RESEARCH PAPER

5-HT_{1B} receptors inhibit glutamate release from primary afferent terminals in rat medullary dorsal horn neurons

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BACKGROUND AND PURPOSE

Although 5-HT_{1B} receptors are expressed in trigeminal sensory neurons, it is still not known whether these receptors can modulate nociceptive transmission from primary afferents onto medullary dorsal horn neurons.

EXPERIMENTAL APPROACH

Primary afferent-evoked EPSCs were recorded from medullary dorsal horn neurons of rat horizontal brain stem slices using a conventional whole-cell patch clamp technique under a voltage-clamp condition.

KEY RESULTS

CP93129, a selective 5-HT_{1B} receptor agonist, reversibly and concentration-dependently decreased the amplitude of glutamatergic EPSCs and increased the paired-pulse ratio. In addition, CP93129 reduced the frequency of spontaneous miniature EPSCs without affecting the current amplitude. The CP93129-induced inhibition of EPSCs was significantly occluded by GR55562, a 5-HT_{1B/1D} receptor antagonist, but not LY310762, a 5-HT_{1D} receptor antagonist. Sumatriptan, an anti-migraine drug, also decreased EPSC amplitude, and this effect was partially blocked by either GR55562 or LY310762. On the other hand, primary afferent-evoked EPSCs were mediated by the Ca²⁺ influx passing through both presynaptic N-type and P/Q-type Ca²⁺ channels. The CP93129-induced inhibition of EPSCs was significantly occluded by ω-conotoxin GVIA, an N-type Ca²⁺ channel blocker.

CONCLUSIONS AND IMPLICATIONS

The present results suggest that the activation of presynaptic 5-HT_{1B} receptors reduces glutamate release from primary afferent terminals onto medullary dorsal horn neurons, and that 5-HT_{1B} receptors could be, at the very least, a potential target for the treatment of pain from orofacial tissues.

LINKED ARTICLE

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Abbreviations

ω-AgTx, ω-agatoxin IVA; APV, DL-2-amino-5-phosphonovaleric acid; ω-CgTx, ω-conotoxin GVIA; CP93129, 3-(1,2,3,6-tetrahydropyridin-4-yl)-1,4-dihydropyrrolo[3,2-b]pyridine-5-one; GIRK, G-protein-coupled inwardly rectifying K⁺; GR55562, 3-(3-dimethylaminopropyl)-4-hydroxy-N-(4-pyridin-4-ylphenyl)benzamide; I-V, current-voltage; K-S, Kolmogorov-Smirnov; LY310762, 3,3-dimethyl-1-(2-[4-(4-fluorobenzoyl)piperidin-1-yl]-1-ethyl)-1,3-dihydro-2H-indol-2-one; mEPSCs, miniature EPSCs; PPR, paired-pulse ratio; SB224289, 1'-methyl-5-[[2'-methyl-4'-(5-methyl-1,2,3-oxadiazol-3-yl)biphenyl-4-yl]carbonyl]-2,3,6,7-tetrahydrospiro[furo[2,3-f]indole-3,4'-piperidine hydrochloride; SG, substantia gelatinosa; SR95531, 6-imino-3-(4-methoxyphenyl)-1(6H)-pyridazinebutanoic acid HBr; SQ22536, 9-(tetrahydro-2-furanyl)-9H-purin-6-amine; TG, trigeminal ganglia; TTX, tetrodotoxin; Vc, trigeminal subnucleus caudalis; VDCCs, voltage-dependent Ca²⁺ channels

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Introduction

Neurons within the trigeminal subnucleus caudalis (Vc) receive primary afferent A δ - and C-fibres from orofacial tissues and process orofacial nociceptive transmission, including migraine (Jacquin *et al.*, 1986; Ambalavanar and Morris, 1992; Crissman *et al.*, 1996). Among them, substantia gelatinosa (SG, or lamina II) neurons also receive these afferent fibres and project their axon terminals to the SG and adjacent laminae (Li *et al.*, 1999), suggesting that changes in the excitability of SG neurons via primary afferents as well as local interneurons play crucial roles in the processing of pain signals (Furue *et al.*, 2004). Nociceptive transmission can also be regulated by descending inhibitory pathways, which are mediated by descending noradrenergic and 5-hydroxytryptaminergic projections from the brain stem to spinal or medullary dorsal horn areas (Sandkühler, 1996; Millan, 2002). For example, the intrathecal administration of either hydroxytryptaminergic or noradrenergic agents induces anti-nociceptive actions (Yaksh and Wilson, 1979; Reddy and Yaksh, 1980; DeLander and Hopkins, 1987; Obata *et al.*, 2004). In addition, either NA or 5-HT hyperpolarizes SG neurons (Grudt *et al.*, 1995) and inhibits glutamatergic excitatory transmission onto SG neurons in the Vc (Travagli and Williams, 1996).

