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

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Summary

Background and purpose

Hypoxic effects on neuronal functions drastically vary with experimental conditions, but its mechanism is unclear. Adenosine has been reported to play a key role in depression of neuronal activities in the CNS during acute hypoxia. In this study, 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 (P5-8) rats at 27ºC, acute hypoxia released adenosine and depressed three reflex potentials. However, in postnatal day 0-3 (P0-3) rats at 27ºC, the hypoxic adenosine release and depression of MSR were negligible, while the depression of sVRP and DRP were perceptible responses. In P0-3 rats at 33ºC, hypoxia evoked adenosine release and depression of MSR. An adenosine A_1

receptor selective antagonist and a high $[Ca^{2+}]_o$, which suppressed adenosine release, abolished the hypoxic depression of MSR but not those of sVRP and DRP.

Conclusions and implications

These results indicate that the hypoxic depression of MSR depends on adenosine release, which is highly susceptible to age, temperature and $[Ca^{2+}]_o$. On the other hand, a large part of the depressions of DRP and sVRP were mediated via adenosine-independent mechanisms. This differential contribution of adenosine to depression is suggested to be an important factor for the varying influence of hypoxia on neuronal functions.

Key words

Adenosine, hypoxia, adenosine A_1 receptors, reflex potentials, spinal cord,

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_2. 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 at each of the neurons 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 ischemia-like conditions (oxygen-glucose depletion) are largely suppressed by adenosine A_1 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_1 receptor antagonist (Park *et al.*, 2002). In the isolated spinal cord of neonatal rat, it is reported that hypoxia depresses monosynaptic reflex potential (MSR), which is greatly prevented by an adenosine A_1 receptor antagonist (Lloyd *et al.*, 1988). Recently, we found that hypoxia released adenosine from the spinal cord (Takahashi *et al.*, 2010). It has been also reported that hypercapnia and an adenosine kinase inhibitor release

adenosine, resulting in depressing not only MSR but also a slow ventral root potential (sVRP), which is associated with nociceptive reflex (Otsuguro *et al.*, 2006b; 2009). However, the effects of hypoxia on sVRP remained unknown.

In the isolated spinal cord of neonatal rat, electrical stimulation of the lumber dorsal root gives rise to reflex potentials at an ipsilateral ventral and adjacent dorsal root via synaptic pathways as shown in Fig. 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 $GABA_A$ receptors (Seno and Saito, 1985). Each reflex potential has a different sensitivity to drugs or external ions (Brockmeryer 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 demonstrated that hypoxia rapidly depressed not only MSR but also sVRP and DRP. The hypoxic depression of MSR reflected the hypoxic adenosine release, which was susceptible to various factors such as age, temperature and $[Ca^{2+}]_o$. 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 experimental procedures 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.

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^{-1}. 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±2ºC but raised to 33ºC in some experiments. The composition of 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; glucose 10; gassed with 95% O_2 and 5% CO_2; pH~7.3. Hypoxic ACSF was prepared by gassing with 95% N_2 and 5% CO_2 (pH~7.3) from at least 1 hr before the

experiments were started.

Electrophysiology

The spinal lumber roots were sucked into the electrodes, and the reflex potentials were extracellularly recorded as shown in Fig. 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 μs), and a monosynaptic reflex potential (MSR) and a slow ventral root potential (sVRP) were recorded from the ventral root. In separate experiments, the dorsal root was electrically stimulated (30 V, 50-100 μs), and a dorsal root potential (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 were 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 2nd 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 arraycorder (WR7900, Graftec, Yokohama, Japan) with a sampling time of 80 μs. DRP and sVRP were digitized by an analog/digital converter (PowerLab, ADInstruments, Castle Hill, Australia) with a sampling time of 10 ms. Data were stored in a personal computer and analyzed 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 a method of Dale *et al.* (2000) with some modifications. The tip of the adenosine/inosine sensor was Pt wire (50 μm 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 hemisected 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 Δpurine (μM) after subtracting the change in the signal of the null sensor.

Data analysis

Results are expressed as the mean±SEM. The IC_{50} 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

Fig. 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 (Fig. 2B). In separate experiments, hypoxia also depolarized the baseline potential of the dorsal root (data not shown) and reversibly depressed DRP (Fig. 2B). Hypoxia (10 min) repeatedly depressed these reflex potentials (Fig. 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; Fig. 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±0.05 mV, n=5, 100 nM: 0.32±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±0.04 mV, n=5, 100 nM: 0.19±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±0.06 mV, n=4, 100 nM: 0.19±0.05 mV, n=4). In the presence of TTX, hypoxia failed to suppress the responses to substance P (30 nM: 0.09±0.05 mV, n=4, 100 nM: 0.20±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 μM) for 20 min slightly increased MSR (123.9±7.1% of control, n=6), sVRP (126.8±5.0% of control, n=6) and DRP (104.6±1.3% of control, n=5; Fig. 3A). Then, CPT almost abolished the hypoxic depression of MSR (control: 72.6±6.6 % inhibition, n=6 *vs.* CPT: 9.6±3.1% inhibition, n=6, $P<0.01$; Fig. 3B). On the other hand, a large part of the 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_1 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 $GABA_A$ receptor antagonist), strychnine (1 μM, a glycine receptor antagonist), naloxone (3 μM, an opioid receptor antagonist), L-NAME (100 μM, an NO synthase inhibitor), AM251 (3 μM, a

CB_1 receptor antagonist), ketanserin (3 μM, a 5-HT_{2A} receptor antagonist), atipamezole (3 μM, an α_2 adrenoceptor antagonist), LY341495 (1 μM, a metabolic glutamate receptor antagonist) and SCH23390 (1 μM, a D_1-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_1 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).

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 Fig. 4A, the change in $[Ca^{2+}]_o$ from 1.25 mM to 2.5 mM increased the magnitude of MSR (128.9±7.3% of control, n=6), sVRP (125.3±12.0% of control, n=6) and DRP (193.4±15.9% of control, n=5). In addition, the high $[Ca^{2+}]_i$ increased spontaneous activities of the basal ventral and dorsal root potentials as shown in the traces of sVRP of Fig 4A. A high $[Ca^{2+}]_o$ almost abolished the hypoxic depression of MSR (1.25 mM Ca^{2+}: 31.2±4.1% inhibition, n=6 *vs.* 2.5 mM Ca^{2+}: 5.6±1.0% inhibition, n=6, $P<0.01$; Fig. 4B). The depression of DRP was significantly

decreased by high $[Ca^{2+}]_o$ (1.25 mM Ca^{2+}: 23.9±3.7% inhibition, n=5 *vs.* 2.5 mM Ca^{2+}: 15.5±2.5% inhibition, n=5, $P<0.05$), while the depression of sVRP was not affected (1.25 mM Ca^{2+}: 51.6±5.8% inhibition, n=6 *vs.* 2.5 mM Ca^{2+}: 60.1±1.4% inhibition, n=6, $P>0.05$, paired Student's *t*-test).

