Activation of metabotropic GABA receptors increases the energy barrier for vesicle fusion

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Summary

Neurotransmitter release from presynaptic terminals is under the tight control of various metabotropic receptors. We report here that in addition to the regulation of Ca^{2+} channel activity, metabotropic $GABA_B$ receptors ($GABA_BRs$) at murine hippocampal glutamatergic synapses utilize an inhibitory pathway that directly targets the synaptic vesicle release machinery. Acute application of the $GABA_BR$ agonist baclofen rapidly and reversibly inhibits vesicle fusion, which occurs independently of the SNAP-25 C-terminus. Using applications of hypertonic sucrose solutions, we find that the size of the readily releasable pool remains unchanged by $GABA_BR$ activation, but the sensitivity of primed vesicles to hypertonic stimuli appears lowered as the response amplitudes at intermediate sucrose concentrations are smaller and release kinetics are slowed. These data show that presynaptic $GABA_BRs$ can inhibit neurotransmitter release directly by increasing the energy barrier for vesicle fusion.

Key words: $GABA_B$ receptor, Metabotropic, Neurotransmission, Energy barrier, SNARE, Presynaptic inhibition

Introduction

Metabotropic signalling through γ-aminobutyric acid B receptors ($GABA_BR$) has been implicated in a number of physiological and pathophysiological processes, such as hippocampal rhythmic activity (Scanziani, 2000), seizure disorders and anxiety and depression (Cryan and Kaupmann, 2005). On the cellular level, $GABA_BRs$ are found to be both postsynaptically and presynaptically localized. On the postsynaptic side, they mediate the slow component of the inhibitory postsynaptic potential through activation of K^+ conductances (Luscher et al., 1997; Newberry and Nicoll, 1984). On the presynaptic side, they can act as auto- or hetero-receptors on GABAergic and glutamatergic terminals, respectively. In both cases activation of presynaptic $GABA_BRs$ leads to a decrease in transmitter release (Bowery, 2006). The reduction of transmitter release is for the most part achieved by the closing of voltage-dependent Ca^{2+} channels (VDCCs) (Dunlap and Fischbach, 1981). This conversion of VDCCs into the 'reluctantly opening state' occurs by G-protein βγ-subunit-mediated signal transduction in a membrane-delimited pathway, as has been directly demonstrated for the calyx of Held (Kajikawa et al., 2001; Takahashi et al., 1998).

However, there are also indications that $GABA_BRs$ can modulate transmitter release independently of VDCCs. In hippocampal neuronal cell cultures, it has been shown that both adenosine receptor and $GABA_BR$ activation can reduce the frequency of action potential (AP)-independent miniature excitatory postsynaptic currents (mEPSCs) in pyramidal neurons (Scanziani et al., 1992; Scholz and Miller, 1992). Furthermore, these receptors can inhibit artificially induced transmitter release evoked by ionomycin and α-latrotoxin, which occurs independently of VDCCs (Capogna et al., 1996). It has been argued that this form of $GABA_BR$-mediated inhibition acts 'downstream' of VDCCs, but a possible molecular mechanism for this modulation in the mammalian central nervous system has so far remained elusive. Studies in the lamprey spinal cord provided evidence that the SNARE complex member SNAP-25 is important for presynaptic serotinergic modulation of transmission (Blackmer et al., 2001). These authors showed that this modulation was largely independent of VDCCs but involved a direct $G_{\beta\gamma}$-subunit-mediated pathway acting on the SNAP-25 C-terminus (Gerachshenko et al., 2005).

In the present study, we investigated the mechanism by which $GABA_BRs$ modulate synaptic transmission at hippocampal pyramidal neurons. We hypothesized that a direct interference of $GABA_BR$ signalling with transmitter release at the level of the release machinery would increase the energy barrier for vesicle fusion (Rosenmund and Stevens, 1996). We found that $GABA_BRs$ indeed affect transmission through two G-protein dependent pathways: besides the classical pathway involving reduction of Ca^{2+}-influx through VDCCs, a second, downstream pathway leads to an increase of the energy barrier for vesicle fusion. Notably, this form of inhibition does not require the C-terminus of the SNARE complex member SNAP-25, as it is not affected by Botulinum neurotoxin-A (BoNT-A) treatment.

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Results

Activation of GABA$_B$Rs increases the energy barrier for vesicle fusion

In order to assess the involvement of VDCCs in GABA$_B$R-mediated modulation of transmission, we monitored AP-triggered presynaptic Ca^{2+} transients in the Schaffer-collateral pathway and recorded field excitatory postsynaptic potentials (fEPSPs) in area CA1 in acute hippocampal slice preparations (Fig. 1A). GABA$_B$R activation with baclofen (3–30 μM) profoundly inhibited fEPSP amplitudes. At the same time, baclofen reduced the presynaptic Ca^{2+} signals by a maximum of 28%. In a parallel set of experiments, we reduced the Ca^{2+} concentration in the external solution stepwise and monitored presynaptic Ca^{2+} signals or postsynaptic fEPSPs, while $CaCl_2$ was replaced by equimolar concentrations of $MgCl_2$. A data fit revealed a power function for transmitter release at CA1 synapses with an exponent of 4.1. We correlated postsynaptic responses and presynaptic Ca^{2+} signals from the baclofen experiments with the power function and found that the baclofen effect on synaptic transmission was greater than what would be expected if the drug was acting exclusively on VDCCs (Fig. 1A2). We also recorded mEPSCs from CA1 pyramidal neurons in both standard (2.5 mM) extracellular Ca^{2+}, as well as in Ca^{2+}-free conditions (i.e. 0 mM Ca^{2+} and EGTA in the extracellular solution, data not shown). In both conditions, baclofen reduced the frequency of mEPSCs to a similar degree (reduction in 2.5 mM Ca^{2+}, 26.9±2.6%, n=6, P<0.01; in 0 mM Ca^{2+}, 28.5±3.9%, n=7, P<0.01), whereas the mEPSC amplitude remained unchanged. In summary, the data from the slice experiments indicate that GABA$_B$R activation has an additional effect downstream of the modulation of VDCCs.

