Mechanisms Underlying Enhancement of Spontaneous Glutamate Release by Group I mGluRs at a Central Auditory Synapse

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One emerging concept in neuroscience states that synaptic vesicles and the molecular machinery underlying spontaneous transmitter release are different from those underlying action potential-driven synchronized transmitter release. Differential neuromodulation of these two distinct release modes by metabotropic glutamate receptors (mGluRs) constitutes critical supporting evidence. However, the mechanisms underlying such a differential modulation are not understood. Here, we investigated the mechanisms of the modulation by group I mGluRs (mGluR Is) on spontaneous glutamate release in the medial nucleus of the trapezoid body (MNTB), an auditory brainstem nucleus critically involved in sound localization. Whole-cell patch recordings from brainstem slices of mice of both sexes were performed. Activation of mGluR I by 3,5-dihydroxyphenylglycine (3,5-DHPG; 200 μ M) produced an inward current at -60 mV and increased spontaneous glutamate release in MNTB neurons. Pharmacological evidence indicated involvement of both mGluR1 and mGluR5, which was further supported for mGluR5 by immunolabeling results. The modulation was eliminated by blocking Na_v channels (tetrodotoxin, 1 μ M), persistent Na^+ current (I_{NaP} ; riluzole, 10 μ M), or Ca_v channels (CdCl_2 , 100 μ M). Presynaptic calyx recordings revealed that 3,5-DHPG shifted the activation of I_{NaP} to more hyperpolarized voltages and increased I_{NaP} at resting membrane potential. Our data indicate that mGluR I enhances spontaneous glutamate release via regulation of I_{NaP} and subsequent Ca^{2+} -dependent processes under resting condition.

Key words: auditory; EPSC; mGluR; MNTB; spontaneous glutamate release; voltage-gated sodium channel

Significance Statement

For brain cells to communicate with each other, neurons release chemical messengers, termed neurotransmitters, in response to action potential invasion (evoked release). Neurons also release neurotransmitters spontaneously. Recent work has revealed different release machineries underlying these two release modes, and their different roles in synaptic development and plasticity. Our recent work discovered differential neuromodulation of these two release modes, but the mechanisms are not well understood. The present study showed that activation of group I metabotropic glutamate receptors enhanced spontaneous glutamate release in an auditory brainstem nucleus, while suppressing evoked release. The modulation is dependent on a persistent Na^+ current and involves subsequent Ca^{2+} signaling, providing insight into the mechanisms underlying the different release modes in auditory processing.

Introduction

Spontaneous neurotransmitter release occurs in the absence of presynaptic action potentials (APs; Fatt and Katz, 1950). The traditional views assumed that spontaneous neurotransmitter release shares the same pool of synaptic vesicles with evoked neurotransmitter release and serves no physiological functions. Recent works challenge these views and support the alternative notions that the vesicle pool responsible for spontaneous release may be different from the pool for evoked release and that spontaneous synaptic events play physiological roles in neural signaling that lead to synaptic maturation and homeostasis (for review,

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see Kavalali, 2015). Differential modulation of these two distinct transmitter release modes supports these new concepts, but the underlying mechanisms are not well understood.

The medial nucleus of the trapezoid body (MNTB) is a critical nucleus in the auditory brainstem circuits involved in sound localization (for review, see Grothe et al., 2010). MNTB neurons are excited by well timed and strong glutamatergic inputs from bushy cells in the contralateral anteroventral cochlear nucleus (AVCN) via the giant calyx of Held synapse, ensuring high-speed signal transmission and precise temporal coding of acoustic stimuli for computation of sound localization (for review, see Joris and Trussell, 2018). MNTB neurons also receive strong inhibitory inputs primarily from the ventral nucleus of the trapezoid body (Albrecht et al., 2014), the synaptic strength of which is comparable to the powerful glutamatergic input at the equivalent absolute driving force (Awatramani et al., 2004). We previously showed that group I mGluRs (mGluR I) exert neurotransmitter-specific and release mode-specific modulation of synaptic inhibition in MNTB. Activation of mGluR I increases the frequency and amplitude of glycinergic but not GABAergic spontaneous IPSCs (sIPSCs) and suppresses the GABAergic but not glycinergic evoked IPSCs (Curry et al., 2018). While activation of mGluR I inhibits the evoked glutamatergic transmission via retrograde endocannabinoid signaling in MNTB (Kushmerick et al., 2004), it remains unknown whether and how the spontaneous glutamate release is modulated by mGluR I. Most neurons, potentially including those in MNTB (Awatramani et al., 2004; Albrecht et al., 2014), receive equalizing excitatory and inhibit inputs (Wehr and Zador, 2003; Xue et al., 2014; for review, see Froemke, 2015). Because the balance of excitation and inhibition is a critical feature of sound localizing circuits, as shown in the lateral superior olive (LSO; Magnusson et al., 2008), we hypothesized that spontaneous excitatory transmission in MNTB was enhanced by mGluR I, mirroring the differential modulation of mGluR I on the inhibitory inputs to MNTB (Curry et al., 2018).

If so, what could be the underlying mechanisms? mGluR I enhancement of spontaneous glycine release is dependent on voltage-gated sodium (Na_v) channels (Curry et al., 2018). In addition to the transient Na^+ current underlying spike generation, a noninactivating persistent sodium current (I_{NaP}) activated at subthreshold voltages impacts the steady state, subthreshold neuronal excitability (for review, see Crill, 1996). Indeed, activation of I_{NaP} and interaction between I_{NaP} and a depolarizing glycinergic input increases spontaneous glutamate release in MNTB (Turecek and Trussell, 2001; Huang and Trussell, 2008). Additionally, exaggerated mGluR5 (one of the two members of mGluR I) activity in a fragile X syndrome mouse model enhances I_{NaP} in entorhinal cortical cells (Deng and Klyachko, 2016). These studies strongly suggest a critical interaction between mGluR I and I_{NaP} in the regulation of spontaneous neurotransmitter release, leading to our hypothesis that mGluR I enhances spontaneous glutamate release at MNTB via regulation of presynaptic I_{NaP} in the calyx. Using presynaptic and postsynaptic recordings and immunocytochemistry, we investigated how mGluR I and I_{NaP} interact to modulate the spontaneous excitatory transmission in MNTB.

Materials and Methods

Animals. All animal procedures were approved by the Institutional Animal Care and Use Committees at the Northeast Ohio Medical University (NEOMED), Florida State University, and Tulane University, and were performed in accordance with the National Institutes of Health policies on animal use. C57BL/6J mice were originally purchased from

The Jackson Laboratory and were bred at the universities cited above. Mice were housed in a vivarium with a normal 12 h light/dark cycle.

Postsynaptic in vitro whole-cell recordings. Coronal brainstem slices (~250 μm in thickness) were prepared from 2- to 3-week-old mice of both sexes, as previously described (Lu et al., 2007; Curry et al., 2018). Mice were deeply anesthetized with isoflurane and rapidly decapitated. The brainstem was removed and sliced in ice-cold low- Na^+ artificial CSF (ACSF) containing the following (in mM): 250 glycerol, 3 KCl, 1.2 KH_2PO_4 , 20 NaHCO_3 , 3 HEPES, 1.2 CaCl_2 , 5 MgCl_2 , and 10 glucose, pH 7.4 (when gassed with 95% O_2 and 5% CO_2). Slices were incubated in an interface chamber at 34–36°C for ~1 h in normal ACSF containing the following (in mM): 130 NaCl, 20 NaHCO_3 , 3 KCl, 2.4 CaCl_2 , 1.3 MgSO_4 , 1.2 KH_2PO_4 , and 10 glucose, pH 7.4. For recording, slices were transferred to a 0.5 ml chamber mounted on a Zeiss Axioskop 2 FS Plus Microscope with a 40 $\times$ or 63 $\times$ water-immersion objective and infrared differential interference contrast optics. The chamber was continuously superfused with ACSF (2–5 ml/min) by gravity.