Age-dependent depression during hypoxia

In the rat hippocampus, it is reported that the influence of hypoxia/ischemia varies with age (Ben-Ari, 1992; 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 P0-3 rats, the hypoxia-evoked depression of MSR could hardly be observed (2.9±1.6% inhibition, n=8), while the depression of DRP was small but detectable (11.5±3.3% inhibition, n=11; Fig. 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±8.7% inhibition, n=9) was less than that in P5-8 rats (57.9±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 μM – 1 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 (Fig. 6). The IC_{50} value for sVRP was the smallest among 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±4.6 μM, n=6 *vs.* P5-8: 186.4±22.6 μM, n=6, $P<0.01$, unpaired Student's *t*-test) but not for sVRP (P0-3: 5.7±2.4 μM, n=6 *vs.* P5-8: 36.8±17.2 μM, n=6) and DRP (P0-3: 149.0±14.9 μM, n=5 *vs.* P5-8: 236.9±50.4 μM, 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 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 (Fig. 7A). The time course of the sensor response was slower than that of the inhibition of reflex potentials by hypoxia. The difference in the signal between the adenosine/inosine and inosine sensors, which was considered as adenosine signal, was quite small, if any, indicating that most of the signal from the adenosine/inosine sensor was originated from inosine. This result

consisted with our previous findings with HPLC method that most of adenosine was converted to inosine by endogenous adenosine deaminase and that amount of inosine released during hypoxia was about 10-fold greater than that of adenosine in neonatal rat spinal cord (Takahashi *et al.*, 2010). Therefore, the changes of the signal from adenosine/inosine sensors during hypoxia at 10 and 20 min were expressed as Δpurine, and these sensors were calibrated with known concentrations of inosine (Fig. 7B). In P0-3 rats at 27°C, hypoxia failed to increase signal currents from all sensors. These results indicate that hypoxia increases extracellular purine concentration in P5-8, but not in P0-3 rats at 27°C. We also inserted the sensors into both 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 temperature from 27 to 33°C decreased the magnitude of MSR, sVRP and DRP by 8.9±8.9% (n=5), 45.0±8.5 % (n=5) and 11.7±6.3% (n=6), respectively (Fig. 8A). As shown in Fig. 8, the hypoxic depression of MSR, which could hardly be observed at 27°C (5.3±1.2% inhibition, n=5), appeared at 33°C (67.9±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±7.0% inhibition, n=5 *vs.* 33°C: 63.9±7.8% inhibition, n=6, $P<0.05$) and DRP (27°C: 7.6±1.8% inhibition, n=6 *vs.* 33°C: 18.8±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±4.0% of control, n=5), sVRP (122.7±14.6% of control, n=5) and DRP (111.3±3.1% of control, n=6). Then, CPT almost abolished the hypoxic depression of MSR (4.8±2.0% inhibition, n=5, $P<0.01$ *vs.* 33°C) and significantly reversed the hypoxic depression of DRP (6.8±0.9% inhibition, n=6, $P<0.01$ vs. 33°C) but not that of sVRP (55.5±4.3% inhibition, n=6, $P>0.05$ *vs.* 33°C, Dunnett's test). Similarly to the depression of MSR, the hypoxia-evoked increase in purine concentration, which could hardly be observed at 27°C, emerged at 33°C (Fig. 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 hr after the insertion.

Discussion and conclusions

Hypoxic depression of MSR via adenosine A_1 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_1 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_1 receptors in the isolated spinal cord of P6-10 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_1 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 difficult to correctly detect the onset of increase in purine. Adenosine is believed to depress synaptic transmission by presynaptically inhibiting Ca^{2+} channels or postsynaptically activating K^+ channels via adenosine A_1 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. It is thus suggested that MSR is depressed by the presynaptic inhibitory effect of adenosine on transmitter release from primary afferent

endings.

The $[Ca^{2+}]_o$ in CSF is around 1.25 mM (Hunter and Smith, 1960) and would further decrease during hypoxia (Martin *et al.*, 1994). At 2.5 mM $[Ca^{2+}]_o$, hypoxic depression of MSR was completely masked. It is reported that elevating the electrochemical gradient driving the Ca^{2+} influx counteracts the depression of MSR (Czéh and Somjen, 1990). In our study, the high $[Ca^{2+}]_o$ increased the spontaneous neuronal activities and augmented the magnitude of reflex potentials under normoxic conditions. Therefore, it is likely that high $[Ca^{2+}]_o$ enhances Ca^{2+} influx and thus synaptic transmission. However, an increase in the driving force of Ca^{2+} 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 $[Ca^{2+}]_o$. High $[Ca^{2+}]_o$ 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). On the contrary, the removal of external Ca^{2+} enhances adenosine release during hypoxia or ischemia-like conditions (Pedeta *et al.*, 1993; Frenguelli *et al.*, 2007; Takahashi *et al.*, 2010). From these results, it is suggested that Ca^{2+} limits the adenosine release during hypoxia and thus the depression of MSR. This idea is supported by the fact that the hypoxic depression of sVRP, largely independent of

adenosine release, is mostly unaffected by the increase in $[Ca^{2+}]_o$. The inhibitory effects of Ca^{2+} on the adenosine release remain unclear. The manipulation of external Ca^{2+} concentration also changes the amount of inosine release in proportion to that of adenosine release (Pedeta *et al.*, 1993; Takahashi *et al.*, 2010), suggesting that the activity of adenosine deaminase is unchanged. Therefore, Ca^{2+} is speculated to inhibit the adenosine production by acting upstream processes of adenosine deaminase. Alternatively Ca^{2+} may inhibit the purine transport mechanisms.

The susceptibility of MSR to hypoxia drastically changed during the neonatal period. In P6-10 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 at those ages. The influence of hypoxia/ischemia 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 hemoglobins 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 the increase in the release and/or intracellular production of adenosine because brief hypoxia releases adenosine *per se* (Takahashi *et al.*, 2010).

Reflex potentials were depressed by an increase of temperature alone. It does not seem to be due to the change of extracellular adenosine concentration because there was little difference in the effect of CPT on reflex potentials during normoxia between 27 and 33ºC. In rat hippocampus, it is reported that increasing the temperatures from 32.5 to 38.5ºC causes adenosine release, which depresses synaptic transmission (Masino and Dunwiddie, 1999; Masino *et al.*, 2001), while the basal concentration of extracellular adenosine at 21ºC is twice that at 32ºC (Dunwiddi and Diao, 2000), suggesting a U-shaped temperature-response curve for extracellular adenosine that is lowest at around 32ºC. If this is the case in the spinal cord, it is also likely that the change of temperature from 27 to 33ºC 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 the synaptic transmission complex matter.

Adenosine A_1 receptor-independent depression during hypoxia

In contrast to its effect on MSR, CPT did not have great 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. We cannot completely exclude the possibility that CPT fail to inhibit the effect of endogenous adenosine; however, CPT abolishes 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 were under the control of tonic inhibition by adenosine. Although the high $[Ca^{2+}]_o$-evoked increase in the reflex potentials appears to be larger than that by CPT, it is speculated to be due to the increase in driving force of Ca^{2+}, in addition to the inhibition of adenosine release, as described above. Therefore, less sensitivity of the hypoxic sVRP depression to CTP suggests the significant accumulation of adenosine does not occur near to the site of transmissions producing sVRP. There was no difference in the hypoxic purine increase

between the dorsal and ventral horn in this study. More detailed analysis is needed to determine the release site of adenosine.

MSR is evoked by direct transmissions from primary afferent fibers to motoneurons in the ventral horn, whereas DRP and sVRP are evoked by transmissions from primary afferent fibers 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).

There are several reports about the contribution of inhibitory transmitters besides adenosine to hypoxic/ischemic depression in the CNS (de Mendonça and Ribeiro, 1997; Coelho *et al.*, 2000; Deshpande and Jha, 2004; Youssef *et al.*, 2007). However, antagonists for inhibitory transmitters used in the present study had no effect on hypoxic 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 suggested that the adenosine A_1 receptor-independent mechanism is less susceptible to environmental conditions, at least compared to adenosine release, and that this gives rise to the different responses to hypoxia among neuronal pathways in spinal cords.

In conclusion, the contribution of adenosine to hypoxic depression differs among neuronal pathways in the spinal cord. This feature is important for the differences in hypoxic effects, because hypoxic adenosine release is highly susceptible to various conditions, such as age, temperature and $[Ca^{2+}]_o$.

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Conflict of interest

None.

Figure Legends

Figure 1. Diagram illustrating the pathways for reflex potentials and the experimental setup.

