

## Changes in brain content of nociceptin/orphanin FQ and endomorphin 2 in a rat model of neuropathic pain

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### Abstract

Orphanin FQ (OFQ) and endomorphins (EM) are newly characterized members of opioid peptide family. OFQ has been shown to antagonize morphine analgesia at supraspinal level, whereas endomorphins are highly selective endogenous ligands for mu receptor, showing analgesic effect at both spinal and supraspinal level. OFQ and EM-2 (EM2) immunoreactivity (ir) was measured by radioimmunoassay in nociception-related brain areas of rats subjected to L5/L6 spinal nerve ligation, using sham operated rats as control. It was found that: (1) the content of EM2-ir of spinal nerve ligated rats showed a significant increase (778%) in periaqueductal gray (PAG), and a significant decrease (43%) in striatum, compared with the control group. (2) a significant increase of the content of OFQ-ir was found in amygdala (+841%) and PAG (+459%), respectively in spinal nerve ligated rats. High pressure liquid chromatography showed that the EM2-ir and OFQ-ir were both heterogeneous with the major part eluting at the position of EM2 and OFQ standard, respectively. These results suggest that spinal nerve ligation induces significant changes in the content of EM2-ir and OFQ-ir in some discrete brain areas, which may play a role in nociceptive modulation. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

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Orphanin FQ (OFQ) is an endogenous ligand for orphanin opioid-like receptor. While OFQ structurally resembles the existing endogenous opioid peptides, especially dynorphin A (1–17), it does not bind to the classical opioid receptors with high affinity. In vivo pharmacological study revealed that OFQ played a role different from traditional opioid peptides. Mogil et al. demonstrated that intracerebroventricular (i.c.v.) injection of OFQ antagonized  $\mu$ -,  $\kappa$ -, and  $\delta$ - receptor-mediated opioid analgesia and opioid-mediated stress-induced analgesia, suggesting that OFQ is an anti-opioid peptide at supraspinal level [8]. Our previous study in rats demonstrated that i.c.v. OFQ antagonized systemic morphine analgesia and opioid-mediated electroacupuncture (EA)-induced analgesia, whereas i.c.v. OFQ antibody produced a partial reversal of morphine tolerance and EA tolerance [15,16]. It was also shown that the biosynthesis and release of OFQ were accelerated in the brain of morphine tolerance rats [17]. These results demonstrated that OFQ is likely to be an anti-opioid peptide at supraspinal level, and involved in the development of morphine tolerance.

Endomorphins, consisting of two members: endomorphin 1 (EM1) and endomorphin 2 (EM2), are endogenous ligands for mu-opioid receptor with high affinity and selectivity. Endomorphins show similar distribution with the  $\mu$  opioid receptor in central nerve system [18]. In acute pain model, both intrathecal or i.c.v. injection of EM1 and EM2 can increase the tail flick latency and the paw pressure latency. In neuropathic pain model, allodynia could be dose dependently reversed by EM1 and EM2, although morphine was not effective in this regard [13].

Acute pain and chronic pain can be modulated by opioids and anti-opioid peptides in the central nerve system [20]. The present work was designed to study whether there is a change occurred in the content of EM2 (typical opioid peptides) and OFQ (putative anti-opioid peptide) in brain areas related to pain control in a rat model of neuropathic pain.

Female Sprague–Dawley rats, bred by the Institute of Animal Research, Chinese Academy of Science, weighing 230–250 g at the start of the experiment, were used throughout. The experimental protocols were approved by the Animal Use Committee of Peking University Health Science Center. The neuropathic pain model was made by ligation of the right L5 and L6 spinal nerves as described in detail by Kim and Chung [6]. Under 10% chlorohydrate

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anesthesia (0.3 ml/100 g body weight), the right L5 and L6 spinal nerves were exposed and tightly ligated with 6–0 silk thread, whereas the control animals were subjected to sham operation with the same surgical procedure without the spinal nerve ligation.

