Depression of Release by mGluR8 Alters Ca\(^{2+}\) Dependence of Release Machinery

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The ubiquitous presynaptic metabotropic glutamate receptors (mGluRs) are generally believed to primarily inhibit synaptic transmission through blockade of Ca\(^{2+}\) entry. Here, we analyzed how mGluR8 achieves a nearly complete inhibition of glutamate release at hippocampal synapses. Surprisingly, presynaptic Ca\(^{2+}\) imaging and miniature excitatory postsynaptic current recordings showed that mGluR8 acts without affecting Ca\(^{2+}\) entry, diffusion, and buffering. We quantitatively compared the Ca\(^{2+}\) dependence of the inhibition of release by mGluR8 with the inhibition by \(\omega\)-conotoxin GVIA. These calculations suggest that the inhibition produced by mGluR8 may be explained by a decrease in the apparent Ca\(^{2+}\) affinity of the release sensor and, to a smaller extent, by a reduction of the maximal release rate. Upon activation of mGluR8, phasic transmitter release toward the end of a train of action potentials is greater as compared with presynaptic inhibition induced by blocking Ca\(^{2+}\) entry, which is consistent with the important role of Ca\(^{2+}\) in accelerating the replenishment of released vesicles. The action of mGluR8 was resistant to blockers of classical G-protein transduction pathways including inhibition of adenylate cyclase and may represent a direct effect on the release machinery. In conclusion, our data identify a mode of presynaptic inhibition which allows mGluR8 to profoundly inhibit vesicle fusion while not diminishing vesicle replenishment and which thereby differentially changes the temporal transmission properties of the inhibited synapse.

Keywords: calcium, metabotropic glutamate receptor, presynaptic, release machinery, transmitter release

Introduction

Metabotropic glutamate receptors (mGluRs) are as widely distributed as their ionotropic counterparts and represent an additional mode of glutamatergic signaling. The most prominent effect of group II and group III mGluRs is to profoundly inhibit transmitter release as auto- and heteroreceptors at many synapses throughout the brain. It is generally believed that these presynaptic mGluRs reduce action potential-driven release primarily by lowering presynaptic Ca\(^{2+}\) entry (Conn and Pin 1997; Wu and Saggau 1997; Anwyl 1999; El Far and Betz 2002), but this has been directly tested at only a few synapses (Takahashi et al. 1996; Czehilla and Alford 1998; Kamiya and Ozawa 1999; Rusakov et al. 2004). In addition to the reduction of Ca\(^{2+}\) entry via inhibition of Ca\(^{2+}\) channels or via activation of K\(^+\) channels, various parts of the release machinery could also be directly targeted by the metabotropic signaling. Evidence from the analysis of the modulation of miniature release suggested that certain subtypes of mGluRs also have a second-

ary effect downstream of Ca\(^{2+}\) entry (e.g., Gereau and Conn 1995; Poncer et al. 1995; Sanzianii et al. 1995; Tyler and Lovingier 1995; Manzoni et al. 1997; Schoppa and Westbrook 1997), but its relevance for action potential-driven release is unclear (Wu and Saggau 1997; Anwyl 1999). Furthermore, it remains unknown which functional properties of the release machinery might be modified by this downstream signaling.

The identification of the targets of this downstream signaling by metabotropic receptors is important for several reasons. First, presynaptic Ca\(^{2+}\) triggers a multitude of processes besides vesicle fusion, including various forms of short- and long-term synaptic plasticity (Nicoll and Malenka 1995; Zucker and Regehr 2002) as well as the accelerated replenishment of released synaptic vesicles (Dittman and Regehr 1998; Stevens and Wesseling 1998; Wang and Kaczmarek 1998). Thus, a metabotropic receptor acting on Ca\(^{2+}\) entry is likely to also concomitantly inhibit other Ca\(^{2+}\)-dependent processes while a receptor directly modulating the release machinery will bypass such side effects. Second, because transmitter release is a highly nonlinear and saturating process (Heidelberger et al. 1994; Bollmann et al. 2000; Scheggenburger and Neher 2000; Lou et al. 2005), the efficacy of inhibitory mechanisms which lower Ca\(^{2+}\) binding by the release machinery will strongly depend on the amount of Ca\(^{2+}\) entry. As a result, the inhibition by such mechanisms will be significantly weakened by, for example, activity-dependent action potential broadening, while the potency of other mechanisms, which, for example, decrease the maximal fusion rate, will be unaffected. Therefore, the transduction target of presynaptic metabotropic receptors plays a pivotal role for the temporal and dynamic transmission properties of the inhibited synapse.

In the hippocampus, there is a strictly pathway-specific expression of different subtypes of mGluRs (Shigemoto et al. 1997). In particular, the 2 projections from the medial and lateral entorhinal cortex to hippocampal granule cells are decorated exclusively with mGluR2/3 and mGluR8, respectively, both of which potently depress glutamate release (Macek et al. 1996; Dietrich et al. 1997). In the present study, we analyze the transduction target of mGluR8, report that this receptor reduces transmitter release entirely without affecting entry and propose mechanistically which functional properties of the release machinery may be modulated by mGluR8.

Materials and Methods

Slice Preparation

Male Wistar rats aged 20–40 days were decapitated, and the brains were rapidly removed and sliced in ice-cold oxygenated solution containing (in mM): 90 NaCl, 3 KCl, 2 MgCl\(_2\), 2 CaCl\(_2\), 1 Na-pyruvate, 10 glucose, 10...
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 90 sucrose, pH was adjusted to 7.4 or containing (in mM): 87 NaCl, 2.5 KCl, 7 MgCl₂, 0.5 CaCl₂, 25 NaHCO₃, 25 glucose, and 75 sucrose (95% O₂, 5% CO₂). Afterward, slices were stored at room temperature (22-24 °C) in solution containing the following (in mM): 124 NaCl, 3 KCl, 2 MgCl₂, 2 CaCl₂, 26 NaHCO₃, and 10 glucose (95% O₂, 5% CO₂). For recording (field excitatory postsynaptic potentials [fEPSPs], patch clamp), slices were transferred to a Haas-type interface chamber and perfused (2-3 mL/min) with artificial cerebrospinal fluid (ACSF) containing (in mM, 35 °C): 125 NaCl, 3 KCl, 2 CaCl₂, 20.3 NaHCO₃, 10 glucose except when extracellular Ca²⁺ concentration was altered (see below). For confocal imaging slices were transferred to a submerged chamber and perfused with ACSF containing (in mM, room temperature): 121 NaCl, 3 KCl, 2 MgCl₂, 2 CaCl₂, 26 NaHCO₃, 10 glucose (95% O₂, 5% CO₂).

