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In vivo pain-inhibitory role of nociceptin/orphanin FQ in spinal cord

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In vivo pain-inhibitory role of spinal N/OFQ

ABBREVIATIONS: Nociceptin/orphanin FQ, N/OFQ; N/OFQ peptide receptor, NOP; algogenic-induced nociceptive flexion, ANF; intraplantar injection, i.pl.; intrathecal injection, i.t.; scratching, biting and licking, SBL responses

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ABSTRACT

As nociceptin/orphanin FQ (N/OFQ) has both pronociceptive (hyperalgesia) and antinociceptive actions in pharmacological experiments, and there is no significant difference in the nociceptive responses between $NOP^{-/-}$ mice and their wild-type ($NOP^{+/+}$) littermates, the physiological role of N/OFQ in pain regulation has remained to be determined. Under the hypothesis that the use of molecularly distinct nociception test may reveal the pain modality-specific role of N/OFQ, we attempted to examine the physiological role of N/OFQ in pain transmission by using newly developed algogenic-induced nociceptive flexion (ANF) test in $NOP^{-/-}$ and $NOP^{+/+}$ mice or NOP antagonist-treated mice. The nociceptive flexor responses upon intraplantar injection of bradykinin or substance P, which stimulates polymodal substance P-ergic fibers, were markedly potentiated in $NOP^{-/-}$ mice, compared to those in its $NOP^{+/+}$ mice. However, there were no significant changes in $NOP^{-/-}$ mice with adenosine triphosphate or prostagrandin I_2 agonist, which stimulates glutamatergic but not substance P-ergic fibers. The nocifensive responses induced by substance P (i.t.) were also potentiated in $NOP^{-/-}$ mice. On the other hand, there were no significant differences in NK1-like immunoreactivity, [^{3}H] substance P binding or NK1 gene expression in the dorsal horn of the spinal cord between $NOP^{-/-}$ and $NOP^{+/+}$ mice. In addition, N/OFQ antagonists decreased the threshold in nociception tests driving spinal substance P neurotransmission. All these findings suggest that the N/OFQ-ergic neuron may play an *in vivo* inhibitory role on the second-order neurons for primary polymodal substance P-ergic fibers in the spinal cord.

Keywords: **nociceptin/orphanin FQ; NOP knock-out mice; NOP antagonists; spinal cord; physiological role, pain transmission.**

Since the discovery of nociceptin or orphanin FQ (N/OFQ), the endogenous ligand for opioid-like orphan receptor (ORL1), there have been many reports that N/OFQ has both pronociceptive (or hyperalgesic) and antinociceptive properties (Inoue et al., 1999; see review, Mogil and Pasternak, 2001). On the other hand, the supraspinal administration of antisense oligodeoxynucleotide or antagonist for N/OFQ receptor (NOP) caused an increase in nociceptive threshold (Meunier et al., 1995; Zhu et al., 1997; Rossi et al., 1997; Calo' et al., 2000; Shinkai et al., 2000; Calo' et al., 2002), while $NOP^{-/-}$ mice displayed normal baseline nociceptive responses in some analgesic paradigms (Nishi et al., 1997; Mamiya et al., 1998; Ozaki et al., 2000). These findings suggest that N/OFQ might play differential pain modulatory roles. In most popular analgesic paradigms we use, various nociceptive thermal, mechanical and chemical stimulations might activate distinct types of fibers at the same time. These fibers might include both pain-stimulatory and inhibitory ones, according to the gate control theory (Melzack and Wall, 1965). The late nociceptive responses in some paradigms might be modulated to some extent by descending pain-inhibitory mechanisms secondary to the initial nociceptive input (Fields, 1987). Thus, it is important to use nociception tests based on the molecularly distinct nociceptive stimulation, which causes rapid nociceptive behaviors, in the attempt to characterize the modality (or nociceptor)-specific role of specific neurotransmitters or neuropeptides. Algogenic-induced nociceptive flexion (ANF) test in mice would be the one we have recently developed to clarify the distinct roles of such neurotransmitters or neuropeptides in the nociceptor-specific pain regulation. Here we report the *in vivo* inhibitory role of spinal N/OFQ-ergic neurons for the pain following polymodal substance P-ergic fiber stimulation, by use of $NOP^{-/-}$ mice and selective NOP antagonists in the ANF test and other known analgesiometric assays such as paw pressure,

Hargreaves thermal nociception and capsaicin tests.

METHODS

Animals. Male ddY mice weighing 20 - 22 g were used. Mutant mice were homozygotes ($NOP^{-/-}$) lacking the genomic NOP gene, heterozygotes ($NOP^{+/-}$) and its wild-type ($NOP^{+/+}$) mice which have been developed previously (Nishi et al., 1997), and housed in a group of 10 animals. They were kept in a room maintained at 21 ± 2. with free access to a standard laboratory diet and tap water. Procedures were approved by Nagasaki University Animal Care Committee and complied with the recommendations of the International Association for the Study of Pain (Zimmerman et al., 1983).

Drugs. The following drugs were used: N/OFQ (Sawady Technology, Tokyo), substance P (Peptide Institute, Osaka, Japan), bradykinin (Sigma, St. Louis, MO, USA), adenosine triphosphate (Nacalai Tesque, Kyoto, Japan), capsaicin (Nacalai Tesque, Kyoto, Japan) and MK-801 (Research Biochemical). ONO-54918-07 (a stable prostaglandin I_2 agonist) was gift from Ono Pharmaceutical Co.,Ltd (Tokyo, Japan). CP-99994 was generously provided by Pfizer Pharmaceuticals (Sandwich, Kent, UK). J-113397 (1-[(3R,4R)-1-cyclooctylmethyl-3-hydroxymethyl-4-piperidyl]-3-ethyl-1,3-dihydro-2H-benzimidazole-2-one) was generously provided by Banyu (Tsukuba, Japan) and [$Nphe^1$]N/OFQ(1-13)NH_2 was a gift from Prof. S. Salvadori and Dr R. Guerrini of the Department of Pharmaceutical Sciences of the University of Ferrara (Ferrara, Italy). All drugs except capsaicin were dissolved in physiological saline. Capsaicin was dissolved in 10 % ethanol and 10 % Tween 80 in physiological saline.

Intrathecal injection. The intrathecal injection (i.t.) was adopted according to the method of Hylden and Wilcox (1980). A-28 gauge stainless steel needle attached to a 50 μl Hamilton microsyringe was inserted between lumbar 5 and 6 in unanaesthetized mice, and drugs were given slowly in a volume of 5 μl.

***In vivo* nociception test.**

Tail-flick test. Animals were gently restrained by hand, and a light beam adjusted for 10- to 12-sec latency in naive mice was focused onto the blackened dorsal surface of the tail. Latency up to a cut-off time of 20 sec was measured (Ueda et al, 1986).

