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Endogenously released 5-HT inhibits A and C fiber-evoked synaptic transmission in the rat spinal cord by the facilitation of GABA/glycine and 5-HT release via 5-HT_{2A} and 5-HT_3 receptors

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ABSTRACT

Serotonin (5-HT) released from descending fibers plays important roles in spinal functions such as locomotion and nociception. 5-HT_{2A} and 5-HT_3 receptors are suggested to contribute to spinal antinociception, although their activation also contributes to neuronal excitation. In the neonatal spinal cord, DL-p-chloroamphetamine (pCA), a 5-HT releaser, inhibited both A fiber-evoked monosynaptic reflex potential (MSR) and C fiber-evoked slow ventral root potential (sVRP). The pCA-mediated inhibition was reversed by ketanserin (a 5-HT_{2A} receptor antagonist) and tropisetron (a 5-HT_3 receptor antagonist). Bath-applied 5-HT also inhibited MSR and sVRP; in this case, the actions of 5-HT were antagonized by ketanserin, but not by tropisetron. The pCA-evoked inhibition of sVRP was reduced by bicuculline (a $GABA_A$ receptor antagonist) and strychnine (a glycine receptor antagonist). Furthermore, ketanserin inhibited the pCA-evoked release of gamma-aminobutyric acid (GABA) and glycine, while tropisetron inhibited the pCA-evoked release of 5-HT. These results suggest that 5-HT released by pCA activates 5-HT_{2A} receptors, which in turn stimulates the release of GABA/glycine and thereby blocks the spinal nociceptive pathway. 5-HT_3 receptors may be involved in the facilitation of 5-HT release via a positive feedback process.

Keywords: 5-HT; spinal reflex potential; GABA; glycine; autoreceptor

1. Introduction

The descending serotonergic system originating in the brain stem plays important roles to modulate nociceptive pathways in the spinal cord. Serotonin (5-HT, or 5-hydroxytryptamine) and its analogues show both facilitatory and inhibitory effects on spinal functions, including locomotion and nociception (Jordan et al., 2008; Millan, 2002; Vanegas and Schaible, 2004; Yoshimura and Furue, 2006). At least four subtypes (5-HT_1, 5-HT_2, 5-HT_3, and 5-HT_7) are reportedly expressed in the rat spinal cord (Doly et al., 2005; Fonseca et al., 2001; Kidd et al., 1993; Marlier et al., 1991). Most 5-HT receptor subtypes are G-protein coupled receptors (i.e., $G_{i/o}$ (5-HT_1), $G_{q/11}$ (5-HT_2) and G_s (5-HT_7)), whereas 5-HT_3 receptors are ligand-gated cation channels (Fink and Göthert, 2007). In general, 5-HT_1 receptor-mediated signal transduction pathways are inhibitory in regard to neuronal activity, whereas 5-HT_2, 5-HT_3 and 5-HT_7 receptor-mediated signal transduction pathways are excitatory. Thus, receptor subtype-specific mechanisms result in the complex actions of 5-HT in the spinal cord.

The isolated spinal cord of neonatal rat has been widely used for the investigation of spinal neuronal transmission (Akagi and Yanagisawa, 1987; Faber et al.,

1997; Nussbaumer et al., 1989; Woodley and Kendig, 1991). Electrical stimulation of the lumbar dorsal root elicits a reflex potential at the corresponding ipsilateral ventral root. The monosynaptic reflex potential (MSR) is an early part of the reflex potential and is mediated via monosynaptic transmission from primary afferent A fibers to motoneurons in the ventral horn. The input from primary afferent C fibers to interneurons in the dorsal horn is also transmitted to motoneurons. These signals can be measured as a slow ventral root potential (sVRP), which is a late part of the reflex potential and is believed to reflect spinal nociceptive transmission (Akagi et al., 1985; Kendig et al., 1991; Otsuguro et al., 2005; Yanagisawa et al., 1984).

Endogenously released 5-HT has been shown to inhibit MSR, which is reversed by ketanserin, a 5-HT_{2A} receptor antagonist (Crick and Wallis, 1991; Wallis et al., 1993a, 1993b). However, ketanserin failed to reverse MSR blockade by bath-applied 5-HT in previous reports (Crick and Wallis, 1991; Elliott and Wallis, 1992; Manuel et al., 1995). Ketanserin also failed to reverse the inhibition of sVRP by bath-applied 5-HT, which is mediated by the 5-HT_{1B} receptor (Hedo and Lopez-Garcia, 2002). Although several reports have implied that endogenously released 5-HT activates 5-HT_2 and 5-HT_3 receptors, resulting in inhibition of spinal nociceptive transmission (Courade et al.,

2001; Honda et al., 2006; Ochi and Goto, 2000; Seyerk et al., 2010), the effect of endogenous 5-HT on sVRP is still poorly understood.

The present study examined the effects of DL-p-chloroamphetamine (pCA), a 5-HT releaser, on reflex potentials in the spinal cord isolated from neonatal rats. We also measured the release of 5-HT and GABA /glycine from the spinal cord so as to investigate the underlying mechanisms for the actions of 5-HT.

2. Material and methods

2.1. Spinal cord preparation

All experimental protocols were approved by the Institutional Animal Care and Use Committee, Graduate School of Veterinary Medicine, Hokkaido University. Every effort was made to minimize animal suffering and to reduce the number of animals employed in the experiments. Wistar rats (0–5 days old) of both genders were used in the study.

Neonatal rats were euthanized by decapitation, and the spinal cords were removed. Isolated spinal cord preparations were generated as previously described (Otsuguro et al., 2006, 2011). The hemisected spinal cord was superfused in a recording chamber with artificial cerebrospinal fluid (aCSF) at a flow rate of approximately 3

ml/min. The temperature of the chamber was kept at 27±2ºC. The composition of the aCSF was as follows (mM): NaCl 138; $NaHCO_3$ 21; NaH_2PO_4 0.6; KCl 3.5; $CaCl_2$ 1.25; $MgCl_2$ 1.2; and glucose 10. The aCSF was gassed with 95% O_2 and 5% CO_2; pH~7.3.

2.2. Electrophysiological measurements

Stimulating and recording suction electrodes were placed on the dorsal and ipsilateral ventral roots (L3-L5), respectively. The dorsal root was stimulated every 2 min by a single square wave pulse (40 V, 200 μs). MSR and sVRP were recorded from the segmental ventral root. The depolarizing response to 5-HT was also recorded from the ventral root. The magnitudes of the MSR and the depolarizing response were expressed as the peak amplitude (mV), and the magnitude of the sVRP was expressed as the depolarization integral (mV·s) over the resting potential of the ventral root.

In most experiments, the inhibitory effects of pCA and 5-HT on spinal reflex potentials were evaluated by measuring the mean of three maximal responses to either agent, expressed as a percentage of the mean of three responses just before the application of pCA or 5-HT. The time course of the magnitude of the MSR and the sVRP was expressed as a percentage of the mean of the first five responses. Electrical responses were detected by using a high gain amplifier (MEZ-8300, Nihon Kohden,

Tokyo, Japan) equipped with a low-pass filter at 10 kHz. MSR was recorded by using a thermal arraycorder (WR7900, Graftec, Yokohama, Japan) with a sampling time of 80 μs. sVRP was digitized by using an analog/digital converter (PowerLab, ADInstruments, Castle Hill, Australia) with a sampling time of 25 ms. Data were stored in a personal computer and analyzed with LabChart 6 software (ver. 6.0, ADInstruments).

