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Postsynaptic and Presynaptic Group II Metabotropic Glutamate Receptor Activation Reduces Neuronal Excitability in Rat Midline Paraventricular Thalamic Nucleus

M. L. H. J. Hermes and L. P. Renaud

Neuroscience Program, Ottawa Hospital Research Institute and Department of Medicine, University of Ottawa, Ottawa, Ontario, Canada

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ABSTRACT

Drugs that interact with group II metabotropic glutamate receptors (mGluRs) are presently being evaluated for a role in the treatment of anxiety disorders and symptoms of schizophrenia. Their mechanism of action is believed to involve a reduction in excitatory neurotransmission in limbic and forebrain regions commonly associated with these mental disorders. In rodents, the glutamatergic neurons in the midline paraventricular thalamic nucleus (PVT) provide excitatory inputs to the limbic system and forebrain. PVT also displays a high density of group II mGluRs, predominantly the metabotropic glutamate 2 receptor (mGluR2). Because the role of group II mGluRs in regulating cellular and synaptic excitability in this location has yet to be determined, we used whole-cell patch-clamp recording and acute rat brain slice preparations to evaluate PVT neuron responses to a selective group II mGluR agonist, (1*R*,4*R*,5*S*,6*R*)-4-amino-2-oxabicyclo[3.1.0]hexane-4,6-dicarboxylic acid (LY 379268). LY 379268 consistently induced membrane hyperpolarization and suppressed firing by postsynaptic receptor-mediated activation of a barium-sensitive background K^+ conductance. This effect could be blocked by (2*S*)-2-amino-2-[(1*S*,2*S*)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl)propanoic acid (LY 341495), a selective group II mGluR antagonist. In addition, LY 379268 acted at presynaptic receptors to reduce ionotropic glutamate receptor-mediated excitatory synaptic transmission. An mGluR2-positive allosteric modulator, 2,2,2-trifluoro-*N*-[4-(2-methoxyphenoxy)phenyl]-*N*-(3-pyridinylmethyl)ethanesulfonamide hydrochloride (LY 487379), resulted in leftward shifts of the LY 379268 dose-response curve for both postsynaptic and presynaptic actions. The data demonstrate that activation of postsynaptic and presynaptic group II (presumably mGluR2) mGluRs reduces neuronal excitability in midline thalamus, an action that may contribute to the effectiveness of mGluR2-activating drugs in rodent models of anxiety and psychosis.

Introduction

Metabotropic glutamate receptors (mGluRs) are a heterogeneous collection of C-type G protein-coupled receptors that are currently divided into three groups (I, II, and III) based on amino acid sequence homology, signal transduction mechanism, and pharmacological profile (Niswender and Conn, 2010). mGluRs have distinct localizations in the central nervous system (CNS) and reside on neuronal presynaptic, postsynaptic, and extrasynaptic structures and non-neuronal elements, where their activation can result in an acute reduction in synaptic neurotransmitter release, a decrease or increase in somatic membrane excitability through modulation of a variety of postsynaptic ionic conductances, and long-term potentiation or long-term depression of synaptic transmission (Anwyl, 1999, 2009; Cartmell and Schoepp, 2000; Ferraguti and Shigemoto, 2006; Gladding et al., 2009; Niswender and Conn, 2010).

The discrete CNS distribution pattern of the various mGluRs has been an incentive to the development of pharmacological agents that target glutamatergic transmission

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ABBREVIATIONS: mGluR, metabotropic glutamate receptor; PVT, paraventricular thalamic nucleus; mGluR2, metabotropic glutamate 2 receptor; LY 379268, (1*R*,4*R*,5*S*,6*R*)-4-amino-2-oxabicyclo[3.1.0]hexane-4,6-dicarboxylic acid; LY 341495, (2*S*)-2-amino-2-[(1*S*,2*S*)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl)propanoic acid; PAM, positive allosteric modulator; LY 487379, 2,2,2-trifluoro-*N*-[4-(2-methoxyphenoxy)phenyl]-*N*-(3-pyridinylmethyl)ethanesulfonamide hydrochloride; ACSF, artificial cerebrospinal fluid; V_M, resting membrane potential; R_M, input resistance; TTX, tetrodotoxin; NBQX, 2,3-dioxo-6-nitro-1,2,3,4-tretahydrobenzo(f)quinoxaline-7-sulfonamide disodium salt; BIC, bicuculline methochloride; gabazine, SR 95531 hydrobromide; V_H, holding potential; EPSC, excitatory postsynaptic current; mEPSC, miniature EPSC; DCG-IV, (2*S*,2′*R*,3′*R*)-2-(2′,3′-dicarboxycyclopropyl)glycine; I_M, membrane current; g_M, membrane conductance; I_H, hyperpolarization-activated inward current; K-S test, Kolmogorov-Smirnov test; GIRK channel, G-protein-coupled inwardly rectifying K^+ channel; K2P channel, two-pore domain K^+ channel; EPSP, excitatory postsynaptic potential; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CNS, central nervous system.

selectively in brain regions where it is believed to be aberrant or excessive in certain neurological and psychiatric illnesses. Thus, because of their high density in limbic and forebrain regions (Ohishi et al., 1993, 1998; Gu et al., 2008), group II mGluRs [the metabotropic glutamate 2 receptor (mGluR2) in particular] have been targeted in possible therapies for stress and anxiety disorders and circumscribed symptoms of schizophrenia. Indeed, compounds interacting with these receptors are effective in animal models predictive of anxiolytic and antipsychotic activity (Swanson et al., 2005; Conn et al., 2009; Niswender and Conn, 2010) and result in an inhibition of glutamatergic transmission in relevant limbic and forebrain areas, including the amygdala, bed nucleus of the stria terminalis, and prefrontal cortex (Lin et al., 2000; Marek et al., 2000; Grueter and Winder, 2005; Muly et al., 2007).

Anatomical tracer and lesion studies in rat and monkey involving the midline and intralaminar thalamus have revealed that an important source of the glutamatergic drive to select limbic and forebrain regions are the neurons in the midline paraventricular thalamic nucleus (PVT) (Marek et al., 2001; Van der Werf et al., 2002; Hur and Zaborszky, 2005; Huang et al., 2006; Hsu and Price, 2009). Moreover, PVT neurons projecting to these areas display robust cellular activation after stress and administration of psychostimulant drugs that induce schizophrenic-like symptoms, conditions that are known to profoundly influence its target areas (Deutch et al., 1998; Bubser and Deutch, 1999; Gozzi et al., 2008; Hackler et al., 2010). These data point to an important role of PVT neurons in regulating excitability levels in the limbic system and forebrain. It is noteworthy that PVT also displays high expression levels of group II mGluRs, predominantly mGluR2; however, their function at this location remains to be determined (Ohishi et al., 1993, 1998; Gu et al., 2008).

We used patch-clamp recording in rat brain slice preparations to test the hypothesis that activation of group II mGluRs could reduce the excitability of PVT neurons. The data reveal that (1*R*,4*R*,5*S*,6*R*)-4-amino-2-oxabicyclo[3.1.0]hexane-4,6-dicarboxylic acid (LY 379268), a selective group II mGluR orthosteric agonist, activates a postsynaptic barium-sensitive resting K^+ conductance that hyperpolarizes the cells. LY 379268 also acts at presynaptic receptors to decrease glutamatergic synaptic transmission. Moreover, we noted that 2,2,2-trifluoro-*N*-[4-(2-methoxyphenoxy)phenyl]-*N*-(3-pyridinylmethyl)ethanesulfonamide hydrochloride (LY 487379), an mGluR2-positive allosteric modulator (PAM), increases the sensitivity of both postsynaptic and presynaptic receptors to orthosteric activation. A preliminary account of these findings was presented previously (Hermes et al., 2009).

Materials and Methods

Animals. Experiments used male and female Wistar rats (age 21–40 days) bred in-house and maintained on a 12-h light/12-h dark schedule (lights on at 6:00 AM). Experimental protocols conformed to the Canadian Council for Animal Care guidelines and were approved by the Ottawa Hospital Research Institute Animal Care and Use Committee.