Patch pipettes were drawn on a PP-830 Microelectrode Puller (Narishige) to a 1–2 μm tip diameter using borosilicate glass micropipettes (inner diameter, 0.84 mm; outer diameter, 1.5 mm; World Precision Instruments). The electrodes had resistances between 3 and 6 M Ω when filled with internal solution. For current clamp, the internal solution contained the following (in mM): 130 K-gluconate, 4.5 MgCl_2 , 4.4 Tris-phosphocreatine, 9 HEPES, 5 EGTA, 4 Na-ATP, and 0.48 Na-GTP, with pH 7.3, adjusted with KOH, and osmolality of ~290 mOsm/L. For voltage clamp, 5 mM QX-314 was added to the internal solution to block Na_v channels. The liquid junction potential was 10 mV, and data were corrected accordingly. Voltage-clamp and current-clamp experiments were performed with AxoPatch 200B and AxoClamp 2B amplifiers (Molecular Devices), respectively. Recordings were performed under near physiological temperatures (34–36°C) and were obtained at a holding potential of –60 mV for voltage clamp and at the resting membrane potential (RMP) for current-clamp experiments. Data were low-pass filtered at 5 or 3 kHz and digitized with a Data Acquisition Interface ITC-18 (InstruTech) at 50 kHz. Recording protocols were written and run using the acquisition and analysis software AxoGraph X (AxoGraph Scientific).

In all recordings, EPSCs were isolated pharmacologically with GABA_A receptor antagonist gabazine (10 μM ; SR95531) and glycine receptor antagonist strychnine (1 μM ; Stry). All chemicals were purchased from Sigma-Aldrich except for gabazine, riluzole, and (RS)-3,5-dihydroxyphenylglycine (3,5-DHPG), which were obtained from Tocris Bioscience. 3,5-DHPG was prepared in ACSF at a working concentration of 200 μM , which is at least threefold higher than its EC_{50} value (0.7–60 μM depending on animal tissues; for review, see Cartmell and Schoepp, 2000). This was expected to achieve a saturating concentration and thus full activation of group I mGluRs in our experiments.

To record evoked EPSCs (eEPSCs), extracellular stimulation was performed using concentric bipolar electrodes with a tip core diameter of 127 μm (World Precision Instruments). The stimulating electrode was placed using an MN-25 Micromanipulator (Narishige) and was positioned at the ventral brainstem midline to activate the excitatory afferent fibers (Kushmerick et al., 2004). All-or-none eEPSCs were recorded. The stimulus intensity at which maximal response was elicited was chosen to perform experiments with a single-pulse paradigm at a frequency of 0.1 Hz. Group I mGluR agonist 3,5-DHPG (200 μM) was bath applied for 2–5 min to one cell per slice. Puff application of 3,5-DHPG (200 μM , prepared in ACSF) was done with a glass pipette electrode (2–4 μm tip diameter), the tip of which was placed at a distance of 50–100 μm from the recorded cell using an MN-25 Micromanipulator (Narishige). The shank of the puff electrode was connected to a Picospritzer (General Valve) for pressure ejection (at ~68.9 kPa; i.e., 10 psi) of the ACSF containing 3,5-DHPG. Other pharmacological agents were also bath applied. Typically, responses were averaged from a minimum of six eEPSC traces per drug condition for further data analyses.

Spontaneous EPSCs (sEPSCs) are defined as recorded events that occur in the absence of external (electrical) stimulation, and therefore consist of both miniature EPSCs (mEPSCs), which are AP-independent events because of univesicular release (UVR), and additional sEPSC

events that may be AP- or depolarization-dependent events including primarily both UVR and possibly multivesicular release (MVR; for review, see Rudolph et al., 2015; for possible MVR at MNTB, see Taschenberger et al., 2002; see Discussion below). mEPSCs were recorded in the presence of tetrodotoxin (TTX; $1\ \mu\text{M}$), a blocker for Na_v channels. In a subset of experiments, the following additional antagonists were bath applied in the presence of 3,5-DHPG: mGluR1a antagonist (LY367385, $50\ \mu\text{M}$), mGluR5 antagonist (MPEP, $10\ \mu\text{M}$), voltage-gated Ca^{2+} channels blocker (CdCl_2 , $100\ \mu\text{M}$), and I_{NaP} blocker (riluzole, $10\ \mu\text{M}$).

Presynaptic (calyx of Held) in vitro whole-cell recordings. The giant glutamatergic terminal calyx of Held renders direct recordings of presynaptic terminals possible (Forsythe, 1994; Borst et al., 1995), including direct recording of presynaptic I_{NaP} (Huang and Trussell, 2008). Brainstem slices containing the MNTB were prepared from P8–P10 and P14–18 mice of either sex as previously described (Zhang and Huang, 2017). Briefly, mouse brainstems were dissected and $210\text{-}\mu\text{m}$ -thick sections were sliced using a vibratome (VT1200S, Leica) in ice-cold, low- Ca^{2+} , low- Na^+ saline, which contained the following (in mM): 230 sucrose, 10 glucose, 2.5 KCl, 3 MgCl_2 , 0.1 CaCl_2 , 1.25 NaH_2PO_4 , 25 NaHCO_3 , 0.4 ascorbic acid, 3 *myo*-inositol, and 2 Na-pyruvate, bubbled with 95% O_2 and 5% CO_2 . Slices were immediately incubated at 32°C for 20–40 min and subsequently stored at room temperature in normal ACSF containing the following (in mM): 125 NaCl, 10 glucose, 2.5 KCl, 1.8 MgCl_2 , 1.2 CaCl_2 , 1.25 NaH_2PO_4 , 25 NaHCO_3 , 0.4 ascorbic acid, 3 *myo*-inositol, and 2 Na-pyruvate, pH 7.4 (when bubbled with 95% O_2 and 5% CO_2).

Slices were transferred to a recording chamber and were continually perfused with ACSF (2–3 ml/min) warmed to $\sim 32^\circ\text{C}$ (P8–P10 mice) and $34\text{--}36^\circ\text{C}$ (P14–P18 mice) by an in-line heater (Warner Instruments). Neurons were visualized using an Olympus BX51 microscope with a $60\times$ water-immersion objective and custom infrared Dodt gradient contrast optics. Whole-cell patch-clamp recordings were performed with a Multiclamp 700B amplifier (Molecular Devices). Pipettes pulled from thick-walled borosilicate glass capillaries (World Precision Instruments) had open tip resistances of 3–5 $\text{M}\Omega$ for the presynaptic recording. Series resistance (R_s , 6–25 $\text{M}\Omega$) was compensated by up to 70% (bandwidth, 3 kHz). The R_s was of a large variation, perhaps because of intrinsic properties of the presynaptic terminals and likely associated with technically challenging presynaptic recordings. However, the R_s values in this study are not different from those obtained from the same type of preparations in previous reports (Huang and Trussell, 2008, 2011). Signals were filtered at 10 kHz and sampled at 20 kHz.

For presynaptic voltage-clamp experiments, pipettes contained the following (in mM): 120 Cs-methanesulfonate, 10 CsCl, 10 TEA-Cl, 1 MgCl_2 , 10 HEPES, 5 EGTA, 0.4 Tris-GTP, 3 Mg-ATP , and 5 Na_2 -phosphocreatine, 292 mOsm/L, and pH 7.3 with CsOH. To isolate presynaptic Na^+ currents in response to voltage steps or ramps, TEA-Cl (10 mM), and 4-AP (2 mM) and CdCl_2 ($200\ \mu\text{M}$) were added to ACSF, substituting for NaCl with equal osmolarity. Liquid junction potential was measured ($\sim 10\text{ mV}$) and reported voltages were appropriately adjusted. Drugs were applied by bath perfusion. 3,5-DHPG and TTX were stored as aqueous stock solutions at -20°C .

Immunocytochemistry and confocal microscopy imaging. P14 mice ($n = 5$) were anesthetized with a mixture of ketamine and xylazine, and were transcardially perfused with 0.9% saline followed by 4% paraformaldehyde in phosphate buffer (PB). Brains were removed from the skull, postfixed overnight in the same fixative and then transferred to 30% sucrose in PB until they sank.

Brains were sectioned in the coronal plane at $30\ \mu\text{m}$ on a freezing sliding microtome. Each section was collected in $0.01\ \text{M}$ PBS. Alternate serial sections were immunocytochemically double stained for anti-mGluR5 (1:1000; rabbit monoclonal, catalog #ab76316, Abcam; RRID: AB_1523944) or anti-mGluR5 (1:500; rabbit polyclonal, catalog #ab5675, Millipore; RRID: AB_2295173), with anti-synaptic vesicle glycoprotein 2 (SV2; 1:1000; mouse monoclonal, catalog #SV2, Developmental Studies Hybridoma Bank; RRID: AB_2315387) antibodies. Briefly, free-floating sections were incubated with primary antibody solutions diluted in PBS with 0.3% Triton X-100 overnight at 4°C , followed by Life Technologies

Alexa Fluor secondary antibodies (1:1000 overnight at 4°C ; Thermo Fisher Scientific). Sections were then mounted on gelatin-coated slides and coverslipped with Fluoromount-G mounting medium (Southern Biotech). Images were captured with a Leica SP8 and an Olympus FV1200 confocal microscope. Image brightness, γ , and contrast adjustments were performed in Adobe Photoshop (Adobe Systems). All adjustments were applied equally to all images of the same set of staining from the same animal.

