Original Paper

Endomorphin-2 Inhibits the Activity of the Spinoparabrachial Projection Neuron through Presynaptic Mechanisms in the Spinal Dorsal Horn in Rats

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Key Words

Endomorphin • Spinal dorsal horn • Parabrachial nucleus • Projection neuron • Presynaptic effect • Rat

ABSTRACT

Background/Aims: Spinal dorsal horn (SDH) is one of the most important regions for analgesia produced by endomorphin-2 (EM2), which has a higher affinity and specificity for the μ -opioid receptor (MOR) than morphine. Many studies have focused on substantia gelatinosa (SG, lamina II) neurons to elucidate the cellular basis for its antinociceptive effects. However, the complicated types and local circuits of interneurons in the SG make it difficult to understand the real effects of EM2. Therefore, in the present study, we examined the effects of EM2 on projection neurons (PNs) in lamina I. **Methods:** Tracing, immunofluorescence, and immunoelectron methods were used to examine the morphological connections between EM2-immunoreactive (-ir) terminals and PNs. By using *in vitro* whole cell patch clamp recording technique, we investigated the functional effects of EM2 on PNs. **Results:** EM2-ir afferent terminals directly contacted PNs projecting to the parabrachial nucleus in lamina I. Their synaptic connections were further confirmed by immunoelectron microscopy, most of which were asymmetric synapses. It was found that EM2 had a strong inhibitory effect on the frequency, but not amplitude, of the spontaneous excitatory postsynaptic current (sEPSC) of the spinoparabrachial PNs in lamina I, which could be reversed by MOR antagonist CTOP. However, their spontaneous inhibitory postsynaptic current (sIPSC) and intrinsic properties

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were not changed after EM2 application. **Conclusion:** Applying EM2 to the SDH could produce analgesia through inhibiting the activities of the spinoparabrachial PNs in lamina I by reducing presynaptic neurotransmitters release from the primary afferent terminals.

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Introduction

Endomorphin (EM), which could be divided into EM1 and EM2 subtypes, possesses the highest affinity and specificity for the μ -opioid receptor (MOR) among different endogenous opioid peptides [1]. Furthermore, the similar analgesic effects, but much weaker side effects, of EM compared with morphine raise the possibility that EM could be a good candidate for clinical antinociceptive drug [2-4]. Differences have been noted for the distributions of EM1 and EM2 in the brain and spinal cord. EM1 is widely and densely distributed throughout the brain and upper brainstem, whereas EM2 is more prevalent in the spinal cord and lower brainstem [5]. Therefore, EM1 is considered to play more important roles in supraspinal structures such as the periaqueductal gray [6], while EM2 mainly functions in the spinal cord [7-9].

The observation that EM2 produced greater analgesia upon *i.t.* compared to *i.c.v.* injections [1, 10] also raises the possibility that the spinal dorsal horn (SDH) may represent the anatomic site upon which EM2 exerts its analgesic effects. EM2-immunoreactive (-ir) fibers and terminals are localized in the superficial laminae (lamina I and lamina II) of the spinal dorsal horn (SDH), which originate mainly from the primary sensory neurons in the dorsal root ganglion (DRG) [5, 7, 11-13]. Obvious antinociception could be produced by *i.t.* administration of EM2 in the tail-flick, paw-withdrawal, tail pressure and flexor-reflex tests on adult rodents [10, 14-18]. Moreover, it has also been demonstrated that a reduction of EM2 is involved in the chemotherapy-induced neuropathic pain, cancer-induced bone pain, thermal hyperalgesia and inflammatory pain in ovariectomized female rats and painful diabetic neuropathy [14, 17, 19, 20]. However, the underlying mechanisms for these analgesic effect of EM2 in the SDH primarily focused on the morphological and functional studies of the substantia gelatinosa (SG, lamina II) interneurons. EM2 in substance P (SP)-containing axons contacts MOR-ir neural cell bodies in lamina II [21], and most of the axon terminals containing EM2 make asymmetrical synapses with MOR-ir profiles in the rat SDH [22]. EM2 could hyperpolarize membrane potentials by opening inwardly-rectifying K^+ channels and attenuate the spontaneous release of glutamate from primary afferent terminals in the SG, both of which are mediated by MOR [13, 23-25].

The SDH is considered to be an important site for processing nociceptive information, in which there are complicated local neural circuits and various interneurons [26-28]. The projection neurons (PNs) are the center where nociceptive information is processed. Regardless of the amount of the various types of interneurons and how many local neural circuits are involved in the receiving, processing and integrating the perception information, the signal outputs from the SDH are transmitted only by the PNs. Therefore, the study regarding the influences of EM2 on PNs could directly provide the evidence for its analgesic effect and reveal the underlying mechanisms completely. Morphological evidence indicating that EM2 fibers directly make synapses on PNs in the SDH and the functional effects of EM2 on PNs were also investigated in this study.