Thalamic Slice Preparation. After decapitation between 9:00 and 11:00 AM, brains were removed from the skull and immersed in an oxygenated (95% O_2, 5% CO_2) sucrose-based slicing solution of the following composition: 200 mM sucrose, 2.5 mM KCl, 1.2 mM NaH_2PO_4, 26 mM $NaHCO_3$, 11 mM D-glucose, 6 mM $MgCl_2$, and 1 mM $CaCl_2$. Coronal sections containing PVT were sliced at 400 μm using a vibrating blade microtome (Leica VT1000S; Leica Microsystems, Richmond Hill, ON, Canada) and maintained for 30 to 45 min at 30°C, followed by >30 min at room temperature in a storage chamber containing oxygenated artificial cerebrospinal fluid (ACSF) of the following composition: 124 mM NaCl, 3 mM KCl, 1.2 mM NaH_2PO_4, 26 mM $NaHCO_3$, 10 mM D-glucose, 1.3 mM $MgCl_2$, and 2.4 mM $CaCl_2$, with an osmolality of 300 to 305 mOsm/kg. In all experiments, ACSF of the same composition was used, except when assessing the involvement of K^+ conductances, in which case the concentration of KCl in the ACSF was increased by 7 mM and NaCl was equally reduced to maintain similar ionic strength.

Electrophysiological Recordings. For recording, slices were transferred to a custom-build submersion chamber and superfused continuously with oxygenated ACSF at 30 to 32°C. Recording patch pipettes pulled from thin-walled borosilicate glass capillaries (Sutter Instrument Company, Novato, CA) with a Flaming-Brown P-87 horizontal puller (Sutter Instrument Company) were fire-polished and filled with a solution of the following composition: 135 mM K-gluconate, 10 mM KCl, 10 mM HEPES, 0.1 mM EGTA, 0.2 mM $MgCl_2$, and 2 mM Na_2ATP, pH adjusted to 7.2 with KOH (osmolality 285–295 mOsm/kg). When the pipette was filled with the recording solution, the open pipette resistance was 4 to 7 MΩ. Whole-cell current- and voltage-clamp recordings were obtained using the "blind" method of patch-clamp recording. Electrical activity was registered using an Axopatch 200B amplifier, a Digidata 1200 analog-to-digital converter, and pClamp 9 software (including Clampex and Axoscope: MDS Analytical Technologies, Sunnyvale, CA). Data were filtered at 1 or 2 kHz and sampled at 5 kHz. The uncompensated access resistance of 8 to 30 MΩ was compensated by 40 to 50% and periodically monitored during the experiment. Recordings were terminated when changes in the access resistance >20% occurred.

Experimental Protocols. Neurons were recorded throughout the rostrocaudal extent of PVT, from bregma −1.30 mm to −3.60 mm (Paxinos and Watson, 1998). The influence of group II mGluR agonists and antagonists was initially assessed in current-clamp recording mode. To determine a direct, postsynaptic effect of group II mGluR activation on resting membrane potential (V_M) and input resistance (R_M: measured by subjecting the cell to hyperpolarizing current pulses), recordings were also made in ACSF containing 1 μM tetrodotoxin (TTX), 5 μM 2,3-dioxo-6-nitro-1,2,3,4-tretahydrobenzo-(f)quinoxaline-7-sulfonamide disodium salt (NBQX), and 10 μM bicuculline methochloride (BIC) or 10 μM SR 95531 hydrobromide (gabazine) to block action potentials and fast synaptic potentials. To analyze the membrane mechanism underlying the postsynaptic influence of group II mGluR activation, voltage-clamp recordings were made in ACSF containing TTX, NBQX, and BIC or gabazine, and a pulse protocol was applied that stepped the holding potential (V_H) from −65 to −115 mV in 10-mV steps.

The (presynaptic) influence of group II mGluR agonists and antagonists on excitatory glutamatergic synaptic transmission was investigated by measuring miniature excitatory postsynaptic currents (mEPSCs) in ACSF containing TTX, BIC or gabazine, and barium (200–500 μM: to block any induced postsynaptic K^+ conductance). To control for effectiveness of TTX in blocking action potentials, EPSCs were evoked by placing a platinum/iridium bipolar concentric electrode (tip diameter 125 μm; FHC Inc., Bowdoinham, ME) ventral to PVT to deliver constant voltage pulses (5–25 V, 0.1 ms) generated by an isolated stimulation unit (model DS2A; Digitimer, Welwyn Garden City, UK) driven by the pClamp software.

The effect of an mGluR2 PAM on group II mGluR-activated postsynaptic and presynaptic effects was assessed in voltage-clamp mode, by applying the compound before and during orthosteric activation while continuously monitoring membrane currents and mEPSCs, respectively.

Drugs and Solutions. TTX, NBQX, BIC, gabazine, LY 379268, (2*S*,2′*R*,3′*R*)-2-(2′,3′-dicarboxycyclopropyl)glycine (DCG-IV), (2*S*)-2-amino-2-[(1*S*,2*S*)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl)propanoic

acid (LY 341495), LY 487379, and tertiapin-Q were purchased from Tocris Bioscience (Ellisville, MO). Stock (for freezing) solutions (1000×) of TTX, NBQX, BIC, gabazine, DCG-IV, and tertiapin-Q were prepared in deionized water. Stock solutions (10 mM) of LY 379268 and LY 341495 were prepared in deionized water, and the pH was adjusted to 7 to 8 with 5 N NaOH. Stock solutions (10 mM) of LY 487379 were prepared in dimethyl sulfoxide. All drugs were bath-applied with a flow rate of 4 ml/min. The time necessary for a solution change to reach the brain slice was approximately 30 s.

Data Analysis and Statistics. All measured voltages in current-clamp mode were corrected for a liquid junction potential of 13.6 mV calculated using the liquid junction potential calculator software of Clampex. Command potentials in voltage-clamp mode were corrected by 15 mV. Membrane potential and membrane current (I_M) were measured and analyzed using Clampfit 9.2 (part of pClamp) and Prism 4.0 (GraphPad Software Inc., San Diego, CA), and mEPSCs were detected, measured, and analyzed using Minianalysis 6.0.7 software (Synaptosoft, Decatur, GA).

In current-clamp recordings, drug-induced changes in V_M were assessed by averaging 30- to 60-s sections of traces captured with Axoscope 9.2. The effect of NBQX on firing properties of PVT neurons was determined from 2- to 6-min periods. Apparent R_M was measured from averages of at least three consecutive sweeps of the voltage response to repeated injections of small hyperpolarizing currents. In voltage-clamp recordings, drug-induced changes in I_M were determined at a V_H of −65 mV, whereas changes in membrane conductance (g_M) were calculated from slope conductances derived from a linear regression of the relationship between I_M and V_H between −65 to −85 and −95 to −115 mV. The rectification ratio was calculated as the slope conductance at −65 to −85 mV divided by the slope conductance at −95 to −115 mV. The amplitude of the hyperpolarization-activated inward current (I_H) was calculated as the difference between I_M after the settling of the capacitive transient (instantaneous) and I_M at the end of the voltage step from −65 to −115 mV (steady state). Activation kinetics of I_H were estimated by fitting the I_M response by a single exponential function. Changes in the frequency and amplitude of mEPSCs were analyzed at −75 mV over 1-min periods. Depending on the noise level of the recording, a 4- to 8-pA detection threshold was applied.

To determine EC_{50} values of the postsynaptic and presynaptic effects of LY 379268, increasing (1, 3, 10, 30, 100, 300, and 1000 nM) concentrations of the compound were sequentially applied, allowing for stabilization of maximal effects (usually after 6 min into the continuous application of a certain concentration). Data points were fitted to the four-parameter logistic equation: $Y = B + (A - B)/(1 + (x/EC_{50})^D)$, where B is the estimated response at infinite concentration, A is the estimated response at zero concentration, and D is the slope.

