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ORIGINAL ARTICLE

The mu opioid receptor activation does not affect ischemia-induced agonal currents in rat spinal ventral horn

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Abstract
Purpose Opioid-induced spastic paraplegia after transient spinal cord ischemia during aortic surgery has been reported. Opioids modulate neurotransmission through mu (μ) opioid receptors (MORs) in the spinal ventral horn. However, their effects during ischemic insult are not understood.
Methods The effects of the selective μ agonist [D-Ala^2,-*N*-Me-Phe^4, Gly^5-ol]enkephalin (DAMGO) on ischemia-induced agonal currents were examined in the spinal lamina IX neurons of neonatal rats by using the whole-cell patch-clamp technique. Ischemia was simulated in vitro by oxygen/glucose deprivation.
Results DAMGO (1 μM) produced outward currents in ~60 % of spinal lamina IX neurons at a holding potential of −70 mV. Superfusion with ischemia-simulating medium elicited an agonal current. The latency was 457 ± 18 s. Despite its neuromodulatory effects, DAMGO did not significantly change the latencies of the agonal currents with (440 ± 23 s) or without (454 ± 33 s) DAMGO-induced currents.
Conclusion Activation of MORs does not influence ongoing ischemia-induced neuronal death. Our findings indicate that MOR agonist administration should be suitable as an anesthetic during aortic surgery.

Keywords μ Opioid · Oxygen and glucose deprivation · Spinal cord ischemia · Spinal motoneuron

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Introduction

One of the most worrisome complications of thoracoabdominal aneurysm repair is paraplegia caused by spinal cord ischemia because it results in serious physical disability [1]. Because the extent of aortic cross-clamping time is related to the incidence of paraplegia [2], there is a concern about whether the drugs administered during surgery affect the permissible ischemic time. During ischemic insult, glutamate, a major excitatory neurotransmitter, accumulates excessively in extracellular space and excites postsynaptic neurons by allowing Ca^{2+} influx through activation of ionotropic glutamate receptors, leading to excitotoxic cell death [3–5]. Therefore, agents that modulate glutamatergic synaptic transmission have been considered to attenuate such an excitotoxic neuronal injury. Indeed, the neuroprotective efficacy of numerous interventions has been reported in experimental spinal cord ischemia [6].

Opioid is known as an important neuromodulator in the central nervous system. Opioids have primarily inhibitory effects, which include postsynaptic hyperpolarization through the opening of several types of K^+ channels, which causes a reduction in neuronal excitability, and presynaptic inhibition of excitatory neurotransmitter release [7, 8]. Agonists of μ opioid receptor (MOR) such as morphine, fentanyl, and remifentanil are administered during the perioperative period to provide adequate analgesia and stabilize hemodynamic status. The powerful analgesic effect of MOR agonists is partially mediated by their suppressive effects in the spinal dorsal horn [9–11]. Meanwhile, the MOR is also distributed in spinal ventral horn [12–14], and we reported that its activation produce significant neuromodulatory effects on spinal motoneurons [15]. As with the use of opioids in spinal cord ischemia, it has been reported that MOR agonists were shown to cause paraplegia in a model of

transient spinal cord ischemia [16, 17], and the MOR antagonist naloxone ameliorated motor deficits [18]. However, the in vivo experiments described did not assess the effect of MOR activation during an ischemic insult. In this respect, Shirasawa et al. [19] reported high-dose opioid anesthesia did not exacerbate spinal motoneuronal injury during spinal cord ischemia. However, they pointed out the limitation that the influence of coadministered anesthetics or postoperative analgesics could not be completely excluded.

Therefore, to elucidate the effects of MOR stimulation on the spinal motoneurons in the acute spinal cord ischemia at the cellular level, we investigated the actions of MOR agonist in ventral horn neurons of spinal cord slices using the whole-cell patch-clamp method.

Materials and methods

All experimental procedures involving the use of animals were approved by the Animal Care and Use Committee at Niigata University Graduate School of Medical and Dental Sciences (Niigata, Japan).