To investigate whether GABA$_B$Rs trigger a signalling cascade by acting directly on the release machinery, we used autaptic cultures of hippocampal pyramidal neurons. Combined with techniques that allow for rapid application of hypertonic solutions, this system enabled us to study the efficiency of vesicle exocytosis in presence and absence of GABA$_B$R activation (Basu et al., 2007; Gerber et al., 2008), while bypassing Ca^{2+} triggering of neurotransmitter release (Rosenmund and Stevens, 1996). We first determined the sensitivity of autaptic EPSCs to baclofen treatment, which gave similar effects

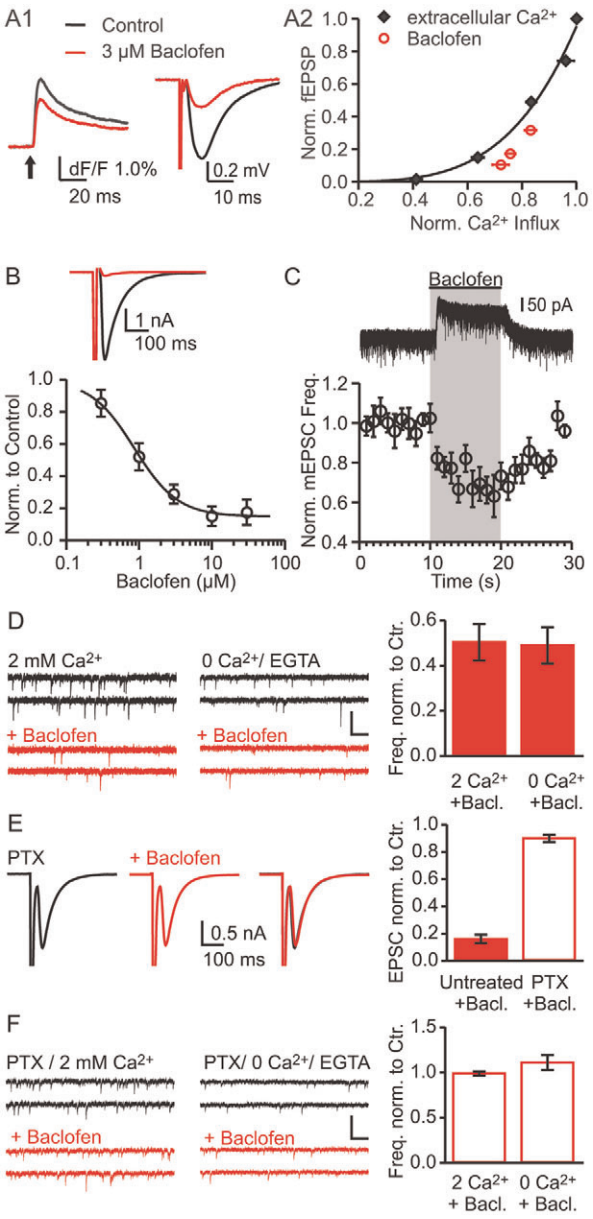

Fig. 1. Activation of GABA$_B$Rs at glutamatergic terminals inhibits transmission in both VDCC-dependent and -independent manner. (**A1**) Traces on the left-hand side show presynaptic Ca^{2+} transients in Schaffer-collaterals determined from wide-field epifluorescence measurements with a single photodiode. Transients evoked with a single extracellular stimulus were reduced by the GABA$_B$R agonist baclofen (3 μM). Traces on the right-hand side show extracellularly recorded fEPSPs in the stratum radiatum of area CA1. Baclofen (3 μM) also decreases the fEPSPs but to a greater extent. (**A2**) Power–function relationship for transmitter release at the Schaffer-collateral synapse, describing the relationship between presynaptic Ca^{2+}-influx and postsynaptic response. Black diamonds denote measurements in which the extracellular Ca^{2+} concentration was changed (2.5, 2, 1.5, 1 and 0.5 mM). Values are normalized to control condition with 2.5 mM Ca^{2+}. Red circles depict the effect of baclofen (3, 10 and 30 μM) on Ca^{2+}-influx and fEPSP. Baclofen has a stronger effect on transmission than expected from the changes of presynaptic Ca^{2+} influx (all n=5 or 6). (**B**) Dose–response curve for baclofen effects on EPSCs in autaptic cultures. Traces show EPSCs in control conditions and upon treatment with 30 μM baclofen (n=4–7). (**C**) Activation of GABA$_B$Rs with baclofen (30 μM) reversibly reduces the frequency of mEPSCs in autaptic cultures. The graph plots the timecourse of the effect for n=9 experiments. A representative trace (top) depicts both mEPSCs and an outward current (upward deflection) induced by baclofen, mediated by postsynaptic GIRK channels. Note the similar timecourse of mEPSC reduction and GIRK channel current activation by baclofen. (**D**) Traces of mEPSCs recorded in 2 mM Ca^{2+} and Ca^{2+}-free (+1 mM EGTA) conditions. In both cases, baclofen reduced the frequency of mEPSCs. The bar graph on the right-hand side summarizes the baclofen effect for both conditions (n=7 and 8). Values are normalized to the mEPSC frequency before baclofen application. (**E**) Incubation of cultures with pertussis toxin (PTX) prevents inhibition of autaptic EPSC by baclofen (black trace, control; red trace, 30 μM baclofen; the rightmost traces are an overlay). The bar graph summarizes the results on AP-evoked EPSCs from control and PTX-incubated cultures for n=6 experiments, indicating the involvement of G_i or G_o in GABA$_B$R-mediated modulation. (**F**) Likewise, the frequency of mEPSCs in autaptic cultures is insensitive to baclofen after incubation with PTX. Traces on the left show mEPSCs in PTX-treated cultures in 2 mM Ca^{2+} and Ca^{2+}-free conditions (upper traces, black) and after addition of 30 μM baclofen (lower traces, red). The bar graph on the right-hand side shows as summary of the insensitivity of mEPSCs to baclofen after PTX treatment (n=8 and n=7). Scale bars: 100 ms (x-axis), 100 pA (y-axis) (D,F).

Table 1. Frequency, amplitude and kinetics of mEPSCs measured in autaptic cultures in Ca^{2+} and Ca^{2+}-free conditions.

