Volatile anesthetic sevoflurane pretreatment alleviates hypoxia-induced potentiation of excitatory inputs to striatal medium spiny neurons of mice

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Volatile anesthetic sevoflurane pretreatment alleviates hypoxia-induced potentiation of excitatory inputs to striatal medium spiny neurons of mice

Running title: Preconditioning effects of sevoflurane on hypoxia

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Abstract

Sevoflurane, a commonly used anesthetic in surgery, has drawn attention because of its preconditioning effects in hypoxic conditions. To investigate the preconditioning effects in the striatum, a common site for ischemic stroke, we collected whole-cell current-clamp recordings from striatal medium spiny neurons. In our *in vitro* brain slice experiments, deprivation of oxygen and glucose depolarized the striatal neurons to subthreshold potentials, and the pre-administration of sevoflurane (4%, 15 min) prolonged the time to depolarization. Furthermore, transient hypoxia induced the potentiation of excitatory postsynaptic potentials, which play a part in post-ischemic excitotoxicity. Glibenclamide, a K_{ATP} channel inhibitor, reversed the prolonged time to depolarization and the prevention of the pathological potentiation of excitatory responses, indicating that the short exposure to sevoflurane likely participates in neuroprotection against hypoxia via activation of K_{ATP} channels. A monocarboxylate transporter blocker, 4-CIN, also depolarized striatal neurons. Interestingly, the blockade of monocarboxylate transporters that supply lactate to neurons caused the pathological potentiation, even in the presence of enough oxygen and glucose. In this case, sevoflurane could not prevent the pathological potentiation, suggesting the involvement of monocarboxylate transporters in the sevoflurane-mediated effects. These results indicate that sevoflurane protect striatal neurons from hypoxic damage and alleviate the pathological potentiation. Under these conditions, sevoflurane may become an effective intervention for patients undergoing surgery.

1. Introduction

Hypoxia is a serious perioperative complication. Clinically, humans succumb to brain disorders within three to five minutes of interrupted oxygen supply, which affects patient prognosis (Gisvold *et al.*, 1996). Sevoflurane, a commonly used inhalation anesthetic in surgical procedures, has drawn attention due to its preconditioning effects on hypoxia and ischemia (Kevin *et al.*, 2003; Wang *et al.*, 2007; Wang *et al.*, 2012; Ye *et al.*, 2012; Yang *et al.*, 2015). Although used as an anesthetic, sevoflurane preconditioning is known to have valid protective effects on the heart, brain, and other organs (Kevin *et al.*, 2003; Mullenheim *et al.*, 2003; Kehl *et al.*, 2004; Swyers *et al.*, 2014). These effects are referred to as anesthetic-induced preconditioning. Recent studies have indicated that preconditioning effects are partly mediated by ATP-sensitive potassium (K_{ATP}) channels and are neuroprotective (Zaugg *et al.*, 2002; Ishiwa *et al.*, 2004; Kehl *et al.*, 2004; Adamczyk *et al.*, 2010). K_{ATP} channels, which are inward rectifier potassium channels, are a clinical target of therapeutic agents in diabetes and heart disease (Nakaya, 2014; Rubaiy, 2016). K_{ATP} channels are activated by a decrease in intracellular ATP concentration and hyperpolarize neurons, thereby maintaining their cellular metabolism and membrane potentials. When patients become hypoxemic and ischemic during surgery, these channels may provide protection and serve as a remedy.

Hypoxia and ischemia-induced depolarization leads to excitotoxicity caused by excessive glutamate release, which plays an important role in apoptotic process and neuronal death. Previous *in vitro* studies have reported that oxygen and glucose deprivation potentiate excitatory postsynaptic currents (EPSCs) and potentials (EPSPs) in the hippocampus (Hammond *et al.*, 1994; Hsu & Huang, 1997; Zhou *et al.*, 2015) and the striatum (Bagetta *et al.*, 2008; Calabresi *et al.*, 2002). The excitatory postsynaptic

responses are depressed during transient ischemic conditions and then paradoxically potentiated after reperfusion of glucose and oxygen. This process is considered to be a pathological form of potentiation because of a lack of input specificity, which therefore exerts excitotoxicity on surviving neurons. However, studies have indicated that pathological potentiation and physiological long-term potentiation (LTP) share some common mechanisms, including depolarization of membrane potentials, activation of NMDA receptors, and an elevation of intracellular calcium concentrations during the induction phase. However, volatile anesthetics can reduce neural excitability by stabilizing resting membrane potentials and synaptic transmissions and by inhibiting the induction of LTP (Ishizeki *et al.*, 2008; Wang *et al.*, 2009). In earlier work, we reported that the application of the volatile anesthetic sevoflurane reduced the evoked EPSCs in the hippocampus and the striatum (Oose *et al.*, 2012). Considering the actions of volatile anesthetics, it is worth investigating the preconditioning effects of sevoflurane on the pathological potentiation of EPSPs under conditions of energy deprivation such as during hypoxia and ischemia.

In addition to a supply of glucose and oxygen, lactate transportation is important when neurons become energy deprived (Herzog *et al.*, 2013). In the central nervous system (CNS), astrocytes transfer lactate to neurons as an energy source for controlling neural activity and metabolism (Serres *et al.*, 2003; van Hall *et al.*, 2009; Suzuki *et al.*, 2011; Machler *et al.*, 2016). Monocarboxylate transporters (MCTs) are one group of membrane proteins that transport the lactate to neurons (Haydon & Carmignoto, 2006; Bergersen, 2015). There are four MCT isozymes (MCT1-4) in the CNS. Astrocytes have MCT1 and MCT4, whereas the MCT2 is mainly expressed in neurons. Blockade of MCTs interferes with lactate transport and may deplete the energy supply, especially at the

synaptic sites. A recent study reported that excitatory synaptic transmissions in the brain stem are partly maintained by lactate transportation via MCTs, even when a sufficient supply of glucose and oxygen is present (Nagase *et al.*, 2014). These studies raise the possibility that lactate deprivation might lead to the pathological potentiation of EPSPs, as with post-ischemic potentiation. Interestingly, inhalation of general anesthetics such as isoflurane and sevoflurane increase brain lactate concentrations, particularly in the basal ganglia (Boretius *et al.*, 2013). A higher lactate level has been detected in peripheral blood when inhalation anesthetic is administered compared to intravenous anesthetics (Horn & Klein, 2010). However, at present, lactate deprivation-induced potentiation has been little studied, and the effects of sevoflurane on it are mostly unknown.

The striatum, a basal ganglia structure, is susceptible to failure, which consumes large amounts of oxygen (Nishizaki *et al.*, 1988; Arakawa *et al.*, 2006; Tisserand *et al.*, 2014). The transient hypoxia and blockade of MCTs may induce similar ischemic conditions, except for the lactate deficiency, because partial energy restriction may occur to various degrees in the penumbra, the surrounding region of the ischemic core. If sevoflurane has preconditioning effects on striatal neurons under hypoxic conditions, this indicates that the K_{ATP} channel is a likely sevoflurane target, because of the importance of the K_{ATP} channel in the protective effect against ischemia in the heart. In this study, we examined (1) whether the pre-administration of sevoflurane protects the striatal neurons from hypoxia-induced depolarization and alleviates the post-ischemic potentiation of excitatory synaptic transmission, and (2) whether the pathological potentiation is induced by the blockade of lactate transporter MCTs and relieved by sevoflurane pre-administration.