Paw-pressure (digital von Frey) test. Mice were placed in a Plexiglas chamber on a 6x6 mm wire mesh grid floor and were allowed to accommodate for a period of one hour. A mechanical stimulus was then delivered onto the middle of the plantar surface of the right hind paw by using a 0.8- to 0.9-mm diameter filament connected to an automatic Transducer Indicator (model 1601, IITC Inc., Woodland Hills, CA), as described by Doboly et al. (2002). The filament used produces 10 g of force at 5 sec, when paw withdrawal is elicited in naive mice. A 20-sec cut-off time was used to avoid tissue damage.

Hargreaves thermal nociception test. A thermal beam was focused on the hind limb footpads of mice placed on a glass surface and the withdrawal response latency measured, with a 20-sec cut-off time, as described by Hargreaves et al. (1998).

Capsaicin-induced ABL test. The algogenic (i.pl.)-induced biting and licking test was carried out by use of 0.4 or 0.8 μg of capsaicin, as reported elsewhere (Tan-No et al., 1998). A 28-gauge stainless steel needle attached to a 50 μl Hamilton microsyringe was inserted into the foot pad in unanesthetized mice, and capsaicin was given slowly in a volume of 20 μl. Total duration time showing these behaviors during 5 min after i.pl. injection was summed and used as biting and licking responses (sec).

Algogenic-induced nociceptive flexion (ANF) test. Experiments were performed, as described earlier (Inoue et al., 1998, Ueda, 1999, Doboly et al. 2002). Briefly, mice were held in a cloth sling with their 4 limbs hanging free through holes. The sling was

suspended on a metal bar. All limbs were tied with strings, and 3 were fixed to the floor, while the other one was connected to an isotonic transducer and recorder. A polyethylene cannula (0.61 mm in outer diameter) filled with drug solution was connected to microsyringe and then carefully inserted into the undersurface of the right hind paw. As we used light and soft polyethylene cannula, it did not fall off the paw during the experiments. As the intensity of flexor responses differs from mouse to mouse, we used the biggest response among spontaneous and non-specific flexor responses occurring immediately after cannulation as the maximal reflex. Algogenic substance-injection was intraplantarly (i.pl.) given every 5 min unless otherwise stated. Algogenic substance-induced nociceptive activity was expressed as the ratio of maximal reflex in each mouse, and in the dose-response experiments, increasing doses of compound were given every 5 min interval. Average of responses by twice repeated challenges per each dose was evaluated.

Central algogenic-induced SBL test. The nocifensive behaviors characterized by reciprocal hind limb scratching, caudally directed biting and licking (SBL behavior) during 5 min following intrathecal injection of algogenic were evaluated (Hylden and Wilcox, 1981; Inoue et al., 1998). Before experiments mice were adapted to an individual plastic cage for 1 h. Immediately after i.t. injection of algogenic (substance P), each mouse was placed into the transparent cage for behavioral test. All mice were used for only one experiment by the observer who did not know what kind of pretreatments had been given.

Immunohistochemistry. Immunohistochemistry for NK1 tachykinin receptor using free-floating 30 μm section of spinal cord from 4 % paraformaldehyde-perfused $NOP^{+/+}$ and $NOP^{-/-}$ mice was performed as described (Mantyh et al., 1995).

Western blot analysis. SDS-PAGE by using 12 % polyacrylamide gel and immunoblot analysis were performed as described (Yoshida and Ueda, 1999). Thirty μg of protein extracted from the dorsal horn of the spinal cord was used. To get equal transfer efficiency, we have applied all samples to the same gel and carried out the immunoblot transfer using the same membrane. Visualization of immunoreactive bands was performed by using an enhanced chemiluminescent substrate for detection of horseradish peroxidase, Super Signaling Substrate (PIERCE, Rockford, IL). The intensities of immunoreactive bands were analyzed by NIH imaging for Machintosh after scanning exposed films.

Receptor binding. The dorsal horn of spinal cord was isolated from the mouse and the synaptic membranes were prepared, and membrane binding study using [^{3}H]-substance P was carried out, according to Inoue et al. (1988). In saturation binding experiments the membranes were incubated with [^{3}H]-substance P at concentrations varying from 0.1 to 1.2 nM in a final volume of 500 μl for 1 hr at 25 .. Binding reaction was terminated by rapid filtration of the incubation mixture through Whatman GF/B glass filter presoaked with 0.1 % polyethyleneimine. The radioactivity content of the filter was determined using a liquid scintillation counter (Aloka LSC-5100) at the efficacy of 50 %. Nonspecific binding was determined using 1 μM unlabeled substance P.

RT-PCR. Total RNA was isolated from mouse spinal cord with TRIzol (Gibco-BRL), and 1 μg was used for cDNA synthesis with Superscript II reverse transcriptase and random hexamer primers (Gibco-BRL). The cDNA was used as a template for PCR amplification with Taq DNA polymerase (Takara) and NK1 primers (5'-CAT CAA CCC AGA TCTC TACC-3' and 5'-AGC TGG AGC TTT CTG TCA TGG-3') or GAPDH primers (5'- GTG AAG GTC GGT GTG AAC GGA TTT-3' and

5'-CAC AGT CTT CTG GGT GGC AGT GAT-3'). PCR amplification was carried out under the condition of 28 cycles (for NK1) at 94. for 30 sec, 51.for 1 min and 72.for 1 min or 24 cycles (for GAPDH) at 94. for 30 sec, 60.for 1 min, and 72.for 1 min. Cycle number was optimized for each primer set to ensure that amplifications using template from spinal dorsal horn of $NOP^{+/+}$ mice were in the linear amplification range (data not shown). The photograph of electrophoresis of PCR products were analyzed by NIH for Machintosh imaging after scanning exposed films.

Statistical analysis. In the experiment using three types of mice, statistical evaluations were performed using the Dunnett test for multiple comparisons, after one way ANOVA. In other experiments, statistical evaluations were performed using Student's *t*-test. The criterion of significance was set at $p<0.05$. All results are expressed as the mean ± S.E.M.