2.3. Measurement of 5-HT and GABA/glycine concentrations

After removal of all roots and the dorsal root ganglion, the isolated spinal cord was sliced into several pieces, and the pieces were then equilibrated in aCSF for more than 30 min at 35°C. Incubation media from before and after treatment with pCA were collected and stored on ice. For 5-HT measurement, collected samples were acidified with perchloric acid (0.2 M), and isoproterenol (1 μM) was added as an internal standard. For GABA/glycine measurement, DL-aminoadipic acid (10 μM) was added as an internal standard, and each sample was then mixed (3:1) with o-phthalaldehyde (0.54 mg/ml)/2-mercaptoethanol (0.4 μl/ml) in 0.1 M K_2CO_3 (pH 9.5).

The samples were applied to a high-performance liquid chromatography system with an octadecylsilyl (ODS) column (EICOMPAK SC-5ODC, 3.0 × 150 mm, EICOM, Kyoto, Japan) equipped with an electrochemical detector (ECD-300, EICOM,

Kyoto, Japan). The mobile phase for 5-HT measurement consisted of 100 mM phosphate buffer (pH 6.0), 16% methanol, 50 mg/l EDTA Na_2 and 500 mg/l sodium octasulfonic acid, whereas the mobile phase for GABA/glycine measurement consisted of 100 mM phosphate buffer (pH 6.0), 35% methanol and 5 mg/l EDTA Na_2. The flow rate was 0.5 ml/min. The amounts of 5-HT and GABA/glycine were expressed relative to the tissue wet weight (fmol/mg).

2.4. Drugs

Atropine sulfate, (S),9(R)-(-)-bicuculline methobromide, 5-HT creatinine sulfate, α-methylserotonin maleate salt (α-Me-5-HT), pCA and tropisetron hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO, USA).

1-(6-Chloro-2-pyridinyl)-4-piperidinamine (SR 57227) hydrochloride, ketanserin tartrate and

N-[4-methoxy-3-(4-methyl-1-piperazinyl)phenyl]-2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl)-1,1'-biphenyl-4-carboxamide (GR 127935) hydrochloride were purchased from Tocris (Bristol, UK). Strychnine sulfate was purchased from Wako (Osaka, Japan).

2.5. Data analysis

Results are expressed as mean ± standard error of the mean. The half maximal inhibitory concentration (IC_{50}) value was calculated by fitting the data to a sigmoidal logistic curve with Origin 8.6J software (OriginLab, Northampton, MA, USA). Statistical comparisons between the two groups were performed by using the paired or unpaired Student's *t*-test, and multiple comparisons were performed by using ANOVA followed by Dunnett's test. A P-value of less than 0.05 was considered significant.

3. Results

3.1. Effects of pCA on the isolated spinal cord

Electrical stimulation of the lumbar dorsal root (L3–L5) every 2 min evoked MSR followed by sVRP at the corresponding ipsilateral ventral root. As shown in Fig. 1A, the application of pCA (3 μM) for 10 min inhibited both reflex potentials. The inhibitory effect of pCA reached a maximum at around 20 min after the washout, and then the reflex potentials reversed to control levels at around 100 min after the washout (Fig. 1B). The second 10 min application of pCA (3 μM) at 130 min after the washout resulted in a reproducible inhibition of the MSR (first application: 39.0±13.1% inhibition; second application: 39.0±16.3% inhibition; n=3,) and the sVRP (first

application: 40.7±4.2% inhibition; second application: 43.8±9.4% inhibition; n=3). The inhibition of MSR and sVRP occurred with pCA (1–10 μM) in a concentration-dependent manner (Fig. 1C).

An application of pCA (3–30 μM) for 10 min also released 5-HT from the isolated spinal cord in a concentration-dependent manner (Fig. 1D). The resting level of 5-HT release was undetectable, while the amount of 5-HT released by pCA (3 μM) reached a peak at around 20 min after the washout and then gradually declined. Higher concentrations of pCA (10 and 30 μM) released 5-HT in a more rapid fashion.

3.2. Effects of 5-HT receptor antagonists on the pCA-evoked inhibition and 5-HT release

The effects of 5-HT receptor antagonists on the pCA-evoked inhibition of MSR and sVRP were next investigated (Fig. 2). Although the extent of depression by pCA varied from spinal cord preparation to preparation, it was reproducible within the same preparation, as described above. Therefore, the MSR and sVRP recordings during the first 10 min exposure to pCA (3 μM) were used as the control response. The spinal cord was pretreated with antagonists at 1 μM, a commonly used effective concentration, for 30 min, and pCA was again applied for 10 min in the presence of the antagonists.

GR127935 (1 μM), a 5-$HT_{1B/1D}$ receptor antagonist, had no effect on the pCA (3

μM)-evoked maximum inhibition of the MSR (control: 52.7±10.5%; GR127935: 53.4±7.2%; n=5) or the sVRP (control: 45.1±7.9%; GR127935: 52.4±8.2%; n=4), although the MSR inhibition did not recover in the presence of GR127935 (Fig. 2A). Ketanserin (1 μM), a 5-HT_{2A} receptor antagonist, significantly decreased the pCA-evoked inhibition of the MSR (control: 70.0±10.5% vs. ketanserin: 7.6±3.9%; n=9, $P<0.01$) and the sVRP (control: 35.5±6.4% vs. ketanserin: 21.3±10.2%; n=9, $P<0.05$) (Fig. 2B). In addition, ketanserin reversed the sustained MSR inhibition by pCA in the presence of GR127935 (data not shown). Tropisetron (1 μM), a 5-HT_3 receptor antagonist, also attenuated the pCA-evoked inhibition of the MSR (control: 60.2±5.5% vs. tropisetron: 40.4±5.8%; n=8, $P<0.01$) and the sVRP (control: 45.2±3.4% vs. tropisetron: 28.8±5.5%; n=9, $P<0.05$) (Fig. 2C).

Then the release of 5-HT for 30 min was measured after treatment of the spinal cord with pCA (30 μM) for 10 min in the presence or absence of 5-HT receptor antagonists. As shown in Fig. 3, the pCA-evoked release of 5-HT was inhibited by tropisetron (1 μM), but not by GR127935 (1 μM) or ketanserin (1 μM). These results indicate that tropisetron attenuated the pCA-evoked inhibition of spinal neurotransmission by decreasing the amount of 5-HT release.

3.3. Effects of exogenous 5-HT on reflex potentials

To further investigate the contribution of 5-HT_{2A} and 5-HT_3 receptors to the inhibition of MSR and sVRP, the effects of exogenous 5-HT were examined in the presence of ketanserin (1 μM) or tropisetron (1 μM). In this experiment, 5-HT (3 nM–10 μM) was cumulatively applied to the isolated spinal cord. The applied 5-HT inhibited both reflex potentials (Fig. 4A) and depolarized the basal ventral root potentials (data not shown) in a concentration-dependent manner. As shown in Fig. 4B and C, the concentration-response curves for the 5-HT-evoked inhibition of MSR (IC_{50} = 1.77±0.57 μM; n=6) and sVRP (IC_{50} = 0.36±0.13 μM; n=6) were significantly ($P<0.01$, Dunnett's test) shifted to the right in the presence of ketanserin (MSR: IC_{50} = 12.7±2.08 μM; sVRP: IC_{50} = 4.23±0.83 μM; n=6), but not tropisetron (MSR: IC_{50} = 1.51±0.82 μM; sVRP: IC_{50} = 1.41±0.48 μM; n=5–6).

Next, 5-HT_2 and 5-HT_3 receptor agonists were cumulatively applied to the isolated spinal cord (Fig. 5). The 5-HT_2 receptor agonist α-Me-5-HT (0.1–10 μM) inhibited the MSR and sVRP in a concentration-dependent manner. Like 5-HT, α-Me-5-HT also depolarized the basal ventral root potentials (data not shown). On the other hand, SR 57227 (0.1–30 μM), a 5-HT_3 receptor agonist, enhanced the sVRP in a concentration-dependent manner. SR 57227 also enhanced the MSR at lower

concentrations, but at higher concentrations (>3 μM) the MSR was inhibited. Moreover, SR 57227 had no effect on the basal ventral root potentials. Brief applications (1 min) of 5-HT (1 μM) or α-Me-5-HT (1 μM) also depolarized the basal root potentials (5-HT: 0.92±0.17 mV; α-Me-5-HT: 0.83±0.24 mV; n=4), while SR 57227 (1 μM) had no such effect.