2. Materials and Method

2.1. Slice preparation

All experimental procedures were approved by the Animal Care and Use Committee of the Tokyo Metropolitan Institute of Gerontology and carried out in accordance with the Guidelines for Animal Experimentation of the Ministry of Education, Culture, Sports, Science, and Technology of Japan and the guidelines of the NIH in the USA. At postnatal day (P) 7-35, 88 male C57BL/6J mice were anesthetized with ether and decapitated. The brains were rapidly removed and put into ice-cold artificial cerebrospinal fluid (ACSF) containing 124 mM NaCl, 3 mM KCl, 1 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 2.4 mM $CaCl_2$, and 10 mM glucose, buffered to pH 7.4 with $NaHCO_3$ (26 mM) and saturated with 95% O_2 and 5% CO_2. Coronal brain slices (300-μm thick) were prepared with a Pro 7 Liner Microslicer (Dosaka, Kyoto, Japan), incubated in ACSF at 30°C for 60 min, and then maintained in ACSF at room temperature (21-25°C) before use.

2.2 Electrophysiology

The slices were placed on a recording chamber, which was continuously perfused with ACSF, and incubated with 95% O_2 and 5% CO_2 at rate of 1.5-2 mL/min at 30°C. A whole-cell patch-clamp technique was performed using an EPC9/2 amplifier (HEKA Elektronik, Lamrecht/Pfalz, Germany). Patch pipettes (4-6 MΩ) were made from borosilicate glass capillaries and filled with (in mM): 129 mM K-gluconate, 11 mM KCl, 2 mM $MgCl_2$, 10 mM HEPES, 0.1 mM EGTA, 0.3 mM GTP, 0.1 mM spermine, and 0.5% biocytin, which was brought to 280 mOsm and pH 7.3 with KOH. In the EPSP experiments, the internal solution contained 0.4 mM Na_2ATP to avoid unexpected depolarization that can reduce EPSP amplitude. To block synaptic transmissions, 6-

cyano-7-nitroquinoxa-line-2,3-dione (CNQX, 10 μM), 2-amino-5-phosphonovaleric acid (AP5, 25 μM), and picrotoxin (100 μM) were added to the external solution. For the EPSP experiments, only picrotoxin was used to observe excitatory responses. When the EPSP potentiation was examined under hypoxic conditions, Mg^{2+} was removed from the external solution. The striatal medium spiny (MS) neurons were identified according to their morphology using an infrared differential interference contrast video microscope (BX50WI; Olympus, Tokyo, Japan) and their characteristic firing patterns in response to depolarizing and hyperpolarizing step currents. After recordings, the slice was immunostained for biocytin to confirm a recording site (Ando *et al.*, 2014; Sugasawa *et al.*, 2017). Measurements were not corrected for liquid junction potentials of 10 mV.

2.3. Drugs

Sevoflurane was manufactured by Maruishi Pharmaceutical Co., Ltd (Osaka, Japan). 2-amino-5-phosphonovaleric acid (AP5) was obtained from Tocris Bioscience (Bristol, UK). Picrotoxin, 6-cyano-7-nitroquinoxa-line-2,3-dione (CNQX), the K_{ATP} channel blocker glibenclamide, the MCT inhibitor α-cyano-4-hydroxycinnamic acid (4-CIN), and all other drugs were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.4. Volatile anesthetic sevoflurane application and measurement

The volatile anesthetic sevoflurane was applied into ACSF with 95% O_2 and 5% CO_2 at twice the clinically effective concentration, 4%, equal to two minimum alveolar concentration (MAC), via a Meratec (Senko Medical Instrument, Japan). Many studies show that sevoflurane preconditioning effects require a sufficient concentration, roughly 1.5-2 MAC of sevoflurane. The concentration was continuously monitored using a Life

Scope (Nihon Kohden, BSM-5132, Japan). Sevoflurane in the ACSF was sampled at the outlet of the perfusion system, and the sevoflurane concentration was measured using gas chromatography (Oose *et al.*, 2012).

2.5. Creation of a hypoxic and hypoglycemic environment

In this study, we used a hypoxic environment that was made from sucrose extracellular fluid containing 124 mM NaCl, 3 mM KCl, 1 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 2.4 mM $CaCl_2$, and 10 mM sucrose, buffered to pH 7.4 with $NaHCO_3$ (26 mM) and saturated with 95% N_2 and 5% CO_2.

2.6. Statistics

All data are shown as means ± SEM. The statistical significance of the difference was assessed using a Student's paired or unpaired *t* test, Chi-squared test, and one-way ANOVA with *post hoc* Bonferroni correction (Prism version 6.0, GraphPad software, La Jolla, CA, USA).

3. Results

3.1. Sevoflurane pre-administration extended the time to depolarization in MS neurons, and glibenclamide reversed the extended time

Figure 1A shows the membrane potentials recorded from MS neurons (P14-35). Under hypoxic conditions, MS neurons were suddenly depolarized by 20-40 mV with 5-15 min latency. Pre-administration of sevoflurane (4%, 15 min) shifted the potential curve toward the right and extended the time for depolarization to the subthreshold potential (-50 mV). When neurons recorded without sevoflurane were considered as a control group, the prolongation of the time to depolarization with sevoflurane pre-administration was significant compared with the control (Fig. 1B, 1C; control, 5.62 ± 1.00 min, n = 10; sevoflurane, 9.65 ± 0.75 min, n = 11; one-way ANOVA with *post hoc* test, $F_{(3, 37)} = 7.06$, *$P = 0.007$). The application of glibenclamide reversed the sevoflurane-extended time to depolarization at -50 mV (Fig. 1B), although the time to depolarization was unaffected by glibenclamide alone (sevoflurane and glibenclamide, 4.56 min ± 1.10, n = 8; glibenclamide, 5.18 min ± 0.72, n = 12). These results indicate that sevoflurane might have the effect of slowing hypoxia-induced depolarization via the activation of K_{ATP} channels.

In contrast, MS neurons (P7) kept their membrane potential under the 15-min hypoxic condition (Supplemental Fig. 1A). Sevoflurane hardly changed the membrane potential of MS neurons in P7 (Supplemental Fig. 1B). The depolarization caused by 5-min hypoxia (ΔVm) of P7 neurons was smaller than that of P14-35 neurons, Supplemental Fig. 1C, D). The P7 neurons could generate action potentials after 15 min of hypoxia (data not shown). Although there might be a difference in the mechanism of reaction to hypoxia between P7 and P14-35 neurons, we did not further examine the P7 neurons and

conducted subsequent experiments only in P14-35 MS neurons.

3.2. The MCT inhibitor 4-CIN shortened the time to depolarization in MS neurons

Recent studies have demonstrated that neurons express MCT2, which transports lactate from glia to utilize it as an energy source, suggesting that lactate might serve to stabilize membrane potentials during deprivation of glucose and oxygen (Haydon & Carmignoto, 2006; Bergersen, 2015). We evaluated whether sevoflurane pre-administration could prevent hypoxia-induced depolarization under the blockade of MCTs. As seen in the Fig. 2A, B, a bath-applied MCT inhibitor, 4-CIN (1 mM), shortened the sevoflurane-prolonged time to depolarization at -50 mV (sevoflurane, 9.65 ± 1.00 min, n = 11; sevoflurane and 4-CIN, 5.25 ± 0.93 min, n = 9; one-way ANOVA, $F_{(3, 40)} = 6.11$, *$P = 0.0016$). Without the sevoflurane pretreatment, 4-CIN slightly shortened the time to depolarization compared to the control group, but there was no significant difference between them (4-CIN group, 4.42 ± 1.06 min, n = 14; control group, 5.62 ± 1.00 min, n = 10).

