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\textsuperscript{2+} channel activity, metabotropic GABA\textsubscript{B} receptors (GABA\textsubscript{B}R) at murine hippocampal glutamatergic synapses utilize an inhibitory pathway that directly targets the synaptic vesicle release machinery. Acute application of the GABA\textsubscript{B}R 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\textsubscript{B}R 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\textsubscript{B}Rs can inhibit neurotransmitter release directly by increasing the energy barrier for vesicle fusion.

Key words: GABA\textsubscript{B} receptor, Metabotropic, Neurotransmission, Energy barrier, SNARE, Presynaptic inhibition

Introduction
Metabotropic signalling through \textgamma-aminobutyric acid B receptors (GABA\textsubscript{B}R) 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\textsubscript{B}Rs 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\textsuperscript{+} conductances (Luscher et al., 1997; Dunlap and Fischbach, 1981). 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\textsubscript{B}Rs 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\textsuperscript{2+} channels (VDCCs) (Dunlap and Fischbach, 1981). This conversion of VDCCs into the ‘reluctantly opening state’ occurs by G-protein \textbeta\gamma-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\textsubscript{B}Rs can modulate transmitter release independently of VDCCs. In hippocampal neuronal cell cultures, it has been shown that both adenosine receptor and GABA\textsubscript{B}R 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 \textalpha-latrotoxin, which occurs independently of VDCCs (Capogna et al., 1996). It has been argued that this form of GABA\textsubscript{B}R-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\textbeta-\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\textsubscript{B}Rs modulate synaptic transmission at hippocampal pyramidal neurons. We hypothesized that a direct interference of GABA\textsubscript{B}R 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\textsubscript{B}R activation does affect transmission through two G-protein dependent pathways: besides the classical pathway involving reduction of Ca\textsuperscript{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.
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$_B$R agonists 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).
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 GABABR 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 Gβγ subunits.

We also found that GABABR activation decreased mEPSC frequency in Ca2+-free (+1 mM EGTA) conditions to a similar extent as in standard extracellular Ca2+, 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 Gi or Go 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 Gi or Go (Dutar and Nicoll, 1988; Thompson and Gahwiler, 1992), these results argue for a Gi or Go-dependent effect of GABA BRs.

Table 1. Frequency, amplitude and kinetics of mEPSCs measured in autaptic cultures in Ca2+ and Ca2+-free conditions.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n=7)</th>
<th>Baclofen</th>
<th>P-value</th>
<th>Control (n=8)</th>
<th>Baclofen</th>
<th>P-value</th>
</tr>
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<tbody>
<tr>
<td>Frequency (Hz)</td>
<td>17.51±3.9</td>
<td>8.6±2.8</td>
<td>P&lt;0.05</td>
<td>9.1±2.8</td>
<td>5.3±2.2</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Amplitude (pA)</td>
<td>21.6±2.6</td>
<td>22.1±2.9</td>
<td>NS</td>
<td>18.1±1.4</td>
<td>16.9±1.5</td>
<td>NS</td>
</tr>
<tr>
<td>Rise time (milliseconds)</td>
<td>0.35±0.03</td>
<td>0.33±0.02</td>
<td>NS</td>
<td>0.35±0.02</td>
<td>0.35±0.02</td>
<td>NS</td>
</tr>
<tr>
<td>Half width (milliseconds)</td>
<td>2.62±0.10</td>
<td>2.59±0.11</td>
<td>NS</td>
<td>2.62±0.06</td>
<td>2.65±0.11</td>
<td>NS</td>
</tr>
<tr>
<td>Decay time constant (milliseconds)</td>
<td>4.61±0.35</td>
<td>4.3±0.19</td>
<td>NS</td>
<td>4.28±0.21</td>
<td>4.35±0.24</td>
<td>NS</td>
</tr>
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Statistics were evaluated using paired two-tailed Student’s t-test. NS, not significant.

Fig. 2. GABABR 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 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 presence 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 GABABR activation with baclofen. (C1) The effect of baclofen on the 250 mM sucrose response is specifically blocked by preincubation with the GABABR 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.
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 GABABRs, 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(±2.2)×10³ compared with + baclofen, 15.4(±2.1)×10³ vesicles; n=17, P>0.05], whereas responses to 250 mM sucrose were significantly reduced by baclofen [Fig. 2B, 250 mM control, 5.9(±0.8)×10³ vesicles compared with + baclofen: 3.9(±0.6)×10³; n=17, P<0.001]. Consequently, the fraction of vesicles released by 250 mM sucrose significantly decreased from 38.8(±3.5)% to 26(±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 GABABR 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(±0.26) nC compared with + baclofen, 0.97(±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 GABABR 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 GABABRs.

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(±3.9)% compared with + baclofen, 97(±22)%; n=17, P<0.001]. In addition, the onset kinetics were significantly slower upon GABABR activation [Fig. 3C1, 500 mM control, 0.52(±0.01) seconds compared with + baclofen, 0.64(±0.02) seconds, n=17, P<0.0001]; 250 mM control, 0.91(±0.06) seconds compared with + baclofen: 1.2(±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(±0.03) seconds + baclofen 0.51(±0.02) seconds; n=17, P<0.001]. As expected, co-application of the GABABR 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 GABABR 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 GABABR 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 GABABR activation? Considering the

![Graph](image)
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_B receptor-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_B receptor 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_ARs (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)%], 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. Hypertonic solutions provide energy to vesicles primed for fusion, comparable to the effect of Ca\(^{2+}\)-binding to synaptotagmin. When the tonicinity 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 hypertonicity-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
establish a role for a direct modulatory influence on GABARRs 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 protein-βγ-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 modulator. The completeness of the BoNT-A block might also be attributed to the increased energy barrier seen in our study?

Materials and Methods

All experiments were performed according to the rules of Berlin authorities and the animal-welfare committee of the Charite Berlin. For field potential, whole-cell and Ca2+-imaging experiments, sagittal slices (300 μm) were prepared from C57BL/6 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 NaHCO3, 75 mM sucrose, 25 mM glucose, 2.5 mM KCl, 1.25 mM Na2HPO4, 0.5 mM CaCl2 and 1 mM MgCl2, saturated with 95% O2 and 5% CO2. Slices were cut on a vibratome (VT 1200S, Leica) and incubated at 34°C for 30 minutes. They were transferred into the recording solution containing: 119 mM NaCl, 26 mM NaHCO3, 10 mM glucose, 2.5 mM KCl, 1 mM Na2HPO4, 2.5 mM CaCl2 and 1 mM MgCl2, equilibrated with 95% O2 and 5% CO2 at room temperature.

For photodiode Ca2+ measurements (Gundlfinger et al., 2007; Regehr and Tank, 1991), slices were placed under an upright microscope (BX51WI, Olympus) equipped with an Olympus Lumarpl FL 60 × 0.9 NA water-immersion objective. Schaffer collaterals were locally labelled with the low-affinity Ca2+ indicator Mag-Fura-2 AM (100 μM; Molecular Probes) dissolved in 200 mM 10% 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/F) relative to the baseline intensity of fluorescence (F) was calculated as ΔF/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 CaCl2 was replaced by equimolar concentrations of MgCl2 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 × 104 astrocytes from precultures were seeded per well and grown until fully covering the microfluids. Before plating hippocampal neurons (3 × 103 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/mg, 1 mM L-cysteine, 0.5 mM EDTA in EBSS) for 60 minutes at 37°C.

To determine the efficacy of Botulinum neurotoxin A (BoNT-A), protein lyastes 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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