5-HT acts on 5-HT receptors to exert a variety of pathophysiological functions, including depression, anxiety, schizophrenia, aggression and migraine, in the CNS (for review, see Lucki, 1998). Most 5-HT receptors, except 5-HT₃ receptors, belong to the GPCR superfamily and, with at least fourteen distinct members, represent one of the most complex families of neurotransmitter receptors (Hoyer *et al.*, 1994; Barnes and Sharp, 1999). Of them, the 5-HT₁ receptor subtype, such as 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E} and 5-HT_{1F} receptors, is widely distributed in the CNS. They are expressed on the neuronal membrane, including presynaptic terminals, and regulate neuronal excitability via either the direct hyperpolarization of postsynaptic neurons or the presynaptic modulation of neurotransmitter release (Barnes and Sharp, 1999; Fink and Göthert, 2007; Feuerstein, 2008). 5-HT₁ receptors are known to modulate transmission of noxious sensory information in the orofacial region (Deseure *et al.*, 2002; Kayser *et al.*, 2002; Okamoto *et al.*, 2005). Among them, 5-HT_{1B} and 5-HT_{1D} receptors are expressed on sensory neurons such as dorsal root ganglia (Nicholson *et al.*, 2003; Classey *et al.*, 2010), suggesting that 5-HT_{1B} and 5-HT_{1D} receptors are involved in the modulation of pain information from peripheral tissues. Similarly, a recent study has shown that i.t. administration of sumatriptan, which is used for the treatment of migraine and activates 5-HT_{1B} and 5-HT_{1D} receptors (Tepper *et al.*, 2002; Ahn and Basbaum, 2005), profoundly reduces somatic and visceral pain (Nikai *et al.*, 2008). On the other hand, 5-HT_{1B} and 5-HT_{1D} receptors are also expressed on trigeminal ganglia (TG) (Hou *et al.*, 2001; Ma *et al.*, 2001). Although a previous study has shown that 5-HT_{1D} receptors inhibit glutamate release onto SG neurons of the Vc (Jennings *et al.*, 2004), it is still not known whether 5-HT_{1B} receptors can modulate glutamatergic transmission. In the present study, therefore, we investigated the 5-HT_{1B} receptor-mediated presynaptic regulation of primary afferent synaptic transmission and mechanisms underlying this type of presynaptic inhibition.

Methods

Preparations

All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). The experiments were approved by the Kyungpook National University Institutional Animal Care and Use Committee and were carried out in accordance with the guiding principles for the care and use of animals approved by the Council of the Physiological Society of Korea and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Every effort was made to minimize both the number of animals used and their suffering.

Sprague-Dawley rats (12–16 days old) were decapitated under pentobarbital anaesthesia (50 mg·kg⁻¹, i.p.). The brain stem was dissected and horizontally sliced at a thickness of 400 μ m by use of a microslicer (VT1000S; Leica, Nussloch, Germany) in a cold artificial CSF (ACSF; 120 NaCl, 2 KCl, 1 KH₂PO₄, 26 NaHCO₃, 2 CaCl₂, 1 MgCl₂ and 10 glucose, saturated with 95% O₂ and 5% CO₂). Slices were kept in an ACSF saturated with 95% O₂ and 5% CO₂ at room temperature (22–25°C) for at least 1 h before electrophysiological recording. On the other hand, the strong stimulation of trigeminal tract might elicit trigeminal subnuclei-evoked EPSCs as well as primary afferent-evoked EPSCs, as glutamatergic transmission between trigeminal subnuclei has been demonstrated (Han *et al.*, 2008). Immediately before recording, therefore, a surgical cut was made between trigeminal subnuclei interpolaris and caudalis without cutting the trigeminal tract to exclude possible activation of inter-subnuclei-evoked glutamate release. Thereafter, the slices were transferred into a recording chamber, and both the superficial dorsal horn of the Vc and trigeminal root was identified under an upright microscope (E600FN, Nikon, Tokyo, Japan) with a water-immersion objective ($\times 40$). The ACSF routinely contained 10 μ M SR95531, 1 μ M strychnine, 50 μ M DL-2-amino-5-phosphonovaleric acid (APV) to block GABA_A, glycine and NMDA receptors, respectively. In experiments with Ba²⁺ and Cd²⁺, KH₂PO₄ in ACSF was replaced with equimolar KCl. The bath was perfused with ACSF at 2 mL·min⁻¹ by the use of a peristaltic pump (MP-1000, EYELA, Tokyo, Japan).

Electrical measurements

All electrical measurements were performed by use of a computer-controlled patch clamp amplifier (MultiClamp 700B; Molecular Devices; Union City, CA). For whole-cell recording, patch pipettes were made from borosilicate capillary glass (1.5 mm outer diameter, 0.9 mm inner diameter; G-1.5; Narishige, Tokyo, Japan) by use of a pipette puller (P-97; Sutter Instrument Co., Novato, CA). The resistance of the recording pipettes filled with internal solution (in mM; 140 CsMeHSO₃, 5 TEA-Cl, 5 CsCl, 2 EGTA, 2 Mg-ATP and 10 HEPES, pH 7.2 with Tris-base) was 4–6 M Ω . Membrane currents were filtered at 2 kHz (MultiClamp Commander; Molecular Devices), digitized at 5 kHz (Digidata 1322A, Molecular Devices) and stored on a computer equipped with pCLAMP 10.0 (Molecular Devices). In whole-cell recordings, 10 mV hyperpolarizing step pulses (30 ms in duration) were periodically delivered to monitor the access resistance (15–

Figure 1

Properties of primary afferent-evoked glutamatergic EPSCs. (A) A photograph of the horizontal brainstem slice. A surgical cut was made between the trigeminal subnuclei interpolaris and caudalis without cutting the trigeminal tract. Sites of the recording and stimulation were indicated (see also Methods for details). Vi; interpolaris, Vo; oralis. (B) A typical time course of EPSC amplitude before and during the application of 50 μM APV and 10 μM CNQX in the presence of both 10 μM SR95531 and 1 μM strychnine. Note that primary afferent-evoked glutamatergic EPSCs were completely blocked by CNQX but not APV. (Ca) Typical traces of primary afferent-evoked glutamatergic EPSCs at various holding potentials (V_H). In these experiments, primary afferent-evoked glutamatergic EPSCs were recorded in the presence of 50 μM APV, 10 μM SR95531 and 1 μM strychnine. (Cb) A plot of the mean amplitude of EPSCs at various V_H values. The continuous line is the least-squares linear fit to the mean EPSC values at each V_H ($r^2 = 0.90$). The calculated reversal potential was 2.9 mV. Each point represents the mean and SEM from seven experiments.