	2 mM Ca^{2+} (n=7)			0 Ca^{2+} (n=8)		
Parameter	Control	Baclofen	P-value	Control	Baclofen	P-value
Frequency (Hz)	17.51±3.9	8.6±2.8	$P<0.05$	9.1±2.8	5.3±2.2	$P<0.001$
Amplitude (pA)	21.6±2.6	22.1±2.9	NS	18.1±1.4	16.9±1.5	NS
Rise time (milliseconds)	0.35±0.03	0.33±0.02	NS	0.35±0.02	0.35±0.02	NS
Half width (milliseconds)	2.62±0.10	2.59±0.11	NS	2.62±0.06	2.65±0.11	NS
Decay time constant (milliseconds)	4.61±0.35	4.31±0.19	NS	4.28±0.21	4.35±0.24	NS

Statistics were evaluated using paired two-tailed Student's t-test. NS, not significant.

to those from the fEPSP recordings in hippocampal slices (Fig. 1B). Next, we applied pulses of baclofen (30 μM) to investigate the kinetics of the $GABA_BR$ effect on neurotransmission (Fig. 1C). A reduction of mEPSC frequency was already present within the first second of baclofen application, which is similar to the outward current by postsynaptic G-protein gated inward rectifier (GIRK) channels. The speed of this effect renders phosphorylation-dependent processes rather unlikely, as it displays comparable latencies to the postsynaptic activation of GIRK channels, which is mediated by $G_{\beta\gamma}$ subunits.

We also found that $GABA_BR$ activation decreased mEPSC frequency in Ca^{2+}-free (+1 mM EGTA) conditions to a similar extent as in standard extracellular Ca^{2+}, further indicating a signalling cascade acting downstream of VDCCs (Fig. 1D, Table 1). It has recently been shown that activation of G-protein βγ-subunits alters vesicles fusion properties and thereby changes the kinetics and amplitudes of EPSCs (Photowala et al., 2006). In our recordings, we did not detect differences in the kinetics or amplitudes of mEPSC following application of baclofen (Table 1), which suggests that $GABA_BR$-mediated presynaptic inhibition does not alter the fusion mode of synaptic vesicles. We confirmed that this effect involves G_i or G_o proteins, as preincubation of cultures with 0.5 μg/ml pertussis toxin (PTX) abolished baclofen-mediated inhibition of EPSCs (Fig. 1E) and mEPSCs (Fig. 1F). In contrast to earlier reports indicating that presynaptic GABA effects are independent of G_i or G_o (Dutar and Nicoll, 1988; Thompson and Gahwiler, 1992), these results argue for a G_i or G_o-dependent effect of $GABA_BRs$

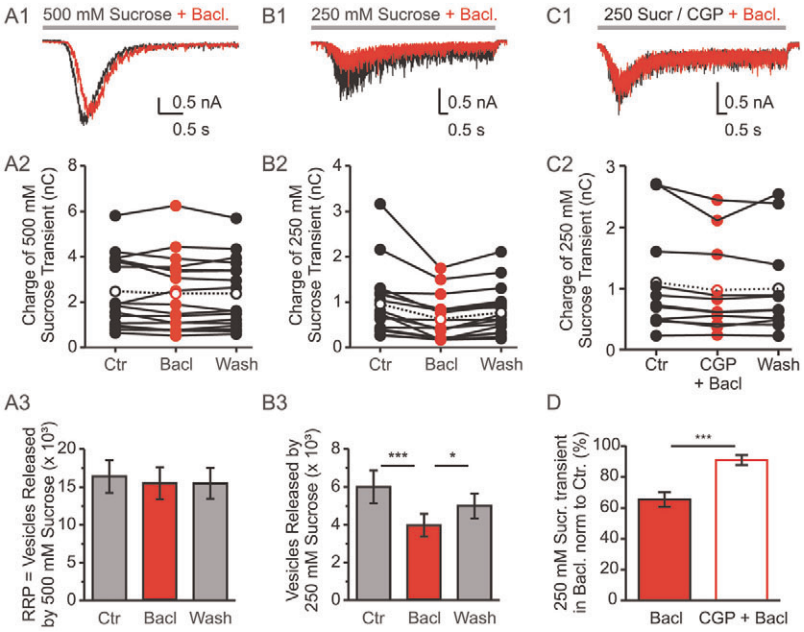

Fig. 2. $GABA_BR$ activation does not change the size of the RRP, but decreases the number of vesicles released by 250 mM sucrose. (**A1**) Current responses in autapses to 500 mM sucrose solutions applied for 4 seconds with a fast application system in control conditions (black trace) and in the presence of 30 μM baclofen (red trace). (**A2**) Scatter plot showing the charge evoked by 500 mM sucrose for n=17 experiments in control conditions, upon baclofen (30 μM) treatment and after wash out. Individual experiments (filled circles) are connected by lines, open circles denote average values for all experiments. Baclofen has no effect on the charge with this pool-depleting stimulus. (**A3**) The number of vesicles released by 500 mM sucrose, which by definition equals the RRP, is unaffected by addition of baclofen as depicted by the bar graph. (**B1**) Currents evoked with a submaximal concentration of 250 mM sucrose (black trace). In the presences of baclofen (red trace), the current response to 250 mM sucrose is clearly reduced. (**B2**) The plot shows the reduction of charge evoked with 250 mM sucrose by baclofen for n=17 experiments (filled circles). Average values for all such experiments are depicted with open circles. (**B3**) Bar graph summarizing the significant reduction of the number of vesicles released with submaximal sucrose application upon $GABA_BR$ activation with baclofen. (**C1**) The effect of baclofen on the 250 mM sucrose response is specifically blocked by preincubation with the $GABA_BR$ antagonist CGP 55845 (2 μM). (**C2**) Scatter plot of n=11 recordings where baclofen was applied in the presence of CGP 55845. (**D**) Direct comparison of the baclofen effect on currents evoked by 250 mM sucrose in absence or presence of the antagonist. *$P<0.05$, ***$P<0.0001$.

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on transmitter release, both at the level of VDCCs and further downstream.

