

## RESEARCH PAPER

# Differential contributions of adenosine to hypoxia-evoked depressions of three neuronal pathways in isolated spinal cord of neonatal rats

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### Keywords

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## BACKGROUND AND PURPOSE

Hypoxic effects on neuronal functions vary significantly with experimental conditions, but the mechanism for this is unclear. Adenosine has been reported to play a key role in depression of neuronal activities in the CNS during acute hypoxia. Hence, we examined the effect of acute hypoxia on different spinal reflex potentials and the contribution of adenosine to them.

## EXPERIMENTAL APPROACH

Spinal reflex potentials, monosynaptic reflex potential (MSR), slow ventral root potential (sVRP) and dorsal root potential (DRP), were measured in the isolated spinal cord of the neonatal rat. Adenosine release was measured by using enzymatic biosensors.

## KEY RESULTS

In the spinal cord preparation isolated from postnatal day 5–8 rats at 27°C, acute hypoxia induced adenosine release and depressed three reflex potentials. However, in postnatal day 0–3 rats at 27°C, the hypoxic-induced adenosine release and depression of MSR were negligible, while the depression of sVRP and DRP were perceptible responses. In postnatal day 0–3 rats at 33°C, hypoxia evoked adenosine release and depression of MSR. An adenosine A<sub>1</sub> receptor selective antagonist and a high [Ca<sup>2+</sup>]<sub>o</sub>, which suppressed adenosine release, abolished the hypoxic-induced depression of MSR but not those of sVRP and DRP.

## CONCLUSIONS AND IMPLICATIONS

Hypoxic-induced depression of MSR depends on adenosine release, which is highly susceptible to age, temperature and [Ca<sup>2+</sup>]<sub>o</sub>. However, a large part of the depressions of DRP and sVRP are mediated via adenosine-independent mechanisms. This differential contribution of adenosine to depression is suggested to be an important factor for the variable effects of hypoxia on neuronal functions.

## Abbreviations

ACSF, artificial cerebrospinal fluid; CPT, 8-cyclopentyl-1,3-dimethylxanthine; DRP, dorsal root potential; MSR, monosynaptic reflex potential; sVRP, slow ventral root potential

## Introduction

Neuronal function in the CNS is extremely sensitive to decreases in partial pressure of O<sub>2</sub>. In general, hypoxia

depresses neuronal activities, but its effect varies with brain region or experimental conditions (Peña and Ramirez, 2005). This variation in effect appears to be due to the differing sensitivities to hypoxia of each of the neurones that compose the neuronal networks of the CNS.

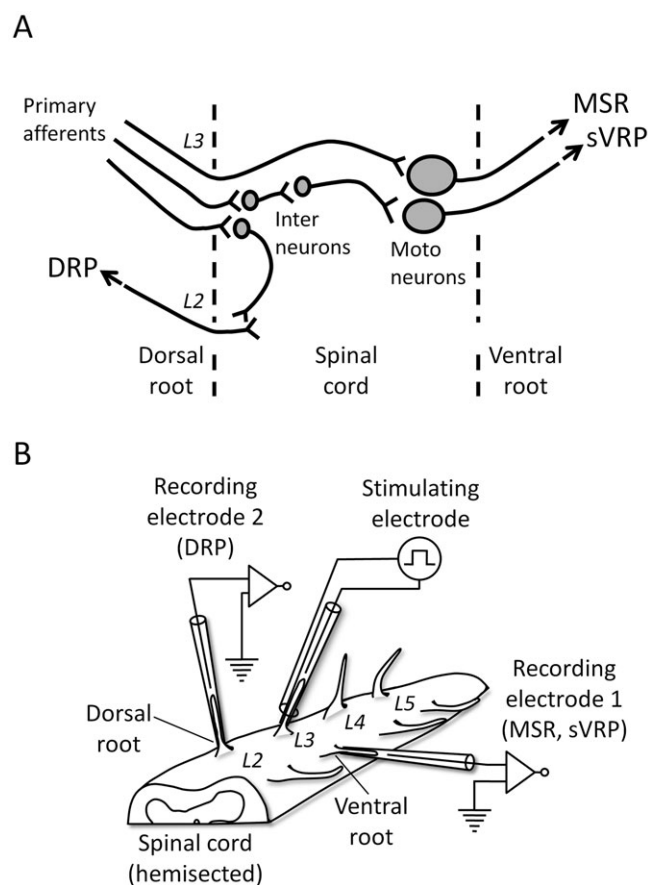
Adenosine is a central mediator of the inhibitory effect of acute hypoxia on synaptic transmission. In rat hippocampal slices, synaptic depression during acute hypoxia or ischaemia-like conditions (oxygen-glucose depletion) are largely suppressed by adenosine A<sub>1</sub> receptor antagonists (Gribkoff and Bauman, 1992; Latini *et al.*, 1999; Pearson *et al.*, 2001; Martín *et al.*, 2007) and temporally correlated with adenosine release (Latini *et al.*, 1998; Dale *et al.*, 2000). In rat spinal cord slices, hypoxic depression of excitatory postsynaptic current is also abolished by an adenosine A<sub>1</sub> receptor antagonist (Park *et al.*, 2002). In the isolated spinal cord of neonatal rat, it has been reported that hypoxia depresses the monosynaptic reflex potential (MSR), and this effect of hypoxia is significantly inhibited by an adenosine A<sub>1</sub> receptor antagonist (Lloyd *et al.*, 1988). Recently, we found that hypoxia induced the release of adenosine from the spinal cord (Takahashi *et al.*, 2010). Also hypercapnia and an adenosine kinase inhibitor have been shown to release adenosine, resulting in a depression not only of the MSR but also of a slow ventral root potential (sVRP), which is associated with the nociceptive reflex (Otsuguro *et al.*, 2006b; 2009). However, the effects of hypoxia on sVRP remain unknown.

In the isolated spinal cord of neonatal rat, electrical stimulation of the lumbar dorsal root gives rise to reflex potentials at an ipsilateral ventral and adjacent dorsal root via synaptic pathways as shown in Figure 1A (Akagi and Yanagisawa, 1987; Nussbaumer *et al.*, 1989; Woodley and Kendig, 1991). The early part of the ventral root potentials is an MSR mediated mainly by non-NMDA receptors, which is followed by an sVRP mediated by NMDA and various metabolic receptors, such as neurokinin and metabolic glutamate receptors. The dorsal root potential (DRP) is mediated by GABA<sub>A</sub> receptors (Seno and Saito, 1985). Each reflex potential has a different sensitivity to drugs or external ions (Brockmeyer and Kendig, 1995; Kurihara and Yoshioka, 1996; Faber *et al.*, 1997; Kubota *et al.*, 1998; Otsuguro *et al.*, 2005; 2006a). In this study, we examined the effects of acute hypoxia on these different spinal reflex potentials. Our results demonstrate that hypoxia rapidly depresses not only MSR but also sVRP and DRP. The hypoxic depression of MSR reflected the hypoxia-induced release of adenosine, which was susceptible to various factors such as age, temperature and [Ca<sup>2+</sup>]<sub>o</sub>. On the other hand, the contribution of adenosine to the hypoxic depressions of sVRP and DRP was limited. The differential contribution of adenosine is suggested to be an important factor for the diversity of hypoxic responses in neuronal functions.

## Methods

### Preparations

All animal care and experimental procedures complied with the Guidelines of the AAALAC and SOP in Japan and were approved by the Animal Care and Use Committee of the Graduate School of Veterinary Medicine, Hokkaido University. Every effort was made to minimize animal suffering and to reduce the number of animals used. Both male and female neonatal rats (Wistar, 0–8 days old) were used.