Experimental design and statistical analyses. sEPSCs were detected by a template using a function for product of exponentials, $f(t) = [1 - \exp(-t/\text{rise time})] \times \exp(-t/\text{decay tau})$, where t stands for time and tau for time constant. The values of the parameters for the template are as follows: amplitude of $-30\ \text{pA}$, rise time of 0.3 ms, decay tau of 0.5 ms, with a template baseline of 1 ms and a template length of 3 ms. These parameters were determined based on the averaged trace of visually detected synaptic events. The detection threshold is typically 2.5-fold the noise SD, which detects most of the events with the least number of false-positive results. The average of detected events for each cell was obtained using AxoGraph to measure the amplitude, 10–90% rise time, and decay tau. These parameters were typically averaged from 60 s periods from each condition: control (Ctrl), drug, and wash. For eEPSC experiments, the peak amplitude of each eEPSC was measured after each stimulus. Averages were obtained from the first minute of the control period, the last minute of the group I mGluR agonist application (3,5-DHPG), and the last minute of the wash period, and were normalized for individual experiments by dividing the peak amplitude of individual eEPSCs by the average eEPSC amplitude under the control condition.

Voltage-clamp data obtained from calyx recordings were analyzed using Clampfit (Molecular Devices) and Igor (WaveMetrics). The detection of the activation threshold for I_{NaP} was determined from 1 kHz-filtered ramp data by extrapolating a line fitted between -100 and -90 mV ; the point of deviation from this line (typically by several picoamperes to be obvious by eye; see Fig. 6) was considered as the point of detectable activation of I_{NaP} . Boltzmann functions were used to describe I_{NaP} activation, as follows: $G = G_{\text{MAX}}/(1 + \exp(-(V - V_{\text{half}})/k))$, where G is conductance in nanosiemens, G_{MAX} is the maximal conductance, V is the potential in millivolts, V_{half} is the voltage for half-maximal activation in millivolts, and k is the slope factor in millivolts.

Graphs were made in Igor (WaveMetrics) and GraphPad Prism8 (GraphPad Software). Mean $\pm$ SEM values are reported. The nonparametric Kolmogorov–Smirnov test was used to assess the significance of shifts in cumulative probability distributions of interevent intervals (IEIs) and amplitude of sEPSC. Data were subject to either repeated-measures (RM) one-way ANOVA followed by Holm–Sidak or Tukey’s multiple-comparisons test if they passed the Kolmogorov–Smirnov normality test, or Friedman test (nonparametric alternative to the one-way ANOVA with repeated measures) followed by Dunn’s multiple-comparisons test if they failed the normality test. Kendall’s W value was used to measure the effect size of the Friedman test: $W = \chi^2/n(k - 1)$, where χ^2 is the Friedman test statistic value, n is the sample size, and k is the number of measurements per subject. For significant differences observed in RM one-way ANOVA, a Geisser–Greenhouse correction was conducted for individual sample comparisons. A paired t test was also used for experiments as indicated in the Results. Cohen’s d (d_{Cohen}) was used to measure the effect size of the paired t test: $d_{\text{Cohen}} = (M_1 - M_2)/\text{SD}_{\text{pooled}}$, where M_1 and M_2 are the mean of group 1 and group 2, respectively, and $\text{SD}_{\text{pooled}}$ is the pooled SD for the two groups. A p value of <0.05 is considered statistically significant. Statistical analyses were performed using GraphPad Prism 8.

Results

Activation of mGluR I increases frequency and amplitude of sEPSC

Activation of mGluR I by a selective agonist 3,5-DHPG inhibits the evoked glutamate release in MNTB (Kushmerick et al., 2004). We hypothesized that 3,5-DHPG enhanced spontaneous glutamate release. This is exactly the opposite modulatory

direction for the same neurotransmitter in the same neuron, so it is necessary to confirm the effect of 3,5-DHPG on the evoked glutamate release under our recording conditions. In the presence of antagonists for GABA_A receptors (gabazine, 10 μ M) and glycine receptors (strychnine, 1 μ M), activation of mGluR I with bath application of 3,5-DHPG (200 μ M) decreased the amplitude of eEPSCs in all five cells tested (Fig. 1*A,B*), similar to the previous observations in rats (Kushmerick et al., 2004).

We then examined the effect of mGluR I on sEPSCs. Bath application of 3,5-DHPG (200 μ M) increased the frequency and amplitude of sEPSCs. The effect usually lasted for minutes after washout of the drug (Fig. 1*C*), a similar observation as for spontaneous glycine release (Curry et al., 2018). We also observed an inward current induced by 3,5-DHPG, with an average of 31.6 ± 5.9 pA at the holding potential of -60 mV (Fig. 1*D*; $n = 15$). Under 3,5-DHPG, the distribution of sEPSC IELs was narrower than the control condition (Fig. 1*E*), and there was a significant shift of the distribution toward shorter IELs (Fig. 1*F*; Kolmogorov–Smirnov test: $p < 0.0001$; $n = 15$), reflecting a substantial increase in sEPSC frequency. Normalized sEPSC frequency was significantly increased by 3,5-DHPG (Fig. 1*G*; DHPG: 7.90 ± 2.94 ; Wash: 1.31 ± 0.28 ; Friedman test: $\chi^2(2) = 10.13$, $p = 0.0063$, $W = 0.343$; Dunn's multiple-comparisons test: Ctrl vs DHPG, $p = 0.0105$; Ctrl vs Wash, $p > 0.9999$; DHPG vs Wash, $p = 0.0318$; $n = 15$). Under 3,5-DHPG, the distribution of sEPSC amplitude was broader than the control condition. Not only did the mean sEPSC amplitude increase during 3,5-DHPG application, but large sEPSCs (>200 pA) that did not exist in the recordings under the control conditions were observed in some neurons, suggesting possible MVR (Fig. 1*H*). 3,5-DHPG caused a shift of the distribution toward larger amplitudes (Fig. 1*I*; Kolmogorov–Smirnov test: $p < 0.0001$; $n = 15$). Normalized data showed that sEPSC amplitude was significantly greater under 3,5-DHPG than control (Fig. 1*J*; DHPG: 1.45 ± 0.14 , Wash: 1.03 ± 0.06 ; RM one-way ANOVA: $F_{(1,207,16,89)} = 8.421$, $p = 0.0074$, $\eta^2 = 0.3756$; Holm–Sidak multiple-comparisons test: Ctrl vs DHPG, $p = 0.0163$; Ctrl vs Wash, $p = 0.6289$; DHPG vs Wash, $p = 0.0303$; $n = 15$). At the same time, 3,5-DHPG did not change the rise time or the decay time constant (τ) of sEPSCs (Fig. 1*K*; rise time, Ctrl: 0.20 ± 0.02 ms; DHPG: 0.22 ± 0.02 ms; Wash: 0.22 ± 0.03 ms; Friedman test: $\chi^2(2) = 3.959$, $p = 0.1381$, $W = 0.131$; Dunn's multiple-comparisons test: Ctrl vs DHPG, $p = 0.2485$; Ctrl vs Wash, $p > 0.9999$; DHPG vs Wash, $p = 0.6037$; $n = 15$; Fig. 1*L*; decay τ , Ctrl: 0.66 ± 0.12 ms; DHPG: 0.71 ± 0.18 ms; Wash: 0.70 ± 0.16 ms; Friedman test: $\chi^2(2) = 1.661$, $p = 0.4358$, $W = 0.055$; Dunn's multiple-comparisons test: Ctrl vs DHPG, $p > 0.9999$; Ctrl vs Wash, $p > 0.9999$; DHPG vs Wash, $p = 0.6037$; $n = 15$). Because a relatively large range of animal ages was used for this experiment, we performed an analysis to examine whether there was any age effect. The normalized sEPSC frequency or amplitude in response to 3,5-DHPG was plotted against the age of animals. Linear regression analysis revealed no significant correlations ($R = 0.07$, $p = 0.802$ for frequency; $R = 0.06$, $p = 0.835$ for amplitude; data not shown). These data support the idea that the evoked glutamatergic transmission in MNTB is inhibited, whereas the spontaneous glutamatergic transmission is facilitated by 3,5-DHPG, demonstrating differential modulation of evoked and spontaneous glutamate release in MNTB neurons by activation of mGluR I.

mGluR I located at the excitatory synapse mediate the modulation

MNTB receives its major excitatory input from contralateral AVCN globular bushy cells. Bath application of 3,5-DHPG (200

