RESEARCH PAPER

Inhibitory effects of dopamine on spinal synaptic transmission via dopamine D₁-like receptors in neonatal rats

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BACKGROUND AND PURPOSE

Dopamine released from the endings of descending dopaminergic nerve fibres in the spinal cord may be involved in modulating functions such as locomotion and nociception. Here, we examined the effects of dopamine on spinal synaptic transmissions in rats.

EXPERIMENTAL APPROACH

Spinal reflex potentials, monosynaptic reflex potential (MSR) and slow ventral root potential (sVRP), were measured in the isolated spinal cord of the neonatal rat. Dopamine release was measured by HPLC.

KEY RESULTS

Dopamine at lower concentrations (<1 µM) depressed sVRP, which is a C fibre-evoked polysynaptic response and believed to reflect nociceptive transmission. At higher concentrations (>1 µM), in addition to a potent sVRP depression, dopamine depolarized baseline potential and slightly depressed MSR. Depression of sVRP by dopamine was partially reversed by dopamine D₁-like but not by D₂-like receptor antagonists. SKF83959 and SKF81297, D₁-like receptor agonists, and methamphetamine, an endogenous dopamine releaser, also caused the inhibition of sVRP. Methamphetamine also depressed MSR, which was inhibited by ketanserin, a 5-HT₂ₐ/₂c receptor antagonist. Methamphetamine induced the release of dopamine and 5-HT from spinal cords, indicating that the release of endogenous dopamine and 5-HT depresses sVRP and MSR respectively.

CONCLUSION AND IMPLICATIONS

These results suggested that dopamine at lower concentrations preferentially inhibited sVRP, which is mediated via dopamine D₁-like and other unidentified receptors. The dopamine-evoked depression is involved in modulating the spinal functions by the descending dopaminergic pathways.

Abbreviations

ACSF, artificial cerebrospinal fluid; DRG, dorsal root ganglion; MSR, monosynaptic reflex potential; sVRP, slow ventral root potential

Introduction

Dopamine is a neurotransmitter in the CNS. Dopamine receptors are classified into five subtypes, referred to as either D₁-like (D₁ and D₅) or D₂-like (D₂, D₃ and D₄) receptors (Missale *et al.*, 1998; receptor nomenclature follows

Alexander *et al.* 2011). Dopamine concentration is a key factor in the activation of different receptor subtypes. In the prefrontal cortex, low concentrations of dopamine act on D₁-like receptors, while higher concentrations act on D₂-like receptors (Zheng *et al.*, 1999; Trantham-Davidson *et al.*, 2004).

In the spinal cord, the descending dopaminergic fibre projects from the hypothalamic A11 region (Björklund and Skagerberg, 1979; Skagerberg and Lindvall, 1985; Millan, 2002; Benarroch, 2008), and dopamine can modulate locomotion and nociception (Clemens and Hochman, 2004; Han et al., 2007; Lapointe et al., 2009). In the rat spinal cord, dopamine at concentrations greater than 10 µM activates K^+ channels, producing hyperpolarization via D_2-like receptors, but not D_1-like receptors in substantia gelatinosa neurones, which are located in the superficial laminae of the dorsal horn receiving nociceptive inputs (Tamae et al., 2005; Taniguchi et al., 2011). D_1-like receptors are also expressed in the spinal cord (Levant and McCarson, 2001; Zhu et al., 2007). Although it has been reported that dopamine increases AMPA currents via D_1-like receptors in the mouse motoneurones (Han and Whelan, 2009), the role of D_1-like receptors in afferent transmission remains unclear.

The spinal cord isolated from neonatal rats is a widely used preparation for investigating spinal functions and drug actions. Electrical stimulation of the dorsal root elicits a monosynaptic reflex potential (MSR) followed by a slow ventral root potential (sVRP) at the corresponding ventral root. The MSR is a fibre-evoked response mainly mediated by non-NMDA receptors. On the other hand, sVRP is a C fibre-evoked polysynaptic response mediated by NMDA and various metabolic receptors such as tachykinin NK receptors in the spinal cord (Akagi et al., 1985; Nussbaumer et al., 1989; Brockmeyer and Kendig, 1995; Faber et al., 1997; Kocsis et al., 2003). The sVRP is believed to reflect nociceptive transmission in the spinal cord based on its electrophysiological and pharmacological features (Akagi and Yanagisawa, 1987; Nussbaumer et al., 1989; Woodley and Kendig, 1991; Faber et al., 1997; Otsuguro et al., 2005). Our laboratory previously reported that dopamine at concentrations of >1 µM depolarized the ventral root and suppressed the MSR (Kitazawa et al., 1985). In the mouse spinal cord, dopamine depresses MSR via D_2-like receptors (Clemens and Hochman, 2004). In contrast, there is no information on the effects of dopamine on sVRP. In the current study, the effects of dopamine on spinal cord isolated from the neonatal rat were examined. The results have demonstrated that dopamine at lower concentrations, as well as endogenous dopamine, preferentially depresses sVRP via D_1-like receptors.

Methods

Spinal cord preparation

All animal care and experimental protocols were approved by the Committee on Animal Experimentation, Graduate School of Veterinary Medicine, Hokkaido University. Every effort was made to minimize animal suffering and to reduce the number of animals used. Wistar rats (0–5 days old) of either sex were used.

Neonatal rats were killed by decapitation and the spinal cords isolated. Isolated spinal cord preparations were prepared as previously described (Otsuguro et al., 2006; 2011). The hemisected spinal cord from the lower thoracic through sacral regions was superfused in a recording chamber of 0.5 mL volume with artificial cerebrospinal fluid (ACSF) at a

flow rate of approximately 2.5 mL·min^{-1}. The temperature of the bath was monitored before and after each recording using a thermometer (CT-1200D, Custom, Tokyo, Japan) and was maintained at 27 ± 2°C. The composition of the ACSF was as follows (mM): NaCl 138; NaHCO$_3$ 21; NaH$_2$PO$_4$ 0.6; KCl 3.5; CaCl$_2$ 1.25; MgCl$_2$ 1.2; glucose 10; gassed with 95% O$_2$ and 5% CO$_2$; pH ~ 7.3.

Electrophysiological measurement

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, and the magnitudes of each were expressed as peak amplitude (mV) and depolarization integral (mV·s) over the resting potential of the ventral root respectively (Figure 1A). The preparation was allowed to equilibrate for 1 h before recordings. In most of the experiments, the inhibitory effects of dopamine, SKF83959, SKF81297 and methamphetamine on spinal reflex potentials were evaluated by measuring the mean of three responses around their maximal effects and the data were expressed as a percentage of the mean of three responses just before application. The time course of the magnitude of the MSR and sVRP was expressed as a percentage of the mean of the first five responses. Electrical responses were detected by a high gain amplifier (MEZ-8300, Nihon Kohden, Tokyo, Japan) with low-pass filter at 10 kHz. MSR was recorded using a thermal arraycorder (WR7900, Graftec, Yokohama, Japan) with a sampling time of 80 µs. sVRP was digitized by an analogue/digital converter (Power-Lab, ADInstruments, Castle Hill, Australia) with a sampling time of 10 ms. Data were stored in a personal computer and analysed with LabChart 6 software (ver. 6.0, ADInstruments).