**Figure 1**

Diagram illustrating the pathways for reflex potentials and the experimental setup. (A) Diagram of the neuronal pathways for the reflex potentials evoked in the isolated spinal cord as shown in (B). The monosynaptic reflex potential (MSR) and slow ventral root potential (sVRP) evoked by stimulation of primary afferents are mediated via a monosynaptic and a polysynaptic pathway to motoneurons, respectively. The dorsal root potential (DRP) is mediated via interneurons to adjacent primary afferents. (B) The hemisected spinal cord isolated from a neonatal rat. The lumbar dorsal root (L3–L5) is placed on a stimulating electrode. This picture shows a case where L3 is used for stimulation. The ipsilateral ventral root is placed on 'recording electrode 1' for MSR and sVRP. In a separate experiment, the adjacent dorsal root is placed on 'recording electrode 2' for DRP.

Neonatal rats were killed by decapitation, and then the isolated spinal cord preparation was prepared as previously described (Otsuguro *et al.*, 2006a). The hemisected spinal cord was superfused with artificial cerebrospinal fluid (ACSF) at a flow rate of approximately 2.5 mL·min<sup>-1</sup>. The temperature of the bath was monitored by a thermometer (CT-1200D, Custom, Tokyo, Japan) before and after each recording and was generally kept at 27 ± 2°C but increased to 33°C in some experiments. The composition of ACSF was as follows (mM): NaCl 138, NaHCO<sub>3</sub> 21, NaH<sub>2</sub>PO<sub>4</sub> 0.6, KCl 3.5, CaCl<sub>2</sub> 1.25, MgCl<sub>2</sub> 1.2, glucose 10, gassed with 95% O<sub>2</sub> and 5% CO<sub>2</sub>, pH~7.3. Hypoxic ACSF was prepared by gassing with 95% N<sub>2</sub> and 5% CO<sub>2</sub> (pH~7.3) from at least 1 h before the experiments were started.

## Electrophysiology

The spinal lumbar roots were sucked into the electrodes, and the reflex potentials were extracellularly recorded as shown in Figure 1B. Suction stimulating and recording 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  $\mu$ s), and a MSR and an sVRP were recorded from the ventral root. In separate experiments, the dorsal root was electrically stimulated (30 V, 50–100  $\mu$ s), and a DRP was recorded from an adjacent dorsal root. The magnitudes of MSR and DRP were expressed as the peak amplitude (mV), and that of sVRP was expressed as the depolarization integral (mV·s) over the resting potential. The time course of these responses was expressed as a percentage of the mean of the first three responses. The effects of hypoxia for 10 min on these spinal reflex potentials were evaluated by the mean of three responses showing maximal depression during hypoxia, and the data are expressed as a percentage of the mean of three responses just before hypoxia. Although the extent of depression by hypoxia varied from preparation to preparation, it was reproducible in the same preparation. Therefore, in most of the experiments, the preparation was exposed to hypoxia twice to confirm the control response, and the reflex potentials during the second hypoxic episode were used as a control response and compared with the responses during the following hypoxic episodes under the various conditions. Electrical responses were detected with a high gain amplifier (MEZ-8300, Nihon Kohden, Tokyo, Japan). MSR was recorded using a thermal array recorder (WR7900, Graftec, Yokohama, Japan) with a sampling time of 80  $\mu$ s. DRP and sVRP were digitized by an analogue/digital converter (PowerLab, 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).

## Adenosine/inosine biosensor

Hypoxia-evoked adenosine release was measured by using enzymatic biosensors (Sarissa Biomedical, Coventry, UK) according to the method of Dale *et al.* (2000) with some modifications. The tip of the adenosine/inosine sensor was Pt wire (50  $\mu$ m diameter, 0.5 mm length) coated with active enzymes (adenosine deaminase, nucleoside phosphorylase and xanthine oxidase). This series of enzymes results in breakdown of adenosine to inosine and then inosine to hydrogen peroxide, which is oxidized on the Pt wire to yield a current. The inosine sensor lacked adenosine deaminase in its coating enzymes, resulting in breakdown of inosine, but not adenosine, to hydrogen peroxide. Therefore, the difference in the signal between the adenosine/inosine and inosine sensors is considered as adenosine signals. The null sensor lacked enzymes and was thus used to measure background signals. Each sensor was polarized to +500 mV by using a potentiostat (Model 3104, Pinnacle Technology, Lawrence, KS, USA) with software (PAL, ver. 1.5.10, Pinnacle Technology). Adenosine/inosine, inosine and null sensors were inserted into an isolated spinal cord preparation from the hemisectioned surface so that the entire enzyme-coating areas for each sensor were within the preparation, and then were allowed to stabilize for at least 1 h before the experiments were started. In most of the experiments, all three sensors

were inserted into the dorsal horn. Adenosine/inosine and inosine sensors were calibrated with known concentrations of adenosine and inosine before and after insertion. The change in the signal of the adenosine/inosine sensor during hypoxia was expressed as  $\Delta$ purine ( $\mu$ M) after the change in the signal of the null sensor had been subtracted.

## Data analysis

Results are expressed as the mean  $\pm$  SEM. The IC<sub>50</sub> value was calculated by fitting the data to a sigmoidal logistic curve with software (Origin, ver. 7.5J, OriginLab, Northampton, MA, USA). Statistical comparisons between two groups were performed by paired or unpaired Student's *t*-test. For multiple comparisons, ANOVA following Dunnett's or Tukey's test were used. A *P*-value of less than 0.05 was considered significant.

## Materials

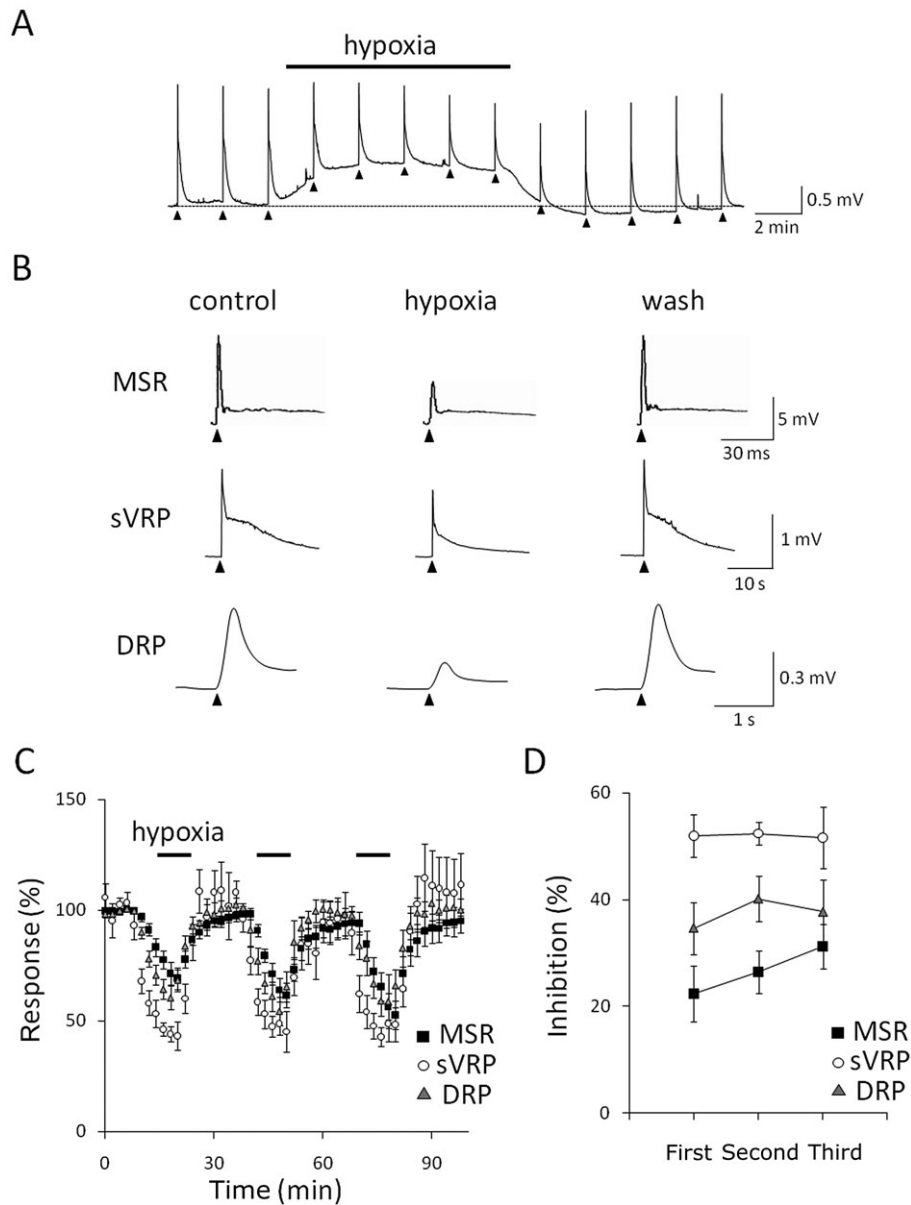
Adenosine, 8-cyclopentyl-1,3-dimethylxanthine (CPT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Substance P was from Peptide Institute (Minoh, Japan). Tetrodotoxin (TTX) was from Wako Pure Chemical (Osaka, Japan). All drugs were mixed in ACSF and applied to preparations. Drugs and molecular target nomenclature follows Alexander *et al.* (2009).

