J Tokyo Wom Med Univ
73 (6) 176～187 (2003)

Orphanin FQ Blocks Mechanical Nociceptive Responses in the Nucleus Gigantocellularis

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(Received Feb. 17, 2003)

We studied pain information induced by mechanical noxious stimulation in the brain stem with electrophysiological and immunohistochomical methods. Nocicsptive responses evoked by mechanical stimulation were analyzed in the gigantocellular reticular neucleus (Gi). Moduratory effects of orphanin FQ or morphine on the nociceptive unit activities were also investigated. Single or multiple extracellular unit discharges were evoked by mechanical stimulation to the tail in Gi, a component of the medial pain pathway. High-intensity mechanical stimulation of 2-sec duration elicited two types of high-threshold unit discharges in the Gi. One, a phasic discharge, was attenuated during stimulation. The other, a tonic discharge, frequently persisted past the end of stimulation without adaptation. Orphanin FQ (100 ng) or morphine (50 μg), injected via the fourth ventricle through a catheter, immediately and completely blocked long-lasting tonic type discharges which could be accounted for by C-fiber activity. The onset burst of the phasic discharge, which was assumed to at least partly reflect Aδ activity, showed a reduced firing rate but was not completely inhibited. Using immunohistochemical methods, C-fiber inputs to the Gi were confirmed by showing Fos expression induced by peripheral C-fiber activation with subcutaneously-injected capsaicin. The present study confirms that C-fiber mediated pain information reaches the Gi and that orphanin FQ as well as morphine can potently block C-fiber mediated noxious responses.

Key words: pain, mechanical stimulation, orphanin FQ/nociceptin, brain stem, reticular formation

Introduction

Opioid receptor-like 1 (ORL 1), a member of the opiate receptor family, was cloned in 1994. Durring the process of cloning of the opiate receptor, four different sequences were identfied[1)2)]. Three, mu, delta, and kappa opiate receptors, had been identified with various ligands in experiments conducted before cloning. In contrast, one of the cloned receptors did not have a known specific ligand at that time, and was therefore called the orphanin receptor. Although no specific ligand was known, a universal ligand (etorphine) activated the orphanin receptor, inducing a decrease in cAMP and an increase in potassium conductance. This receptor was named ORL1 by Mollereau and others who cloned it from human tissue. Expression of ORL1 receptors in the central nervous system by in situ hybridization

analysis revealed[2)~4)] an endogenous ligand which numerous investigators have sought with great effort.

The endogenous ligand of the orphanin receptor was found in 1995 to consist of 17 amino acids and to have a sequence resembling that of dynorphin A. It was named nociceptin by Meunier[5)]. It produced hyperalgesia on tail-flick and hot-plate tests following intracerebroventricular injection into mice. In contrast, Reinscheid et al[6)] reported the ligand to have an antinociceptive effect and named it orphanin FQ. Intrathecal administration of orphanin FQ was shown to reduce pain-induced behaviors in the tail flick test[7)], attenuate thermal hyperalgesia[8)9)] and reduce formalin-induced pain in mice[10)]. As in the first reports by Meunier, intracerebroventricular injection of orphanin FQ also produced hyperalgesia[10)] and blunted the analgesia induced by morphine[11)]. These opposing effects with different routes of administration indicate that orphanin FQ has complex modifiable effects on pain information at different sites in various brain areas.

In the brain stem trigeminal nucleus (SNT), orphanin FQ reportedly to inhibits nociceptive responses evoked by mechanical and thermal stimulation[11)]. ORL 1 immunoreactivity was observed in all brain stem nuclei including SNT[12)13)]. Thus, orphanin FQ might have similar inhibitory effects on other parts of the brain stem. Strong ORL1 reactivity[13)] was detected, especially in the somata of Gi neurons, suggesting orphanin FQ to affect nociceptive responses in the Gi.

Repetitive stimulation of C-fibers elicits a summation of responses[14)], which consequently induces long-lasting post-stimulus discharges and a wind-up discharge. Glutamate release following NMDA receptor activity at the C-fiber terminal is thought to mediate these prolonged responses of dorsal horn nociceptive neurons[15)]. Orphanin FQ reduced glutamatergic excitatory postsynaptic potentials (EPSPs)[16)] and winding-up discharges in the spinal cord[17)]. These results indicate that orphanin FQ blocks C-fiber mediated post-stimulus discharges. Gi neurons receive C-fiber mediated nociceptive inputs from the peripheral nerves[18)~21)]. A substantial number of Gi neurons contain glutamate[22)], and a portion of these neurons may transmit nociception. Thus, orphanin FQ may reduce C-fiber responses via inhibition of glutamatergic EPSPs in the Gi as well as in the dorsal horn. A large population of Gi neurons responds to mechanical noxious stimulation[18)23)], although the evoked discharges reveal no somatotopic organization[19)]. The spinothalamic pathway, which sends collateral projections to the Gi, reaches the limbic cortex via the intralaminar thalamic nucleus[24)]. In addition, the Gi, which is a part of the ascending reticular activating system, projects to the intralaminar nucleus[25)]. Hence, the Gi may transmit diffuse complex information associated with pain and orphanin FQ may modulate this nociceptive information.

