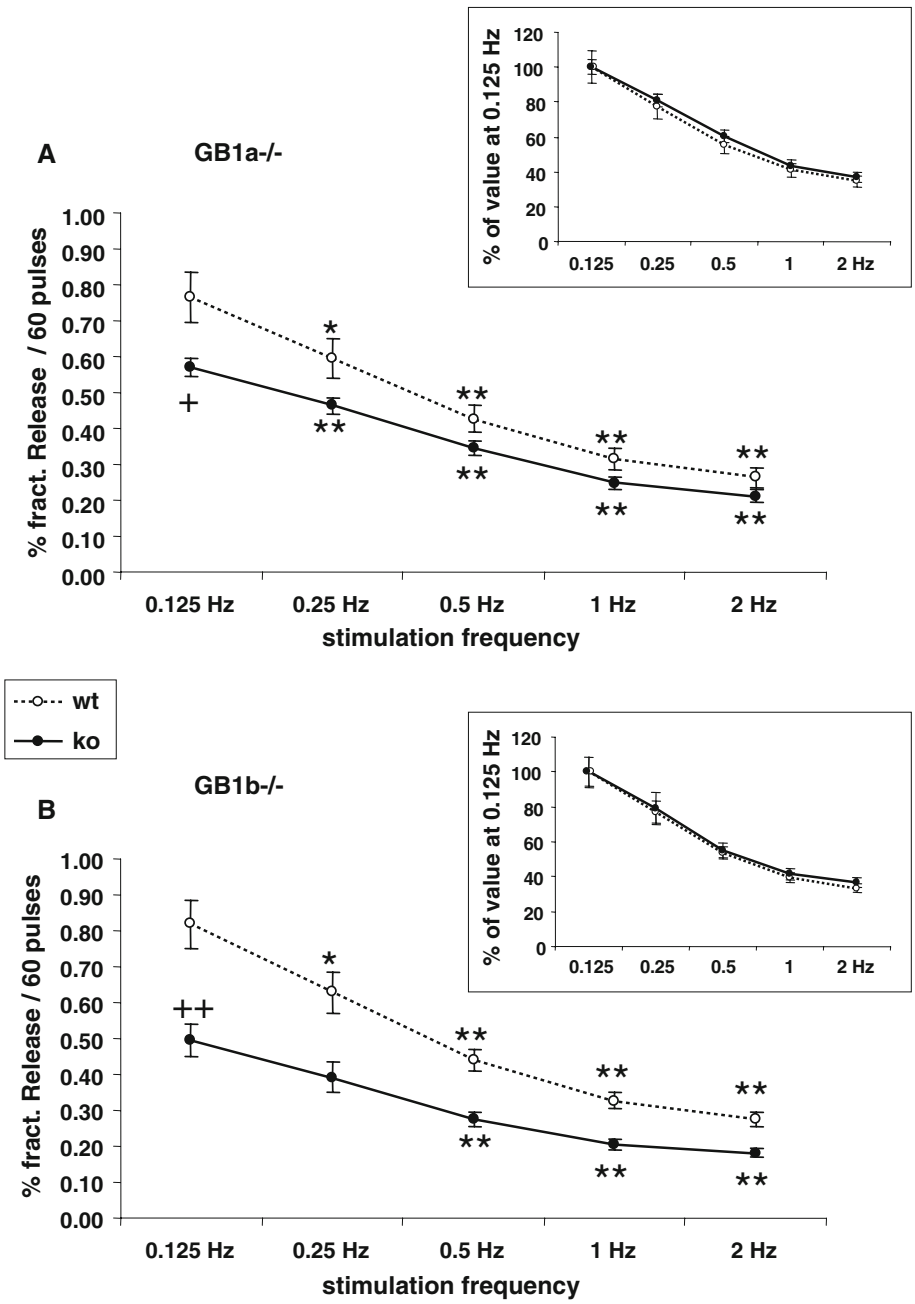

Fig. 3 Comparison of frequency dependence of [^{3}H]GABA release in cortical slices from wt, GB1a−/−, and GB1b−/− mice. **a** Slices were prepared from three female wt (age 10 weeks) or three female GB1a−/− (age 24 weeks) mice and distributed into six different superfusion chambers for each group (wt or ko). The slices were stimulated five times with the same number of pulses (60), but progressively increasing frequency. A second experiment with slice preparations from a male and a female wt mouse (age 41 and 22 weeks, respectively) or a male and a female GB1a−/− mouse (age 41 and 22 weeks, respectively) was performed in which the frequency sequence was reversed (degressive). The results were pooled. Data represent the means ± SEM ($n = 12$) of the stimulated fractional release at the different frequencies ($S_{1[0.125\ Hz]} - S_{5[2\ Hz]}$). The *inset* shows the same data expressed as percent of S_1 of the respective groups. **b** Slices were prepared from two male wt mice (age 14 and 41 weeks) and two male GB1b−/− mice (age 11 and 21 weeks) and distributed into six different superfusion chambers for each group. The slices were stimulated five times with the same number of pulses (60), but progressively increasing frequency. A second experiment with slice preparations from two male wt mice (age 14) and two male GB1b−/− mice (age 14 weeks) was performed in which the frequency sequence was reversed (degressive). The results were pooled. Data represent the means ± SEM ($n = 12$) of the stimulated fractional release at the different frequencies ($S_{1[0.125\ Hz]} - S_{5[2\ Hz]}$). The inset shows the same data expressed as percent of S_1 of the respective groups. +/++ significantly lower ($P < 0.05/P < 0.01$) than the corresponding wt group; */** significantly different from the corresponding S_1 value ($P < 0.05/P < 0.01$, ANOVA/Dunnett's test)