The mechanical allodynia was determined by measuring the 50% paw withdrawal threshold in response to von Frey hairs probing, using the up-down method [1]. Beginning with the 2.0 g hair, in the middle of the series of eight von Frey hairs with logarithmically incremental stiffness ranging from 0.41 to 15.1 g (#s 3.61–5.18), each hair was pressed perpendicularly against the paw with sufficient force to cause slight bending and held for approximately 6–8 s. A positive response was recorded if the paw was sharply withdrawn. The pattern of positive and negative responses was converted to a 50% threshold value by using the formula given by Dixon [2].

Cold-induced ongoing pain was determined as described by Jasmin [5]. Each rat was placed on a brass plate kept at a cold temperature ( $5 \pm 1^\circ\text{C}$ ), and covered by a transparent plastic box ( $21 \times 21 \times 28$  cm). After 5 min of adaptation, the cumulative numbers and duration of time that the rat held its foot off the floor for the next 5 min was recorded.

Rats were divided into two groups, 12 in sham operation group and 15 in L5/L6 spinal nerve ligation (SNL) group. Rats with the paw withdrawal threshold less than 4 g and with the number of foot lifts in  $5^\circ\text{C}$  cold plate more than eight times during 5 min on 8th postoperative day were recruited into the SNL group (about 60% of operative rats were in this category). The animals were assessed 36 days after operation, where marked signs of spontaneous pain, cold-induced ongoing pain and mechanical allodynia were present in the SNL group but not in sham operative group. The rats were rapidly decapitated and the brains were removed promptly and dissected on an ice-cold plate. The amygdala, hypothalamus, periaqueductal gray (PAG) and striatum were dissected according to rat brain stereotaxic atlas [12], then quickly put into the Eppendorf tube containing 1 ml of 0.5 M acetic acid solution, and heated in a boiling water bath for 10 min. After cooling, the brain tissue was supersonically homogenated at  $4^\circ\text{C}$  for 1 min, the homogenate was centrifuged at  $4000 \times g$  ( $4^\circ\text{C}$ ) for 25 min. The supernatant was stored at  $-70^\circ\text{C}$ , and reconstituted in equal volume of 0.5 M NaOH solution to neutralize acetic acid before the assay. The protein content was assayed by Coomassie Brilliant blue method.

The contents of EM2-immunoreactivity (ir) and OFQ-ir were assayed by radioimmunoassay (RIA) using  $^{125}\text{I}$ -EM2 and  $^{125}\text{I}$ -Tyr-OFQ as radio ligands, respectively (EM2 and OFQ RIA kits were products of Phoenix Pharmaceuticals, USA), according to the protocol suggested by Phoenix Pharmaceuticals. On the basis of 11 consecutive assay, the sensitivity of the EM2 assay was 100 pg/ml, non-specific binding was 2%, and total binding was 53%, with 9.49% interassay-variation; the corresponding data for the OFQ assay were 10 pg/ml, 3%, 41%, and 9.86%, respectively. Cross-reactivity

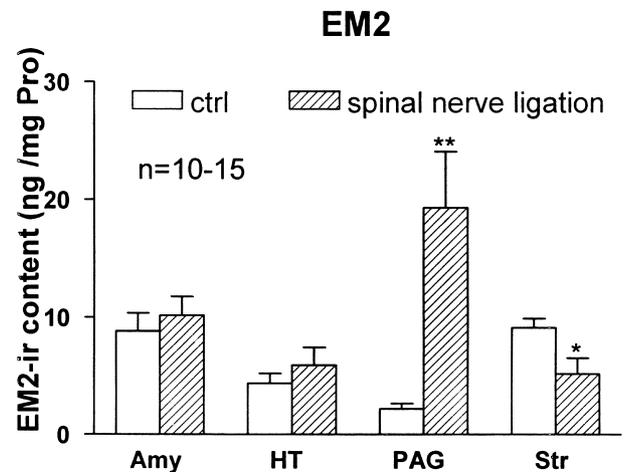


Fig. 1. The change of EM2-ir in brain areas of rats subjected to nerve injury on 36th post-operative day. Amy, amygdala; HT, hypothalamus; Str, striatum. Data are expressed as mean  $\pm$  SEM of 10–15 rats in each group. \* $P < 0.05$ , \*\* $P < 0.01$ , compared with control group. All the data were assessed by *t*-test.