All data were expressed as mean ± S.E.M., and group statistical significance ($p < 0.05$) was assessed using two-tailed paired and unpaired t tests. Statistically significant ($p < 0.05$) differences in frequency and amplitude distributions of mEPSCs were determined with the Kolmogorov-Smirnov test (K-S test).

Results

Group II mGluR Agonist LY 379268 Hyperpolarizes PVT Neurons. All eight cells tested responded to a 2-min bath application of the potent and highly selective group II mGluR agonist LY 379268 (100 nM) (Monn et al., 1999) with a gradual membrane hyperpolarization of −13.0 ± 1.3 mV ($p < 0.01$) from a V_M of −69.5 ± 1.3 mV. This effect was accompanied by cessation of spontaneous action potential discharge in seven active cells and only partially reversed (by 38 ± 10%) after a 12- to 30-min washout (Fig. 1, A and D). Similar responses were obtained with applications of DCG-IV (1 μM), another selective but less potent group II

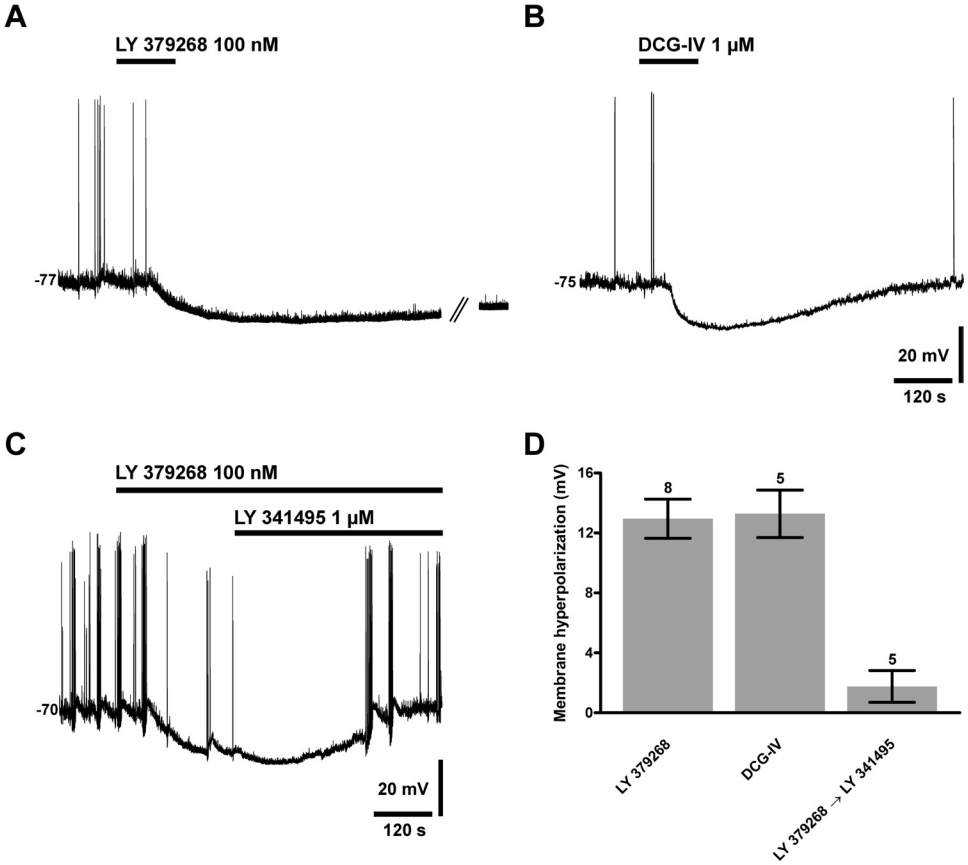

Fig. 1. Activation of group II mGluRs hyperpolarizes PVT neurons. A, current-clamp trace showing a typical slowly developing and largely irreversible hyperpolarization of V_M and concomitant cessation of spontaneous action potential discharge in a PVT neuron after a 2-min bath application of the group II mGluR agonist LY 379268 (100 nM). Trace interruption (//) represents 6 min. B, the less potent group II mGluR agonist DCG-IV (1 μM) results in a membrane hyperpolarization of similar magnitude, but its effect is more rapid in onset and quickly reversed upon washout. C, the sustained membrane potential hyperpolarization by continuous application of LY 379268 can be reversed by secondary application of the group II mGluR antagonist LY 341495 (1 μM). D, histogram depicting the magnitude of membrane hyperpolarization by LY 379268, DCG-IV, and LY 379268 followed by LY 341495.

mGluR agonist, achieving a membrane hyperpolarization of -13.3 ± 1.6 mV ($n = 5$; $p < 0.01$) from a V_M of -71.0 ± 2.0 mV, an effect not significantly different from that of LY 379268 ($p = 0.88$). By contrast, the response to DCG-IV was always more rapid in onset and fully reversed with 7- to 10-min wash (Fig. 1, B and D). We investigated whether the irreversibility of the effect of LY 379268 could be abrogated by LY 341495 (1 μM), a moderately selective group II mGluR antagonist (Kingston et al., 1998). At low micromolar concentrations, LY 341495 has been reported to antagonize group III mGluRs (notably mGluR7 and mGluR8); however, these receptors show low expression (mGluR7) or no expression (mGluR8) in PVT (Ferraguti and Shigemoto, 2006 and references therein). The application of LY 341495 (1 μM) rapidly reversed the membrane hyperpolarization induced by continuous perfusion of the slice with LY 379268 (100 nM), such that a -15.4 ± 1.1-mV hyperpolarization was diminished by $90 \pm 7\%$ (to -1.8 ± 1.1 mV) within 5 min after the start of application of LY 341495 ($n = 5$; $p < 0.01$) (Fig. 1, C and D). These data suggest that the largely irreversible membrane hyperpolarization by brief applications of LY 379268 is presumably caused by the continuing binding of this compound to the receptor, rather than a continuous activation of second-messenger pathways.

In ACSF containing TTX (1 μM), NBQX (5 μM), and BIC or gabazine (10 μM) to block action potential-evoked and spontaneous fast synaptic transmission, 6/6 neurons responded to LY 379268 (100 nM) with a membrane hyperpolarization of -9.6 ± 2.3 mV from a V_M of -67.0 ± 2.8 mV, an effect not significantly different from that of LY 379268 in control ACSF ($p = 0.20$) (Fig. 2A). Intracellular injection of hyperpolarizing current pulses revealed that the LY 379268-induced hyperpolarization was accompanied by a reduction in R_M to $77 \pm 4\%$ of a control value of 490 ± 68 MΩ ($n = 6$; $p < 0.01$) (Fig. 2, A and B). In a separate set of cells, an initial 5-min bath application of LY 341495 (1 μM) did not change V_M (by 1.0 ± 0.6 mV from a V_M of -73.3 ± 2.0 mV; $n = 5$; $p = 0.20$) or R_M (to $100 \pm 2\%$ of a control value of 487 ± 77 MΩ; $n = 5$; $p = 0.75$). A subsequent 2-min bath application of LY 379268 (100 nM) was now without effect on V_M (change by 0.8 ± 0.5 mV; $n = 5$; $p = 0.20$) and R_M (change to $101 \pm 3\%$ of control value; $n = 5$; $p = 0.95$). These results imply that the LY 379268-induced hyperpolarization is mediated by postsynaptic group II mGluRs and presumably mGluR2, because mGluR3 can not be detected in PVT (Gu et al., 2008). Moreover, the data indicate that the membrane mechanism underlying the hyperpolarization comprises a decrease in R_M.