To gain further insight into the mechanism of VDCC-independent modulation of transmission by GABA$_B$Rs, we used pulsed application of hypertonic solutions onto autaptic cultures. Application of 500 mM sucrose can be used to test the readily releasable pool (RRP) of vesicles (Rosenmund and Stevens, 1996), whereas the sensitivity of the stimulated release upon application of intermediate hypertonicity probes the energy barrier for vesicle fusion (Basu et al., 2007; Gerber et al., 2008). Application of 30 µM baclofen did not alter the size of the RRP [Fig. 2A, 500 mM control, $16.3(\pm 2.2)\times 10^3$ compared with + baclofen, $15.4(\pm 2.1)\times 10^3$ vesicles; $n=17$, $P>0.05$], whereas responses to 250 mM sucrose were significantly reduced by baclofen [Fig. 2B, 250 mM control, $5.9(\pm 0.8)\times 10^3$ vesicles compared with + baclofen: $3.9(\pm 0.6)\times 10^3$; $n=17$, $P<0.001$]. Consequently, the fraction of vesicles released by 250 mM sucrose significantly decreased from $38.8(\pm 3.5)$% to $26(\pm 2.5)$% (values normalized to the RRP size determined with 500 mM sucrose), which amounts to a reduction of ~33%. We verified the specificity of the baclofen effect on hypertonicity-evoked release by performing sucrose applications in neurons preincubated with 2 µM CGP 55845, a high-affinity GABA$_B$R antagonist. In presence of the antagonist, inhibition of EPSCs by 30 µM baclofen was essentially abolished (supplementary material Fig. S1A). CGP 55845 also strongly reduced the inhibitory effect of baclofen on release evoked by 250 mM sucrose [Fig. 2C1–C2, 250 mM control, $1.09(\pm 0.26)$ nC compared with + baclofen, $0.97(\pm 0.22)$ nC; $n=11$, $P<0.05$]. The decrease in CGP 55845 was significantly smaller than the effect of baclofen seen in absence of the GABA$_B$R antagonist (Fig. 2D, $P<0.001$, unpaired two-sided Student's *t*-test), confirming that the baclofen-induced increase of the energy barrier for vesicle fusion is specifically mediated by activation of GABA$_B$Rs.

Furthermore, we found that the peak vesicular release rate was significantly reduced in the presence of baclofen for both 500 and 250 mM sucrose applications [Fig. 3A,B, values normalized to 500 mM control, 500 mM sucrose + baclofen, $80.5(\pm 3.9)$%, $n=17$, $P<0.0001$; 250 mM control, $13.1(\pm 1.1)$% and 250 mM sucrose + baclofen, $7.0(\pm 0.6)$%, $n=17$, $P<0.0001$]. In addition, the onset kinetics were significantly slower upon GABA$_B$R activation [Fig. 3C1, 500 mM control, $0.52(\pm 0.01)$ seconds compared with + baclofen, $0.64(\pm 0.02)$ seconds, $n=17$, $P<0.0001$; 250 mM control, $0.91(\pm 0.06)$ seconds compared with + baclofen: $1.2(\pm 0.10)$ seconds, $n=17$, $P<0.001$]. In line with these results, the decay kinetics of the sucrose-evoked current transients were slower for 500 mM sucrose [Fig. 3C1, 500 mM control $0.42(\pm 0.03)$ seconds + baclofen $0.51(\pm 0.02)$ seconds; $n=17$, $P<0.001$]. As expected, co-application of the GABA$_B$R antagonist CGP 55845 blocked the decelerating effect of baclofen on hypertonicity-evoked release for both 500 and 250 mM sucrose (supplementary material Fig. S1C). In summary, these findings indicate that GABA$_B$R signalling directly affects vesicle release, independently of VDCCs. As the RRP size is unaltered, it rather seems that the fusion process itself is affected.

BoNT-A treatment does not prevent VDCC-independent inhibition

The above data strongly argue for a scenario in which GABA$_B$R activation affects the release machinery in a way that increases the energy demand for overcoming the fusion barrier for vesicle release. Which specific components of the release apparatus might be affected by GABA$_B$R activation? Considering the