of EM2 with EM1 was 0.47%, and less than 0.001% with dynorphin A, OFQ, Met-enkephalin, Leu-enkephalin and  $\beta$ -endorphin. Cross-reactivity of OFQ with dynorphin A, EM1, EM2, Met-enkephalin, Leu-enkephalin,  $\beta$ -endorphin was less than 0.001%.

The experimental data were expressed as mean  $\pm$  SEM. Group differences were tested by the *t*-test, taking  $P < 0.05$  as the significant level of difference.

The contents of EM2 and OFQ-ir in various brain areas of SNL rats and control rats are shown in Figs. 1 and 2. Compared with the control group, the content of EM2-ir in PAG of SNL rats showed an increase of 778% ( $P < 0.01$ ). In contrast, the content of EM2-ir in the striatum

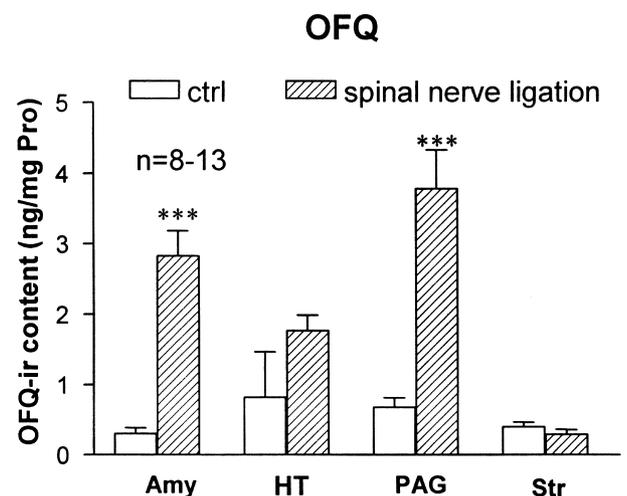


Fig. 2. The change of OFQ-ir in brain areas of rats subjected to nerve injury on 36th post-operative day. Amy, amygdala; HT, hypothalamus; Str, striatum. Data are expressed as mean  $\pm$  SEM of 8–13 rats in each group. \*\*\* $P < 0.01$ , compared with control group. All the data were assessed by *t*-test.

tum showed a decrease of 43% ( $P < 0.05$ ) (see Fig. 1). No significant changes were observed in amygdala and hypothalamus.

Change in the content of OFQ-ir revealed a different profile, i.e. an increase of 841% and 459% in amygdala and PAG, respectively ( $P < 0.001$ ). The mean content was doubled in hypothalamus, although the difference did not reach statistically significant level ( $P > 0.05$ ) (Fig. 2).

In order to validate whether the immunoreactivity from RIA represents the authentic peptide, we used reversed phase (RP)-high pressure liquid chromatography-RIA analysis. PAG of rat was separated and homogenized in 10 volumes of 0.5 N acetic acid. The homogenates were centrifuged at  $4000 \times g$  at  $4^\circ\text{C}$ . The EM2 and OFQ standard and supernatants of the four samples (each sample from six rats) was pooled on a reversed phase Zorbax C8 column (Rockland Technologies, Inc.) Elution was performed using a 80 min acetonitrile gradient (5–100–5%) in 0.1% trifluoroacetic acid (TFA) and  $1.5 \times 10^{-5}$  M glycylglycine at a flow rate of 1 ml/min. The eluates at the position of the standard EM2 and OFQ were lyophilized, taken up in RIA buffer and assayed. The EM2-ir eluting at the position of the EM2 standard was  $46.15 \pm 5.83\%$  of the total EM2-ir in samples, The OFQ-ir eluting at the position of the OFQ standard was  $56.99 \pm 7.19\%$  of the total OFQ-ir in samples. The results suggest that although EM2 and OFQ antibodies may recognize other peptides in addition to EM2 and OFQ, the greater part of EM2-ir and OFQ-ir may represent authentic peptide.