LY 379268 Activates a Barium-Sensitive Nonrectifying K^+ Conductance. To further assess the membrane mechanism underlying the hyperpolarizing action of LY 379268, neurons were tested in voltage-clamp mode in ACSF containing TTX, NBQX, and BIC or gabazine. PVT neurons, clamped at a holding potential of −65 mV, responded to a 2-min bath application of LY 379268 (100 nM) with an outward current of 37.4 ± 6.3 pA ($n = 6$; $p < 0.01$) that was slow in onset and prolonged and only partially reversed after a 15-min wash (Fig. 3, A and E). Current-voltage (*I-V*) relationships obtained at the peak of the response indicated that the outward current was accompanied by an increase in g_M to $145 \pm 10\%$ of a control value of 2.00 ± 0.25 nS ($n = 6$; $p < 0.01$) (Fig. 3, B and E). The net LY 379268-induced outward current obtained by subtraction of the *I-V* relationships during control and at the peak of the drug-induced response showed a marginal rectification ratio of 1.20 ± 0.42 and reversal of polarity at a membrane potential of -105.7 ± 5.2 mV ($n = 6$), approximating the calculated K^+ reversal potential of −102.2 mV (Fig. 3, C and D). Consistent with a role for K^+ channels, when tested in ACSF containing 10 mM K^+ (up from 3 mM) the outward current (at −65 mV) was diminished to 4.7 ± 4.1 pA and reversed at -68.1 ± 2.8 mV ($n = 5$), close to the calculated K^+ reversal potential of −70.7 mV (Fig. 3D). Collectively, these observations suggest that LY 379268 activates a nonrectifying or weakly rectifying K^+ conductance in PVT neurons.

To mediate their postsynaptic effects, group II mGluRs have been shown, in the majority of cases investigated, to activate G protein-coupled inwardly rectifying K^+ channels (GIRK channels) in in vitro expression systems and to functionally couple to GIRK channels in select CNS neurons (Saugstad et al., 1996; Knoflach and Kemp, 1998; Watanabe and Nakanishi, 2003; Irie et al., 2006). The possible lack of

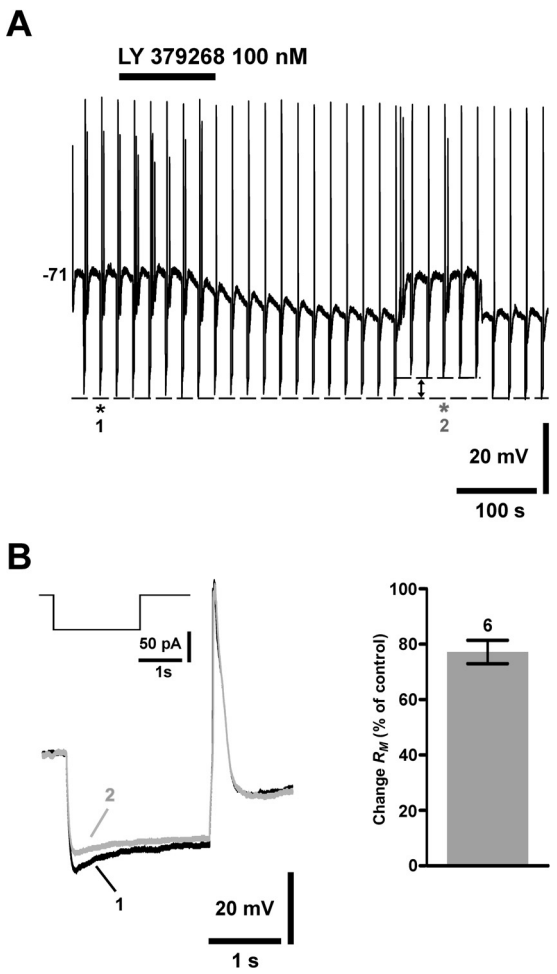

Fig. 2. Activation of group II mGluRs opens a hyperpolarizing membrane conductance in PVT neurons. A, trace illustrating that the magnitude of LY 379268-induced membrane hyperpolarization is unchanged in the presence of TTX, NBQX, and BIC that blocks synaptic transmission. Vertical lines are transients from a recurring (every 20.67 s) intracellular 2-s hyperpolarizing current pulse: note the drug-induced decrease in R_M (indicated by the difference between the two dashed lines during manual repolarization to the predrug V_M, vertical arrow). B, superimposed expansions of the membrane potential deflections at the points indicated in the trace in A. Inset, pulse protocol. The histogram expresses the overall reduction in R_M by LY 379268 (in %).