RESULTS

Altered nociceptive responses upon various nociceptive stimuli in $NOP^{+/+}$ mice. When tail-flick test, a very popular thermal nociception test was adopted, there was no significant change in nociception among $NOP^{+/+}$, $NOP^{+/-}$ and $NOP^{-/-}$ mice (Fig. 1A), being consistent with previous reports (Nishi et al., 1997). In this test, radiant heat stimulus was adjusted for $NOP^{+/+}$ mice to show 10-11s latency. Similar degree of tail-flick latency and nociception were observed in standard mice (Data not shown). In the paw pressure test, the average of threshold pressure (g) to induce withdrawal response in control $NOP^{+/+}$ mice was 10.4 ± 0.7 g (n=6). As shown in Fig. 1B, there was no significant change in the threshold between $NOP^{+/+}$ and $NOP^{-/-}$ mice. In the Hargreaves test, however, the latency for paw withdrawal in $NOP^{-/-}$ mice was significantly lowered (hyperalgesic) to that in $NOP^{+/+}$ mice, which show the average latency of 9.2 ± 0.3 s (n=6, Fig. 1C). Similarly the capsaicin (0.4 or 0.8 μg)-induced ABL test also showed the hyperalgesia in $NOP^{-/-}$ mice, compared with $NOP^{+/+}$ mice (Fig. 1D).

In the ANF test in $NOP^{+/+}$ mice, N/OFQ dose-dependently induced nociceptive flexor responses from 0.1 to 100 fmol (i.pl.), as shown in Fig. 1E. As expected, the N/OFQ-induced nociceptive flexor responses were completely abolished in $NOP^{-/-}$ mice, while there was no significant change between heterozygous $NOP^{+/-}$ and $NOP^{+/+}$ mice (Fig. 1E). In this test, the nociceptive dose showing 50% effective dose (ED_{50}) of N/OFQ in $NOP^{+/+}$ mice was 0.52 ± 0.10 fmol, i.pl. (n=6), which is consistent with our previous report using ddY mice (Inoue et al., 1998). On the other hand, the dose-response curve of bradykinin (i.pl.)-induced flexor responses in $NOP^{-/-}$ mice was shifted to the left, compared to $NOP^{+/+}$ mice (Fig. 1F). The ED_{50} in $NOP^{-/-}$ mice was 205.2 ± 31.2 amol (i.pl.), 500-times lower than that (110.3 ± 24.2 fmol) in $NOP^{+/+}$ mice. Quite similar

hyperalgesia was also observed when used substance P for i.pl. injection (Fig. 1G). However, there was no significant difference between $NOP^{-/-}$ and $NOP^{+/+}$ mice in the nociceptive flexor responses by intraplantar injection of adenosine triphosphate or ONO-54918-07, a stable prostagrandin I_2 agonist (Iguchi et al., 1987; Terawaki et al., 1988), as shown in Fig. 1H,I.

Selective enhancement of spinal substance P responses in $NOP^{-/-}$ mice. The substance P (i.t.)-induced nocifensive SBL responses, characterized by scratching, biting and licking to hind paw were mainly observed at the period of 0-5 min after injection. The time period showing the SBL responses during 5 min after the substance P-injection was evaluated as the central nociception. In $NOP^{+/+}$ mice, marked nocifensive responses were observed with 100 pmol (i.t.) of substance P, and similar results were also obtained in heterozygous $NOP^{+/-}$ mice (Fig. 2A). In $NOP^{-/-}$ mice, however, markedly enhanced nocifensive responses were observed. The SBL responses by 10 pmol (i.t.) of substance P in $NOP^{-/-}$ mice were equivalent to those by 100 pmol (i.t.) in $NOP^{+/+}$ mice.

Mice were neonatally pretreated with 50 mg/kg s.c. of capsaicin to degenerate polymodal substance P-ergic C fiber neurons (Hiura and Ishizuka, 1989; Inoue et al., 1999). As shown in Fig.2B, the nocifensive responses by 30 pmol (i.t.) of substance P were slightly, but significantly increased to 28.0 $\pm$ 5.8 sec by the neonatal capsaicin pretreatment, compared with 8.8 $\pm$ 1.0 sec in mice without capsaicin pretreatment (Fig. 2A). This hyperalgesia has previously been well discussed as a denervation-induced supersensitivity due to upregulation of NK1 receptor in the spinal cord (Mantyh and Hunt, 1985). These responses were markedly enhanced in $NOP^{-/-}$ mice (Fig.2B).

On the other hand, NMDA-induced SBL responses were also significantly

increased in $NOP^{-/-}$ mice, compared with $NOP^{+/+}$ or $NOP^{+/-}$ mice (Fig.2C). The NMDA-induced SBL responses in $NOP^{+/+}$ mice were partially, but significantly blocked by pretreatment with CP-99994 (10 nmol, i.t.), which completely abolished substance P (100 pmol)-induced nociception (Inoue et al., 1998,1999), as shown in Fig. 2C. These results suggest that NMDA (i.t.)-induced nocifensive responses are mediated at least through substance P release from central terminal of primary afferent neurons, as elsewhere reported (Liu et al., 1997). When the NMDA was intrathecally injected in the presence of CP-99994, however, there were no significant differences in the spinal NMDA receptor-mediated nocifensive responses without substance P-mediated mechanisms in the among $NOP^{-/-}$, $NOP^{+/-}$ and $NOP^{+/+}$ mice. Thus, all these results suggest that the enhancement of NMDA-induced SBL responses in $NOP^{-/-}$ mice was mediated through substance P release.

Lack of NK1 receptor upregulation in $NOP^{-/-}$ mice. The NK1-like immunoreactivity was intensely found in the laminae I of the dorsal horn of spinal cord, but there was no significant change between $NOP^{-/-}$ and $NOP^{+/+}$ mice (Fig. 3A,B). As shown in Fig. 3C, no significant change was also observed in the immunoblot analysis using the dorsal horn region of spinal cord (see Fig. 3A). The [^{3}H]-substance P binding experiments using dorsal horn membranes revealed that Kd 0.69±0.10 nM, Bmax 27.79±2.34 pmol/mg protein for $NOP^{-/-}$ mice were quite similar to those for $NOP^{+/+}$ mice (Kd 0.66±0.05 nM, Bmax 24.26±1.57 pmol/mg protein), as shown in Fig. 3D. Furthermore, there was also no significant difference in the NK1 gene expression in the dorsal horn by RT-PCR between $NOP^{-/-}$ and $NOP^{+/+}$ mice (Fig.3E).

Enhanced nociception in NOP antagonist (i.t.)-treated mice. The hyperalgesia was also observed in the ANF test with bradykinin and substance P, when 1 pmol of J-113397 (Ozaki et al., 2000; Ueda et al., 2000) or 1 nmol of [Nphe^{1}]N/OFQ(1-13)NH2 (Calo' et al., 2000) was i.t. pretreated 20 min prior to the test (Fig. 4A, B). The ED_{50} for bradykinin and substance P in antagonist-treated mice was 100-10,000-times lower than those in vehicle-control mice. However, the i.t. injection of either antagonist alone did not show any gross behavioral changes nor nocifensive responses without stimuli. On the other hand, the SBL responses by i.t. injection of substance P were markedly enhanced by the treatment of these antagonists (Fig.4C).