Because 5-HT depolarized the basal ventral root potentials, we then investigated the effects of 5-HT receptor antagonists on the 5-HT-induced depolarization. The repetitive application of 5-HT (3 μM) for 1 min at intervals of 20 min evoked reproducible depolarization at the ventral root (first application: 1.44±0.28 mV; second application: 1.39±0.29 mV; n=6). Next, 5-HT was applied to the isolated spinal cord in the presence of the 5-HT receptor antagonists. As shown in Fig. 6, the 5-HT-evoked depolarization was inhibited by ketanserin (1 μM), but not by tropisetron (1 μM) or GR127935 (1 μM).

3.4. Contribution of GABA and glycine on the pCA-evoked inhibition of reflex potentials

It has been reported that inhibitory amino acids such as GABA contribute to the effects of 5-HT on neuronal transmission in the spinal cord (Fukushima *et al.*, 2009; Song *et al.*, 2011). Thus we examined the contribution of endogenous inhibitory

transmitters to the pCA-evoked inhibition. The pretreatment of bicuculline (5 μM), a $GABA_A$ receptor antagonist, for 30 min markedly increased sVRP (295±36.3%, n=13) but not MSR (110.5±2.6%, n=13). As shown in Fig 7A, in the presence of bucuculline, the inhibitory effects of pCA (3 μM) on sVRP (control: 43.6±6.2% vs. bucuculline: 4.4±8.9%; n=13, $P<0.01$) but not MSR (control: 81.6±8.5% vs. bucuculline: 80.6±6.0%; n=13) were significantly reduced. The pretreatment of strychnine (1 μM), a glycine receptor antagonist, also increased sVRP (175.2±15.5%, n=9) but not MSR (115.5±4.9%, n=9). In the presence of strychnine (Fig. 7B), the inhibitory effects of pCA (3 μM) on sVRP (control: 22.5±6.2% vs. strychnine: 5.8±6.9%; n=9, $P<0.05$) but not MSR (control: 39.1±5.6 % vs. strychnine: 39.8±6.0%; n=13) were significantly reduced. Atipamezole (an α_2-adrenoceptor antagonist), naloxone (an opioid receptor antagonist) and atropine (a muscarinic receptor antagonist) had no effect on the pCA-evoked inhibition of either reflex potential (data not shown).

Then the release of GABA and glycine over a period of 30 min was measured before and after a 10 min pCA (30 μM) treatment. The release of GABA from the isolated spinal cord was significantly increased by pCA (Fig. 8A). The release of glycine also tended to be enhanced by pCA, but the effect was not significant ($P=0.077$) (Fig. 8C). The basal release of GABA and glycine was decreased by application of tropisetron (1

μM), but not by GR127935 (1 μM) or ketanserin (1 μM) (Fig. 8A and B). On the other hand, the pCA-evoked increase in GABA and glycine release was inhibited by ketanserin, but not by GR127935 and tropisetron (Fig. 8C and D).

4. Discussion

The present study demonstrated that A fiber-evoked MSR and C fiber-evoked sVRP were inhibited by both endogenously released and exogenously applied 5-HT. The amount of endogenous 5-HT released by pCA reached nanomolar concentrations in the incubation media. Therefore, the local concentrations of 5-HT in the spinal cord (e.g. 5-HT release site) are expected to be more than nanomolar levels, which are enough to inhibit reflex potentials. This result was in agreement with the effects of exogenous 5-HT, in which 5-HT at more than 10 nM inhibited reflex potentials. The inhibitory effects of 5-HT were reversed by ketanserin, a 5-HT_{2A} receptor antagonist. The results regarding sVRP blockade are somewhat inconsistent with a previous report, which showed that bath-applied 5-HT-evoked sVRP inhibition was insensitive to ketanserin (Hedo and Lopez-Garcia, 2002). The discrepancy between the previous report and the current study might be due to differences in experimental design. For example, in the previous report, ketanserin was only tested against a single, maximally inhibitory

concentration of 5-HT.

5-HT_{2A} receptors are $G_{q/11}$-coupled receptors that increase neuronal activity. Exogenously applied 5-HT has been shown to activate GABAergic and/or glycinergic interneurons via 5-HT_2 receptors in the CNS including the spinal cord (Abi-Saab et al., 1999; Lewis et al., 1993; Shen and Andrade, 1998). GABA and glycine are the major inhibitory neurotransmitters in the spinal cord, and thus, the inhibition of their release results in the marked enhancement of sVRP but not MSR (Akagi and Yanagisawa, 1987; Otsuguro et al., 2006). In the present study, the inhibitory effects of pCA on sVRP but not MSR were significantly reduced in the presence of antagonists for $GABA_A$ and glycine receptors. In addition, ketanserin blocked the pCA-evoked release of GABA/glycine from the spinal cord. Our results therefore suggest that the activation of 5-HT_{2A} receptors by endogenous 5-HT evokes the release of GABA and glycine from inhibitory interneurons, thereby inhibiting sVRP. Furthermore, ketanserin also blocked 5-HT and α-Me-5-HT (a 5-HT_2 receptor agonist)-evoked depolarization of the basal ventral root, suggesting that these excitatory effects on motoneurons were also mediated via 5-HT_{2A} receptors. The depolarization of motoneurons may, at least in part, contribute to the inhibitory effects of 5-HT because marked depolarization in the spinal networks can inhibit signal transduction (Hochman et al., 2010).

Tropisetron, a 5-HT_3 receptor antagonist, reversed the inhibition of reflex potentials by pCA but not by bath-applied 5-HT. In addition, tropisetron decreased pCA-evoked 5-HT release. 5-HT_3 receptors are ligand-gated cation channels, the activation of which can excite neurons and release their neurotransmitters. We suggest that 5-HT_3 receptors serve as autoreceptors to enhance 5-HT release through a positive feedback process, inhibiting reflex potentials via stimulation of 5-HT_2 receptors and subsequent GABA/glycine release. This may explain why tropisetron does not influence the actions of bath-applied 5-HT.

The detection of 5-HT_3 receptor mRNA in the brain stem, from which descending serotonergic fibers originate (Fonseca et al., 2001), supports our hypothesis that 5-HT_3 receptors exist in the nerve terminals of descending fibers in the spinal cord. Furthermore, tropisetron decreased the basal release of GABA/glycine without inhibiting the GABA/glycine release by pCA. These results indicate that, unlike the pCA-evoked release of GABA/glycine, the basal release of GABA/glycine is modulated by 5-HT_3 rather than 5-HT_{2A} receptors.

Interestingly, SR 57227, a 5-HT_3 receptor agonist, enhanced the reflex potentials. Similar controversial results were also reported for pain sensation, i.e., 5-HT_3 receptors were shown to mediate both pronociceptive (Ali et al., 1996; Oyama et

al., 1996) and antinociceptive effects in the spinal cord (Alhaider et al., 1991; Bardin et al., 2000; Giordano, 1997). 5-HT_3 receptors are also expressed in the terminals of afferent C fibers in the dorsal horn (Hamon et al., 1989; Kidd et al., 1993; Morales et al., 2001). It is likely that bath-applied 5-HT and its analogues affect 5-HT_3 receptors in different regions from those affected by endogenously released 5-HT, which may be a crucial factor behind the conflicting reports on the facilitatory and inhibitory effects of 5-HT in the spinal cord.

