Mu and delta, but not kappa, opioid agonists induce spastic paraparesis after a short period of spinal cord ischaemia in rats

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Background. Intrathecal (IT) morphine given after a short interval of aortic occlusion in a rodent model induced transient spastic paraparesis via opioid receptor-predicted actions in spinal cord. To determine the role(s) of spinal opioid receptor subtypes we investigated whether IT administration of various selective opioid receptor agonists can induce paraparesis following a short period of spinal cord ischaemia in rats.

Methods. In Sprague–Dawley rats implanted with an IT catheter, spinal cord ischaemia was induced for 6 min using an intraaortic balloon. Mu ([D-Ala2,N-Me Phe4,Gly-ol5] enkephalin), kappa (U50488H) or delta ([D-Pen2,5] enkephalin) selective agonists were injected intrathecally 30 min after reperfusion. A separate group of animals was used to investigate the dose–response effect on this motor dysfunction. For this purpose, three doses of mu, kappa, or delta agonists were injected intrathecally after ischaemia. After IT injection, recovery of motor function was assessed periodically using the motor deficit index (0 = complete recovery; 6 = complete paraplegia).

Results. IT administration of mu and delta but not kappa agonists produced dose-dependent effects in the induction of spastic paraparesis. In addition, this spasticity induced by IT mu and delta agonists was reversed completely by IT naloxone and naltrindole, respectively.

Conclusion. These results suggest that the effect of various opioids on motor function after a short period of spinal cord ischaemia depends upon individual opioid receptor subtypes.


Keywords: agonists, DAMGO, DPDPE, U50488H; complications, paraparesis; complications, spinal cord ischaemia; receptors, opioid

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Introduction

Intrathecal (IT) morphine has been used for pain control after thoracoabdominal aortic aneurysm repair surgery,1 in which aortic cross-clamping is essential although it may induce spinal ischaemia. β-Endorphin levels in cerebrospinal fluid were found to be increased during the reperfusion period after spinal ischaemia in the dog.2 Based on evidence that IT or i.v. naloxone can attenuate neurological consequences of spinal injury,3 it can be suggested that endogenous opioids could be involved in neuronal injury in the spinal cord after aortic cross-clamping.

Although neuronal injury after spinal trauma or ischaemia appeared to be worsened by endogenous opioids, the safety of exogenous opiates in central nervous injury is unclear. We recently reported that IT morphine following a short interval of aortic occlusion in the rodent induced transient spastic paraparesis via opioid receptor-coupled effects in the spinal cord.45 Histopathological analysis revealed the possibility that IT morphine could induce degeneration of spinal ventral neurones even after a short period of spinal cord ischaemia, and this degeneration was associated with the activation of spinal N-methyl-D-aspartate receptors by elevation of glutamate release in cerebrospinal fluid after IT morphine.6

This work has been attributed mainly to Department of Anesthesiology, Faculty of Medicine, University of the Ryukyus.
At present, agents classified as opioids are believed to exert their effect by a specific interaction with one or more subclasses of three opiate receptors, designated as mu (MOP), delta (DOP), and kappa (KOP). In the present study we compared the dose-dependent effects produced by IT administration of selective mu-, delta- and kappa-opioid agonists following a short period of spinal cord ischaemia on neurological function during reperfusion in the rat.

**Material and methods**

The following investigations were carried out under a protocol approved by the Institutional Animal Care Committee, University of the Ryukyus, Okinawa, Japan. Male Sprague–Dawley rats (350–400 g; n=156) were used in this study.

**General preparation—implantation of IT catheters**

Animals were implanted with IT catheters by the method described by Yaksh and Rudy. Briefly, animals were anaesthetized with isoflurane 2.5% in a room air/oxygen mixture (1:1) and the back of the head and neck shaved. The animals were then placed in a stereotaxic head holder with the head flexed forward. Anaesthesia was maintained with isoflurane 1.5% delivered by mask. A midline incision was made on the back of the neck. The muscle was freed at the attachment to the skull and retracted with a flat elevator, exposing the cisternal membrane. The membrane was opened with a stab blade and modestly retracted with a small dural hook. The PE-10 catheter was then inserted through the cisternal opening and passed slowly and carefully caudally 9 cm into the IT space. All animals displayed normal feeding and drinking behaviour.