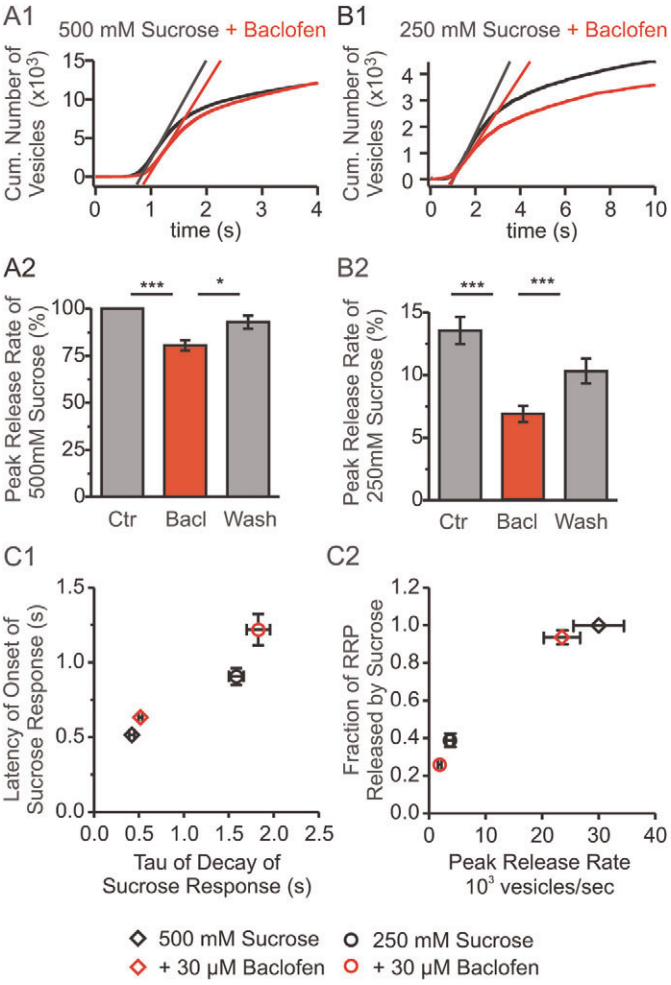

Fig. 3. GABA$_B$R activation alters the kinetics of sucrose-evoked release. (**A1**) Cumulative number of vesicles released in 500 mM sucrose (black curve) from a representative experiment. Baclofen application (red curve) slows the number of vesicles released with respect to time but leaves the total number of released vesicles unaltered. The peak release rate is determined from the maximum slope of the curves (straight lines in black and red, respectively). The bar graph (**A2**) summarizes the significant effect of baclofen application on the peak release rate evoked with 500 mM sucrose for $n=17$ experiments. Values are normalized to the release rate before baclofen application. (**B1**) Example experiment demonstrating the cumulative release rate for a submaximal stimulus with 250 mM sucrose (black curve). In the presence of baclofen (red) both the release rate and the maximum number of released vesicles are reduced. (**B2**) The effect of baclofen on the peak release rate, as determined from the straight line fits in B1, is summarized in the bar graph for all experiments, normalized to the release rates in 500 mM sucrose ($n=17$). The peak release rate is again significantly reduced by baclofen. (**C1**) Correlation plot showing the effect of baclofen on the response latency and decay time constant of sucrose-induced current transients. For 500 mM (black diamond) and 250 mM (black circle) sucrose stimuli, addition of baclofen (red diamond and circle, respectively) prolongs the onset latency and the decay time constant of the current transients. (**C2**) Graph plotting the fraction of vesicles released from the RRP against the peak release rate in the absence or presence of baclofen. As GABA$_B$R activation decreases the peak release rate, it also decreases the fraction of vesicles released from the RRP during submaximal hypertonic stimuli ($n=17$ for all panels). *$P<0.05$, ***$P<0.0001$.

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literature, a probable candidate would be a member of the proteins forming the SNARE complex, specifically SNAP-25 (Blackmer et al., 2001; Gerachshenko et al., 2005). We incubated cultures with BoNT-A, which cleaves nine C-terminal amino acids from SNAP-25 and could thereby block SNAP-25-dependent effects of baclofen. To achieve full cleavage of the protein in neuronal cell culture, a 48-hour incubation with 500 ng/ml BoNT-A was required, as observed by western blotting (Fig. 4A). Cleavage of SNAP-25 in autaptic neurons resulted in complete silencing of transmission with respect to both AP-evoked and spontaneous release (Fig. 4B,C). Moreover, incubation with BoNT-A also prevented vesicle release with hyperosmotic solutions (Fig. 4B). In order to test the possible block of $GABA_BR$-mediated reduction of vesicle release by SNAP-25 cleavage, we used 2 μM ionomycin to restore release by introducing artificial Ca^{2+}-permeable pores in BoNT-A-treated cultures (Capogna et al., 1997). Application of ionomycin led to a steady inward current with multiple superimposed release events (Fig. 4C). All ionomycin-induced currents were completely blocked by 10 μM NBQX [an 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid (AMPA)-receptor antagonist], confirming that this procedure evoked no other conductances except for AMPA-receptor-mediated currents (Fig. 4C, Fig. 5A). We calculated the total charge of release over 4 seconds before, during and after baclofen application and found that baclofen reduced the ionomycin-evoked charge by 27% in control cultures [Fig. 4D,E, untreated ionomycin + baclofen, 73.4(±6.5)% of control; $n=11$, $P<0.05$] and by 38% in BoNT-A treated cultures [Fig. 4D,E, BoNT-A-treated ionomycin + baclofen, 61.5(±5.7)% of control; $n=9$, $P<0.05$]. This effect was not significantly different between the two conditions ($P=0.2$). Therefore, we conclude that $GABA_BR$ activation increases the barrier for vesicle fusion independently of SNAP-25. This set of experiments was performed in the continuous presence of SCH 23390 (20 μM) in order to block postsynaptic GIRK channels activated by $GABA_BRs$ (supplementary material Fig. S2). The effectiveness of SCH 23390 was verified in all experiments. In another series of experiments, we applied baclofen in the presence and absence of 10 μM NBQX instead of SCH 23390. This enabled us to subtract the baclofen-induced GIRK channel currents from the ionomycin experiments (Fig. 5A–C). In addition, baclofen reduced the ionomycin-induced transmitter release in control [Fig. 5B,D, 66.6(±7.2)%, $n=9$, $P<0.001$], as