As previously reported (Kawamoto et al., 2011; Wallis et al., 1993a, 1993b; Yomono et al., 1992), endogenous 5-HT inhibited MSR in the neonatal rat spinal cord, and the inhibition was reversed by ketanserin. Ketanserin failed to overturn MSR inhibition by bath-applied 5-HT in several previous studies (Crick and Wallis, 1991; Elliott and Wallis, 1992; Manuel et al., 1995), but in our study, MRS inhibition in response to bath-applied 5-HT was also reversed by ketanserin. 5-$HT_{1D\text{-like}}$ receptors rather than 5-HT_{2A} receptors have been proposed to mediate ketanserin-sensitive MSR inhibition (Manuel et al., 1995). Interestingly, pCA provoked a particularly long blockade of MSR in the presence of GR 127935 (a 5-$HT_{1B/D}$ receptor antagonist), which was reversed by ketanserin. This suggests that GR 127935 has an agonistic effect on 5-HT_{1D} receptors (Manuel et al., 1995; Pauwels et al., 1996). Therefore, in this study, we

have not examined the effect of GR 127935 at higher concentrations.

On the other hand, 5-HT appears to inhibit MSR at least in part via 5-HT_{2A} receptors, as assessed by the blocking actions of α-Me-5-HT, a 5-HT_2 receptor agonist. Moreover, the depolarizing response to 5-HT was inhibited by ketanserin. Therefore, it seems likely that depolarization readily blocks monosynaptic transmission. In addition, our results indicate that 5-HT_3 receptors also contribute to pCA-evoked MSR inhibition. Thus, 5-HT released via a 5-HT_3 receptor-mediated positive feedback loop may modulate not only the sVRP pathway, but also the MSR pathway. MSR was reported to be inhibited via the activation of 5-HT_{1A} receptors (Crick et al., 1994; Hedo et al., 2002). Thus 5-HT_{1A} receptors might be also involved in the MSR inhibition in the present study. In addition, 5-HT_7 receptors have been reported to have both pronociceptive (Rocha-González et al., 2005) and antinociceptive effects (Dogrul et al., 2009) in the spinal cord. Further experimentation will be required to fully characterize the 5-HT receptor subtypes that are responsible for spinal modulation.

5. Conclusions

We have demonstrated that excitatory 5-HT_{2A} and 5-HT_3 receptors contribute to the endogenous 5-HT-evoked inhibition of spinal reflex potentials in neonatal rats.

The activation of 5-HT_{2A} receptors elicits the release of GABA/glycine, which in turn impairs C fiber-evoked synaptic transmission. Moreover, 5-HT_3 receptors apparently act as autoreceptors to provide feedback facilitation of 5-HT release.

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FIGURE LEGENDS

Fig. 1. Effects of pCA on spinal reflex responses and 5-HT release. (A) The isolated spinal cord was exposed to pCA (3 μM) for 10 min. Representative traces of MSR and sVRP before (control), 30 min (pCA 3 μM), and 100 min (wash) after the application of pCA. (B) Time course of the effects of pCA (3 μM) on MSR and sVRP (n=39). (C) The pCA (1–10 μM)-evoked MSR and sVRP inhibitions are summarized (n=4–39). (D) Time course of 5-HT release by pCA (3–30 μM) (n=3–9). *N.D.*: not detected.

Fig. 2. Effects of 5-HT receptor antagonists on the pCA-evoked inhibition of MSR and sVRP. pCA (3 μM) was repeatedly applied for 10 min at intervals of 2 h. GR 127935 (1 μM, A), ketanserin (1 μM, B) or tropisetron (1 μM, C) were applied for 130 min beginning at 30 min before the second application of pCA (n=4–9).

Fig. 3. Effects of 5-HT receptor antagonists on the pCA-evoked 5-HT release. pCA (30 μM) was applied for 10 min in the absence (control) or presence of GR 127935 (1 μM), ketanserin (1 μM), or tropisetron (1 μM). The isolated spinal cord was pretreated with each 5-HT receptor antagonist beginning at 30 min before the application of pCA (n=5–

9). **P<0.01 vs. control.

Fig. 4. Effects of 5-HT on spinal reflex responses. (A) 5-HT (3 nM–10 μM) was cumulatively applied to the isolated spinal cord. Concentration-response curves are shown for MSR (B) and sVRP (C) in the absence (control) or presence of ketanserin (1 μM) or tropisetron (1 μM) (n=6).

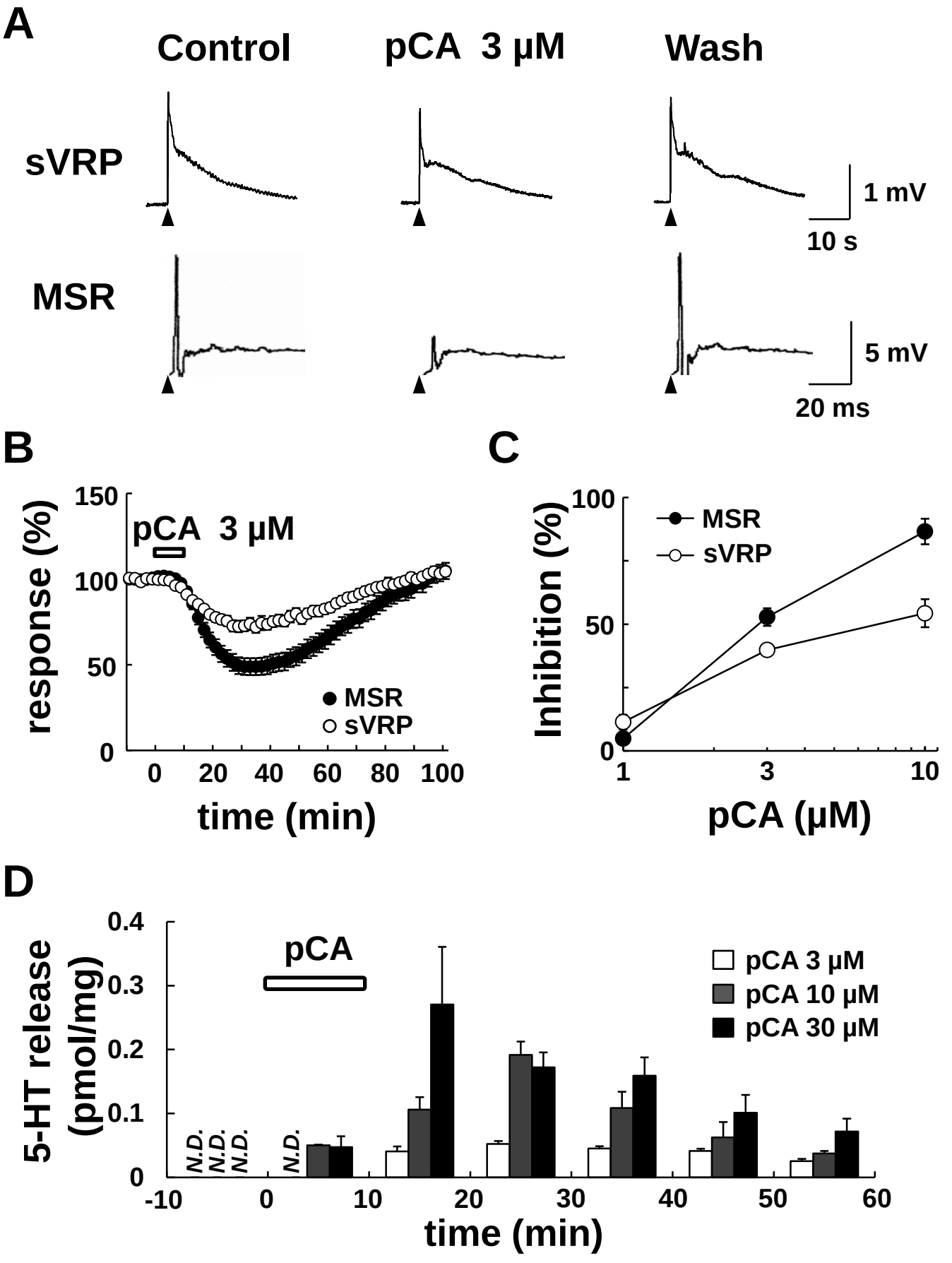
Fig. 5. Effects of 5-HT receptor agonists on spinal reflex responses. α-Me-5-HT (0.1–10 μM) and SR 57227 (30 nM–30 μM) were cumulatively applied to the isolated spinal cord. Concentration-response curves are shown for MSR (A) and sVRP (B) (n=4). The concentration-response curves for 5-HT utilized the same data as employed in Fig. 3.