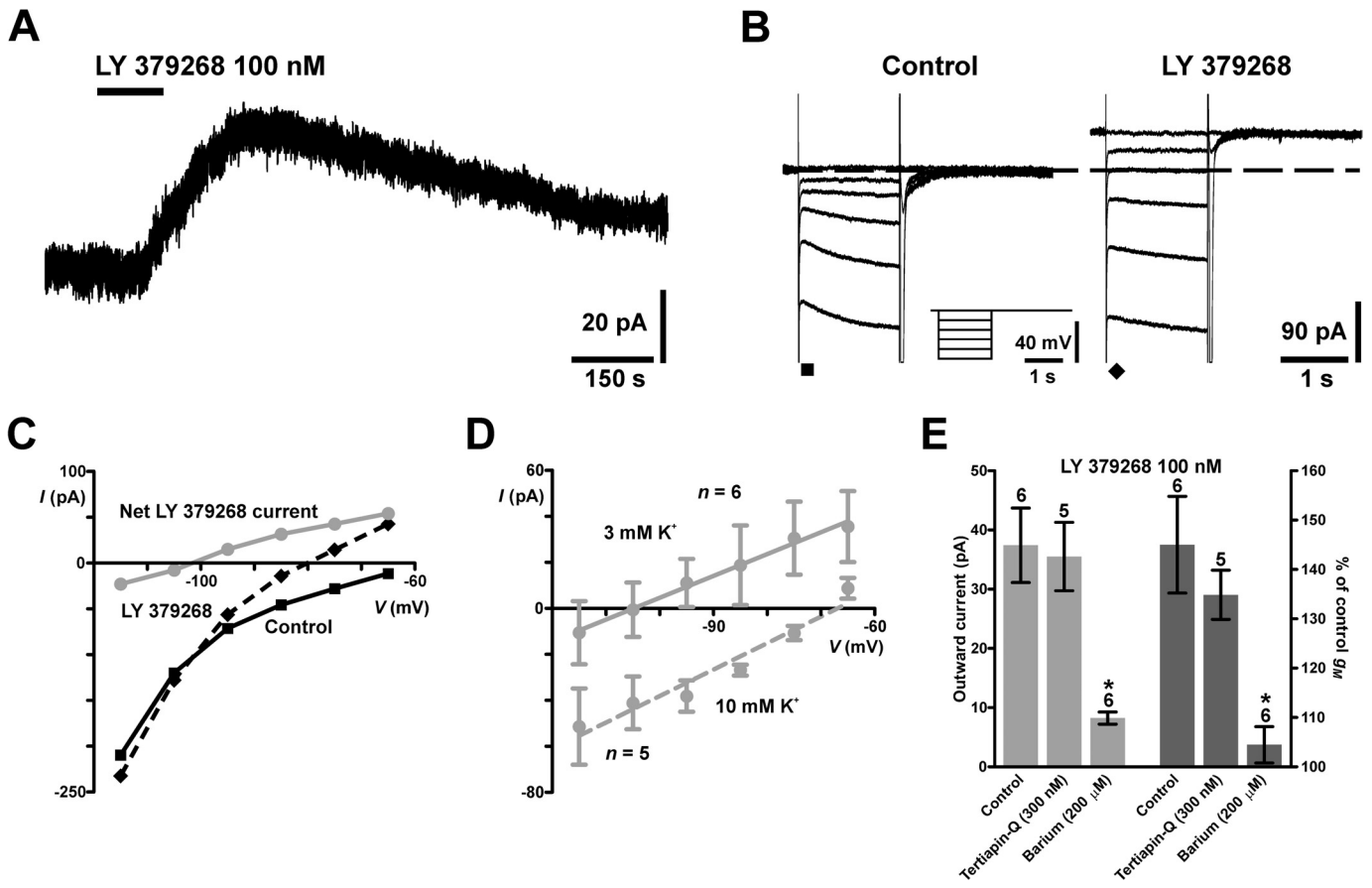

Fig. 3. Activation of group II mGluRs generates an outward current in PVT neurons by opening a barium-sensitive nonrectifying K^+ conductance. A, voltage-clamp trace (V_H −65 mV) depicting a partially reversible outward current after a 2-min bath application of the group II mGluR agonist LY 379268 (100 nM). B, *I-V* relationships demonstrate the increase in membrane current flows (i.e., increase in g_M) at the peak of the LY 379268-activated outward current. Filled symbols indicate the time point in response where instantaneous membrane currents are measured. Insets, pulse protocol with V_H −65 mV. C, graph of the relationship to transmembrane potential of the instantaneous membrane current of the cell in B in control (■) and LY 379268 (▲) and of the net LY 379268-induced current (◍). The LY 379268-induced current has no apparent rectifying properties and reverses polarity close to the K^+ reversal potential. D, an increase in extracellular K^+ concentration from 3 to 10 mM shifts the reversal potential of the LY 379268-induced current in a depolarizing direction by 34 mV, consistent with features of a pure K^+ conductance. E, histogram summary showing the LY 379268-induced outward current and increase in g_M are not blocked by tertiapin-Q (300 nM), a specific blocker of GIRK channels, but are largely diminished in 200 μM barium, a nonspecific blocker of K^+ channels. ∗, $p < 0.01$ compared with control.

adequate voltage clamp over parts of the cellular membrane that may contain these GIRK channels could have obscured any rectifying properties of the LY 379268-activated K^+ current that we observed in PVT neurons. Therefore, in ACSF containing TTX, NBQX, and BIC or gabazine, we next evaluated the efficacy of two K^+ channel blockers to suppress the LY 379268-induced outward current and increase in g_M. Tertiapin-Q is a bee venom peptide that effectively blocks GIRK channels (Jin and Lu, 1998). By contrast, barium blocks multiple types of K^+ channels. In PVT neurons, tertiapin-Q (300 nM) itself induced no significant change in holding current, g_M, or rectifying properties ($n = 5$). In addition, in the presence of tertiapin-Q, LY 379268 (100 nM) induced an outward current of 35.5 ± 5.8 pA that was not significantly different from its effect in control ACSF ($n = 5$; $p = 0.83$) (Fig. 3E). An increase in g_M to 135 ± 5% of control values was also not different from observations in control ACSF ($n = 5$; $p = 0.41$) (Fig. 3E). In contrast to tertiapin-Q, bath application of low concentrations of barium (200 μM) did influence membrane properties of PVT neurons, resulting in an inward current of −25.5 ± 2.3 pA, a reduction in g_M to 90 ± 8% of control values, and abolition of inward rectification (as indicated by a change of the rectification ratio from 0.69 ± 0.18 to 1.22 ± 0.07) ($n = 6$). In the presence of barium, the outward current activated by LY 379268 (100 nM) was reduced to 8.2 ± 1.0 pA, and the increase in g_M diminished to 105 ± 4% of control values ($n = 6$; $p < 0.01$ versus control ACSF) (Fig. 3E). Taken together, the pharmacology data substantiate the observed absence of significant rectifying properties of the LY 379268-induced membrane current and suggest that GIRK channels are not involved in the postsynaptic effect of group II mGluR activation in PVT neurons. Alternative candidates may include members of the mammalian family of two-pore domain K^+ channels (K2P channels), which are expressed in midline thalamus and exhibit sensitivity to micromolar concentrations of barium (Talley et al., 2001; Lotshaw, 2007).

LY 379268 Has No Effect on the Hyperpolarization-Activated Inward Current, I_H. Voltage-clamp recordings reveal that PVT neurons display the characteristics of a modest I_H-type conductance, expressed as a slowly developing inward current with hyperpolarizing voltage steps (Fig. 3B, Left). I_H may be modulated by activation of neurotransmitter receptors that influence intracellular cAMP produc-

tion (Pape, 1996), including group II mGluRs (Niswender and Conn, 2010). Indeed, LY 379268 (100 nM) reduced the amplitude of I_H to 69 ± 5% of a control value of −51.0 ± 10.5 pA (stepping V_H from −65 to −115 mV; $n = 6$; $p < 0.01$) (Fig. 3B). Because the result may be caused by a shunting effect of the described LY 379268-activated K^+ conductance, the effect was retested in the presence of nonspecific K^+ channel blocker barium (200–500 μM). In barium, the amplitude of I_H with a voltage step to −115 mV was increased to −124.7 ± 22.8 pA ($n = 5$), indicating that in control conditions I_H in PVT is partly shunted by barium-sensitive K^+ conductances. As expected, LY 379268 (100 nM) now induced only a small outward current of 7.0 ± 1.2 pA and a negligible change in g_M to 99 ± 2% of control values ($n = 5$). The amplitude of the isolated I_H was unaffected by LY 379268, changing to 94 ± 5% of control values ($n = 5$; $p = 0.28$). Moreover, the activation kinetics of I_H, estimated from the time constant of the current response to the voltage step to −115 mV, were unchanged, to 102 ± 6% of a control value of 907 ± 169 ms ($n = 5$; $p = 0.96$). Collectively, these data indicate that the apparent LY 379268-induced reduction in I_H is secondary to the activated K^+ conductance.

Synaptic Input to PVT Neurons Is Dominated by NBQX-Sensitive, TTX-Insensitive Excitatory Postsynaptic Potentials That Influence Action Potential Discharge. In acute thalamic slice preparations, a majority of PVT neurons (23/29 cells recorded in current-clamp) displayed many (in 12 cells, 5–10 Hz; in 11 cells, > 10 Hz) excitatory postsynaptic potentials (EPSPs) that appear to trigger action potentials or low threshold calcium potentials supporting bursts of action potentials. The sole application of NBQX (5 μM) blocking α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate-type ionotropic glutamate receptor-mediated synaptic transmission reversibly abolished these EPSPs and significantly reduced the mean frequency of action potential discharge in 6/6 active cells from 0.51 ± 0.12 to 0.22 ± 0.10 Hz ($n = 6$; $p < 0.05$) (Fig. 4A). It is noteworthy that, in ACSF containing BIC or gabazine (10 μM) to block any $GABA_A$ receptor-mediated spontaneous inhibitory postsynaptic currents, voltage-clamp recordings of NBQX-sensitive spontaneous EPSCs in six cells revealed no significant change in their frequency or amplitude upon addition of TTX (1 μM) ($p > 0.05$; K-S test, all cells) (Fig. 4, B and C). Overall, in TTX the mEPSC frequency changed to 99 ± 2% of a control value of 7.5 ± 0.6 Hz, and the mEPSC amplitude changed to 99 ± 1% of a control value of −12.7 ± 1.6 pA. However, TTX effectively blocked the NBQX-sensitive EPSC that could be evoked in four cells by electrical stimulation ventral to PVT (Fig. 4B).

Taken together, these data imply a significant role for glutamatergic EPSPs in regulating action potential discharge of PVT neurons. Moreover, these EPSPs are independent of TTX-sensitive action potential discharge of cells presynaptic to PVT neurons and arise from spontaneous or stochastic release of glutamate from nerve terminals.