A, diagram of the neuronal pathways for the evoked reflex potentials in the isolated spinal cord as shown in B. The monosynaptic reflex potential (MSR) and slow ventral root potential (sVRP) evoked by primary afferents stimulation 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 lumber 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.

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 with 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±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±SEM (n=4-6).

Figure 3. Effect of adenosine A_1 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).

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 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±SEM (n=5-6).

Figure 5. Effect of age on hypoxic 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±SEM (n=8-11).

Figure 6. Concentration-dependent depression by adenosine in P0-3 and P5-8 rats

Adenosine was cumulatively applied to spinal cords isolated from postnatal day 0-3 (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± SEM (n=5-6).

Figure 7. Adenosine release from spinal cord during hypoxia

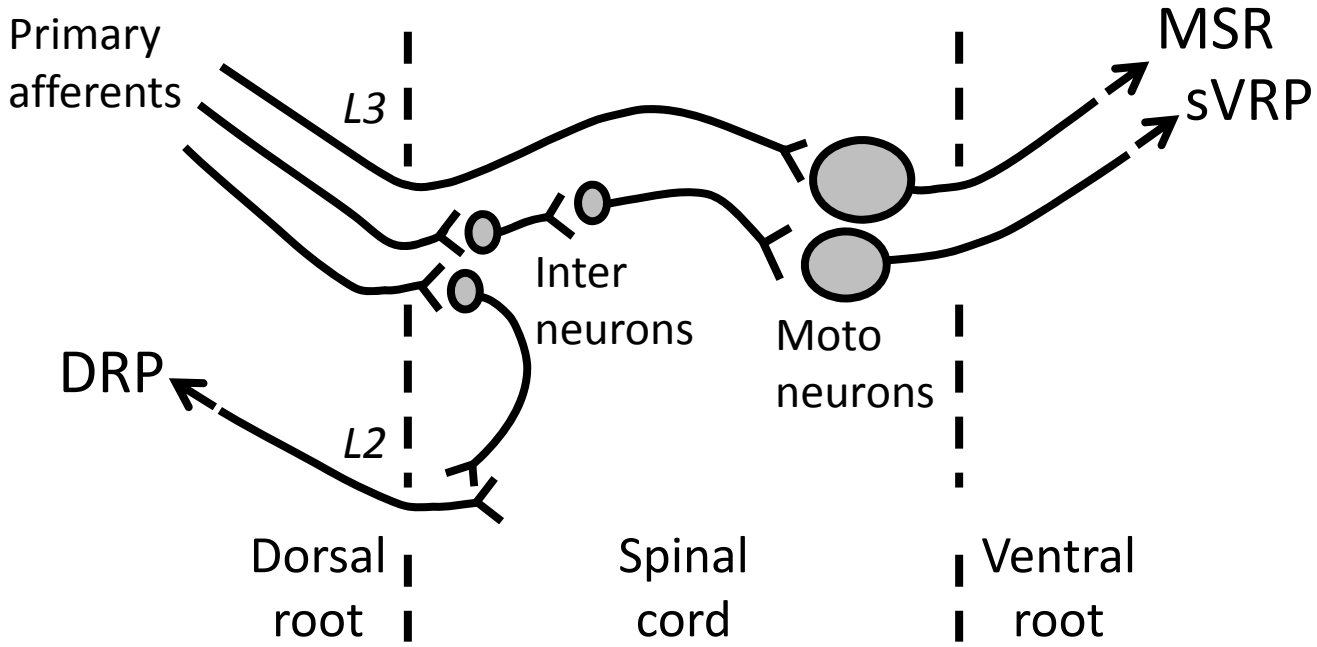
Adenosine/inosine (ADO/INO), inosine (INO) and null sensors were inserted into the dorsal horn of the spinal cord and then hypoxia was applied for 20 min. A, the representative traces of signal current for each sensor observed in a postnatal day 8 (P8) rat at 27°C (left panel), postnatal day 3 (P3) rats at 27°C (middle panel) and 33°C (right panel) are shown. B, the hypoxia-evoked change in adenosine/inosine concentration (Δpurine) 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±SEM (n=5-6). *P<0.05 *vs.* P0-3 rats at 27°C (Dunnett's test).

Figure 8. Effect of temperature on hypoxic 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).

Fig. 1

A


Primary
afferents
L3
MSR
sVRP
Inter
neurons
Moto
neurons
DRP
L2
Dorsal
root
Spinal
cord
Ventral
root