Materials and Methods

Animals

Male Sprague-Dawley (SD) rats were used. Adult rats (weighing 250-300 g) were adopted for morphological investigations, while young rats (aged 21-24 d) were used for whole-cell patch clamp

recording. The animals were housed in a temperature-controlled environment under a 12 h light-dark cycle (8 a.m. to 8 p.m. light), with food and water freely available. All procedures were approved by the Committee of Animal Use for Research and Education of The Fourth Military Medical University (Xi'an, China). All efforts were made to minimize animal suffering and the number of animals used.

Intra-lateral parabrachial nucleus (LPB) stereotaxic microinjections

The injection procedures have been described in our previous study [29]. In brief, adult or young rats were anesthetized with 2% sodium pentobarbital [40 mg/kg, intraperitoneal (*i.p.*)]. A midline opening was made in the skull with a dental drill to insert a glass micropipette (tip diameter 40–60 μ m) connected to a microsyringe (1 μ l, Hamilton, Reno, NV, USA) into the target site. A volume of 0.04 μ l of 4% Fluoro-Gold (FG; Fluorochrome; 80014; Biotium; Hayward, CA, USA) dissolved in normal saline was pressure-injected into the LPB, according to the following coordinates: 9.0 mm caudal to Bregma, 2.3 mm lateral to the midline and 7.0 mm ventral to the surface of the cranium [30]. For electron microscopy analysis, 0.1 μ l of 2% (w/v) wheat germagglutinin-horseradish peroxidase (WGA-HRP) conjugate (Toyobo, Osaka, Japan) dissolved in normal saline was injected into the LPB with the same coordinates used as the FG injection. For whole-cell patch clamp recording, 0.1 μ l of 10% tetramethylrhodamine (TMR, 3000 MW, Molecular Probe, Eugene, OR, USA) distilled in 0.1 M citrate-NaOH (pH 3.0) [31] was injected into the LPB (7.8 mm posterior to Bregma, 1.7 mm lateral to the midline, and 6.1 mm ventral to the surface of the cranium) of young rats. Each injection was made slowly over 10 min and the injection needle was then kept in place for another 10 min. After the retrograde tracing operation, the adult rats that were injected with 4% FG were allowed to survive for 7 d, the adult rats that were injected with 2% (w/v) WGA-HRP were allowed to survive for 60 h, and the young rats that were injected with 10% TMR were allowed to survive for 3–5 d.

Immunofluorescent histochemical staining

Five adult rats receiving FG injection were anesthetized with an overdose of 2% sodium pentobarbital (100 mg/kg, *i.p.*), and perfused through the ascending aorta with 100 ml of 0.9% (w/v) saline, followed by 500 ml of 4% (w/v) paraformaldehyde and 30% (v/v) saturated picric acid in 0.1 M phosphate buffer (PB, pH 7.4). After perfusion, the brains and lumbar segments of the spinal cord (SC) were immediately removed, immersed in the same fixative for 4 h at 4°C, and then transferred to 30% (w/v) sucrose in 0.1 M PB overnight at 4°C until they completely sunk to the bottom. After being embedded in an inert mounting medium (OCT; Tissue-Tek; Sakura; Torrance, CA, USA), coronal sections of the brain containing LPB and lumbar SC were cut at 50 μ m-thick and 30 μ m-thick on a freezing microtome (Kryostat 1720; Leitz, Germany) respectively, and collected into eight dishes containing 0.01 M phosphate-buffered saline (PBS, pH 7.4). The sections containing the LPB regions or lumbar SC in the first dish were mounted onto gelatin-coated glass slides, air dried, cover-slipped with a mixture of 50% (v/v) glycerin and 2.5% (w/v) triethylenediamine (anti-fading agent) in 0.01 M PBS and then observed using a fluorescence microscope (Olympus BX-60; Tokyo, Japan) for investigating the injection sites in the LPB or distribution patterns of the FG retrogradely labeled neurons in the lumbar SC.