Characterization of spinal transmission in several nociception tests. We tested the spinal antagonism using substance P and glutamate receptor antagonists in various nociception tests. As shown in Table 1, the i.t. injection (3 nmol each) of MK801 (NMDA receptor antagonists) or CNQX (AMPA/kainate receptor antagonist) markedly inhibited the tail-flick responses, while there was only a little change with CP-99994 (3 nmol). Quite similar results were obtained with C57/Black mice (data not shown). The paw pressure response in standard mice was blocked by MK801, but not by CNQX or CP-99994. On the other hand, the Hargreaves thermal nociception was equally and significantly inhibited by i.t. injection with CP-99994 or MK801, but not by CNQX. Quite similar results were obtained in capsaicin test. Capsaicin-induced nociception was also equally blocked by CP-99994 or MK801, but not by CNQX. In the ANF test, lower doses of antagonists (100 pmol., i.t.) were used, since the pain intensity used in this test is much weaker than other tests (tail-flick, hargreaves, paw pressure and capsaicin tests). Bradykinin- and substance P-induced nociception was blocked by CP-99994, but not by

MK801 and CNQX. On the other hand, adenosine triphosphate- or prostagrandin I_2 agonist-induced nociception was blocked by MK801, but not by CNQX or CP-99994. All the cases with lack of antagonism (100 pmol, i.t.) in ANF tests were reproduced when the antagonist doses were increased to 3 nmol (data not shown).

DISCUSSION

It has remained to be determined how N/OFQ-ergic neurons play roles in the pain regulation. Several pharmacological analyses revealed that N/OFQ showed both anti- and pro-nociceptive actions in *in vivo* studies, while it mostly did inhibitory actions in *in vitro* studies (Meunier 1997; Borgland et al., 2001; Vaughan et al., 2001). Very small amounts of N/OFQ mRNA are observed in the dorsal root ganglion neurons (Pettersson et al., 2002), and this activity in the spinal cord is reported to originate from intrinsic spinal neurons, rather than primary afferent neurons (Riedl et al., 1996). These findings suggest that the *in vivo* role of N/OFQ in the spinal cord seems to play a role as an interneuronal transmitter to regulate some modalities of pain.

In the ANF test, we used several algogenics to stimulate distinct nociceptive fibers. This ANF test is more sensitive to produce algogenic-induced nociception, because we observed nociceptive responses in much lower doses compared with that in another test (Kato et al., 2002). From a series of experiments using ANF test (Inoue et al., 1999; Ueda et al., 2000; Rashid et al., in press), we have proposed three different types of nociceptive fibers based on the sensitivity to neonatal capsaicin and spinal antagonism. In this diagram, bradykinin and substance P stimulate neonatal capsaicin-sensitive polymodal C (we call it type I) fibers, which use substance P and NK1 receptor for primary afferent pain transmission in the spinal cord, while adenosine triphosphate stimulates the capsaicin-sensitive (we call it type II) fibers, which use glutamate and NMDA receptor for the pain transmission. On the other hand, prostagrandin I_2-agonist stimulates the capsaicin-insensitive (we call it type III) fibers, which use glutamate and NMDA receptor for the pain transmission. These type I and II fibers may be suitable for the substance P-containing, NGF-sensitive neurons and $P2X_3$ receptor expressing,

GDNF-sensitive fiber, which are proposed by Snider and McMahon (1998), respectively. As expected, N/OFQ (i.pl.)-induced flexor responses were abolished in $NOP^{-/-}$ mice (Fig. 1E). The nociceptive responses by both bradykinin and substance P, on the other hand, were markedly potentiated in $NOP^{-/-}$ mice (Fig. 1F,G). On the other hand, there was no significant change in the responses by adenosine triphosphate or prostagrandin I_2-agonist, which do not use the substance P-transmission, but glutamate-one (Fig. 1F,G, Table 1). These results might suggest the view that substance P-mediated nociception is negatively regulated by spinal N/OFQ-ergic system, rather than glutamate-nociception is, as shown in the working hypothesis (Fig. 5). To prove this hypothesis, the peripheral stimulation-selective release of N/OFQ from the spinal cord should be detected as a future subject.

In addition, our hypothesis was also supported by the results (Fig. 1A-D) that the hyperalgesia in $NOP^{-/-}$ mice was observed in the Hargreaves thermal nociception test and capsaicin tests, which drive spinal substance P system in part for pain transmission, while not in the tail-flick and paw pressure test without substance P system (Table 1). The lack of hyperalgesia in the tail-flick test in $NOP^{-/-}$ mice is consistent with the previous report (Nishi et al., 1997). In the previous study (Nishi et al., 1997), $NOP^{-/-}$ mice did not show the hyperalgesia in acid-induced writhing responses, which are sensitive to neonatal capsaicin treatment (Ikeda et al., 2001). However, as the acid-induced writhing responses were not affected in mice lacking the gene encoding tachykinin 1 (Zimmer et al., 1998), spinal substance P-transmission is unlikely involved in this test. All these results strongly suggest that the involvement of spinal substance P-transmission in the nociception test is closely related to the hyperalgesia in $NOP^{-/-}$ mice.

It should be important how spinal N/OFQ-ergic system acts on the substance

P-mediated pain transmission. One of these questions is which presynaptic (primary afferent) nerve terminal or/and postsynaptic (second order) spinal neuron is the site for N/OFQ, since we have previously reported that N/OFQ given i.t. exerts nocifensive actions in the fmol dose range through an substance P release from primary substance P fibers, while analgesic actions in the nmol dose range through an inhibition of substance P-actions on the second-order neuron (Inoue et al., 1998, 1999). These findings raise the question which pain inhibitory or stimulatory responses are observed in $NOP^{-/-}$ mice. However, as far as we have observed in the present study, there is no evidence for the pain inhibitory responses obtained. All the data we obtained using various nociception tests show the hyperalgesia in Hargreaves, capsaicin and ANF tests using bradykinin and substance P, all of which use spinal substance P transmission, while no significant changes in tail-flick, paw pressure and ANF tests using adenosine triphosphate and prostagrandin I_2 agonist, which do not use the substance P transmission. On the other hand, the nocifensive responses by substance P (i.t.) were also potentiated in $NOP^{-/-}$ mice with or without neonatal capsaicin pretreatment to degenerate C-fibers. All results strongly suggest that there is a discrepancy between pharmacological actions and physiological roles of spinal N/OFQ in the pain regulation.