The present study was designed to examine the effects of orphanin FQ, as compared with morphine, on nociceptive responses of Gi neurons.

Materials and Methods

Animal preparation

Experiments conformed to the experimental guidelines for animal care of the Animal Experiments Committee of Tokyo Women's Medical University. We used 76 male Wistar rats weighing 280~360 g (Sankyo Lab Co, Tokyo) The animals were anesthetized with urethane (1~1.5 g/kg) for experiments. Respiration was controlled by a respirator (NK-55, Natsume Co, Tokyo) through an endotracheal tube. Body temperature was maintained at 37.0~37.8 ℃ with a chemical thermomat.

Electrophysiology and drug application

Urethane-coated stainless steel needles (impedance 5~10 MΩ) and screw electrodes were used

for recording and reference electrodes, respectively. Unit discharges were recorded at 11.8 mm posterior 0.8 mm lateral to the bregma and, depth 7.0～9.0 mm[22] with a 500 Hz～1.5 kHz frequency band (Vc-11, ABV-11, Nihon-Koden Co, Tokyo) and led to a histogram analyzer (QC-111J, Nihon-Koden Co, Tokyo) on line.

An epidural catheter (Hakko Co, Tokyo) was inserted into the fourth ventricle via the foramen magnum for microinjection (microinjection syringe, Hamilton Co, Nevada, USA). First, we injected 50 μl of artificial liquor as a control. After a 30-min observation period as a baseline control, orphanin FQ (Alexis Co, San Diego, USA) at 100 ng/50 μl (artificial liquor) or 50 μg/50 μl morphine was injected at a rate of 10 μl/min. Unit discharges were continuously recorded 120 min after the injections to detect recovery from the effects of the drugs. Before the experiments, we examined spread of the solution in the ventricle using radiopaque material (Omnipaque, Daiichi Seiyaku Co, Tokyo) which was injected via the catheter at the same rate (10 μl/min). At 5 min and 30 min after 50 μl of radio opaque medium had been injected, X-rays were taken of the whole animal. At 5 min, the radiopaque medium was concentrated around the tip of the catheter in the fourth ventricle but the solution had spread to the cervical spinal level (C2) by 30 min.

Mechanical stimulation to the tail

A mechanical stimulator (DPS-260, Diamedical Co, Saitama, Japan) was used to apply mechanical pressure stimulation, with a 0.1-mm-tip-diameter circle probe, to the tail. Constant force (50 or 200 gw, duration of 2 s) controlled by a feedback system was delivered every 1 min to the tail. We also manually applied 300 mmHg pressure (2 s duration) using a 0.5-mm square probe with a syringe containing water (Fijio-Tech Co, Tokyo). The pressure in the syringe led to a high pressure transducer monitor (Nihon-Koden Co). The stimuli used in our experiments elicited a pain sensation when applied to our own fingertips, indicating both stimulus methods to be adequate for nociceptive stimulation.

Histological examination

Fos induction following subcutaneous injection of capsaicin was detected in the Gi. A 1 ml volume of 1 mM capsaicin was injected subcutaneously at the coudal region of the tail under urethane anesthesia. Animals were perfused intracardially with 200 ml of saline and then 200 ml of fixative (4% paraformaldehyde and 0.25% glutaraldehyde in PBS) at 90 min after injection. The brain stem was sectioned at 30 μm after overnight postfixation. We used commercial anti- Fos antibody (Cat#PC38 polyclonal Ab-5, Calbiochem, USA) diluted 10,000× in PBS. The antibody was detected by the ABC method (Vectastain ABC kit, Vector Lab Inc, USA) following conventional staining with methylviolet.

At completion of the electrophysiological experiments, brain tissue was removed to examine recording locations under light microscopy. Brain samples were sectioned at 50-μm after transcardial perfusion and stained by conventional methods.

Results

Unit discharges evoked by mechanical stimulation in Gi

We recorded 41 cells which responded to pressure stimulation, and 22 cells located in the Gi (18/22) or PGi (4/22) were treated with drugs. A total of 17 of 22 cells were tested with orphanin FQ (Fig. 1 filled circles), the other 5 cells with morphine (Fig. 1A, B cross). All cells recorded were located between 11.5 and 12.0 mm posterior to the bregma[26].