The procedures for immunofluorescent histochemical staining were the same as those previously described [7, 32, 33]. The second set of the serial SC sections were used to evaluate the double staining of EM2/FG. In brief, free-floating lumbar sections were blocked with 10% normal donkey serum (NDS) in 0.01 M PBS for 0.5 h. Then the sections were subjected to the following sequential incubations with (1) rabbit antiserum against EM2 (1:200, AB5104, Chemicon; Temecula, CA, USA) and guinea pig antiserum against FG (1:500, NM-101, Protos Biotech Corp; New York, NY, USA) in the antibody dilution medium (0.01 M PBS containing 5% (v/v) NDS, 0.3% (v/v) Triton X-100, 0.05% (w/v) Na₃N and 0.25% (w/v) λ -carrageenan (PBS-NDS, pH 7.4)) overnight at room temperature (RT) and then 72 h at 4°C; (2) a mixture of biotinylated donkey anti-rabbit IgG (1:500, AP182F, Millipore; Billerica, MA, USA) and Alexa594-donkey anti-guinea pig IgG (1:500, 706-585-148, Jackson ImmunoResearch; Suffolk, UK) in PBS-NDS for 6 h at RT; (3) fluorescence isothiocyanate (FITC)-conjugated avidin (1:1000, A-2001, Vector; Burlingame, CA, USA) in PBS containing 0.3% Triton X-100 (PBS-X, pH 7.4) for 3 h at RT.

The third set of serial SC sections was used for a control test, in which the primary antisera were omitted or replaced with normal serum and the other procedures were as the same as those in the above groups. Finally, all of the sections were then mounted onto glass slides and observed with laser scanning

confocal microscopy (FV1000, Olympus, Japan) under appropriate filters for FITC (excitation 492 nm; emission 520 nm) and Alexa594 (excitation 590 nm; emission 618 nm). Forty-five sections were selected from 3 rats and were scanned through a 60 X water-immersion lens to generate z-stacks with a z-separation of 1 μ m. The number of PNs with EM2 contacts was carefully checked. Sixty-four PNs-ir neurons were selected from 3 rats and scanned by digital zoom on the previous lens. For boutons contacting the neurons, the cell body surface areas were measured and the surface areas of dendrites were estimated from their lengths and diameters, based on the assumption that they were cylindrical [34, 35]. For the contacts of EM2-ir terminals with the PNs-ir neurons, the density per 100 μ m² of combined somatic and dendritic surface was determined.

Electron microscopy

After the WGA-HRP injection, three SD rats were deeply anesthetized with an overdose of sodium pentobarbital and transcardially perfused with 100 ml of 0.9% saline, followed by 500 ml of 4% (w/v) paraformaldehyde, 0.1% (w/v) glutaraldehyde, and 15% (v/v) saturated picric acid in 0.1 M PB. Subsequent to perfusion, the brains were quickly removed, and the pons containing LPB and lumbar SC were then serially cut into 50 μ m thick coronal sections using a vibratome (Microslicer DTM-1000, DSK, Kyoto, Japan). The sections were consecutively collected into 0.1 M PB. All sections in the dishes were processed for the histochemical detection of WGA-HRP using the tetramethylbenzidine-sodium tungstate (TMB-ST) method [36, 37]. The WGA-HRP reaction products were intensified using a diaminobezidin (DAB)/cobalt/H₂O₂ solution [38]. The sections containing LPB were then mounted onto gelatin-coated slides and were used to observe the WGA-HRP injection sites. The sections containing many WGA-HRP-labeled neurons in the SDH were selected under a light microscope by being placed on glass slides. Then, these selected sections were collected in vials containing a mixture of 25% (w/v) sucrose and 10% (v/v) glycerol in 0.05 M PB, and treated by using the freeze-thaw method in liquid nitrogen for enhancement of the penetration of the antibody in the immunohistochemical reaction [6, 39]. Subsequently, the sections were washed three times in 0.05 M Tris-buffered saline (TBS; pH 7.4), incubated with 20% (v/v) NDS in 0.05 M TBS for 30 min to block the non-specific immunoreactivity.

The procedures for immunoelectron microscopy histochemical staining of EM2-ir profiles were the same as those previously described [6, 39, 40]. Briefly, the sections were incubated for 24 h at RT and then for 72 h at 4°C with rabbit antiserum against EM2 (1:100), which was diluted in 0.05 M TBS (pH 7.4) containing 2% (v/v) NDS (TBS-NDS). Then, these sections were incubated overnight in biotinylated donkey anti-rabbit IgG (1:200) and finally with the ABC kit (1:100; Vector) for 4 h at RT. After incubation, the sections were placed in 0.05 M Tris-HCl (pH 7.6) containing 0.02% DAB and 0.003% H₂O₂ for 10-20 min.

