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Identification of the G-protein-coupled ORL1 receptor in the mouse spinal cord by [^{35}S]-GTPγS binding and immunohistochemistry

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1 Although the ORL1 receptor is clearly located within the spinal cord, the functional signalling mechanism of the ORL1 receptor in the spinal cord has not been clearly documented. The present study was then to investigate the guanine nucleotide binding protein (G-protein) activation mediated through by the ORL1 receptor in the mouse spinal cord, measuring the modulation of guanosine-5′-*o*-(3-[^{35}S]-thio) triphosphate ([^{35}S]-GTPγS) binding by the putative endogenous ligand nociceptin, also referred as orphanin FQ. We also studied the anatomical distribution of nociceptin-like immunoreactivity and nociceptin-stimulated [^{35}S]-GTPγS autoradiography in the spinal cord.

2 Immunohistochemical staining of mouse spinal cord sections revealed a dense plexus of nociceptin-like immunoreactive fibres in the superficial layers of the dorsal horn throughout the entire length of the spinal cord. In addition, networks of fibres were seen projecting from the lateral border of the dorsal horn to the lateral grey matter and around the central canal.

3 *In vitro* [^{35}S]-GTPγS autoradiography showed high levels of nociceptin-stimulated [^{35}S]-GTPγS binding in the superficial layers of the mouse dorsal horn and around the central canal, corresponding to the areas where nociceptin-like immunoreactive fibres were concentrated.

4 In [^{35}S]-GTPγS membrane assay, nociceptin increased [^{35}S]-GTPγS binding of mouse spinal cord membranes in a concentration-dependent and saturable manner, affording maximal stimulation of 64.1 ± 2.4%. This effect was markedly inhibited by the specific ORL1 receptor antagonist [Phe$^{1}\Psi$ (CH_2-NH) Gly^{2}] nociceptin (1–13) NH_2. None of the μ-, δ-, and κ-opioid and other G-protein-coupled receptor antagonists had a significant effect on basal or nociceptin-stimulated [^{35}S]-GTPγS binding.

5 These findings suggest that nociceptin-containing fibres terminate in the superficial layers of the dorsal horn and the central canal and that nociceptin released in these areas may selectively stimulate the ORL1 receptor to activate G-protein. Furthermore, the unique pattern of G-protein activation in the present study provide additional evidence that nociceptin is distinct from the μ-, δ- or κ-opioid system.

Keywords: Nociceptin; ORL1 receptors; [^{35}S]-GTPγS binding; G-proteins; dorsal horn; immunohistochemistry; autoradiography

Abbreviations: cc, central canal; CTOP, D-Phe-Cys-D-Tyr-Orn-Thr-Pen-Thr-NH_2; DA, dopamine; DAMGO, [D-Ala^{2},NH-Phe^{4},Gly-ol]enkephalin; DPDPE, [D-Pen^{2,5}]enkephalin; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; $GABA_B$, γ-amino-n-butyric $acid_B$; GDP, guanosine-5′-diphosphate; G-protein, guanine nucleotide binding protein; GTP, guanosine-5′-triphosphate; GTPγS, guanosine-5′-*o*-(3-thio)triphosphate; IgG, γ immunoglobuline; M, muscarinic acetylcholine; mRNA, messenger ribonucleic acid; nor-BNI, nor-binaltorphimine; NTI, naltrindole hydrochloride; PBS, phosphate-buffered saline; [^{35}S]-GTPγS, guanosine-5′-*o*-(3-[^{35}S]-thio)triphosphate; U50,488H, (±)-trans-U50,488 hydrochloride

Introduction

ORL1 receptor that shows approximately 50% homology to the cloned μ-, δ- or κ-opioid receptors has been identified (Mollereau *et al*., 1994; Lachowicz *et al*., 1995). In spite of this homology to opioid receptors, its affinity for opioid peptides and opiate alkaloids is low (Fukuda *et al*., 1994; Mollereau *et al*., 1994; Wang *et al*., 1994; Lachowicz *et al*., 1995). *In situ* hybridization analysis revealed that ORL1 receptor messenger ribonucleic acid (mRNA) is densely expressed in the cerebral cortex, thalamus, subfornical organ, habenulae, hypothalamus, central grey, dorsal raphe, locus coeruleus, and also in the dorsal horn of the spinal cord and dorsal root ganglia (Bunzow *et al*., 1994; Wick *et al*., 1994). Thus, ORL1 receptors may be involved in a wide variety of physiological functions.

The relatively large number of opioid peptides isolated in recent years clearly reflects the complexity of the endogenous opioid system. Nociceptin, also known as orphanin FQ, is thought to be the naturally occurring ligand of ORL1 receptor (Meunier *et al*., 1995; Reinscheid *et al*., 1995). Nociceptin, a 17-amino acid peptide, although shows homology with the endogenous κ-opioid receptor peptide dynorphin $A_{1\text{-}17}$ displays very low binding affinity for the cloned μ-, δ- and κ-opioid receptors.