We observed two types of high-threshold responses evoked by mechanical stimulation. One was a tonic-type discharge which persisted past the end of stimulation (post stimulus discharge;

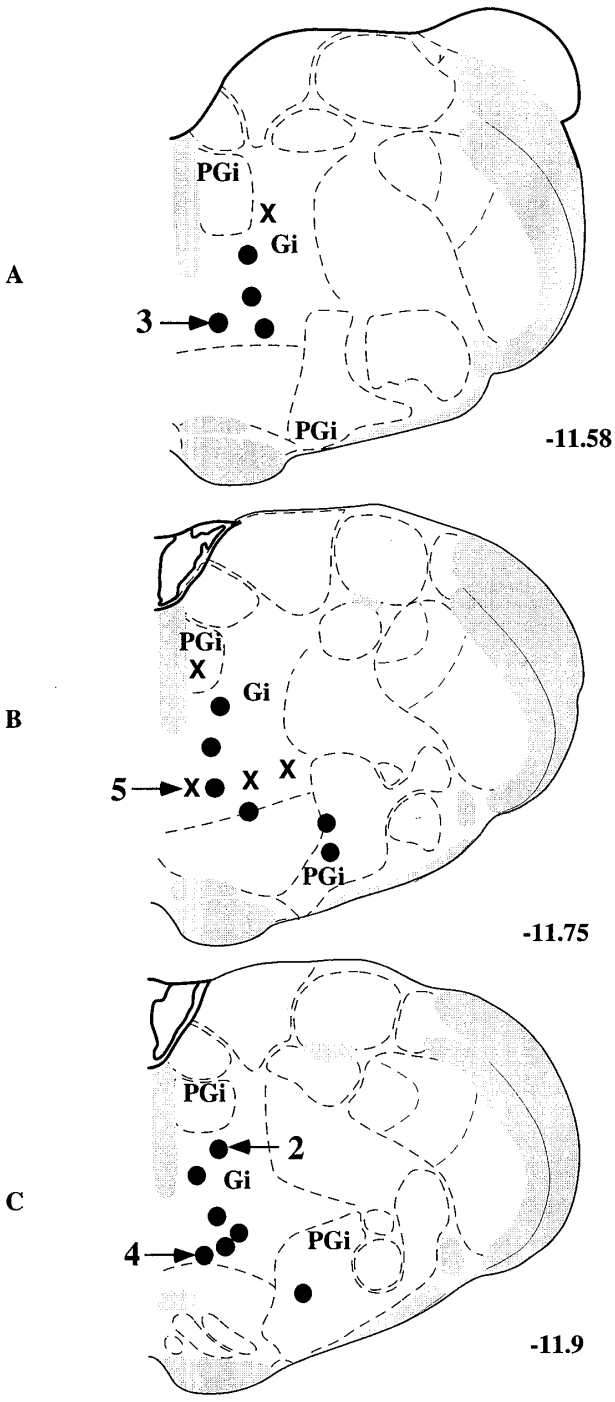

Fig. 1 Locations of unit recording sites
Representation of the recording sites in a coronary section of the rat brain stem. The numbers on the right represent caudal distances (mm) from the bregma. Arrows and numbers represent recording locations of the units in the figures 2, 3, 4 and 5.
● : neurons inhibited by orphanin FQ injection, X : neurons inhibited by morphine injection.
The figures in the coronal section were copied from Brain Maps (Swanson, 1998[26]).

Fig. 2A). The other response, which was seen throughout the application of strong pressure, gradually adapted to the stimulation (phasic type, Fig. 3). Post-stimulus histograms, which were constructed from single unit activities, frequently contained these two components (Fig. 3A). The early component corresponded to unit discharges during pressure stimulation and post-stimulus discharges constituted the late component. We detected no low-threshold cells which responded to weak pressure or wide dynamic-type discharges under our experimental conditions. Post-stimulus discharges were observed in 15 of the 22 neurons. The discharge patterns evoked by mechanical stimulation were the same in the Gi and the PGi; thus, we could not distinguish these two areas on the basis of electrophysiological properties during the control stage.

Orphanin FQ and morphine

Orphanin FQ and morphine inhibited discharge frequency in the high-threshold response (Figs. 2B, 3B, 5B). The depressive effects of orphanin FQ were reversed, restoring the control level, within 60 min (Fig. 2C). In contrast, the reduction of discharge frequency by produced morphine persisted longer and the control level had not been reached even at 120 min (Fig. 5C). In phasic adaptive type units, discharge frequency was reduced but persisted after injection (Fig. 3 B). In contrast, tonic post-stimulus discharges were completely blocked immediately after injection (Fig. 2B). In the post-stimulus histograms, the first component of the histogram was reduced but persisted. In contrast, the late component was completely inhibited (Fig. 3B) in all neurons. Both peaks returned to the control level within 90 min after injection. The frequency of background discharges occasionally increased after orphanin FQ injection (7 animals, Fig. 4B), while the evoked nociceptive responses were impaired. This enhancement lasted throughout the