20 MΩ), and recordings were discontinued if the access resistance changed by more than 15%. All electrophysiological experiments were performed at room temperature (22–25°C). To record action potential-dependent glutamatergic EPSCs, a glass stimulation pipette (~10 μm diameter) filled with a bath solution, was positioned around the spinal trigeminal tract (3–6 mm rostral to the border between the trigeminal subnuclei interpolaris and caudalis; see Figure 1A). Brief paired pulses (500 μs, 100–200 μA, 10 Hz) were applied by the stimulation pipette at a frequency of 0.1 Hz using a stimulator

(SEN-7203, Nihon Kohden, Tokyo, Japan) equipped with an isolator unit (SS-701J, Nihon Kohden).

Data analysis

The amplitudes of action potential-dependent glutamatergic EPSCs were calculated by subtracting the baseline from the peak amplitude. The conduction velocity of primary afferents innervating SG neurons of the Vc was calculated by dividing the distance between stimulation and recording sites by the latency of EPSCs. Since the latency of EPSCs consists of the conduction time of action potential and the synaptic delay, the EPSC latency was further compensated by subtracting 0.6 ms, which is the experimentally calculated synaptic delay at thalamocortical excitatory synapses (Salami *et al.*, 2003), from the onset time of EPSCs. However, the influence of synaptic delay on the conduction velocity of primary afferents in the spinal cord might be negligible (Nakatsuka *et al.*, 2000). The effect of drugs on EPSCs was quantified as a percentage change in EPSC amplitude compared with the control values. Spontaneous miniature EPSCs (mEPSCs) were counted and analysed using the MiniAnalysis programme (Synaptosoft, Inc., Decatur, GA) as described previously (Jang *et al.*, 2002). Briefly, mEPSCs were screened automatically using an amplitude threshold of 10 pA and then visually accepted or rejected based upon the rise and decay times. Basal noise levels during voltage-clamp recordings were less than 10 pA. The average values of both the frequency and amplitude of mEPSCs during the control period (10–20 min) were calculated for each recording, and the frequency and amplitude of all the events during the CP93129 application (5 min) were normalized to these values. The effects of these different conditions were quantified as a percentage increase in mEPSC frequency compared with the control values. The inter-event intervals and amplitudes of a large number of synaptic events obtained from the same neuron were examined by constructing cumulative probability distributions and compared using the Kolmogorov–Smirnov (K-S) test with Stat View software (SAS Institute, Inc., Cary, NC). The continuous curve for the concentration–inhibition relationship was fitted using a least-squares fit to the following equation:

$$I = 1 - [C^{n_H} / (C^{n_H} + EC_{50}^{n_H})],$$

where I is the inhibition ratio of CP93129-induced EPSC amplitude, C is the concentration of CP93129, EC_{50} is the concentration for the half-effective response and n_H is the Hill coefficient. Numerical values are provided as the mean and SEM using values normalized to the control. Significant differences in the mean amplitude and frequency were tested using Student's two-tailed paired t -test, using absolute values rather than normalized ones. Values of $P < 0.05$ were considered significant.

Drugs

The drugs used in the present study were 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), DL-2-amino-5-phosphonopivalic acid (APV), strychnine, nifedipine, forskolin (from Sigma, St. Louis, MO), CP93129, GR55562, LY310762, SQ22536, SR95531, SB224289, tetrodotoxin (TTX) (from Tocris, Bristol, UK) and ω-agatoxin IVA (ω-AgTx),

Figure 2

Effect of CP93129 on primary afferent-evoked glutamatergic EPSCs. (A) A typical time course of the EPSC₁ amplitude (a) and PPR (EPSC₂/EPSC₁; b) before, during and after application 1 μ M CP93129. The amplitudes of all EPSCs were plotted. Insets represent typical traces of the numbered region. (B) CP93129-induced changes in the EPSC₁ amplitude (a) and PPR (b). Each column was normalized to the control and represents the mean and SEM from 20 experiments. * P < 0.05, ** P < 0.01. (C) Concentration–response relationship of CP93129. The EC₅₀ value calculated from curve fitting result was 220 nM. Each point and error bar represents the mean and SEM from six to eight experiments. (D) A scatter plot of the extent of CP93129 (1 μ M)-induced inhibition of EPSCs against the calculated conduction velocity of primary afferents innervating SG neurons of the Vc. The continuous line is the least-squares linear fit (r^2 < 0.001, n = 60).

ω -conotoxin GVIA (ω -CgTx) (from Peptide institute, Osaka, Japan). Sumatriptan was kindly gifted from Yuyu Pharma. Inc. (Seoul, Korea). All drugs were applied by bath application (2 mL·min⁻¹). The drug/molecular target nomenclature conforms to *BJP's Guide to Receptors and Channels* (Alexander *et al.*, 2011).

Results

CP93129 acts presynaptically to inhibit glutamate release in Vc neurons

In the presence of 1 μ M strychnine and 10 μ M SR95531, which blocks glycine and GABA_A receptors, action potential-dependent synaptic currents were recorded from SG neurons of the Vc at a V_H of -60 mV by electrical stimulation through a glass pipette placed to the spinal trigeminal tract (Figure 1A). In all SG neurons tested, these synaptic currents were not affected by 50 μ M APV, a selective NMDA receptor antagonist, but they were completely blocked by 10 μ M CNQX, a selective AMPA/K_A receptor antagonist (Figure 1B).

Figure 1C shows typical synaptic currents at various V_H conditions and their current–voltage (I – V) relationship (n = 7). The reversal potential of synaptic currents estimated from the I – V relationship was 2.9 mV, which was very similar to the theoretical equilibrium potential of monovalent cations. These results indicate that the synaptic currents are glutamatergic EPSCs mediated by Ca²⁺-impermeable AMPA/K_A receptors based on their linear I – V relationship (Burnashev *et al.*, 1995). On the other hand, the calculated conduction velocity of primary afferents innervating SG neurons of the Vc was 1.45 ± 0.87 m·s⁻¹ (SD in the range 0.19–4.44 m·s⁻¹, n = 60; see also Figure 2D). This calculated conduction velocity is similar to that estimated from A δ - and/or C-fibres of rat sciatic nerve at 22°C (Pinto *et al.*, 2008), indicating that most of EPSCs recorded in the present study are likely to have originated from A δ - and/or C-fibres.