The ORL1 receptors are seven-transmembrane domain receptors whose actions are mediated through activation of heterotrimeric guanine nucleotide binding protein (G-protein). In an *in vitro* assay system, replacing the guanosine-5′-triphosphate (GTP) with the non-hydrolyzable analogue,

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guanosine-5′-*o*-(3-[^{35}S]-thio) triphosphate ([^{35}S]-GTPγS), allows the measurement of the exchange process in the presence of added guanosine-5′-diphosphate (GDP). Unlike GTP, [^{35}S]-GTPγS addition results in accumulation of a stable Gα-[^{35}S]-GTPγS complex in brain membranes. Namely, if the receptor is coupled to G-proteins, the receptor agonist increases the amount of [^{35}S]-GTPγS binding (Sim *et al*., 1995; Traynor & Nahorski, 1995). In our laboratory, the ability of μ-, δ- and κ-opioid receptors agonist-stimulated [^{35}S]-GTPγS bindings have been reported in membranes prepared from the mouse brainstem and spinal cord (Narita & Tseng, 1998; Narita *et al*., 1998, 1999). It has been recently reported that nociceptin increases [^{35}S]-GTPγS binding in rat brain (Sim *et al*., 1996), guinea-pig brain (Sim *et al*., 1997) and mouse brain (Shimohira *et al*., 1997). Nociceptin-stimulated [^{35}S]-GTPγS binding in isolated membranes thus provide a functional measurement of agonist occupation of ORL1 receptors and its efficacy in activating G-proteins. Although ORL1 receptor transcript (Wick *et al*., 1994) and ORL1 receptor-like immunoreactivity (Anton *et al*., 1996) are clearly detected within the spinal cord, which is considered to be an important site for the processing of sensory information including pain, the ability of nociceptin to activate G-protein in the spinal cord has not been clearly documented.

The present study was undertaken to investigate the effects of nociceptin on [^{35}S]-GTPγS binding to membranes from the mouse spinal cord. We also report here the anatomical distribution of nociceptin-like immunoreactivity and nociceptin-stimulated [^{35}S]-GTPγS autoradiography in the mouse spinal cord. This information provides an anatomical basis of the activation of ORL1 receptors by endogenously released nociceptin in the mouse spinal cord.

Methods

Animals

Male ICR mice weighing 23–30 g (Charles River Breeding Laboratories, Wilmington, MA, U.S.A.) were used. Animals were housed five per cage in a room maintained at $22 \pm 0.5^{\circ}$C with an alternating 12 h light-dark cycle. Food and water were available *ad libitum*.

Immunohistochemical approach

Mice anaesthetized with pentobarbital (50 mg kg^{-1}, i.p.) were intracardially perfused with 0.1 M phosphate-buffered saline (PBS) followed by freshly prepared 4% paraformaldehyde in PBS (pH 7.4). Spinal cords were removed, postfixed in the same fixative for 2 h, and cryoprotected in 30% sucrose/PBS solution overnight. Spinal cords were sectioned to 40 μm with the use of a Vibratome. Sections were processed for nociceptin-like immunoreactivity by means of the standard avidin-biotin complex techniques as described (Lai *et al*., 1997). Sections were first treated with 3% H_2O_2 to quench endogenous peroxidase, washed several times and blocked with 10% normal goat serum. Tissues were then incubated in nociceptin antisera, which were a rabbit polyclonal directed against the synthetic peptide of nociceptin (BioTrend Chemikalien Gmbh, Koln, Germany), for 48 h at 4°C with gentle agitation. The antisera, which were used at a dilution of 1 : 10,000 with 0.4% Triton X-100 and 1% bovine serum albumin in PBS, exhibited no cross-reactivity with other opioid peptides (BioTrend). After thorough rinsing, sections were incubated with biotinylated anti-rabbit γ immunoglobuline (IgG) (1 : 200, Vector Laboratories, Burlingame, CA, U.S.A.) for 2 h. Sections were rinsed with PBS and incubated in avidin-biotin complex solution for 1 h (1 : 100, Vector Laboratories). After several rinses in Tris-buffered saline, sections were developed in diaminobenzidine-H_2O_2 solution and washed for at least 2 h with Tris-buffered saline. Sections were mounted on slides with 0.25% gel alcohol, air-dried, dehydrated with absolute alcohol followed by xylene and coverslipped with Permount.

Two sets of control experiments were performed. First, nociceptin antisera were omitted in the staining procedures from randomly selected sections. Second, spinal sections were processed with nociceptin antisera pre-absorbed overnight with the peptide nociceptin (10 μg ml^{-1}; Peninsula Laboratories, Belmont, CA, U.S.A.).