μ M) would activate all mGluR I wherever the receptors are expressed in various nuclei regardless of their locations in the brain slice, including AVCN. There is evidence that activation of mGluR I depolarizes bushy cells in mouse AVCN (Chanda and Xu-Friedman, 2011). The voltage change in the cell body may conduct to terminals and trigger or modulate transmitter release (Alle and Geiger, 2006; Shu et al., 2006). Therefore, to determine whether the modulatory effect of mGluR I is mediated by the excitatory synapse at MNTB, we made local puff application of 3,5-DHPG (200 μ M) to the recorded MNTB neurons, which elicited the same effects on sEPSCs as bath application (Fig. 2*A*). An inward current was induced (Fig. 2*B*; 15.6 ± 2.8 pA, $n = 11$). sEPSC frequency and amplitude were significantly increased (Fig. 2*C*; for normalized frequency, DHPG: 17.38 ± 9.84 , Wash: 0.99 ± 0.16 ; Friedman test: $\chi^2(2) = 14.36$, $p = 0.0074$, $W = 0.652$; Dunn's multiple-comparisons test: Ctrl vs DHPG, $p = 0.0167$; Ctrl vs Wash, $p > 0.9999$; DHPG vs Wash, $p = 0.0009$; $n = 11$; Fig. 2*D*; for normalized amplitude, DHPG: 2.00 ± 0.37 , Wash: 1.05 ± 0.10 ; Friedman test: $\chi^2(2) = 9.60$, $p = 0.0075$, $W = 0.436$; Dunn's multiple-comparisons test: Ctrl vs DHPG, $p = 0.0315$; Ctrl vs Wash, $p > 0.9999$; DHPG vs Wash, $p = 0.0315$; $n = 11$), and no significant changes in sEPSC kinetics were detected (Fig. 2*E,F*; $n = 11$).

3,5-DHPG modulation of sEPSC is via both mGluR1 and mGluR5

Group I mGluRs include two members, mGluR1 and mGluR5. 3,5-DHPG can activate both, and selective agonists for each member are not yet available. To determine activation of which members enhanced sEPSC, we applied mGluR1a antagonist LY367385 (50 μ M) or mGluR5 antagonist MPEP (10 μ M) separately for ~ 5 –10 min, during which 3,5-DHPG was applied. Either LY367385 or MPEP eliminated the effects of 3,5-DHPG on sEPSCs (Fig. 3*A–F*), with relatively large variations among different cells when MPEP was applied. There were no significant differences in sEPSC frequency and amplitude between control and LY367385 + 3,5-DHPG (Fig. 3*B*; for normalized frequency, LY367385: 0.94 ± 0.06 ; LY367385 + DHPG: 0.98 ± 0.10 ; Wash: 1.10 ± 0.06 ; RM one-way ANOVA: $F_{(1,719,10,31)} = 1.212$, $p = 0.3290$, $\eta^2 = 0.1681$; Holm–Sidak multiple-comparisons test: Ctrl vs LY367385, $p = 0.8014$; Ctrl vs LY367385 + DHPG, $p = 0.8864$; $n = 7$; Fig. 3*C*; for normalized amplitude, LY367385: 1.08 ± 0.13 ; LY367385 + DHPG: 1.09 ± 0.16 ; Wash: 0.94 ± 0.14 ; Friedman test: $\chi^2(3) = 1.435$; $p = 0.6974$, $W = 0.068$; Dunn's multiple-comparisons test: Ctrl vs LY367385, $p > 0.9999$; Ctrl vs LY367385 + DHPG, $p > 0.9999$; $n = 7$). Similarly, no significant differences were detected between control and MPEP + 3,5-DHPG (Fig. 3*E*; for normalized frequency, MPEP: 1.02 ± 0.10 ; MPEP + DHPG: 2.51 ± 0.63 ; Wash: 1.14 ± 0.18 ; Friedman test: $\chi^2(3) = 8.309$; $p = 0.04$, $W = 0.197$; Dunn's multiple-comparisons test: Ctrl vs MPEP, $p > 0.9999$; Ctrl vs MPEP + DHPG, $p = 0.3422$; $n = 14$; Fig. 3*F*; for normalized amplitude, MPEP: 0.96 ± 0.04 ; MPEP + DHPG: 1.11 ± 0.07 ; Wash: 0.97 ± 0.07 ; RM one-way ANOVA: $F_{(1,757,22,84)} = 1.865$, $p = 0.1808$, $\eta^2 = 0.1255$; Holm–Sidak multiple-comparisons test: Ctrl vs MPEP, $p = 0.7789$; Ctrl vs MPEP + DHPG, $p = 0.4181$; $n = 14$). While the averaged data showed that either LY367385 (50 μ M) or MPEP (10 μ M) abolished the modulation, in some individual cells, neither of the antagonists completely blocked the 3,5-DHPG effects. This was especially obvious in a few cells, in which the sEPSC frequency was increased substantially by 3,5-DHPG in the presence of MPEP (Fig. 3*E*). Therefore, we applied both antagonists for ~ 5 –10 min, during which we applied 3,5-DHPG (200 μ M; Fig. 3*G*). Application of

Figure 1. Differential mGluR modulation of eEPSC and sEPSC. **A, B,** An agonist for mGluR I, 3,5-DHPG (200 μ M) inhibited eEPSCs in MNTB neurons ($n = 5$), consistent with published results (Kushmerick et al., 2004). Amp, Amplitude; Norm, normalized. **C,** Bath application of 3,5-DHPG (200 μ M) produced an inward current and increased sEPSCs in the sample neuron. The instantaneous frequency and amplitude of sEPSC reaches the highest during 3,5-DHPG application. Inset, Averaged sEPSC traces from the three conditions superimposed at a larger time scale. **D,** Inward current induced by 3,5-DHPG ($n = 15$). **E,** Under 3,5-DHPG (red), the distribution of sEPSC IELs was narrower than the control (black) condition. **F,** Cumulative probability analysis of the IELs ($n = 15$). The leftward shift of the distribution under 3,5-DHPG reflects the increase in frequency compared with control. Kolmogorov–Smirnov analysis, $p < 0.0001$. **G,** 3,5-DHPG significantly increased sEPSC frequency ($n = 15$). Freq, Frequency. **H,** Under 3,5-DHPG, the distribution of sEPSC amplitude of a different sample cell is wider than the control condition, extending well beyond the dashed vertical line that indicates the maximal sEPSC amplitude under the control condition, suggesting possible multivesicular release under 3,5-DHPG. **I,** Cumulative probability analysis of the amplitude of sEPSCs ($n = 15$). The rightward shift of the distribution under 3,5-DHPG reflects the increase in amplitude compared with control (cutoff, 250 pA). Kolmogorov–Smirnov analysis, $p < 0.001$. **J,** 3,5-DHPG significantly increased the sEPSC amplitude ($n = 15$). **K, L,** 3,5-DHPG did not affect the kinetics of sEPSCs ($n = 15$). In this and subsequent figures, the mean $\pm$ SEM values are shown, with each dot representing the value of one individual cell. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. n.s.: not significant, $p > 0.05$.

Figure 2. mGluR I located at the excitatory synapse mediates the modulation. **A**, Left, Schematic diagram showing puff application to the recorded MNTB neuron. Right, Puff application of 3,5-DHPG (200 μ M) produced an inward current and a robust enhancement of sEPSCs in the recorded MNTB neuron. **B**, Inward current induced by puff application of 3,5-DHPG ($n = 11$). **C**, The frequency of sEPSCs was significantly increased by puffed 3,5-DHPG ($n = 11$). **D**, The amplitude of sEPSCs was also significantly increased ($n = 11$). **E**, **F**, Puffed 3,5-DHPG did not affect the kinetics of sEPSCs ($n = 11$).

both antagonists completely abolished the 3,5-DHPG effects (Fig. 3H; for normalized frequency, LY367385 + MPEP: 0.72 ± 0.16 ; LY367385 + MPEP + DHPG: 0.69 ± 0.19 ; Wash: 0.72 ± 0.17 ; RM one-way ANOVA: $F_{(1,583,6,330)} = 2.217$, $p = 0.1873$, $\eta^2 = 0.3566$; Holm–Sidak multiple-comparisons test: Ctrl vs LY367385 + MPEP, $p = 0.6626$; Ctrl vs LY367385 + MPEP + DHPG, $p = 0.6626$; $n = 5$; Fig. 3I; for normalized amplitude, LY367385 + MPEP: 0.87 ± 0.06 ; LY367385 + MPEP + DHPG: 0.82 ± 0.05 ; Wash: 0.86 ± 0.06 ; RM one-way ANOVA: $F_{(1,428,5,714)} = 6.575$; $p = 0.0384$, $\eta^2 = 0.6248$; Holm–Sidak multiple-comparisons test: Ctrl vs LY367385 + MPEP, $p = 0.2825$; Ctrl vs LY367385 + MPEP + DHPG, $p = 0.1189$; $n = 5$). Based on these pharmacological results, we conclude that the modulation involves both mGluR1 and mGluR5, and a functional cross talk between the two members may exist.