In order to investigate whether presynaptic 5-HT_{1B} receptors modulate action potential-dependent glutamatergic synaptic transmission onto SG neurons, all the following experiments were performed in the presence of 1 μ M strychnine, 10 μ M SR95531 and 50 μ M APV. In these conditions,

the effect of CP93129 on glutamatergic EPSCs evoked by pairing stimulation at an interval of 50 ms (20 Hz) was observed. CP93129 (1 μ M), a selective 5-HT_{1B} receptor agonist [EC_{50} = 56 nM in rat substantia nigra (Macor *et al.*, 1990); see also Chopin *et al.*, 1994], inhibited primary afferent-evoked glutamate release in more than half of SG neurons tested (148 of 255 neurons, 58%). In 20 SG neurons, in which the inhibitory effect of CP93129 on glutamatergic EPSCs was fully analysed, CP93129 (1 μ M) reversibly decreased the first EPSC (EPSC₁) amplitude to $57.3 \pm 3.7\%$ of the control ($n = 20$, $P < 0.01$; Figure 2A and B) and increased the paired-pulse ratio (PPR; EPSC₂/EPSC₁) from 0.68 ± 0.10 to 1.07 ± 0.20 ($n = 20$, $P < 0.01$; Figure 2A and B), suggesting that CP93129 acts presynaptically to decrease the probability of glutamate release. In addition, CP93129 clearly inhibited glutamatergic EPSCs in a concentration-dependent manner with an EC_{50} value of 220 nM (Figure 2C). On the other hand, there is no relationship between the extent of CP93129-induced inhibition of EPSCs and the calculated conduction velocity of primary afferents innervating SG neurons of the Vc ($r^2 < 0.001$, $n = 60$; Figure 2D).

In a subset of experiments, we examined the effect of CP93129 on glutamatergic mEPSCs. Glutamatergic mEPSCs were recorded from SG neurons in the presence of 300 nM TTX, which completely blocks voltage-dependent Na⁺ channels, 50 μ M APV, 10 μ M SR95531 and 1 μ M strychnine. In 8 of 10 SG neurons tested, in which the effect of CP93129 was fully analysed, CP93129 (1 μ M) decreased the mean mEPSC frequency to $72.6 \pm 3.7\%$ of the control ($n = 8$, $P < 0.01$), without affecting the mean mEPSC amplitude ($99.7 \pm 1.6\%$ of the control, $n = 8$, $P = 0.63$; Figure 3A and B insets). CP93129 significantly shifted the distribution of the inter-event interval to the right ($P < 0.01$, K-S-test; Figure 3Ba), indicating a decrease in mEPSC frequency. However, CP93129 did not change the distribution of the current amplitude ($P = 0.69$, K-S test; Figure 3Bb). In addition, CP93129 (1 μ M) decreased the mean mEPSC frequency even in the presence of 100 μ M Cd²⁺, a general voltage-dependent Ca²⁺ channel (VDCC) blocker ($63.4 \pm 7.7\%$ of the Cd²⁺ condition, $n = 5$, $P < 0.01$), without affecting the mean mEPSC amplitude ($100.1 \pm 2.9\%$ of the Cd²⁺ condition, $n = 5$, $P = 0.73$). The results again suggest that CP93129 acts presynaptically to reduce the probability of glutamate release onto SG neurons of the Vc.

5-HT_{1B} receptors are responsible for the CP93129-induced inhibition of glutamate release

In order to verify whether presynaptic 5-HT_{1B} receptors are responsible for the CP93129-induced inhibition of glutamate release, we examined the effect of GR55562, a 5-HT_{1B/1D} receptor antagonist (pK_i = 7.3 and 6.3 for 5-HT_{1B} and 5-HT_{1D} receptors, respectively; Connor *et al.*, 1995), on the CP93129-induced decrease in EPSCs. The extent of CP93129-induced inhibition of EPSCs ($55.6 \pm 8.9\%$ of the control, $n = 6$) was greatly reduced in the presence of 30 μ M GR55562 ($88.0 \pm 0.7\%$ of the GR55562 condition, $n = 6$, $P < 0.01$; Figure 4A and B). In addition, the CP93129-induced inhibition of EPSCs was significantly reduced in the presence of 30 μ M SB224289, a more selective 5-HT_{1B} receptor antagonist (pK_i = 8.0 and 6.2 for 5-HT_{1B} and 5-HT_{1D} receptors, respectively; Roberts *et al.*,

Figure 3

Effect of CP93129 on glutamatergic mEPSCs. (A) An all-point scatter plot of the amplitude of glutamatergic mEPSCs before, during and after the application of 1 μ M CP93129; 2157 events were plotted. Insets represent typical traces with an expanded time scale. (B) Cumulative probability distributions for the inter-event interval (a; $P < 0.01$, K-S test) and current amplitude (b; $P = 0.69$, K-S test) of glutamatergic mEPSCs shown in (A); 468 for the control and 351 events for CP93129 were plotted. Insets, CP93129 (1 μ M)-induced changes in mEPSC frequency (left) and amplitude (right). Each column represents the mean and SEM from eight experiments. ** $P < 0.01$, n.s., not significant.

1997) ($64.0 \pm 8.8\%$ of the control and $87.3 \pm 8.0\%$ of the SB224289 condition, $n = 5$, $P < 0.05$; data not shown). In addition, CP93129 failed to decrease glutamatergic EPSCs in the presence of both GR55562 and SB224289 ($98.7 \pm 3.2\%$ of the GR55562 and SB224289 condition, $n = 4$, $P = 0.10$; data not shown). Neither GR55562 nor SB224289 had an effect on either the EPSC amplitude or the PPR (Figure 4Bb and Table S1), indicating that there is little tonic activation of presynaptic 5-HT_{1B} receptors. In contrast, CP93129 (1 μ M) still decreased EPSCs in the presence of 10 μ M LY310762, a selective 5-HT_{1D} receptor antagonist (IC_{50} = 31 nM, Pullar *et al.*, 2004) ($61.3 \pm 8.5\%$ of the LY310762 condition, $n = 5$, $P < 0.05$; Figure 4C and D), suggesting that 5-HT_{1B} receptors are responsible for the CP93129-induced inhibition of glutamate release.

