Effects of delta-opioid receptor stimulation and inhibition on hippocampal survival in a rat model of forebrain ischaemia

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Background. It has been reported that delta-opioid (DOP) receptor agonists may be neuroprotective in the central nervous system. However, the DOP agonist [D-Ala², D-Leu⁵]enkephalin (DADLE) does not produce neuroprotection in severe forebrain ischaemia. The aim of this study was to examine the effects of DADLE on hippocampal neurone survival against less severe forebrain ischaemia.

Methods. Intraperitoneal injection of DADLE (0 or 16 mg kg⁻¹) in male Sprague–Dawley rats was performed 30 min before ischaemia. Severe (10 min), moderate (8 min), or mild (6 min) forebrain ischaemia was produced by bilateral carotid occlusion combined with hypotension (35 mm Hg) under isoflurane (1.5%) anaesthesia. Naltrindole (10 mg kg⁻¹) (DOP antagonist) was administered 30 min before DADLE in order to confirm DOP receptor activation in the neuroprotective efficacy of DADLE. Naltrindole alone was also administered 30 min before ischaemia to examine endogenous DOP agonism as a self-protecting mechanism against ischaemia. All animals were evaluated neurologically and histologically after a 1 week recovery period.

Results. DADLE improved neurone survival in hippocampal CA3 and dentate gyrus (DG) sectors. CA1 neurones were not protected against moderate and mild ischaemia. Naltrindole abolished DADLE neuroprotection in the CA3 and DG after both moderate and mild ischaemia. Interestingly, regardless of co-administration of DADLE, naltrindole significantly worsened neuronal injury in the CA1 region after mild ischaemia.

Conclusions. These results suggest that DADLE provides limited neuroprotection to relatively ischaemia-resistant regions but not to selectively vulnerable regions. This was probably mediated by DOP stimulation. Pre-ischaemic treatment with a DOP antagonist, regardless of co-administration of DADLE, worsened neuronal damage at the selectively vulnerable regions only after mild forebrain ischaemia. These data suggest that DOP activation with endogenous DOP ligand may be involved in self-protecting ischaemia-sensitive regions of the brain.

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forebrain ischaemia. The differences between the experimental designs (focal vs forebrain ischaemia) might contribute to the failure to find any protection in the hippocampus because the pathophysiological responses to focal stroke and forebrain ischaemia are inherently different. A protective effect of DADLE may have been observed if a less severe ischaemic insult had been used.

This study, in rats, was initially conducted to test the hypothesis that pre-ischaemic treatment with DADLE would attenuate neuronal death produced by experimental forebrain ischaemia, when the ischaemic insult is not severe. Severe (10 min), moderate (8 min), or mild (6 min) forebrain ischaemia was produced by bilateral carotid artery occlusion (BCAO) combined with hypotension (35 mm Hg). DOP stimulation by DADLE was confirmed using naltrindole, which is a DOP antagonist. In addition, we examined any role of endogenous DOP stimulation (self-protecting) using naltrindole alone.

Methods

Surgical preparation

All experimental protocols were approved by the Animal Care and Use Committee of Nara Medical University. Male Sprague–Dawley rats (Japan SLC, Inc., Shizuoka, Japan), weighing 270–330 g, were fasted for 12 h. Anaesthesia was induced with isoflurane 5% with balance oxygen and the trachea was intubated orotracheally. Isoflurane concentration was reduced to 1.5–2.0%. Lungs were ventilated mechanically with a gas mixture of oxygen and air to achieve an \( F_{1_{\text{O}_2}} \) of 0.3. A needle thermometer was inserted between the temporal muscle and the skull, and the pericranial temperature was controlled to 37.5 (±0.5)°C by surface heating or cooling. Needle EEG electrodes were inserted in a biparietal configuration, and the EEG was monitored continuously (EEG-4217; Nihon Kohden, Tokyo, Japan). A cannula was inserted in the tail artery using PE-50 tubing. The mean arterial pressure (MAP) was monitored continuously. A cannula was inserted in the extra-jugular vein using PE-60 tubing for withdrawing blood.