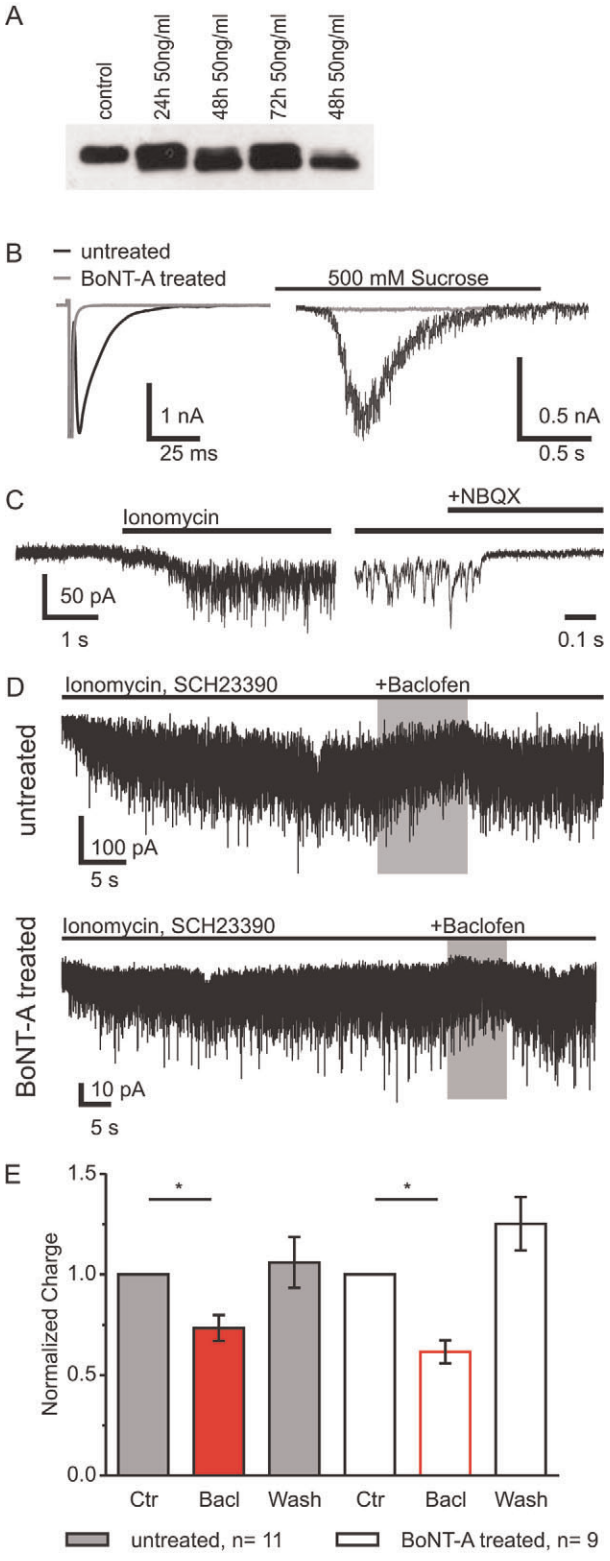

Fig. 4. Cleavage of SNAP-25 with BoNT-A does not abolish Ca^{2+}-independent presynaptic inhibition by $GABA_BRs$. (**A**) Western blot demonstrating the efficacy of SNAP-25 cleavage by BoNT-A. A 48-hour incubation of neuronal cultures with 500 ng/ml BoNT-A is sufficient for full cleavage of SNAP-25 (rightmost lane). The toxin cleaves the C-terminal nine amino acids, reducing the molecular mass from 25 to 22.5 kDa. Double bands for 24- to 72-hour incubations with 50 ng/ml BoNT-A indicate incomplete cleavage. (**B**) BoNT-A treatment completely abolishes AP-triggered and sucrose-evoked transmitter release in autaptic cultures (grey traces). (**C**) Ionomycin (2 μM) was used to restore transmitter release in BoNT-A-treated cultures. A steady inward current is superimposed with multiple individual release events. The trace on the right-hand side shows, on an expanded timescale, the individual ionomycin-induced currents that are completely blocked by the AMPA receptor antagonist NBQX. (**D**) The upper trace depicts the effect of baclofen applied during the steady state of ionomycin-induced vesicle fusion in a control cell. The lower trace shows the effect of baclofen on ionomycin-induced currents in a BoNT-A-treated neuron. In both cases, baclofen reduced the currents to the same extent. (**E**) Bar graph summarizing the reduction of ionomycin-induced currents by baclofen. For quantification the charge was calculated over 4 seconds and normalized to the control. Baclofen significantly reduced the ionomycin-induced currents in control ($n=11$, filled bars) and BoNT-A incubated cultures ($n=9$, open bars). The amount of reduction was not significantly different between the two conditions. *$P<0.05$.

well as in BoNT-A-treated cultures [Fig. 5C,D: 75.6(±10.8)%, $n=10$, $P<0.05$], further corroborating our evidence for a SNAP-25-independent mode of action for GABA$_B$Rs.

Discussion

In the present study, we investigated the effects of GABA$_B$R activation on evoked and spontaneous release from glutamatergic hippocampal neurons and found that, in addition to the VDCC-mediated effects, the activation of presynaptic GABA$_B$Rs leads to a substantial increase in the energy barrier for vesicle fusion. We furthermore demonstrate that, in contrast to other synapses studied previously, this modulation of the release machinery is independent of the C-terminus of SNAP-25.

What are the mechanistic consequences of a release modulation downstream of VDCCs? We addressed this question by challenging autaptic neurons with hypertonic solutions. Similar to high frequency 'trains' of APs, applications of 500 mM sucrose allow to estimate the number of vesicles in the RRP (Rosenmund and Stevens, 1996; Stevens and Williams, 2007; Moulder and Mennerick, 2005). In addition, hypertonic solutions have the advantage of providing a Ca^{2+}-independent stimulus to induce transmitter release, which is indispensable when investigating VDCC-independent inhibition of transmitter release. Hypertonic solutions provide energy to vesicles primed for fusion, comparable to the effect of Ca^{2+}-binding to synaptotagmin. When the tonicity at synapses increases, more primed vesicles reach the energy threshold for fusion. If the energy threshold for primed vesicles is increased by a G_i or G_o-dependent mechanism, responses to subsaturating tonicities, such as 250 mM added sucrose, will decrease in size relative to the maximal response induced by 500 mM sucrose. In addition, the onset of hypertonically induced transmitter release will be delayed and the maximal release rate will decrease. We found that this was indeed the case, as GABA$_B$R activation substantially slows the kinetics of sucrose-evoked fusion and reduces the total number of vesicles released by intermediate hypertonic stimuli. With similar methods, it was previously possible to identify mutations that change the energy barrier for fusion independently of the priming processes further upstream in the presynaptic vesicle cycle. For example, the gain-of-function mutation H567K in the vesicle priming factor Munc13-1 lowers the energy barrier for vesicle fusion, resulting in an increased sensitivity to sucrose challenge and increased spontaneous vesicle release, whereas it does not affect the RRP size (Basu et al., 2007). Conversely, complexin-deficient synapses have a normal RRP size, but show a comparable increase of the energy barrier and reduction in spontaneous release (Xue et al., 2010), as seen for GABA$_B$R activation in the current work. So, our results