Preparation of spinal cord slices

Slices of rat spinal cord were prepared as previously described [20–22]. In brief, neonatal Wistar rats (8–12 days old) were anesthetized with urethane (1.2–1.5 g/kg, intraperitoneally). Dorsal laminectomy was performed, and the lumbosacral segment of the spinal cord was removed. The rats were immediately killed by exsanguination. The spinal cord was placed in preoxygenated, ice-cold artificial cerebrospinal fluid (ACSF). After all the ventral and dorsal roots near the root entry zone were cut, the pia-arachnoid membrane was removed. The spinal cord was mounted on the metal stage of a microslicer (DTK-1500; Dosaka, Kyoto, Japan) and cut into 500-μm-thick transverse slices. Each spinal cord slice was transferred to a recording chamber and placed on the stage of an upright microscope equipped with an infrared-differential interference contrast (IR-DIC) system (E600FN; Nikon, Tokyo, Japan). The slices were fixed by an anchor and superfused at 5 ml/min with ACSF solution equilibrated with a gas mixture of 95 % O_2 and 5 % CO_2, and they were maintained at 36 °C using a temperature controller (TC-324B; Warner Instruments, Hamden, CT, USA) (Fig. 1a). The ACSF solution contained (in mM) 117 NaCl, 3.6 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 25 $NaHCO_3$, and 11.5 D-glucose (pH = 7.4).

Patch-clamp recordings from spinal lamina IX neurons

Lamina regions were identified under low magnification (5× objective lens), and individual neurons were detected using a 40× objective lens under an IR-DIC microscope and monitored by a charge coupled device camera (C2400-79H; Hamamatsu Photonics, Hamamatsu, Japan) on a video monitor screen (Fig 1b). The size of each neuron was calculated from the arithmetic mean diameter of the long and short axes of the soma intersecting at right angles. Whole-cell patch-clamp recordings were made from large lamina IX neurons (size, 15–25 μm) [23, 24]. After the whole-cell configuration was established, voltage-clamped neurons were held at −70 mV. Whole-cell patch pipettes were constructed from borosilicate glass capillaries (1.5 mm OD; World Precision Instruments, Sarasota, FL, USA). The resistance of a typical patch pipette was 4–8 MΩ when filled with internal solution. The potassium gluconate-based internal patch pipette solution was used containing (in mM) 135 potassium gluconate, 5 KCl, 0.5 $CaCl_2$, 2 $MgCl_2$, 5 ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 5 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), and 5-adenosine 5′-triphosphate magnesium salt (ATP-Mg) (pH = 7.2). Signals were amplified by an Axopatch 200B amplifier (Molecular Devices, Union City, CA, USA), filtered at 2 kHz, and digitized at 5 kHz. Data were stored and analyzed using a pCLAMP 9.1 data acquisition program (Molecular Devices). Membrane capacitance was measured by integrating the transient capacitative currents evoked during the voltage-clamp steps. The holding potential at which the holding current became 0 was considered the resting membrane potential.

Drug application and ischemia simulation

DAMGO was obtained from Sigma-Aldrich (St. Louis, MO, USA). Ischemia was simulated by superfusing the slices with an ACSF solution equilibrated with a gas mixture of 95 % N_2 and 5 % CO_2, in which glucose was replaced with an equimolar concentration of sucrose [ischemia-simulating medium (ISM)]. The partial pressure of oxygen was measured using a PO_2 monitor (POG-5500; M.T. Giken, Tokyo, Japan). Drugs and ISM were applied by perfusion via a three-way stopcock without change in the perfusion rate or temperature. After applying the ischemia-simulating medium, the slices were discarded.

Statistical analysis

Numerical data are expressed as mean ± SEM. Statistical significance was defined as $P < 0.05$. Student's paired *t* test or one-way analysis of variance (ANOVA) was used as indicated for statistical analyses. In the electrophysiological data, *n* refers to the number of neurons studied.