To determine the presynaptic or postsynaptic localization of mGluR I, we examined the distribution pattern of mGluR5 immunoreactivity (for mGluR1, see Discussion) relative to the localization of a presynaptic marker SV2. Using a monoclonal antibody (ab76316), mGluR5 immunoreactivity exhibited a punctate and partially membrane-associated pattern in MNTB (Fig. 4A,B). First, we identified mGluR5 immunoreactivity in a subset of SV2-labeled presynaptic structures (Fig. 4B1–B3, yellow arrows). Second, mGluR5-immunoreactive puncta were detected within the cell bodies of the principle MNTB neurons, as outlined by SV2 staining. Some puncta were localized immediately adjacent to SV2-labeled presynaptic terminals (Fig. 4B1–B3, white arrows), implicating a postsynaptic localization. Other cell groups of the superior olivary complex, such as the LSO, exhibited a more granular appearance of mGluR5 immunoreactivity without large puncta (Fig. 4C1–C3), indicating that the observed mGluR5 puncta in MNTB are not nonspecific background staining.

mGluR5 localization in SV2-labeled presynaptic terminals was also detected using a polyclonal anti-mGluR5 antibody (ab5675; Fig. 4D1–D3, yellow arrows). The somatic localization

of mGluR5 appeared more prominent with this antibody compared with ab76316, while presynaptic localization of mGluR5 was also evident (Fig. 4D3, inset). Together, our results demonstrate that mGluR5 is localized in both presynaptic terminals and the cell bodies of postsynaptic neurons in MNTB at P14.

3,5-DHPG modulation of sEPSCs depends on Na_V channels, and the signaling pathway involves Ca_V channels

3,5-DHPG modulation of glycinergic sEPSCs is dependent on Na_V channels (Curry et al., 2018). To determine whether 3,5-DHPG modulation of sEPSCs also depends on Na_V channels, a Na_V channel blocker, TTX (1 μ M), was applied before and during 3,5-DHPG application. Without TTX, activation of mGluR I by 3,5-DHPG (200 μ M) increased sEPSC frequency and amplitude (Fig. 5A). In the same cell, TTX application resulted in the recoding of mEPSCs, and TTX completely eliminated the effect of 3,5-DHPG (Fig. 5B). Normalized population data showed that 3,5-DHPG application in the presence of TTX did not cause significant changes in mEPSC frequency or amplitude (Fig. 5C; for normalized frequency, DHPG with TTX: 1.11 ± 0.29 ; Wash: 1.94 ± 0.88 ; Friedman test: $\chi^2(2) = 4.80$; $p = 0.1242$, $W = 0.48$; Dunn's multiple-comparisons test: Ctrl vs DHPG, $p > 0.9999$; $n = 5$; Fig. 5D; for normalized amplitude, DHPG with TTX: 1.01 ± 0.16 ; Wash: 1.02 ± 0.05 ; RM one-way ANOVA: $F_{(1,155,4,621)} = 0.008973$, $p = 0.9491$, $\eta^2 = 0.0022$; Holm–Sidak multiple-comparisons test: Ctrl vs DHPG, $p = 0.9962$; $n = 5$). These results indicate that the 3,5-DHPG modulation is Na_V channel dependent.

In calyx of Held, small depolarization induced by glycine enhances presynaptic Ca^{2+} concentration, leading to increased frequency of mEPSCs (Turecek and Trussell, 2001). To test whether 3,5-DHPG modulation of sEPSCs involves Ca^{2+} signaling, a nonspecific Ca_V channel blocker, CdCl_2 , was applied. Without CdCl_2 , activation of mGluR I by 3,5-DHPG increased sEPSC frequency and amplitude (Fig. 5E). In the same cell, CdCl_2 (100 μ M) eliminated the effect of 3,5-DHPG (Fig. 5F).

Figure 3. Both mGluR1 and mGluR5 are involved in modulation of sEPSCs, with possible cross talk between the two subtypes. **A–C**, An antagonist for mGluR1a (LY367385, 50 μ M) prevented the effect of 3,5-DHPG on sEPSCs ($n = 7$). **D–F**, An antagonist for mGluR5 (MPEP, 10 μ M) prevented the effect ($n = 14$), with greater variations. **G–I**, When combined, the mGluR1a and mGluR5 antagonists completely prevented 3,5-DHPG modulation ($n = 5$).

Normalized population data showed that sEPSC frequency and amplitude were not affected by 3,5-DHPG in the presence of CdCl₂ (Fig. 5G; for normalized frequency, CdCl₂: 0.68 ± 0.14 ; CdCl₂ + DHPG: 0.75 ± 0.21 ; RM one-way ANOVA: $F_{(1,059,5,296)} = 5.800$, $p = 0.1880$, $\eta^2 = 0.5370$; Holm–Sidak multiple-comparisons test: Ctrl vs CdCl₂, $p = 0.1352$; Ctrl vs CdCl₂ + DHPG, $p = 0.5179$; $n = 6$; Fig. 5H; for normalized amplitude, CdCl₂: 1.15 ± 0.10 ; CdCl₂ + DHPG: 1.05 ± 0.11 ; Friedman test: $\chi^2(2) = 4.333$, $p = 0.11416$, $W = 0.361$; Dunn's multiple-comparisons test: Ctrl vs CdCl₂, $p = 0.4467$; Ctrl vs CdCl₂ + DHPG, $p > 0.9999$; $n = 6$). These results support that the signaling pathway underlying the enhancement of sEPSCs by 3,5-DHPG involves Ca_v channels.

Activation of mGluR I depolarizes membrane and enhances the I_{NaP} of calyx of Held

In the calyx of Held, I_{NaP} amplifies the glycine-induced enhancement of glutamate release (Huang and Trussell, 2008). It is possible that the activation of mGluR I depolarizes the membrane;

enhances the I_{NaP} of calyx of Held, which further depolarizes the membrane; and leads to the enhancement of spontaneous glutamate release. To directly test these ideas, calyx (presynaptic) recordings were performed. The success of such recordings was confirmed by filling the calyx of Held with Alexa Fluor 488 (Fig. 6A, inset). Because of the high difficulty level achieving calyx recordings, we first performed the experiments in younger animals (P8–P10) and under lower temperature (32°C), to achieve sufficient voltage clamp. Under current-clamp configuration, 3,5-DHPG (200 μ M) depolarized the membrane of calyx of Held (Fig. 6A) by an average of 3.9 ± 0.5 mV (Fig. 6B; $n = 7$), similar to a previous report in rats (Kushmerick et al., 2004).

I_{NaP} in the calyx of Held terminal was then studied using voltage-ramp and voltage-step protocols in the presence of K_v and Ca_v channel blockers (see Materials and Methods). In calyces obtained from P8–P10 mice and recorded at 32°C, a voltage ramp (70 mV/s) evoked an inward current, without escaping action current. Bath application of 3,5-DHPG (200 μ M) shifted the I_{NaP} activation to more hyperpolarized voltages. The current

Figure 4. Subcellular localization of mGluR5 in MNTB. Double immunostaining against mGluR5 (red) and presynaptic marker SV2 (green) was performed on P14 transverse brainstem sections containing MNTB. Sections were also counterstained with DAPI (blue). **A1–A3**, **B1–B3** and **C1–C3** were taken from sections stained with a monoclonal anti-mGluR5 (ab76316), while **D1–D3** was with a polyclonal anti-mGluR5 (ab5675). **A1–A3**, Low-magnification image of MNTB (outlined by dashed lines). **B1–B3**, High-magnification images of MNTB. Yellow arrows point to SV2-labeled presynaptic terminals that contain mGluR5 immunoreactivity. White arrows point to examples of mGluR5 puncta that were apposed to the presynaptic structure. Insets are closer views of the boxes in B3. **C1–C3**, High-magnification images of LSO. Note the lack of large mGluR5 puncta that were observed in MNTB. **D1–D3**, High-magnification image of ab5675 showing mGluR5 localization in SV2-labeled terminals (yellow arrows) in MNTB. The inset is a closer view of the box in D3, showing colocalization of SV2 and mGluR5. Scale bars: **A1** (for **A1–A3**), 100 μ m; **B1** (for **B1–B3**, **C1–C3**, **D1–D3**), 5 μ m; insets (all insets in **B3** and **D3**), 1 μ m.