**Field Excitatory Postsynaptic Potentials**

fEPSPs were recorded as described (Dietrich et al. 1997). As criterion for the specificity of fiber tracts stimulated medial molecular layer (MML) versus outer molecular layer (OML), we accepted fEPSPs as originating from the lateral perforant path if they showed paired-pulse facilitation > 120% and as originating from the medial perforant path in case of paired-pulse depression of ~80% (McNaughton 1980; Dietrich et al. 1997).

**Extracellular Solutions Containing Different Ca²⁺ Concentrations**

For Ca²⁺ concentrations < 4 mM, the Mg²⁺ concentration of the ACSF was adjusted to maintain the total concentration of these 2 divalent cations at 4 mM. For 4 mM Ca²⁺, the concentration of Mg²⁺ was lowered to 0.1 mM and 25 μM 2-aminosuccinopentanoic acid (α-APV) was added to limit the activation of NMDA receptors.

We employed a HEPES-buffered saline to reliably avoid the partial precipitation of Ca²⁺ when using 10 and 20 mM Ca²⁺. Initial experiments were carried out to find the Ca²⁺ and Mg²⁺ concentration required in HEPES to produce an equivalent paired-pulse facilitation because the activity of these ions is expected to be higher in the absence of NaH₂CO₃. We found the following "normal HEPES” saline to be "paired-pulse equivalent” to the above-mentioned ACSF (in mM): 133 NaCl, 3 KCl, 2 CaCl₂, 1 MgCl₂, 19 glucose, 15 HEPES (pH 7.4, NaOH). The inhibition of fEPSPs by 3 μM 4-phosphonophenylglycine (PPG) was also similar in both solutions to 60 ± 2.4% (n = 21) and to 58 ± 4.2% (n = 14) in ACSF and normal HEPES, respectively. To reliably identify fibers of the lateral perforant path, each experiment applying 10 or 20 mM Ca²⁺ was started in normal HEPES to verify paired-pulse facilitation and the inhibition of fEPSPs by PPG before high Ca²⁺ concentrations were applied to conduct the experiment. Ten millimolar Ca²⁺ HEPES contained (in mM): 133 NaCl, 3 KCl, 10 CaCl₂, 1 MgCl₂, 8 glucose, 7 HEPES, 0.025 mM α-APV (pH 7.4, NaOH). Twenty millimolar Ca²⁺ HEPES contained (in mM): 120 NaCl, 3 KCl, 20 CaCl₂, 1 MgCl₂, 5 glucose, 6 HEPES, 0.025 mM α-APV (pH 7.4, NaOH).

For the experiments involving application of a low (20 μM) concentration of Cd²⁺ to achieve a rapid and partial block of transmission (Fig. 4B), we also used HEPES saline to prevent complexation of Cd²⁺ with bicarbonate.

**Miniature Excitatory Postsynaptic Currents**

"Blind" whole-cell patch-clamp recordings were obtained from hippocampal granule cells as described previously (Dietrich et al. 2002; Podlogar and Dietrich 2006) with a pipette solution containing (in mM): 125 potassium gluconate, 10 HEPES, 0.5 ethylene glycol-bis(2-aminoethylether)-N,N,N’,N’-tetra-acetic acid (EGTA), 2 MgCl₂, 23 KCl, 3 NaCl, pH adjusted to 7.3. Voltages were corrected for a liquid junction potential by offsetting the amplifier to -10 mV before seal formation. Cells were held in voltage clamp at -70 mV, and membrane current was recorded nearly continuously in 10 s long sweeps. Miniature excitatory postsynaptic current (mEPSCs) were recorded from hippocampal granule cells at 35 °C using the ACSF described above. Series resistance ranged between 15 and 25 MΩh. Signals were filtered at 1 kHz and sampled at 10 kHz. mEPSCs were analyzed by the sliding template algorithm (Clements and Bekkers 1997) provided by NeuroMatic (V1.71) for Igor Pro (V5) with a threshold of 4. For each cell a template was created by averaging ~50 manually selected very slow currents. All detected events were visually inspected and discarded in case of doubt (~20% in each cell).

**Confocal Calcium Imaging**

For imaging of intraterminal Ca²⁺ transients, fibers were loaded with the low-affinity Ca²⁺ indicator Mg-Green acetoxyxymethyl ester (Molecular Probes, the Netherlands) as described previously (Dietrich et al. 2003). Alternatively, slices were dye labeled on the stage of the confocal microscope according to the protocol described in Kukley et al. (2007). A glass pipette containing the Ringer-diluted dye was placed in stratum molecular along the course of the axons for 30–45 min while applying a slight pressure to the back of the pipette. For data acquisition, scans were obtained perpendicular to the orientation of the molecular layers at 1-3 kHz. To evoke action potentials and Ca²⁺ entry, fibers were stimulated with a glass electrode as for fEPSPs. Excitation wavelength was 488 nm, and the emitted fluorescence was collected by a 20× objective lens long-pass filtered at 505 nm. The pinhole was set to maximal size. Changes in fluorescence were quantified as ΔF/ΔF. For illustration, the inhomogeneity of fluorescence along the line scan was removed by normalizing on an average fluorescence line profile taken before stimulation (cf. Supplementary Figs 1 and 2). Calcium imaging experiments and electrophysiological recordings of synaptic transmission were always executed in different slices and not performed simultaneously to avoid any alterations of transmitter release by the presence of the presynaptic calcium indicator dye.

**Drugs**

The following drugs were bath applied: (from Tocris): (RS)-PPG, (S)-3,4-dicarboxyphenylglycine (DCGP), 6-cyano-7-nitroquinoline-2,3-dione (CNQX), di-APV, (RS)-¿-cyclopropyl-¿-phosphonophenylglycine (CPPG), (RS)-¿-methyl-¿-phosphonophenylglycine, (2X2R®)-¿-cyclopropyl-¿-phosphonophenylglycine (DCG), 4-(4-octadecyl)- ¿-oxobenzenebutenoic acid (OOAB), staurosporine, forskolin, tetrodotoxin, ZD7288, and U73122 (from Alexis): H-7, 1H-[1,2,4-oxadiazolo]4-[3-[4-quinolinol-1-one (ODQ), mastoparan, ryanodine, lavendustin A, H-89, 2,5-dideoxyadenosine (DDA), and cис-N-(2-phenylcyclopropyl)-azacyclotridec-1-¿-amine (MDL 12,330A); (from Calbiochem): 9-(tetrahydro-2-furanyl)-9H-purin-6-amine (SQ22536), pertussis toxin, and 2-(2-amino-3-methoxyphenox)ethylamine (H-1- benzopryran-1-one (PD 98059); (from Sigma): ophiobolin A, baclofen, adenosine, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), calmidazolium, ¿-aminopropyl-¿-thiacetone; 2-(trifluromethyl)phenylace- tonitrile (SL327), and MRS1191. Substances with intracellular targets were preapplied for at least 30 min to allow for transmembraneous diffusion.