We next examined whether the MCT blockade itself can depolarize MS neurons, even in the presence of a sufficient supply of oxygen and glucose. Membrane potentials were recorded using an internal solution containing 0.4 mM ATP to satisfy experimental conditions in subsequent EPSP experiments. As shown in Figure 3A, B, 4-CIN application induced transient depolarization in some MS neurons. Sevoflurane pre-administration did not change the integrated value of the depolarized area during 4-CIN application (Fig. 3C; sevoflurane and 4-CIN, n = 13; 4-CIN, n = 14; $P = 0.646$; Student's t test) or the number of depolarized cells (Fig. 3D, Chi-squared test). In addition, glucose depletion also depolarized MS neurons (Supplemental Fig. 2A, B), and the depolarization

seemed to be irreversible. Sevoflurane pre-administration had no effect on the integrated value of the depolarized area during glucose depletion or on the number of depolarized MS neurons (Supplemental Fig. 2C, D).

3.3. Sevoflurane prevented the oxygen and glucose deprivation-induced excessive plasticity of EPSPs

In addition to hypoxia-induced depolarization, hypoxia induces pathologic potentiation of excitatory synaptic transmission. When cells fall into short-term hypoxia and then oxygen is given again, the EPSPs become larger than normal and may cause reperfusion injury (Gao *et al.*, 1998; Gajendiran *et al.*, 2001; Haghani *et al.*, 2016). We investigated the effect of sevoflurane pretreatment on the oxygen and glucose deprivation-induced EPSP potentiation. Evoked EPSPs were recorded using an internal solution containing 0.4 mM ATP to avoid irregular depolarization that can reduce EPSP amplitude. As shown in Fig. 4A, the 3-min deprivation of oxygen and glucose reversibly depolarized the MS neurons by -6.7 ± 1.1 mV in the control group, -6.7 ± 2.8 mV in the sevoflurane group, and -4.7 ± 0.6 mV in the sevoflurane + glibenclamide group. The amplitude of the EPSPs became smaller immediately after the deprivation of oxygen and glucose, and then gradually increased during the reperfusion period (Fig. 4A1, 4B). Sevoflurane pre-administration (4%, 15 min) inhibited the potentiation of EPSP after hypoxia (Fig. 4A2, 4B). Glibenclamide administration canceled the effect of sevoflurane (Fig. 4C). There were significant differences between the control, sevoflurane administration, and sevoflurane administration with glibenclamide groups (Fig. 4D; control group, 202.6 ± 34.7 %, n = 7; sevoflurane group, 88.2 ± 10.6 %, n = 5; sevoflurane and glibenclamide group, 263.6 ± 15.2 %, n = 8; one-way ANOVA, $F_{(2, 17)} = 12.2$, *P =

0.0005). Sevoflurane inhibited hypoxia-induced excessive EPSPs, and glibenclamide abolished its effects.

3.4. 4-CIN application potentiated the evoked EPSPs despite the pre-administration of sevoflurane

Next, we measured the EPSP under administration of 4-CIN for 10 min. Figure 5A and 5B show that the EPSP amplitude of the 4-CIN group increased over time. The 10-min application of 4-CIN caused subtle depolarization in both groups (4-CIN, -2.7 ± 0.9 mV; 4-CIN and sevoflurane, -4.2 ± 1.1 mV). The EPSP amplitude gradually increased after the 4-CIN application regardless of whether sevoflurane was used. Figure 5C shows a summary of EPSP amplitude in each group at 15 min after the 4-CIN application. There was no significant difference in the EPSP potentiation among the groups (control, 202.6 ± 34.7 %, n = 7; 4-CIN, 173.0 ± 31.5 %, n = 6; 4CIN and sevoflurane, 186.8 ± 41.9 %, n = 6; one-way ANOVA, $F_{(3, 20)}$ = 2.09, P = 0.1852). Sevoflurane hardly influenced the 4-CIN-induced potentiation, whereas lactate deprivation might be a factor in inducing EPSP potentiation.

Finally, we examined the requirement of NMDA receptor activity for the 4-CIN-induced EPSP potentiation. Continuous application of the NMDA receptor antagonist AP-5 prevented 4-CIN-induced EPSP potentiation (4-CIN and AP-5, 101.8 ± 12.6%, n = 8; Student's t test, P = 0.0257).

4. Discussion

4.1. Sevoflurane alleviated hypoxia-induced EPSP potentiation, and glibenclamide abolished this effect

It is well known that the myocardium, brain, and other organs have the capability, called ischemic preconditioning (IPC), to protect themselves from ischemic events (Nakano *et al.*, 2000; Perez-Pinzon, 2007; Steiger & Hanggi, 2007). The mechanism of IPC is a series of molecular pathways in which K_{ATP} channels play a part. These channels are found in various tissues, including the brain. Anesthetic-induced preconditioning has effects similar to IPC, showing neuroprotection against hypoxia. Some theorists argue that the sevoflurane preconditioning mechanism also involves intracellular signaling via K_{ATP} channels. In this study, we investigated sevoflurane preconditioning in striatal neurons and found that 4% sevoflurane pretreatment delayed subsequent hypoxia-induced depolarization in MS neurons (Fig. 1A).

This effect was blocked by the K_{ATP} channel antagonist glibenclamide, whereas glibenclamide itself did not shorten the time to depolarization (Fig. 1C). It may be that sevoflurane has a protective effect by delaying depolarization under hypoxic environments, and the preconditioning effects of sevoflurane seem to include the activation of K_{ATP} channels. However, it is unclear whether the sevoflurane effects mainly involve the activation of membrane K_{ATP} channels or mitochondrial K_{ATP} channels. Previous research indicates that mitochondrial K_{ATP} channels are mainly related with neuroprotection in cerebral ischemia (Adamczyk *et al.*, 2010). Detailed study is needed to evaluate the effects of sevoflurane on membrane compared to mitochondrial K_{ATP} channels. We also tested the effects of 5 and 10 min pre-administration of sevoflurane. In

those experiments, sevoflurane did not show consistent results. Therefore, 15 min pre-administration of 4% sevoflurane is considered the minimal dose for protective effects against ischemic conditions.

Reperfusion injury is a complication that can occur during surgery and can progress to organ failure. Excessive excitatory input to neurons is assumed to play an important role in neuronal cell death triggered by reperfusion injury. We observed prominent EPSP potentiation after short hypoxia. Calabresi *et al*. (2002) reported that oxygen and glucose deprivation induce post-ischemic long-term synaptic potentiation (i-LTP) at corticostriatal synapses. They argued that i-LTP may trigger apoptotic neuronal death by increasing neuronal excitability. However, the induction of i-LTP requires depolarization, activation of NMDA receptors, and Ca^{2+} influx into neurons, sharing common features with physiological LTP. Our results also confirmed that EPSPs, which were made temporarily smaller by hypoxia, began subsequently to increase and lasted throughout the period of the experiment. As we expected, the pre-administration of sevoflurane delayed the hypoxia-induced depolarization and inhibited the pathological EPSP potentiation. These effects of sevoflurane were abolished by glibenclamide (Fig. 4). This could mean that sevoflurane prevented excessive EPSPs and contributed to reducing the excitability of cells via activation of K_{ATP} channels. In this regard, sevoflurane may reduce the reperfusion injury caused by cerebral infarction and hypoxia in perioperative periods via the activation of K_{ATP} channels. To be consistent with previous studies (Calabresi *et al.*, 2002; Bagetta *et al.*, 2008), we removed Mg^{2+} from ACSF in the hypoxia-induced EPSP potentiation experiments. We also tried using Mg^{2+}-containing standard ACSF, and observed the hypoxia-induced potentiation in three out of five neurons (156.3 ± 12.4%, $n = 3$). Thus, it seems reasonable to suppose that hypoxia induces

the pathological potentiation in patients under ischemia.