Since a previous study has shown that sumatriptan acts on 5-HT_{1D} receptors to inhibit the primary afferent-evoked glutamate release onto SG neurons of the Vc (Jennings *et al.*, 2004), we further examined the effect of sumatriptan, a 5-HT_{1B/1D} receptor agonist (pK_i = 7.8 and 8.1 for 5-HT_{1B} and 5-HT_{1D} receptors, respectively; Dupuis *et al.*, 1999), on glutamatergic EPSCs. In 29 of 34 neurons tested, sumatriptan (3 μ M) decreased glutamatergic EPSCs to $65.6 \pm 3.5\%$ of the control ($n = 29$, $P < 0.01$; Figure 5A and B), and increased the

Figure 4

Effects of 5-HT_{1B} and 5-HT_{1D} receptor antagonists on the CP93129-induced decrease in EPSCs. (A) A typical time course of the EPSC₁ amplitude before, during and after application of 1 μM CP93129 in the absence or presence of 30 μM GR55562. All points and error bars represent the mean and SEM of six EPSCs. Insets represent typical traces of the numbered region. (B) CP93129-induced changes in the EPSC₁ amplitude (a) and PPR (b) in the absence or presence of 30 μM GR55562. Each column was normalized to the control and represents the mean and SEM from six experiments. ** $P < 0.01$. (C) A typical time course of the EPSC₁ amplitude before, during and after application of 1 μM CP93129 in the absence or presence of 10 μM LY310762. All points and error bars represent the mean and SEM of six EPSCs. Insets represent typical traces of the numbered region. (D) CP93129-induced changes in the EPSC₁ amplitude (a) and PPR (b) in the presence of 10 μM LY310762. Each column was normalized to the control and represents the mean and SEM from six experiments. * $P < 0.05$.

PPR from 0.57 ± 0.05 to 0.94 ± 0.07 ($n = 29$, $P < 0.01$, data not shown), suggesting that sumatriptan also acts presynaptically to decrease the probability of glutamate release. In 22 of 29 neurons responding to sumatriptan, CP93129 (1 μM) also decreased glutamatergic EPSCs to $52.7 \pm 3.7\%$ of the control ($n = 22$, $P < 0.01$; Figure 5Aa and B). In the remaining 7 of 29 neurons responding to sumatriptan, however, CP93129 had no inhibitory effect ($<10\%$ inhibition) on glutamatergic EPSCs (Figure 5Ab and B). On the other hand, both sumatriptan and CP93129 had no inhibitory effect on glutamatergic EPSCs in 5 of 34 neurons tested (Figure 5B). The extent of sumatriptan-induced decrease in EPSCs ($54.4 \pm 5.6\%$ of the control, $n = 8$) was significantly but not completely blocked by either 10 μM LY310762 ($77.4 \pm 7.8\%$ of the LY310762 condition, $n = 6$, $P < 0.05$) or 30 μM GR55562 ($76.4 \pm 7.3\%$ of the GR55562 condition, $n = 8$, $P < 0.05$) (Figure 5C). In addition, sumatriptan failed to decrease glutamatergic EPSCs in the presence of both LY310762 and GR55562 ($95.2 \pm 7.2\%$ of the LY310762 and GR55562 condition, $n = 6$, $P = 0.23$; Figure 5C and Table S2). These results suggest that sumatriptan acts on both 5-HT_{1B} and 5-HT_{1D} receptors to inhibit glutamate release.

Mechanisms underlying the 5-HT_{1B} receptor-mediated presynaptic inhibition of primary afferent-evoked glutamate release

5-HT_{1B} receptors are seven-transmembrane proteins coupled to G_{i/o} proteins and negatively coupled to AC, which increases

intracellular cAMP concentration (Barnes and Sharp, 1999; Brown and Sihra, 2008). If presynaptic 5-HT_{1B} receptors decrease glutamate release by inhibiting AC, the direct blockade of AC should decrease the glutamatergic EPSC amplitude. However, this was not the case because SQ22536, a selective AC inhibitor (Turcato and Clapp, 1999; Yum *et al.*, 2008; Choi *et al.*, 2009), even at a 100 μM concentration, had no effect on EPSCs ($93.5 \pm 12.0\%$ of the control, $n = 5$, $P = 0.47$; Figure 6A). In addition, the extent of CP93129-induced inhibition of EPSCs ($64.8 \pm 9.6\%$ of the control, $n = 5$) was not affected by 100 μM SQ22536 ($62.5 \pm 12.3\%$ of the SQ22536 condition, $n = 5$, $P = 0.66$; Figure 6A and Da). We also examined the effect of forskolin, an AC activator (Laurenza *et al.*, 1989; Yum *et al.*, 2008; Choi *et al.*, 2009), on the CP93129-induced inhibition of glutamatergic EPSCs. Forskolin at a 10 μM concentration did not change EPSCs ($91.7 \pm 8.0\%$ of the control, $n = 9$, $P = 0.38$; Figure 6B). In addition, forskolin had no effect on the CP93129-induced inhibition of EPSCs ($64.1 \pm 5.7\%$ of the control and $61.9 \pm 6.5\%$ of the forskolin condition, $n = 9$, $P = 0.85$; Figure 6B and Db). We next examined the effect of Ba²⁺, a G-protein-coupled inwardly rectifying K⁺ (GIRK) channel blocker (Gerber *et al.*, 1989; Yum *et al.*, 2008), on the CP93129-induced inhibition of EPSCs. Ba²⁺ at a 500 μM concentration did not change EPSCs ($105.1 \pm 5.2\%$ of the control, $n = 7$, $P = 0.47$; Figure 6C). In addition, Ba²⁺ had no effect on the CP93129-induced inhibition of EPSCs ($57.9 \pm 4.7\%$ of the control and $65.3 \pm 3.6\%$ of the Ba²⁺ condition, $n = 7$, $P = 0.11$; Figure 6C and Dc). The results