Induction of forebrain ischaemia using bilateral carotid artery occlusion

The animal was placed in the supine position. Anaesthetic concentration was changed to isoflurane 1.5%. A 10–15 min period was allowed for anaesthetic concentrations to equilibrate. Blood samples were drawn for pre-ischaemic values of haematocrit (Hct), glucose, pH, arterial partial pressure of carbon dioxide (\( P_{a_{\text{CO}_2}} \)), and arterial partial pressure of oxygen (\( P_{a_{\text{O}_2}} \)). Ventilation was adjusted for a \( P_{\text{CO}_2} \) of 35–40 mm Hg with a \( P_{a_{\text{O}_2}} \) of 100 mm Hg or greater. MAP and heart rate were monitored throughout the experiment. Heparin, 100 U kg\(^{-1}\), was administered i.v. Five minutes later, hypotension was induced by withdrawal of blood from the superior vena cava catheter into a pre-warmed syringe. Once the MAP decreased to 35 mm Hg, both carotid arteries were occluded with vascular clamps for a period of 10, 8, or 6 min. Ischaemia was confirmed by the observation of an isoelectric EEG. During the occlusion period, MAP was maintained at 35 mm Hg by either withdrawal or re-infusion of blood. After the determined period of ischaemia, reperfusion of the brain was established by removal of the vascular clamps and re-infusion of the withdrawn blood. Vascular catheters were then removed and the wounds were closed after infiltration with bupivacaine 0.25%. Isoflurane was discontinued, when the wound closure was completed. Heparin was reversed with intraperitoneal administration of protamine (0.3 mg). Ventilation was continued with \( O_2 \) 100%, and the animals were allowed to recover from anaesthesia. A rectal probe was used for intermittent temperature monitoring in the first 3 h after operation and the rectal temperature was recorded every hour and controlled to 37.5 (1.0)°C by surface heating or cooling or managing the temperature in the recovery chambers. DOP stimulation can affect body temperature, usually inducing hypothermia. Once the animal resumed spontaneous ventilation, which was usually observed immediately after discontinuation of isoflurane, it was transferred to a pre-warmed oxygen-rich humidified recovery chamber (environmental \( F_{1_{\text{CO}_2}}=0.4 \)). The trachea was extubated once spontaneous movement was observed. Animal care in the recovery chamber was performed for 3 h.

Neurological assessment

At 7 days post-ischaemia, a neurological examination that consisted of an assessment of motor activity according to the modified method of Sano and colleagues\(^{10}\) was performed by an investigator blinded to group assignment. The rat was placed on a 50×50 cm metal screen (2 cm mesh), which was rotated slowly from a horizontal to a vertical plane. A score was given according to the length of time that the animal was able to cling to the screen. Each animal was given two trials with a 15 min interval, and an averaged score was recorded as a motor activity score.

Brain harvesting and analysis

Animals were weighed and brains were removed 7 days post-ischaemia. After evaluation of weight loss compared with the pre-ischaemic state, the animal was re-anaesthetized using isoflurane 2.5–3% through the mask followed by an injection of 2–3 mg kg\(^{-1}\) of sodium thiopental intraperitoneally. For histological experiments, after loss of consciousness and absence of a pain response, the rat underwent transcardial perfusion with 150 ml of saline at a rate of 40 ml min\(^{-1}\), followed by 200 ml of...
4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, at a rate of 20 ml min$^{-1}$. The brain was removed and stored in 4% paraformaldehyde in 0.1 M PB for later dissection. The brains then were prepared for histological analysis. After dehydration in graded concentrations of ethanol and butanol, the brains were embedded in paraffin. Coronal sections of 5 μm thickness were obtained and stained with haematoxylin and eosin. With the investigator blinded to group assignment, the CA1 and CA3 sectors and the dentate gyrus (DG) of the hippocampus were evaluated in a coronal plane 3300 μm posterior to the bregma. These regions were determined on each slice by referring to the rat brain atlas of Palkovis and Brownstein. The frequency of ischaemic neurones was calculated by dividing the number of acidophilic or pyknotic neurones by the total number of neurones to obtain the percentage necrosis in a counting frame (0.5×0.5 mm) at a magnification of 200-fold.