4.2. MCT inhibition promoted EPSP potentiation, and sevoflurane did not suppress it

Neurons are supported by surrounding glial cells that have various functions, including controlling neuronal energy and metabolism. It has been proposed that lactate transported by MCTs might be involved in the maintenance of synaptic transmission. Nagase *et al.* (2014) demonstrated that lactate plays an important role in synaptic transmission in the nucleus of the solitary tract, even in a state where there is oxygen and glucose. Considering the involvement of MCTs in energy balance and synaptic transmissions, the blockade of MCTs could induce pathological EPSP potentiation, as hypoxia does in the striatum. In this study, 10-min administration of 4-CIN reduced the EPSP amplitude immediately and reversibly, indicating that lactate made by the astrocyte-to-neuron shuttle maintains synaptic transmissions also in the striatum. Importantly, the amplitude of EPSPs became larger after the administration of 4-CIN in the presence of glucose and oxygen. Our study demonstrated, for the first time to our knowledge, that the blockade of MCTs can induce EPSP potentiation in the striatum, providing valuable evidence for the involvement of lactate deficiency in the mechanisms of excitotoxicity.

Sevoflurane pre-administration could not suppress the EPSP potentiation caused by 4-CIN, though hypoxia-induced EPSP potentiation was inhibited. One simple explanation for this difference is that sevoflurane may facilitate the MCT-maintaining energy supply pathway and/or act upstream of MCTs. Considering that lactate produced and stored in glial cells is transported to neurons via MCTs (Serres *et al.*, 2003; Haydon & Carmignoto, 2006; Suzuki *et al.*, 2011; Bergersen, 2015; Machler *et al.*, 2016), 4-CIN may interfere with all the phenomena that depend on lactate transportation. If this is the

case, sevoflurane might activate mitochondrial K_{ATP} channels. However, striatal neurons express numerous types of potassium channels, including K_{IR} and K_{AS} as well as K_{ATP} channels. As many of these potassium channels are affected by volatile anesthetics, sevoflurane might have a broad effect on various types of potassium channels. Another possible reason for the ineffectiveness of sevoflurane is the magnitude of depolarization during the induction phase. According to previous studies and this one, there might be a number of differences between the 4-CIN- and hypoxia-induced responses; 4-CIN application causes relatively small depolarization and reduces NMDA current (Nagase *et al.*, 2014), whereas hypoxia effectively depolarizes neurons and activates NMDA channels (Hammond *et al.*, 1994; Hsu & Huang, 1997; Calabresi *et al.*, 2002; Bagetta *et al.*, 2008; Zhou *et al.*, 2015). As shown in Fig. 5, 4-CIN application depolarized cells, but the depolarization was transient and small compared with those in the hypoxia and the glucose deprivation groups (Fig. 4, Supplemental Fig. 2). Hypoxia depolarized neurons by -6.7 mV for 3 min, whereas 10-min 4-CIN application caused -2.7 to -4.2 mV depolarization. In our preliminary experiments, 5-min 4-CIN application caused subtle changes and could not induce the potentiation (data not shown). In general, K_{ATP} channels effectively exert their effects at more depolarized potentials, which is apart from the equilibrium potential of potassium (-98 mV in our study). In this regard, at a moderately depolarized potential, the effects of sevoflurane via K_{ATP} channels would be small. Furthermore, the MCT blockade reduced NMDA receptor-mediated EPSCs and delimited postsynaptic depolarization, causing momentary accumulation of Na^+ that reduced the driving force for EPSPs (Nagase *et al.*, 2014), suggesting different induction mechanisms from those of the hypoxia-induced potentiation. If so, it is unlikely that sevoflurane has a common action on the two forms of potentiation. In any case, the pretreatment effects of

sevoflurane on the pathological potentiation and the energetic interaction between neurons and astrocytes require additional studies. On the other hand, in the recordings made in Mg^{2+}-containing ACSF (Fig. 2), 4-CIN did not depolarize the membrane potential. A possible explanation for this discrepancy is that Mg^{2+} might stabilize the membrane potential by blocking NMDA receptors and/or by directly acting on the membrane, although future studies are needed to elucidate this.

A previous study has shown that higher lactate levels are detected in peripheral blood under inhalation anesthetics (Horn & Klein, 2010). In addition, sevoflurane increases the concentration of brain lactate levels, particularly in the basal ganglia (Boretius *et al.*, 2013). Although sevoflurane did not modulate the 4-CIN-induced phenomena in our *in vitro* brain slice experiments, there is a need for further investigation of systemic lactate metabolism under anesthesia. However, our current research revealed that the sevoflurane preconditioning effects are partly related to K_{ATP} channels under hypoxic conditions. Short exposure to sevoflurane may protect striatal MS neurons from depolarization and from excessive EPSP potentiation via activation of K_{ATP} channels. These results indicate the possibility that sevoflurane has a neuroprotective function against hypoxic conditions and can serve as an effective adjuvant for patients undergoing surgery.

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Conflict of interest statement

The authors declare no conflicts of interest.

Author contributions

MF, MM and KN designed the research. MF, NA, YS, RI and KN collected electrophysiological data. SN and MM performed histological experiments. MF and MM wrote the manuscript.

Data Accessibility

Data will be available upon request to the corresponding author.

Abbreviations

4-CIN, α-cyano-4-hydroxycinnamic acid; ACSF, artificial cerebrospinal fluid; AP5, 2-amino-5-phosphonovaleric acid; ATP-sensitive potassium channels; CNQX, 6-cyano-7-nitroquinoxa-line-2,3-dione; GABA, γ-aminobutyric acid; K_{ATP} channels, MCT, monocarboxylate transporters; MS, medium spiny; P, postnatal day.

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Figure legends

Fig. 1. Sevoflurane pre-administration extended the time to depolarization in MS cells, and glibenclamide reversed the extended time

(A, B) Resting membrane potentials recorded from MS neurons (P14-35) with an ATP-depleted internal solution. To block excitatory and inhibitory synaptic transmissions, CNQX, AP5, and picrotoxin were added to the external solution. Solid and dashed lines indicate the groups with and without the sevoflurane pre-administration (sevoflurane 4%, 15 min). Glibenclamide was added in (B). (C) Summary data of the time required for the subthreshold potential at -50 mV. One-way ANOVA with *post hoc* test, $^*P < 0.05$.

Fig. 2. Bath-applied 4-CIN restored the time to depolarization in MS cells

(A) Resting membrane potentials recorded from MS cells with an ATP-depleted internal solution. To block excitatory and inhibitory synaptic transmissions, CNQX, AP5, and picrotoxin were added to the external solution. Solid and dashed lines indicate the groups with and without the application of 4-CIN (1 mM), respectively. Sevoflurane was applied in both groups. (B) Summary data of the time to depolarization in MS neurons. One-way ANOVA with *post hoc* test, $^*P < 0.05$.