[^{35}S]-GTPγS autoradiography

The spinal cords at the lumbar level were quickly removed from each animal after decapitation, and frozen with the powder of dry ice for 10–15 min. The spinal cords were cut into 20 μm sections on a cryostat maintained at -20°C, and cross sections of the spinal cord were collected and thaw-mounted onto gelatin-coated slides. Slides were collected in racks in a humidified chamber on ice, desiccated under vacuum, and stored overnight at 4°C. On the next day, tissue sections on glass slides were placed in slide boxes with desiccant pellets, and stored at -20°C until use. Tissue sections on glass slides were incubated in an assay buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), and 100 mM NaCl at 25°C for 15 min, then in 2 mM GDP with protease inhibitors (10 μl ml^{-1} of a solution containing 0.2 mg ml^{-1} each of bestatin, leupeptin, pepstatin A and aprotinin) in assay buffer at 25°C for 45 min. Tissue sections on glass slides were then incubated with 20 pM [^{35}S]-GTPγS (1000 Ci mmol^{-1}; Amersham, Arlington Heights, IL, U.S.A.), 2 mM GDP and 10 μM nociceptin in the above-mentioned assay buffer at 25°C for 2 h. Basal activity was assessed in the absence of nociceptin, and non-specific binding was assessed in the presence of 10 μM unlabelled guanosine-5′-*o*-(3-thio)triphosphate (GTPγS). Tissue sections on glass slides were then rinsed twice for 2 min each in 50 mM Tris-HCl buffer (pH 7.4) on ice and once for 30 s in deionized H_2O on ice. Tissue sections on glass slides were dried overnight at room temperature, lined up on cardboard and then apposed to hyperfilm (Amersham) for an 48 h autoradiographic exposure. The optical density from the autoradiogram films was measured by a computer-assisted densitometer.

Membrane tissue preparation

The spinal cord was quickly removed after decapitation of mice and homogenized in 15 volumes of ice-cold 0.32 M sucrose using a Potter-Elvehjem tissue grinder with a Teflon pestle. The homogenate was centrifuged $1000 \times g$ for 10 min and the supernatant was centrifuged at $20{,}000 \times g$ for 20 min. The supernatant was discarded, and the pellet was resuspended in 15 volumes of 50 mM Tris-HCl (pH 7.4) and recentrifuged at $20{,}000 \times g$ for 20 min. The resulting pellet was resuspended in assay buffer containing (mM): Tris-HCl (pH 7.4) 50, $MgCl_2$ 5, EGTA 1 and NaCl 100 and stored at -70°C until used.

[^{35}S]-GTPγS membrane assay

The reaction was initiated by the addition of membrane suspension (3–8 μg protein assay) into the assay buffer with

various concentrations of the agonist, various concentrations of GDP and 50 pM [^{35}S]-GTPγS (Amersham) in a total volume of 1 ml. The suspensions were incubated at 25°C for 2 h. The reaction was terminated by filtering through Whatman GF/B glass filters, which had been previously soaked in 50 mM Tris-HCl (pH 7.4) and 5 mM $MgCl_2$. The filters were then washed three times with 5 ml of Tris-HCl buffer (pH 7.4) at 4°C and transferred to scintillation counting vials. Subsequently, 0.5 ml of Soluene-350 (Packard Instrument Company, Inc., Meriden, CT, U.S.A.) and 4 ml of Hionic Fluor Cocktail (Packard Instrument Company) were added to the vials. After a 12 h equilibration period, the radioactivity in the samples was determined with a liquid scintillation analyzer (Model 1600 CA, Packard Instrument Company). Non-specific binding was measured in the presence of 10 μM unlabelled GTPγS. Comparable results were obtained from more than three independent sets of experiments.

Drugs

The drugs used were: nociceptin (Peninsula Laboratories); [Phe$^1\Psi$(CH_2-NH) Gly^2] nociceptin (1–13) NH_2 (Tocris Cookson Inc, Ballwin, MO, U.S.A.); [D-Ala^2,NHPhe^4,Gly-ol]enkephalin (DAMGO; Bachem California, Torrance, CA, U.S.A.); D-Phe-Cys-D-Tyr-Orn-Thr-Pen-Thr-NH_2 (CTOP; Bachem California), [D-Pen^{2,5}] enkephalin (DPDPE; Bachem California), naltrindole hydrochloride (NTI; Toray Industries Inc.); (±)-trans-U50,488 hydrochloride (U50,488H; Toray Industries Inc., Komakura, Japan); nor-binaltorphimine (nor-BNI; Toray Industries Inc.); atropine (RBI, Natick, MA, U.S.A.), haloperidole (RBI), phaclofen (RBI), propranolol (RBI), yohimbine (RBI), GTPγS (RBI); and GDP (Sigma Chemical Company, St. Louis, MO, U.S.A.).

Statistical analysis

The data are expressed as the mean ± s.e.mean. The statistical significance of differences between the groups was assessed with the Newman-Keuls multiple comparison test.

Results

Distribution of nociceptin-immunoreactivity

Nociceptin-like immunoreactivity was detected in nerve fibres of the superficial layers of the mouse dorsal horn and in a network of fibres projecting from the lateral border of the dorsal horn to the lateral grey matter throughout the entire length of the spinal cord (Figure 1A,B,C). Some immunoreactive fibres were also noted around the central canal (Figure 1A). However, nociceptin-like immunoreactive somata were not observed in the grey matter.