LY 379268 Inhibits mEPSCs by Activating Presynaptic Receptors. In various CNS regions, group II mGluRs have been reported to inhibit fast excitatory glutamatergic synaptic transmission by acting on presynaptic receptors to reduce glutamate release (Anwyl, 1999; Cartmell and Schoepp, 2000; Niswender and Conn, 2010). We examined TTX-insensitive mEPSCs to analyze the effect of activation of group II mGluRs on excitatory synaptic transmission in PVT. It is generally accepted that a change in the frequency of TTX-insensitive synaptic events reflects a change in the excitability of the synaptic terminal, whereas a change in their amplitude indicates an alteration in the sensitivity of the postsynaptic receptor. To inhibit the occurrence of an outward current and increase in g_M caused by activation of postsynaptic group II mGluRs, we recorded the effect of group II mGluR activation on mEPSCs in ACSF containing barium (200–500 μM). In a separate set of experiments it was determined that barium (200–500 μM) had no significant effect on the frequency or amplitude of mEPSCs. A 2-min bath application of LY 379268 (100 nM) resulted in a progressive and prolonged decrease in the frequency of NBQX-sensitive mEPSCs to a minimum of 58 ± 3% of a control value (at $t = 0$) of 8.8 ± 1.2 Hz ($n = 8$) 9 min after cessation of the application (Fig. 5). In contrast, there was little change in mEPSC amplitude, which ranged between 95 ± 2% (at $t = 15$ min) and 100 ± 4% (at $t = 18$ min) of control values (−12.1 ± 1.3 pA; $n = 8$) during the course of the experiment (Fig. 5). These observations were confirmed with statistical testing: at $t = 11$ min all eight cells showed a significant change in the cumulative distribution of mEPSC interevent intervals ($p < 0.05$; K-S test), whereas the cumulative distribution of mEPSC amplitudes was unaltered ($p > 0.05$; K-S test, all cells) (Fig. 5B). The effect of LY 379268 showed poor recovery, even after a 16-min washout (Fig. 5C). However, at that point application of the group II mGluR antagonist LY 341495 (1 μM) in three cells fully reversed the diminished frequency to 107 ± 10% ($t = 25$ min) of control values (Fig. 5, A and C).

Although the high frequency of mEPSCs may suggest considerable synaptic glutamate release and possible tonic activation of group II mGluRs, an initial application of LY 341495 for 5 min was largely without effect and changed the frequency of mEPSCs to 103 ± 2% of a control value of 8.1 ± 1.5 Hz ($p > 0.05$; K-S test, 4/5 cells). It prevented the inhibitory effect of a subsequent 2-min application of LY 379268 in 4/4 cells, resulting in a change in mEPSC frequency to 96 ± 3% of a control value of 8.3 ± 1.5 Hz ($p > 0.05$; K-S test, all cells) 9 min after cessation of LY 379268.

Finally, we investigated the influence of group II mGluR activation on fast inhibitory, $GABA_A$ receptor-mediated synaptic transmission. Inhibition of $GABA_A$ receptor-mediated synaptic transmission by group II mGluR agonists has been reported in another thalamic nucleus to result from activation of mGluR3, which are largely absent in PVT (Turner and Salt, 2003; Gu et al., 2008). In view of the lack of substantial spontaneous inhibitory synaptic transmission in PVT, we tested the effect of LY 379268 (in the presence of NBQX and barium) on BIC-sensitive inhibitory postsynaptic currents that could be evoked by electrical stimulation of the dorsal surface of PVT. A 2-min bath application of LY 379268 (100 nM) had no significant effect on the amplitude of the evoked inhibitory postsynaptic current in the 10-min period after the application, and 9 min after cessation of the application the amplitude was 91 ± 5% of a control value of 175 ± 31 pA ($n = 5$; $p = 0.14$).

Collectively, these data indicate that LY 379268 activates presynaptic group II mGluRs to selectively decrease fast excitatory, AMPA/kainate glutamate receptor-mediated synaptic transmission to PVT neurons.

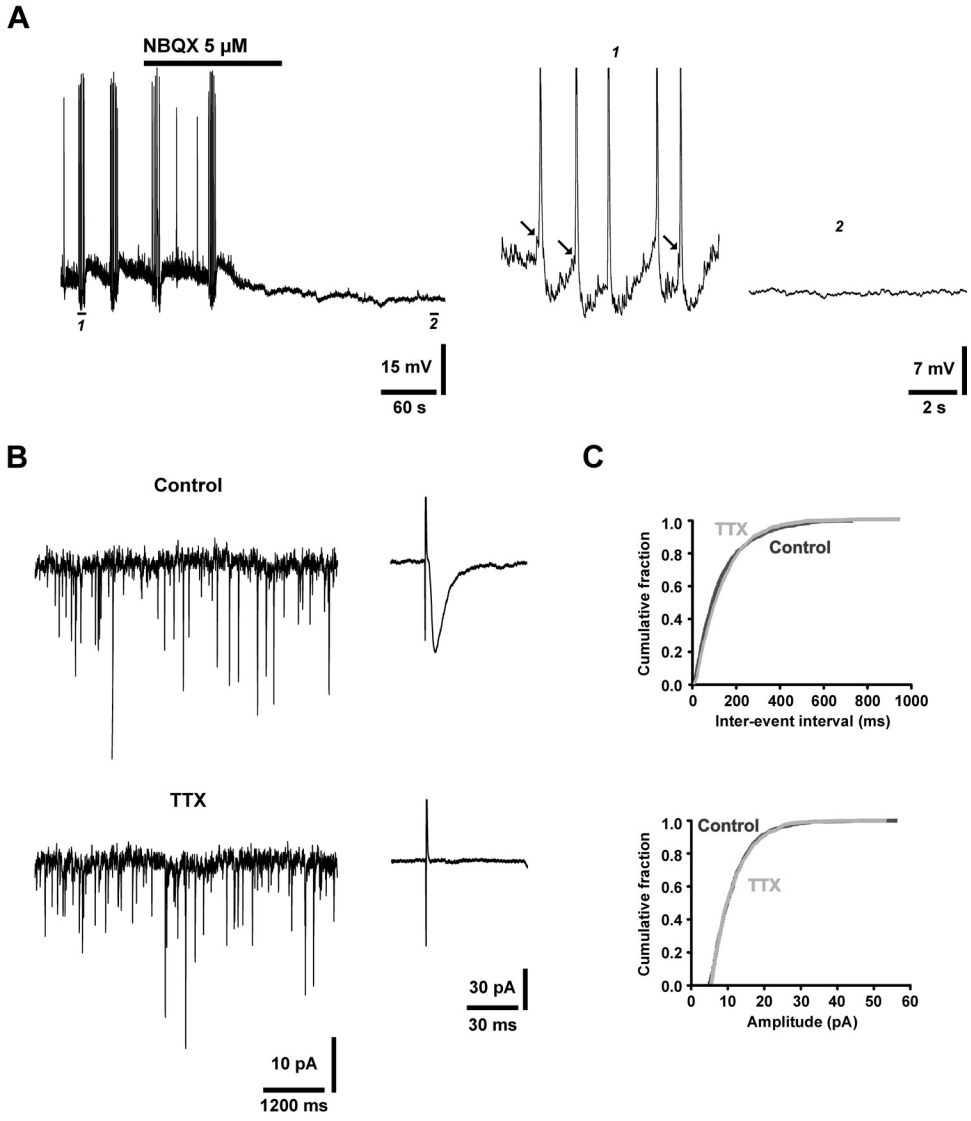

Fig. 4. PVT neurons receive an active excitatory input from spontaneous synaptic glutamate release that determines their pattern of action potential discharge. A, current-clamp trace showing the abolition of spontaneous action potential discharge in a PVT neuron after bath application of the AMPA/kainate ionotropic glutamate receptor antagonist NBQX (5 μM). Expansion of the sections indicated in the left trace show EPSPs (arrows) that trigger bursts of action potentials (1) that are abolished in the presence of NBQX (2). Action potentials are truncated. B, the frequency and amplitude of spontaneous NBQX-sensitive EPSCs recorded in voltage-clamp mode (left traces) are not visibly changed by the application of TTX that blocks EPSCs evoked by electrical stimulation (right traces). C, cumulative plots of the events recorded in the cell depicted in B demonstrate no significant changes in interevent interval or amplitude distribution of spontaneous EPSCs after TTX administration, as was similarly observed in five other cells tested.