RT-PCR

The oligonucleotide primers used for amplifying dopamine receptor subtype gene sequences (GenBank accession number) and its expected product size were as follows: D_1 (M35077) forward: 5′-CAGTCCATGCCAAGAATTGCC-3′ and reverse: 5′- AATCGATGCAGAATGGCTGGG-3′ (225 bp); D_2 (D2S: M36831, D2L: X53278) forward: 5′-GCAGTCGAGC TTTCAGAGCC-3′ and reverse: 5′-TCTGCGGCTCATCGT CTTAGG-3′ (317 and 404 bp, respectively); D_3 (X53944) forward: 5′- TCCTGTCTGAGGCTGCATCC-3′ and reverse: 5′-TCGAAGTGGTACTCCCCGAG-3′ (381 bp); D_4 (M84009) forward: 5′-GATGTGTTGGACGCCTTTTCT-3 and reverse: 5′-TCGGCATTGAAGATGGTGTA-3′ (150 bp); D_5 (NM_012768) forward: 5′-ACCAAGACACGGTCTTCCAC-3′ and reverse: 5′-CACAGTCAAGCTCCCAGACA-3′ (189 bp); β-actin (V01217) forward: 5′-TGTCACCAACTGGGACGATA-3′ and reverse: 5′-ACCCTCATAGATGGGCACAG-3′ (280 bp). Total RNA was extracted from the lumber region of the spinal cord and its dorsal root ganglion (DRG) using TRI Reagent (Sigma-Aldrich, Saint Louis, MO, USA) and then treated with DNase I (Invitrogen, Carlsbad, CA, USA). First strand cDNA synthesis and subsequent amplification were performed using a Prime-Script One Step RT-PCR Kit (ver. 2, Takara Bio, Otsu, Japan). PCR reactions were preceded by incubation at 94°C for 2 min and consisted of 94°C for 30 s, followed by 57°C (D2, D5 and β-action), 60°C (D4) or 65°C (D1, D3) for 30 s, and 72°C for
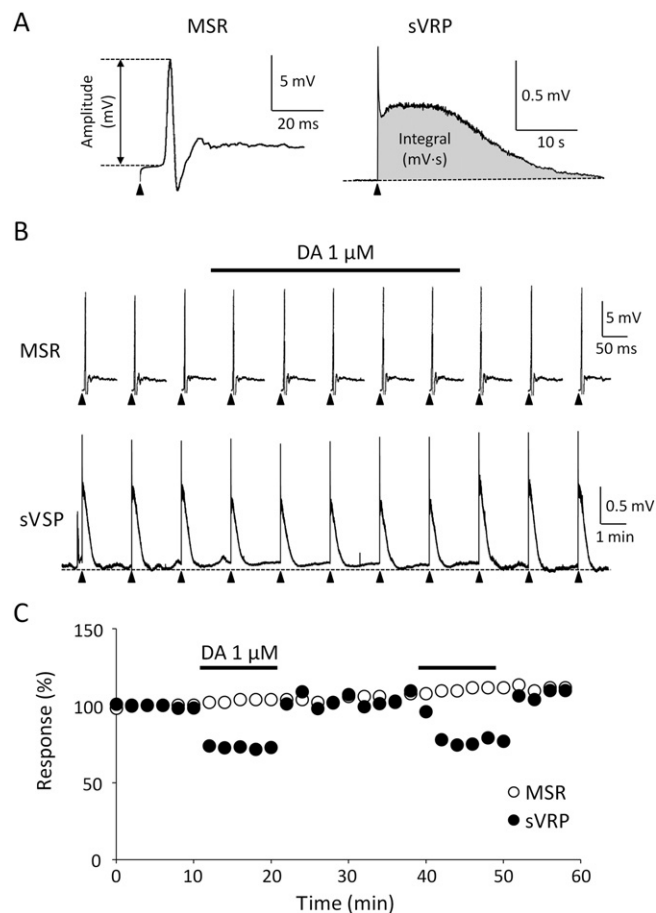
Figure 1

Effect of dopamine (DA) on spinal reflex potentials in spinal cords from neonatal rats. (A) Representative traces of reflex potentials evoked by electrical stimulation (arrowhead). The magnitude of MSR and sVRP was measured as the peak amplitude (mV) and the integral of depolarization (mV·s) over the resting potential respectively. (B) Representative traces of MSR (upper panel) and sVRP (lower panel) evoked by electrical stimulation every 2 min (arrowheads). DA (1 μM) was applied for 10 min. Dotted line indicates the baseline ventral root potential. (C) Depression of sVRP but not MSR by repeated application of dopamine (1 μM).

60 s for 30 (D1, D2, D5 and β-actin), 34 (D3) and 36 (D4) cycles. Amplified products were separated and analysed by 2.0% agarose gel electrophoresis containing ethidium bromide and visualized under UV light.

Measurement of dopamine and 5-HT concentration

The concentrations of dopamine and 5-HT were determined according to the method of Ito *et al.* (2001) with some modifications. Rat spinal cord from the lower thoracic through lumber regions was isolated from five littermates. After removal of all roots and DRGs, the spinal cord was sliced into several pieces and then they were equilibrated in ACSF for 1 h at 35°C. After incubation for 30 min with fresh ACSF, the tissues were treated for 10 min with methamphetamine (30 μM) and then incubated for an additional 30 min with fresh ACSF. Incubation media from before and after treatment with methamphetamine was stored on ice for the measurement of 5-HT. For the measurement of dopamine, the sample solution was treated with alumina to purify and concentrate the dopamine (Anton and Sayre, 1962). Isoprenaline (1 μM) was used as an internal standard.

The samples were applied to an HPLC system with an ODS column (EICOMPAK SC-5ODC, 3.0 × 150 mm, EICOM, Kyoto, Japan) equipped with an electrochemical detector (ECD-300, EICOM). The mobile phase consisted of 100 mM citrate buffer (pH 3.5), 19% methanol, 5 mg·L^{-1} EDTA Na$_2$ and 190 mg·L^{-1} sodium octasulfonic acid. The flow rate was 0.5 mL·min^{-1}. The amounts of dopamine and 5-HT were expressed relative to tissue wet weight (fmol·mg^{-1}).

Data analysis

Results were expressed as means ± SEM. Statistical comparisons between two groups were performed by paired or unpaired Student's *t*-test. A *P*-value of less than 0.05 was considered significant.

Materials

Haloperidol was purchased from Pfizer Japan (Tokyo, Japan). (S),9(R)-(-)-bicuculline methobromide and 5-HT creatinine sulfate were from Sigma-Aldrich. Dopamine (3-hydroxy tyramine hydrochloride) was from Tokyo Chemical (Tokyo, Japan). dl- Isoprenaline hydrochloride and strychnine sulfate were from Wako Pure Chemical (Osaka, Japan). Ketanserin tartrate, LE300, raclopride, SCH23390 hydrochloride, SKF81297 hydrobromide and SKF83959 hydrobromide were from Tocris Bioscience (Bristol, UK). Methamphetamine hydrochloride was from Dainippon Sumitomo Pharma (Osaka, Japan). Atipamezole hydrochloride was supplied from Orion (Espoo, Finland). Naloxone hydrochloride was from Daiichi Sankyo (Tokyo, Japan).

Results

Effects of dopamine on reflex potentials in rat spinal cord

The effects of dopamine on spinal reflex potentials evoked by electrical stimulation were measured every 2 min. Bath application of dopamine (1 μM) rapidly suppressed the sVRP without any effect on MSR (Figure 1B). This inhibitory effect of dopamine was often accompanied by suppression of spontaneous activity without changes in baseline ventral root potential. The dopamine-evoked depression of sVRP was maintained during application for 10 min followed by immediate recovery after washout of the drug. As shown in Figure 1C, repeated application of dopamine inhibited sVRP to the same extent (1st: 64.5 ± 3.9%, n = 6; 2nd: 64.5 ± 3.7%, n = 6).