## Results

### Effects of hypoxia on spinal cord isolated from postnatal day 5–8 rats

To investigate the effect of hypoxia on neuronal activity, spinal cords isolated from postnatal day 5–8 (P5–8) rats were used. The dorsal root was electrically stimulated every 2 min and the preparation was exposed to hypoxic ACSF for 10 min. As shown in Figure 2A, the baseline potential of the ventral root (the resting level was expressed as a broken line) was depolarized during hypoxia. A small hyperpolarization was often observed when normoxic ACSF was re-superfused. In addition, hypoxia reversibly depressed MSR and sVRP (Figure 2B). In separate experiments, hypoxia also depolarized the baseline potential of the dorsal root (data not shown) and reversibly depressed DRP (Figure 2B). Hypoxia (10 min) repeatedly depressed these reflex potentials (Figure 2C). There was no significant difference in the hypoxia-evoked depression of MSR, sVRP and DRP among three repetitive hypoxic episodes (*P* > 0.05, Tukey's test; Figure 2D).

We also examined whether hypoxia depressed the postsynaptic depolarizing response to substance P. Bath-applied substance P (30 and 100 nM) for 1 min depolarized the ventral root (30 nM:  $0.20 \pm 0.05$  mV, *n* = 5; 100 nM:  $0.32 \pm 0.08$  mV, *n* = 5). Exposure to hypoxia (10 min) significantly (*P* < 0.01, paired Student's *t*-test) suppressed the responses to substance P (30 nM:  $0.05 \pm 0.04$  mV, *n* = 5; 100 nM:  $0.19 \pm 0.07$  mV, *n* = 5). TTX (3 nM), which inhibited synaptic transmission by suppressing action potential generation, abolished the electrically evoked reflex potentials (data not shown) and decreased the responses to substance P (30 nM:  $0.12 \pm 0.06$  mV, *n* = 4; 100 nM:  $0.19 \pm 0.05$  mV, *n* = 4). In the



**Figure 2**

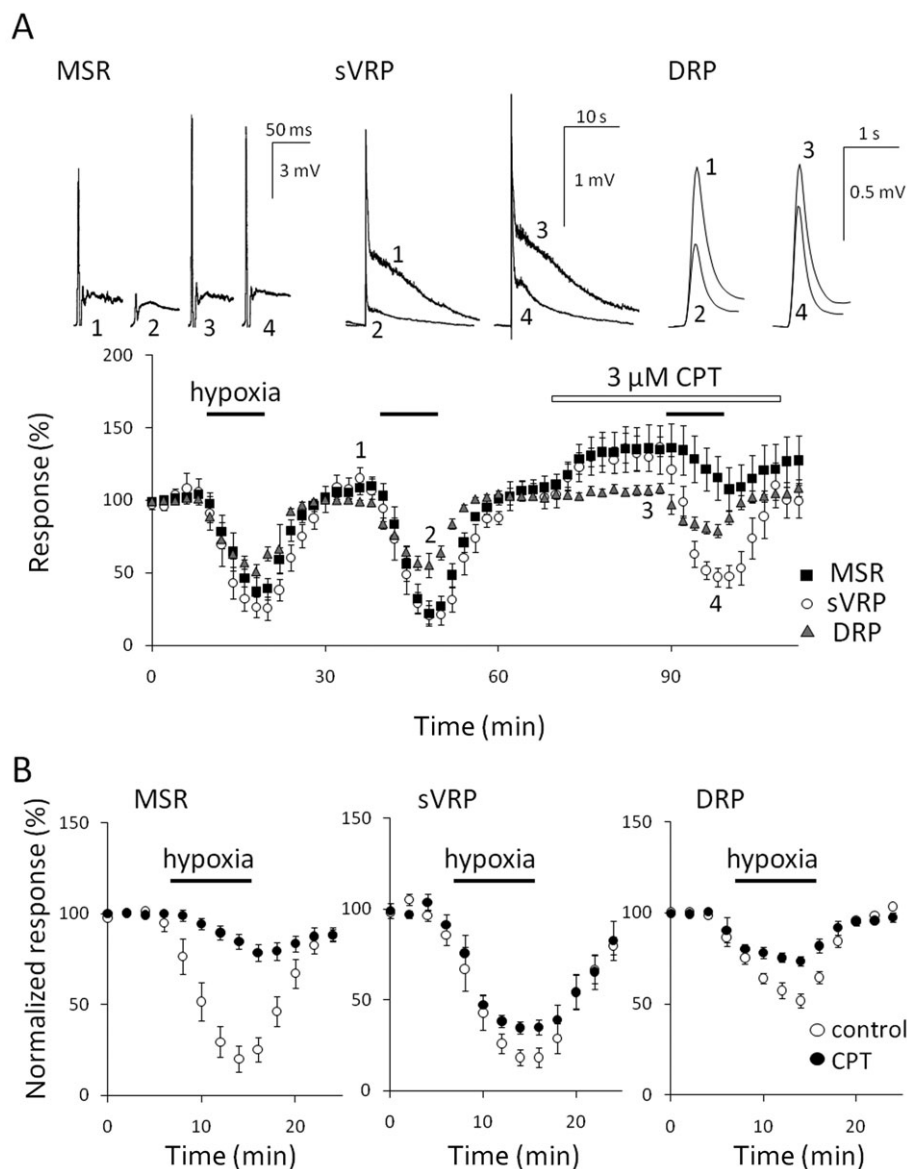
Effect of hypoxia on isolated spinal cord in neonatal rat. (A) The slow ventral root potential (sVRP) was evoked by electrical stimulation every 2 min (arrow heads). The preparation isolated from postnatal day 5–8 (P5–8) rats was exposed to hypoxia for 10 min at 27°C. The broken line shows the resting level of the ventral root potential. (B) Representative traces of monosynaptic reflex potential (MSR), sVRP and dorsal root potential (DRP). The left, middle and right traces show the responses before (control), during (hypoxia) and after hypoxia (wash) respectively. (C) The time course of the peak amplitude (MSR and DRP) and the area under the curve (sVRP) of depolarization. Each symbol and error bar represents the mean  $\pm$  SEM ( $n = 4-6$ ). (D) The depression of reflex potentials by repetitive exposure to hypoxia in P5–8 rats at 27°C. Each symbol and error bar represents the mean  $\pm$  SEM ( $n = 4-6$ ).

presence of TTX, hypoxia failed to suppress the responses to substance P (30 nM:  $0.09 \pm 0.05$  mV,  $n = 4$ ; 100 nM:  $0.20 \pm 0.06$  mV,  $n = 4$ ).