Fig. 6. Depolarizing responses to 5-HT in the isolated spinal cord. (A) Representative traces of the 5-HT (3 μM)-evoked depolarization in the absence (control) or presence of GR127935 (1 μM), tropisetron (1 μM) or ketanserin (1 μM). 5-HT was repetitively applied for 1 min at intervals of at least 20 min following pretreatment of the spinal cord with each antagonist for 20 min. (B) Summary of the effects of 5-HT receptor antagonists on 5-HT-evoked depolarization. The amplitude of each depolarizing

response was normalized to the response to the first application of 5-HT (n=4–6). **P<0.01 vs. control.

Fig. 7. Effects of antagonists for $GABA_A$ and glycine receptors on the pCA-evoked inhibition of MSR and sVRP. pCA (3 μM) was repeatedly applied for 10 min at intervals of 2 h. Bicuculline (5 μM, A) or strychnine (1 μM, B) were applied for 130 min beginning at 30 min before the second application of pCA (n=6–8).

Fig. 8. Effects of 5-HT receptor antagonists on GABA and glycine release by pCA. pCA (30 μM) was applied for 10 min in the absence (control) or presence of GR 127935 (1 μM), ketanserin (1 μM), or tropisetron (1 μM). The isolated spinal cord was pretreated with 5-HT receptor antagonists beginning at 30 min before the application of pCA. The summarized data of GABA and glycine release before (open column) and after (filled column) application of pCA (A and C) and the extent of the pCA-evoked increase (Δ GABA, Δ glycine) (B and D) are shown (n=5–8). *P<0.05, **P<0.01 vs. before the application of pCA; #P<0.01, ##P<0.01 vs. control.


A
Control
pCA 3 μM
Wash
sVRP
MSR
1 mV
10 s
5 mV
20 ms
B
pCA 3 μM
response (%)
150
100
50
0
0
20
40
60
80
100
time (min)
MSR
sVRP
C
Inhibition (%)
100
50
0
MSR
sVRP
1
3
10
pCA (μM)
D
5-HT release (pmol/mg)
0.4
0.3
0.2
0.1
0
pCA
pCA 3 μM
pCA 10 μM
pCA 30 μM
N.D.
N.D.
N.D.
N.D.
-10
0
10
20
30
40
50
60
time (min)

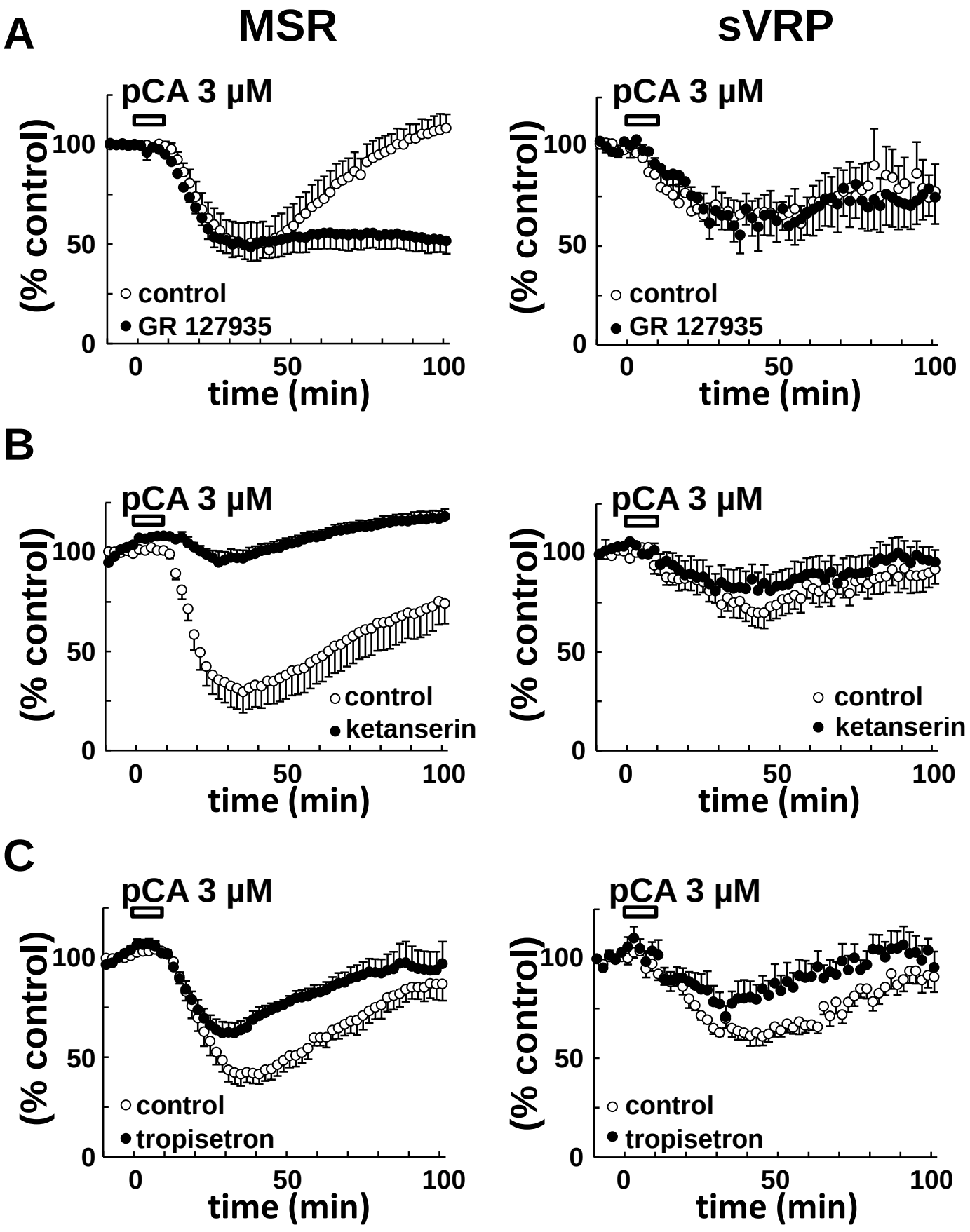
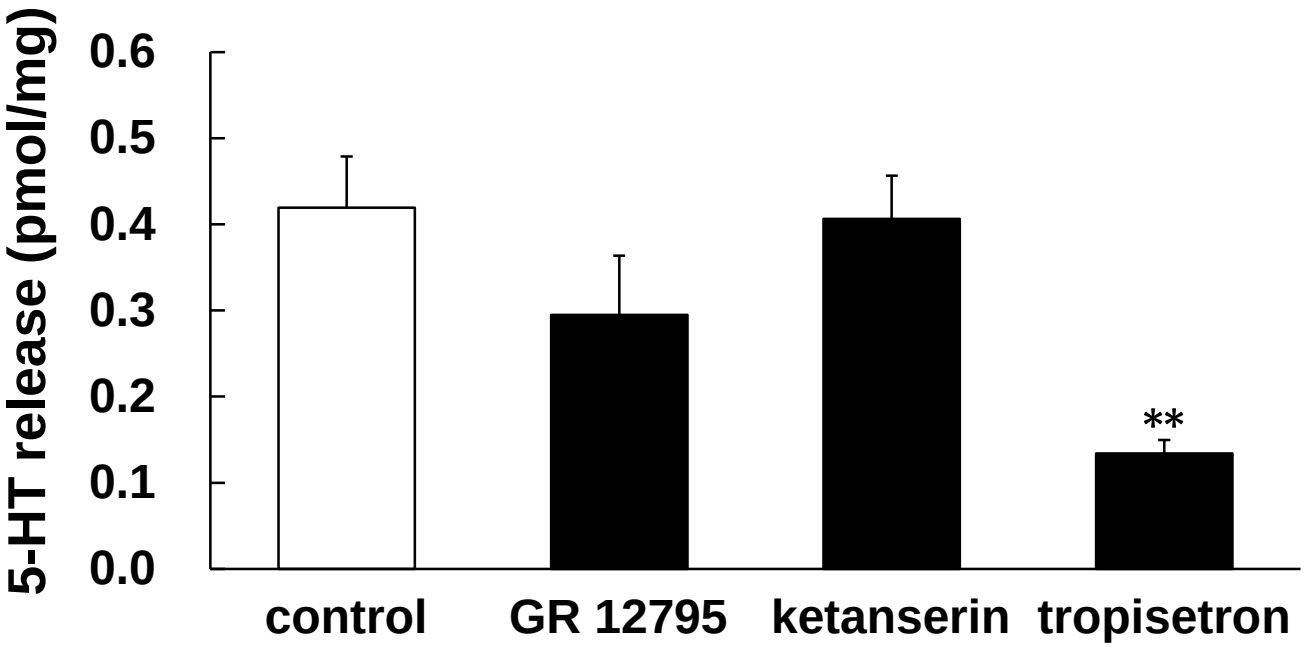