Statistical significance was tested by the 2-tailed students t-test (α = 0.05) unless stated differently. All data are given as mean ± standard error of the mean.

**Results**

fEPSPs of the lateral entorhinal cortex to hippocampal granule cell projection were evoked by stimulation and recorded in the outer molecular layer of the dentate gyrus. fEPSPs were highly sensitive to the group III mGlur agonist (RS)-PPG. A Hill fit to the concentration response curve revealed that PPG depresses synaptic transmission maximally by 79% with an EC₅₀ (concentration of half-maximal effect) of 0.53 μM and a slope of 0.89 (Fig. 1A, B, n = 6). The action of 3 μM PPG was blocked in a concentration-dependent manner by the group III mGlur- specific antagonist (RS)-¿-CPPG with an IC₅₀ of 3.5 μM (Fig. 1B). The inhibition of fEPSPs was also seen when applying the mGlur8-specific agonist DCPG (1 μM, Supplementary Fig. 3). These data, together with previous work in mGlur8 knockout mice, using mGlur8-specific antibodies and pharmacological characterization (Dietrich et al. 1997; Shigemoto et al. 1997; Zhai et al. 2002), strongly suggest that the stimulated synapses
in the outer molecular layer contain a high density of presynaptic mGluR8.

Since it is a common scenario that presynaptic neurotransmitter receptors reduce transmitter release by inhibition of voltage-gated Ca\textsuperscript{2+} channels (Wu and Saggau 1997), we analyzed presynaptic Ca\textsuperscript{2+} entry by confocal Ca\textsuperscript{2+} imaging as described (Dietrich et al. 2003) (for details and controls, see Supplementary Figs 1 and 2). Lateral perforant path fibers were loaded with the low-affinity Ca\textsuperscript{2+} indicator Mg-Green. Fibers were stimulated as for fEPSPs, and fluorescent transients were selectively recorded in the OML in line scan mode (Fig. 2A). The response amplitudes to 3 stimulations at 25 Hz did not diminish (Fig. 2A, 101 ± 2% and 101 ± 2% for the second and third amplitude, respectively, n = 4) suggesting that the indicator dye was far from saturation. Combined application of 10 μM CNQX and 50 μM APV did not affect the fluorescent transient (not shown, n = 3) consistent with the presynaptic origin of the signals. Furthermore, we used patch-clamp and fluorescent recordings from dentate granule cells to verify the absence of a postsynaptic Ca\textsuperscript{2+} signal (Supplementary Fig. 2).

Contrasting with many other presynaptic receptors, activation of mGluR8 by 3 μM PPG did not affect presynaptic Ca\textsuperscript{2+} entry (Fig. 2B, n = 6) while the same concentration of PPG significantly reduced transmitter release (Fig. 2C, n = 6). Identical results were obtained with the specific mGluR8 agonist DCG: At 1 μM, DCG reduced fEPSPs to 30 ± 2% (n = 4) but did not notably decrease presynaptic Ca\textsuperscript{2+} entry (n = 4, Supplementary Fig. 3). To verify that we were able to track changes in presynaptic Ca\textsuperscript{2+} entry, we performed imaging experiments and fEPSP recordings in the MML, where fibers of the medial perforant pathway carrying group II mGluRs are found (Macek et al. 1996; Dietrich et al. 1997; Shigemoto et al. 1997). As shown in Figure 2D, the group II mGluR-specific agonist DCG profoundly reduced presynaptic Ca\textsuperscript{2+} entry at the medial perforant path-granule cell synapse. With the published value of Ca\textsuperscript{2+} cooperativity of transmitter release at this synapse (3.5, Qian and Noebs 2001) the reduction of presynaptic Ca\textsuperscript{2+} entry by DCG almost completely explains the amount of depression of synaptic transmission at medial perforant path synapses (Fig. 2D,E).

To substantiate the conclusion that mGluR8 inhibits transmitter release independently of voltage-gated Ca\textsuperscript{2+} channels, we tested whether PPG would modulate the frequency of mEPSCs when Ca\textsuperscript{2+} channels are already blocked by Cd\textsuperscript{2+}. Nearly complete blockade of Ca\textsuperscript{2+} channels was obtained by perfusion of 100 μM Cd\textsuperscript{2+} (Fig. 3A,B). In the presence of Cd\textsuperscript{2+}, 1 μM tetrodotoxin and 50 μM picrotoxin mEPSCs were frequently observed in whole-cell recordings from granule cells in hippocampal slices (Fig. 3C). Because only synapses contacting the most distal portion of the dendrites of granule cells carry mGluR8, we aimed at identifying those synaptic currents that originated in this distal area (Macek et al. 1996; Dietrich et al. 1997; Shigemoto et al. 1997). Due to electrotonic filtering, these distal synaptic currents can be assumed to show a particularly slow rise time when compared with currents arising more proximally (Schmidt-Hieber et al. 2007). Therefore, we designed a detection template (see Materials and Methods) to preferentially identify currents originating from lateral perforant path synapses by averaging ~50 mEPSCs which showed the slowest rise times during an initial screening analysis in each cell (Fig. 3D, rise time\textsuperscript{10-90%} = 2.6 ± 0.1 ms, t\textsubscript{decay} = 11 ± 1.9 ms, n = 4). mEPSCs identified with this template were clearly reduced in frequency (to 47 ± 7%, n = 4) but not in amplitude (105 ± 4%, n = 4) by the application of PPG despite the preblock of Ca\textsuperscript{2+} channels by Cd\textsuperscript{2+} (Fig. 3D,E). For comparison, we verified in the same cells that the frequency of synaptic currents detected with fast rising and decaying templates (rise time\textsuperscript{10-90%} = 0.7 ± 0.15 ms, t\textsubscript{decay} = 3.9 ± 0.6 ms) was not reduced by PPG (114 ± 30% of control, not significant, n = 4).
We further explored the inhibition of action potential-stimulated release by relating the depression of fEPSPs by PPG to that by the well-known blockers of voltage-gated Ca\(^{2+}\) channels: α-conotoxin GVIA (1 μM) and Cd\(^{2+}\) (20 μM, yielding a partial block). In order to compare the dependence of release inhibition on the local intracellular Ca\(^{2+}\) concentration produced by the presynaptic action potential, we varied the amount of Ca\(^{2+}\) entry by altering the extracellular Ca\(^{2+}\) concentration. As can be seen in Figure 4A,B, α-conotoxin GVIA (n = 6 and 4 for high and low Ca\(^{2+}\)) and Cd\(^{2+}\) (n = 7 and 4) are much less effective at inhibiting release in 4 mM extracellular Ca\(^{2+}\) when compared with 1/0.85 mM Ca\(^{2+}\). In contrast, the potency of PPG only weakly, though statistically significantly, depended on the extracellular Ca\(^{2+}\) concentration (Fig. 4C,D, n = 11 and 9). We further analyzed this differential dependence on extracellular Ca\(^{2+}\) more quantitatively.