### Contribution of adenosine to hypoxic depression

Hypoxia results in the accumulation of adenosine in the extracellular space of the CNS, including the spinal cord (Latini and Pedata, 2001; Pearson *et al.*, 2003). Therefore, we

examined the effect of CPT, an adenosine  $A_1$  receptor selective antagonist, on the hypoxia-evoked depression of reflex potentials in P5–8 rats. The treatment with CPT (3  $\mu$ M) for 20 min slightly increased MSR ( $123.9 \pm 7.1\%$  of control,  $n = 6$ ), sVRP ( $126.8 \pm 5.0\%$  of control,  $n = 6$ ) and DRP ( $104.6 \pm 1.3\%$  of control,  $n = 5$ ; Figure 3A). Then, CPT almost abolished the hypoxic depression of MSR (control:  $72.6 \pm 6.6\%$  inhibition,  $n = 6$  vs. CPT:  $9.6 \pm 3.1\%$  inhibition,  $n = 6$ ,  $P < 0.01$ ; Figure 3B). On the other hand, a large part of the



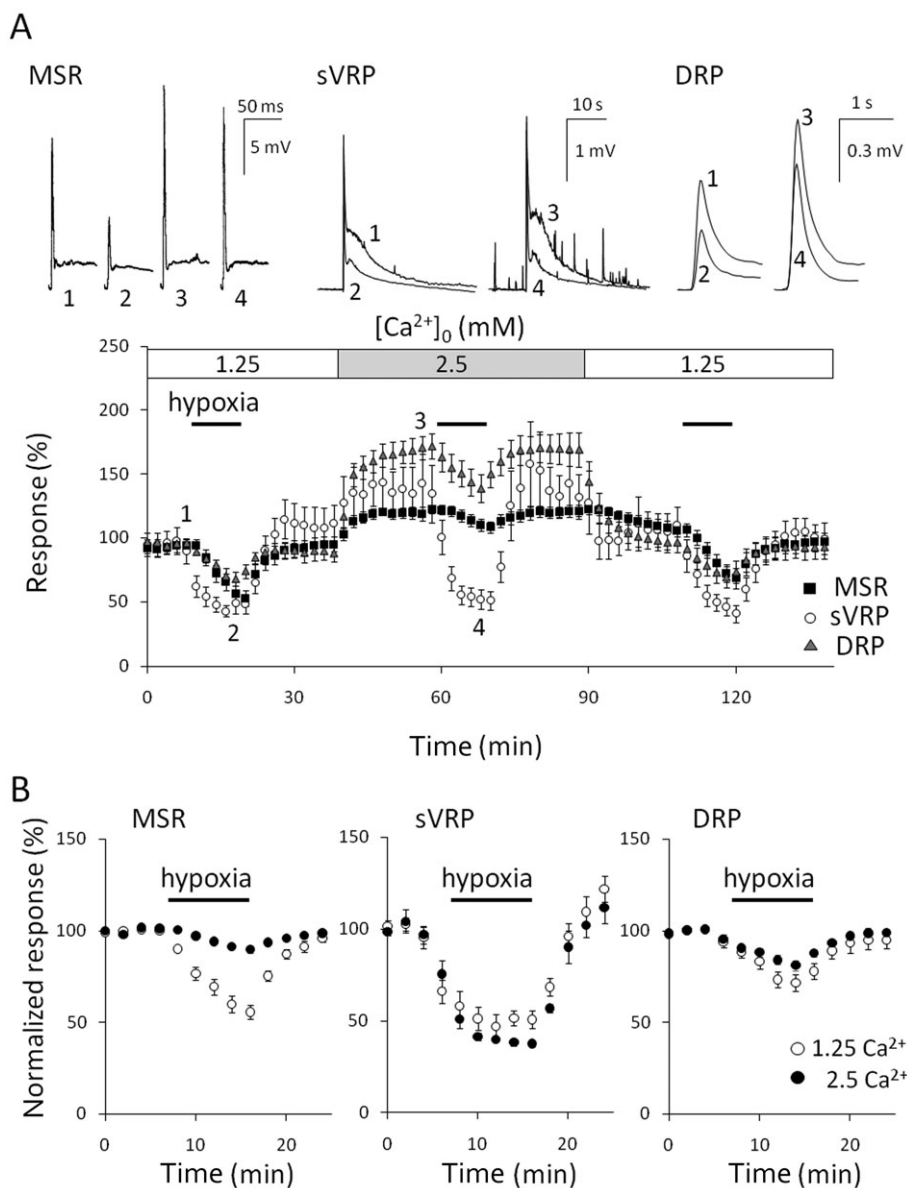
**Figure 3**

Effect of an adenosine A<sub>1</sub> receptor selective antagonist on hypoxic depression. (A) The preparation isolated from postnatal day 5–8 (P5–8) rats was repeatedly exposed to hypoxia for 10 min each at 27°C and pretreated with 3 μM 8-cyclopentyl-1,3-dimethylxanthine (CPT) for 20 min before the third exposure. Then effects of hypoxia on monosynaptic reflex potential (MSR), slow ventral root potential (sVRP) and dorsal root potential (DRP) were observed in the presence of CPT. The number in the representative traces of reflex potentials (upper panel) correspond to those in the lower panel. (B) The effects of CPT on the hypoxia-evoked depression of MSR, sVRP and DRP in P5–8 rats at 27°C. Each symbol and error bar represents the mean ± SEM (*n* = 5–6).

depression of sVRP (control: 73.1 ± 6.0% inhibition, *n* = 6 vs. CPT: 59.4 ± 4.5% inhibition, *n* = 6, *P* < 0.01) and DRP (control: 42.1 ± 3.7% inhibition, *n* = 5 vs. CPT: 24.2 ± 2.6% inhibition, *n* = 5, *P* < 0.01, paired Student's *t*-test) was insensitive to CPT. A higher concentration of CPT (10 μM) or another adenosine A<sub>1</sub> receptor antagonist, 8-cyclopentyl-1,3-dipropylxanthine (1 μM), did not show any further inhibitory effect. Atropine (10 μM, an M receptor antagonist), bicuculline (5 μM, a GABA<sub>A</sub> receptor antagonist), strychnine (1 μM, a glycine receptor antagonist), naloxone (3 μM, an opioid receptor antagonist), NG-nitro-L-arginine methyl ester

(100 μM, an NOS inhibitor), AM251 (3 μM, a CB<sub>1</sub> receptor antagonist), ketanserin (3 μM, a 5-HT<sub>2A</sub> receptor antagonist), atipamezole (3 μM, an α<sub>2</sub> adrenoceptor antagonist), LY341495 (1 μM, a metabolic glutamate receptor antagonist) and SCH23390 (1 μM, a D<sub>1</sub>-like receptor antagonist) also did not show any inhibitory effect on the hypoxia-evoked depression of sVRP in the presence or absence of adenosine A<sub>1</sub> receptor antagonists (data not shown). The hypoxia-evoked depolarization of ventral root potential was not affected by CPT (control: 0.32 ± 0.06 mV, *n* = 6; CPT: 0.37 ± 0.21 mV, *n* = 6).