Fig. 3. Partial energy restriction by 4-CIN transiently depolarized the membrane potential in striatal MS neurons

(A, B) Resting membrane potentials recorded from MS neurons with the internal solution containing 0.4 mM ATP. Averaged values are overlaid as thick lines. Bath-applied 4-CIN (1 mM) caused transient depolarization (thin lines) regardless of the application of

sevoflurane (4%, 15 min). (C) Integrated values of the depolarized area measured during the period of 4-CIN application. There was no significant difference between both groups; Student's *t* test. (D) The numbers of depolarized and non-depolarized neurons were counted during the period of 4-CIN administration. No significant difference was observed; Chi-squared test; depo (+): depolarization more than 20 mV from the resting membrane potential during the period of 4-CIN administration; Chi-squared test.

Fig. 4. Sevoflurane pre-administration prevented hypoxia-induced potentiation of excitatory postsynaptic potentials

(A1, A2) Lower traces are continuously recorded membrane potentials and evoked EPSPs in MS neurons using an internal solution containing 0.4 mM ATP in the absence of external Mg^{2+}. EPSPs were evoked every 15 sec using a glass electrode placed 200 µm apart from the neurons recorded. Upper traces are representative EPSPs at the time points (1, 2, 3) indicated in the lower traces. Sevoflurane was pre-administrated in A2. The EPSP amplitude decreased during the 3-min deprivation of oxygen and glucose (hypoxia), and then gradually increased after reperfusion of oxygen and glucose (control, closed circle). (B) Summary of EPSP amplitude normalized to pre-hypoxic values (control, closed circle; Sevoflurane pre-administration, open circle). (C) EPSPs recorded with (closed circle) and without (open circle, same data as in (B)) the application of glibenclamide. (D) Summary of EPSP amplitude in each group at 45 min. One-way ANOVA, $*P < 0.05$.

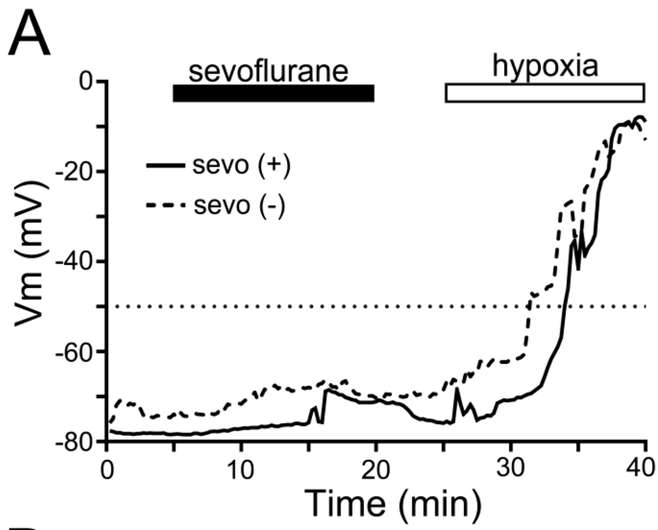
Fig. 5. Administration of 4-CIN increased the EPSP amplitude, while sevoflurane did not influence EPSP potentiation

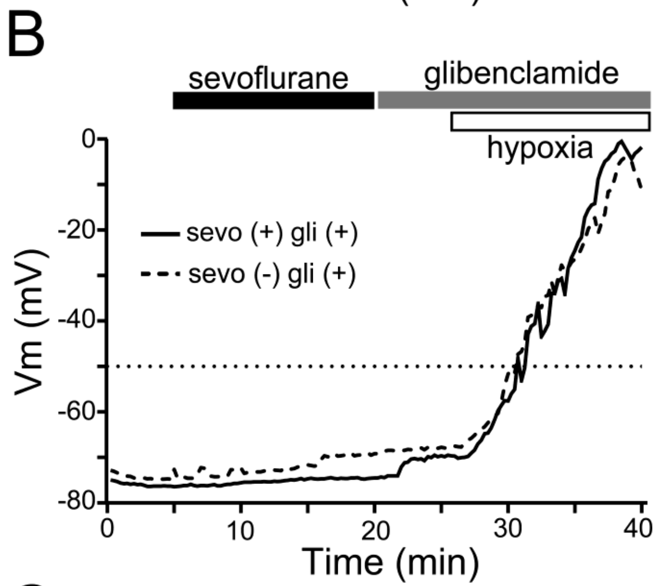
(A1, A2) Lower traces are continuously recorded membrane potentials and EPSPs in MS

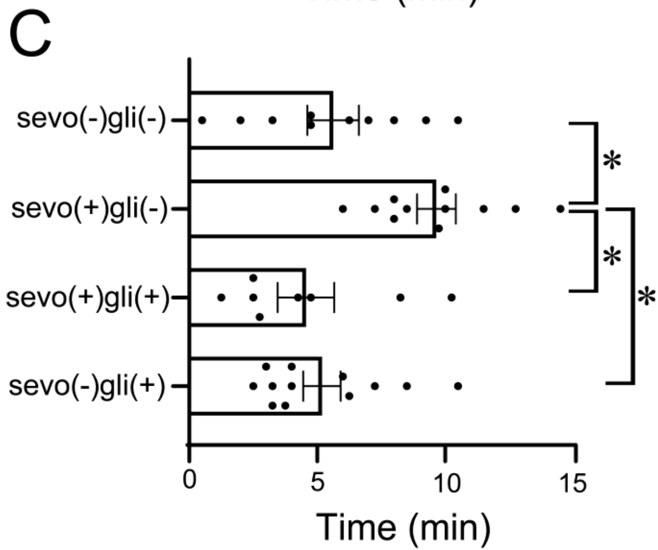
neurons using internal solution containing 0.4 mM ATP in the absence of external Mg^{2+}. (B) Amplitude of evoked EPSPs recorded in the absence of external Mg^{2+}. The EPSPs were increased by 4-CIN application (1 mM, 10 min). The potentiation of EPSPs in 4-CIN-administered MS cells was not affected by the pre-administration of sevoflurane. (C) Summary of EPSP amplitude in each group at 45 min. One-way ANOVA, $*P < 0.05$.

Fig. 6. Application of the NMDA receptor antagonist AP-5 prevented 4-CIN-induced EPSP potentiation

(A) The lower trace is the continuously recorded membrane potentials and EPSPs in MS neurons in the presence of an antagonist of the NMDA receptor, AP-5 (25 μM). (B) The 4-CIN-induced EPSP potentiation (closed circle, same data as in Fig. 5B) was completely blocked by AP-5 (open circle).