Mechanistically, there are 3 general categories by which a presynaptic metabotropic receptor can achieve an inhibition of transmitter release. First, it can decrease the local free Ca\(^{2+}\) concentration at the Ca\(^{2+}\) sensor of vesicle fusion, either by directly affecting Ca\(^{2+}\) channels or indirectly, for example, by modulation of action potential properties. Second, it can reduce the Ca\(^{2+}\) sensitivity of the Ca\(^{2+}\) sensor without changing the free Ca\(^{2+}\) concentration. Third, it can reduce the maximal release probability, for example, by reducing the overall number of docked vesicles or by decreasing the maximal fusion willingness of docked vesicles. Whereas the first mechanism seems ruled out based on the above data, the latter 2 are possible modes of action for mGluR8 to depress transmitter release. In the following, we use the fact that these 2 mechanisms show a differential dependence on Ca\(^{2+}\) entry to identify their contribution to the transduction of mGluR8.

The Ca\(^{2+}\) dependence of action potential-induced transmitter release is well described by an n-step, cooperative Ca\(^{2+}\)-binding scheme (n ~ 4 to 5) (Heidelberger et al. 1994; Bollmann et al. 2000; Schneggenburger and Neher 2000; Lou et al. 2005). The release resulting from such binding schemes at various Ca\(^{2+}\) concentrations can be fitted by a Hill function with an apparent cooperativity of n\(_{app}\) < n:

\[
\text{norm.release} = \frac{[\text{Ca}]^{n_{app}}}{[\text{Ca}]^{n_{app}} + K_A^{n_{app}}} \tag{1}
\]

where K\(_A\) indicates the Ca\(^{2+}\) concentration leading to half-maximal release (Segel 1976). The graph of this function is shown in Figure 5A on a double logarithmic scale (n\(_{app}\) = 3.5). For this plot, the peak of the local Ca\(^{2+}\) concentration reached at the sensor during action potentials (plotted on the abscissa)
is expressed relative to the $\text{Ca}^{2+}$ concentration at which half-maximal release is obtained (indicated by the vertical dashed line), therefore $K_A = 1$. This plot illustrates why blocking the same number of $\text{Ca}^{2+}$ channels, for example, by applying $\omega$-conotoxin GVIA, is less effective in elevated extracellular $\text{Ca}^{2+}$ (as found in Fig. 4A). When perfusing a higher $\text{Ca}^{2+}$ concentration, the single channel $\text{Ca}^{2+}$ current is larger and increases the local $\text{Ca}^{2+}$ concentration seen by the release sensor during an action potential. As a result, the sensor is closer to saturation and operates in a region of the release graph with a more shallow slope (right most black dot). A reduction of this local $\text{Ca}^{2+}$ concentration by a given factor through a block of a fraction of channels by the toxin (category 1), for example, by 0.63-fold, decreases transmitter release much more weakly (dashed arrow to red dot in Fig. 5A) than if the experiment was started at an initial lower $\text{Ca}^{2+}$ concentration (left most black dot). More generally, if we start an experiment with any given level of peak $\text{Ca}^{2+}$ concentration reached at the sensor during an action potential, the fraction of transmitter release remaining after an $a$-fold reduction of the local $\text{Ca}^{2+}$ concentration (i.e., the shifts of the dots on the ordinate along the dashed arrows) is given by (cf. eq. 1):

$$\text{fractional release (\%)} = \frac{(a \ast [\text{Ca}])_{\text{app}}}{(a \ast [\text{Ca}])_{\text{app}} + K_{\text{app}}}$$

Equation (2) is plotted in the lower panel of Figure 5A for 3 different values of $a$ (i.e., different degrees of reduction in

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**Figure 3.** mGluR8 depresses mEPSCs independently of $\text{Ca}^{2+}$ channels to a similar extent as action potential-evoked transmission. (A) Presynaptic $\text{Ca}^{2+}$ imaging experiments verify that the application of 100 $\mu\text{M}$ Cd$^{2+}$ under our conditions greatly reduces $\text{Ca}^{2+}$ entry ($n = 4$). (B) Left panel, averaged line scan profiles from time points indicated by lower case letters in A, illustrating the amount of the decrease of the fluorescent signals. Right panel, example line scans before and during application of Cd$^{2+}$. Time runs from top to bottom, asterisk indicates time points of stimulation in both line scans. Line scans were normalized on prestimulus line scans (unit of color scale is relative fluorescence). Note that almost no fluorescent increase is visible in the Cd$^{2+}$ trace. Horizontal scale bar 5 $\mu\text{m}$, vertical scale bar 20 ms. (C) Whole-cell patch-clamp recording of mEPSCs from a hippocampal granule cell ($V_{\text{hold}} = -70$ mV). Glutamatergic currents were pharmacologically isolated. (D) Quantification of the interevent intervals and amplitudes of spontaneous currents putatively arising from lateral perforant path synapses of the recording shown in C. PPG shifts the cumulative probability curve of the intervals significantly to the right but does not affect the distribution of the amplitudes of the currents. The top traces in the right panel show averaged spontaneous currents before and during activation of mGluR8. Vertical scale bar: 4 pA, horizontal scale bar: 5 ms. Lower panel shows the template current used to identify currents putatively arising from lateral perforant path synapses (for details, see Materials and Methods). Template is shown at the same time scaling as the averaged mEPSCs shown above. (E) Summary bar graph of the percentage change of frequency and amplitudes of template-selected mEPSCs by activation of mGluR8.
channels can be completely over-currents (Muller et al. 2007) + concentration in contrast to the potency of 6 and 4 for high and low 1): (\[\frac{1}{\sqrt{C_3}}\) at the release sensor. \[\frac{1}{\sqrt{C_3}}\ex\] concentration seen by the release sensor by varying the 0.81, 2 2 n 3 and imaging to register Ca 1.5 = n concentration (before a channel