In control experiments, nociceptin-like immunoreactivity was not detected in tissue sections processed with antisera pre-absorbed with nociceptin (Figure 1D) or in tissue sections where nociceptin-antisera were omitted from the staining procedures (data not shown).

Nociceptin-stimulated [^{35}S]-GTPγS autoradiography in the spinal cord

In vitro [^{35}S]-GTPγS autoradiography was performed in cross sections of lumbar spinal cords of the mice to map the anatomical distribution of nociceptin-stimulated [^{35}S]-GTPγS binding. Representative sections are illustrated in Figure 2, in which the basal [^{35}S]-GTPγS binding (Figure 2A) and nociceptin-stimulated [^{35}S]-GTPγS binding (Figure 2B) were shown at the mouse lumbar spinal cord. Nociceptin stimulated high levels of [^{35}S]-GTPγS binding in the superficial layers of the mouse dorsal horns. Nociceptin-stimulated [^{35}S]-GTPγS binding was also noted around the central canal.

Nociceptin-stimulated [^{35}S]-GTPγS binding in spinal cord membranes

Results obtained from isolated spinal cord membranes have shown that opioid-receptor agonists produce significant stimulation of [^{35}S]-GTPγS binding when assays were performed in the presence of a large excess of GDP in order to ensure that G-proteins were present in the inactivated state (Narita & Tseng, 1998; Narita *et al*., 1998, 1999). The appropriate concentrations of GDP for detecting nociceptin-stimulated [^{35}S]-GTPγS binding were determined in spinal cord

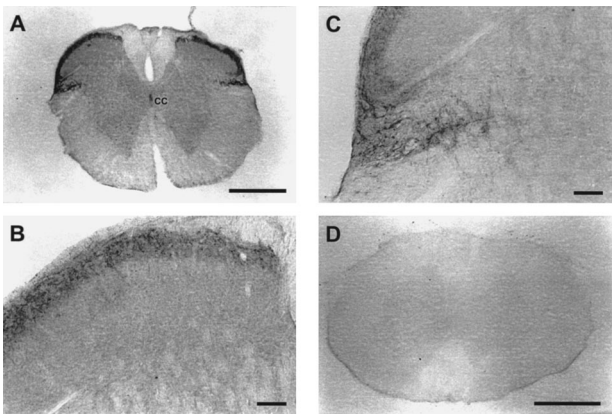

Figure 1 Photomicrographs of a mouse cervical spinal cord labelled with nociceptin-antisera or antisera pre-absorbed with the peptide. (A) Low magnification showing nociceptin-like immunoreactivity is concentrated in dense networks of fibres in the superficial layers (I and II) and in the lateral border of the dorsal horn. A few nociceptin-like immunoreactivity fibres can be seen around the central canal (cc). (B) A higher magnification showing nociceptin-like immunoreactivity fibres occupying layers I and II. (C) A higher magnification showing dense networks of nociceptin-like immunoreactivity fibres in the lateral border of the dorsal horn. (D) A section of thoracic spinal cord processed with nociceptin-antisera pre-absorbed with the peptide overnight. Positive labelling is not seen in this section. Calibration bar: 500 μm for A and D; 50 μm for B and C.

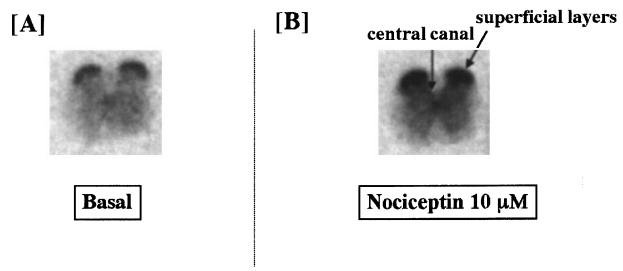

Figure 2 Representative sections at the level of the mouse lumbar spinal cord showing the distributions of nociceptin-stimulated [^{35}S]-GTPγS binding. Sections were incubated with 2 mM GDP, then [^{35}S]-GTPγS (20 pM) with 2 mM GDP and 10 μM nociceptin. Basal binding was assessed in the absence of nociceptin (A). Nociceptin stimulated high levels of [^{35}S]-GTPγS binding in the superficial layers of the mouse dorsal horns and around the central canal as compared to basal (B).

membranes by incubating with various concentrations of GDP with and without nociceptin (Figure 3A,B). GDP inhibited [^{35}S]-GTPγS binding in a concentration-dependent manner (Figure 3A). Nociceptin produced little significant effect on binding in the absence of GDP, and relative stimulation by nociceptin increased with increasing concentration of GDP (Figure 3B). In spinal cord membranes assayed in the presence of 30 μM GDP, nociceptin stimulated [^{35}S]-GTPγS binding in a concentration-dependent and saturable manner, reaching maximal stimulation of 64.1$\pm$2.4% at 1 μM as compared to basal (Figure 3C).