B

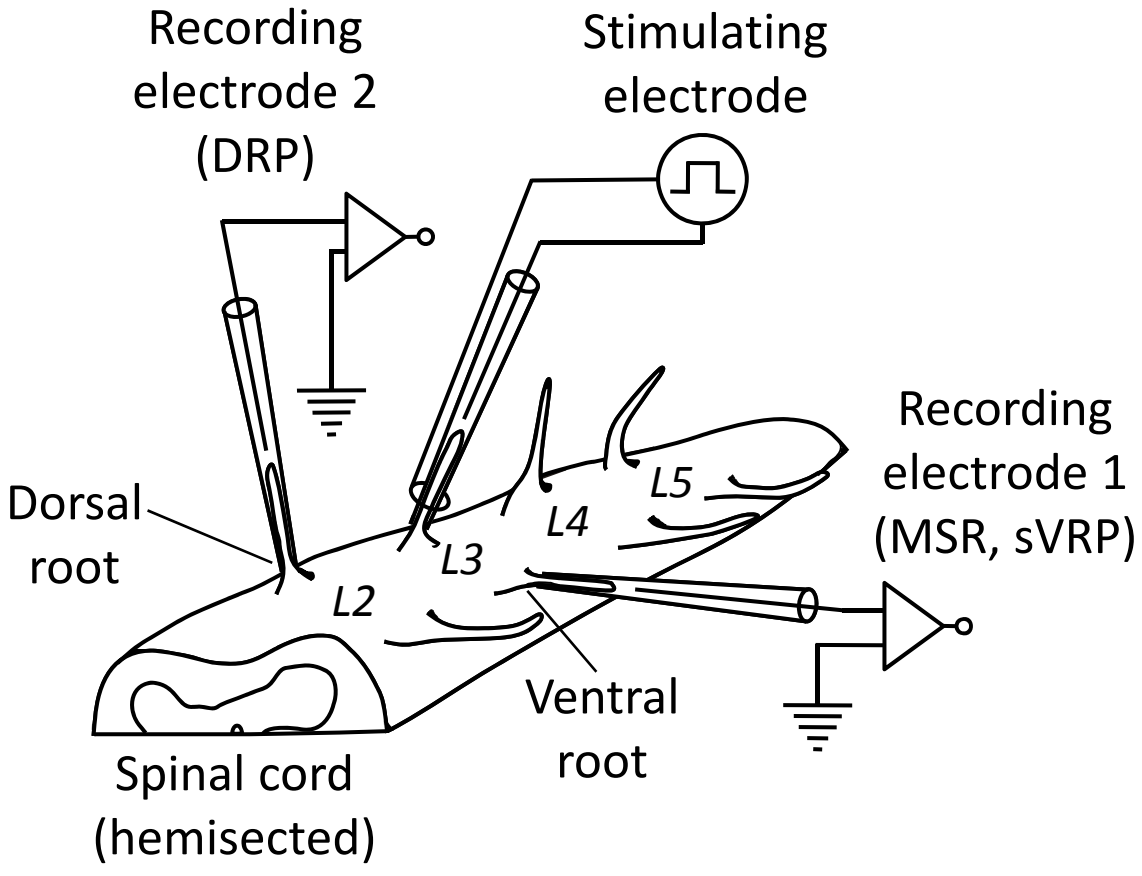
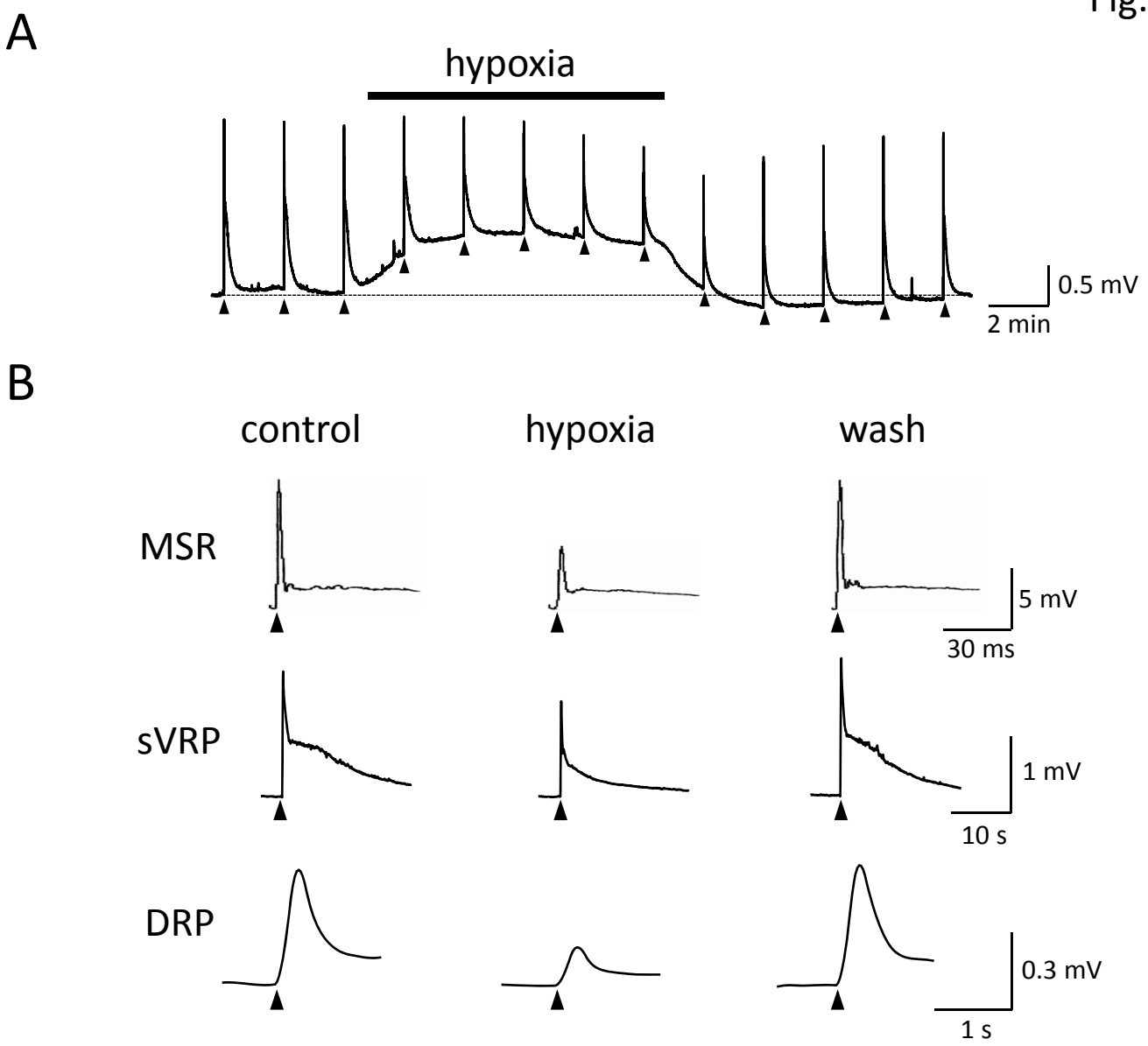

Recording
electrode 2
(DRP)
Stimulating
electrode
Recording
electrode 1
(MSR, sVRP)
Dorsal
root
L5
L4
L3
L2
Ventral
root
Spinal cord
(hemisected)

Fig. 2


A
hypoxia
0.5 mV
2 min
B
control
hypoxia
wash
MSR
5 mV
30 ms
sVRP
1 mV
10 s
DRP
0.3 mV
1 s

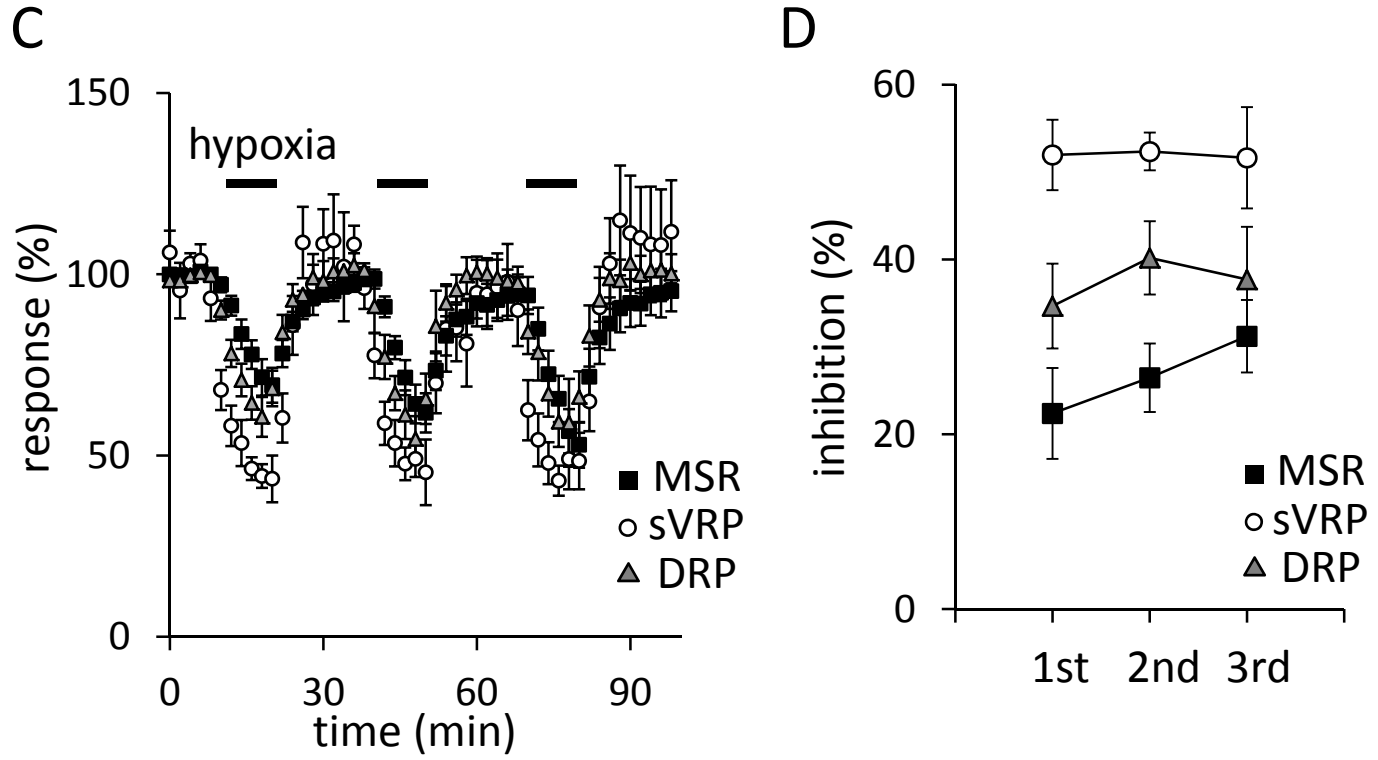

C
hypoxia
response (%)
150
100
50
0
0
30
60
90
time (min)
MSR
sVRP
DRP
D
inhibition (%)
60
40
20
0
1st
2nd
3rd
MSR
sVRP
DRP