D 2 5 (\[\frac{2}{3}\)] as reducing the increase in free Ca (Kukley et al. 2007; Qian and Noebels 2001) are well fit by a function for a bi-molecular reaction scheme describing Ca 2+ channel pore occupancy by extracellular Ca 2+ (Ks set to 3.3 mM, Akaike et al. 1978; Carbone and Lux 1987; Hille 1992) (Fig. 5B):

\[
\text{norm. Ca entry} = 2.7 \times \frac{[\text{Ca}]_{\text{ex}}}{([\text{Ca}]_{\text{ex}} + 3.3 \text{ mM})} \quad (4)
\]

The changes in whole-cell Ca 2+ currents (Muller et al. 2007) and in global intracellular Ca 2+ (Mintz et al. 1995; Qian and Noebels 2001; Kukley et al. 2007) described by equation (4) reflect variations in single channel current (as reasoned in Sinha et al. 1997; Muller et al. 2007). Furthermore, because microdomain Ca 2+ is proportional to the single channel Ca 2+ current under most conditions (as shown in Supplementary Fig. 4), the output of equation (4) also sufficiently describes the relative changes in local Ca 2+ at the release sensor.

We measured the reduction of fEPSPs by \(\alpha\)-conotoxin GVIA at 6 different extracellular Ca 2+ concentrations and plotted the fractional fEPSP amplitudes remaining after toxin application versus the relative changes in the local Ca 2+ concentration calculated from equation (4). For this, the local Ca 2+ concentration achieved under control conditions (2 mM [Ca 2+] ex) was set to 1 (Fig. 5C, by scaling the output of eq. 4 by 1.11). As predicted, the elevation of extracellular Ca 2+ can almost completely overcome the inhibition by the toxin (red dots). The data points are well fitted by equation (2) with \(\alpha = 0.81, n_{\text{app}} = 3.26, K_a = 1.25\) (red line). This indicates that transmitter release at the lateral perforant path synapse can be adequately approximated by equation (1) and that equation (2) well models the effect of a category 1 mechanism. The fit parameters indicate that \(\alpha\)-conotoxin GVIA-sensitive (N-type) Ca 2+ channels contribute ~19% (1–0.81) to the total action potential-induced elevation of the local Ca 2+ concentration at the sensor. Furthermore, \(n_{\text{app}}\) and \(K_a\) provide estimates of the Ca 2+ cooperativity and affinity of the release machinery at this synapse.

Next, a similar series of experiments was performed for activation of mGluR8 by PPG. As shown in Figure 5C, PPG, though to a smaller degree, also becomes less effective in depressing transmitter release when Ca 2+ influx is strongly elevated (black dots). Since experiments were performed at

\[\frac{[\text{Ca}]_{\text{app}}}{[\text{Ca}]_{\text{app}} + (b + K_a)_{\text{app}}} = \frac{[\text{Ca}]^\text{app}_{\text{app}} + K_a^\text{app}}{[\text{Ca}]^\text{app}_{\text{app}} + (b + K_a)^\text{app}} \quad (3)
\]

It is important that equation (3) is formally equivalent to equation (2) if \(a = 1/b\). Therefore, altering Ca 2+ sensitivity by multiplying \(K_a\) with \(b\) shows the same dependence on local Ca 2+ as reducing the increase in free Ca 2+ by a factor \((a = 1/b)\).

In contrast, a reduction in maximal release probability (category 3) cannot be overcome (by definition) and will reduce overall transmitter release to a certain fraction irrespective of the local Ca 2+ concentration seen by the sensor. A category 3 mechanism can be modeled by multiplying equation (1) with a positive factor, for example, \(\max \leq 1\). Taken together, constructing an experimental fractional release curve as exemplified in Figure 5A, lower panel, should allow us to decide whether mGluR8 depresses transmitter release by a category 2 or 3 mechanism (category 1 being excluded experimentally by Ca 2+ imaging).

We performed a series of experiments with \(\alpha\)-conotoxin GVIA to test the predictions of our model more comprehensively and to verify that the effect of \(\alpha\)-conotoxin GVIA on fEPSPs follows equation (2). We systematically altered the local Ca 2+ concentration seen by the release sensor by varying the extracellular Ca 2+ concentration. To construct a fractional release curve as shown in Figure 5A, changes in extracellular Ca 2+ concentration have to be converted into the respective relative changes of the local intracellular Ca 2+ concentration. For this, we first measured how Ca 2+ entry depends on extracellular Ca 2+. We have previously used whole-cell recordings and axonal Ca 2+ imaging to register Ca 2+ influx under various concentrations of extracellular Ca 2+ (Kukley et al. 2007; Muller et al. 2007). These values as well as that of other investigators in related slice preparations (Mintz et al. 1995; Qian and Noebels 2001) are well fit by a function for a bi-molecular reaction scheme describing Ca 2+ channel pore occupancy by extracellular Ca 2+ (Ks set to 3.3 mM, Akaike et al. 1978; Carbone and Lux 1987; Hille 1992) (Fig. 5B):

\[\text{norm. Ca entry} = 2.7 \times \frac{[\text{Ca}]_{\text{ex}}}{([\text{Ca}]_{\text{ex}} + 3.3 \text{ mM})} \quad (4)\]
The mGluR8 effect is best explained by a dual effect on the Ca\textsuperscript{2+} affinity and on the maximal rate of transmitter release. (A) Upper panel, plot of equation (1). The Ca\textsuperscript{2+} concentration seen by the release sensor is normalized on that concentration achieved during an action potential under control conditions, that is, in 2 mM extracellular Ca\textsuperscript{2+}. The degree of inhibition of release is dependent on the Ca\textsuperscript{2+} concentration achieved at the sensor under control conditions, that is, it is dependent on the horizontal position of the black dots (for details, see text). Lower panel, plot of equation (2) for 3 different values of reduction of Ca\textsuperscript{2+} entry (a). Ca\textsuperscript{2+} concentration is taken from various previous publications working in similar preparations and fitted by equation (4). As the affinity value (K\textsubscript{A}), we used 3.3 mM extracellular Ca\textsuperscript{2+} concentration, a value previously derived by (Carbone and Lux 1987) using an extended range of Ca\textsuperscript{2+} concentrations. (C) Fractional release curves calculated from the fractional fEPSP amplitudes remaining after application of \(\omega\)-conotoxin GVIA (red dots) and PPG (black dots), respectively. \(\omega\)-conotoxin GVIA data are well fitted by equation (2) (red line). In contrast, this equation does not acceptably fit the PPG data (gray continuous line). However, PPG data are well fitted by equation (5), which assumes a dual action on Ca\textsuperscript{2+} affinity and maximal release probability (black line). The dotted and the dashed gray lines represent the components of inhibition by mGluR8 mediated by an effect on maximal release probability and on Ca\textsuperscript{2+} affinity, respectively.