To standardize, a counting frame was placed across the middle zone of each field of the hippocampus which was targeted for the assessment of the neuronal damage. By convention, values from the hemisphere with the worst damage were used for the final analysis.

Experiment 1: the effects of DADLE on severe, moderate, and mild forebrain ischaemia

The animals were allocated to 10, 8, or 6 min BCAO groups (n=14 per group). Saline 1.0 ml kg$^{-1}$ including 0 or 16 mg kg$^{-1}$ of DADLE was administered intraperitoneally 30 min before forebrain ischaemia (n=7 per group). The administration dose was determined based on previous studies. Sham operations were also performed in additional animals using the same procedures as in the groups receiving saline 1.0 ml kg$^{-1}$ including 0 or 10 mg kg$^{-1}$ of DADLE without ischaemia (n=7).

Experiment 2: the role of DOP in neuroprotective efficacy of DADLE after moderate and mild forebrain ischaemia

We hypothesized that treatment with a DOP antagonist would attenuate DADLE neuroprotection against moderate and mild forebrain ischaemia. In Experiment 1, the length of the BCAO was either 8 or 6 min. We used naltrindole, a highly selective non-peptide DOP antagonist. Saline 1.0 ml kg$^{-1}$ including 0 or 10 mg kg$^{-1}$ of naltrindole was administered intraperitoneally 60 min before forebrain ischaemia, and saline 1.0 ml kg$^{-1}$ including 16 mg kg$^{-1}$ of DADLE was administered intraperitoneally 30 min before forebrain ischaemia. As the control, two intraperitoneal injections of saline 1.0 ml kg$^{-1}$ 30 and 60 min before forebrain ischaemia were performed (n=7–8 per group).

Experiment 3: the role of DOP after moderate and mild forebrain ischaemia

We hypothesized that treatment with the DOP antagonist without co-administration of DADLE would worsen the neuronal cell survival against mild but not moderate forebrain ischaemia. In Experiments 1 and 2, the length of the BCAO was either 8 or 6 min. Saline 1.0 ml kg$^{-1}$ including 0 or 10 mg kg$^{-1}$ of naltrindole was administered intraperitoneally 30 min before forebrain ischaemia (n=7–8 per group).

Experiment 4: evaluation of neurotoxicity induced by naltrindole

We hypothesized that treatment with naltrindole alone would worsen neuronal survival as this treatment would inhibit the actions of endogenous DOP peptides which may produce a self-protecting response. Thus, saline 1.0 ml kg$^{-1}$ including 0 or 10 mg kg$^{-1}$ of naltrindole was administered intraperitoneally 30 min before sham operation (n=7 per group).

Statistical analysis

The physiological values, neurological assessment, and cell count were compared among groups using analysis of variance (ANOVA) or ANOVA for repeated measures. If the ANOVA identified significant differences, unpaired t-tests with Bonferroni corrections were used for inter-group comparisons. Mortality rate comparison was made using the χ² test or Fisher’s exact test. All data except for mortality rate are presented as the mean (SD).

Results

$P_{aO_2}$, $P_{aCO_2}$, pericranial, and rectal temperatures were maintained at target values as described in the Methods. Pre-ischaemic physiological parameters, including Hct, glucose, arterial pressure, and heart rate, were similar among the experimental groups. Weight loss after ischaemia in all the groups was negligible (data are not shown). Several rats died during the 7 day recovery period. Some animals died in the early recovery period with apparent upper airway obstruction or seizure. However, the cause of the other deaths was not identified because those deaths were not witnessed. The mortality rate in each group for each experiment was 0–14% and not different among the groups.