Figure 5

Effects of sumatriptan on glutamatergic EPSCs. (A) A typical time course of the EPSC₁ amplitude before, during and after application of 3 μ M sumatriptan (Suma) and 1 μ M CP93129. All points and error bars represent the mean and SEM of six EPSCs. Insets represent typical traces of the numbered region. Note that both sumatriptan and CP93129 had an inhibitory effect on glutamatergic EPSCs in most of neurons tested (a). However, only sumatriptan had an inhibitory effect on glutamatergic EPSCs in a subset of neurons tested (b). (B) The extent of sumatriptan- and CP93129-induced decrease in glutamatergic EPSCs in the same neurons. Results from 34 neurons were plotted. Dotted lines; 10% inhibition. (C) Sumatriptan-induced changes in the EPSC₁ amplitude in the absence and presence of 30 μ M GR55562, 10 μ M LY310762 or both antagonists. Each column was normalized to the control and represents the mean and SEM from six to eight experiments. * $P < 0.05$, ** $P < 0.01$.

suggest that neither AC-cAMP pathways nor GIRK channels are involved in the CP93129-induced inhibition of glutamatergic EPSCs.

Multiple types of VDCCs control the neurotransmitter release, and especially, N- and P/Q-type Ca^{2+} channels are closely involved in the action potential-dependent neurotransmitter release (Wu and Saggau, 1997). In addition, the activation of presynaptic 5-HT_{1B} receptors inhibits VDCCs to decrease neurotransmitter release at central synapses (Mizutani *et al.*, 2006; Xiao *et al.*, 2008). Therefore, we examined the effects of specific VDCC blockers on the CP93129-induced inhibition of EPSCs. Nifedipine (10 μ M), a selective L-type VDCC blocker, had little effect on EPSCs ($92.4 \pm 6.6\%$ of the control, $n = 6$, $P = 0.38$; Figure 7A), suggesting that L-type VDCCs do not contribute to glutamate release from primary afferent terminals. In the presence of 10 μ M nifedipine, the CP93129-induced inhibition of EPSCs was not affected ($46.6 \pm 11.6\%$ of the control and $52.5 \pm 8.4\%$ of the nifedipine condition, $n = 6$, $P = 0.57$; Figure 7A and Ba). ω -AgTx (500 nM), a selective P/Q-type VDCC blocker, significantly decreased EPSC amplitude ($35.9 \pm 6.5\%$ of the control, $n = 4$, $P < 0.01$; Figure 7B). After treatment with 500 nM ω -AgTx, however, the CP93129-induced inhibition of EPSCs was not affected ($51.4 \pm 3.9\%$ of the control and $52.4 \pm 6.9\%$ of the ω -AgTx condition, $n = 4$, $P = 0.58$; Figure 7B and Bb), indicating that P/Q-type VDCCs might be not involved in the

CP93129-induced inhibition of EPSCs. ω -CgTx (2 μ M), a selective N-type VDCC blocker, also significantly decreased EPSCs ($33.4 \pm 7.6\%$ of the control, $n = 7$, $P < 0.01$; Figure 7C). After the treatment with 2 μ M ω -CgTx, the CP93129-induced inhibition of EPSCs was significantly reduced ($38.9 \pm 9.3\%$ of the control and $68.3 \pm 6.6\%$ of the ω -CgTx condition, $n = 7$, $P < 0.01$; Figure 7C and Bc). SNX-482 (1 μ M), a selective R-type VDCC blocker, had little effect on EPSCs ($99.5 \pm 1.0\%$ of the control, $n = 4$, $P = 0.97$). In the presence of 1 μ M SNX-482, the CP93129-induced inhibition of EPSCs was not affected ($45.1 \pm 5.2\%$ of the control and $55.8 \pm 10.2\%$ of the SNX-482 condition, $n = 4$, $P = 0.54$). On the other hand, the cumulative application of both 500 nM ω -AgTx and 2 μ M ω -CgTx abolished all glutamatergic EPSCs (Figure 7B), suggesting that primary afferent-evoked EPSCs were mediated by the Ca^{2+} influx passing through both presynaptic N-type and P/Q-type Ca^{2+} channels.

Discussion

5-HT₁ receptors are known to inhibit the release of a number of neurotransmitters, such as glutamate, GABA, glycine, catecholamines and 5-HT itself, as heteroreceptors or autoreceptors at central synapses (Feuerstein, 2008; Jeong *et al.*, 2008; Guo and Rainnie, 2010). Although 5-HT₁ receptors are further

Figure 6

Effects of SQ22536, forskolin and Ba²⁺ on the CP93129-induced decrease in EPSCs. (A) A typical time course of the EPSC₁ amplitude before, during and after application of 1 μM CP93129 in the absence or presence of 100 μM SQ22536. All points and error bars represent the mean and SEM of six EPSCs. Insets represent typical traces of the numbered region. (B) A typical time course of the EPSC₁ amplitude before, during and after application of 1 μM CP93129 in the absence or presence of 10 μM forskolin. All points and error bars represent the mean and SEM of six EPSCs. Insets represent typical traces of the numbered region. (C) A typical time course of the EPSC₁ amplitude before, during and after application of 1 μM CP93129 in the absence or presence of 500 μM Ba²⁺. The amplitudes of 6 EPSCs were averaged and plotted. Insets represent typical traces of the numbered region. (D) Changes in the CP93129-induced decrease in EPSC₁ amplitude in the absence and presence of SQ22536 ($n = 5$, a), forskolin ($n = 9$, b), and Ba²⁺ ($n = 7$, c). Each column was normalized to the control and represents the mean and SEM. * $P < 0.05$, ** $P < 0.01$.