In order to verify the specificity of this effect of nociceptin, [^{35}S]-GTPγS binding assay was conducted with nociceptin in the presence and absence of the specific ORL1 receptor antagonist [Phe$^1\Psi$ (CH_2-NH) Gly^2] nociceptin (1–13) NH_2 (Guerrini *et al*., 1998). As shown in Figure 4A, the increase of [^{35}S]-GTPγS binding by nociceptin was inhibited by co-incubation with [Phe$^1\Psi$ (CH_2-NH) Gly^2] nociceptin (1–13) NH_2 in a concentration-dependent manner. [Phe$^1\Psi$ (CH_2-NH) Gly^2] nociceptin (1–13) NH_2 had no effect on the basal [^{35}S]-GTPγS binding level when it was added alone (Figure 4B). In addition, [Phe$^1\Psi$ (CH_2-NH) Gly^2] nociceptin (1–13) NH_2 at 10 μM did not cause the significant changes in the increased [^{35}S]-GTPγS binding by each selective opioid μ-(DAMGO), δ-(DPDPE)- or κ-(U50,488H) receptor agonist (Figure 5).

In order to confirm the ORL1 receptor specificity of nociceptin-stimulated [^{35}S]-GTPγS binding, membranes were also incubated with nociceptin in the presence and absence of selective μ- (CTOP), δ-(NTI) or κ-(nor-BNI) opioid receptor antagonists. Concentrations of antagonist were chosen so that >90% of the corresponding agonist-stimulated [^{35}S]-GTPγS binding was selectively inhibited by the antagonist (Narita & Tseng, 1998). Under these conditions, neither CTOP, NTI nor nor-BNI had any effect on the stimulation of [^{35}S]-GTPγS binding by 1 μM nociceptin (Figure 6A). Furthermore, 1 μM of the muscarinic acetylcholine receptor antagonist atropine, the dopamine receptor antagonist haloperidole, γ-amino-n-butyric $acid_B$ ($GABA_B$) receptor antagonist phaclofen, β-adrenergic receptor antagonist propranolol or α_2-adrenergic receptor antagonist yohimbine had no significant effect on basal (data not shown) or 1 μM nociceptin-stimulated [^{35}S]-GTPγS binding in this study (Figure 6B).

Discussion and conclusions

Several recent studies have revealed that in the rat spinal cord nociceptin-like immunoreactivity is concentrated in dense networks of nerve fibres innervating the superficial layers (laminae I and II) of the dorsal horn (Reidl *et al*., 1996; Schulz *et al*., 1996; Lai *et al*., 1997; Schuligoi *et al*., 1997). More interestingly, nociceptin-like immunoreactivity in the rat superficial dorsal horn resists unilateral dorsal rhizotomy, indicating that nociceptin may not originate from primary afferent neurons whose cell bodies are located in dorsal root ganglia (Reidl *et al*., 1996).

We reported here for the first time that in the mouse spinal cord nociceptin-like immunoreactive fibres were abundant in the superficial layers of the dorsal horn and in a network of fibres projecting from the lateral border of the dorsal horn to the lateral grey matter. Furthermore, moderately dense networks of nociceptin-like immunoreactive fibres were also identified around the central canal. It has been reported that immunoreactive fibres for three endogenous opioid peptides, endomorphin, enkephalin and dynorphin are heavily concentrated in the superficial dorsal horn of rat spinal cord (Reidl *et*