No animal died during the recovery period in the sham group. The motor activity score for the sham group was 58 (23). The numbers of histologically injured neurones (the percentages of histologically injured neurones relative to total neurones) for the sham groups were 11 (5)/0.25 mm² [5 (2)%] for CA1, 7 (3)/0.25 mm² [4 (2)%] for CA3, and 11 (4)/0.25 mm² [2 (1)%] for DG, respectively. The sham group was not included in the statistical analysis for Experiment 1.
**Experiment 1**

Of a total of 42 animals studied, five rats in the DADLE- and saline-treated groups died after ischaemia. The percentages of the histologically injured neurones in the CA1 sector, CA3 sector, and DG after 10, 8, and 6 min ischaemia are shown in Figure 1A–C. The percentages of injured neurones in CA1, CA3, and DG after 10 min ischaemia were not different among the groups (Fig. 1A). Significant differences were detected in the CA3 and DG but not in the CA1 regions with both 8 and 6 min ischaemia (Fig. 1B and C). There were no differences in motor activity scores between the DADLE- and saline-treated groups with any degree of insult [45 (26) vs 41 (28); \( P=0.71 \), 49 (29) vs 30 (17); \( P=0.19 \), 62 (22) vs 64 (23); \( P=0.90 \), after 10, 8, and 6 min ischaemia, respectively].

**Experiment 2**

Of a total of 45 animals studied, five rats died after ischaemia. Regarding motor activity assessment, there were no differences in motor activity scores between the DADLE- and saline-treated groups with any degree of insult [45 (26) vs 41 (28); \( P=0.71 \), 49 (29) vs 30 (17); \( P=0.19 \), 62 (22) vs 64 (23); \( P=0.90 \), after 10, 8, and 6 min ischaemia, respectively].

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**Fig 1 (A–C)** The effects of DADLE on percentage of hippocampal dead neurones after 10, 8, and 6 min forebrain ischaemia. DADLE, \([\alpha\text{-Ala}^2, \text{D-Leu}^5]\)enkephalin. Significant differences were detected in CA3 and DG but not in CA1 with both 6 and 8 min ischaemia.
differences among the saline+DADLE, naltrindole+DADLE, and saline+saline groups [49 (29) vs 57 (26) vs 28 (16); \(P=0.10\), 62 (22) vs 70 (21) vs 64 (23); \(P=0.77\), after 8 and 6 min ischaemia, respectively].

The percentages of histologically injured neurones in the CA1 sector, CA3 sector, and DG after 8 and 6 min ischaemia are shown in Figure 2. After 8 min ischaemia, the percentage of injured neurones in the CA1 sector was similar among the groups. In contrast, regarding the CA3 and DG, the naltrindole+DADLE group had similar numbers of injured neurones to the saline+saline group. The saline+DADLE group had a lower percentage of injured neurones compared with the saline+saline group. After 6 min ischaemia, in the CA3 and DG, the naltrindole+DADLE group had similar percentage of injured neurones to the saline+saline group. The saline+DADLE group still had a lower percentage of injured neurones compared with the saline+saline group. However, the naltrindole+DADLE group had a significantly increased percentage of injured neurones at the CA1 sector compared with the other groups.

**Experiment 2**

Of a total of 30 animals studied, three rats died after ischaemia. Regarding motor activity assessment, there were no differences between the naltrindole and saline groups [47 (29) vs 29 (18); \(P=0.20\), 49 (35) vs 64 (23); \(P=0.28\), after 8 and 6 min ischaemia, respectively]. The percentages of histologically injured neurones in the CA1 sector, CA3 sector, and DG after 8 and 6 min ischaemia are presented in Figure 3. There were no significant differences after 8 min ischaemia. After 6 min ischaemia, the percentage of histologically injured neurones was...
significantly increased by naltrindole in CA1 but not in CA3 and DG.