The application of dopamine (0.01–1 μM) inhibited sVRP but not MSR, in a concentration-dependent manner without any effect on baseline potential (Figure 2). As previously reported (Kitazawa *et al.*, 1985), at a higher concentration (3 μM), dopamine slightly suppressed MSR and depolarized the baseline ventral root potential, and this was accompanied by increases in spontaneous activity (Figure 2B). The
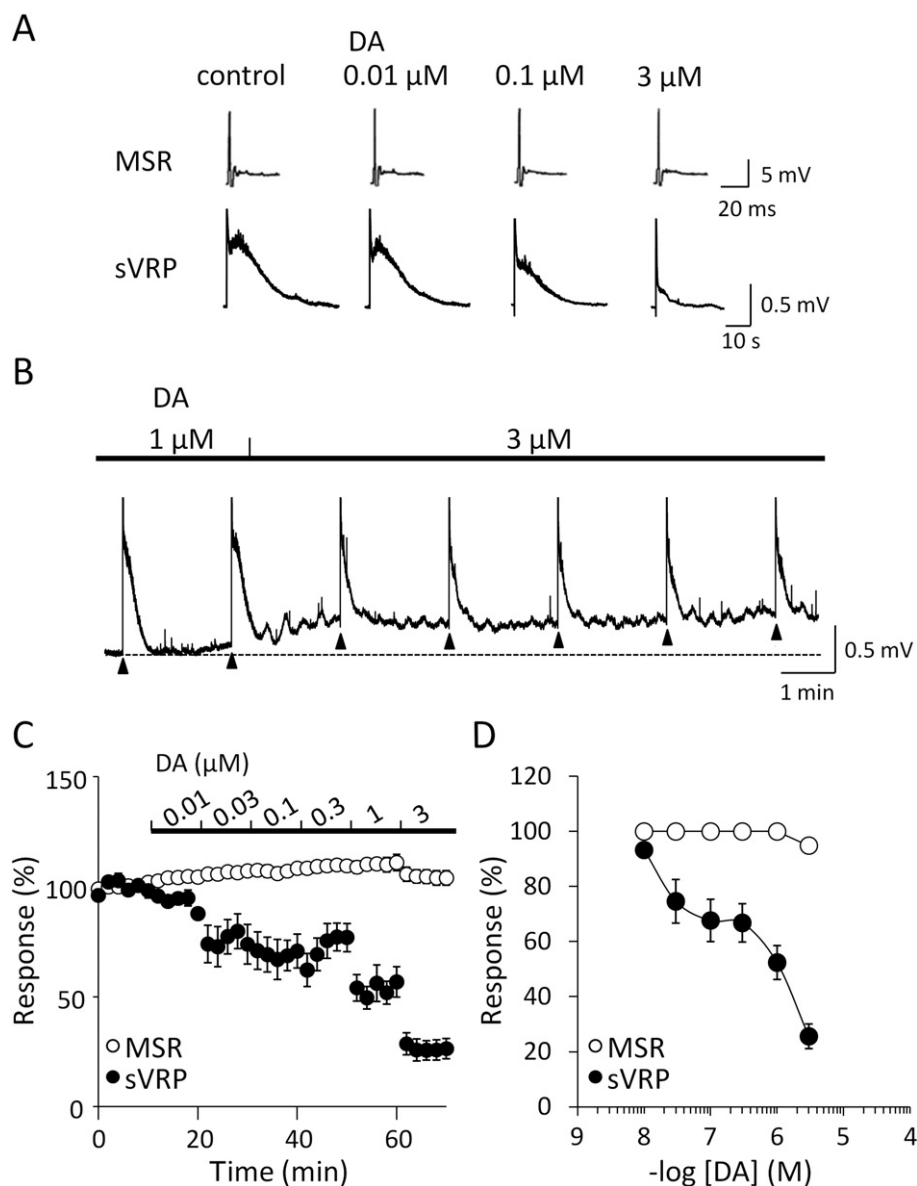
Figure 2
Concentration–response relationship of effects of dopamine (DA) on spinal reflex potential. (A) Representative traces of MSR (upper panel) and sVRP (lower panel) in the presence of dopamine. Dopamine (0.01–3 µM) was cumulatively applied to the spinal cord. (B) Depolarization of ventral root potential evoked by dopamine (3 µM). Dotted line indicates baseline ventral root potential. (C) Time course of dopamine-evoked depression of sVRP. (D) Concentration–response curves for MSR and sVRP in the presence of dopamine. Data represent means ± SEM ($n = 6$).

concentration–response curve for sVRP inhibition by dopamine was biphasic with a first phase at concentrations of 300 nM or less and a second phase at concentration of more than 300 nM (Figure 2C, D).

Dopamine-evoked depression of sVRP was mediated by D₁-like receptors

The expression of dopamine receptor subtypes in the spinal cord and DRG of neonatal rats was examined by RT-PCR. The mRNA expression of all dopamine receptor subtypes (D_1, D_2, D_3, D_4 and D_5) was detected in the spinal cord. In the DRG, on the other hand, only D_2 and D_4 receptor mRNA was detected (Figure 3).
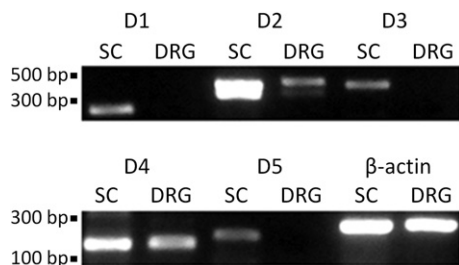
The effects of dopamine receptor antagonists on the depression of sVRP in response to dopamine were investigated (Figure 4). Pretreatment with SCH23390 (1 µM), a D_1-like receptor antagonist, for 20 min resulted in gradual decrease in MSR ($71.0 \pm 10.7\%$, $n = 8$) but not sVRP ($110.5 \pm 5.0\%$, $n = 8$). In the presence of SCH23390, the inhibition of sVRP induced by dopamine (1 µM) was largely attenuated (Figure 4A). Dopamine-evoked depression of sVRP was also attenuated by LE300 (5 µM), another D_1-like receptor antagonist (Figure 4B). Although treatment with raclopride (5 µM), a D_2-like receptor antagonist, also resulted in a significant decrease of the dopamine-evoked depression of sVRP, haloperidol (1 µM), another D_2-like receptor antagonist, had no

effect on it (Figure 4C). LE300, haloperidol and raclopride had no effect on MSR (data not shown). These results suggested that D1-like receptors are involved in sVRP inhibition in response to dopamine. It was next examined whether the effect of dopamine on the spinal cord was mimicked by SKF83959 and SKF81297, D_1-like receptor agonists. Similar to dopamine, SKF83959 (1 µM) and SKF81297 (1 µM) suppressed the sVRP without any effects on MSR (Figure 5A, B) or baseline ventral root potential. Inhibition of sVRP in

response to SKF83959 was also effectively decreased by SCH23390 (Figure 5C, D). Unlike dopamine, however, the effect of SKF81297 was irreversible until 1 h after washout (data not shown). Therefore, we examined the effect of SCH23390 on SKF81297-evoked depression in separate preparations. Inhibition of sVRP in response to SKF81297 was abolished by SCH23390 (Figure 5D).