Fig. 3

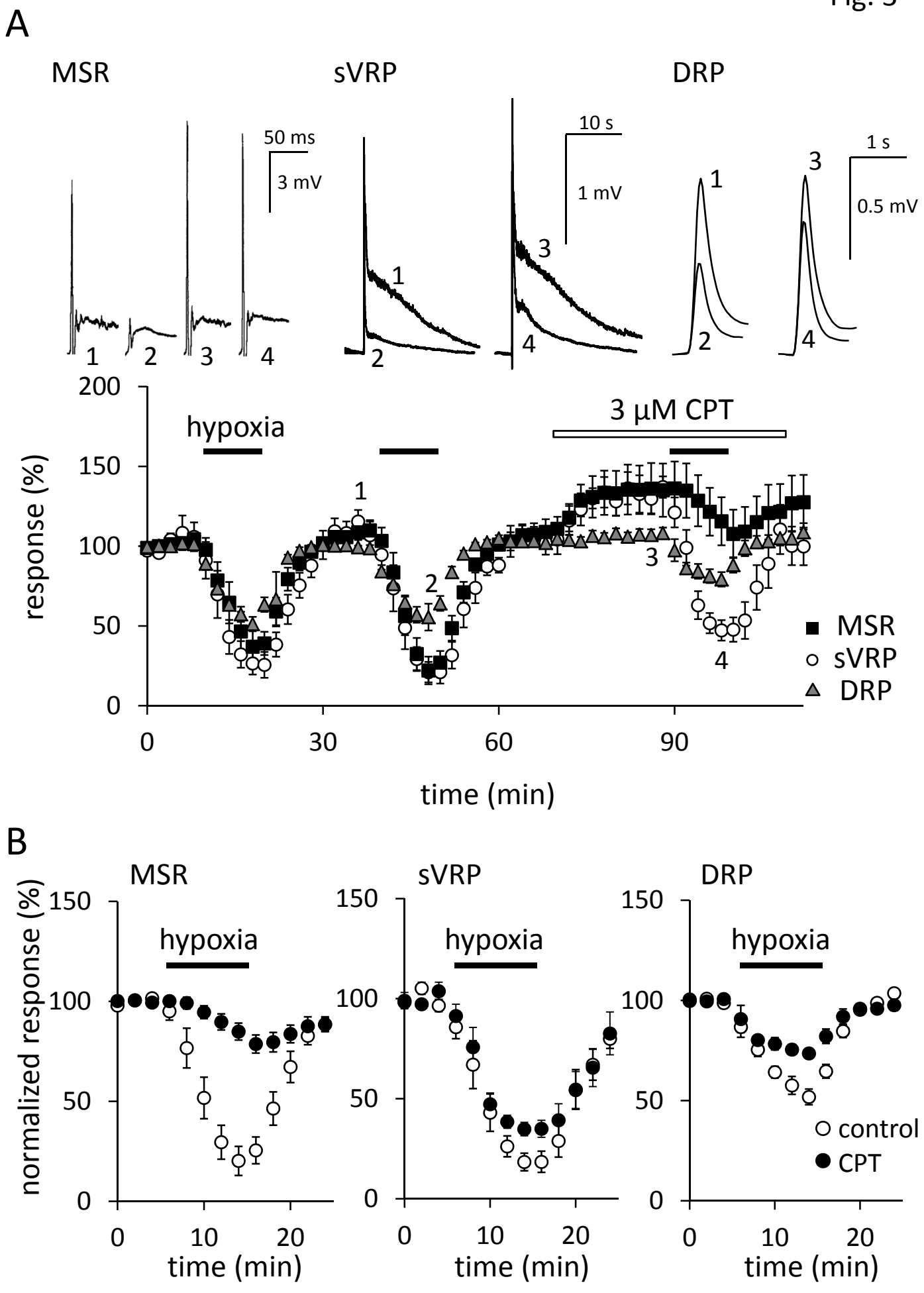
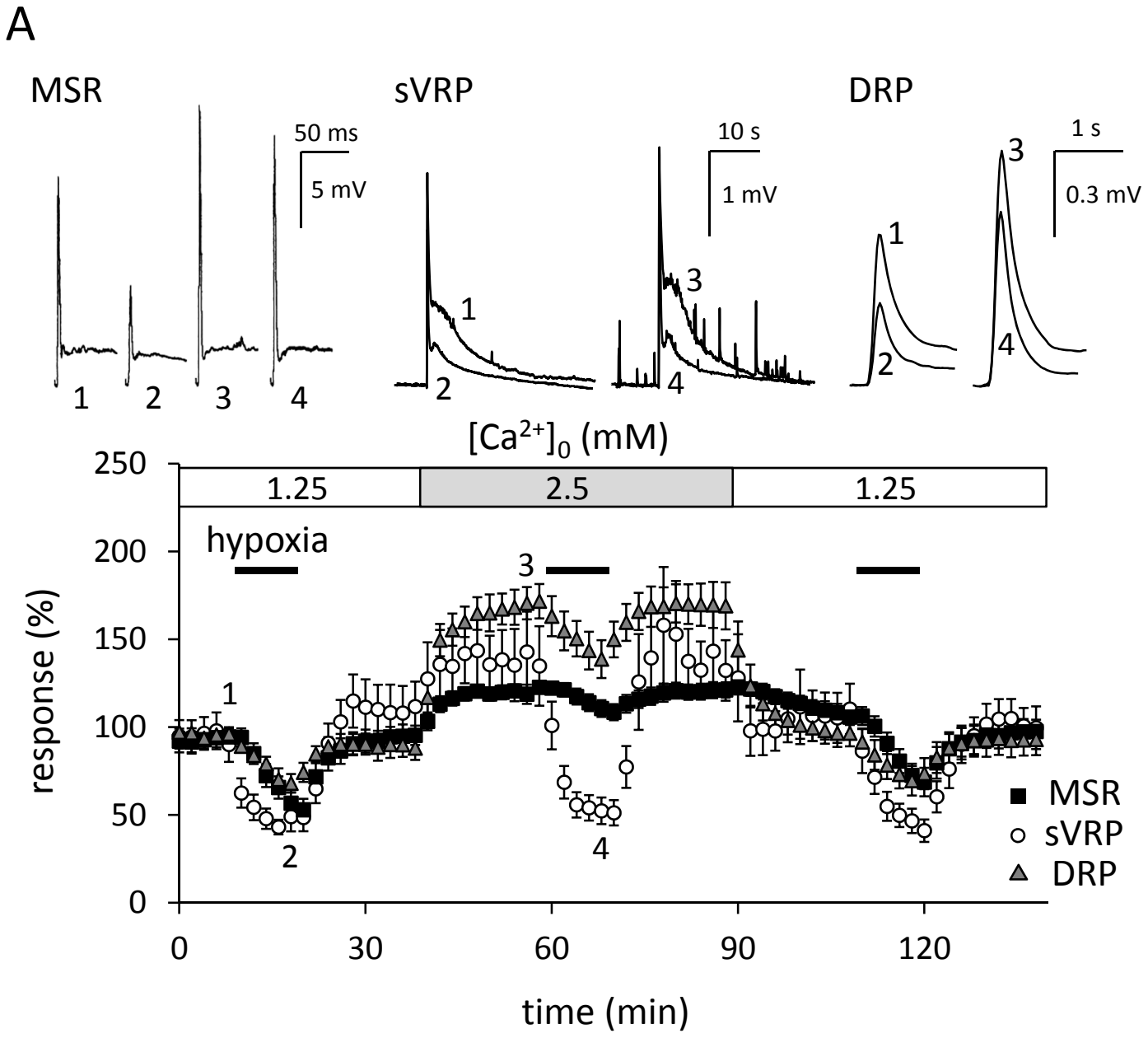

A
MSR
sVRP
DRP
50 ms
3 mV
10 s
1 mV
1 s
0.5 mV
1
2
3
4
hypoxia
3 μM CPT
response (%)
time (min)
MSR
sVRP
DRP
B
normalized response (%)
control
CPT