evoked with ramps was fully blocked by TTX (500 nM; Fig. 6C, blue trace), confirming it is a Na^+ current (Fig. 6C). By subtraction of the I_{NaP} traces from the trace with TTX (500 nM), the current–voltage relation for I_{NaP} and the activation threshold were determined (as defined in the Materials and Methods). The enlarged I_{NaP} traces showed that 3,5-DHPG shifted the activation threshold from -85.3 ± 0.8 to -88.0 ± 0.7 mV and largely increased the I_{NaP} at voltages around RMP (Fig. 6D), the value of which was reported to be -75 mV for immature calyces at 32°C (Huang and Trussell, 2011, 2014) and at approximately -74 mV at a more physiological temperature (Sierksma and Borst, 2017). To further explore these activation characteristics, conductance versus voltage plots were constructed using voltage steps. Boltzmann fits revealed that the V_{half} shifted from -52.7 ± 4.1 to -56.2 ± 3.9 mV (Fig. 6E; paired t test; $d_{\text{Cohen}} = -0.396$, $p = 0.002$, $n = 5$). At -75 mV, DHPG increased the I_{NaP} amplitude from 43.7 ± 15.6 to 70.6 ± 21.4 pA (Fig. 6F; paired t test; $d_{\text{Cohen}} = 0.642$, $p = 0.04$, $n = 5$).

The above voltage-clamp experiments were performed in calyces obtained from prehearing (P8–P10) mice, and the recordings were done at 32°C for practical reasons: relatively higher feasibility in younger animals and sufficient voltage clamp. To be consistent with the conditions for our postsynaptic recordings, we performed the same experiments described above (Fig. 6C–F) in posthearing (P14–P18) mice at more physiological temperatures (34 – 36°C). Under these conditions, three of five recorded calyces generated escaping action currents (data not shown), because of fast activation kinetics of the transient Na_V currents (Huang and Trussell, 2008; Hu and Bean, 2018). Nonetheless, the effects of 3,5-DHPG on the I_{NaP} were similar to those observed in prehearing mice (Fig. 6G–J). 3,5-DHPG shifted the activation threshold of I_{NaP} from -87.5 ± 2.6 to -90.4 ± 1.7 mV (Fig. 6G,H) and shifted the V_{half} of I_{NaP} from -42.6 ± 3.8 to -48.6 ± 3.3 mV (Fig. 6I; paired t test, $d_{\text{Cohen}} = -0.79$, $p = 0.005$, $n = 5$). At -75 mV, 3,5-DHPG increased the I_{NaP} amplitude from 107.6 ± 69.1 to 173.1 ± 70.0 pA (Fig. 6J; paired t

Figure 5. mGluR I modulation of sEPSCs is Na_V channel dependent, and the signaling pathway involves Ca_V channels. **A, B**, Under control conditions, 3,5-DHPG increased the frequency and amplitude of sEPSCs. In the presence of TTX ($1 \mu\text{M}$), 3,5-DHPG did not affect mEPSCs. **C, D**, Summary of population data with the presence of TTX ($n = 5$). **E**, In another group of MNTB neurons, under control conditions, 3,5-DHPG increased the frequency and amplitude of sEPSCs. **F**, In the presence of a generic blocker for Ca_V channels (CdCl_2 , $100 \mu\text{M}$), 3,5-DHPG did not affect sEPSCs. **G, H**, Population data for the change in frequency and amplitude of sEPSC in the presence of CdCl_2 ($n = 6$).

test, $d_{\text{Cohen}} = 0.471$, $p = 0.0417$, $n = 4$; one cell was excluded because the escaping action current prevented measurement of the I_{NaP} amplitude). These results indicate that mGluR I activation substantially increases the I_{NaP} in posthearing mice, confirming mGluR modulation of the spontaneous glutamate release in both age groups.

To further support our conclusion, we tested the effect of riluzole, a potent I_{NaP} inhibitor (Urbani and Belluzzi, 2000), on

sEPSC. Applied at least 5 min before 3,5-DHPG application, riluzole ($10 \mu\text{M}$) prevented the effects of 3,5-DHPG on sEPSCs (Fig. 6K). We detected no significant differences in the frequency and amplitude of sEPSCs between control and riluzole + 3,5-DHPG conditions (Fig. 6L; for normalized frequency, riluzole: 1.11 ± 0.15 ; riluzole + DHPG: 1.58 ± 0.27 ; Wash: 1.24 ± 0.18 ; Friedman test: $\chi^2(3) = 2.356$, $p = 0.0519$, $W = 0.523$; Holm–Sidak multiple-comparisons test: Ctrl vs riluzole, $p > 0.9999$; Ctrl vs riluzole + DHPG, $p > 0.9999$; $n = 15$; Fig. 6M; for normalized amplitude, riluzole: 1.05 ± 0.07 ; riluzole + DHPG: 1.14 ± 0.11 ; Wash: 1.05 ± 0.06 ; Friedman test: $\chi^2(3) = 0.68$, $p = 0.8779$, $W = 0.0151$; Dunn’s multiple-comparisons test: Ctrl vs riluzole, $p > 0.9999$; Ctrl vs riluzole + DHPG, $p > 0.9999$; $n = 15$). These results suggest that mGluR I-mediated enhancement of spontaneous glutamate release occurs via enhancing the presynaptic I_{NaP} .

Enhancement of sEPSCs by mGluR I does not involve CB1 cannabinoid receptors activated via retrograde signaling

In calyx of Held, activation of postsynaptic mGluR I produces second messengers that diffuse retrogradely into the presynaptic terminal, and it inhibits evoked glutamate release via activation of presynaptic CB1 cannabinoid receptors (CB1Rs; Kushmerick et al., 2004). We designed a set of control experiments to test the involvement of this retrograde signaling pathway in the mGluR I modulation of sEPSCs. First, we tested whether postsynaptic Ca^{2+} was involved in mGluR I modulation of sEPSCs. The intracellular Ca^{2+} concentration could be raised because of mGluR I activation through release from internal stores via the phospholipase pathway (for review, see Niswender and Conn, 2010) and was involved in generation of depolarization-induced suppression of excitation (Glitsch et al., 2000). We included a fast Ca^{2+} chelator (BAPTA, 40 mM) in the recording electrodes (the concentration of K-gluconate was reduced accordingly from 130 to 90 mM, to maintain proper osmolarity), and obtained the effects of 3,5-DHPG ($200 \mu\text{M}$) on sEPSCs. Kushmerick

et al. (2004) showed that including 40 mM BAPTA in the recording electrodes eliminated the inhibitory effects of 3,5-DHPG on eEPSCs caused by postsynaptic mGluR I activation. In our present study, with 40 mM BAPTA in the recording electrodes, the modulatory effects of 3,5-DHPG on sEPSCs were still observed (Fig. 7A–C; $n = 9$), same as those recorded with the regular internal solution (Figs. 1, 2). sEPSC frequency and amplitude were significantly

Figure 6. 3,5-DHPG enhances the activation of persistent sodium current (I_{NaP}) of calyx of Held, in both prehearing (P8–P10) mice at 32°C and posthearing (P14–P18) mice at a more physiological temperature (34–36°C). **A**, 3,5-DHPG depolarized the membrane potential of calyx of Held. Inset, Presynaptic recording was confirmed by filling the calyx of Held with Alexa Fluor 488. **B**, Depolarization of calyx of Held induced by 3,5-DHPG ($n = 7$). **C**, In prehearing (P8–P10) mice, at 32°C, a voltage ramp (70 mV/s) evoked an inward current in a calyx of Held terminal in control (black) and in the presence of 3,5-DHPG (200 μ M, red). Subsequent addition of TTX (0.5 μ M) fully blocked the inward current (blue). **D**, TTX-subtracted traces, with the inset showing the expanded view of activation of the TTX-sensitive current in control (black) and in the presence of 3,5-DHPG (red). **E**, Boltzmann fitting of the conductance voltage curves revealed that 3,5-DHPG shifted the V_{half} to the left toward a more negative voltage. Inset, Representative currents to voltage steps from -80 to -40 mV (increment of 10 mV, duration of 1 s). The current amplitude was measured at 750–1000 ms. **F**, At -75 mV, around the resting membrane potential for calyx of Held, bath application of 3,5-DHPG increased the I_{NaP} amplitude ($n = 5$). **G–J**, In posthearing (P14–P18) mice, at 34–36°C, the same experiments and analyses as described above (**C–F**) were repeated, and the observations were confirmed ($n = 4–5$). **K–M**, An antagonist for I_{NaP} (riluzole, 10 μ M) largely prevented the effect of 3,5-DHPG on sEPSCs ($n = 15$).