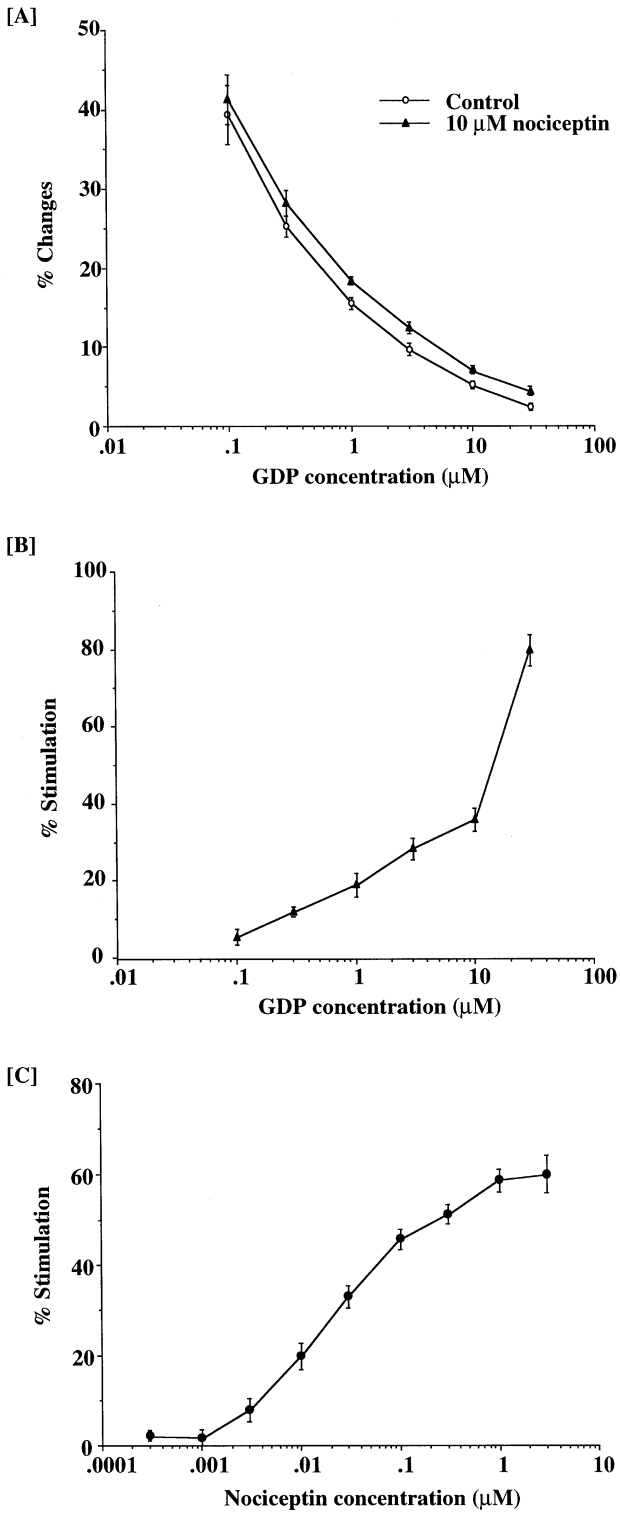

Figure 3 (A) Influence of GDP concentration (0.1–30 μM) on the binding of [^{35}S]-GTPγS to mouse spinal cord membranes in the absence or presence of 10 μM nociceptin. Incubations were performed at 25°C for 2 h in the presence of 50 pM [^{35}S]-GTPγS. Data are expressed as the mean$\pm$s.e.mean of the per cent total [^{35}S]-GTPγS binding in the absence of GDP. Comparable results were obtained from more than three independent sets of experiments. (B) Nociceptin (10 μM)-stimulated [^{35}S]-GTPγS binding to mouse spinal cord membranes, expressed as the mean$\pm$s.e.mean of the per cent stimulation over basal [^{35}S]-GTPγS binding at each concentration of GDP. (C) Concentration-effect curve of nociceptin-stimulated [^{35}S]-GTPγS binding in the mouse spinal cord. Data are expressed as the mean$\pm$s.e.mean of the per cent stimulation over basal [^{35}S]-GTPγS binding in the presence of 30 μM GDP and absence of nociceptin.

al., 1996; Pierce *et al*., 1998), however, nociceptin-like immunoreactive nerve fibres do not overlap with enkephalin- or dynorphin-containing fibres in this area (Reidl *et al*., 1996; Schulz *et al*., 1996). Furthermore, immunoreactivities for endomorphin, enkephalin and dynorphin are significantly reduced following dorsal rhizotomy (Reidl *et al*., 1996; Pierce *et al*., 1998). Taken together, these observations suggest that nociceptin production probably occurs predominantly within the central rather than within nociceptive primary afferent neurons of the dorsal root ganglia which can express other types of opioids, such as endomorphin, enkephalin and dynorphin.

The identification of nociceptin-stimulated [^{35}S]-GTPγS binding in mouse spinal cord membranes and sections provides further evidence for the biological importance of ORL1 receptor function in the spinal cord region. In membrane assay with 30 μM GDP, nociceptin produced concentration-dependent increase in [^{35}S]-GTPγS binding. The increase of [^{35}S]-GTPγS binding by nociceptin was markedly suppressed by co-incubation with a specific ORL1 receptor antagonist [Phe$^1\Psi$ (CH_2-NH) Gly^2] nociceptin (1–13) NH_2. Consistent with the finding using brain membranes (Sim *et al*., 1996), nociceptin-stimulated [^{35}S]-GTPγS binding to mouse spinal

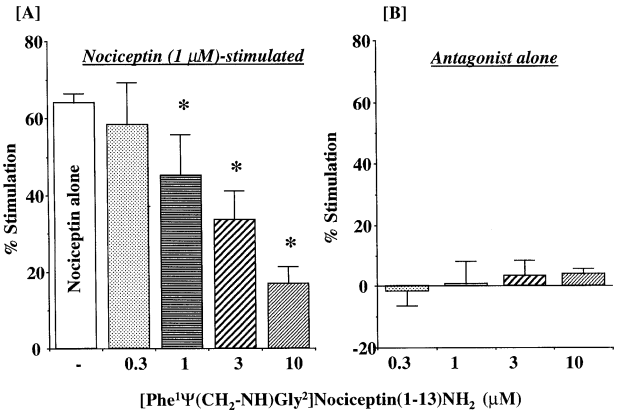

Figure 4 Effect of the selective ORL1 receptor antagonist [Phe$^1\Psi$ (CH_2-NH) Gly_2] nociceptin (1–13) NH_2 (0.3–10 μM) on nociceptin-stimulated (A) and basal (B) [^{35}S]-GTPγS binding in the mouse spinal cord. Incubations were performed at 25°C for 2 h in the presence of 50 pM [^{35}S]-GTPγS and 30 μM GDP. Data are expressed as the mean ± s.e.mean of the per cent stimulation over basal [^{35}S]-GTPγS binding in the absence of agonist and/or antagonist. Comparable results were obtained from more than three independent sets of experiments. *$P<0.01$ vs nociceptin alone.