Characterization of dopamine-evoked depression of sVRP

Several reports have indicated that dopamine enhances inhibitory transmissions such as those mediated by GABA and glycine in the CNS (Porras and Mora, 1993; Radnikow and Misgeld, 1998; Seamans et al., 2001; Trantham-Davidson et al., 2004). We next examined the effects of bicuculline and strychnine, GABA$_A$ and glycine receptor antagonists, respectively, on sVRP inhibition in response to dopamine (Figure 6). As strychnine and bicuculline markedly increased the amplitude of the sVRP, the inhibitory effect of dopamine was evaluated as a percentage of the responses just before the first application. Strychnine (0.5 µM) increased sVRP to $130.6 \pm 0.4\%$ ($n = 6$). In the presence of strychnine, dopamine (1 µM) decreased sVRP by $18.7 \pm 0.4\%$ ($n = 6$), which was less than the control ($38.0 \pm 2.8\%$, $n = 6$, $P < 0.01$, paired Student's t-test). Bicuculline (3 µM) also increased
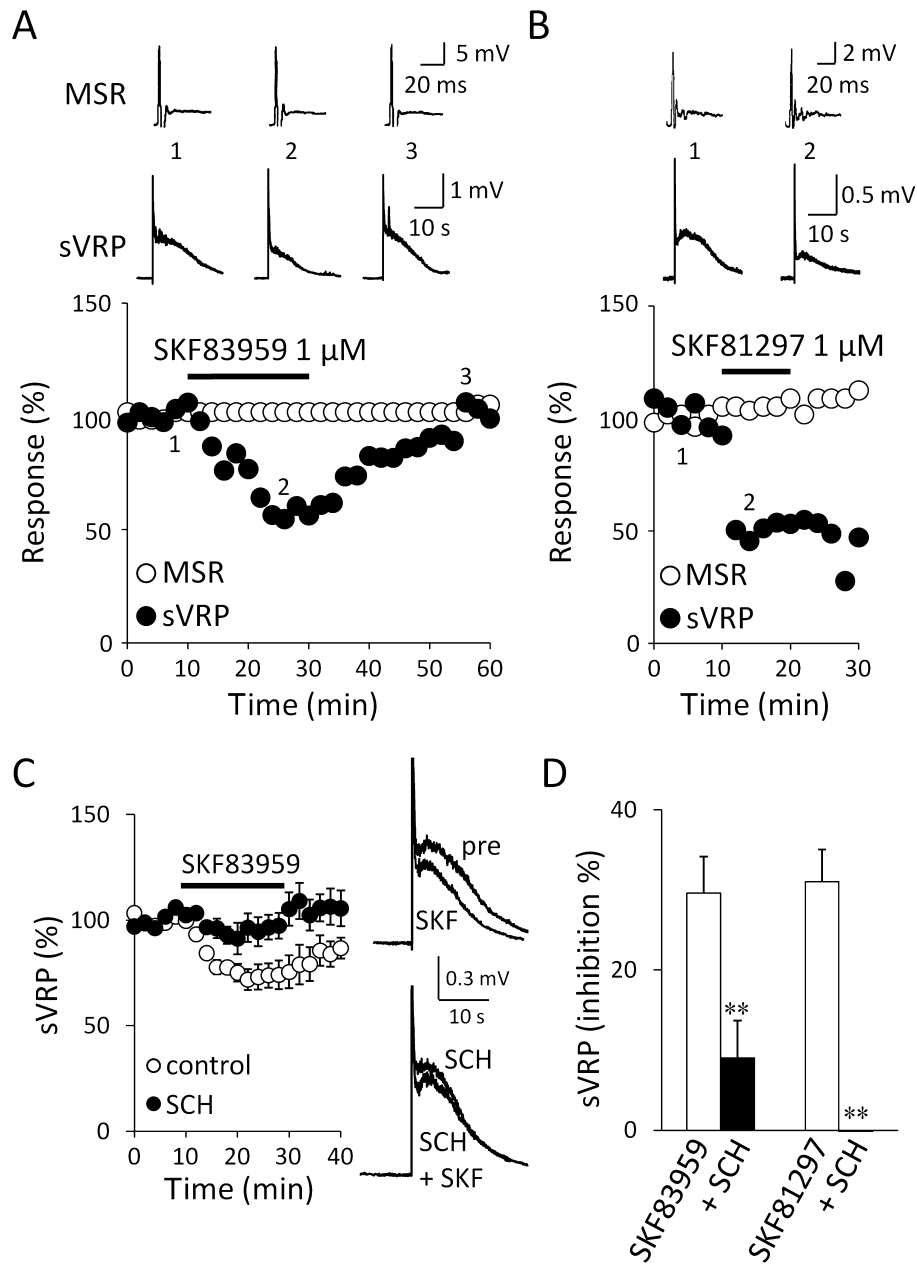

Figure 3

Expression of dopamine receptor subtypes in spinal cord. RT-PCR analysis of dopamine D_1, D_2, D_3 (upper panel), D_4, D_5 and β-actin mRNA (lower panel) in rat spinal cord (SC) and DRG.
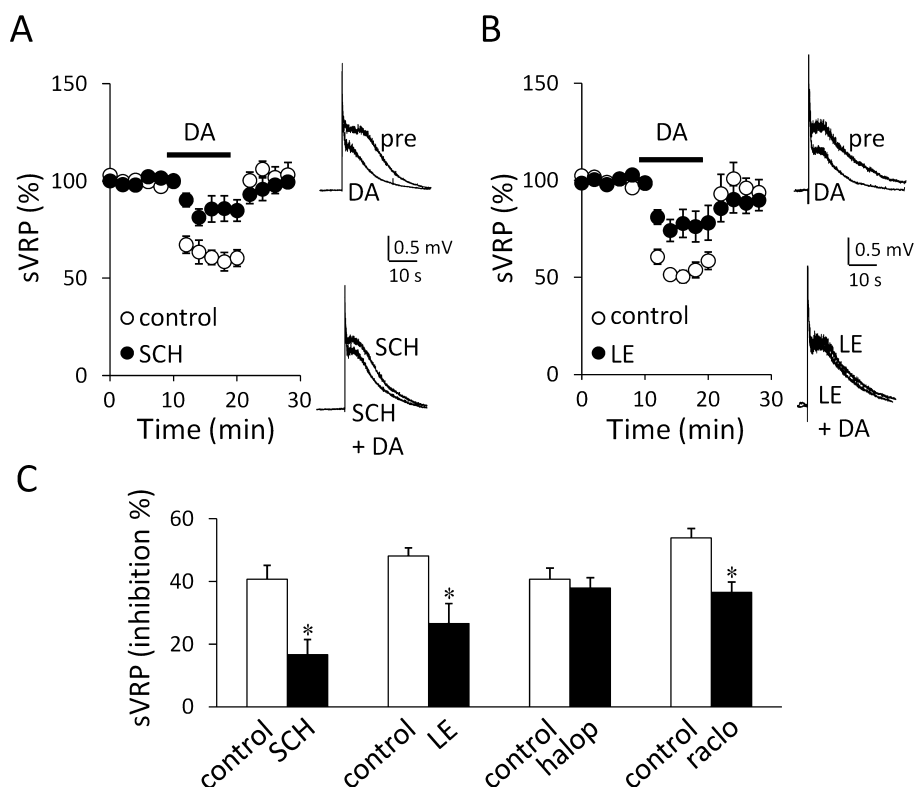
Figure 4