A
MSR
sVRP
pCA 3 μM
(% control)
100
50
0
control
GR 127935
0
50
100
time (min)
B
ketanserin
C
tropisetron

Fig. 3


5-HT release (pmol/mg)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
**
control
GR 12795
ketanserin
tropisetron

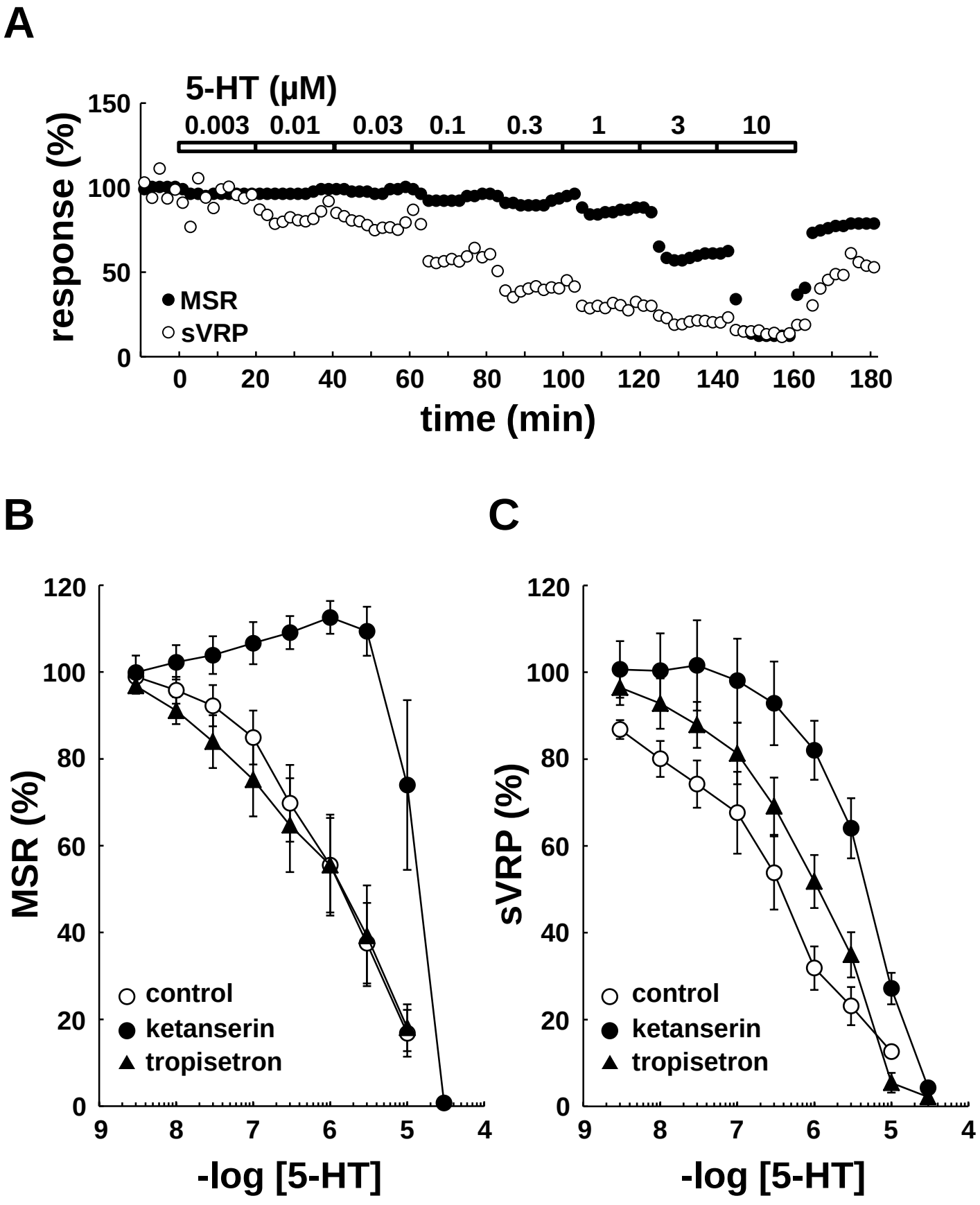
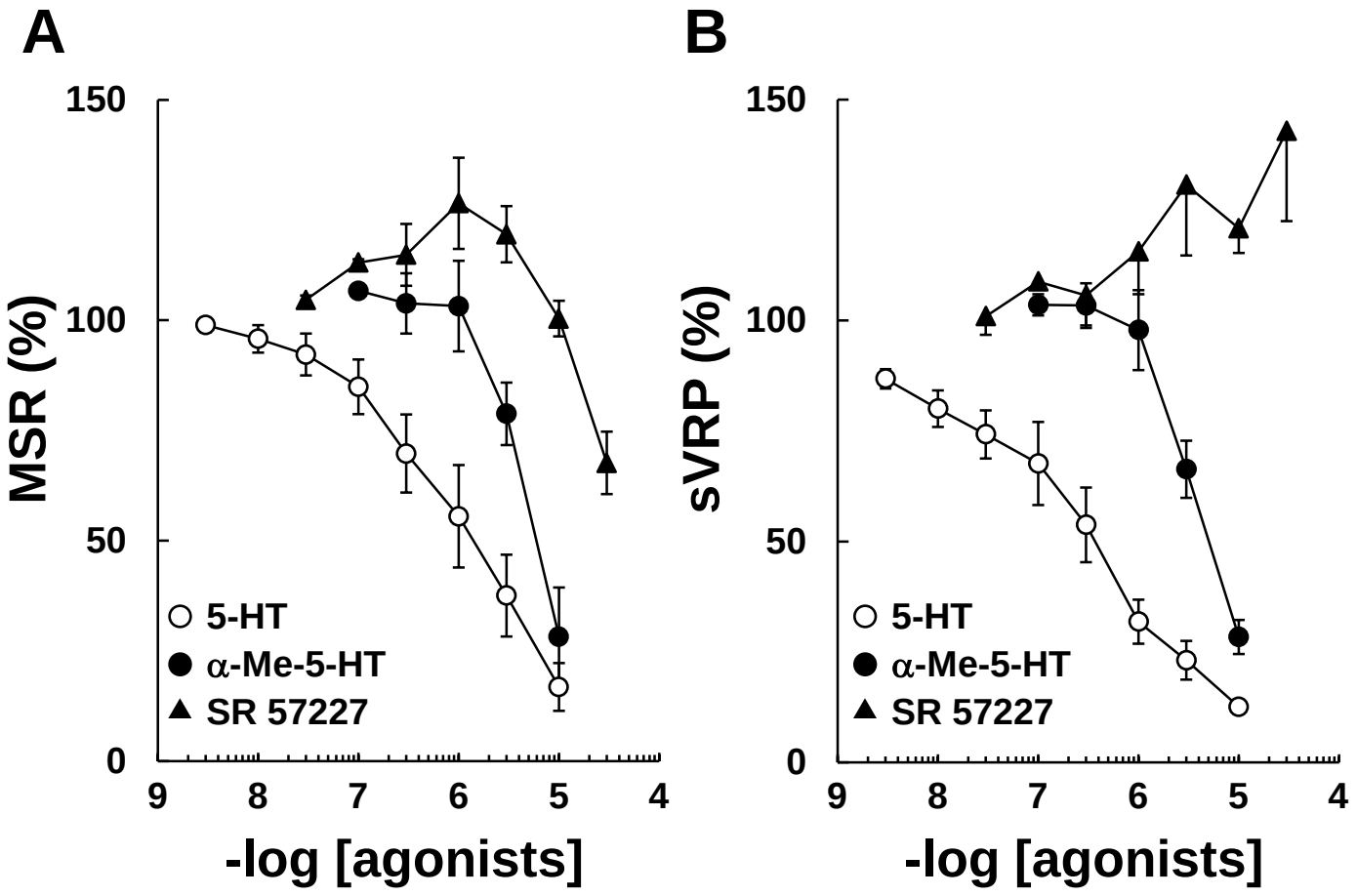
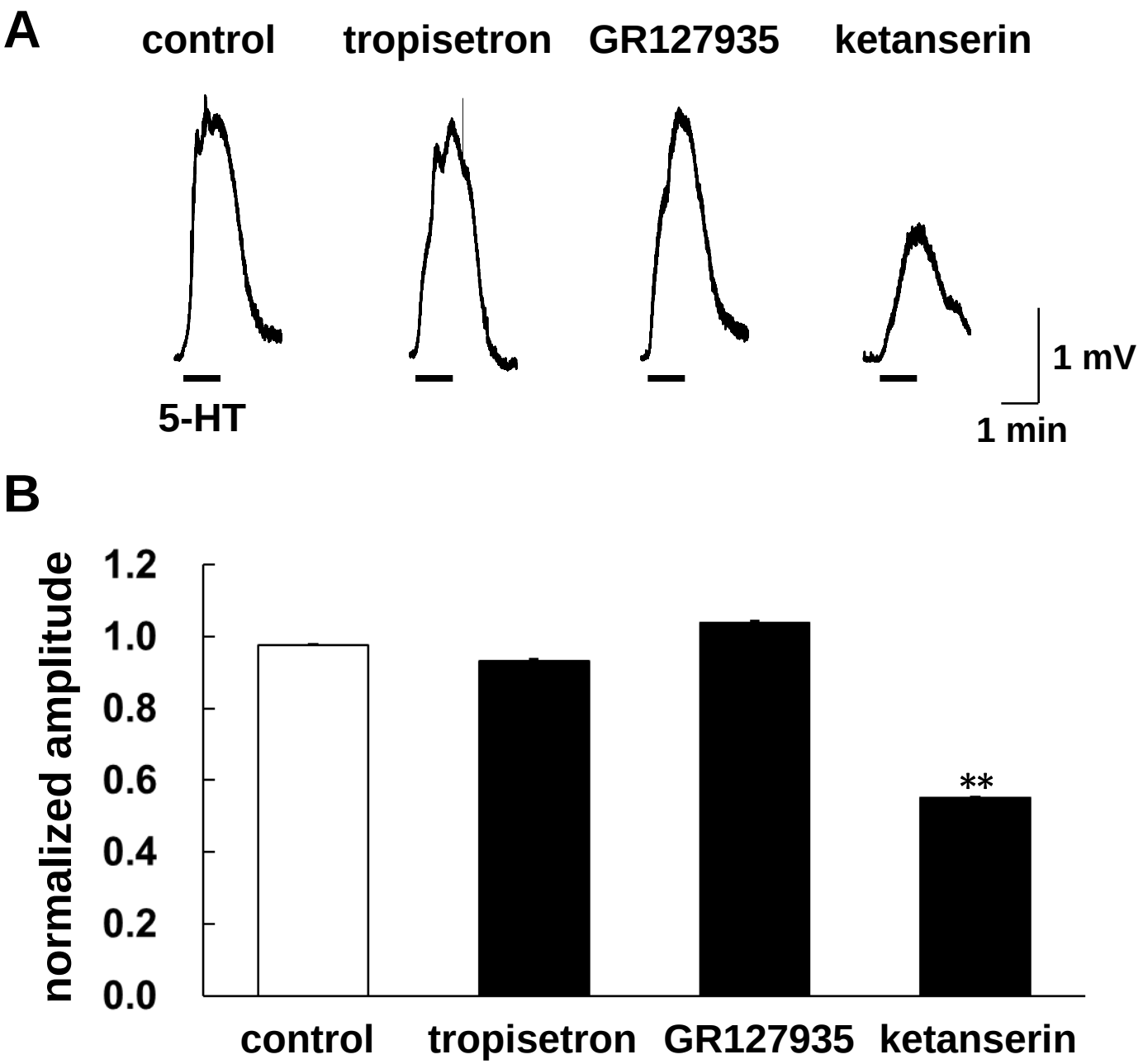
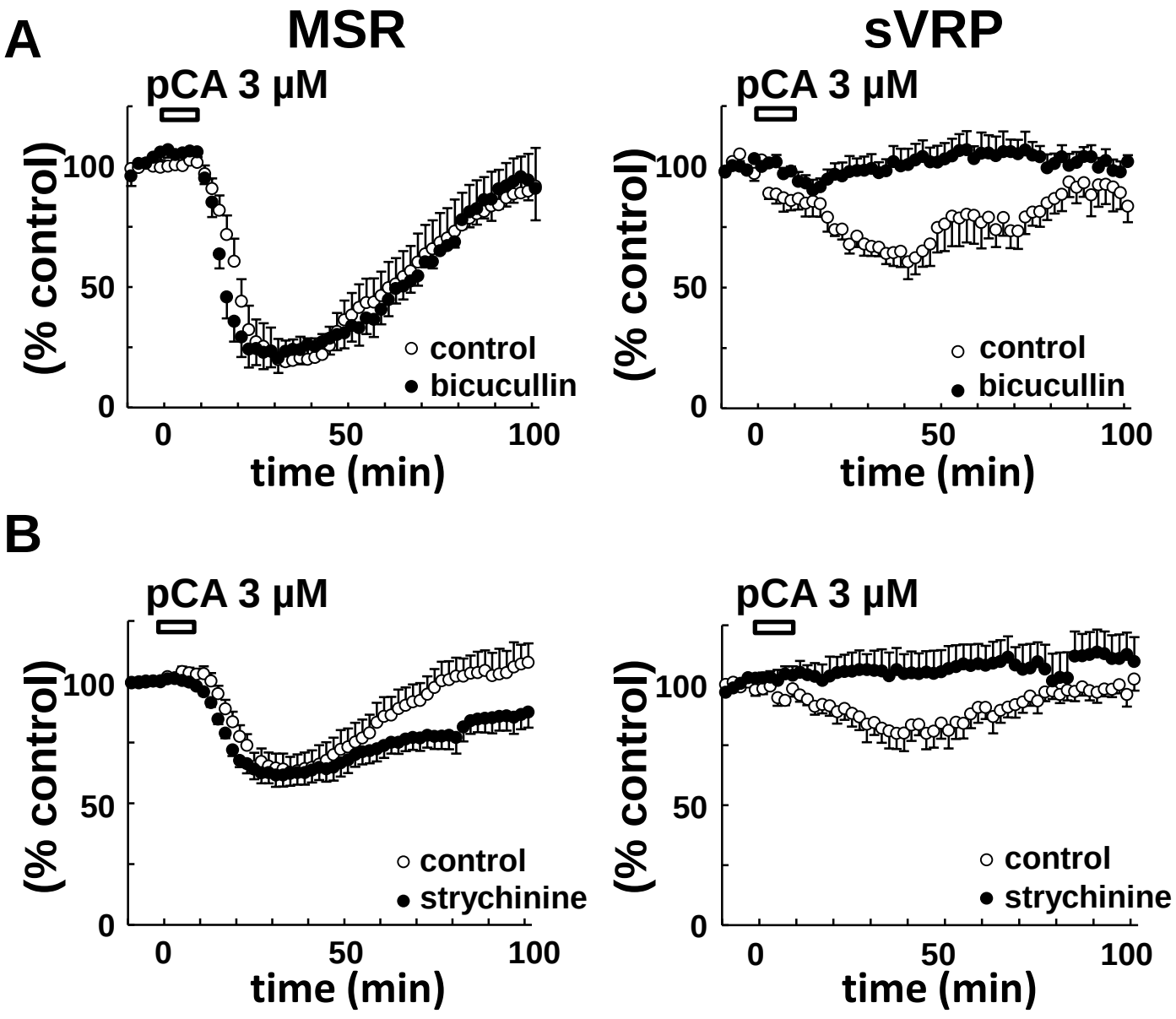
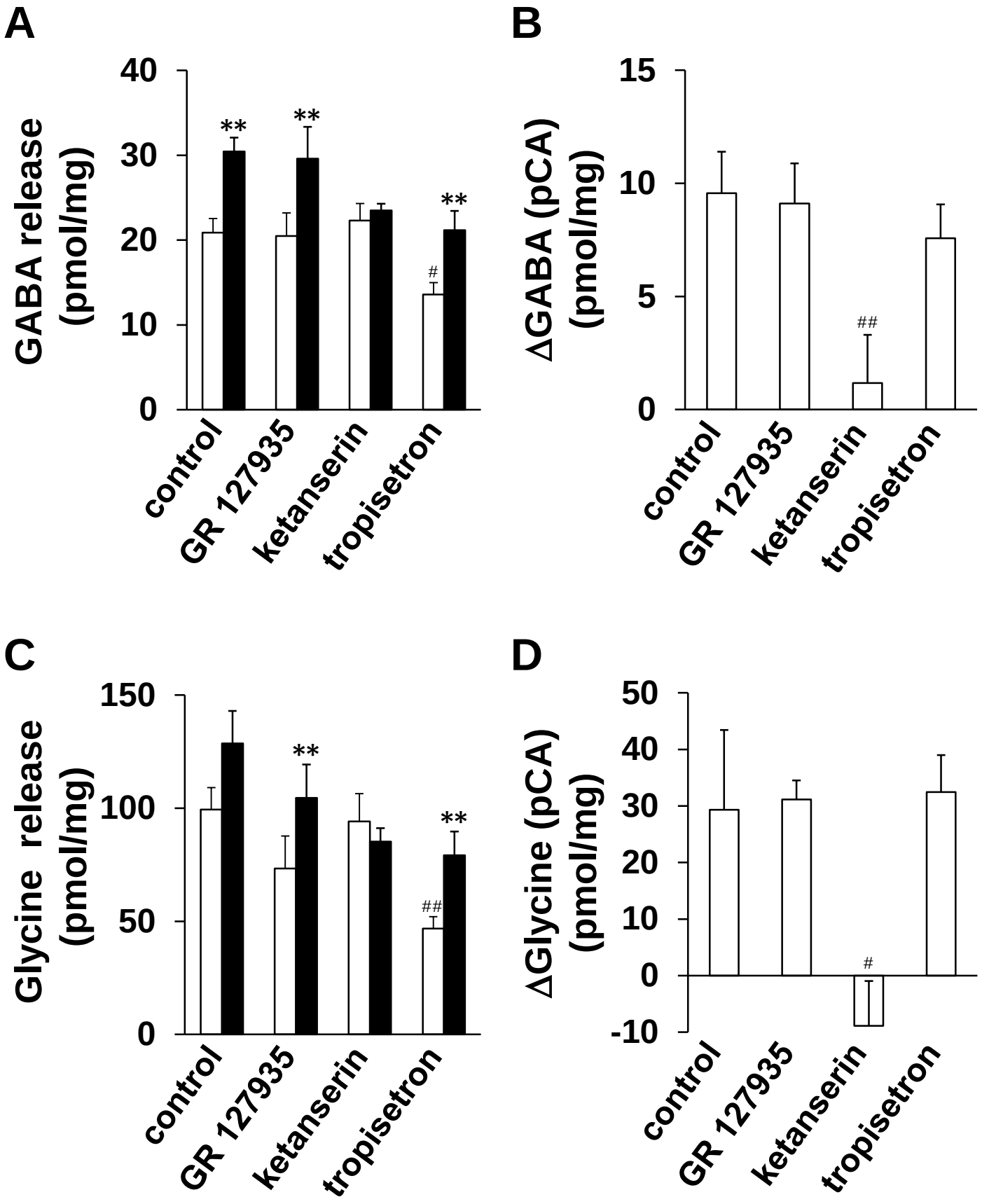