**Induction of spinal ischaemia**

Details of the aortic occlusion model have been reported previously. In brief, animals previously implanted with IT catheters were anaesthetized in a Plexiglas box with isoflurane 4% in room air. Following induction, anaesthesia was maintained with isoflurane 1–2% delivered by an inhalation mask. For monitoring of distal arterial pressure and injection of heparin, a polyethylene catheter (PE-50) was inserted into the tail artery. For induction of spinal ischaemia, the left femoral artery was isolated and a 2 Fr Fogarty catheter was placed into the descending thoracic aorta so that the catheter tip reached the level of the left subclavian artery. To control proximal arterial blood pressure to 40 mm Hg during the period of aortic occlusion, a 20 G Teflon catheter connected to an external blood reservoir (37.5°C) was inserted into the left carotid artery. To maintain spinal cord normothermia during aortic occlusion, water (38.5–38.8°C) was perfused through a heat exchanger at 100 ml min

**Experimental groups and design**

**Agonist effects**

The first series of experiments determined the time course and dose–response of IT [D-Ala², N-Me Phe³, Gly-ol⁵] enkephalin (DAMGO), U50488H and [D-Pen²,⁵] enkephalin (DPDPE) on neurological outcome after a short period of spinal cord ischaemia. For assessment of the time-course effect of the IT opioid agonists DAMGO, U50488H or DPDPE after 6 min of aortic occlusion, the animals were assigned to one of the following eight groups. Animals subjected to spinal cord ischaemia and IT treatment were divided into subgroups treated with IT saline 0.9% (group IS: n=6), DAMGO 2.5 µg (group IM: n=6), U50488H 150 µg (group IK: n=6) or DPDPE 45 µg (group ID: n=6) 30 min after exposure to 6 min of aortic occlusion. The sham-operated and IT treatment group was divided into subgroups treated with IT saline 0.9% (group SS: n=6), DAMGO 2.5 µg (group SM: n=6), U50488H 150 µg (group SK: n=6) or DPDPE 45 µg (group SD: n=6) 30 min after exposure to sham treatment. Assessment of neurological function was performed at pre-ischaemia, 30 min of reperfusion, and 5, 15, and 30 min and 1, 2, 4, 8, and 24 h after IT injection.

To investigate the dose–response relationship, the following doses of each opioid agonist were examined with regard to neurological function following a short period of spinal cord ischaemia: DAMGO (2.5, 0.5, 0.1 µg, saline; n=24; 6/dose), U50488H (300, 150, 50 µg, saline; n=24; 6/dose), and DPDPE (45, 15, 5 µg, saline; n=24; 6/dose). All drugs were injected 30 min following the 6 min of aortic occlusion.

**Opioid agonist–antagonist interaction**

For evaluation of specific spinal opioid receptors in neurological dysfunction, each opioid receptor’s antagonist or a saline control was injected IT at the time of maximal effect induced by IT opioid agonists. Naloxone and naltrindole were used as antagonists of DAMGO and DPDPE, respectively. The study protocol was as follows.

**Opioid mu agonist–antagonist interaction**

Group MS (n=6): IT 2.5 µg of DAMGO was injected at 30 min of reperfusion (first IT injection) followed by
rats were killed with pentobarbital (100 mg kg\(^{-1}\)). At the end of the survival period (24 h) in the agonist study, perfusion fixation and histopathological analysis by a grading system used previously. Motor function was assessed during reperfusion. Recovery of motor function was assessed by a grading system used previously. A motor deficit index (MDI) was calculated for each rat at each time interval. The final MDI was the sum of the scores (walking with lower extremities plus placing and stepping reflex).