Effects of D_1-like and D_2-like receptor antagonists on dopamine (DA)-evoked depression of sVRP. (A and B) Dopamine (1 µM) was applied to the spinal cord for 10 min (control). Dopamine was again applied in the presence of SCH23390 (SCH, 1 µM, A) and LE300 (LE, 5 µM, B) after pretreatment for 20 min. Representative traces of sVRP are shown in the right panels. (C) Summary of the effects of D_1-like and D_2-like receptor antagonists, SCH23390, LE300, haloperidol (halop, 1 µM) and raclopride (raclo, 5 µM), on dopamine-evoked depression of sVRP. Data represent means ± SEM ($n = 6$–8). *$P < 0.05$ versus control (paired Student's t-test).
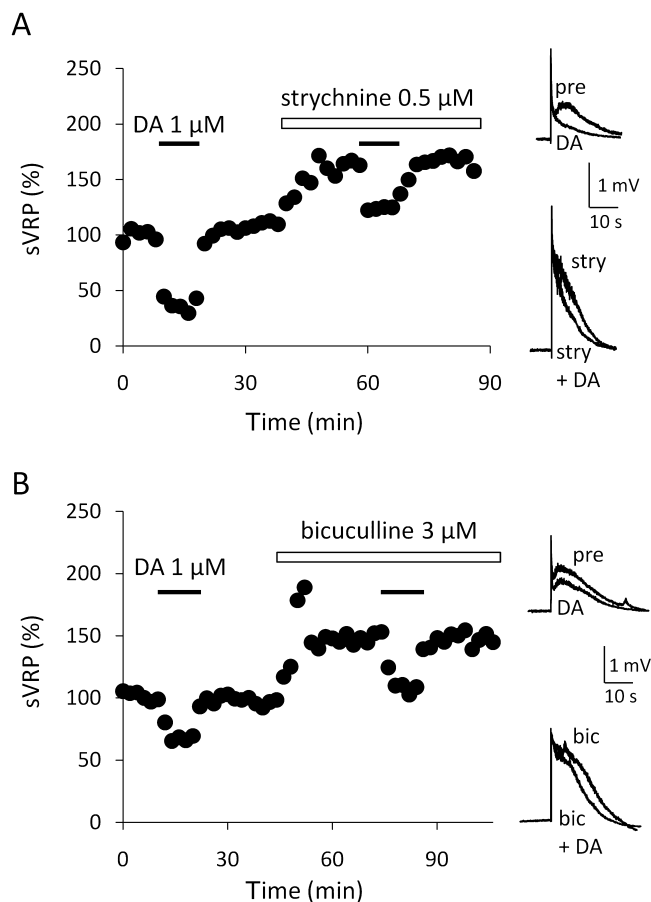
Figure 5

Depression of sVRP by the D_1-like receptor agonist SKF83959. (A and B) SKF83959 (1 μM, A) and SKF81297 (1 μM, B) were applied to the spinal cord. The numbers in the representative traces of MSR (upper panel) and sVRP (middle panel) correspond to those in the lower panel. (C) SKF83959 (SKF, 1 μM)-evoked depression of sVRP in the presence or absence of SCH23360 (SCH, 1 μM). (D) Summary of the effect of SCH23360 (SCH, 1 μM) on SKF83959 (1 μM)- and SKF81297 (1 μM)-evoked depression of sVRP. Data represent means $\pm$ SEM (n = 6). **P < 0.01 versus control in the absence of SCH23360 (paired Student's t-test).

sVRP to 188.2 $\pm$ 20.4% (n = 6). In the presence of bicuculline, dopamine decreased sVRP by 21.2 $\pm$ 5.6% (n = 6), which was not significantly different from the control (34.5 $\pm$ 2.2%, n = 6). Naloxone (1 μM), an opioid receptor antagonist, also increased sVRP to 117.4 $\pm$ 3.7% (n = 6). Dopamine-evoked depression of sVRP (41.3 $\pm$ 2.8% inhibition, n = 6) was unaffected by naloxone (42.7 $\pm$ 7.7% inhibition, n = 6).

Methamphetamine-evoked depression of reflex potentials via dopamine and 5-HT release

To investigate the effect of endogenous dopamine on the spinal cord, the spinal cord preparations were treated with methamphetamine, an endogenous dopamine releaser. Treatment with methamphetamine (10 μM) for 10 min gradually depressed sVRP (Figure 7A). Unlike dopamine, methamphet-

A

B

Figure 6

Effects of glycine and GABA$_A$ receptor antagonists on dopamine (DA)-evoked depression of sVRP. (A and B) Effects of dopamine (1 µM) in the presence and absence of strychnine (stry, 0.5 µM, A) and bicuculline (bic, 3 µM, B). Representative traces of sVRP are shown in the right panels.

amine also depressed MSR, and these inhibitory effects on MSR and sVRP continued even after washout of the drug. The level of depression reached a trough, followed by gradual recovery to control levels, approximately 10 and 20 min after washout respectively. Methamphetamine (3, 10 and 30 µM) depressed both MSR and sVRP in a concentration-dependent manner (Figure 7B, C). Methamphetamine at a low concentration (3 µM) had little, if any, effect on reflex potentials; the highest concentration (30 µM) caused more potent and long-lasting depressions of MSR and sVRP than 10 µM methamphetamine.

Repeated application of methamphetamine (10 µM) for 10 min after an interval of 40 min depressed reflex potentials to the same extent for MSR (first: 57.1 ± 12.0%; second: 52.8 ± 11.6%, $n = 6$) and sVRP (first: 61.6 ± 9.3%; second: 55.8 ± 11.5%, $n = 6$). As shown in Figure 8, the inhibition of sVRP by methamphetamine was attenuated by the D$_1$-like receptor antagonists SCH23390 (1 µM) and LE300 (5 µM) but not by the D$_2$-like receptor antagonists haloperidol (1 µM) and raclopride (5 µM). On the other hand, the depression of MSR by methamphetamine was abolished by LE300 and attenuated

by haloperidol but not by raclopride (Figure 9). We could not analyse the effect of SCH23390 on methamphetamine-evoked depression of MSR because SCH23390 by itself depressed the MSR as mentioned above.

In addition to dopamine, methamphetamine releases other monoamines such as 5-HT and noradrenaline (Ono and Fukuda, 1984; Seiden *et al.*, 1988; Ono *et al.*, 1991; Flecken-stein *et al.*, 2000). Therefore, the effects of ketanserin, a 5-HT$_{2A/2C}$ receptor antagonist, and atipamezole, an α$_2$-adrenoceptor antagonist, on MSR inhibition in response to methamphetamine were examined. Ketanserin (1 µM) inhibited the methamphetamine-evoked depression of MSR but not sVRP (Figure 10), while atipamezole (1 µM) had no effect on the depression of either reflex potentials by methamphetamine. Ketanserin and atipamezole by themselves had no effect on MSR and sVRP (data not shown). We also examined whether methamphetamine induced the release of these monoamines from the spinal cord. As shown in Figure 11, methamphetamine (30 µM) significantly increased the release of dopamine and 5-HT. The amount of 5-HT released was at least five-fold greater than that of dopamine. These results suggest that methamphetamine released 5-HT and dopamine, and these monoamines depressed MSR and sVRP respectively.

Discussion and conclusions

In the current study, dopamine depressed sVRP in the isolated spinal cords of neonatal rats via the activation of D$_1$-like receptors. Methamphetamine also depressed sVRP through the release of endogenous dopamine. These effects are suggested to contribute to functional regulation of spinal cord by dopamine released from descending nerve fibres.