Large body of evidence has shown that OFQ is an anti-opioid peptide at the supraspinal level. Morgan et al. demonstrated that microinjection of OFQ into PAG blocked the antinociceptive effects of morphine applied at the same site, suggesting that PAG is an important place for OFQ to exert an anti-opioid effect [9]. Amygdala has been considered to be involved in morphine analgesia, since microinjections of morphine into amygdala and PAG showed analgesic synergy [11]. Pavlovic et al. reported that microinjection of morphine or beta-endorphin into amygdala elicited analgesia, which could be reversed by microinjection of naloxone into PAG suggesting that the analgesic effect of opioid in amygdala is mediated by an opioid synapse in PAG [11]. Tian et al. [16] and Yuan et al. [17] in our lab demonstrated that the contents of OFQ-ir in amygdala and PAG increased in morphine tolerant rats, and i.c.v. OFQ antibody reversed morphine tolerance. In the present study, we demonstrated that the content of OFQ-ir in amygdala and PAG of SNL rats increased significantly, which is similar to the change observed in morphine tolerance. These results further support the hypothesis that neuropathic pain and morphine tolerance seem to be underlied by some common mechanism in the central nerve systems [7]. Zhu et al. reported that i.c.v. OFQ could aggravate formalin-induced pain behavior and attenuate morphine analgesia, whereas i.c.v. injection of antisense oligonucleotide complementary to OFQ receptor relieved the pain behavior

[19], further suggesting that endogenous OFQ is likely to potentiate pathological pain at supraspinal level.

However, Rosen et al. recently reported a divergent result. They found no significant changes in the PAG level of OFQ-ir in a neuropathic pain model of chronic constriction injury (CCI) [14]. The discrepancy may be explained by the following: (a) the neuropathic pain model they used (CCI model) was different from ours; (b) the time of detection was also different, in that they found no change in OFQ-ir of PAG 14 days after nerve injury as contrast to 36 post-operative day in the present study; (c) the most important difference lies probably on the existence of neuropathic pain behavior. Every rat used in the present study showed profound neuropathic pain behavior as detected by the van Frey hair and  $5^\circ\text{C}$  cold plate test, that was not mentioned in the Rosen et al. paper.

Zanida et al. discovered two potent and selective endogenous agonists for mu-opioid receptor, EM 1 and EM 2 [18], which declared the end of the notion that 'there was no selective endogenous agonist for mu-opioid receptor'. Peripheral nociceptive afferent impulse such as inflammation and various kinds of stress can activate endogenous antinociceptive system, such as enkephalin and  $\beta$ -endorphin [4,10]. Here we show that peripheral nerve injury can induce an increase of EM 2-ir content in PAG, which most likely presents a compensatory neuronal response to mitigate the full expression of neuropathic pain. However, this compensatory neuronal response might have been masked by the increased expression of OFQ in the same area.

High density mu-opioid receptor in striatum suggests its possible involvement in opioid analgesia. He et al. reported that electroacupuncture stimulation elicited analgesia by increased release of endogenous opioid peptides in the striatum [3]. However, in the present study, we found a significant reduction in the content of EM 2-ir in striatum. Whether this is the result of overwhelming release or a suppression of its biosynthesis needs to be studied when endomorphins gene is characterized.

In conclusion, peripheral nerve injury may activate endogenous nociceptive system (e.g. OFQ) and antinociceptive system (e.g. EM2) successively or simultaneously. Taking into account the enormous increase of OFQ-ir in PAG and amygdala of the rats undergoing neuropathic pain and the unambiguous anti-opioid effect of OFQ in brain, a selective reduction of the effect of OFQ in brain may serve as a new approach for the treatment of neuropathic pain.

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