Another question is whether or not the genetic deletion of NOP causes some changes in the sensitivity to substance P. As shown in Fig. 3B-E, the immunoreactivity for NK1 receptor, substance P-binding activity and gene expression at the dorsal horn of spinal cord showed no significant difference between $NOP^{-/-}$ and $NOP^{+/+}$mice. Taken the fact that N/OFQ exerts inhibitory actions through $G_{i/o}$ mechanisms on various cells *in vitro* (Meis and Pape 1998; Zeilhofer et al., 2000), all these findings strongly suggest that the N/OFQ-ergic interneuron plays a role as a recurrent inhibitory interneuron *in vivo*

(Fig. 5).

Here we demonstrated that intrathecally administrated NMDA caused nocifencive responses through a spinal substance P release (Fig. 2C). This finding raises a question why NMDA (i.t.)-induced nocifensive responses are enhanced in $NOP^{-/-}$ mice, though there is no change in adenosine triphosphate (i.pl.)-induced responses, which are mediated by spinal NMDA receptors. In the previous and present studies, we reported adenosine triphosphate or its analog (i.pl.)-induced responses were blocked by the intrathecal injection of NMDA receptor antagonist, MK-801, but not by substance P receptor antagonist, CP-99994 (Ueda et al., 2000 and Table 1). This finding suggests that glutamate released from nociceptive fibers stimulated by adenosine triphosphate (i.pl.) unlikely presynaptically activates the fibers to be stimulated by substance P (i.pl.). Snider and McMahon (1998) supports this view in the review, in which the nociceptive fibers containing substance P (type I) innervate lamina I and II (outer) regions in the dorsal horn, while $P2X_3$ (adenosine triphosphate receptor)-expressing fibers (type II) do lamina II (inner) region. Although the *in vivo* role of presynaptic NMDA receptor on type I nociceptive fibers remains unclear, the algogenic (i.pl.)-induced nociceptive responses through type I fibers unlikely involve this NMDA mechanisms, since they are blocked by substance P antagonist, but not by NMDA antagonist (Ueda et al., 2000 and Table 1).

In the present study, we demonstrated that the postsynaptic supersensitization of substance P (i.t.)-induced nociceptive responses in $NOP^{-/-}$ mice is attributed to the lack of inhibitory N/OFQ-ergic interneurons downstream to substance P-responsive neurons. Here we used two chemically different NOP antagonists, the non-peptide J-113397 and the N/OFQ related peptide $[Nphe^1]N/OFQ(1\text{-}13)NH_2$ (Nphe1). Although the potency of the peptide antagonist was about 1,000 fold lower than that of J-113397, the two agents

produced superimposable results strongly suggesting that their action are exclusively due to NOP blockade.

In summary, the present study demonstrates that N/OFQ plays an inhibitory role in the pain transmission through polymodal substance P fibers. However, this does not necessarily mean that NOP agonists could behave as potent spinal analgesics, since several potent NK1 receptor antagonists have no significant analgesic actions in clinic (see review Hill, 2000; Villanueva, 2000). Clinical availability of NOP selective and potent ligands should be rather discussed in terms of the potency of chronic pain suppression.

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FOOTNOTES

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Figure 1. Altered responses to different modalities of nociceptive stimuli in $NOP^{+/+}$, $NOP^{+/-}$ or $NOP^{-/-}$ mice. Each point of data in all figures was the mean ± S.E.M. from the experiments using at least 6 mice. Comparison of nociceptive response in various tests among $NOP^{+/+}$, $NOP^{+/-}$ and $NOP^{-/-}$ mice (**A, E**) or between $NOP^{+/+}$ and $NOP^{-/-}$ mice (**B-D, F-I**). E-I: Results represent the percentage of the maximal reflex, which is the biggest response among spontaneous and non-specific flexor responses occurring immediately after cannulation. The increasing doses of compound were given every 5 min and the average of two responses by repeated challenges per each dose was evaluated. *<0.05, vs. corresponding control. n=6. N/OFQ, Nociceptin/orphanin FQ; BK, bradykinin; SP, substance P; ATP, adenosine triphosphate; PGI_2, prostagrandin I_2

Figure 2. Enhanced nociceptive responses in $NOP^{-/-}$ mice to intrathecally administered substance P, but not NMDA with CP-99994. Results represent SBL responses by the i.t. injection of NMDA or substance P. Each point of data in all figures was the mean ± S.E.M. from the experiments using at least 6 mice. **A**: Enhanced substance P-induced SBL responses in $NOP^{-/-}$ mice. **B**: Enhanced substance P-induced SBL responses in capsaicin-pretreated $NOP^{-/-}$ mice. **C**: NMDA-induced SBL responses in the absence or presence of CP-99994 in $NOP^{+/+}$, $NOP^{+/-}$ or $NOP^{-/-}$ mice. Intrathecal injection of saline was performed to assess a control response. CP-99994 (10 nmol, i.t.) was injected 20 min prior to NMDA injection. Capsaicin (50 mg/kg) or vehicle was injected into the back of newborn (P4) ddY mice. n=6-8. *p<0.05, compared to NMDA-treated $NOP^{+/+}$ mice. #p<0.05, compared to saline-treated group in each mice. §p<0.05, compared to NMDA-treated groups in each mice.

Figure 3. No change in NK1-receptor gene expression between $NOP^{+/+}$ and $NOP^{-/-}$ mice. **A**: Schematic representation of the spinal dorsal horn. **B:** NK1-immunohistochemistry of a coronal section of the spinal cord of $NOP^{+/+}$ and $NOP^{-/-}$ mice. Thirty μm sections were used for immunostaining. **C:** Western blot analysis of NK1 protein levels in the spinal cord of $NOP^{+/+}$ and $NOP^{-/-}$ mice. **D:** [^{3}H]-substance P binding in the spinal cord of $NOP^{+/+}$ and $NOP^{-/-}$ mice. There was no significant change between both preparations. **E:** Quantitative RT-PCR for NK1-receptor gene expression. NK1 gene expressions were analyzed, using GAPDH transcript as a reference. The results represent the relative NK1-receptor expression to GAPDH one. There was no significant increase in the expression in $NOP^{-/-}$ mice, compared to that in $NOP^{+/+}$ mice. n=6.

Figure 4. Enhancement of nociception driving spinal substance P neurotransmission or substance P-induced nociception by NOP antagonist treatments. **A,B:** Enhanced bradykinin (i.pl.)- or substance P (i.pl.)-induced nociceptive responses in NOP antagonist-treated mice in ANF test. J-113397 (1 pmol) or Nphe1 (1 nmol) was given by i.t. 20 min prior to several peripheral nociceptive stimulation in the ANF tests. **C:** Enhanced substance P (i.t.)-induced SBL responses in NOP antagonist-treated mice. J-113397 (1 pmol) or Nphe1 (1 nmol) was given by i.t. 20 min prior to substance P injection. Details are indicated in the legend of figure 1. n=6-8.