divided into 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, and 5-HT_{1F} receptors, most of the previous studies have shown that either 5-HT_{1A} or 5-HT_{1B} receptors are mainly involved in the presynaptic inhibition of neurotransmitter release (Feuerstein, 2008). In contrast, a few studies have suggested the involvement of 5-HT_{1D} receptors in the presynaptic inhibition of glutamate release (Travagli and Williams, 1996; Maura *et al.*, 1998). In the present study, several lines of evidence support the conclusion that presynaptic 5-HT_{1B} receptors are responsible for the CP93129-induced inhibition of glutamatergic EPSCs. Firstly, exogenously applied CP93129, a selective 5-HT_{1B} receptor agonist, simultaneously decreased glutamatergic EPSC amplitude and increased the PPR. In addition, CP93129 decreased glutamatergic mEPSC frequency without affecting the current amplitude, although a further study is needed to elucidate whether the origin of mEPSCs is primary afferents. These results suggest that CP93129 acts presynaptically to decrease the probability of glutamate release from primary afferent terminals. Secondly, the CP93129-induced inhibition of glutamatergic EPSCs was significantly blocked by 5-HT_{1B/1D} but not 5-HT_{1D} receptor antagonists. Taken together, our present results provide evidence that functional 5-HT_{1B} receptors are expressed on trigeminal primary afferents, and that their activation decreases action potential-dependent and spontaneous glutamate release onto SG neurons of the Vc.

Travagli and Williams (1996) have suggested that 5-HT_{1D} receptors are responsible for the sumatriptan-induced inhibi-

tion of glutamate release onto medullary dorsal horn neurons of adult guinea-pigs. This conclusion might be derived from the use of sumatriptan as a potent agonist for only 5-HT_{1D} receptors rather than 5-HT_{1B} receptors, although sumatriptan is now regarded as an agonist for 5-HT_{1B/1D} receptors. In addition, since Travagli and Williams (1996) did not examine the effects of 5-HT_{1D} or 5-HT_{1B} receptor antagonists on the sumatriptan-induced inhibition of glutamate release, it is still not clear whether sumatriptan acts only on 5-HT_{1D} receptors to decrease glutamate release. On the other hand, Jennings *et al.* (2004) have shown that sumatriptan, via the activation of 5-HT_{1D} rather than 5-HT_{1B} receptors, reduces either the amplitude of evoked EPSCs or the frequency of mEPSCs in SG neurons of the Vc (Jennings *et al.*, 2004). This conclusion is based on lack of effect of CP93129 on the frequency of glutamatergic mEPSCs, although CP93129 clearly decreased the frequency of mEPSCs in the present study. One difference between the previous and present studies is the position of the stimulating electrodes used to stimulate primary afferents; in the previous study, the trigeminal tract was about 1 mm rostral to the site of recording (Jennings *et al.*, 2004) and, in the present study, the trigeminal tract was about 3–6 mm rostral to the border between the trigeminal subnuclei interpolaris and caudalis. Further, strong stimulation of the trigeminal tract might elicit trigeminal subnuclei-evoked EPSCs as well as primary afferent-evoked EPSCs. Another difference between the previous and present studies is the temperature; in the previous study it was 34°C (Jennings *et al.*,

Figure 7

Effects of specific VDCC antagonists on the CP93129-induced decrease in EPSCs. (A) A typical time course of the EPSC₁ amplitude before, during and after application of 1 μM CP93129 in the absence or presence of 3 μM nifedipine. All points and error bars represent the mean and SEM of six EPSCs. Insets represent typical traces of the numbered region. (B) A typical time course of the EPSC₁ amplitude before, during and after application of 1 μM CP93129 in the absence or presence of 500 nM ω-AgTx. All points and error bars represent the mean and SEM of six EPSCs. Insets represent typical traces of the numbered region. (C) A typical time course of the EPSC₁ amplitude before, during and after application of 1 μM CP93129 in the absence or presence of 2 μM ω-CgTx. All points and error bars represent the mean and SEM of six EPSCs. Insets represent typical traces of the numbered region. (D) Changes in the CP93129-induced decrease in EPSC₁ amplitude in the absence and presence of nifedipine ($n = 6$, a), ω-AgTx ($n = 4$, b), and ω-CgTx ($n = 7$, c). Each column was normalized to the control and represents the mean and SEM. ** $p < 0.01$.

2004) and in our study it was room temperature (22–25°C). Temperature is an important factor that influences the activity of receptors. Although these possibilities were not tested in the present study, further studies should be done to explain the apparent discrepancy between these two studies.

On the other hand, other 5-HT receptor subtypes including 5-HT_{1B} receptors might contribute to the sumatriptan-induced inhibition of glutamatergic EPSCs, because the inhibitory effect of sumatriptan on glutamatergic EPSCs seems to be partially blocked by the 5-HT_{1D} receptor antagonist (Jennings *et al.*, 2004). In addition, Jennings *et al.* did not examine the effect of CP93129 on action potential-dependent EPSCs. In the present study, we also found that sumatriptan has an inhibitory effect on glutamatergic EPSCs in the majority of Vc neurons tested, and that the sumatriptan-induced decrease in glutamatergic EPSCs was partially attenuated by adding either 5-HT_{1B} or 5-HT_{1D} receptor antagonists, and completely blocked by adding both antagonists. Further, since CP93129 decreased glutamatergic EPSCs in most of the Vc neurons responding to sumatriptan, the majority of primary afferent terminals is likely to express both 5-HT_{1B} and 5-HT_{1D} receptors. This conclusion is further supported by previous immunohistochemical studies showing that 5-HT_{1B} and 5-HT_{1D} receptors are extensively expressed in small- and medium-sized TG neurons, and they are even colocalized in the same TG neurons (Wotherspoon and Priestley, 2000; Ma *et al.*, 2001). However, it should be

noted that a subset of primary afferent terminals might express only 5-HT_{1D} receptors, because CP93129 had no inhibitory effect on glutamatergic EPSCs of Vc neurons responding to sumatriptan.