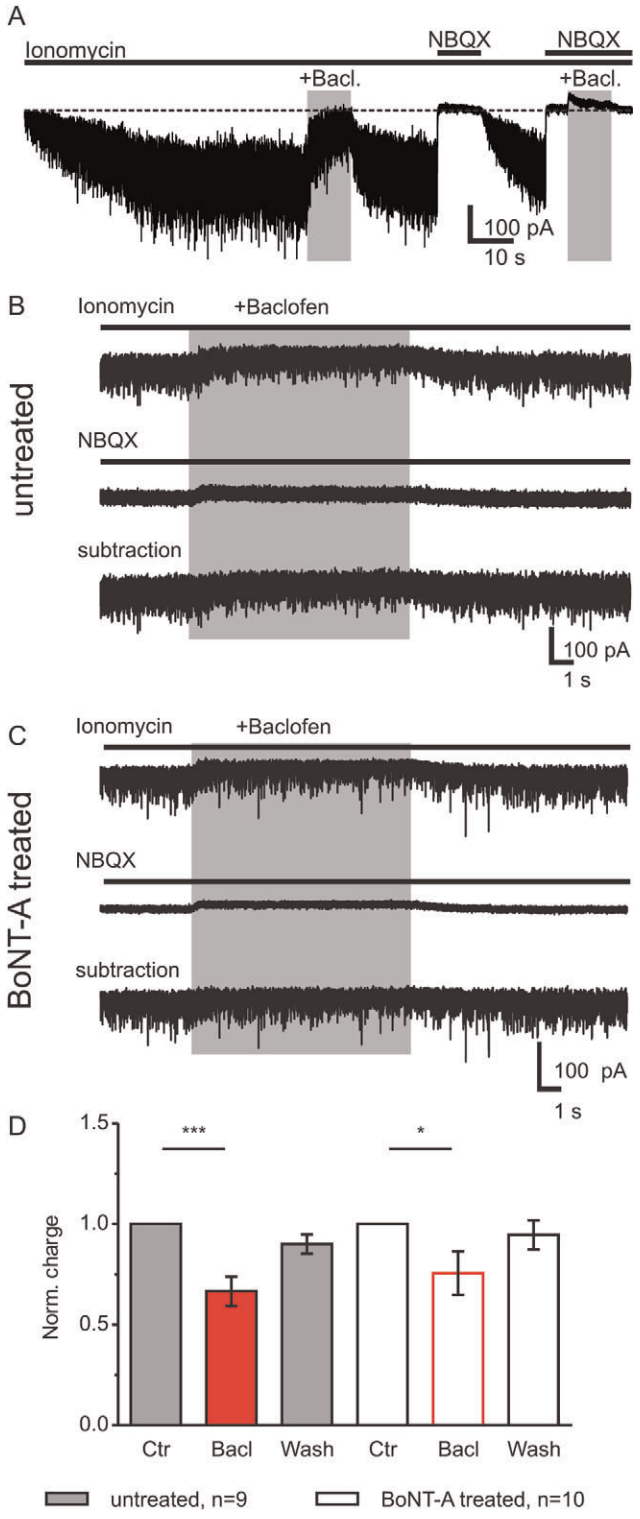

Fig. 5. Subtraction of non-AMPA receptor currents during baclofen application from the ionomycin-induced release reveals that cleavage of SNAP-25 with BoNT-A does not prevent the direct modulation of transmitter release by GABA$_B$Rs. (**A**) Representative experiment with ionomycin application on a control cell and subsequent application of 30 µM baclofen, 10 µM NBQX and baclofen in the presence of NBQX. Block of the AMPA-receptor-mediated currents by NBQX shifts the holding current back to the baseline (broken line). (**B**) Baclofen was applied for 10 seconds during the steady state of ionomycin-triggered release. GIRK-channel-mediated currents activated by postsynaptic GABA$_B$Rs are unveiled by application of baclofen in the presence of NBQX. Post-hoc subtraction of the GIRK channel current reveals a decrease of transmitter release during baclofen application. (**C**) After cleavage of SNAP-25 with BoNT-A, GABA$_B$R activation still decreases ionomycin-induced transmitter release. (**D**) Direct comparison of GABA$_B$R-mediated inhibition of ionomycin-induced release. In both untreated (filled bars, $n=9$) and BoNT-A-treated cultures (open bars, $n=10$), baclofen significantly reduced transmitter release. The effect was not significantly different between control and BoNT-A-treated cells ($P=0.51$). *$P<0.05$, ***$P<0.0001$.

establish a role for a direct modulatory influence on $GABA_BRs$ for the vesicular release machinery in the mammalian synapse.

The C-terminus of the SNARE complex member SNAP-25 has been suggested to be the target for direct modulation of vesicular fusion by GPCRs, as it has been found to be directly affected, through $G_{\beta\gamma}$-subunits, in serotonin-mediated modulation in the spinal cord of lampreys (Blackmer et al., 2001; Gerachshenko et al., 2005). A similar mechanism has been proposed for noradrenaline (norepinephrine)-mediated modulation at glutamatergic fibres onto the central amygdala in rats, also arguing for SNAP-25 as a direct target of metabotropic signalling at mammalian synapses (Delaney et al., 2007). In the case of the lamprey spinal cord, synapse direct modulation was also reported to involve alterations in the opening of the vesicular fusion pore (Photowala et al., 2006; Schwartz et al., 2007). Mechanistically these studies suggested that the interaction of G-protein βγ-subunits with the C-terminus of SNAP-25 competes with synaptotagmin for the binding site to the SNARE complex, thereby hindering tight coupling of the Ca^{2+} sensor to the vesicle (Blackmer et al., 2005). On the basis of three lines of evidence, we rather exclude the SNAP-25 interaction site with synaptotagmin as a target for G-protein βγ-subunit-mediated direct inhibition of neurotransmitter release by $GABA_BRs$. First, we find a reduction of mEPSC frequency by $GABA_BR$ activation in Ca^{2+}-free extracellular solution, arguing against the involvement of the Ca^{2+} sensor. Second, we observe that $GABA_BRs$ cause a reduction in sucrose-evoked transmitter release that is independent of Ca^{2+} (Rosenmund and Stevens, 1996) and synaptotagmin-1 (Xu et al., 2009). Finally, we show that cleavage of SNAP-25 by BoNT-A does not abolish the inhibitory effect of $GABA_BRs$ on ionomycin-evoked release. A possible explanation for the discrepancy between our data and studies that did show an involvement of SNAP-25 is that metabotropic receptors might in fact utilize different pathways at distinct synapses and thereby possess diverse targets for modulation. The completeness of the BoNT-A block might also affect the interpretation of results. We have taken great care to achieve complete cleavage of SNAP-25, necessitating active restoration of release by agents such as ionomycin (Capogna et al., 1997). Therefore, we can exclude the involvement of the SNAP-25 C-terminus in direct inhibition by $GABA_BRs$ at hippocampal synapses. However, our experiments do indicate a $GABA_BR$-mediated direct effect on the core release machinery that is involved in the very late steps of exocytosis. A study in the calyx of Held has demonstrated that $GABA_BR$ activation can directly modulate vesicle priming, leading to a slower refilling of the RRP. This effect, however, was observed only after prolonged stimulation paradigms, and it involved a cAMP-dependent signalling cascade (Sakaba and Neher, 2003). Because we did not detect any changes in the RRP size, the docking and priming steps seem unimpaired by $GABA_BR$ activation during low frequency activity in our preparation. So, direct inhibition of vesicle fusion acts after priming, at a very late step of transmitter release.