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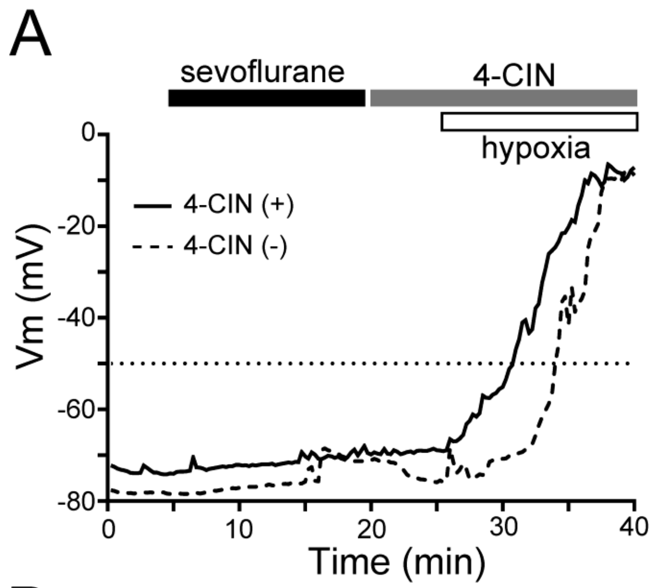

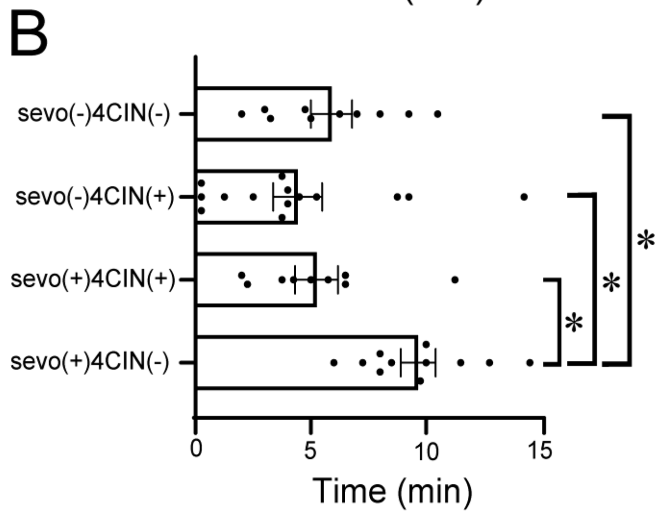

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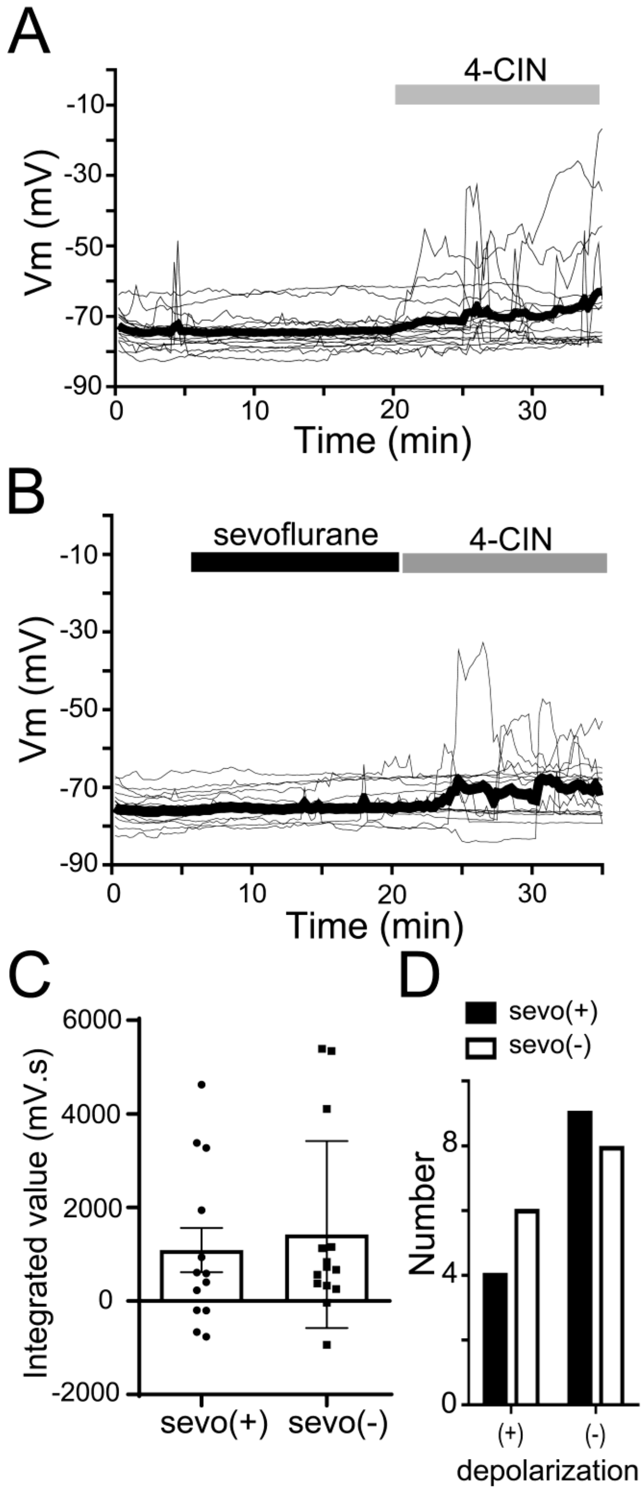

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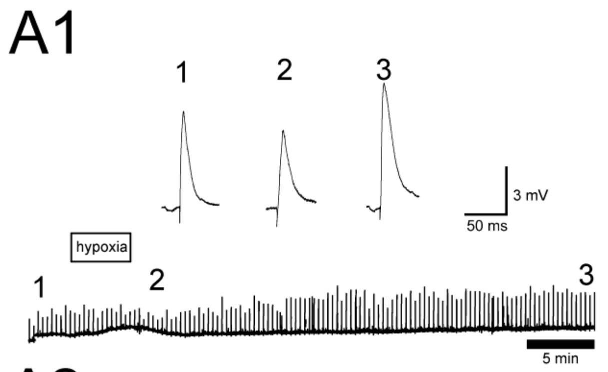