Figure 7. Enhancement of sEPSCs by mGluR I does not involve CB1Rs activated via retrograde signaling. **A–C**, Including a fast Ca^{2+} chelator, BAPTA (40 mM), in the recording internal solution did not change the effect of 3,5-DHPG (200 μM) on sEPSCs ($n = 9$). **D–F**, CB1R agonist (WIN, 5 μM) had no effects on sEPSCs ($n = 9$). **G–I**, CB1R antagonist (AM251, 5 μM) had no effects on sEPSCs ($n = 6$).

increased (Fig. 7B; for normalized frequency, DHPG: 26.20 ± 12.08 ; Wash: 1.33 ± 0.46 ; Friedman test: $\chi^2(2) = 10.89$, $p = 0.0029$; $W = 0.605$; Dunn's multiple-comparisons test: Ctrl vs DHPG, $p = 0.0186$; Ctrl vs Wash, $p > 0.9999$; DHPG vs Wash, $p = 0.0065$; $n = 9$; Fig. 7C; for normalized amplitude, DHPG: 3.21 ± 0.78 ; Wash: 1.21 ± 0.11 ; Friedman test: $\chi^2(2) = 6.89$, $p = 0.0307$; $W = 0.382$; Dunn's multiple-comparisons test: Ctrl vs DHPG, $p = 0.0286$; Ctrl vs Wash, $p = 0.2969$; DHPG vs Wash, $p > 0.9999$; $n = 9$). Second, we tested the effects of CB1R agonist (WIN, 5 μM) and found no effects on sEPSCs (Fig. 7D–F; $n = 9$), indicating that activation of the presynaptic CB1Rs did not affect spontaneous glutamate release. Finally, to exclude the possibility that CB1Rs might be saturated under the rest condition and thus the agonist WIN did not cause further modulation, we tested the effects of CB1R antagonist (AM251, 5 μM). As expected, no significant effects on sEPSCs were detected (Fig. 7G–I; $n = 6$), indicating minimal endogenous activity of CB1Rs in our slice recording conditions. The results of these control experiments further confirmed that presynaptic mGluR I enhanced sEPSCs through signaling pathways inside the presynaptic terminal, without involvement of the CB1Rs that are involved in modulation of eEPSCs and other postsynaptic Ca^{2+} -dependent signaling.

Activation of mGluR I depolarizes membrane potential, increases the frequency and amplitude of spontaneous EPSP, and affects AP generation in MNTB neurons

To investigate the physiological function of the observed modulatory effects of mGluR I activation, current-clamp recordings of MNTB neurons were performed. Consistent with voltage-clamp recordings, puff application of 3,5-DHPG (200 μM) depolarized the membrane potential (Fig. 8A,B; 4.9 ± 0.8 mV; $n = 10$). Spontaneous EPSP (sEPSP) frequency and amplitude were significantly increased (Fig. 8C; for normalized frequency, DHPG: 5.54 ± 1.72 ; Wash: 1.52 ± 0.54 ; Friedman test: $\chi^2(2) = 15.44$, $p < 0.0001$, $W = 0.772$; Dunn's multiple-comparisons test: Ctrl vs DHPG, $p = 0.0016$; Ctrl vs Wash, $p > 0.9999$; DHPG vs Wash, $p = 0.0036$; $n = 10$; Fig. 8D; for normalized amplitude, DHPG: 2.29 ± 0.57 ; Wash: 0.98 ± 0.09 ; Friedman test: $\chi^2(2) = 15.79$, $p < 0.0001$, $W = 0.789$; Dunn's multiple-comparisons test: Ctrl vs DHPG, $p = 0.0024$; Ctrl vs Wash, $p > 0.9999$; DHPG vs Wash, $p = 0.0024$; $n = 10$). In 2 of 10 cells, 3,5-DHPG induced APs (Fig. 8E,F).

Is the AP generation due to the presynaptic enhancement of sEPSPs or the postsynaptic membrane depolarization caused by activation of mGluR I, or both? To test this, we blocked synaptic

Figure 8. mGluR I affect the output of MNTB neurons. **A**, Top, Under current-clamp configuration, 3,5-DHPG depolarized the membrane potential, and increased the frequency and amplitude of sEPSPs in postsynaptic MNTB neurons. Bottom, A small segment of the recording during 3,5-DHPG shown at enlarged scales. Also shown on the right are the averaged sEPSPs under the three conditions. **B**, Depolarizations induced by 3,5-DHPG ($n = 10$). **C**, The frequency of sEPSPs was significantly increased by 3,5-DHPG ($n = 10$). **D**, The amplitude of sEPSPs was also significantly increased ($n = 10$). **E**, In 2 of 10 cells, 3,5-DHPG induced APs (indicated by the red triangles). **F**, The captured sEPSPs (blue) and APs (red) during 3,5-DHPG application shown at an enlarged time scale. **G**, In the presence of a cocktail of synaptic receptor blockers, puffed 3,5-DHPG depolarized the membrane potential of MNTB neurons. **H**, In 2 of 12 cells, APs were generated on the top of the membrane depolarization induced by 3,5-DHPG. **I**, Depolarizations induced by 3,5-DHPG in the presence of synaptic receptor blockers. **J**, The percentages of the tested cells that generated APs during 3,5-DHPG application with (black) or without (red) synaptic receptor blockers.

Figure 9. Hypothetical mechanisms underlying differential mGluR I modulation of eEPSC and sEPSC in MNTB. For evoked glutamatergic transmission, Kushmerick et al. (2004) demonstrated that activation of postsynaptic mGluR1 in rat MNTB neurons produces retrograde signaling molecules, which lead to the activation of CB1Rs. CB1Rs reduce evoked glutamate release potentially via inhibition of presynaptic Ca^{2+} current. Our current study demonstrated that for spontaneous glutamatergic transmission, presynaptic mGluR5, and likely mGluR1 too, depolarize the membrane of calyx of Held and enhance I_{NaP} . We propose that this signaling pathway targets the spontaneous glutamate vesicle pool and facilitates its release via an increase of the stochastic opening probability of presynaptic Ca_v channels that may be different from those mediating the evoked glutamate release.

transmission by applying a cocktail of synaptic receptor blockers (DNQX, 50 μM ; APV, 50 μM ; strychnine, 1 μM ; gabazine, 10 μM) and then tested the effect (supposedly postsynaptic only) of 3,5-DHPG. Under this condition, 3,5-DHPG (200 μM) caused membrane depolarization without generating APs in 10 of 12 cells (Fig. 8G). In the other two cells, APs were observed (Fig. 8H). The average depolarization induced by 3,5-DHPG was 7.2 ± 0.9 mV (Fig. 8I; $n = 12$), similar to most recently published results (5.9–7.5 mV depolarization depending on the age of mice; Dos Santos e Alhadas et al., 2020). The percentage of cells generating APs during 3,5-DHPG application with and without synaptic receptor blockers was about the same (Fig. 8J), suggesting that the firing due to mGluR I activation is largely a postsynaptic effect, and therefore the presynaptic effects of mGluR I activation may primarily affect synaptic integration at subthreshold levels.

Discussion

We first discovered that mGluR I exerted differential modulation on spontaneous versus evoked glutamate release in MNTB neurons. Then we investigated the mechanisms underlying the enhancement of spontaneous glutamate transmission. Local activation of the receptors at the recorded cells revealed that mGluR I at the presynaptic and postsynaptic MNTB components mediated the modulation on spontaneous glutamate release and cell depolarization, respectively. Pharmacological and anatomic data supported involvement of both mGluR1 and mGluR5, with possible functional cross talk between them. More importantly, the presynaptic modulation on spontaneous glutamate release was dependent on Na_v and involved Ca_v channels. The I_{NaP} plays a pivotal role linking mGluR I to the increased spontaneous glutamate release.

Signaling pathway underlying mGluR I enhancement of spontaneous glutamate release

Different signaling pathways underlie modulation of glutamatergic transmission by mGluR I in MNTB neurons depending on

the release mode. For evoked glutamatergic transmission, activation of postsynaptic mGluR1 in MNTB neurons generates retrograde signaling molecules, diffusion of which into the calyx activates CB1Rs, which subsequently reduces eEPSCs via inhibition of presynaptic Ca^{2+} currents (Kushmerick et al., 2004). Here, we propose the following signaling pathway to interpret our observation that 3,5-DHPG enhanced sEPSCs in MNTB neurons (Fig. 9). First, mGluR I depolarizes the presynaptic terminal. Second, the depolarization enhances I_{NaP} of the calyx. Third, the stochastic opening of Ca_v channels at rest is enhanced, leading to increased spontaneous glutamate release. The discussion below centers around interpretations and implications associated with each of these steps.