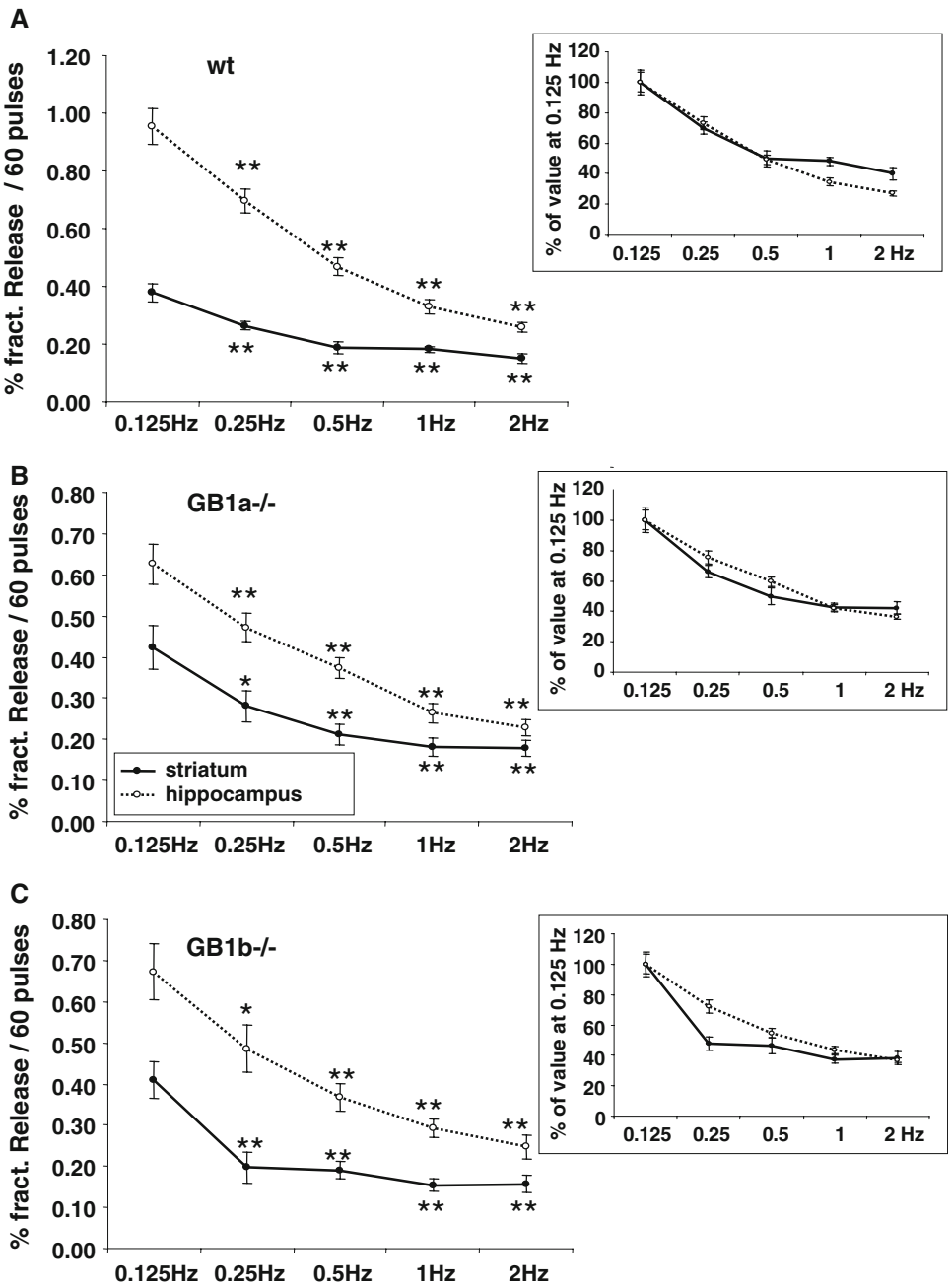

Fig. 4 Comparison of frequency dependence of [^{3}H]GABA release in striatal versus hippocampal slices from wt, GB1a−/− and GB1b−/− mice. Striatal and hippocampal slices were prepared from five male and five female wt mice (age 17–41 weeks) (**a**), five male and five female GB1a−/− mice (age 13–48 weeks) (**b**), or five male and five female GB1b−/− mice (age 49–56 weeks) (**c**) and distributed into six different superfusion chambers for each group. The slices were stimulated five times with the same number of pulses (60), but progressively increasing frequency. Data represent the means ± SEM ($n = 6$) of the stimulated fractional release at the different frequencies ($S_{1[0.125\ Hz]} - S_{5[2\ Hz]}$). The *inset* shows the same data expressed as percent of S_1 of the respective groups. */** significantly different from the corresponding S_1 value ($P < 0.05/P < 0.01$, ANOVA/Dunnett's test)

mice. In GB1a−/− mice, the inhibitory effect of *R*(–)-baclofen was markedly reduced, but not completely abolished as compared to wt mice, whereas in GB1b−/− mice, it was essentially unaltered. The latter result suggests that $GABA_B$ heteroreceptors involved in the regulation of glutamate release are predominantly, but not exclusively, composed of $GABA_{B(1a/2)}$ heterodimers.

Three very recent electrophysiological studies, using the same ko animals as the ones which served for our work, have addressed the role of the two receptor isoforms in pre- and postsynaptic $GABA_B$ receptor functions. Pérez-Garci et al. (2006) have demonstrated that postsynaptic inhibition of dendritic Ca^{2+} spikes in neocortical pyramidal neurons is mediated by GB1b, whereas in their case, somewhat at variance with our findings, presynaptic inhibition of GABA release was mediated by GB1a. At the same time, Vigot et al. (2006), by means of whole cell patch clamp electrophysiology, investigated the presence of functional $GABA_B$ heteroreceptors on excitatory terminals and autoreceptors on GABAergic terminals, in slices from the hippocampus of GB1−/−, GB1a−/−, and GB1b−/− mice. Autoreceptor activation by baclofen in either