Assessment of neurological function
During reperfusion, recovery of motor function was assessed by a grading system used previously. Motor function was quantified by assessment of ambulation and placing and stepping responses. For statistical purposes, ambulation (walking with lower extremities) was graded as follows: 0, normal; 1, toes flat under the body when walking, but ataxia present; 2, knuckle walking; 3, movement in lower extremities but unable to knuckle walk; or 4, no movement, drags lower extremities. The placing/stepping reflex was assessed by dragging the dorsum of the hind paw over the edge of a surface. This normally evokes a coordinating lifting and placing response (e.g. stepping) which was graded as follows: 0, normal; 1, weak; 2, no stepping. A motor deficit index (MDI) was calculated for each rat at each time interval. The final MDI was the sum of the scores (walking with lower extremities plus placing and stepping reflex).

Perfusion fixation and histopathological analysis
At the end of the survival period (24 h) in the agonist study, rats were killed with pentobarbital (100 mg kg\(^{-1}\) i.p.) and phenytoin (25 mg kg\(^{-1}\) i.p.). Animals were then transcardially perfused with heparinized saline 100 ml followed by paraformaldehyde 4% 150 ml in phosphate buffer (pH 7.4). After 24 h, spinal cords were removed and postfixed in the same fixative for 2–14 days. After this period, spinal cords were removed and L3, L4 and L5 spinal segments dissected. A spinal cord segment was embedded in paraffin wax, and serial transverse sections (10 μm) were obtained. Slides were stained using the Nissl method and evaluated for evidence of cellular degeneration and necrosis. Cells that contained Nissl substance in the cytoplasm, loose chromatin, and prominent nucleoli were considered to be normal neurones and ischaemic neurones were identified by loss of Nissl substance and by the presence of pyknotic homogeneous nuclei.

Drugs
Drugs used in this study were obtained from the following sources: DAMGO and U50488H (Sigma-RBI., Natick, MA), DPDPE (Sigma Chemical Co., St Louis, MO), Naloxone HCL (DuPont, Wilmington, DE), and Naltindrolo HCl (Sigma–Aldrich Co., St Louis, MO). All drugs were freshly prepared in sterile physiological saline (0.9% NaCl) in concentrations that allowed IT injections to be made in volumes of 10 μl. All IT injections were performed slowly by a microinjector and were followed by a similarly slow injection of saline (10 μl) to flush the catheter.

Statistics
Statistical analysis of physiological data was performed by one-way analysis of variance (ANOVA) for multiple comparisons followed by Dunnett post hoc test. Differences in MDI over time were determined by one-way repeated-measures ANOVA followed by Fisher’s post hoc test. Specific comparisons between experimental groups at individual time points after reperfusion were made with ANOVA using multiple means analysis followed by the Bonferroni test. A P-value of <0.05 was considered significant.

Results
Opioid agonist effects
All rats exposed to 6 min of spinal cord ischaemia displayed modest motor dysfunction but were able to stand and walk after 30 min of reperfusion. In Group IS, rats had modest and transient motor weakness (median MDI=3) 30 min after reperfusion followed by gradual recovery over 8–24 h of reperfusion. No significant motor deficit was evident at 24 h of reperfusion. IT injection of DAMGO (Group IM) resulted in the gradual development of spasticity and near complete loss of the ability to stand, walk or step. The peak of DAMGO-induced spasticity was observed 15 min after DAMGO administration (median MDI=6) and persisted for ~30–45 min. IT administration of 45 μg DPDPE (Group ID) at 30 min of reperfusion also evoked significant development of spasticity. The peak effect was observed between 30 min and 1 h after IT injection when all of the animals had a significant loss of the ability to walk or to stand. Although several animals displayed ataxia (but with preserved ability to walk) and partial weakness in place-stepping reflex at 24 h after IT injection, the majority of animals in both groups regained motor function. In contrast
to those groups, rats injected IT with 150 μg of U50488H (Group IK) had no significant changes in motor dysfunction compared with the saline group. In sham-operated groups, those receiving saline (Group SS), DAMGO (Group SM), U50488H (Group SK) or DPDPE (Group SD) had no motor dysfunction after IT injection throughout the experiment (Fig. 1).