Figure 5.Working hypothesis of *in vivo* pain-inhibitory role of N/OFQ neuron in the dorsal horn of the spinal cord. The hypothetical diagram is based on the sensitivity to neonatal capsaicin and spinal antagonism. In this diagram, bradykinin and substance P stimulate neonatal capsaicin-sensitive polymodal C (type I) fibers, which use substance

P and NK1 receptor for primary afferent pain transmission in the spinal cord, while adenosine triphosphate stimulates the capsaicin-sensitive (type II) fibers, which use glutamate and NMDA receptor for the pain transmission. On the other hand, prostagrandin I_2-agonist stimulates the capsaicin-insensitive (type III) fibers, which use glutamate and NMDA receptor for the pain transmission. The pain inhibitory role through NOP or N/OFQ-ergic neuron in the dorsal horn of spinal cord could be attributed to the action on the second-order neuron for type I substance P-fibers. Cells indicated with open or closed circle represent stimulatory or inhibitory neurons, respectively. Details are indicated in the text.

Table 1 Characterization of spinal transmission in several nociception tests.

Test	Vehicle	CP-99994	MK-801	CNQX
Tail-flick (sec)	10.4±0.5	13.5±1.4	35.7±4.0*	33.4±4.3*
Hargreaves (sec)	8.7±0.4	13.2±0.9*	12.6±1.3*	9.8±0.9
Paw pressure (g)	10.4±0.7	10.3±0.3	15.6±0.6*	11.0±0.8
Capsaicin (sec)	60.1±3.0	29.2±2.0*	30.9±4.5*	60.0±1.1
ANF				
BK (2 pmol)#	68.9±8.4	14.5±5.6*	70.1±7.1	57.7±6.5
SP (10 pmol)	61.9±11.6	6.7±3.3*	69.0±8.3	54.0±5.9
ATP (100 pmol)	63.3±6.9	66.2±5.2	24.9±6.8*	51.7±8.1
PGI_2(100 pmol)#	60.6±8.9	68.3±5.4	19.8±9.3*	49.3±6.7

Various antagonists (3 nmol) were given intrathecally 20 min prior to tail-flick, paw pressure, Hargreaves or capsaicin test. In ANF test, various antagonists (100 pmol) were given 20 min prior to bradykinin (BK), substance P (SP), adenosine triphosphate (ATP) or prostagrandin I_2 agonist (PGI_2) stimulation. *$P < 0.05$, compared with vehicle treated mice. #These data are quoted from our previous report (Ueda et al.,2000) to compare with other nociception tests.

Fig. 1

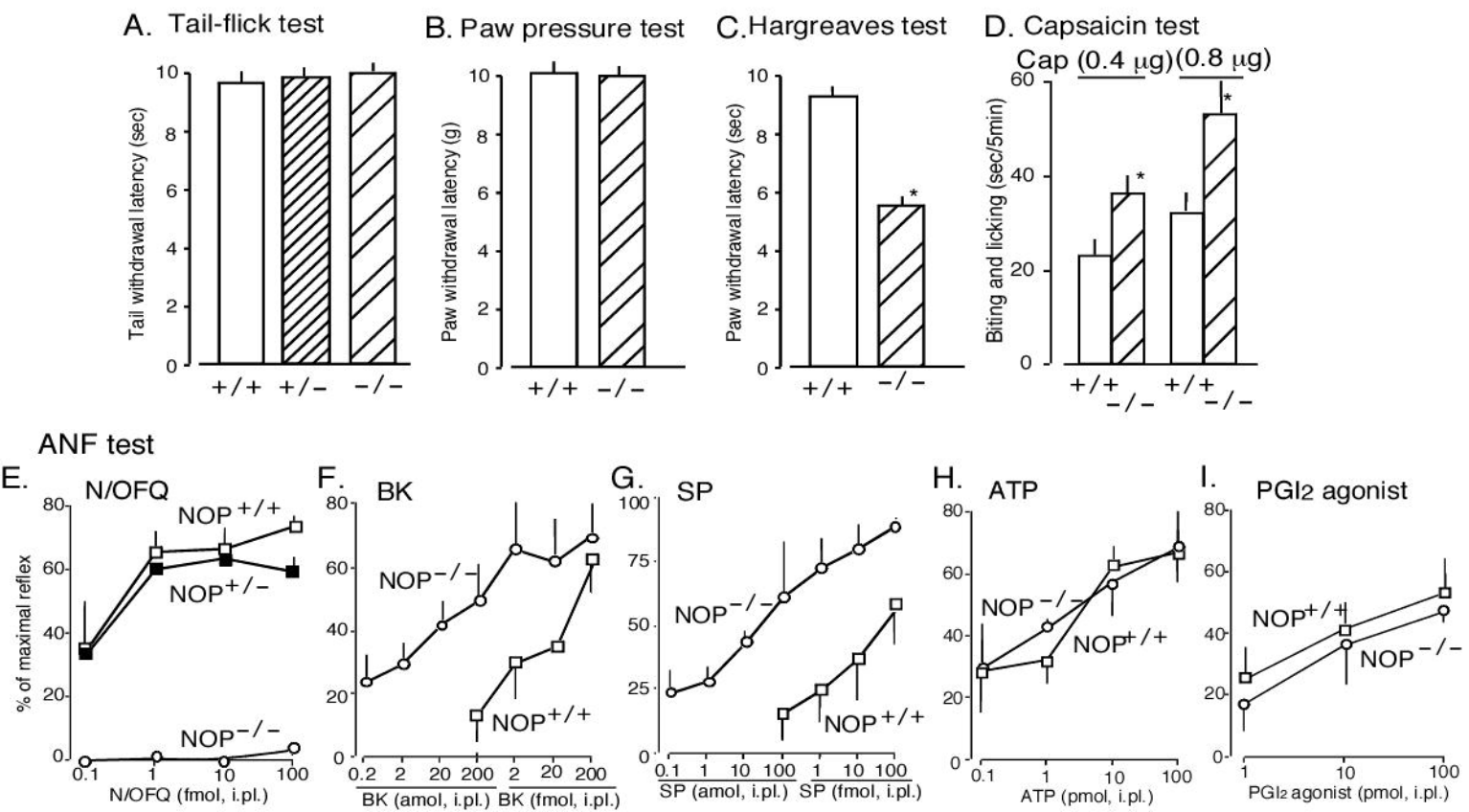

A. Tail-flick test
Tail withdrawal latency (sec)
+/+ +/− −/−
B. Paw pressure test
Paw withdrawal latency (g)
+/+ −/−
C. Hargreaves test
Paw withdrawal latency (sec)
+/+ −/−
D. Capsaicin test
Cap (0.4 μg)(0.8 μg)
Biting and licking (sec/5min)
+/+ −/− +/+ −/−
ANF test
E. N/OFQ
NOP+/+
NOP+/−
NOP−/−
% of maximal reflex
N/OFQ (fmol, i.pl.)
F. BK
NOP−/−
NOP+/+
BK (amol, i.pl.) BK (fmol, i.pl.)
G. SP
NOP−/−
NOP+/+
SP (amol, i.pl.) SP (fmol, i.pl.)
H. ATP
NOP−/−
NOP+/+
ATP (pmol, i.pl.)
I. PGI2 agonist
NOP+/+
NOP−/−
PGI2 agonist (pmol, i.pl.)