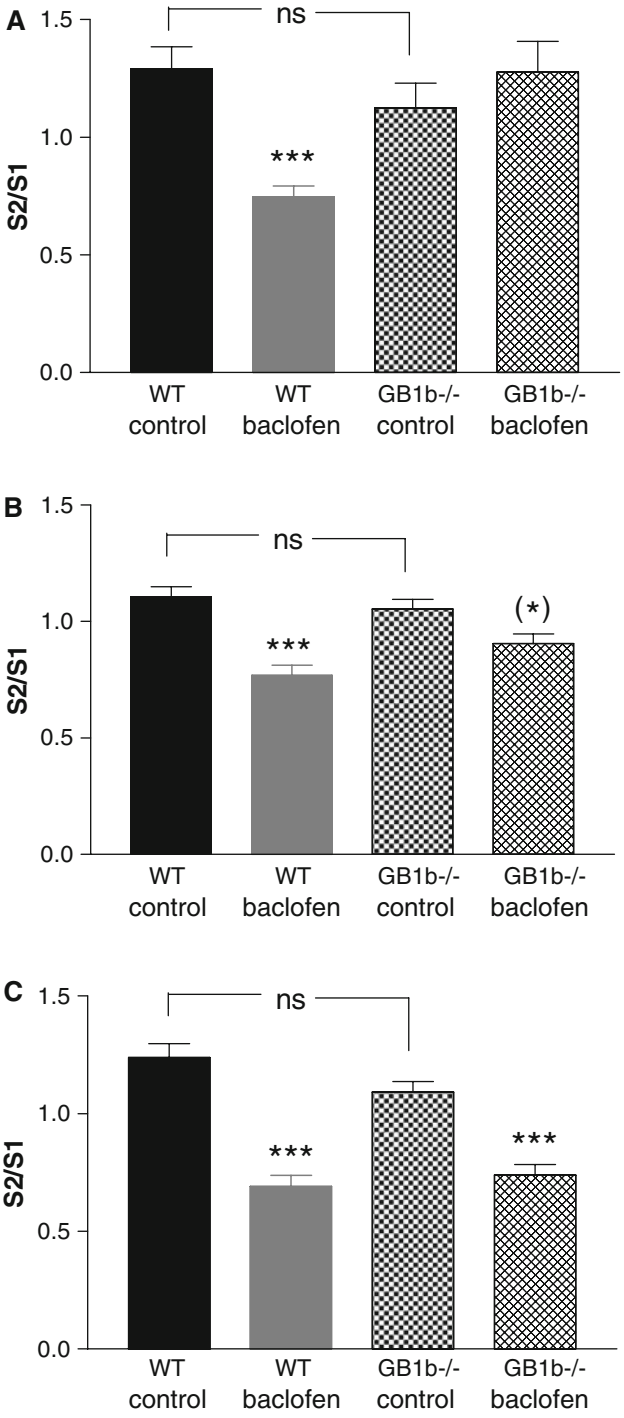

Fig. 5 Baclofen effects on the electrically stimulated release of endogenous glutamate in cortical slices from mice lacking the GB2 (**a**), GB1a (**b**) or GB1b (**c**) receptor subunits, respectively. For each ko type, two or more experiments comprising two groups of six cortical slice preparations from at least one male and one female wt or ko mouse, respectively, were performed and the corresponding data pooled. The animals ranged between 7 and 40 weeks of age, but within each experiment, the age of the animals was comparable. Three wt and 3 ko slice preparations each were used as controls and stimulated twice at 1 Hz for 4 min; to the three others of each type, 10 μM *R*(–)-baclofen was added before the second stimulation. Data represent the means of the S_2/S_1 ratios ± SEM. **a** WT versus GB2−/−: $n = 5$ in all groups; **b** WT versus GB1a−/−: wt controls, $n = 13$; wt baclofen, $n = 11$; ko controls, $n = 12$; ko baclofen, $n = 10$; **c** WT versus GB1b−/−: wt controls, $n = 11$; wt baclofen, $n = 12$; ko controls, $n = 12$; ko baclofen, $n = 9$). *N.s.* not significant. (*) $P < 0.05$ compared to the wt control, but not significant compared to the ko control. ***$P < 0.001$ compared to the corresponding control groups (one way ANOVA followed by Tukey's test)

GB1a−/−, and GB1b−/− mice was similar to wt mice, suggesting that both isoforms can substitute for each other to inhibit GABA release from GABAergic terminals. This corresponds exactly to the results of our direct measurements of GABA release in cortical, hippocampal and striatal slices. With respect to functional heteroreceptors on excitatory terminals, Vigot et al. (2006) found a much smaller inhibition of excitatory synaptic transmission by baclofen in GB1a−/−, mice than in wt or GB1b−/−, mice, suggesting a