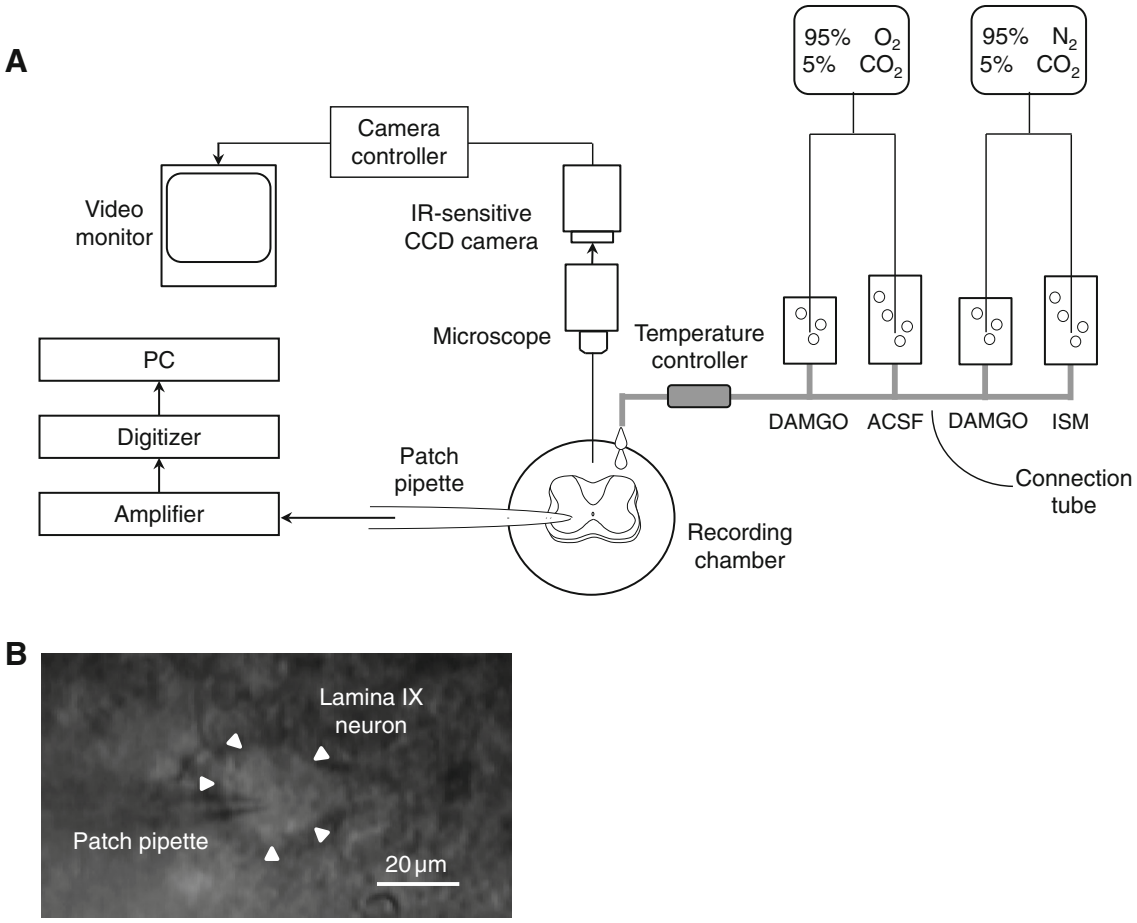

Fig. 1 **a** Schematic representation of the experimental system. *IR-sensitive CCD camera* infrared-sensitive charge-coupled device camera, *ACSF* artificial cerebrospinal fluid, *ISM* ischemia-simulating medium, *PC* personal computer. **b** The spinal cord slice preparation viewed under a 5× objective lens (*left*) and a 40× objective lens (*right*) of an infrared-differential interference contrast microscope. The ventral horn neuron having large soma was identified as a spinal motoneuron (*arrowheads*)

Results

DAMGO produced outward current in spinal lamina IX neurons

Whole-cell patch-clamp recordings were obtained from 71 lamina IX neurons. Bath-applied DAMGO (1 μM; 1–4 min) produced outward current in spinal lamina IX neurons (Fig. 2a). After washout, the currents gradually returned to baseline level in about 6 min. However, sustained application of DAMGO (1 μM; 10 min) produced long-lasting outward currents, and the opioidergic action was poor on washout (Fig. 2b). In this situation, we did not observe desensitization of the current. We previously reported that DAMGO at 1 μM appears sufficient to produce maximal action, because the current observed at this concentration was well above the EC_{50} (0.10 μM) [15]. Hence, in the present study, we used DAMGO at a concentration of 1 μM continuously during ischemic insult to investigate its effects on ischemia-induced excitotoxic cell death.