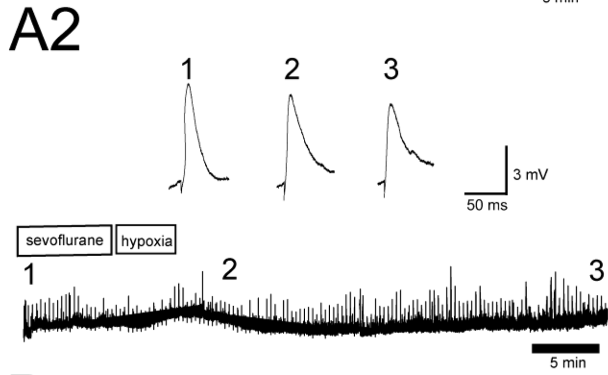

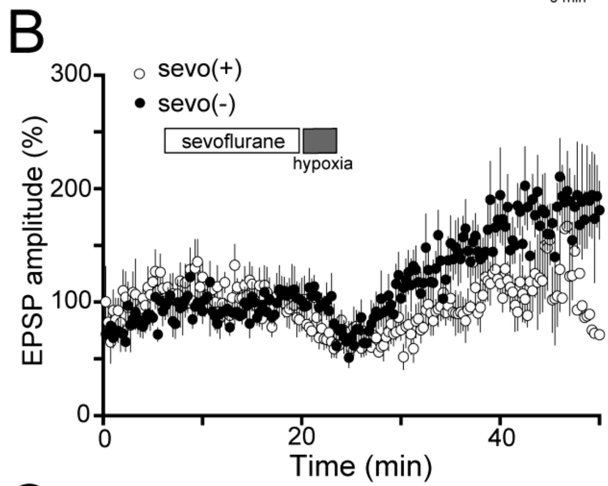

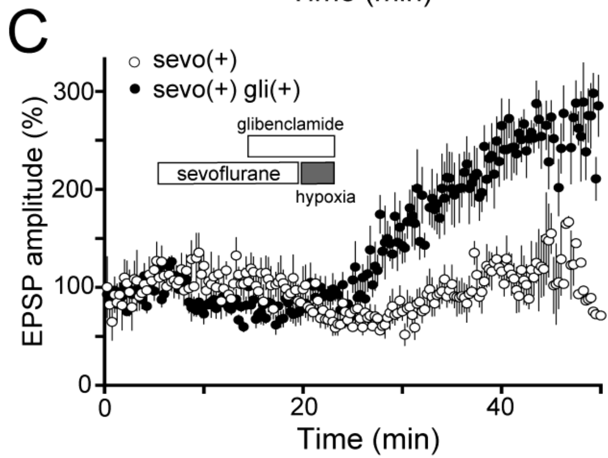

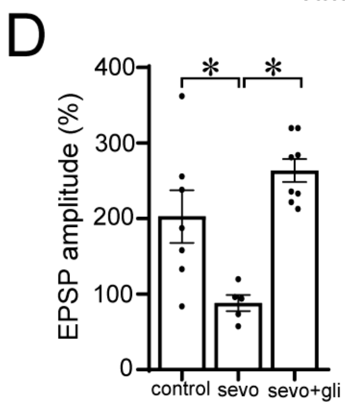

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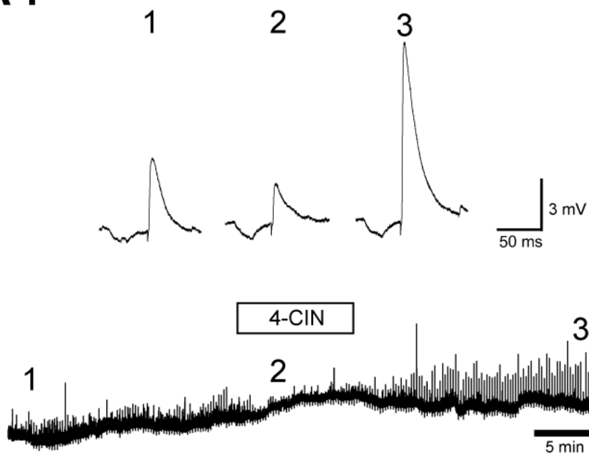

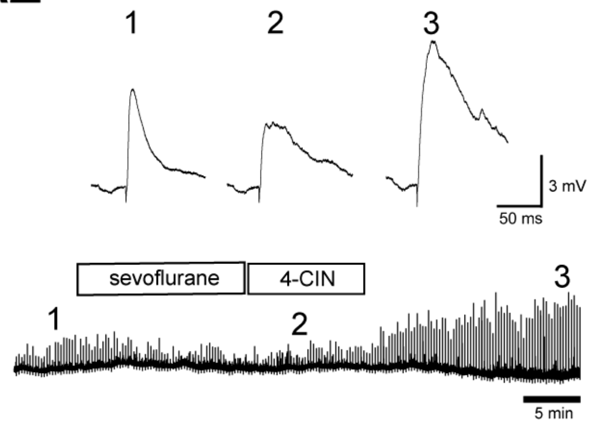

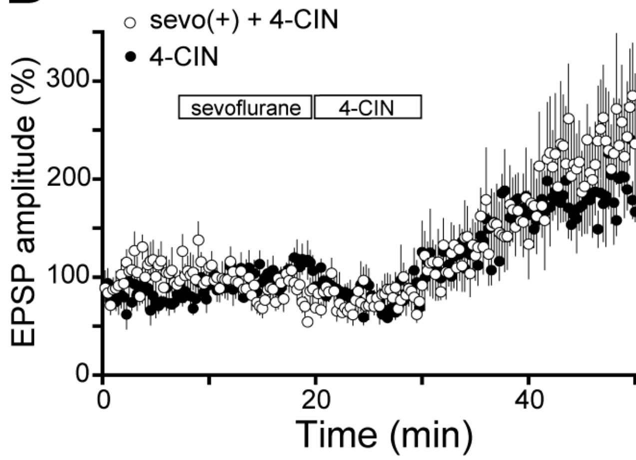

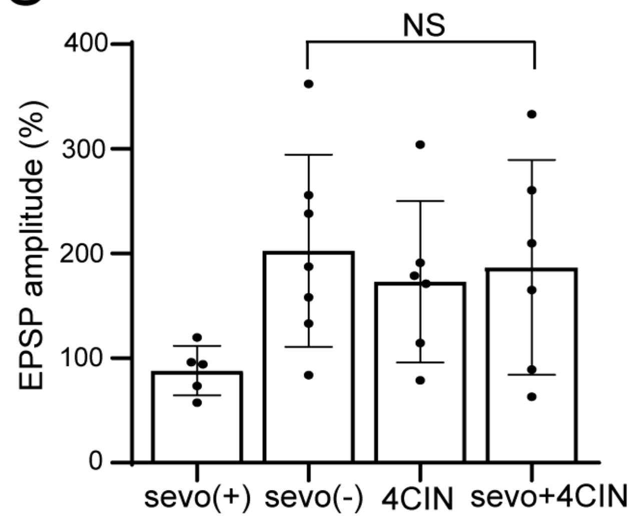

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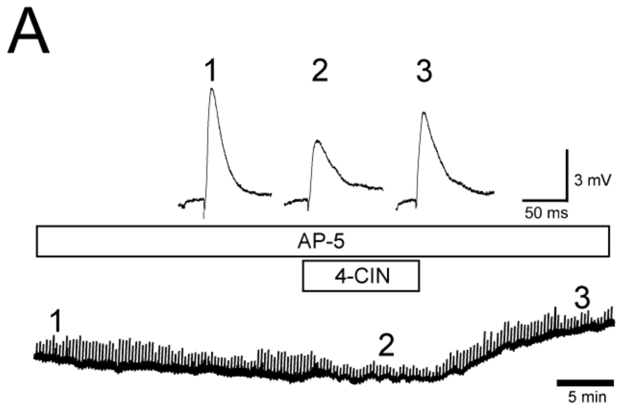