predominant role of GB1a. Again, the results of our measurements of the release of endogenous glutamate in cortical slices lead to exactly the same conclusion. Also, Shaban et al. (2006) have demonstrated that GB1a containing receptors are the key heteroreceptors mediating presynaptic inhibition at glutamatergic cortical and thalamic afferents in the amygdala, whereas $GABA_B$ autoreceptor mediated presynaptic inhibition at GABAergic terminals was not different in GB1a−/− and GB1b−/− mice when compared to wildtypes.

To our knowledge, this is the first study using release experiments to elucidate the role of 1a and 1b isoforms in presynaptic $GABA_B$ receptor function. Whereas results from electrophysiological experiments usually reflect events occurring at the level of individual cells, biochemical observations such as the ones described here integrate over larger brain areas and therefore allow more generalized conclusions. Therefore, we believe that our work nicely confirms and extends the conclusions drawn from the electrophysiological studies mentioned above.

In summary, our study suggests that GB1a and GB1b differentially contribute to $GABA_B$ auto- and heteroreceptor functions. There is as yet no evidence for pharmacological differences between receptor complexes containing the GB1a and GB1b isoforms (Kaupmann et al. 1998a, b; Malitschek et al. 1998). This is not surprising as the two isoforms do not differ in their ligand binding domains to which all of the currently available competitive agonists and antagonists bind (Bettler et al. 2004). The identification of GB1a as a possible molecular target to selectively interfere with $GABA_B$ heteroreceptor function suggests the further search for pharmacological means to selectively target GB1 isoforms may be warranted.

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