At 1 µM, dopamine depresses the MSR, representing monosynaptic transmission evoked by an A fibre activation, in the mouse spinal cord via D$_2$-like receptors (Clemens and Hochman, 2004). At higher concentrations (>1 µM), dopamine depolarizes baseline ventral root potential and depresses MSR in the rat spinal cord (Kitazawa *et al.*, 1985). As shown in the current study, in addition to the inhibition of MSR, lower concentrations (<1 µM) of dopamine depressed sVRP without any effects on MSR and baseline level potential. Methamphetamine, an endogenous dopamine releaser, also depressed sVRP. Inhibition by both agents was reversed by the D$_1$-like receptor antagonists. Moreover, the D$_1$-like receptor agonists mimicked the inhibitory effect of dopamine on sVRP. Taken together, these results indicate that endogenous dopamine effectively depressed sVRP via D$_1$-like receptors. Raclopride, a D$_2$-like receptor antagonist, slightly decreased the effect of dopamine, implying the additional contribution of D$_2$-like receptors to the depression. However, it is unlikely that D$_2$-like receptors were the major contributors to the inhibition by low concentrations (<1 µM) of dopamine because the methamphetamine-evoked depression was only slightly decreased by raclopride. In addition, another D$_2$-like receptor antagonist, haloperidol, failed to attenuate both the dopamine- and methamphetamine-evoked sVRP depression. On the other hand, in the presence of SCH23390, the inhibitory effect of SKF81297 on sVRP was abolished, while the effect of dopamine was only partly blocked. These results also
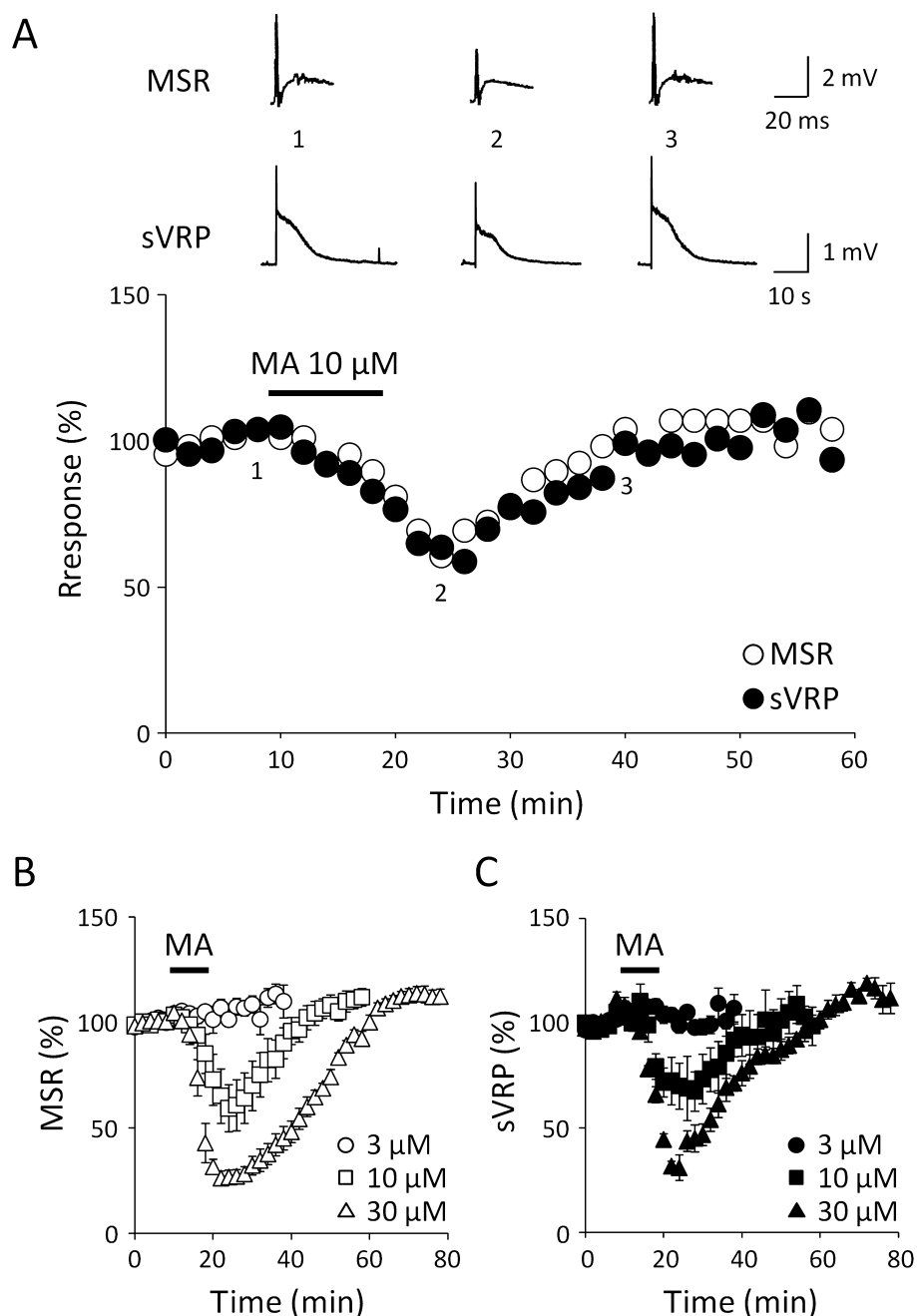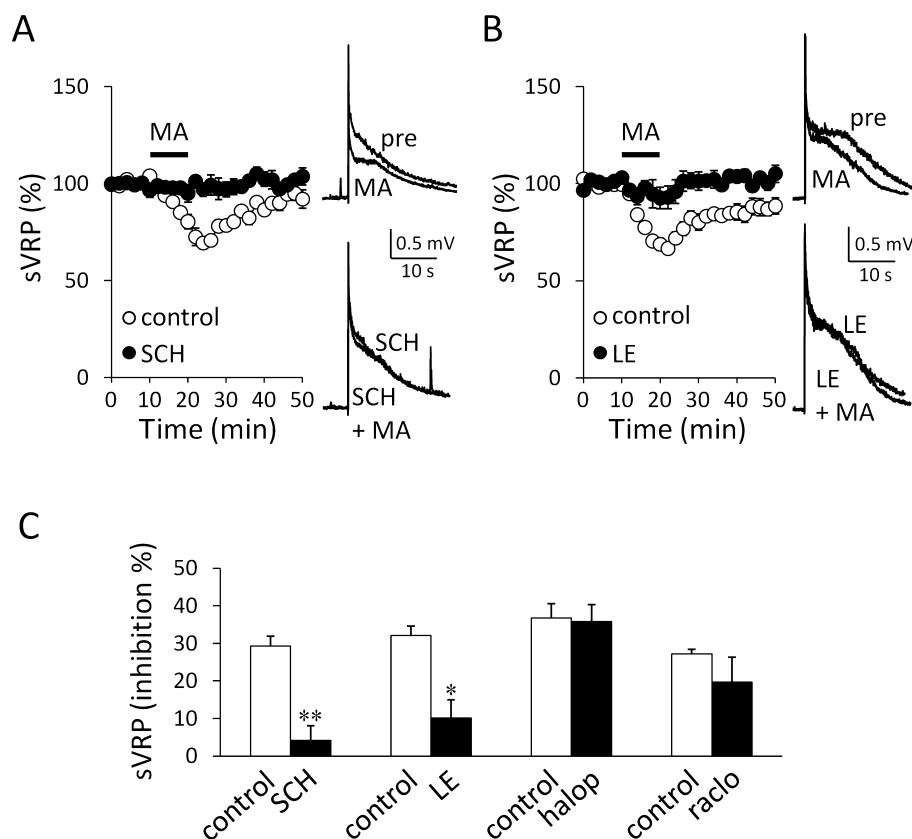
Figure 7

Depression of MSR and sVRP by the endogenous dopamine releaser methamphetamine (MA). (A) MA (10 µM) was applied for 10 min to the spinal cord. The numbers in the representative traces of MSR (upper panel) and sVRP (middle panel) correspond to those in the lower panel. (B and C) Concentration-dependent depression of MSR (B) and sVRP (C) by methamphetamine (3, 10 and 30 µM). Data represent means ± SEM ($n = 6$).

suggest the contribution of other receptors, distinct from D_1- and D_2-like receptors, to the effects of dopamine. Further investigation is needed to determine the nature of these receptors.