Fig. 4


A
MSR
sVRP
DRP
50 ms
5 mV
10 s
1 mV
1 s
0.3 mV
1
2
3
4
[Ca2+]0 (mM)
1.25
2.5
1.25
hypoxia
response (%)
MSR
sVRP
DRP
time (min)

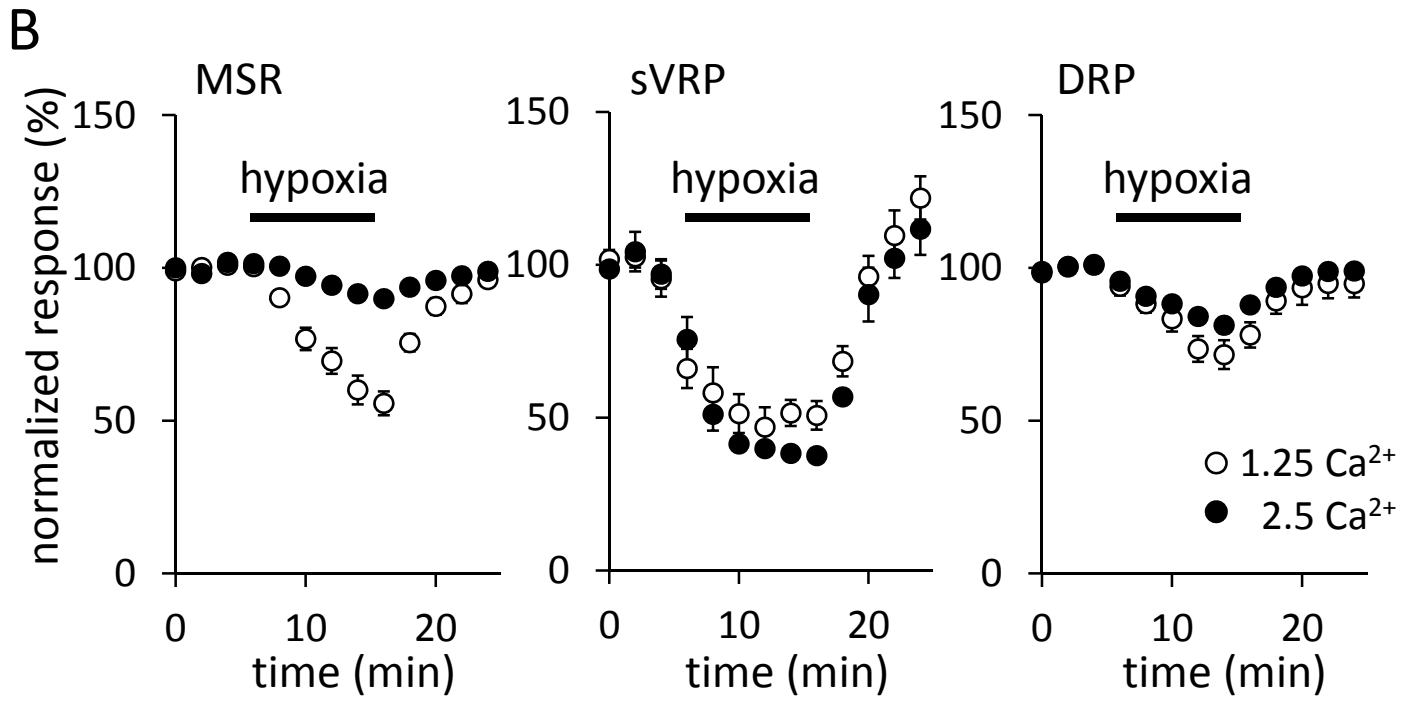

B
MSR
sVRP
DRP
hypoxia
normalized response (%)
1.25 Ca2+
2.5 Ca2+
time (min)

Fig. 5

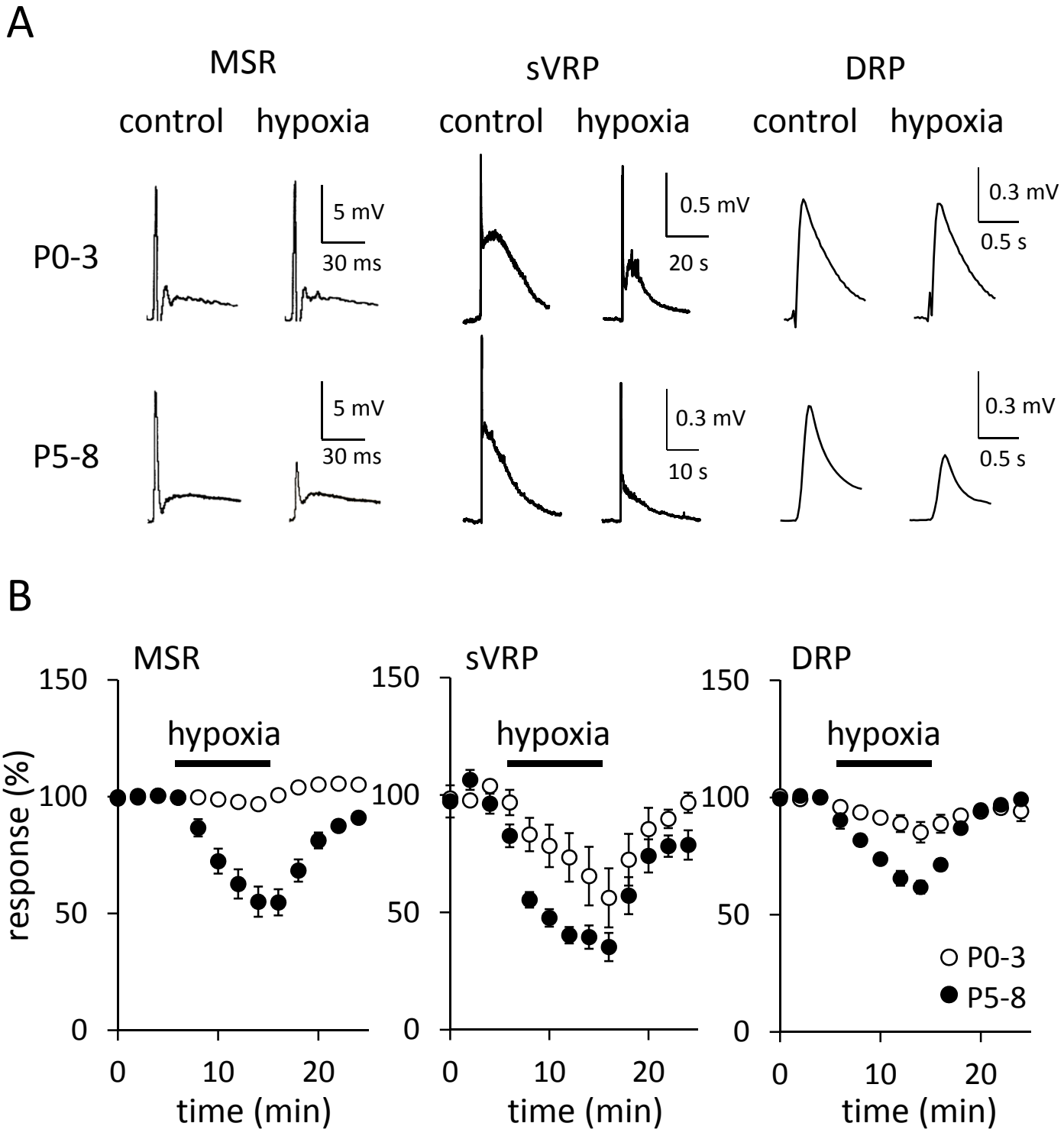

A
MSR
sVRP
DRP
control
hypoxia
control
hypoxia
control
hypoxia
P0-3
P5-8
5 mV
30 ms
0.5 mV
20 s
0.3 mV
0.5 s
5 mV
30 ms
0.3 mV
10 s
0.3 mV
0.5 s
B
MSR
sVRP
DRP
hypoxia
hypoxia
hypoxia
response (%)
150
100
50
0
0
10
20
time (min)
P0-3
P5-8