LY 487379, an mGluR2 PAM, Enhances the Postsynaptic and Presynaptic Sensitivity to Orthosteric Activation. We next assessed the effects of LY 487379, one of two compounds that act as mGluR2 PAMs and have been shown to have significant activity in animal models used to test anxiolytic and antipsychotic activity (Swanson et al., 2005; Conn et al., 2009; Niswender and Conn, 2010). Characteristic of a PAM is that it does not activate the receptor directly but acts at an allosteric site to potentiate the orthosteric agonist response, including a leftward shift of the concentration-response curve. Here, we investigated the influence of LY 487379 on the concentration-dependence of the effects of LY 379268.

Application of a sequence of increasing concentrations of LY 379268 activated outward currents that were concentration-dependent, with an EC_{50} value of 49 ± 10 nM (n = 7) (Fig. 6A). The increase in g_M had an EC_{50} value of 54 ± 14 nM (n = 7) (Fig. 6B). The maximal outward current in these experiments was 32.5 ± 5.7 pA, and the maximal increase in g_M was to 158 ± 7% of control values. Administration of LY 487379 (10 μM) for 10 min resulted in no change in holding current (by 0.4 ± 1.2 pA; n = 9; p = 0.76) or g_M (by 100 ± 1%; n = 9; p = 0.89). Subsequent application of increasing concentrations of LY 379268 in the continuing presence of LY 487379 in five cells showed a leftward shift of the outward current dose-response curve, to an EC_{50} value of 14 ± 2 nM (Fig. 6A). The dose-response curve of the effect of LY 379268 on g_M in the presence of LY 487379 had an EC_{50} value of 17 ± 3 nM (Fig. 6B). Maximal responses to LY 379268 were not significantly different from those in the absence of LY 487379, being 26.7 ± 5.2 pA (p = 0.48) and 139 ± 10% of control values (p = 0.14).

The inhibitory effect of LY 379268 on the frequency of mEPSCs had an EC_{50} value of 41 ± 7 nM (n = 6) (Fig. 6C). The maximal inhibition by LY 379268 was to 24 ± 5% of control values. Application of LY 487379 (10 μM) for 10 min had no major effect of the frequency of mEPSCs, which changed to 95 ± 2% of control values (n = 9; $p > 0.05$; K-S test in 7/9 cells). In the presence of LY 487379 (10 μM), the mean EC_{50} value of inhibition of mEPSCs by LY 3792678 was reduced to 17 ± 9 nM (n = 5) (Fig. 6C). The maximal inhibition in the presence of LY 487379 was to 20 ± 4% of control values, not significantly different from the inhibition in the absence of LY 487379 (p = 0.52).

Taken together, these data indicate that the group II mGluRs in PVT have nanomolar sensitivity to LY 379268 and that both postsynaptic and presynaptic receptors are

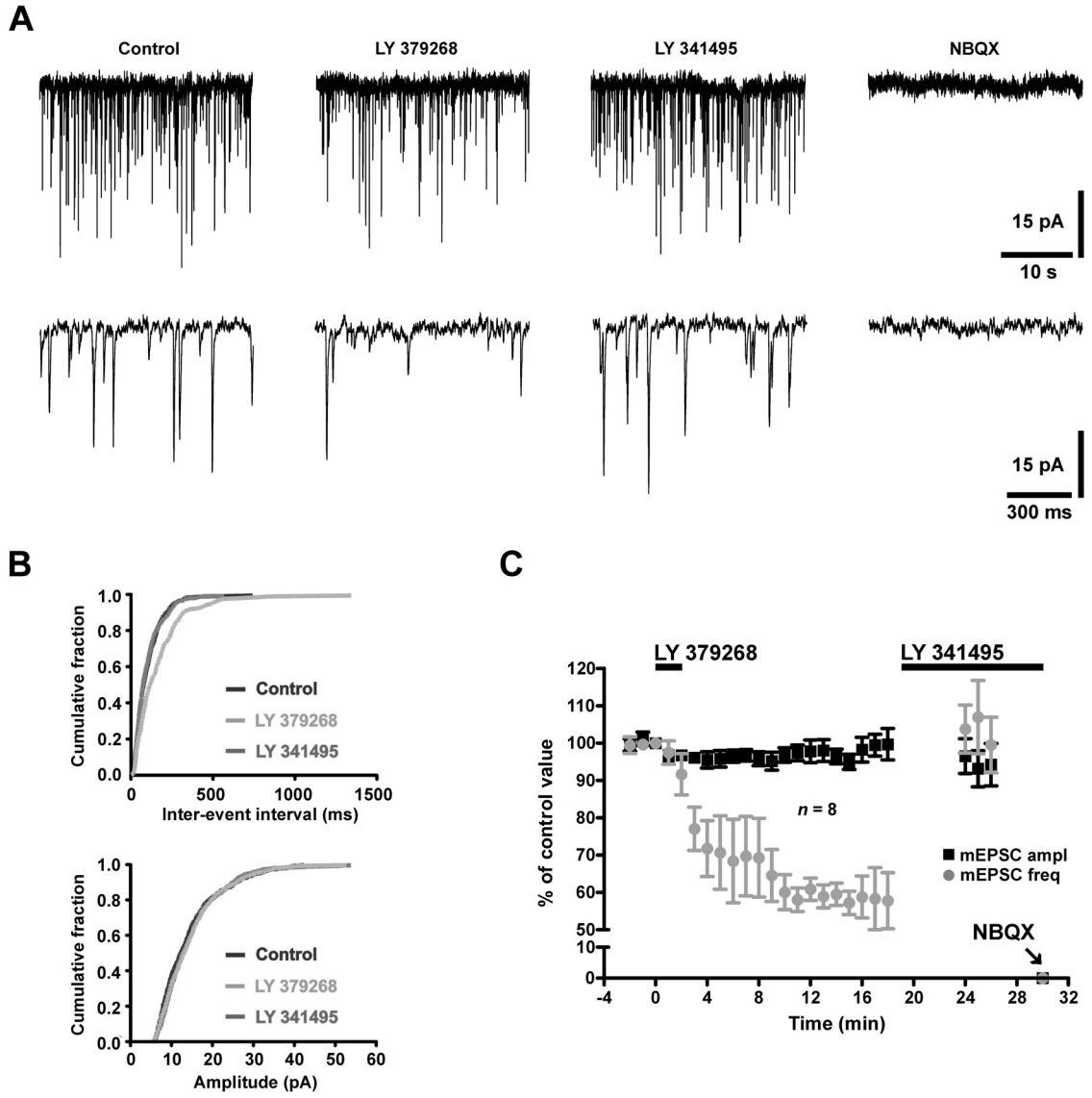

Fig. 5. Activation of presynaptic group II mGluRs inhibits glutamatergic input to PVT neurons. A, sections of a continuous current trace recorded in voltage-clamp mode at two different time scales illustrating the reduction in frequency of NBQX-sensitive mEPSCs after a 2-min bath application of group II mGluR agonist LY 379268 (100 nM). The lasting and largely irreversible effect of LY 379268 is reversed by the group II mGluR antagonist LY 341495 (1 μM). Recordings were made in the presence of TTX to block action potentials, in the presence of BIC or gabazine (10 μM) to block $GABA_A$ receptor-mediated synaptic currents, and in the presence of barium (500 μM) to block the effect of activation of postsynaptic group II mGluRs. B, cumulative plots of the distribution of interevent intervals and amplitude of mEPSCs of the cell depicted in A in control conditions, in LY 379268, and in LY 341495. LY 379268 increases the interevent interval of mEPSCs but has no effect on their amplitude. The increase in interevent interval is fully reversed by LY 341495. C, time course of the influence of LY 379268 and LY 341495 on the frequency of mEPSCs. Neither LY 379268 or LY 341495 affected the amplitude of mEPSCs.

probably mGluR2 in view of the sensitivity of both types of response to the mGluR2 PAM LY 487379 (cf. Ohishi et al., 1993, 1998; Gu et al., 2008).

Discussion

PVT is a midline thalamic structure that shows robust expression of group II mGluRs, predominantly mGluR2 (Ohishi et al., 1993, 1998; Gu et al., 2008). The present data illustrate that orthosteric activation of these receptors with the selective group II mGluR agonist LY 379268 results in a pronounced reduction in the excitability of PVT neurons in rat brain slice preparations via distinct postsynaptic and presynaptic membrane mechanisms. In addition, they show that positive allosteric modulation of mGluR2 with LY 487379 increases the sensitivity of both responses to orthosteric activation.