Effects of DAMGO on membrane dysfunction induced by ISM

All recordings were obtained within <4 h in this experiment. ISM exposure generated an agonal inward current at −70 mV in all lamina IX neurons ($n = 64$; Fig. 3). When ISM superfusion was continued after the appearance of the agonal current, synaptic activity disappeared, and it could not be restored to its previous state despite ACSF reperfusion. The agonal inward current consisted of a slow current followed by a rapid inward current (Fig. 3). The onset of the rapid inward current was estimated by extrapolating the slope of the rapid inward current into the slope of the slow current [25, 26]. Permissible ischemic time was determined by the latency to the onset of the rapid inward current, which was measured from the onset of ISM

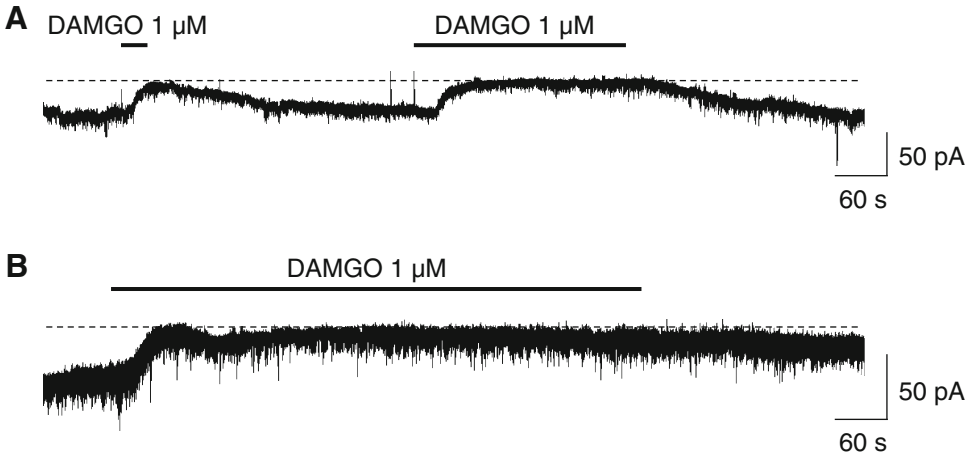

Fig. 2 [D-Ala^2,-*N*-Me-Phe^4, Gly^5-ol] enkephalin (*DAMGO*)-induced outward currents in lamina IX neurons. **a** Representative current traces produced by DAMGO (1 μM). After washout, the amplitude gradually returned to the previous level, and subsequent application of DAMGO elicited the same reaction. In this figure and subsequent figures, the *horizontal bars* above the chart recordings indicate the duration of drug superfusion. **b** Representative current traces produced by sustained application of DAMGO (1 μM). The DAMGO-induced long-lasting outward currents were not easily influenced by washout. Each current trace was recorded from a different neuron

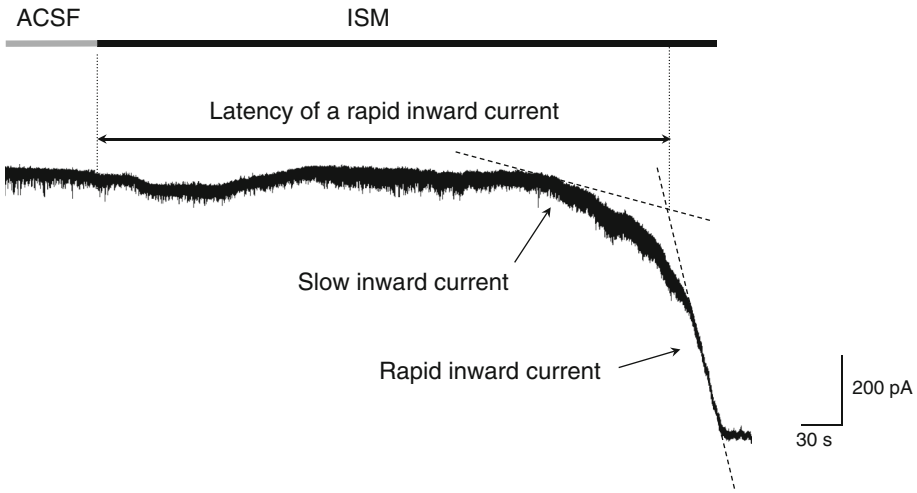

Fig. 3 Agonal inward current induced by ISM in lamina IX neurons. In voltage-clamp mode, perfusion with ISM produced an agonal inward current at −70 mV. The latency of the rapid inward current was measured from the beginning of ISM perfusion to the onset of rapid inward current, which was estimated by extrapolating the slope of the rapid inward current to the slope of the slow inward current

superfusion (Fig. 3). During superfusion with ACSF, the PO_2 in the chamber was 290–320 mmHg. After switching to the ISM, the PO_2 rapidly declined and reached the lowest plateau value (50–60 mmHg) in 3 min, which was sufficient to produce agonal currents under normoglycemic conditions (the average latency of the rapid inward currents was 1,311 ± 108 s, $n = 5$).