Fig. 6

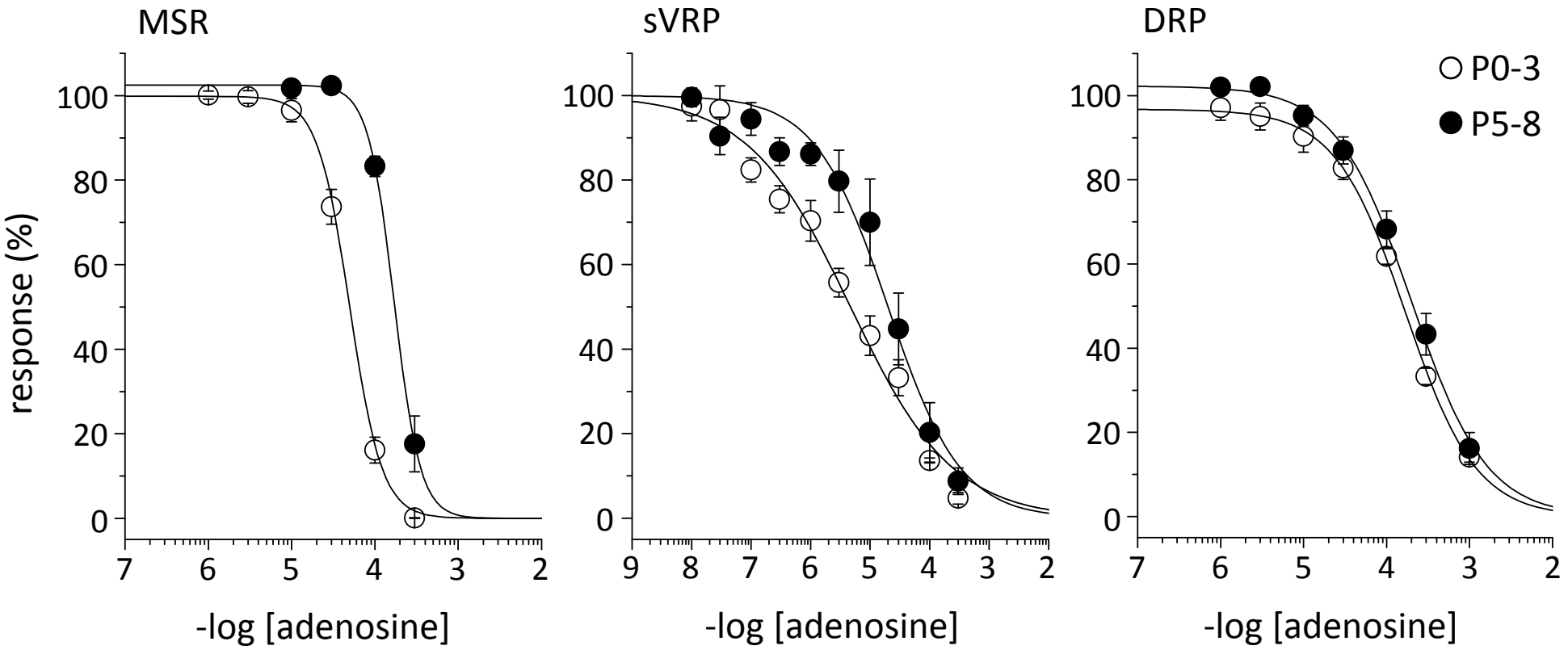

MSR
sVRP
DRP
response (%)
100
80
60
40
20
0
7
6
5
4
3
2
9
8
-log [adenosine]
P0-3
P5-8