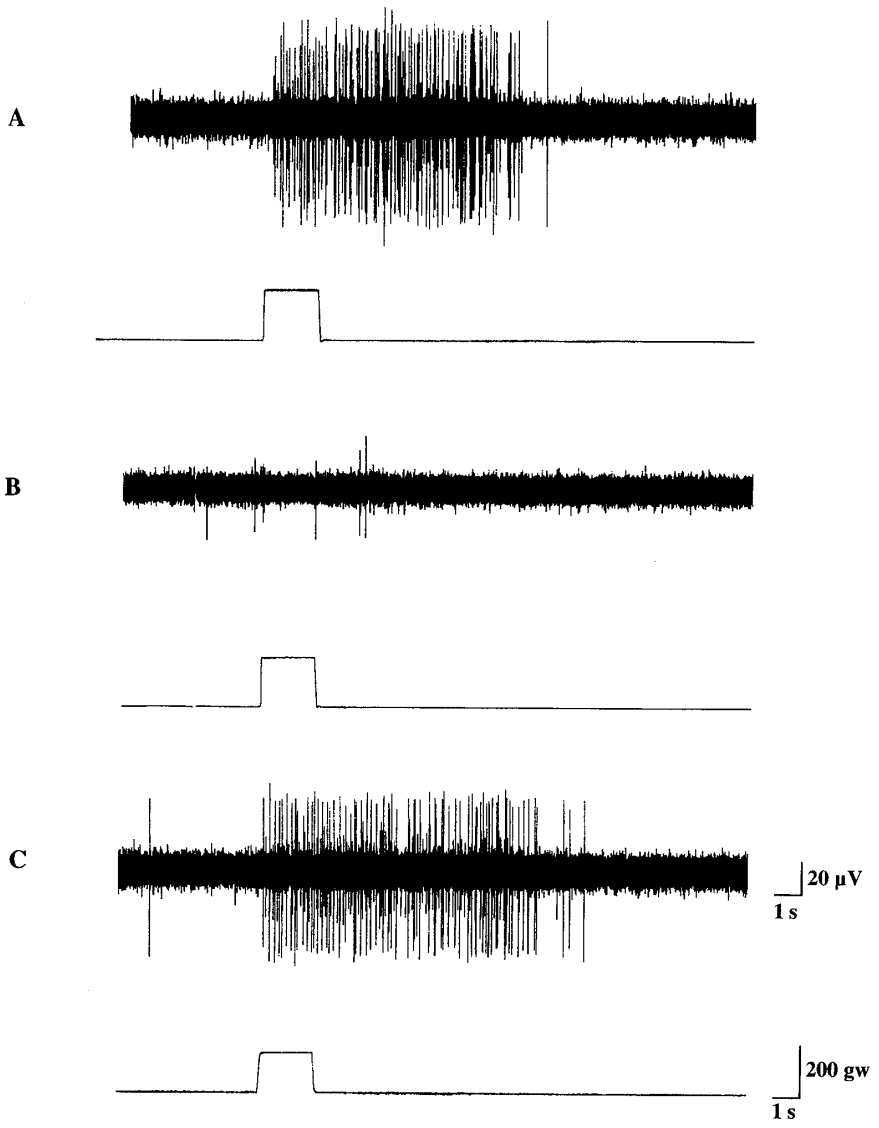

Fig. 2 Post-stimulus discharges blocked by orphanin FQ
Tonic-type unit discharges were recorded beyond the end of stimulation. Orphanin FQ immediately blocked unit activities. Extracellular unit discharges (upper trace) and stimulus pressure curves (lower trace) are present.
A: preinjection control
B: 10 min after orphanin FQ injection
C: 60 min after injection

depression of nociceptive discharges and decreased to preinjection levels when the inhibitory effects disappeared (Fig. 4C).

Peripheral C-fiber activation expressed Fos protein in Gi cells

Cells labeled with Fos antibody were scattered bilaterally in the Gi. Most positive cells were polygonal and medium sized (10～30 μm in diameter, Fig. 6B). They were located in nets of fibers. A small number of large cells, 30～50 μm in diameter, showed light staining (Fig. 6C). In contrast, no Fos-positive cells were observed in the Gi of intact animals not treated with capsaicin (Fig. 6A).