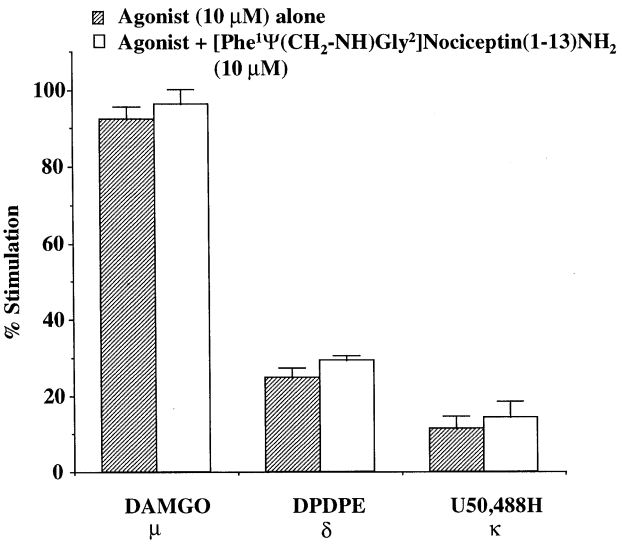

Figure 5 Effect of the selective ORL1 receptor antagonist [Phe$^1\Psi$ (CH_2-NH) Gly^2] nociceptin (1–13) NH_2 on [^{35}S]-GTPγS binding by each selective opioid μ-(DAMGO), δ-(DPDPE)- or κ-(U50,488H) receptor agonist. Assay was performed with 10 μM of each opioid receptor agonist in the presence or absence of 10 μM [Phe$^1\Psi$ (CH_2-NH) Gly^2] nociceptin (1–13) NH_2. Data are expressed as the mean ± s.e.mean of the per cent stimulation over basal [^{35}S]-GTPγS binding in the absence of agonist and/or antagonist. Comparable results were obtained from more than three independent sets of experiments.

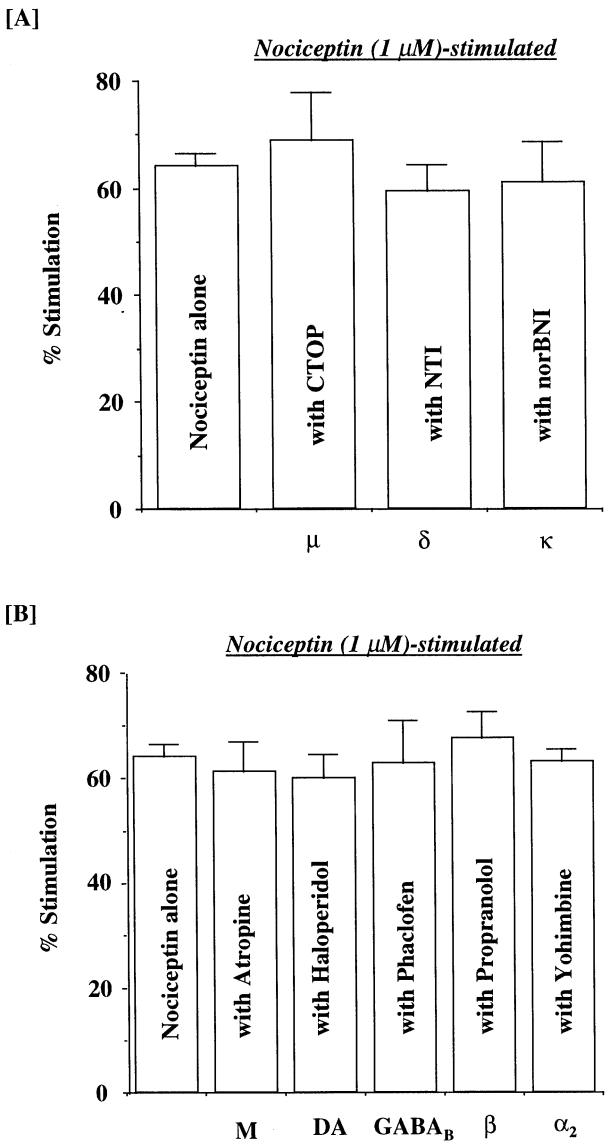

Figure 6 (A) The lack of effect of μ-(CTOP), δ-(NTI) and κ-(nor-BNI) opioid receptor antagonists on [^{35}S]-GTPγS binding by nociceptin. Assay was initiated by incubations with 1 μM nociceptin in the presence or absence of 1 μM of each antagonist. Data are expressed as the mean ± s.e.mean of the per cent stimulation over basal [^{35}S]-GTPγS binding in the absence of agonist and/or antagonist. Comparable results were obtained from more than three independent sets of experiments. (B) Effect of several G-protein-coupled receptor antagonists on [^{35}S]-GTPγS binding by nociceptin. Atropine: muscarinic acetylcholine (M) receptor antagonist; Haloperidole: dopamine (DA) receptor antagonist; Phaclofen: $GABA_B$ receptor antagonist; Propranolol: β-adrenergic receptor antagonist; Yohimbine: α_2-adrenergic receptor antagonist.