**Figure 4**

Effect of  $[Ca^{2+}]_o$  on hypoxic depression. (A) The preparation isolated from postnatal day 5–8 (P5–8) rats was repeatedly exposed to hypoxia for 10 min each at 27°C and pretreated with 2.5 mM  $[Ca^{2+}]_o$  for 20 min before the second exposure. Then the effects of hypoxia on monosynaptic reflex potential (MSR), slow ventral root potential (sVRP) and dorsal root potential (DRP) were observed in 2.5 mM  $[Ca^{2+}]_o$ . The number in the representative traces of reflex potentials (upper panel) correspond to those in the lower panel. (B) The hypoxia-evoked depression of MSR, sVRP and DRP in 1.25 and 2.5 mM  $[Ca^{2+}]_o$  in P5–8 rats at 27°C. Each symbol and error bar represents the mean  $\pm$  SEM ( $n = 5–6$ ).

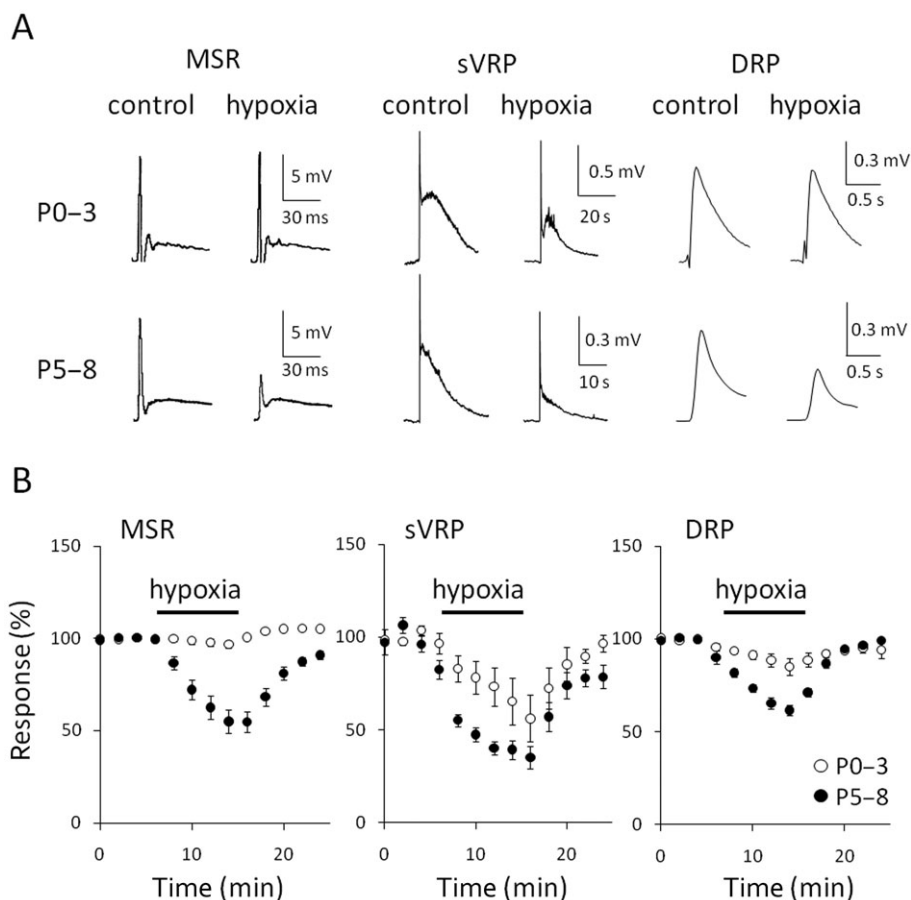
### Influence of extracellular $Ca^{2+}$ on hypoxic depression

It has been reported that an increase in extracellular  $Ca^{2+}$  inhibits adenosine release during hypoxia (Dale *et al.*, 2000; Takahashi *et al.*, 2010). Therefore, the influence of high  $[Ca^{2+}]_o$  was examined in P5–8 rats. As shown in Figure 4A, the change in  $[Ca^{2+}]_o$  from 1.25 mM to 2.5 mM increased the magnitude of MSR ( $128.9 \pm 7.3\%$  of control,  $n = 6$ ), sVRP ( $125.3 \pm 12.0\%$  of control,  $n = 6$ ) and DRP ( $193.4 \pm 15.9\%$  of control,  $n = 5$ ). In addition, the high  $[Ca^{2+}]_o$  increased spontaneous activities of the basal ventral and DRPs as shown in the traces of sVRP of Figure 4A. A high  $[Ca^{2+}]_o$  almost abolished the hypoxic

depression of MSR (1.25 mM  $Ca^{2+}$ :  $31.2 \pm 4.1\%$  inhibition,  $n = 6$  vs. 2.5 mM  $Ca^{2+}$ :  $5.6 \pm 1.0\%$  inhibition,  $n = 6$ ,  $P < 0.01$ ; Figure 4B). The depression of DRP was significantly decreased by high  $[Ca^{2+}]_o$  (1.25 mM  $Ca^{2+}$ :  $23.9 \pm 3.7\%$  inhibition,  $n = 5$  vs. 2.5 mM  $Ca^{2+}$ :  $15.5 \pm 2.5\%$  inhibition,  $n = 5$ ,  $P < 0.05$ ), while the depression of sVRP was not affected (1.25 mM  $Ca^{2+}$ :  $51.6 \pm 5.8\%$  inhibition,  $n = 6$  vs. 2.5 mM  $Ca^{2+}$ :  $60.1 \pm 1.4\%$  inhibition,  $n = 6$ ,  $P > 0.05$ , paired Student's *t*-test).

### Age-dependent depression during hypoxia

In the rat hippocampus, it has been reported that the influence of hypoxia/ischaemia varies with age (Ben-Ari, 1992;



**Figure 5**

Effect of age on hypoxic-induced depression. (A) Representative traces of monosynaptic reflex potential (MSR), slow ventral root potential (sVRP) and dorsal root potential (DRP) in postnatal day 0–3 (P0–3, upper panel) and 5–8 rats (P5–8, lower panel), both before (control) and during hypoxia (hypoxia) at 27°C. (B) The hypoxia-evoked depression of MSR, sVRP and DRP in P0–3 and P5–8 rats at 27°C. Each symbol and error bar represents the mean  $\pm$  SEM ( $n = 8–11$ ).

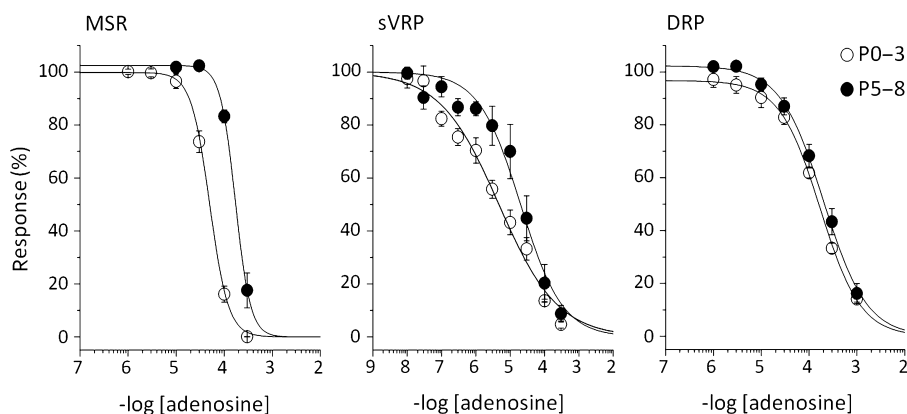
Yager and Thornhill, 1997). We therefore examined the effect of hypoxia on reflex potentials in spinal cord isolated from younger neonatal rats. In the spinal cords from postnatal day 0–3 (P0–3) rats, little hypoxia-evoked depression of MSR was observed ( $2.9 \pm 1.6\%$  inhibition,  $n = 8$ ), while the depression of DRP was small but detectable ( $11.5 \pm 3.3\%$  inhibition,  $n = 11$ ; Figure 5). In contrast to MSR, sVRP was markedly depressed by hypoxia in the spinal cords from P0–3 rats, although the inhibitory effect of hypoxia in P0–3 ( $30.5 \pm 8.7\%$  inhibition,  $n = 9$ ) was less than that in P5–8 rats ( $57.9 \pm 10.8\%$  inhibition,  $n = 9$ ,  $P < 0.01$  vs. P0–3, unpaired Student's *t*-test).