Figure 5. The mGluR8 effect is best explained by a dual effect on the Ca\textsuperscript{2+} affinity and on the maximal rate of transmitter release. (A) Upper panel, plot of equation (1). The Ca\textsuperscript{2+} concentration seen by the release sensor is normalized on that concentration achieved during an action potential under control conditions, that is, in 2 mM extracellular Ca\textsuperscript{2+}. The degree of inhibition of release is dependent on the Ca\textsuperscript{2+} concentration achieved at the sensor under control conditions, that is, it is dependent on the horizontal position of the black dots (for details, see text). Lower panel, plot of equation (2) for 3 different values of reduction of Ca\textsuperscript{2+} entry (a). Ca\textsuperscript{2+} concentration is taken from various previous publications working in similar preparations and fitted by equation (4). As the affinity value (K\textsubscript{A}), we used 3.3 mM extracellular Ca\textsuperscript{2+} concentration, a value previously derived by (Carbone and Lux 1987) using an extended range of Ca\textsuperscript{2+} concentrations. (C) Fractional release curves calculated from the fractional fEPSP amplitudes remaining after application of \(\omega\)-conotoxin GVIA (red dots) and PPG (black dots), respectively. \(\omega\)-conotoxin GVIA data are well fitted by equation (2) (red line). In contrast, this equation does not acceptably fit the PPG data (gray continuous line). However, PPG data are well fitted by equation (5), which assumes a dual action on Ca\textsuperscript{2+} affinity and maximal release probability (black line). The dotted and the dashed gray lines represent the components of inhibition by mGluR8 mediated by an effect on maximal release probability and on Ca\textsuperscript{2+} affinity, respectively.

the same synapse, the fitting procedure with equation (3) was constrained by the values of \(n_{\text{app}}\) and \(K_{A}\) as determined by \(\omega\)-conotoxin GVIA applications. The best fit only poorly matched the data points (Fig. 5C, gray line) indicating that a pure category 2 mechanism cannot explain the effect of mGluR8 at this synapse. Hence, we introduced an additional parameter into equation (5) representing a Ca\textsuperscript{2+}-independent inhibition of maximal release (max, category 3):

\[
\text{fractional release} \left(\%\right) = \text{max} \times \frac{[Ca]^{n_{\text{app}}} + K_{A}^{n_{\text{app}}}}{[Ca]^{n_{\text{app}}} + (b + K_{A})^{n_{\text{app}}}}\tag{5}
\]

Equation (5) represents a dual action on transmitter release via category 2 and 3 and significantly better approximates the data points of PPG in Figure 5C (black line) despite the additional degree of freedom (\(P < 0.001, F\)-test). The best estimates for max and \(b\) were 0.73 and 1.27, respectively. This means that the Ca\textsuperscript{2+} dependence of the PPG effect can be best explained if mGluR8 is assumed to decrease both the overall maximal release probability to 73% and the Ca\textsuperscript{2+} affinity by increasing \(K_{A}\) by 27%. The 2 components of the action of mGluR8 are drawn separately in Figure 5C (dashed lines combine multiplicatively to yield the black line). This graph shows that under most settings of Ca\textsuperscript{2+} entry, the inhibition of fEPSPs by the effect of mGluR8 on Ca\textsuperscript{2+} affinity is clearly more pronounced. This situation only changes if Ca\textsuperscript{2+} entry is increased more than ~1.6-fold (intersection of curves) as compared with basal levels (2 mM [Ca\textsuperscript{2+}]\textsubscript{ext}). Importantly, if we do not constrain the parameters \(n_{\text{app}}\) and \(K_{A}\) with the values of the fit of the toxin data, we still obtain very similar values for max and \(b\) with 0.78 and 1.29, respectively, by fitting equation (5) (Supplementary Fig. 5, red line). Moreover, in this case, even the estimates for the basal release parameters are well comparable to the ones estimated by \(\omega\)-conotoxin GVIA application (\(n_{\text{app}} = 3.23, K_{A} = 1.39\), showing the robustness of our approach.

To further substantiate this robustness, we performed an additional curve fitting analysis in which we fit both the toxin data and the PPG data with equation (5) simultaneously without constraining any parameters. Also in this case, extending the equation by the parameter max led to a significantly better fit (\(P < 0.001, F\)-test), and we derived very similar parameter estimates for \(\omega\)-conotoxin GVIA: \(a = 0.82\) and for PPG: \(b = 1.26, \text{max} = 0.73\) and for the common parameters \(K_{A}\) and \(n_{\text{app}}\) of 1.2 and 3.40, respectively (for details, refer to Supplementary Fig. 6).

The fractional release curves shown in Figure 5C are based on our estimates of the changes in Ca\textsuperscript{2+} entry upon altering the extracellular Ca\textsuperscript{2+} concentration. Because the fit used to calibrate the Ca\textsuperscript{2+} entry strongly extrapolates our data points from 4 mM up to 20 mM of extracellular Ca\textsuperscript{2+} (for norm. [Ca\textsuperscript{2+}]\textsubscript{local,ctrl} = 2.32, compare for Fig. 5B), we asked whether our conclusions regarding the downstream targets of mGluR8 are the same if we assume a different calibration curve. Supplementary Figure 7 shows that the parameters \(b\) max, and \(K_{A}\) are quite insensitive to changes in this calibration because the calibration curve affects both the fraction of release curve of PPG and of conotoxin. The only parameter notably affected by a different calibration is \(n_{\text{app}}\) (Supplementary Fig. 7) which, however, does not influence our conclusions regarding the mechanism of action of mGluR8. Finally, our conclusion is also not affected by the fact that we do not analyze individual
synapses and record population synaptic responses (Supplementary Fig. 8).