Fig. 2

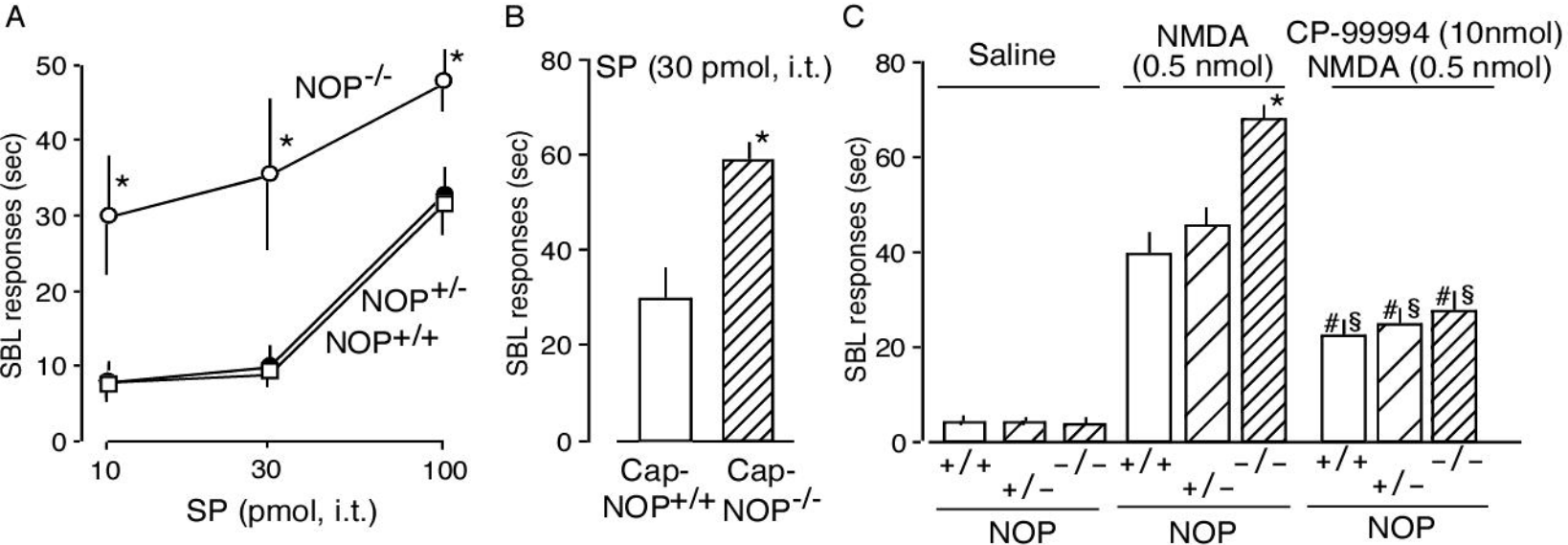

A
B
C
SBL responses (sec)
SP (pmol, i.t.)
NOP-/-
NOP+/-
NOP+/+
SP (30 pmol, i.t.)
Cap-NOP+/+
Cap-NOP-/-
Saline
NMDA (0.5 nmol)
CP-99994 (10nmol) NMDA (0.5 nmol)
NOP