The depression of sVRP by dopamine suggests an antinociceptive effect on the spinal cord, as sVRP is believed to reflect C fibre-evoked nociceptive transmission (Akagi *et al.*, 1985; Faber *et al.*, 1997), which can be depressed by analge-

sics such as opioids or α_2-adrenoceptor agonists (Yanagisawa *et al.*, 1984; Nussbaumer *et al.*, 1989; Kendig *et al.*, 1991; Faber *et al.*, 1998; Otsuguro *et al.*, 2005). However, in pain tests *in vivo*, the antinociceptive effects of D_1-like receptors are inconsistent. In the mouse (Zarrindast *et al.*, 1999) and rat formalin test (Munro, 2007), systemic administration of D_1-like receptor agonists preferentially suppressed nociceptive behaviour in phase II (chronic pain) compared with
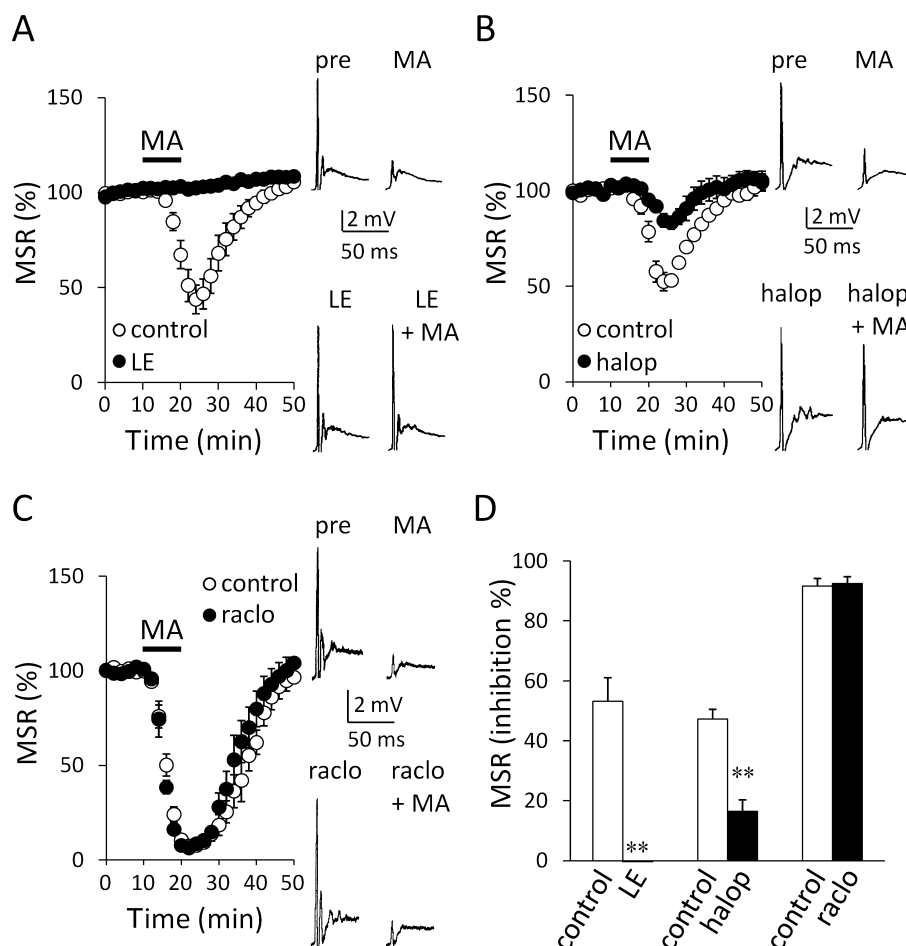
Figure 8

Effects of D_1-like and D_2-like receptor antagonists on methamphetamine (MA)-evoked depression of sVRP. (A and B) Methamphetamine (10 μM) was applied to the spinal cord for 10 min (control). Methamphetamine was again applied in the presence of SCH23390 (SCH, 1 μM, A) or LE300 (LE, 5 μM, B) after pretreatment for 20 min. Representative traces of sVRP are shown in the right panels. (C) Summary of the effects of dopamine D_1-like and D_2-like receptor antagonists, SCH23390, LE300, haloperidol (halop, 1 μM) and raclopride (raclo, 5 μM), on methamphetamine-evoked depression of sVRP. Data represent means ± SEM ($n = 6$–7). *$P < 0.05$, **$P < 0.01$ versus control (paired Student's t-test).

phase I (acute pain), while D_2-like receptor agonists were effective in both phases. On the other hand, dopamine or its analogues have been shown to cause antinociception via D_2-like but not D_1-like receptors in the rat tail-flick test (Barasi and Duggal, 1985; Liu *et al.*, 1992), von Frey test (Tamae *et al.*, 2005) and carrageenan-induced inflammatory pain (Gao *et al.*, 2001). Further studies are needed to define the role of D_1-like receptors in nociceptive transmission in the spinal cord.

Unlike dopamine and the D_1-like receptor agonists, methamphetamine depressed sVRP and MSR to a similar extent. The depression of MSR was inhibited by dopamine receptor antagonists (LE300 and haloperidol). However, it is unlikely that dopamine is involved in this effect because MSR was not inhibited by dopamine and the D_1-like receptor agonists at concentrations that inhibited sVRP. The depression of MSR was inhibited by ketanserin, a $5-HT_{2A/2C}$ antagonist, but not by raclopride, a selective D_2-like receptor antagonist. Methamphetamine releases not only dopamine but also 5-HT (Azzaro and Rutledge, 1973; Seiden *et al.*, 1988; Higuchi *et al.*, 2008), and this was also the case in the current study. The higher concentration of 5-HT than dopamine may be due to the extensive projection of 5-hydroxytryptaminergic fibres

throughout the spinal cord (Millan, 2002). In addition, it has also been reported that 5-HT depresses MSR in the rat spinal cord (Yomono *et al.*, 1992; Wallis *et al.*, 1993). It seems likely, therefore, that 5-HT released by methamphetamine inhibited MSR in the neonatal rat spinal cord. The inhibitory effects of SCH23390 on MSR may be mediated via 5-hydroxytryptaminergic mechanisms because of its agonist effects in 5-hydroxytryptaminergic systems (Briggs *et al.*, 1991; Millan *et al.*, 2001; Zarrindast *et al.*, 2011). Ketanserin is a $5-HT_{2A/2C}$ receptor antagonist (Hartman and Northup, 1996). In addition, the demonstrated affinities of LE300 and haloperidol to $5-HT_{2A}$ receptors (Fontenla *et al.*, 1994; Seeman and Van Tol, 1994; Witt *et al.*, 2000; Rostom *et al.*, 2001; El-Subbagh *et al.*, 2002) suggest that 5-HT released by methamphetamine inhibits MSR via $5-HT_{2A}$ receptors.