Action potential-induced Ca\(^{2+}\) entry into synapses is not only important for glutamate release but also for refilling the pool of releasable vesicles in neuronal synapses (Stevens and Wesseling 1998; Wang and Kaczmarek 1998; Hosoi et al. 2007). It has been shown that the accumulation of presynaptic Ca\(^{2+}\) during repetitive synaptic activity can accelerate vesicle recruitment by as much as 10-fold (Hosoi et al. 2007). Therefore, one functional consequence of mGluR8's lack of effect on Ca\(^{2+}\) entry is that synaptic transmission during a train of action potentials for the period of inhibition by mGluR8 should be greater when compared with a similar train of action potentials undergoing depression of release by inhibition of voltage-gated Ca\(^{2+}\) channels. To test this prediction, we compared repetitive synaptic responses when transmission was depressed to the same degree either by PPG or by the Ca\(^{2+}\) antagonist Co\(^{2+}\), which blocks but does not permeate Ca\(^{2+}\) channels. The low-affinity Ca\(^{2+}\) channel blocker Co\(^{2+}\) was chosen for these experiments because it quickly washes in and out of the slice preparation and facilitates the titration of the concentration to achieve an inhibition equal to that of 3 \(\mu\)M PPG. We applied 600 \(\mu\)M Co\(^{2+}\) which depressed fEPSPs to 56 ± 1\% (n = 11), very similar to PPG (to 58 ± 2\%, n = 9, Fig. 6A,B,D). Once a steady depression of fEPSPs was reached, we stimulated lateral perforant path fibers 35 times at 25 Hz. In the presence of either inhibitor, there was a pronounced initial and transient facilitation of transmission as expected because release probability was decreased (Fig. 6A-C, third pulse: 159 ± 4\% and 155 ± 4\% for PPG, n = 9, and Co\(^{2+}\), n = 11, respectively). This facilitation declined in both groups presumably because the supply of vesicles became a limiting factor. However, after the first ~250 ms (5–7 pulses), the responses under Co\(^{2+}\) clearly depressed more strongly while larger fEPSP amplitudes were maintained in the presence of PPG. At the end of the train relative amplitudes amounted to 89 ± 5\% and 67 ± 5\% under PPG and Co\(^{2+}\), respectively. This difference is consistent with the mGluR8's lack of effect on Ca\(^{2+}\) entry.

It is generally assumed that group II and III mGluRs act through inhibition of adenylate cyclase (Conn and Pin 1997; Anwyll 1999). However, direct evidence that the inhibition of adenylate cyclase is necessary or causative for the reduction of transmitter release is still lacking. To test the involvement of adenylate cyclase in the action of mGluR8 at the lateral perforant path synapse, we inhibited this enzyme by SQ22536 (800 \(\mu\)M) which should block Ca\(^{2+}\) entry if adenylate cyclase is required. To rule out side effects of SQ22536 on adenosine receptors (Schulte and Fredholm 2002), we preapplied DPCPX (3 \(\mu\)M) as well as MRS1191 (2 \(\mu\)M) to block A1 and A2 receptors, respectively. We verified the effectiveness of inhibition of adenylate cyclase with 3-isobutyl-1-methylxanthine (IBMX, 50 \(\mu\)M). IBMX reduces the breakdown of cAMP and elevates intracellular cAMP levels, if adenylate cyclase is active. Under control conditions (in the presence of A1 and A2 blockers), IBMX increased fEPSPs amplitude as expected by an elevation of presynaptic cAMP levels (Weisskopf et al. 1994; Sakaba and Neher 2001) (Fig. 7A, n = 7). The increase of fEPSPs was effectively prevented by 40 min of SQ22536 application (Fig. 7A, n = 5) showing that adenylate cyclase activity is inhibited. However, SQ22536 did not have an effect on fEPSPs itself nor on the inhibition of fEPSPs by PPG (Fig. 7C, n = 6). Similar results were obtained with another adenylate cyclase inhibitor, DDA (500 \(\mu\)M, n = 4, Fig. 7B) making it unlikely that mGluR8 reduces transmitter release via downregulation of adenylate cyclase.

We next examined a series of other membrane-permeable inhibitors of known G-protein pathways for potential interference with the action of mGluR8 on transmitter release. In each experiment, we first tested the response of fEPSPs to PPG, then applied the inhibitor, typically for 30 min, before we retested the response to PPG (cf. Fig. 8B). As a control, we applied PPG twice (right most bar in Fig. 8A, n = 4). With this approach, we tested for the involvement of protein kinases C, A, and G (1 \(\mu\)M staurosporine, n = 5, 100/30 \(\mu\)M H7/H89, n = 4), Calmodulin signaling (3 \(\mu\)M calmidazolium, n = 5; 25 \(\mu\)M ophobolin A, n = 4), phospholipases C and A2 (5 \(\mu\)M U73122, n = 7; 5 \(\mu\)M OBAA, n = 4), tyrosine kinase (10 \(\mu\)M lavendustin A, n = 4), nitric oxide-sensitive guanylate cyclase (10 \(\mu\)M ODQ, n = 4), and the mitogen-activated protein kinase pathway (30 \(\mu\)M PD 98059, n = 6, 10 \(\mu\)M SL 327, n = 6) (Fig. 8A). Except lavendustin...
mGluR8-induced depression of glutamate release is resistant to a wide range of blockers of known G-protein transduction pathways. (A) Summary bar graph showing the depression of fEPSPs by PPG under the respective pathway blockers, normalized to the inhibition by PPG before application of the blocker in the same experiment. Control bar was calculated from 2 successive PPG applications. (B) Example traces taken from experiments summarized in A for U73122 and for the combined application of H7/H89. Control and PPG traces on the left represent fEPSP recordings before the blocker application. Control potentials before application of U73122 and H7/H89 are scaled to the same size to facilitate the judgment of the degree of inhibition by PPG.