Discussion

A newly recognized endogenous ligand of the

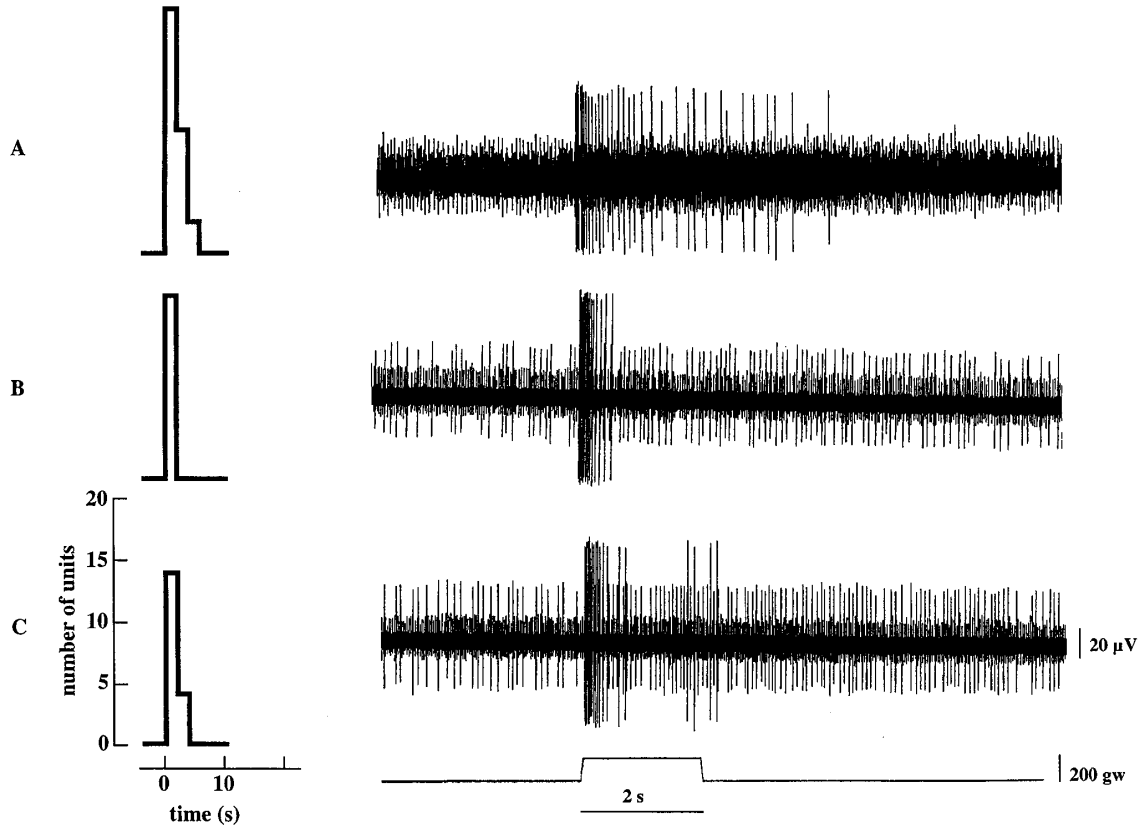

Fig. 3 Post-stimulus histograms of nociceptive responses induced by mechanical stimulation and phasic-type of discharges
Typical post-stimulus histograms (left) calculated from unit activities (right) are shown. The lower trace is a pressure curve of stimulation. Each bar on the histograms represents a 2-sec bin.
A: The frequency rate of discharges was high at the onset of stimulation and thereafter gradually decreased. The histogram consists of two components. The early component represents unit discharges during stimulation, the late component post-stimulus discharges.
B: 10 min after orphanin FQ injection: Only the phasic unit discharge at the onset remains and the late component is completely suppressed.
C: 30 min after orphanin FQ injection: The late component has partially recovered.

opiate receptor-like 1 receptor, orphanin FQ (Nociceptin)[5)6)], is considered to have anti-nociceptive effects[7)~10)27)]. Intraventriculear injection of orphanin FQ blocked nociceptive discharges recorded in Gi neurons supporting the anti-nociceptive effects of orphanin FQ. At the spinal level, C-fiber mediated wind-up discharges[17)] and glutamatergic EPSP[16)] of dorsal horn neurons are reduced by orphanin FQ. Moreover, Orphanin FQ reduced the amplitudes of glutamatergic EPSCs evoked by small fiber activities in the substantia gelatinosa neurons but had few effects on miniature IPSCs[28)]. In the brain stem, nociceptive responses induced by mechanical and thermal stimuli were depressed by injection of orphanin FQ into the trigeminal nucleus[10)].

Mechanical noxious discharges recorded in Gi were characterized by post stimulus discharges persisting for the duration of stimulus delivery[18)~21)29)]. Orphanin FQ, in this study, critically