A
5-HT (μM)
0.003
0.01
0.03
0.1
0.3
1
3
10
response (%)
150
100
50
0
MSR
sVRP
0
20
40
60
80
100
120
140
160
180
time (min)
B
MSR (%)
120
100
80
60
40
20
0
control
ketanserin
tropisetron
9
8
7
6
5
4
-log [5-HT]
C
sVRP (%)
120
100
80
60
40
20
0
control
ketanserin
tropisetron
9
8
7
6
5
4
-log [5-HT]

Fig. 5


A
B
MSR (%)
sVRP (%)
150
100
50
0
9
8
7
6
5
4
-log [agonists]
5-HT
α-Me-5-HT
SR 57227


A
control
tropisetron
GR127935
ketanserin
1 mV
5-HT
1 min
B
normalized amplitude
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
control
tropisetron
GR127935
ketanserin


A
MSR
sVRP
pCA 3 μM
pCA 3 μM
(% control)
100
50
0
control
bicucullin
0
50
100
time (min)
B
pCA 3 μM
pCA 3 μM
control
strychinine


A
GABA release (pmol/mg)
40
30
20
10
0
**
**
**
#
control
GR 127935
ketanserin
tropisetron
B
ΔGABA (pCA) (pmol/mg)
15
10
5
0
##
control
GR 127935
ketanserin
tropisetron
C
Glycine release (pmol/mg)
150
100
50
0
**
**
##
control
GR 127935
ketanserin
tropisetron
D
ΔGlycine (pCA) (pmol/mg)
50
40
30
20
10
0
-10
#
control
GR 127935
ketanserin
tropisetron