**Dose–response effects**

All rats in this study displayed modest motor dysfunction (median MDI=3) at 30 min of reperfusion. In all of the rats receiving IT saline, motor function showed gradual recovery at 2 h of reperfusion. In contrast, IT treatment with 2.5 μg of DAMGO resulted in the gradual development of spasticity and complete loss of the ability to stand, walk or step. Rats that received IT injection of 0.5 μg of DAMGO displayed a gradual increase in spasticity in the hind limbs and a significant loss of motor function ($P<0.05$) between 15 and 30 min of IT injection compared with IT saline. With IT injection of DAMGO 0.1 μg, there were no significant effects on neurological function between 15 and 30 min in comparison with IT saline. IT injection of DPDPE induced increasing spasticity of the hind limbs in a dose-dependent manner; in particular, spastic paraplegia (MDI=5 or 6) occurred with 45 and 15 μg of IT DPDPE, but not 5 μg, 1 h after injection. With U50488H, there were no changes in motor function even with 300 μg dose (Fig. 2).

**Opioid agonist–antagonist interaction**

IT injection of saline had no significant effect on ischaemia/DAMGO (2.5 μg) or /DPDPE (45 μg)-induced spasticity and all animals had a time course for spastic paraplegia

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Fig 1  MDI assessed from 30 min to 24 h in animals after sham operation or 6 min of aortic occlusion and IT injection of saline or DAMGO 2.5 μg, U50488H 150 μg or DPDPE 45 μg. Each time points represent as follows: 1, before spinal cord ischaemia; 2, 30 min of reperfusion; 3, 5 min after IT injection; 4, 15 min after IT injection; 5, 30 min after IT injection; 6, 1 h after IT injection; 7, 2 h after IT injection; 8, 4 h after IT injection; 9, 8 h after IT injection; 10, 24 h after IT injection. There was significant motor dysfunction following IT DAMGO or DPDPE, but not U50488H, when administered after ischaemia ($P<0.01$, compared with the MDI at 30 min of reperfusion (time point 2)).

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comparable to that described in the opioid agonist study. In contrast, IT injection of naloxone (30 \( \mu \)g) 15 min after DAMGO (i.e. during the peak of DAMGO-induced spasticity) led to a significant reversal of spasticity with all animals regaining their ability to walk and step 5 min after naloxone injection (\( P < 0.01 \)). In addition, IT injection of naltrindole (30 \( \mu \)g) 1 h after DPDPE injection also resulted in a complete reversal of spastic paraplegia. All rats could walk and step 30 min after naltrindole injection (\( P < 0.01 \)) (Fig. 3).

**Histopathological analysis**

Systematic analysis of L3–L5 spinal segments at the end of 24 h of survival showed no detectable neurodegenerative changes in all groups in agonist study (Fig. 4A and B). All neuronal pools, including ventral neurones and small- and intermediate-sized interneurones, showed normal structure with well-preserved neuronal membrane, nucleus, and nucleolus.

**Discussion**

In this study, we clearly demonstrated that IT administration of DAMGO and DPDPE, but not U50488H, increased spasticity in a dose-dependent manner after a short period of spinal cord ischaemia in rats. Spasticity evoked by DAMGO or DPDPE was reversed completely by IT naloxone or naltrindole, respectively. These results suggested that opioid-induced spastic paraparesis may be associated with spinal mu- and delta-receptor but not kappa-activation, after a period of aortic occlusion.

Our recent paper is the first clinical and experimental report of paraparesis induced by neuraxial morphine after a short period of spinal cord ischaemia. According to our previous studies, opioid-inducing motor dysfunction featured spastic paraparesis or paraplegia. For normal motor function, balanced tonic activity of excitation and inhibition is needed; excitation can be induced by fiber terminals directly on motor neurones and inhibition can be induced by inhibitory interneurones. In spastic paraplegia or paraparesis, excitatory tonic activity in spinal motor neurones may predominate as the result of a decrease in inhibition mediated by inhibitory interneurones. It is reasonable that...
opioid-induced paraparesis could result from suppression of inhibitory interneurons.