The effects of adenosine on three spinal reflex potentials were examined in P0–3 and P5–8 rats. Adenosine ( $0.01 \mu\text{M}–1 \text{mM}$ ) was cumulatively applied to the spinal cords for 10 min at each concentration. All three reflex potentials in both P0–3 and P5–8 rats were depressed by adenosine in a concentration-dependent manner (Figure 6). The  $\text{IC}_{50}$  value for sVRP was the smallest among the three reflex potentials in both P0–3 and P5–8 rats, and that for MSR in P0–3 rats were significantly smaller than that in P5–8 rats (P0–3:  $47.5 \pm 4.6 \mu\text{M}$ ,  $n = 6$  vs. P5–8:  $186.4 \pm 22.6 \mu\text{M}$ ,  $n = 6$ ,  $P < 0.01$ ,

unpaired Student's *t*-test) but not for sVRP (P0–3:  $5.7 \pm 2.4 \mu\text{M}$ ,  $n = 6$  vs. P5–8:  $36.8 \pm 17.2 \mu\text{M}$ ,  $n = 6$ ) and DRP (P0–3:  $149.0 \pm 14.9 \mu\text{M}$ ,  $n = 5$  vs. P5–8:  $236.9 \pm 50.4 \mu\text{M}$ ,  $n = 5$ ). These results indicate that the lack of hypoxic depression of MSR in P0–3 is not due to the failure of downstream mechanisms of adenosine  $\text{A}_1$  receptor activation.

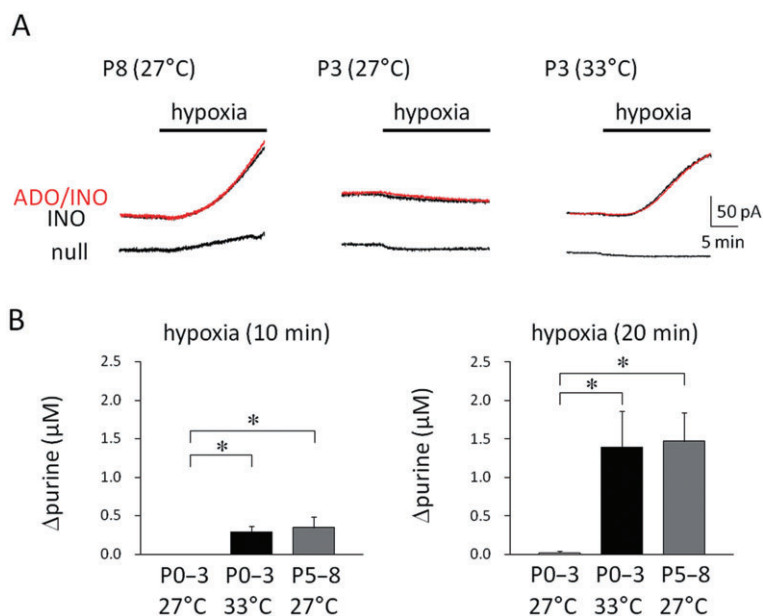
### Measurement of purines by biosensors

We investigated the effects of hypoxia on the extracellular concentration of purines using the biosensors. In P5–8 rats at 27°C, the exposure of spinal cords to hypoxia for 20 min gradually increased signal currents from both adenosine/inosine and inosine but not null sensors in the dorsal horn of the spinal cord (Figure 7A). The time course of the sensor response was slower than that for the inhibition of reflex potentials by hypoxia. The difference in the signal between the adenosine/inosine and inosine sensors, which was considered to represent the adenosine signal, was quite small, if any, indicating that most of the signal from the adenosine/inosine sensor originated from inosine. This result is consistent with our previous findings, obtained by use of HPLC, that most of the adenosine is converted to inosine by endog-



**Figure 6**

Concentration-dependent depression by adenosine in postnatal day 0–3 (P0–3) and 5–8 (P5–8) rats. Adenosine was cumulatively applied to spinal cords isolated from P0–3 and 5–8 (P5–8) rats at 27°C. Concentration–response curves for monosynaptic reflex potential (MSR), slow ventral root potential (sVRP) and dorsal root potential (DRP) in the presence of adenosine. Each symbol and error bar represents the mean  $\pm$  SEM ( $n = 5–6$ ).



**Figure 7**

Adenosine release from spinal cord during hypoxia. Adenosine/inosine (ADO/INO), inosine (INO) and null sensors were inserted into the dorsal horn of the spinal cord and then hypoxia was applied for 20 min. (A) Representative traces of signal current for each sensor observed in a postnatal day 8 (P8) rat at 27°C (left panel), postnatal day 3 (P3) rats at 27°C (middle panel) and 33°C (right panel) are shown. (B) The hypoxia-evoked change in adenosine/inosine concentration ( $\Delta$ purine) at 10 min (left panel) and 20 min (right panel) after hypoxia in postnatal day 0–3 (P0–3) rats at 27 and 33°C and in postnatal day 5–8 (P5–8) rats at 27°C. Each symbol and error bar represents the mean  $\pm$  SEM ( $n = 5–6$ ). \* $P < 0.05$  versus P0–3 rats at 27°C (Dunnett's test).