sVRP is evoked by transmission from primary afferent fibres to motoneurones via interneurones. These neuronal activities are modulated by inhibitory inputs (Akagi and Yanagisawa, 1987; Nussbaumer *et al.*, 1989), a process in which GABAergic and glycinergic interneurones play a key role (Akagi and Yanagisawa, 1987; Otsuguro *et al.*, 2006). There are several possible mechanisms of D_1-like receptor-evoked sVRP inhibition, including pre-synaptic inhibition of

Figure 9

Effects of dopamine D1-like and D_2-like receptor antagonists on methamphetamine (MA)-evoked depression of MSR. (A–C) MA (10 μM) was applied to the spinal cord for 10 min (control). Methamphetamine was again applied in the presence of LE300 (LE, 5 μM, A), haloperidol (halop, 1 μM, B) or raclopride (raclo, 5 μM, C) after pretreatment for 20 min. Representative traces of MSR are shown in the right panels. (D) Summary of the effects of D_1-like and D_2-like receptor antagonists, LE300, haloperidol and raclopride, on methamphetamine-evoked depression of MSR. Data represent means $\pm$ SEM ($n = 6$–7). **$P < 0.01$ versus control (paired Student's t-test).

excitatory transmitter release, pre-synaptic facilitation of inhibitory transmitter release and postsynaptic inhibition. Our results showed that D_1-like receptor mRNA is expressed in the spinal cord but not in the DRG, suggesting that dopamine inhibits sVRP by acting on interneurones and/or motoneurones but not by inhibiting excitatory transmitter release from the endings of primary afferents.

Endogenous opioids have been implicated in spinal antinociception by dopamine (Kang *et al.*, 1998; Hu *et al.*, 1999). However, in the current study, blocking of opioid receptors did not affect the depression of sVRP. D_1-like receptors stimulate adenylyl cyclase (Missale *et al.*, 1998). On the other hand, D_1-like receptors also stimulate phospholipase C (Undie and Friedman, 1990), and SKF83959 has been reported to selectively activate phosphatidylinositol (PI)-linked D_1-like receptors in the rat brain (Jin *et al.*, 2003; Chu *et al.*, 2010). Therefore, PI response may be involved in the sVRP inhibition via D_1-like receptors in the neonatal rat spinal cord. In the current study, blocking $GABA_A$ and glycine receptors

appeared to attenuate the inhibition of sVRP by dopamine, suggesting that the postsynaptic activation of GABAergic and/or glycinergic inhibitory neurones by dopamine might at least partly contribute to the sVRP inhibition. However, we cannot exclude the possibility that the inhibitory effects of dopamine were underestimated due to the substantial increase in the amplitude of sVRP induced by strychnine or bicuculline alone. Alternatively, these antagonists may preferentially enhance the activities of neurones that do not receive dopaminergic depression. Further studies are needed to clarify the cellular mechanism of sVRP inhibition evoked by dopamine in the spinal cord.

Acknowledgement
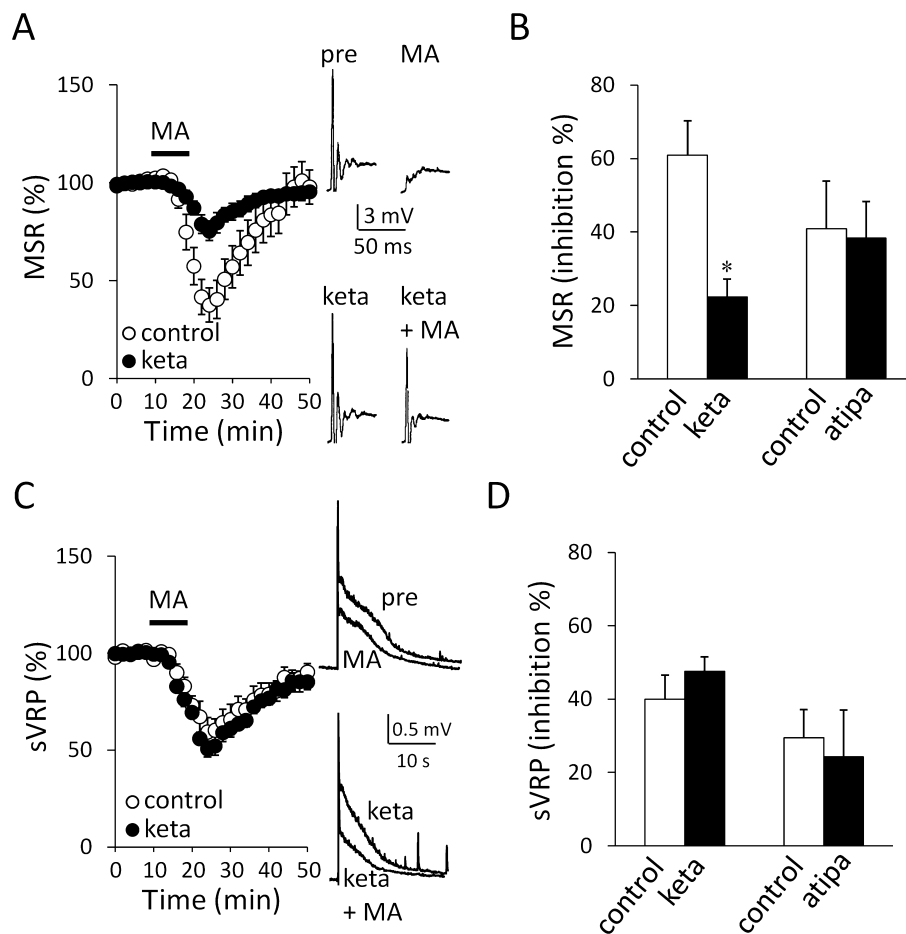
Figure 10

Effects of 5-HT$_2$ and α_2-adrenoceptor antagonists on methamphetamine (MA)-evoked depression of MSR and sVRP. (A and C) Methamphetamine (10 µM) was applied to the spinal cord for 10 min (control). Methamphetamine was again applied in the presence of ketanserin (keta, 10 µM) after pretreatment for 20 min. Representative traces of MSR (A) and sVRP (C) are shown in the right panels. (B and D) Summary of the effects of ketanserin and atipamezole (atipa, 1 µM) on methamphetamine-evoked depression of MSR (B) and sVRP (D). Data represent means $\pm$ SEM ($n =$ 6–8). *$P < 0.05$ versus control (paired Student's t-test).
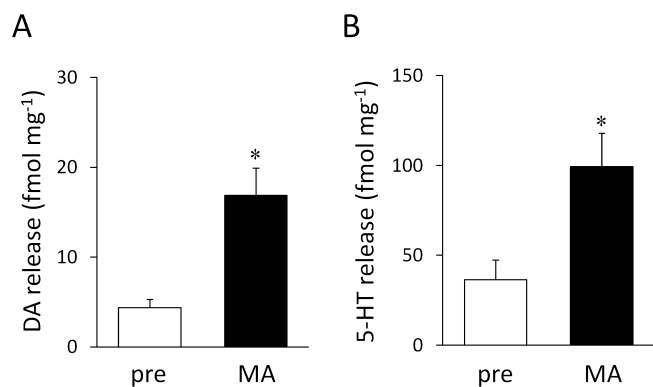
Figure 11

Methamphetamine (MA)-evoked dopamine (DA) and 5-HT release from spinal cord. (A and B) The amount of dopamine (A) and 5-HT (B) release for 30 min before (pre) and after treatment with methamphetamine (30 µM) for 10 min. Data represent means $\pm$ SEM ($n = 3$–4). *$P < 0.05$ versus control (paired Student's t-test).

Conflict of interest

None.

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