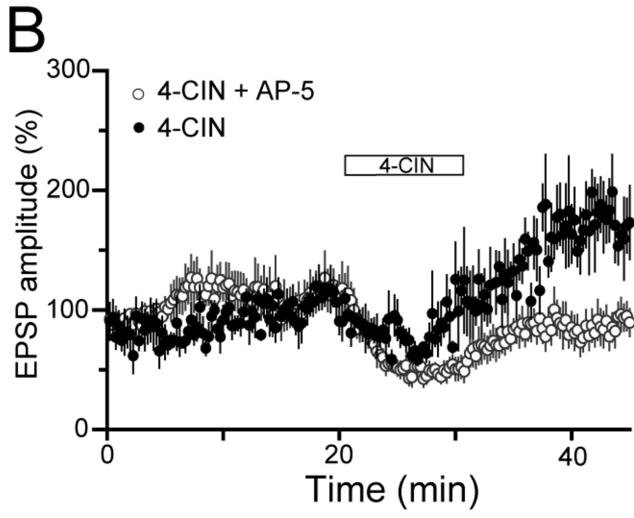

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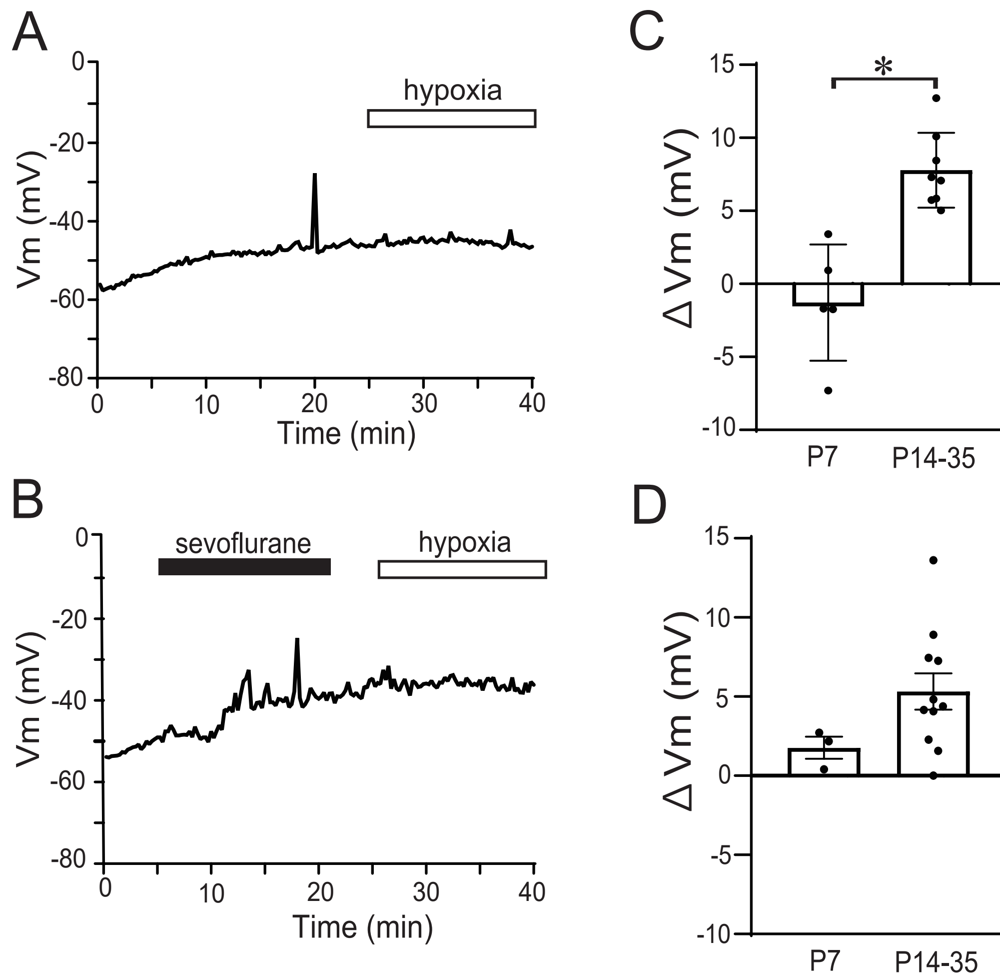
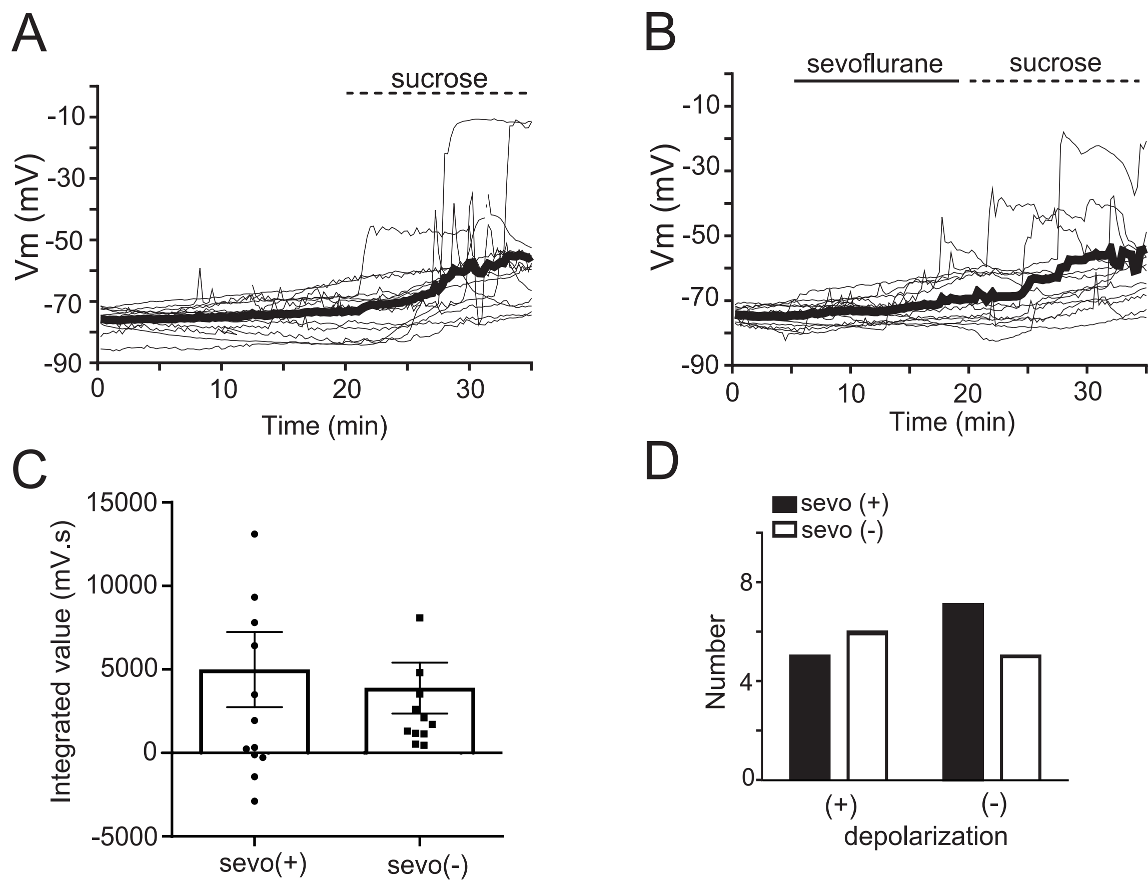


Supplemental figure 1. MS neurons (P7) showed only slow and moderate depolarization under hypoxic conditions.

(A) MS neurons (P7) kept the membrane potential under the depletion of glucose and oxygen. (B) Sevoflurane hardly change the membrane potential of MS neurons (P7). (C) Depolarization caused by 5 -min hypoxia (ΔVm) of MS neurons (P7) was smaller than that of P14-35 neurons (P7; n = 5, ΔVm = -1.28 ± 1.78 mV; P14-35; n = 8, ΔVm = 7.78 ± 0.91 mV, *P = 0.0016; Student's t test). There was significant difference between them. (D) Depolarization caused by 5 -min hypoxia (ΔVm) of MS neurons (P7) under administration of sevoflurane was slightly smaller than that of P14 -35 neurons (P7; n = 3, ΔVm = 1.76 ± 0.69 mV; P14-35; n = 11, ΔVm = 5.32 ± 1.14 mV, P = 0.1264; Student's t test).



Supplemental figure 2. Partial energy restriction by glucose depletion changes the membrane potential in MS neurons.

(A, B) Resting membrane potentials in MS neurons recorded with an internal solution containing 0.4 mM ATP. Potentials of each cell (thin lines) and averaged value (thick line are plotted against time. Replacement of glucose with sucrose depolarized MS neurons. (C, D) Sevoflurane pre-administration did not change the integrated value or the number of MS neurons depolarized more than 20 mV during the depletion of glucose. Student's *t* test and Chi-squared test.