Although it has been well documented that 5-HT_{1B} receptors are coupled to G_{i/o} proteins and that their activation results in a decrease in cAMP production, the increase in K⁺ conductance and the inhibition of VDCCs (Barnes and Sharp, 1999; Brown and Sihra, 2008), mechanisms underlying the 5-HT_{1B} receptor-mediated inhibition of neurotransmitter release are poorly understood. In the present study, the contribution of either cAMP or GIRK channels to the CP93129-induced inhibition of EPSCs would be negligible because both the specific inhibitor and activator of AC and GIRK channel blocker had no effect on the CP93129-induced inhibition of EPSCs. In contrast, we found that the extent of CP93129-induced inhibition of EPSCs was significantly reduced by adding ω-CgTx (N-type blocker), but not nifedipine (L-type blocker), ω-AgTx (P/Q-type blocker) or SNX-482 (R-type blocker), suggesting that the CP93129-induced inhibition of EPSCs is mainly mediated by the inhibition of Ca²⁺ entry through presynaptic N-type Ca²⁺ channels. On the other hand, we also found that CP93129 still decreased the frequency of mEPSCs even in the presence of Cd²⁺. Since miniature currents are generally independent of extracellular Ca²⁺ or VDCCs (Scanziani *et al.*, 1992; Capogna *et al.*, 1993), these results suggest that presynaptic 5-HT_{1B} receptors reduce

action potential-independent glutamate release by acting on the release machinery downstream of the presynaptic Ca²⁺ entry (Wu and Saggau, 1997). These additional direct effects on the presynaptic vesicular release machinery might also contribute to the 5-HT_{1B} receptor-mediated presynaptic inhibition of action potential-dependent glutamate release (see also Wu and Saggau, 1997). However, it should be noted that mechanisms operating at room temperature might be not the same as those prevailing at physiological temperature.

Triptans, such as sumatriptan, naratriptan and zolmitriptan, have a selective analgesic action on some types of cranial pain, including migraine and cluster headache (Ekbom *et al.*, 1995; Ahn and Basbaum, 2005), but not facial or somatic pain (Dao *et al.*, 1995; Antonaci *et al.*, 1998). In peripheral tissues, the anti-migraine action of triptans is mainly mediated by both 5-HT_{1B} and 5-HT_{1D} receptors. For example, smooth muscle cells in cranial blood vessels express 5-HT_{1B} receptors (Hamel *et al.*, 1993; Longmore *et al.*, 1998), and the activation of 5-HT_{1B} receptors directly elicits vasoconstriction (Humphrey and Feniuk, 1991). However, as trigeminovascular sensory neurons in humans express 5-HT_{1D} rather than 5-HT_{1B} receptors (Longmore *et al.*, 1997; Potrebic *et al.*, 2003), 5-HT_{1B} receptors might be not involved in the anti-migraine action of triptans in trigeminovascular sensory neurons. In addition, previous studies have shown that 5-HT_{1D} receptors in humans contribute to the triptan-mediated vasoconstriction by inhibiting the release of vasodilatory neuropeptides, for example, calcitonin gene-related peptide, from the peripheral terminals of trigeminal sensory neurons (Longmore *et al.*, 1997; but see also Shepherd *et al.*, 1997). Similarly, sumatriptan, via the activation of 5-HT_{1D}, rather than 5-HT_{1B}, receptors has been shown to reduce glutamate release onto SG neurons of the Vc (Jennings *et al.*, 2004). Although these previous studies suggest that 5-HT_{1B} receptors are not involved in the anti-migraine action of triptans in trigeminovascular sensory neurons, recent studies have suggested that a central trigeminal action of triptans, which is mediated by 5-HT_{1B} rather than 5-HT_{1D} receptors could be involved in the processing of craniovascular pain (Muñoz-Islas *et al.*, 2006; 2009). Further studies are needed to verify whether 5-HT_{1B} receptors can inhibit glutamate release from trigeminovascular afferents onto Vc neurons. On the other hand, 5-HT_{1B} receptors are expressed in neurons within the TG as well as dorsal root ganglia (Pierce *et al.*, 1996; Hou *et al.*, 2001). In addition, many 5-HT_{1B}-positive neurons are known to be Aδ- or C-fibre neurons, which also contain calcitonin gene-related peptide and substance P (Wotherspoon and Priestley, 2000; Ma *et al.*, 2001), suggesting that 5-HT_{1B} receptors might have an analgesic effect on cranial as well as non-cranial pain by decreasing the excitability of nociceptive afferents. In fact, a recent study has shown that triptans have an analgesic action in animal models of non-cranial pain including somatic and visceral pain (Nikai *et al.*, 2008), although it is not known whether such antinociceptive effects of triptans on non-cranial pain are mediated by 5-HT_{1B} and/or 5-HT_{1D} receptors.

In conclusion, we have shown that the activation of presynaptic 5-HT_{1B} receptors decreases glutamate release from trigeminal primary afferents, presumably nociceptive Aδ- and C-fibres, onto SG neurons of the Vc. Together with previous immunohistochemical studies, the present results suggest

that 5-HT_{1B} receptors could be a potential target for the treatment of pain from orofacial tissues.

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Statement of conflicts of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1 CP93129-induced changes in paired-pulse ratio in the absence and presence of various drugs. Data represent the mean and SEM of paired-pulse ratio

Table S2 Sumatriptan-induced changes in paired-pulse ratio in the absence and presence of 5-HT_{1B} and 5-HT_{1D} receptor antagonists. Data represent the mean and SEM of paired-pulse ratio

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