Experiment 4
No animal died during this experiment. The percentages of histologically injured neurones in the CA1 sector, CA3 sector, and DG were not different between the naltrindole and saline groups [5.5 (0.7)% vs 4.6 (2.1); $P=0.45$, 5.1 (1.2)% vs 4.2 (2.1); $P=0.33$, 2.9 (1.2) vs 2.4 (1.0); $P=0.43$, respectively]. Regarding motor activity assessment, there were no significant differences between the naltrindole and saline groups [50 (31) vs 58 (23); $P=0.58$].

Discussion
We have previously shown that DADLE did not produce neuroprotection against severe forebrain ischaemia. In the present study, we show limited, naltrindole-sensitive, neuroprotection in the CA3 and DG regions of the hippocampus after moderate and mild forebrain ischaemia. Interestingly, regardless of DADLE co-administration, naltrindole significantly worsened CA1 injury after mild forebrain ischaemia. Collectively, these data suggest that DADLE provides limited neuroprotection to relatively ischaemia-resistant regions but not to selectively vulnerable regions, and this is probably mediated by DOP stimulation. That pre-ischaemic DOP antagonism (independent of DADLE) worsens neuronal damage at the
selectively vulnerable regions after mild forebrain ischaemia implies that endogenous DOP ligands exert self-protection roles in ischaemia-sensitive regions. Exogenous ligands, for example, DADLE, produce no further neuroprotective effects.

As mentioned earlier, there are several reports that have demonstrated the protective efficacy of DADLE in the central nervous system.1–3 5 6 12 Zhang and colleagues2 3 investigated the effects of DADLE on glutamate-induced excitotoxic injury of neocortical neurones in vitro and demonstrated that DADLE reduced injury in a naltrindole-sensitive manner. Hayashi and colleagues12 examined the action of DADLE in a serum-deprived pheochromocytoma cell (PC12) and showed that low concentrations of DADLE were anti-apoptotic. In animal experiments, Borlongan and colleagues6 also reported that DADLE enhanced in vitro and in vivo survival of rat dopaminergic neurones in mice. Tsao and colleagues5 reported that DADLE reduced methamphetamine-induced neuronal damage in the striatum in rats. With respect to experimental cerebral ischaemia, Su and colleagues1 successfully demonstrated that treatment with DADLE attenuated the infarct volume after 90 min of transient middle cerebral artery occlusion under normothermic conditions followed by 24 or 72 h of reperfusion in rats. We hypothesized that DADLE would also produce neuroprotection against forebrain ischaemia, although the nature compared with focal stroke is inherently different. Consequently, DADLE failed to show neuroprotection in the ischaemia-sensitive region (CA1), but it was effective in the relatively resistant regions (CA3, DG), if ischaemic insults were not severe. We therefore conclude that neuroprotection provided by DADLE is limited against forebrain ischaemia, although its efficacy might be significant in the case of focal ischaemia. Why was DADLE ineffective against severe (10 min) ischaemia but effective against mild and moderate ischaemia? Ischaemia results in the release of substantial quantities of glutamate13 14 in proportion to the duration of ischaemia.15 It has been demonstrated that DOP activation protects neurones from glutamate-induced excitotoxic injury, which is considered one of the mechanisms of DOP-mediated neuroprotection.3 Accordingly, glutamate release from 10 min severe ischaemia might exceed the capacity of DADLE-induced DOP-mediated neuroprotection. In addition, it should be noted that the limited neuroprotection observed after a recovery period of 1 week in this study might have later faded without supplemental intervention because it has been reported that neuronal cell death continues over weeks even after forebrain ischaemia.16–18 Therefore, we may have still overestimated the limited neuroprotection because we observed signs of necrosis in our histological assessment rather than programmed cell death.