In our previous reports, we suggested the following mechanisms by which IT morphine may induce spasticity after a short period of spinal ischaemia. The first is that sensitivity to morphine should be increased by an ischaemic insult to the spinal cord. One report (not related to the spinal cord) demonstrated a 2- to 3-fold increase in binding sites for brain mu-, kappa-, and delta-agonists during the early reperfusion period following temporary focal cerebral ischaemia in the cat.12

Second, the evidence that a medium-sized interneuronal pool localized predominantly in laminae V–VII is the most vulnerable to spinal cord ischaemia is important. An immunohistochemical study showed that medium-sized interneurones in laminae V–VII are likely to be inhibitory interneurones, containing γ-aminobutyric acid (GABA) and/or glycine. As for the interaction between morphine and GABA or glycine interneurones, it was reported that opiate alkaloids, including morphine, appeared to inhibit GABA and glycine interneuronal function in the spinal cord. Throughout the neuraxis, mu receptors are often found on GABAergic interneurones, and an inhibitory effect of mu opioid receptor activation on these neurones could account for observations of excitatory effects of opioid agonists in vivo. From these results and suggestions, it can be suggested that the increase in sensitivity to opioids in the spinal cord after an ischaemic insult might enhance the effect of IT morphine and that this might block the inhibitory systems’ input (GABA and/or glycine) to motoneurones, leading to increased spasticity in the hind limb.

In general, it is well-known that morphine predominantly binds to mu opioid receptors to produce an antinociceptive effect in the spinal cord, but pentazocine can act at kappa receptors. Our previous study showed that IT morphine, but not pentazocine, could induce neurological dysfunction after a short period of spinal cord ischaemia in the rat. Based on these results, we suggest that the effect of opioids on motor function after a short period of spinal cord ischaemia is opioid receptor-specific. This suggestion is consistent with results in the present study.

ED50 in the spinal cord of DAMGO and DPDPE for analgesia against thermal stimuli (hot plate) is 0.17 nmol (0.087 μg) and 130 nmol (84 μg), respectively. For DAMGO, the present results indicated that ED50 for inducing spastic paraplegia after spinal cord ischaemia was between 0.1 μg (0.19 nmol) and 0.5 μg (0.97 nmol) (Fig. 2), suggesting that this spinal mu agonist at a dose producing analgesia may not be sufficient to induce spastic paraplegia. In contrast, since the ED50 of IT DPDPE for increasing spasticity was between 5 μg (7.7 nmol) and 15 μg (23.2 nmol) (Fig. 2), it is very likely that spastic paraplegia may be induced by spinal DPDPE at a dose producing analgesia.

As shown previously, IT morphine after spinal cord ischaemia produced transient spastic paraparesis, but a few degenerative changes in spinal ventral neurones. Hence, repetitive IT morphine even after a short period of spinal cord ischaemia produced irreversible paraplegia and degenerative changes of almost all ventral neurones. In the present study, spasticity induced by IT DAMGO and DPDPE after spinal cord ischaemia persisted for only 45 min and 1 h after IT injection, respectively. In comparison, spasticity induced by IT morphine after spinal cord ischaemia persisted for ~4 h. The degenerative changes in the spinal ventral neurones appears to be associated with prolonged elevation of the glutamate concentration in cerebrospinal fluid after IT morphine following a short period of spinal cord ischaemia. No detectable neurodegenerative changes in this study (Fig. 4A and B) may result from the duration of effect corresponding to the typical time course of spinally administered DAMGO or DPDPE in rats.

In conclusion, we clearly demonstrated that IT administration of mu and delta, but not kappa agonists, increased spasticity in a dose-dependent manner after a short period of spinal cord ischaemia in rats. The present study in combination with our previous work suggests that the effect of various opioids on motor function after a short period of spinal cord ischaemia should depend upon individual opioid receptor subtypes.

Acknowledgements

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