Fig. 3

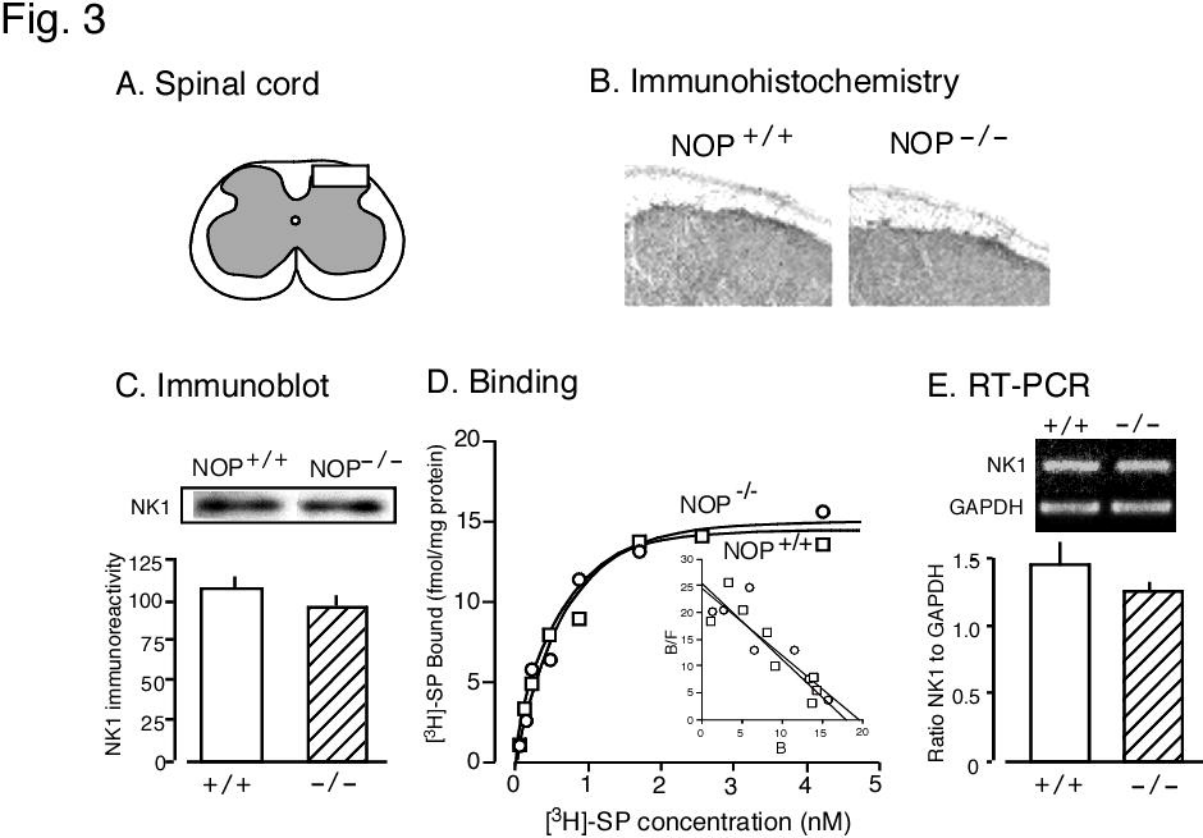

A. Spinal cord
B. Immunohistochemistry
NOP +/+
NOP −/−
C. Immunoblot
NOP+/+
NOP−/−
NK1
NK1 immunoreactivity
125
100
75
50
25
0
+/+
−/−
D. Binding
[3H]-SP Bound (fmol/mg protein)
20
15
10
5
0
NOP -/-
NOP +/+
B/F
B
0
1
2
3
4
5
[3H]-SP concentration (nM)
E. RT-PCR
+/+
−/−
NK1
GAPDH
Ratio NK1 to GAPDH
1.5
1.0
0.5
0
+/+
−/−

Fig. 4

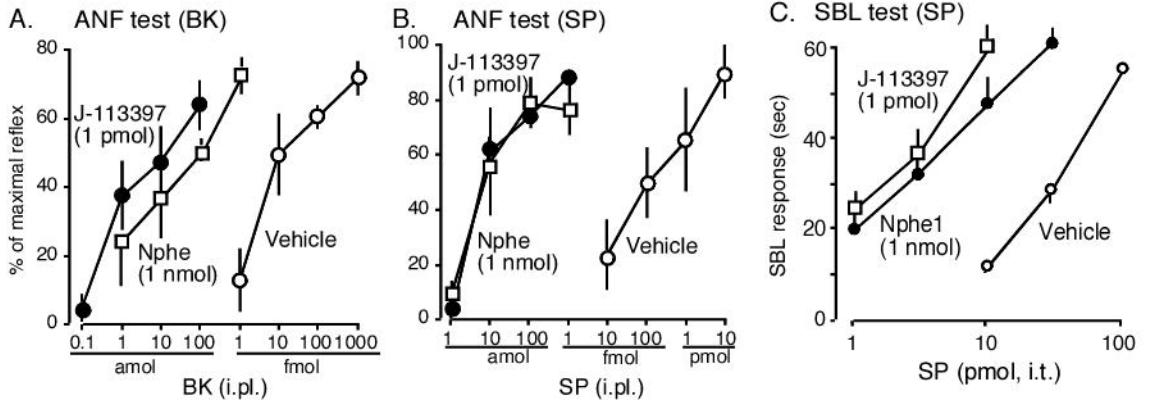
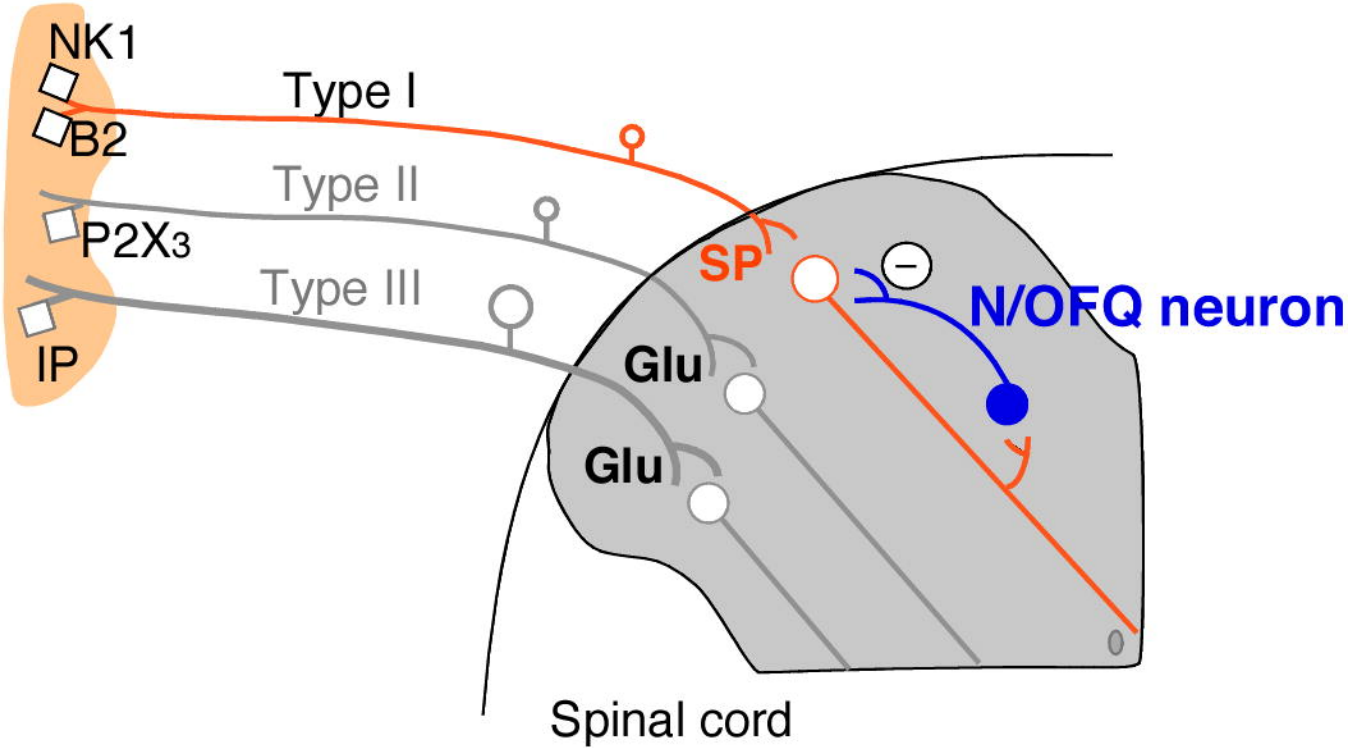

A.
ANF test (BK)
% of maximal reflex
J-113397
(1 pmol)
Nphe
(1 nmol)
Vehicle
amol
fmol
BK (i.pl.)
B.
ANF test (SP)
J-113397
(1 pmol)
Nphe
(1 nmol)
Vehicle
amol
fmol
pmol
SP (i.pl.)
C.
SBL test (SP)
SBL response (sec)
J-113397
(1 pmol)
Nphe1
(1 nmol)
Vehicle
SP (pmol, i.t.)

Fig. 5


NK1
B2
P2X3
IP
Type I
Type II
Type III
SP
N/OFQ neuron
Glu
Glu
Spinal cord