Fig. 7

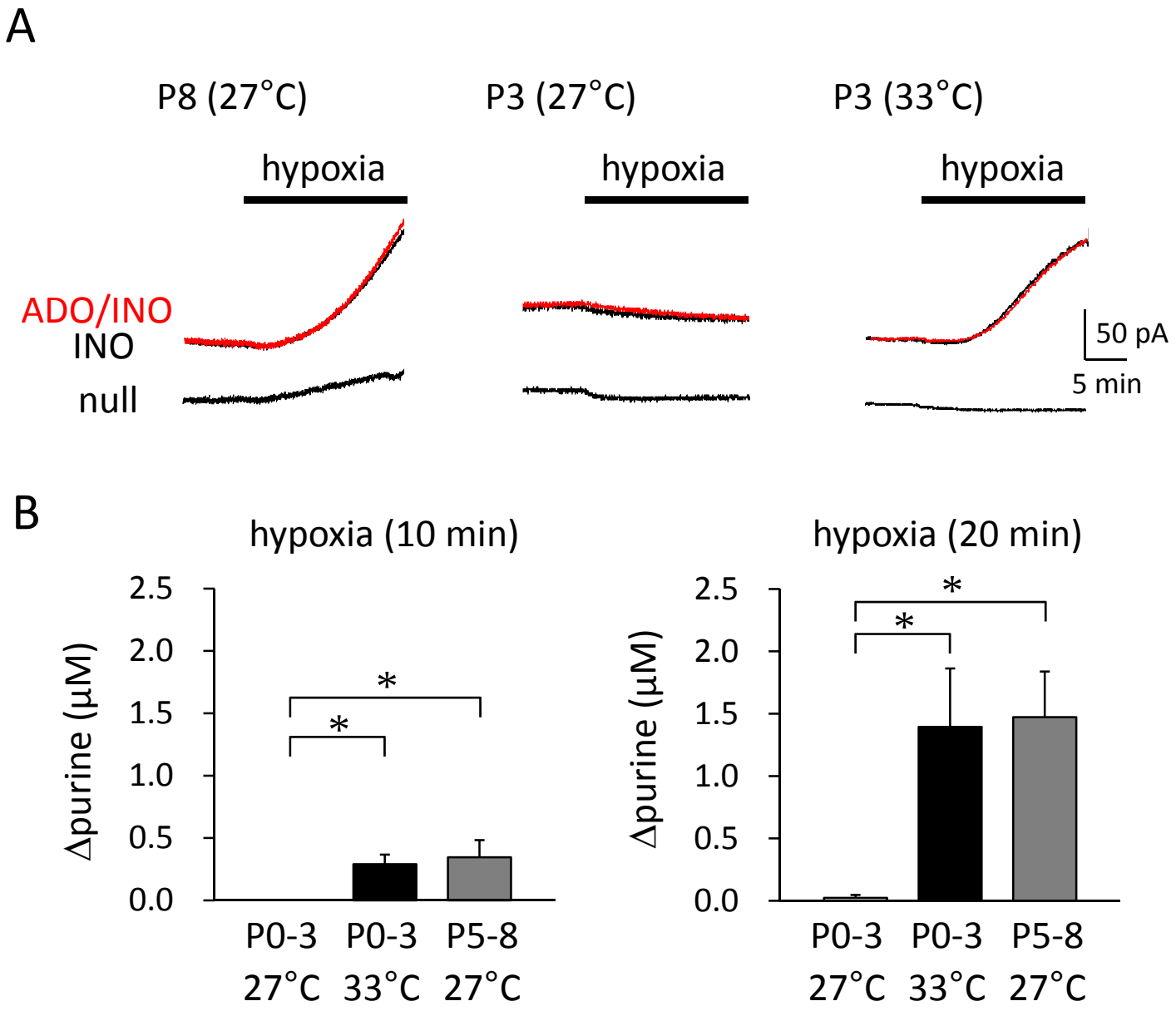
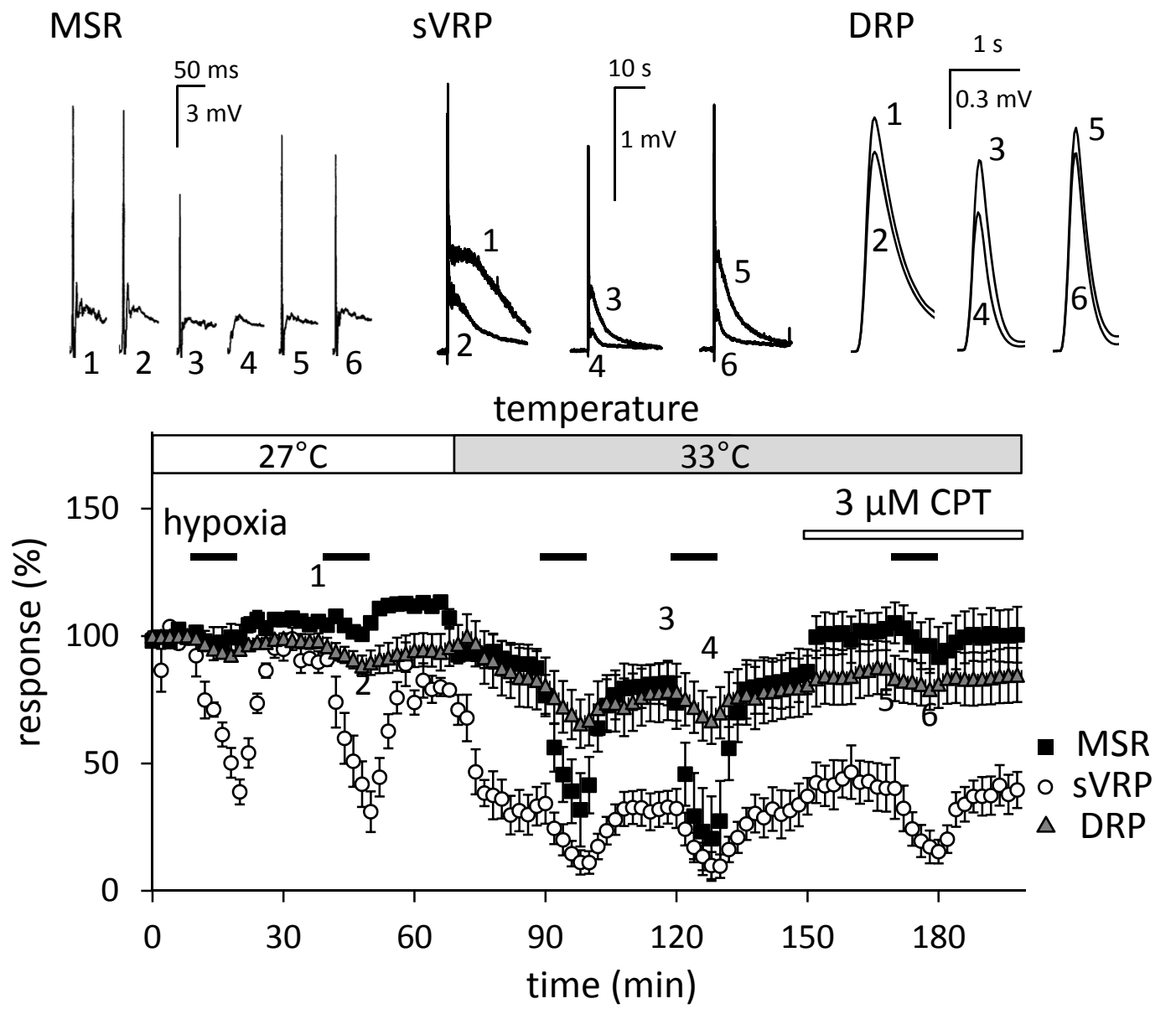
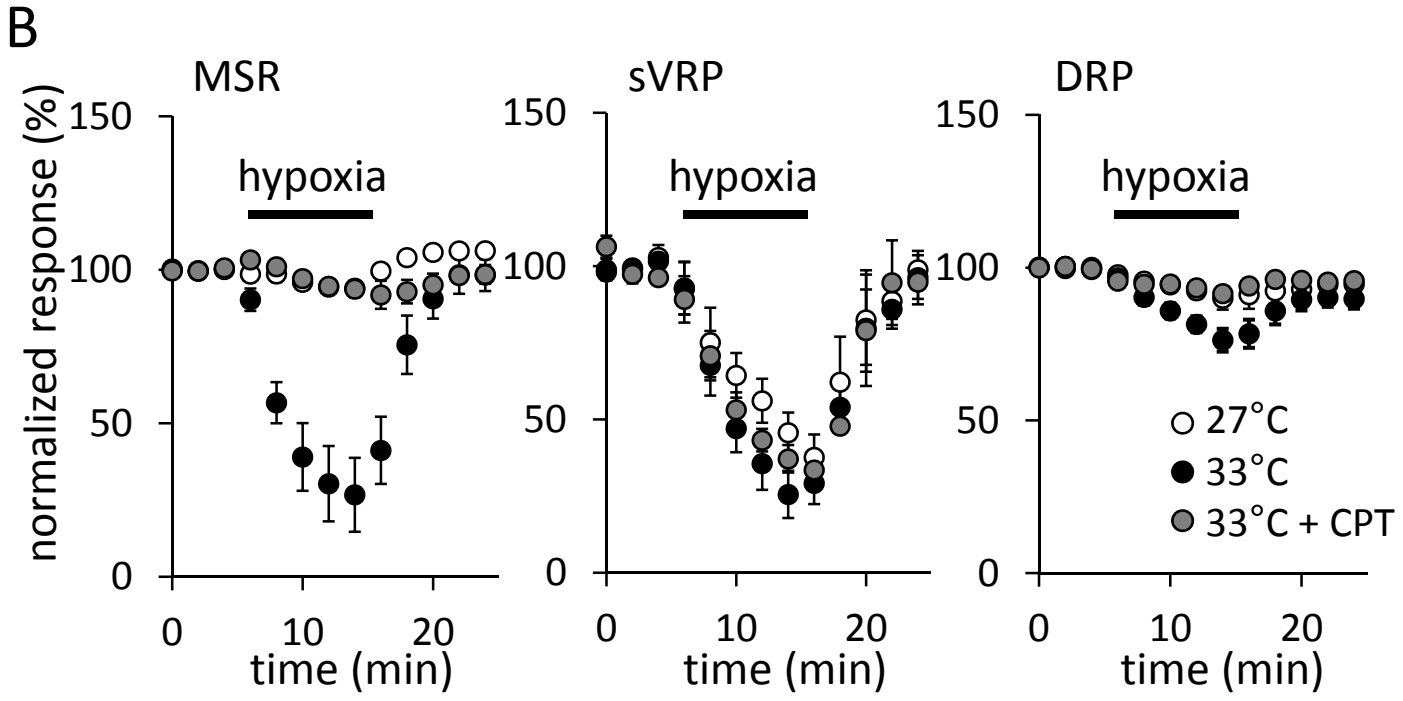

A
P8 (27°C)
P3 (27°C)
P3 (33°C)
hypoxia
hypoxia
hypoxia
ADO/INO
INO
null
50 pA
5 min
B
hypoxia (10 min)
hypoxia (20 min)
Δpurine (μM)
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
*
P0-3 27°C
P0-3 33°C
P5-8 27°C

Fig. 8


A
MSR
sVRP
DRP
50 ms
3 mV
10 s
1 mV
1 s
0.3 mV
temperature
27°C
33°C
3 µM CPT
hypoxia
response (%)
time (min)
MSR
sVRP
DRP


B
MSR
sVRP
DRP
hypoxia
normalized response (%)
time (min)
27°C
33°C
33°C + CPT