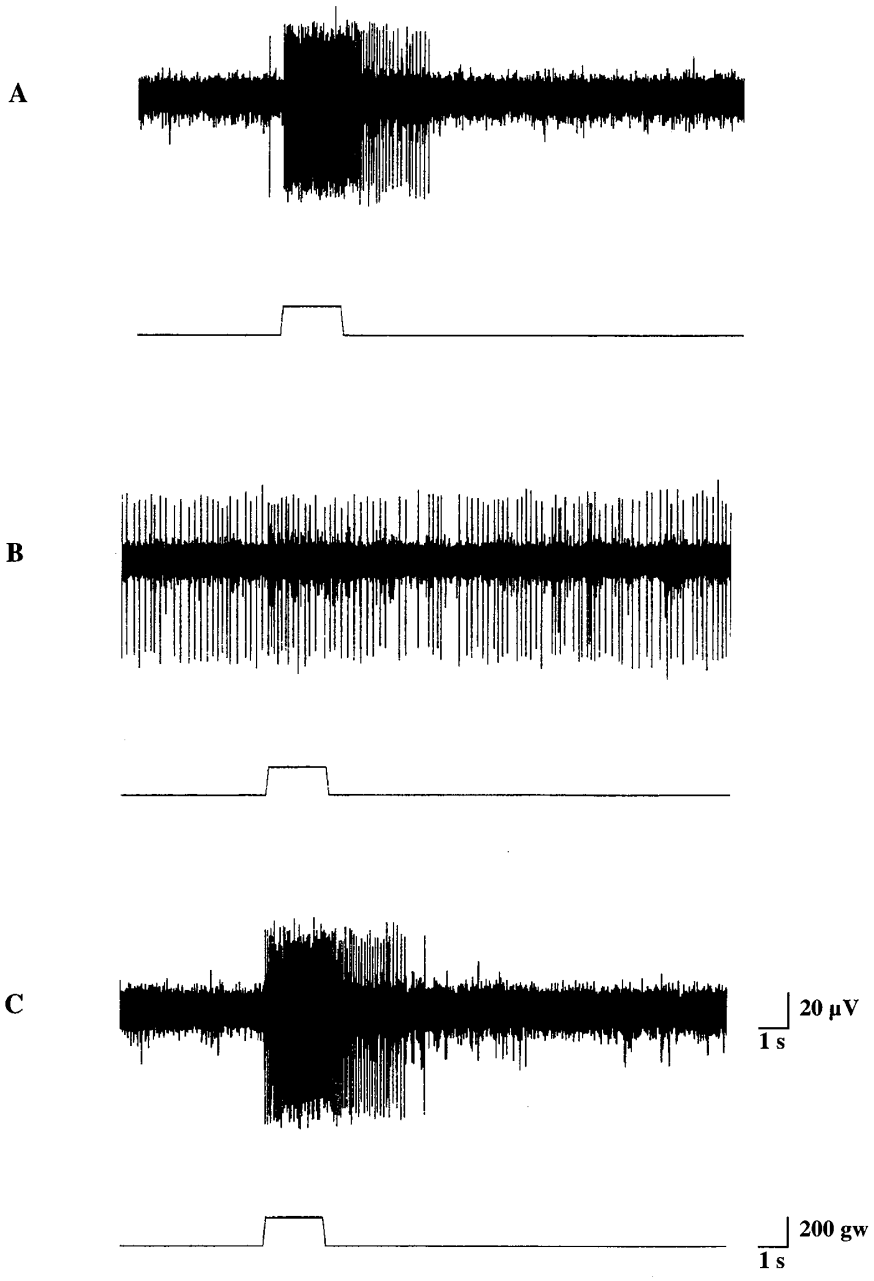

Fig. 4 Orphanin FQ changed the frequency rate of background spontaneous discharges
A: Preinjection control
B: 10 min after orphanin FQ injection: Occasionally, orphanin FQ injection increased the firing frequency of background units which did not respond to noxious stimulation.
C: 90 min after injection: The enhancement had disappeared and the preinjection level was restored by the time nociceptive responses had returned to the control level.

impaired post stimulus discharges, suggesting that it affects the modulation of C-fiber mediated response summation. This temporal summation of discharges without adaptation is a conspicuous feature of C-fiber activation. Repetitive stimulation of C-fibers induced NMDA receptor activa-