Figure 8.

concentrations (Heidelberger et al. 1994; Bollmann et al. 2000; Schneggenburger and Neher 2000; Lou et al. 2005). Furthermore, and as suggested by the same work, we assume that the local intracellular Ca\(^{2+}\) concentration is necessary and sufficient to determine vesicle fusion. Our model does not include any effect of voltage on the release process as suggested by Parnas and Parnas (2007). This neglect may be justified by the observation that mGluR8 is not activated by ambient glutamate (Bushell et al. 1995; Bushell et al. 1996; Friedl et al. 1999) which is required by the “calcium-voltage” hypothesis (Parnas and Parnas 2007). The basic idea of our approach was to use this saturation to distinguish presynaptic mechanisms which act upstream of Ca\(^{2+}\) binding to the release sensor from downstream mechanisms by the fact that the former but not the latter will be rendered ineffective if the release machinery is brought to near-saturation by elevating presynaptic Ca\(^{2+}\) entry. It was found that the Ca\(^{2+}\) dependence of the inhibition of release by mGluR8 could not adequately be explained by a sole reduction in Ca\(^{2+}\) binding to the sensor. Instead, we had to assume a dual action of mGluR8 on both calcium binding to the sensor and the maximal release rate. Separating the 2 components of mGluR8 signaling algebraically showed that under normal conditions and upon low Ca\(^{2+}\) entry the effect of mGluR8 on Ca\(^{2+}\) binding to the release sensor is primarily responsible for the inhibition of glutamate release.

Our formalism predicts that mGluR8 reduces Ca\(^{2+}\) binding to the release sensor but it does not per se resolve whether this is caused by an effect on the Ca\(^{2+}\)-binding properties of the sensor itself, the idea favored by us, or by a change in the very local Ca\(^{2+}\) seen by the sensor. For example, mGluR8 may selectively inhibit only those Ca\(^{2+}\) channels which are directly associated with synaptic vesicles and which may carry only an insignificant fraction of total Ca\(^{2+}\) entry. Alternatively, mGluR8...
may, by some means, increase the diffusional distance between Ca\(^{2+}\) channels and the sensor. However, neither mechanism explains on its own why mGluR8 reduces the frequency of mEPSCs when Ca\(^{2+}\) channels are blocked by Cd\(^{2+}\): under these conditions, channel opening and concomitant Ca\(^{2+}\) diffusion from voltage-gated calcium channels to the release machinery does not occur, consequently mGluR8 must reduce mEPSC frequency in a different way. This reduction could be caused by an mGluR8-dependent modulation of spontaneous calcium release events from presynaptic calcium stores. Such events have been demonstrated to be responsible for a significant fraction of mEPSCs at other synapses (Collin et al. 2005). However, an effect of mGluR8 on calcium stores has not been described and would also not explain the inhibition of release triggered by single action potentials. Taken together, the most parsimonious explanation of our experimental data is that mGluR8 directly modulates the release sensor.

It is important to identify the downstream targets of presynaptic modulation because the functional implications differ for a decrease in affinity versus a change in maximal release rate. The degree of inhibition via the former but not the latter mechanism is dependent on the actual amount of Ca\(^{2+}\) entry. Changes in Ca\(^{2+}\) entry are known to occur during physiological settings when a synapse is repetitively activated: broadening of presynaptic action potentials can increase (Jackson et al. 1991; Byrne and Kandel 1996; Geiger and Jonas 2000), while depletion of extracellular Ca\(^{2+}\) (Rusakov and Fine 2003) or inactivation of presynaptic Ca\(^{2+}\) channels (Forsythe et al. 1998) can decrease Ca\(^{2+}\) entry. Thus, the degree of inhibition via a reduction in Ca\(^{2+}\) binding to the release machinery (category 1 and 2) is dependent on previous activity, whereas further downstream mechanisms (category 3) will proportionally scale down synaptic strength independent of the history of activity. The different functional meanings clearly emerge, when considering natural repetitive patterns of neuronal activity, which involve vesicle replenishment, short-term synaptic plasticity and changes in presynaptic Ca\(^{2+}\) entry.

What could be the signaling pathway from mGluR8 to the components of the release machinery? Classically, group III mGluRs, such as mGluR8, negatively couple to adenylyl cyclase (Conn and Pin 1997). A reduction in cAMP levels by group III mGluRs has been shown in expression systems and in native slices (Bedingfield et al. 1995; Wu et al. 1998) but it was suggested that the inhibition of spontaneous transmitter release by mGluR8 in mitral cells may be independent of presynaptic cAMP levels (Schoppa and Westbrook 1997). This finding is supported by our study 2-fold: First, adenylyl cyclase inhibitors changed neither basal synaptic transmission nor the inhibition of release by mGluR8. We tested various other inhibitors of known G-protein transduction pathways, but none of them prevented the action of mGluR8. A possible alternative explanation may be that activated mGluR8 releases G\(\beta\)/\(\gamma\)-subunits which directly interact with the release machinery as shown for 5-HT receptors at a noncortical synapse (Blackmer et al. 2001). Another scenario of a direct interaction of a G-protein-coupled receptor with the release machinery has been reported at the mouse neuromuscular junction: presynaptic munc18 receptors directly inhibit the release apparatus without an involvement of a G-protein or G\(\beta\)/\(\gamma\)-subunits (Parnas and Parnas 2007; Kupchik et al. 2011).

However, further experiments are required to provide direct evidence for one of these scenarios.

A decrease in Ca\(^{2+}\) affinity likely reflects a direct or indirect modulation of a component of the release machinery which is responsible for Ca\(^{2+}\) binding and initiating the fusion process. A decreased maximal release probability by mGluR8 could also arise by affecting steps not directly involved in Ca\(^{2+}\)-dependent membrane fusion. A recent study indeed reports that noradrenergic presynaptic receptors completely deactivate certain release sites, while others are unaffected (Delaney et al. 2007). This mechanism is, however, unlikely to account for the effect observed in our study because it predicts that paired-pulse facilitation upon activation of the presynaptic receptor remains unchanged (Delaney et al. 2007). In contrast, we observed that in high extracellular Ca\(^{2+}\) concentrations (20 mM), when the effect of mGluR8 is nearly exclusively mediated by the effect on maximal transmitter release (cf. Fig. 5C), there is a strong increase in the paired-pulse index from 76 ± 3% to 103 ± 2% (data not shown, \(n = 5\)). At the calyx of Held, a secondary effect of presynaptic \(\gamma\)-aminobutyric acid (GABA)-B receptors on vesicle recruitment during sustained activity has been shown and provides another example of inhibition of transmission independent of changing Ca\(^{2+}\) binding to the release sensor (Sakaba and Neher 2003). However, considering that sustained phasic release during repetitive stimulation is more prominent when compared with Ca\(^{2+}\) channel blockade, an inhibition of vesicle supply by mGluR8 is also not suggested by our data. Thus, our study suggests a modulation of Ca\(^{2+}\)-sensing components of the fusion machinery and our functional study may provide useful guidelines to identify appropriate candidates.

**Supplementary Material**

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/