Here, we establish a role for a direct modulation of the release machinery during GABAergic inhibition of transmission at glutamatergic synapses, which adds to our understanding of metabotropic signalling. What functional relevance could be attributed to the increased energy barrier seen in our study? Inhibiting release while only slightly reducing Ca^{2+} influx might be advantageous in situations of higher frequency activity, during which a substantial Ca^{2+} influx is required for accelerated replenishing of the RRP (Wang and Kaczmarek, 1998). The exact target of the release machinery that is responsible for this modulation remains to be determined and will be the focus of future studies.

Materials and Methods

All experiments were performed according to the rules of Berlin authorities and the animal welfare committee of the Charité Berlin. For field potential, whole-cell and Ca^{2+}-imaging experiments, sagittal slices (300 μm) were prepared from C57/BL6 mice at postnatal day 19–29. Animals were briefly anesthetized with isofluorane and decapitated. Brains were rapidly removed and chilled in cold solutions containing: 87 mM NaCl, 26 mM $NaHCO_3$, 75 mM sucrose, 25 mM glucose, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 0.5 mM $CaCl_2$ and 7 mM $MgCl_2$, saturated with 95% O_2 and 5% CO_2. Slices were cut on a vibratome (VT 1200S, Leica) and incubated at 34℃ for 30 minutes. They were transferred into the recording solution containing: 119 mM NaCl, 26 mM $NaHCO_3$, 10 mM glucose, 2.5 mM KCl, 1 mM NaH_2PO_4, 2.5 mM $CaCl_2$ and 1.3 mM $MgCl_2$, equilibrated with 95% O_2 and 5% CO_2 at room temperature.

For photodiode Ca^{2+} measurements (Gundlfinger et al., 2007; Regehr and Tank, 1991), slices were placed under an upright microscope (BX51WI, Olympus) equipped with an Olympus LumPlan FI 60× 0.9 NA water-immersion objective. Schaffer collaterals were locally labelled with the low-affinity Ca^{2+} indicator Magnesium Green AM (100 μM, Invitrogen) dissolved in 20% Pluronic/DMSO. Epifluorescence was measured outside of the loading spot at least 30 minutes after labelling. Signals were low-pass filtered at 1 kHz, digitized at 5 kHz and recorded with IGOR Pro (WaveMetrics). AP-triggered change in fluorescence intensity (ΔF) relative to the baseline intensity of fluorescence (F) was calculated as $\Delta F/F$.

Field EPSPs (fEPSPs) were recorded with low-resistance pipettes filled with external solution in the stratum radiatum of CA1 using a Multiclamp 700A (Molecular Devices). Fibres were stimulated at 0.1 Hz. Data were filtered at 2 kHz and digitized at 5 kHz and recorded with IGOR. Extracellular $CaCl_2$ was replaced by equimolar concentrations of $MgCl_2$ to keep the total concentration of divalent cations constant.

Autaptic cultures were prepared as described previously (Bekkers and Stevens, 1991; Pyott and Rosenmund, 2002). For astrocyte precultures, cortices of newborn mice (postnatal day zero, P0) were digested with trypsin. Cells were grown in T75 flasks in BME medium (with 10% fetal calf serum, 1 mM Glutamax, 0.2% penicillin-streptomycin, 10 mM HEPES, 5 mM glucose and 2.5 μg/ml insulin; Invitrogen and Sigma) for 1 week. Growth permissive 200-μm spots of a 1:4 collagen and poly-D-lysine mixture were printed, using a custom-made stamp, on agarose-coated coverslips in six-well plates. 2×10^4 astrocytes from precultures were seeded per well and grown until fully covering the microislands. Before plating hippocampal neurons (3×10^3 cells per well), the medium was changed to Neurobasal A (with 2% B27, 0.2% penicillin-streptomycin; Invitrogen). To obtain neurons, P0 mouse hippocampi were digested using papain (Worthington, 20 units/ml, 1 mM L-cysteine, 0.5 mM EDTA in EBSS) for 60 minutes at 37℃.

Whole-cell voltage clamp recordings were performed with Axopatch 200A or Multiclamp 700B amplifiers using borosilicate electrodes (2–3 mΩ tip resistance) filled with: 146 mM K-gluconate, 17.8 mM Hepes, 1 mM EGTA, 4 mM Mg-ATP, 0.3 mM Na-GTP, 12 mM creatine phosphate and 50 units per ml creatine-phosphokinase (pH adjusted to 7.3 with KOH). Data were filtered at 2–5 kHz and digitized at 5–10 kHz. Fast applications of hypertonic solutions were performed using procedures described previously (Pyott and Rosenmund, 2002; Rosenmund et al., 1995). Sucrose was dissolved in extracellular solution containing: 140 mM NaCl, 2.4 mM KCl, 10 mM HEPES, 10 mM glucose, 2 mM $CaCl_2$ and 4 mM $MgCl_2$ (pH adjusted to 7.3 with NaOH, 300 mOsm).

To determine the efficacy of Botulinum neurotoxin A (BoNT-A), protein lysates of toxin-treated neuronal cultures were analyzed by western blotting using a monoclonal antibody directed against SNAP-25 (Synaptic Systems, Goettingen, Germany).

Data are given as means ± standard error. Significance was determined using paired Student's *t*-test or single-factor ANOVA with Bonferroni's post-hoc test where appropriate. The number of vesicles in the RRP was calculated by normalizing the charge of the 500 mM sucrose transient by the mean mEPSC charge.

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