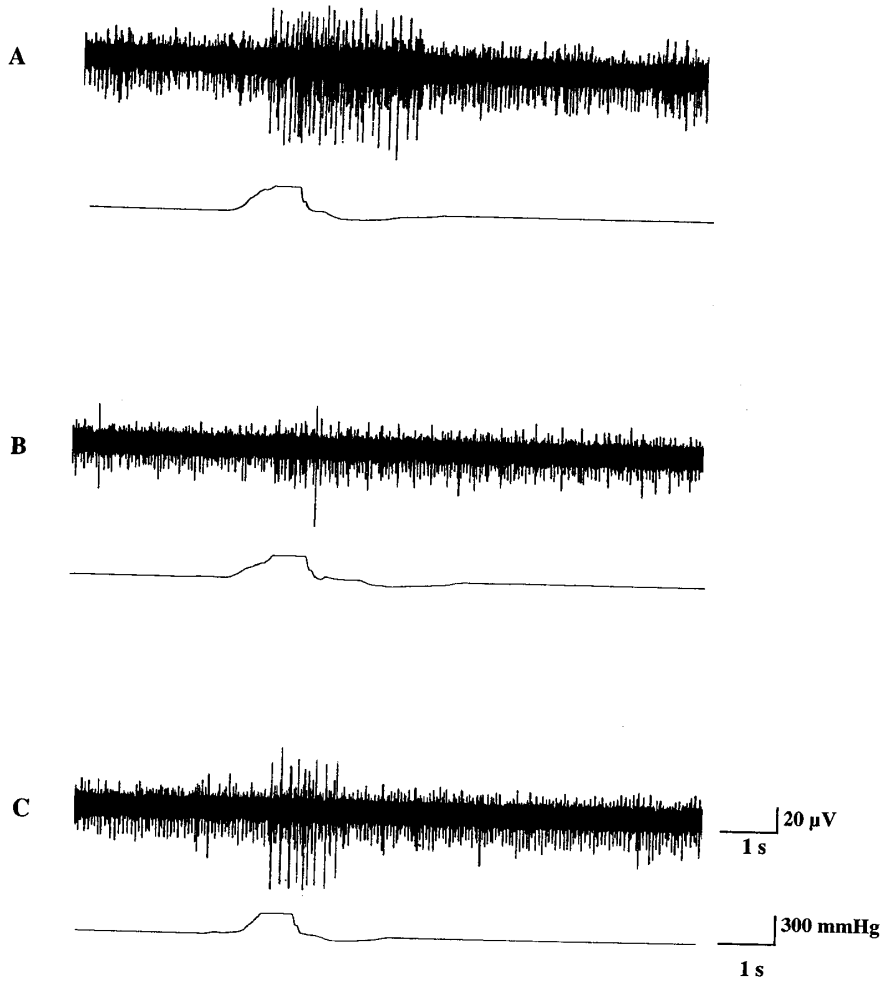

Fig. 5 Effects of morphine on noxious responses in the Gi
A: Preinjection control: The tonic type of discharge was recorded.
B: 10 min after morphine injection: The noxious response was inhibited, while background discharges were unchanged.
C: 120 min after morphine injection: Responses did not fully recover and the duration of the post-stimulus discharge was still shorter than prior to injection.

tion following co-release of glutamate and substance P[30)31)], which produced long-lasting post-stimulus discharges[14)]. As shown in Fig. 3, stimulus onset bursts were not completely blocked as seen with post-stimulus discharges. C-fiber and Aδ-fiber mechanosensitive afferents elicit high threshold responses in neurons[32)]. The first responses may include Aδ-mediated responses, which may be resistant to orphanin FQ. Recently, similar results were obtained using wide dynamic range neurons in the rat dorsal horn[33)].

In contrast to the inhibitory effects on nociceptive responses, orphanin FQ occasionally increased background discharges (Fig. 4) suggesting that it can induce excitatory effects on some neurons. Induction of hyperalgesia by intracerebroventricular injection of orphanin FQ has been observed in the tail flick test[6)], hot plate test[5)] and formalin test[10)]. Biphasic effects on pain modulation of orphanin FQ have also reported[11)29)34)]. Orphanin FQ may affect pain modulation via the neural network and induce final complex effects on pain behavior. High-dose orphanin FQ, injected locally into Gi, decreased the intentional