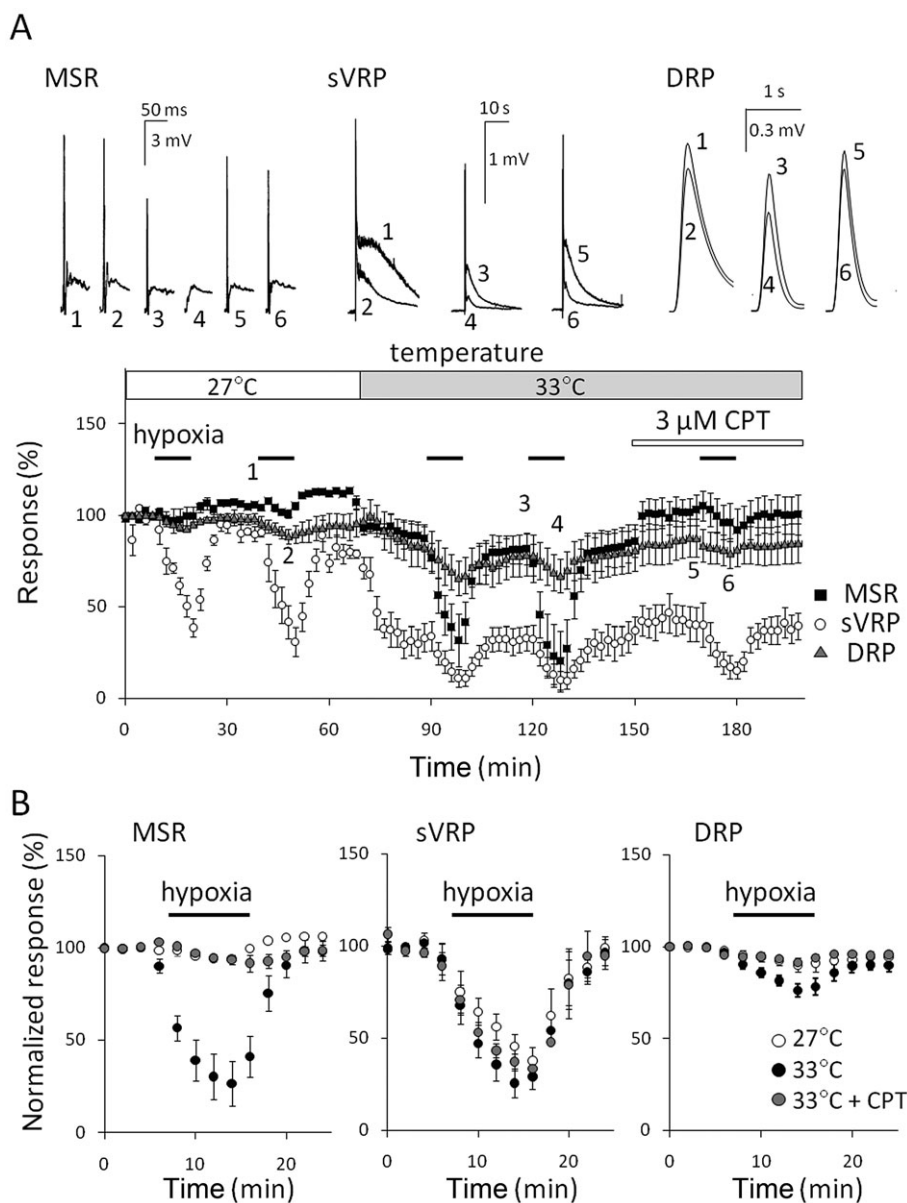
enous adenosine deaminase and that the amount of inosine released during hypoxia is about 10-fold greater than that of adenosine in neonatal rat spinal cord (Takahashi *et al.*, 2010). Therefore, the changes in the signal from adenosine/inosine sensors during hypoxia at 10 and 20 min were expressed as  $\Delta$ purine, and these sensors were calibrated with known concentrations of inosine (Figure 7B). In P0–3 rats at 27°C, hypoxia failed to increase the signal currents from all sensors. These results indicate that hypoxia increases the extracellular

purine concentration in P5–8, but not in P0–3 rats at 27°C. We also inserted the sensors into both the dorsal and ventral horn of a preparation. There was no difference in the signals between them (data not shown).

### Temperature-dependent depression during hypoxia

Next we examined the influence of temperature on the hypoxia-evoked depression in P0–3 rats. The increase in tem-





**Figure 8**

Effect of temperature on hypoxic-induced depression. (A) The preparation isolated from postnatal day 0–3 (P0–3) rats was repeatedly exposed to hypoxia for 10 min each. Temperature was elevated from 27 to 33°C 20 min before the third exposure. Then, the preparation was pretreated with 3 μM 8-cyclopentyl-1,3-dimethylxanthine (CPT) 20 min before the fifth exposure at 33°C. The number in the representative traces of monosynaptic reflex potential (MSR), slow ventral root potential (sVRP) and dorsal root potential (DRP) (upper panel) correspond to those in the lower panel. (B) The hypoxia-evoked depression of MSR, sVRP and DRP in P0–3 rats at 27 and 33°C in the presence and absence of 3 μM CPT. Each symbol and error bar represents the mean ± SEM ( $n = 5–6$ ).

perature from 27 to 33°C decreased the magnitude of MSR, sVRP and DRP by  $8.9 \pm 8.9\%$  ( $n = 5$ ),  $45.0 \pm 8.5\%$  ( $n = 5$ ) and  $11.7 \pm 6.3\%$  ( $n = 6$ ) respectively (Figure 8A). As shown in Figure 8, the hypoxic depression of MSR, which was only just detectable at 27°C ( $5.3 \pm 1.2\%$  inhibition,  $n = 5$ ), appeared at 33°C ( $67.9 \pm 11.8\%$  inhibition,  $n = 5$ ,  $P < 0.01$  vs. 27°C). The increase in temperature significantly enhanced the hypoxic depression of sVRP (27°C:  $44.6 \pm 7.0\%$  inhibition,  $n = 5$  vs. 33°C:  $63.9 \pm 7.8\%$  inhibition,  $n = 6$ ,  $P < 0.05$ ) and DRP (27°C:

$7.6 \pm 1.8\%$  inhibition,  $n = 6$  vs. 33°C:  $18.8 \pm 2.9\%$  inhibition,  $n = 6$ ,  $P < 0.01$ ). At 33°C, the treatment with CPT (3 μM) for 20 min slightly increased MSR ( $115.7 \pm 4.0\%$  of control,  $n = 5$ ), sVRP ( $122.7 \pm 14.6\%$  of control,  $n = 5$ ) and DRP ( $111.3 \pm 3.1\%$  of control,  $n = 6$ ). Then, CPT almost abolished the hypoxic depression of MSR ( $4.8 \pm 2.0\%$  inhibition,  $n = 5$ ,  $P < 0.01$  vs. 33°C) and significantly reversed the hypoxic depression of DRP ( $6.8 \pm 0.9\%$  inhibition,  $n = 6$ ,  $P < 0.01$  vs. 33°C) but not that of sVRP ( $55.5 \pm 4.3\%$  inhibition,  $n = 6$ ,  $P > 0.05$

vs. 33°C, Dunnett's test). Similar to the depression of MSR, the hypoxia-evoked increase in purine concentration, which was only just detectable at 27°C, emerged at 33°C (Figure 7). We could not measure the purine concentration in P5–8 rats at 33°C because the signal current from the sensors did not become steady even more than 2 h after the insertion.

## Discussion and conclusions

### *Hypoxic depression of MSR via adenosine A<sub>1</sub> receptors*

Hypoxia increases extracellular adenosine level in rat hippocampal slices (Dale *et al.*, 2000) and isolated spinal cords (Takahashi *et al.*, 2010). In the rat hippocampus, the depression of synaptic transmission during brief periods of hypoxia is greatly reduced by adenosine A<sub>1</sub> receptor antagonists (Fowler, 1989; Katchman and Hershkowitz, 1993; Martín *et al.*, 2007). In addition, the hypoxic depression of MSR is abolished by the blockade of adenosine A<sub>1</sub> receptors in the isolated spinal cord of postnatal day 6–10 rats (Lloyd *et al.*, 1988). In the present study, CPT also reversed the hypoxic depression of MSR by more than 80% and hypoxia increased the signal currents from the adenosine/inosine sensors in P5–8 rats, suggesting that hypoxia released adenosine and thus depressed MSR via adenosine A<sub>1</sub> receptors. However, the time course of the signal currents was much slower than that of MSR inhibition by hypoxia. It is likely that a small negative shift of signal currents observed with hypoxic ACSF (Dale *et al.*, 2000) make it difficult to correctly detect the onset of increase in purine. Adenosine is believed to depress synaptic transmission by presynaptically inhibiting Ca<sup>2+</sup> channels or postsynaptically activating K<sup>+</sup> channels via adenosine A<sub>1</sub> receptors (de Mendonça *et al.*, 2000; Wardas, 2002). MSR is mediated via a monosynaptic pathway from primary afferent endings to motoneurons. Hypoxia does not seem to impair the postsynaptic activity of motoneurons as it failed to depress the response to substance P in the presence of TTX, reflecting the direct effect on motoneurons. Thus, it is possible that MSR is depressed by a presynaptic inhibitory effect of adenosine on transmitter release from primary afferent endings.