The postsynaptic response to LY 379268 consisted of a prominent membrane hyperpolarization that was largely irreversible, presumably because of continuing binding of the compound to its receptor. The effect was mimicked by DCG-IV and resulted from the activation of a nonrectifying or weakly rectifying tertiapin-Q-resistant K^+ conductance that displayed sensitivity to micromolar concentrations of barium. Although we also observed an apparent reduction in an I_H-type conductance, this was deemed to result from a shunting influence of the activated K^+ conductance. Qualitatively similar responses after activation of postsynaptic group II mGluRs have been described in several other

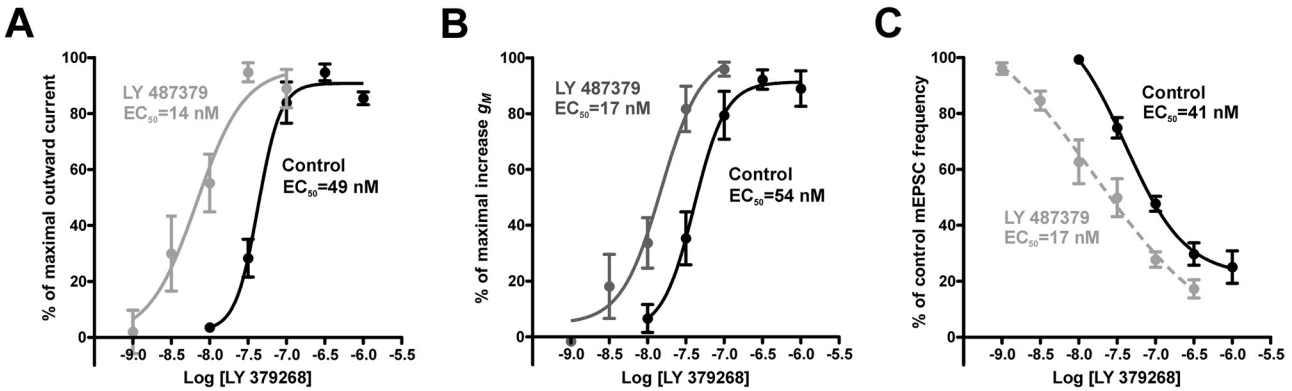

Fig. 6. Positive allosteric modulation of mGluR2 enhances the sensitivity of the postsynaptic and presynaptic response of PVT neurons to group II mGluR agonist. A, dose-response curve of the LY 379268-activated outward current in the absence (Control; $n = 7$) and presence of the mGluR2 PAM LY 487379 (10 μM; $n = 5$). The EC_{50} of the response to LY 379268 was decreased 3.5-fold in the presence of LY 487379. B, the dose-response curve of the LY 379268-induced increase in g_M ($n = 7$) shows a similar leftward shift in the presence of LY 487379 ($n = 5$). C, the decrease in frequency of mEPSCs by LY 379268 has an EC_{50} value similar to the postsynaptic response ($n = 6$). LY 487379 (10 μM) results in a 3-fold decrease in the EC_{50} value of this response ($n = 5$).

areas of the mammalian brain, including the amygdala (Muly et al., 2007), cerebellum (Knoflach and Kemp, 1998; Watanabe and Nakanishi, 2003), reticular thalamic nucleus (Cox and Sherman, 1999), and cochlear nucleus (Irie et al., 2006). It is noteworthy that PVT neurons responded with remarkably large membrane hyperpolarizations that have not been reported before, presumably in part attributable to the high density of group II mGluRs, in particular mGluR2, in midline thalamus (Ohishi et al., 1993, 1998; Gu et al., 2008). We also noted that the affected K^+ conductance in PVT has properties different from GIRK channels that have previously been identified as targets of group II mGluRs (e.g., Knoflach and Kemp, 1998; Watanabe and Nakanishi, 2003; Irie et al., 2006). Among alternative possibilities, the described features suggest that this K^+ conductance may belong to the family of K2P channels. Indeed, midline and intralaminar regions of the thalamus are reported to express transcripts of certain K2P channels that are known to contribute to resting membrane potential and are sensitive to low concentrations of barium (Talley et al., 2001; Lotshaw, 2007).

We additionally observed that LY 379268 selectively reduces the frequency, but not amplitude, of NBQX-sensitive mEPSCs in PVT, implying an activation of group II mGluRs located on glutamatergic synaptic terminals, resulting in an inhibition of spontaneous transmitter release. A presynaptic reduction of excitatory synaptic transmission by activation of group II mGluRs has been described in many brain regions (Anwyl, 1999; Cartmell and Schoepp, 2000). In the thalamus, the data reported here indicate that PVT neurons are subject to a high degree of spontaneous or stochastic release of excitatory (glutamate) neurotransmitter, which in turn can profoundly influence the occurrence of spontaneous action potential discharges. We surmise that the suppressant action of presynaptic group II mGluRs on glutamatergic afferents to PVT would be a significant contributor to the reduction in excitatory drive conveyed to sites targeted by axons of PVT neurons.

A final set of experiments demonstrated that positive allosteric modulation of mGluR2 by LY 487379 potentiates the postsynaptic and presynaptic actions of group II mGluR orthosteric agonists in PVT. These results suggest that the effects of LY 379268 and DCG-IV in PVT are mediated by an activation of mGluR2, consistent with anatomical data showing a selective presence of these receptors in midline and intralaminar thalamus (Ohishi et al., 1993, 1998; Gu et al., 2008). mGluR2 PAMs presently receive much attention because they have potential therapeutic advantages over group II mGluR orthosteric agonists because of their high selectivity, the absence of continuous receptor activation, and the (concomitant) lack of receptor desensitization. These compounds have shown promising results in animal tests used to assess anxiolytic and antipsychotic activity (Swanson et al., 2005; Conn et al., 2009; Niswender and Conn, 2010). The present article provides supporting evidence that mGluR2 PAMs can have the appropriate cellular effects in brain regions relevant to their therapeutic potential (cf. Galici et al., 2006; Benneyworth et al., 2007).

Early clinical trials have indicated that group II mGluR agonists may reduce symptoms of anxiety and schizophrenia (Swanson et al., 2005; Conn et al., 2009; Niswender and Conn, 2010). As mentioned before, animal studies have suggested that group II mGluR- and mGluR2-interacting drugs are believed to have potential therapeutic benefits by reducing excessive excitatory activity in limbic and forebrain areas such as the amygdala, bed nucleus of the stria terminalis and prefrontal cortex (Lin et al., 2000; Marek et al., 2000; Grueter and Winder, 2005; Muly et al., 2007). The present investigation demonstrates that activation of postsynaptic and presynaptic group II mGluRs, presumably mGluR2, produces a major reduction in neuronal excitability in PVT, a midline thalamic nucleus providing robust and mostly excitatory glutamatergic inputs selectively to the above-mentioned brain regions (Marek et al., 2001; Van der Werf et al., 2002; Hur and Zaborszky, 2005; Huang et al., 2006; Hsu and Price, 2009). We hypothesize that orthosteric activation or positive allosteric modulation of mGluR2s in midline thalamus contribute to a reduction in (excessive) excitatory drive to limbic and forebrain regions and constitutes part of the central mechanism underlying the beneficial effects of group II mGluR- and mGluR2-interacting drugs in animal models of anxiety and psychosis.

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Authorship Contributions

Participated in research design: Hermes and Renaud.

Conducted experiments: Hermes.

Performed data analysis: Hermes.

Wrote or contributed to the writing of the manuscript: Hermes and Renaud.

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Address correspondence to: M. L. H. J. Hermes, University of Ottawa, 725 Parkdale Avenue, Ottawa, ON, Canada K1Y 4E9. E-mail: mhermes@ohri.ca