First, while both mGluR1 and mGluR5 were involved, why did the antagonist for each subtype nearly completely block the effect of 3,5-DHPG on sEPSCs? Although homodimers of mGluRs are the common path for each member to function, cross talk between different members has been discovered (Lee et al., 2020). Physical association and functional cooperative signaling between mGluR1 and mGluR5 have been reported (Doumazane et al., 2011; Sevastyanova and Kammermeier, 2014). We showed that mGluR5 was localized both presynaptically and postsynaptically (Fig. 4). In rats, mGluR1 is localized in the cell bodies of MNTB neurons (Kushmerick et al., 2004). Although mixed results of presynaptic mGluR1 in mice were seen (data not shown), our pharmacological results did suggest the existence of mGluR1 in the calyx. Thus, mGluR1 and mGluR5 could potentially form mGluR1–mGluR5 dimer, or interact via functional mGluR1–mGluR1 dimer with mGluR5–mGluR5 dimer. Consequently, blocking either subtype would largely disable the signaling pathway, while application of both antagonists completely prevented the effect.

Second, how does the mGluR I-mediated depolarization cause enhancement of I_{NaP} and spontaneous glutamate release? Small in amplitude, I_{NaP} is functionally significant, regulating

neuronal excitability and AP generation (Thor and Morris, 2016; Browne et al., 2017; Hsu et al., 2018; Müller et al., 2018). In hypothalamus tuberomammillary neurons, I_{NaP} (average, 22.6 pA) is sufficient to drive spontaneous pacemaking firing (Taddese and Bean, 2002). In cortical neurons, an increased I_{NaP} causes hyperexcitability by decreasing AP threshold (Deng and Klyachko, 2016). In MNTB, the presynaptic I_{NaP} augments the depolarizing effect of glycine and increases mEPSC frequency (Huang and Trussell, 2008). I_{NaP} is also highly sensitive to neuromodulation. In glutamatergic external tufted cells in the olfactory bulb, mGluR I increases I_{NaP} and bursting firing (Dong and Ennis, 2014). In nucleus accumbens, mGluR5 increases I_{NaP} , enhances an afterdepolarization, and subsequently increases spike firing (D'Ascenzo et al., 2009). In our presynaptic recordings, 3,5-DHPG shifted the V_{half} of I_{NaP} and increased the amplitude at the RMP (Fig. 6). The resulting Na^+ influx would further depolarize the calyx and facilitate spontaneous glutamate release. It is not uncommon that subthreshold depolarization increases spontaneous neurotransmitter release (Graydon et al., 2011; Trapani and Nicolson, 2011). In MNTB, glycine depolarizes the calyx by 8.7 mV and increases mEPSC frequency (Turecek and Trussell, 2001). In our study, mEPSCs because of UVR were not affected by mGluR I (Fig. 5), suggesting that spontaneous MVR might be emerging because of mGluR modulation. MVR was thought to exist in only some specialized synapses, but indeed it is more common than previously believed (for review, see Rudolph et al., 2015). At the mouse calyx–MNTB synapse, MVR is likely to occur in young animals (P5) and is reduced in older animals (P12–P14; Taschenberger et al., 2002). Based on a binomial model (Oertner et al., 2002), Taschenberger et al. (2002) predicted that ~14% of the successful release events in P12–P14 mouse MNTB are MVR. Such release, however, may not be AP dependent, because activation of mGluR I did not generate APs in AVCN bushy cells (Chanda and Xu-Friedman, 2011). Meanwhile, we cannot exclude AP-dependent release from noncalyceal excitatory inputs to MNTB (Hamann et al., 2003).

Third, the involvement of Ca_v channels in this mGluR-induced enhancement of sEPSCs is intriguing in that these channels usually require high membrane voltages for activation. Spontaneous transmitter release is affected by presynaptic Ca^{2+} , and usually an increased Ca^{2+} concentration results in enhanced spontaneous release (for review, see Williams and Smith, 2018). The presynaptic Ca_v channels have stochastic opening at RMP, and subthreshold depolarizations could enhance their opening probability and increase spontaneous release (for review, see Kavalali, 2020). Consistent with this idea, an increase in the intracellular Ca^{2+} concentration through the opening of Ca_v channels near RMP in the calyx increased mEPSC frequency when glycine causes a presynaptic depolarization (Turecek and Trussell, 2001; Awatramani et al., 2005). A modeling study shows that a 10 mV depolarization increases the likelihood of stochastic activation of Ca_v channels and triggers Ca_v channel-dependent spontaneous release (Ermolyuk et al., 2013). The R-type Ca_v channels have a relatively low activation threshold and may play a prominent role in spontaneous transmitter release (Ermolyuk et al., 2013). These channels are present at the calyx (Iwasaki et al., 2000), suggesting that different Ca_v channels may underlie the differential modulation of mGluR I on spontaneous versus evoked glutamate release. Additionally, while CB1R activation suppresses eEPSCs of MNTB neurons (Kushmerick et al.,

2004), sEPSCs were not affected (Fig. 7), suggesting involvement of different Ca_v channels and/or different signaling pathways for spontaneous versus evoked release. Activation of mGluR I could also increase intracellular Ca^{2+} concentration via release of Ca^{2+} from internal stores (Dong et al., 2009; Pittaluga, 2016). The blockade of 3,5-DHPG effect by Cd^{2+} (Fig. 5) hints that the major pathway for the Ca^{2+} signaling may be through Ca_v channels, which could induce further Ca^{2+} release from internal stores (Ca^{2+} -induced Ca^{2+} release). Finally, low-threshold Ca_v channels could also trigger transmitter release (for review, see Carbone et al., 2014). The existence and properties of such low-threshold Ca_v channels in the calyx await future investigation.

Together, activation of mGluR I generated membrane depolarization of the calyx and shifted activation voltage of I_{NaP} toward more hyperpolarized levels. Consequently, uncorrelated stochastic opening of Ca_v channels at rest may be facilitated, causing Ca^{2+} influx, which may selectively target the spontaneous vesicle pool and might enhance MVR of glutamate. Meanwhile, mGluR I activation, via PKC pathway, may increase Ca^{2+} sensitivity of synaptic vesicle fusion (Lou et al., 2005; Vyleta and Smith, 2011). These mGluR-associated processes may work synergistically to increase spontaneous glutamate release.

Functional implication

Spontaneous and evoked neurotransmission may serve different physiological functions (for review, see Kavalali, 2015). The differential modulation of spontaneous and evoked neurotransmission by mGluRs constitutes one of the mechanisms distinguishing the functions of these two release modes. Our studies (Curry et al., 2018; and the current study) further point to potential yet unrevealed contributions of spontaneous transmitter release to neural function at the circuitry level, especially when spontaneous transmitter release is enhanced by mGluRs. Do the cellular events required for mGluR modulation of the spontaneous glutamatergic transmission at rest condition exist? The well timed excitatory input from the contralateral AVCN to MNTB is of high spiking activity. AVCN bushy cells of gerbils fire spontaneously at a rate of ~50 Hz (Kopp-Scheinflug et al., 2002; Keine and Rübsamen, 2015). The *in vivo* spontaneous firing rates of mouse (CBA/J) AVCN bushy cells vary between 22 and 62 Hz in P12–P18 animals (Müller et al., 2019). MNTB neurons in C57BL/6 mice fire spontaneously at ~70 Hz *in vivo* (Lorteije et al., 2009), suggesting high spiking activity of the calyx. This highly active spiking status may provide the condition for spillover of glutamate and subsequent activation of mGluRs on the calyx. Following mGluR activation, spontaneous glutamate release is enhanced while evoked release is inhibited. This may constitute a mechanism critical for regulating the excitatory tone and the auditory processing at the synapse. Presynaptic enhancement of the spontaneous excitatory transmission and postsynaptic increase of excitability of MNTB neurons by mGluRs (Dos Santos e Alhadas et al., 2020; Fig. 8) may allow relatively small synaptic excitatory events transform into APs. This would minimize synaptic delays, enable fast response, and increase spike reliability. Finally, the matched modulatory direction of mGluR I on the glutamatergic and the glycinergic inputs may assist maintenance of the excitation/inhibition balance for proper circuit function and homeostasis of cellular excitability.

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