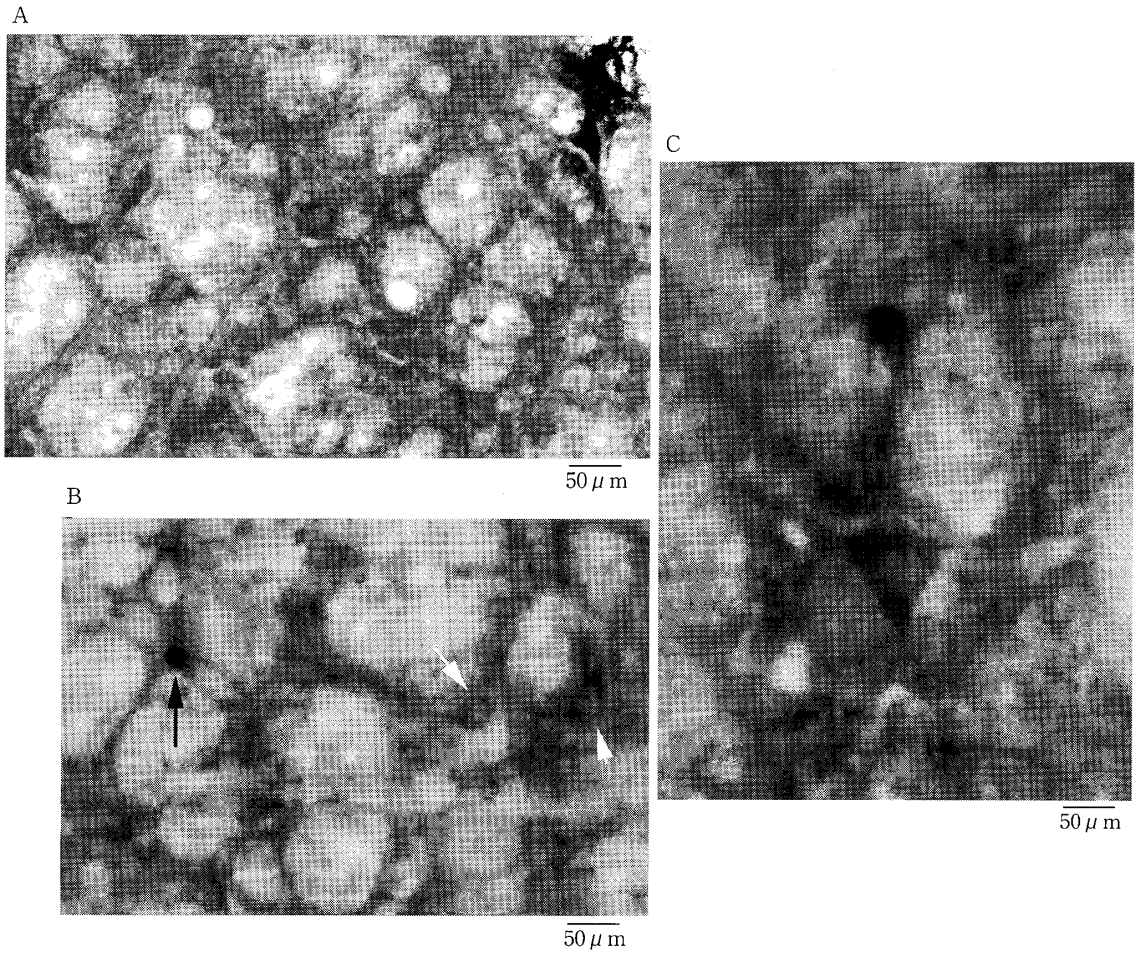

Fig. 6 Fos protein immunoreactive neurons in Gi induced by capsicine injection into the peripheral tissue
A: Control without capsicine
B: Fos positive neurons are observed on the left side (black arrow), negative neurons on the right side (white arrows) in a slice.
C: A Fos positive neuron can be seen on the upper side.
A, B and C are from different samples.

tail flick latency in rats[35]. The discrepancies between our results and those of other investigators may be attributable to the doses administered and injection methods.

Capsicine injection into the peripheral tissue induced Fos protein in the nuclei of Gi neurons indicating that afferent pain information is transmitted to the brain stem Gi. Collateral fibers from the spinothalamic and spinoreticular pathways project to the reticular formation[36)~38)]. The Gi has been regarded as part of the medial pain pathway. Electrical stimulation of Gi induced Fos positive neurons in the median nuclei of the thalamus[39], which are also included in medial pain pathway. Large Fos immunoreactive neurons might be glutamatergic excitatory relay neurons[40]. Collateral fibers from the spinothalamic and spinoreticular pathways project bilaterally in the reticular formation[36)~38)]. This explains why immunolabeled cells were scattered bilaterally. There was no detectable activation of units activated by low-threshold somatic stimulation. Responses of Gi neurons to innocuous stimulation were, however, present in 17.6% of all discharges

recorded in decerebrate cats[22]. The urethane anesthesia used in our experiments may therefore have had some effects on low-threshold type discharges.

The Gi plays a complex role in the affective pain reaction[19] and in sensory motor integration[41)42]. The Gi also participates in blood pressure modulation[43]. Gi neurons respond to blood pressure changes and nociceptive stimulation. Earlier reports suggest pain and autonomic activity integration in the Gi. Intense immunoreactivity for ORL1 in the soma of Gi neurons[44] suggests orphanin FQ to have important effects on the activities of Gi neurons. Orphanin FQ inhibits hippocampal LTP production[45)46] and impairs spatial attention[47] suggesting that it affects more than just pain sensation. These observations suggest that orphanin FQ has more than simple analgesic effects on Gi neurons and may modulate pain behavior associated with nociceptive sensation via the brain stem pain pathway.

Acknowledgments

The valuable suggestions and manuscript reviews of Drs. Hidehiro Suzuki, Makoto Ozaki and Yoriko Kawakami are greatly appreciated. This study was supported in part by the Japanese Ministry of Education, Science and Culture and Sports (Grant No. 12770841)

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ラット巨細胞性網様体核における機械的侵害受容ニューロンに対する orphanin FQ の影響

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オピオイド受容体のクローニングの過程で既知であった受容体の他に新たな受容体の存在が確認され，これに対応する内因性のオピオイドリガンドとして orphanin FQ が 1995 年に生体より抽出された．その作用は投与方法，投与経路，投与量により鎮痛効果が発現するといった報告と hyperalgesia が認められるといった相反する報告がある．著者は脳幹網様体における orphanin FQ の侵害受容ニューロンへの作用を検索した．機械的侵害刺激によって誘発された侵害受容細胞の発火が脳室内投与された orphanin FQ によりどのような影響を受けるかを，痛みの伝達経路の一つである巨細胞性網様体核で観察し morphine と比較検討した．ラットの尾部に与えた 2 秒間の持続する機械的侵害刺激により 2 種類の高閾値作動性ニューロンが記録された．その発火パターンは 1 種類は刺激に対する順応の早い phasic タイプ，もう 1 種類は刺激中に発火が持続する tonic タイプであった．orphanin FQ (100 ng) および morphine (50 μg) は C-fiber の活動を反映すると考えられる tonic タイプの神経細胞の発火を抑制したが，対照的に Aδ-fiber の活動を反映すると考えられる phasic タイプへの効果は弱く反応は消失しなかった．C-fiber を介して伝達される痛みの情報伝達が巨細胞性網様体核を介することを確認するため，カプサイシンを皮下投与し誘導された Fos 陽性細胞の存在を巨細胞性網様体核で確認した．電気生理学的および免疫組織学的な検索の結果より C-fiber を介して伝達される痛みの情報は巨細胞性網様体核に至り，orphanin FQ の脳室内投与によって抑制されることが示唆された．