cord membranes was not affected by any opioid or other G-protein-coupled receptor antagonists examined. These results clearly indicate that G-protein activation by nociceptin is mediated by specifically ORL1 receptors in the mouse spinal cord.

The present autoradiographic analyses disclose interesting aspects of the spatial distribution of nociceptin-stimulated [^{35}S]-GTPγS binding in relation to nociceptin-containing fibres. We found high levels of nociceptin-stimulated [^{35}S]-GTPγS binding in the superficial layers and around the central canal of mouse dorsal horns, which correspond to the areas where nociceptin-like immunoreactive fibres are concentrated. Recently, the immobilized-antibody microprobe technique revealed a release of nociceptin-like substances from the dorsal horn of the rat spinal cord (Williams *et al*., 1998). These findings suggest that nociceptin-containing fibres, which may originate from the supraspinal site, terminate onto the superficial layers and the central canal areas of the dorsal horn, and that nociceptin released in these areas can activate the ORL1 receptor.

It has been proposed that the distribution of nociceptin-stimulated [^{35}S]-GTPγS binding is similar to that of ORL1 receptor mRNA in the rat brain (Sim *et al*., 1996). However, the mRNA for ORL1 receptors appears to be relatively high in the cell bodies of the dorsal root ganglia (Wick *et al*., 1994). It is therefore probable that ORL1 receptors are located in the primary afferent terminals.

The distribution of nociceptin-containing fibres and ORL1 receptors in the superficial dorsal horn (laminae I and II) and central canal (laminae X) is particularly relevant to studies of pain modulation. The superficial dorsal horn is a region known to be important in the processing of nociceptive information. Laminae I neurons may be divided physiologically into three groups: (i) neurons activated by fibres having A-δ/C fibre conduction velocities that respond to intense mechanical stimulation, (ii) neurons activated by innocuous skin cooling with afferents having a conduction velocity akin to those of A-δ fibres, and (iii) a small percentage of neurons activated by C fibre polymodal afferents (Christensen & Perl, 1970; Willis *et al*., 1974; Kumazawa *et al*., 1975; Price & Mayer, 1975; Cervero *et al*., 1976). A significant proportion of laminae II neurons receive A-δ/C fibre input (Kumazawa & Perl, 1976; Cervero *et al*., 1977; Light *et al*., 1979; Wall *et al*, 1979). Neurons located in laminae II tend to be excited by activation of thermal receptive or mechanical nociceptive afferents (Light *et al*., 1979). Although laminae X is a parvecellular region, recent studies have demonstrated that branches of small, lightly myelinated fibres were observed to enter the region (Light & Perl, 1979). Cells in laminae X respond primarily to high-threshold temperature and noxious pinch with small receptive field (Nahin *et al*., 1983; Honda, 1985; Honda & Perl, 1985).

A hyperalgesic response to nociceptin when injected intracerebrovascularly into mouse was demonstrated (Meunier *et al*., 1995; Reinscheid *et al*., 1995). More recent studies, however, showed that intrathecally administered nociceptin produced antinociceptive response (King *et al*., 1997; Yamamoto *et al*., 1997). Interestingly, it has been recently shown that exogenously applied nociceptin inhibits spinal transmission including excitatory synaptic transmission from primary afferent fibres to rat dorsal horn neurons (Faber *et al*., 1996; Stanfa *et al*., 1996; Lai *et al*., 1997). Thus, these recent findings support the hypothesis that nociceptin may function as a putative inhibitory transmitter in the dorsal horn and produce an analgesic, rather than a hyperalgesic, response.

In conclusion, this study is the first to show that opioid-like peptide nociceptin is able to stimulate [^{35}S]-GTPγS binding in the spinal cords of mice. The distribution of nociceptin-stimulated [^{35}S]-GTPγS binding in general overlapped with nociceptin-like immunoreactive fibres in the superficial dorsal horn and central canal. Furthermore, nociceptin-induced G-protein activation in the spinal cord was specifically mediated by ORL1 receptors, but not by opioid and G-protein-coupled receptors. These findings provide no evidence for cross-talk between ORL1 receptors and opioid receptors at the cellular level. The future study is required to further reveal which G-protein (Gs, Gi, Go, Gq or Gx/z) at the cellular level is activated by nociceptin in this region.

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