Sixty-four neurons were divided equally into two groups. In the control group, the average latency of the rapid inward currents was 457 ± 18 s ($n = 32$). To adjust for the effect of the outward current resulting from postsynaptic activation of the MOR, the DAMGO-treated group was separated according to the presence or absence of DAMGO-induced current. In the DAMGO-treated group, 19 (59 %) of 32 neurons exhibited outward currents (33 ± 5 pA) and the remaining 13 neurons did not. There were no significant differences in postnatal age, size, and membrane properties by one-way ANOVA among the three groups (Table 1). DAMGO (1 μM) was applied 2 min before ISM superfusion until an agonal current was observed but did not significantly change the latencies of the rapid inward currents induced by ISM in the neurons either with (440 ± 23 s, $n = 19$) or without (454 ± 33 s, $n = 13$) DAMGO-induced currents ($P = 0.85$, one-way ANOVA; Fig. 4).

Discussion

The present study demonstrated that MOR activation did not affect the latencies of rapid inward currents induced by simulated ischemia.

Table 1 Comparison of postnatal age, size, and electrophysiological membrane properties

	DAMGO			*P* value
	Control ($n = 32$)	Current (+) ($n = 19$)	Current (−) ($n = 13$)	
Age (days)	10.2 ± 0.2	10.3 ± 0.3	9.6 ± 0.4	0.21
Size (μM)	20.4 ± 0.4	20.2 ± 0.4	20.3 ± 0.8	0.95
Cm (pF)	90.8 ± 5.3	89.5 ± 11.9	80.9 ± 12.1	0.69
RMP (mV)	−58.5 ± 1.5	−60.4 ± 1.9	−60.4 ± 2.3	0.69

There was no significant difference in postnatal age, size, and membrane properties by one-way analysis of variance among the three groups. Data are shown as mean ± SEM

DAMGO [D-Ala^2-*N*-Me-Phe^4, Gly^5-ol] enkephalin, *Cm* membrane capacitance, *RMP* resting membrane potential

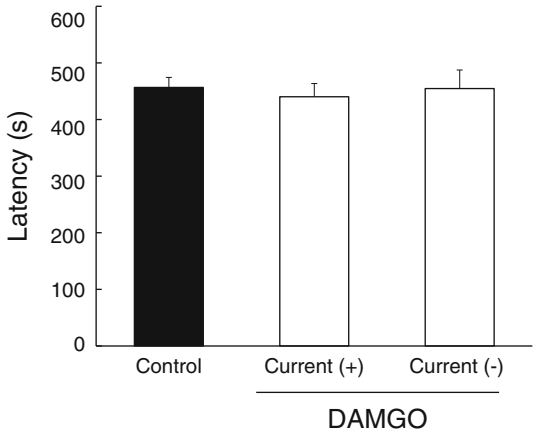

Fig. 4 Comparison of the latency of the agonal inward current produced by ISM in control ($n = 32$) and in the presence of DAMGO with or without outward current at −70 mV ($n = 19$ and 13, respectively). DAMGO (1 μM) did not change the latency of the rapid inward current induced by ISM regardless of whether the DAMGO-induced outward current was present or absent. One-way analysis of variance, $P = 0.85$

Agonal currents induced by ISM

In the present study, ischemia was mimicked with ISM, which removed glucose and oxygen from ACSF. This preparation has been well established in electrophysiological studies using slices from the spinal cord and brain [20, 21, 26–28]. ISM superfusion elicited an outward current followed by an agonal inward current [20, 21]. The agonal inward current consists of a slow and subsequent rapid inward current and has pharmacologically distinct mechanisms [26]. Glutamate accumulates in the interstitial space and mediates the slow inward current via receptor activation [26]. Meanwhile, the rapid inward current is accompanied by a sudden increase in the intracellular Ca^{2+} concentration [26]. In addition to the action of glutamate receptors, the increase in the intracellular Ca^{2+} concentration is associated with Ca^{2+} influx via voltage-dependent Ca^{2+} channels, reversed operation of Na^+/Ca^{2+} transporters, and Ca^{2+} release from intracellular storage sites [29]. When ISM superfusion was continued after the appearance of the agonal inward current, synaptic activity disappeared, and it could not be returned to its previous state despite ACSF reperfusion, indicating that ISM resulted in irreversible membrane dysfunction [25].