There is growing evidence to support opiate-induced neuronal preconditioning and this may involve DOP activation.19 20 Pre-treatment with DADLE before ischaemia in our study might have induced this type of preconditioning. However, DADLE was also likely to be present during ischaemia because of the short time frame (30 min). Therefore, it seems that the neuroprotection observed in this study was mainly provided by direct activation of DOP by DADLE.

The differences between experimental designs (focal vs forebrain ischaemia) appear to contribute to the present failure to demonstrate protection in the CA1 sector. Forebrain ischaemia results in selective neuronal injury within vulnerable brain regions, whereas focal ischaemia typically gives rise to localized brain infarction.21 Therefore, it is speculated that DOP stimulation with DADLE may not be effective in reducing neuronal stimulation in the ischaemia-sensitive regions even against a mild insult. On the other hand, DADLE provides neuroprotection in the relatively resistant regions, if ischaemic insults were not so severe. This neuroprotection was abolished with naltrindole pre-treatment. It is therefore reasonable to suggest that DOP stimulation can rescue some neurones in relatively ischaemia-resistant regions. However, the detailed neuroprotective mechanisms of DADLE are still unclear2 3 5 12 22–26 and further work is required, particularly to understand the discrepancy of the neuroprotective efficacy after experimental focal and forebrain ischaemia.

Interestingly, DOP antagonism not only reversed the limited neuroprotection provided by DADLE in the regions relatively resistant to ischaemia but also worsened ischaemic neuronal injury in the regions vulnerable to ischaemia. As noted earlier, abolition of neuroprotection by naltrindole pre-treatment indicated that DADLE-induced protection was probably mediated by DOP. This is consistent with previous reports.1–3 5 However, naltrindole worsened ischaemic neuronal injury in the ischaemia-sensitive regions regardless of co-administration of DADLE. This phenomenon indicated that stimulation of DOP by endogenous DOP ligands may play a self-protection role in ischaemia-sensitive regions. Exogenous DOP agonists such as DADLE cannot provide further neuroprotective effects. This phenomenon might be based on the distribution of DOPs and their ligands in the hippocampus.27–38 However, further studies are needed.

There are several limitations to this study. Although DADLE has previously been demonstrated to produce neuroprotection against various neurotoxic or ischaemic insults in vivo at doses similar to those used here, the dose used in this study might not have been appropriate in our experimental setting.1 5 6 Intracerebroventricular injection of DADLE might have produced different results as it has been suggested that DADLE passage of the BBB is difficult.29 However, the dose of DADLE used in mild or moderate ischaemia was effective in CA3 and DG but not in CA1. In addition, we previously reported that DADLE did not show any protective tendency in severe ischaemia.8 Considering that similar doses of DADLE in other studies have shown neuroprotection and differences in experimental
settings between this and previous reports, it is reasonable to assume that different doses or alternative methods of administration of DADLE would not have demonstrated better neuroprotection in this setting of forebrain ischaemia. A similar question might be raised regarding whether other non-peptide delta agonists (e.g. SNC-80 or 121), which could penetrate BBB more easily, would have demonstrated better neuroprotection. We do not believe this to be the case as we feel DADLE penetration of the BBB was sufficient. Another concern is that isoflurane was used for basal anaesthesia in this study, which might have affected the study results as isoflurane itself has neuroprotective effects at relatively short recovery periods after global ischaemia.\textsuperscript{40, 41} It is possible that, with isoflurane anaesthesia, it would be difficult for DADLE to exert additional protective effects. The limited protective effects of DADLE may also have been due to basal anaesthesia used in this study.

In summary, DADLE provided limited naltrindole-sensitive neuroprotection to relatively ischaemia-resistant but not to selectively vulnerable hippocampal regions in the setting of forebrain ischaemia. In addition, as DOP antagonism with naltrindole alone worsened ischaemic damage, endogenous DOP agonists may be self-protective. It is likely that the discrepancy regarding the neuroprotective efficacy of DADLE between our results and the results from other researchers is a function of the differences in the experimental models that were used.

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