The [Ca<sup>2+</sup>]<sub>o</sub> in CSF is around 1.25 mM (Hunter and Smith, 1960) and would further decrease during hypoxia (Martín *et al.*, 1994). At 2.5 mM [Ca<sup>2+</sup>]<sub>o</sub>, the hypoxic depression of MSR was completely masked. It has been reported that elevating the electrochemical gradient driving the Ca<sup>2+</sup> influx counteracts the depression of MSR (Czéh and Somjen, 1990). In our study, the high [Ca<sup>2+</sup>]<sub>o</sub> increased the spontaneous neuronal activities and augmented the magnitude of reflex potentials under normoxic conditions. Therefore, it is likely that high [Ca<sup>2+</sup>]<sub>o</sub> enhances Ca<sup>2+</sup> influx and thus synaptic transmission. However, an increase in the driving force of Ca<sup>2+</sup> does not seem to be a major factor in its inhibitory effect on hypoxic depression, because the hypoxic depression of sVRP was not attenuated by the increase in [Ca<sup>2+</sup>]<sub>o</sub>. High [Ca<sup>2+</sup>]<sub>o</sub> inhibits the increase in extracellular adenosine during hypoxia in rat hippocampal slices (Dale *et al.*, 2000) and cortical astrocytes (Martín *et al.*, 2007). This is the case in the spinal cord (Takahashi *et al.*, 2010). In contrast, the

removal of external Ca<sup>2+</sup> enhances adenosine release during hypoxia or ischaemia-like conditions (Pedata *et al.*, 1993; Frenguelli *et al.*, 2007; Takahashi *et al.*, 2010). From these results, it is hypothesized that Ca<sup>2+</sup> limits the adenosine release during hypoxia and thus the depression of MSR. This idea is supported by the finding that the hypoxic depression of sVRP, largely independent of adenosine release, is mostly unaffected by the increase in [Ca<sup>2+</sup>]<sub>o</sub>. The inhibitory effects of Ca<sup>2+</sup> on adenosine release remain unclear. The manipulation of external Ca<sup>2+</sup> concentration also changes the amount of inosine release in proportion to that of adenosine release (Pedata *et al.*, 1993; Takahashi *et al.*, 2010), suggesting that the activity of adenosine deaminase is unchanged. Therefore, it is speculated that Ca<sup>2+</sup> inhibits adenosine production by acting on processes upstream of adenosine deaminase. Alternatively Ca<sup>2+</sup> may inhibit the purine transport mechanisms.

The susceptibility of MSR to hypoxia drastically changed during the neonatal period. In postnatal day 6–10 rats, it has been reported that hypoxia rapidly depresses MSR (Lloyd *et al.*, 1988), which is consistent with our results in P5–8 rats. However, in P0–3 rats, hypoxia failed to depress MSR although bath-applied adenosine more potently depressed MSR in P0–3 than in P5–8 rats. This resistance of MSR to hypoxia in younger rats is suggested to be due to a lack of adenosine release, as hypoxic adenosine release was negligible in these rats. The influence of hypoxia/ischaemia varies with age (Ben-Ari, 1992; Yager and Thornhill, 1997). During fetal and postnatal development, there are marked conversions of some factors, such as the types of haemoglobins and expression pattern of adenosine kinase (Iwahara *et al.*, 1996; Studer *et al.*, 2006). In P0–3 rats, the CPT-sensitive hypoxic depression of MSR at a higher temperature was associated with hypoxic adenosine release. It is therefore suggested that the increase in temperature results in an increase in the release and/or intracellular production of adenosine because acute hypoxia releases adenosine *per se* (Takahashi *et al.*, 2010).

Reflex potentials were depressed by an increase in temperature alone. This effect does not seem to be due to a change in the extracellular adenosine concentration because there was little difference in the effect of CPT on reflex potentials during normoxia between 27 and 33°C. In rat hippocampus, it is reported that increasing the temperatures from 32.5 to 38.5°C causes adenosine release, which depresses synaptic transmission (Masino and Dunwiddie, 1999; Masino *et al.*, 2001), while the basal concentration of extracellular adenosine at 21°C is twice that at 32°C (Dunwiddie and Diao, 2000), suggesting a U-shaped temperature–response curve for extracellular adenosine that is lowest at around 32°C. If this is the case in the spinal cord, it is also likely that the change of temperature from 27 to 33°C does not increase or rather decrease the extracellular adenosine concentration. In experiments using the isolated spinal cord of rats, it is known that the optimum temperature for maximal amplitude and stable recording of MSR is around 25°C, and that both lower and higher temperatures reduce MSR (Harada and Takahashi, 1983), although the mechanism for this remains unclear (Deshpande and Warnick, 1988). Temperature influences various aspects of neuronal function (Thompson *et al.*, 1985; Borst and Sakmann, 1998; Kullmann and Asztely, 1998;

Volgushev *et al.*, 2000), making its effect on synaptic transmission a complex matter.

### Adenosine $A_1$ receptor-independent depression during hypoxia

In contrast to its effect on MSR, CPT did not have a marked inhibitory effect on the hypoxic depression of sVRP and DRP. This was unexpected because sVRP was the most sensitive to exogenous adenosine in the present study. However, the possibility that CPT failed to inhibit the effect of endogenous adenosine cannot be completely excluded, but CPT does abolish the depression of sVRP when endogenous adenosine is released by an adenosine kinase inhibitor (Otsuguro *et al.*, 2006b). In this study, CPT and high  $[Ca^{2+}]_o$  increased MSR and sVRP, indicating that not only MSR but also sVRP was under the control of tonic inhibition by adenosine. As the high  $[Ca^{2+}]_o$ -evoked increase in the reflex potentials appears to be larger than that induced by CPT, it is speculated to be due to an increase in the driving force of  $Ca^{2+}$ , in addition to the inhibition of adenosine release, as described above. Therefore, the lack of sensitivity of the hypoxic-induced sVRP depression to CTP suggests that a significant accumulation of adenosine does not occur near to the site generating sVRP. There was no difference in the hypoxia-induced purine increase between the dorsal and ventral horn in this study. More detailed analysis is needed to determine the release site of adenosine.

The MSR is evoked by direct transmissions from primary afferent fibres to motoneurons in the ventral horn, whereas DRP and sVRP are evoked by transmissions from primary afferent fibres to interneurons in the dorsal horn. It is likely that hypoxia decreases the activities of interneurons in the dorsal horn in an adenosine  $A_1$  receptor-independent manner. Alternatively, hypoxia may selectively suppress the transmitter release from the endings of primary afferents, which project into interneurons at the dorsal horn but not into motoneurons, because the primary afferent neurons projecting into the dorsal horn have different transmitters and receptors from those into the ventral horn (Garry *et al.*, 1989; Gold *et al.*, 1996; Petruska *et al.*, 2000).

Several studies have been done on the contribution of inhibitory transmitters besides adenosine to hypoxic/ischaemic depression in the CNS (de Mendonça and Ribeiro, 1997; Coelho *et al.*, 2000; Deshpande and Jha, 2004; Youssef *et al.*, 2007). However, the antagonists of various inhibitory transmitters used in the present study had no effect on the hypoxia-induced depression. As hypoxia reportedly modulates the activity of several channels (Nieber, 1999; Peña and Ramirez, 2005), the activities of such channels expressed in primary afferent endings or interneurons of the dorsal horn may be directly affected by hypoxia. It is speculated that the adenosine  $A_1$  receptor-independent mechanism is less susceptible to environmental conditions, at least compared with adenosine release, and that this gives rise to the different responses to hypoxia among neuronal pathways in the spinal cord.

In conclusion, the contribution of adenosine to hypoxia-induced depression differs among neuronal pathways in the spinal cord. This feature is important for the differences in hypoxic effects, because hypoxia-induced adenosine release is

highly susceptible to various conditions, such as age, temperature and  $[Ca^{2+}]_o$ .

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## Conflict of interest

None.

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