We previously described postnatal age-dependent shortening of the latency of the rapid inward current in spinal lamina IX neurons [25]. Furthermore, the membrane capacitance and resting membrane potential demonstrated significant negative correlations with the latency of the rapid inward current [25]. To exclude such influences, we confirmed that there were no differences in age, size, or membrane properties between control and DAMGO-treated neurons.

MOR activation did not change the latency of the rapid inward current

The disruption of ionic homeostasis induced by ischemia is known to excite the neuronal membrane and subsequently lead to necrosis [30]. Synaptic-released glutamate is considered to be one of the important mediators in ischemia [3, 31]. Hence, numerous studies have focused on glutamatergic neurotransmission as a therapeutic target and have demonstrated the neuroprotective effects of glutamatergic receptor antagonists [6]. Adenosine, which decreases synaptic glutamate release and hyperpolarizes spinal lamina IX neurons, has been reported to prolong the latency of the agonal inward current elicited by ISM [20]. We previously showed that DAMGO produced an outward current and suppressed excitatory neurotransmitter release in lamina IX neurons [15]. Therefore, the suppressive effects of DAMGO on neuronal excitability appear to protect lamina IX neurons from ischemic insult. Conversely, we formerly demonstrated that DAMGO also reduced inhibitory neurotransmitter release [15]. Inhibitory neurotransmitters suppress motoneuronal excitability [32] and exhibit neuroprotective effects through the activation of the GABA receptor in the spinal cord [33, 34]. MOR activation may therefore precipitate neuronal injury as a result of the suppression of inhibitory neurotransmission.

However, DAMGO did not significantly change the latency of the rapid inward current induced by ISM. The fact that the latency of the rapid inward current did not differ between with and without DAMGO-induced current

indicates that postsynaptic MOR stimulation was ineffective in modulating the latency of the rapid inward current. Furthermore, there was no difference in the latency of the rapid inward current between control and the neurons without DAMGO-induced current, which suggests that presynaptic MOR activation also did not influence the latency. One explanation for these results is that the sum of the precipitating effects and the neuroprotective effects of MOR activation on ischemic neuronal damage was low and was greatly exceeded by other excitotoxic mechanisms. Previous studies showed that not only glutamates and their ionotropic receptors but also glutamate transporters [35], plasmalemmal Na^+/Ca^{2+} exchangers [36], the endoplasmic reticulum [37], and mitochondria [38] contribute to the disruption of ionic homeostasis under ischemic conditions. We did not assess the contribution of each factor, and further investigation is therefore necessary.

Our results are inconsistent with those reported using in vivo spinal cord ischemia models. Several studies have demonstrated that μ opioid exacerbated ischemic spinal cord injury [16, 17] whereas μ antagonists ameliorated it [18]. This discrepancy between our results and those of previous studies could be attributed to differences in the timing of MOR activation, because prior studies estimated the effect of MOR activation after transient spinal cord ischemia. Transient ischemic insult possibly altered the response to MOR agonists and its effects on the neurotransmission to lamina IX neurons. In hippocampal neurons, experimental ischemia altered the activity of the gene-silencing transcription factor repressor element-1 silencing transcription factor (REST), which triggered significant changes in both MOR mRNA and protein expression [39]. Because REST has also been detected in the spinal cord [40], it is possible that MOR expression drastically changes after transient spinal cord ischemia. Such alteration after ischemic insult is likely to modify the response to MOR agonists and affect cell death. In the present study, we observed that MOR activation during experimental ischemia did not affect the latency period to the onset of excitotoxic cell death. This finding is in agreement with a previous report showing that massive intravenous administration of MOR agonists during spinal cord ischemia by means of aortic occlusion did not worsen paralysis of rabbit hindlimbs [19]. Considering these findings, opioidergic neuromodulation after ischemic insult could be a vital component of the exploration of spinal neuroprotection from ischemic injury.

In conclusion, our findings show that treatment with MOR agonists does not alter the permissible ischemic time in motor neurons, which indicates that MOR agonist administration should be suitable as a potential component of anesthetics during aortic surgery.

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Conflict of interest None.

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