Subsequently, the sections were post-fixed for 45 min in 1% solution of osmium tetroxide in 0.1 M PB and then stained with 1% uranyl acetate in 70% ethanol for 1 h. Then, the sections were flat embedded in an Epoxy resin (Fluka Chemie, Buchs, Switzerland) after dehydration in a graded series of ethanol and degreased in propylene oxide. Small pieces of SDH areas containing many labeled elements were selected and cut out from the flat-embedded sections with fresh razor blades under a dissection microscope. The selected tissue pieces were cut into ultrathin sections 70 nm thick with a diamond knife mounted on an ultramicrotome (Reichert-Nissei Ultracut S; Leica, Wein, Austria). The series of ultrathin sections were mounted on single-slot grids coated with pioloform membrane (Agar Scientific, Stansted, UK), stained with 1% (w/v) lead citrate and then examined with an electron microscope (JEM1440, Tokyo, Japan). We chose 60 sections from each rat to check the staining for EM2 and HRP. To ensure the synapses were symmetrical, we attempted to examine all adjacent sections to confirm there was no post-synaptic density on the same postsynaptic membrane.

Whole cell patch clamp recording

Transverse spinal cord slices at the level of the lumbar enlargement were prepared following our previous method [31]. Fifteen rats were perfused transcardially with 100 ml of 4°C sucrose artificial cerebrospinal fluid (sucrose-aCSF) containing the following reagents: 220 mM sucrose, 2.5 mM KCl, 26 mM NaHCO₃, 6.0 mM MgSO₄, 1.2 mM KH₂PO₄, 0.5 mM CaCl₂, 10 mM glucose, 1 mM ascorbate, and 3 mM sodium pyruvate. Transverse slices (300 μ m thick) were cut on a vibrating microtome (Leica VT 1200s,

Heidelberg, Nussloch, Germany) in 4°C sucrose-aCSF bubbled with carbogen gas (95% O₂ and 5% CO₂). The slices were transferred to a submerged recovery chamber containing normal-sodium aCSF (normal-aCSF) (124 mM NaCl, 2.5 mM KCl, 2 mM CaCl₂, 2 mM MgSO₄, 25 mM NaHCO₃, 1 mM NaH₂PO₄, 10 mM glucose, 1 mM ascorbate, and 3 mM sodium pyruvate), which was continuously bubbled with carbogen gas at RT.

The patch pipettes were constructed with a P-97 micropipette puller (Sutter Instruments, Novato, CA, USA) from borosilicate glass capillary tubes (World Precision Instruments, Sarasota, FL, USA). The intrinsic properties of the PNs were recorded in current clamp mode and the spontaneous excitatory post-synaptic current (sEPSC) and spontaneous inhibitory post-synaptic current (sIPSC) were recorded in voltage clamp mode by using Axon 700B amplifier (Axon Instruments, Foster City, CA, USA). Pclamp software (v. 10.02, Axon Instruments) was used to acquire and analyze the data. The recording pipettes (3–6 MΩ) filled with a solution containing: 130 mM potassium-gluconate, 5 mM NaCl, 15 mM KCl, 0.4 mM ethylene glycol tetraacetic acid (EGTA), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 4 mM Mg-ATP, and 0.3 mM Na₂-GTP (adjusted to pH 7.3 with KOH) were used for intrinsic property and sEPSC recording; the pipettes filled with a solution containing: 112 mM Cs-Gluconate, 5 mM TEA-Cl, 3.7 mM NaCl, 0.2 mM EGTA, 10 mM HEPES, 2 mM Mg-ATP, 0.3 mM Na₂-GTP and 5 mM QX-314 (adjusted to pH 7.3 with CsOH) were used for sIPSC recording. For sEPSC recording, cells were voltage clamped at -60 mV and picrotoxin (100 μM) and strychnine (2 μM) were added in the ACSF. For sIPSC recording, cells were voltage clamped at 0 mV and 6-Cyano-7-nitroquinoxaline-2, 3-dione (CNQX, 25 μM) and DL-2-amino-5-phosphonopentanoic acid (AP-5, 50 μM) were added to the ACSF. The initial access resistance was 15–30 MΩ, and this was monitored throughout the experiment. The data were discarded if the access resistance changed >15% during the experiment. The data were filtered at 1 kHz and digitized at 10 kHz. The whole cell patch-clamp recordings were performed on TMR-containing PNs that were visualized under epifluorescence using a tetramethyl rhodamine isothiocyanate (TRITC) filter set (U-HGLGPS, Olympus) with a monochrome CCD camera (IR-1000E, DAGE-MTI, Michigan, USA) and monitor.

The action potentials (APs) were detected in response to supra-threshold current injections in the current clamp mode. Depolarizing currents of 5–80 pA (400 ms duration) were delivered in increments of 5–20 pA. The rheobase was defined as the minimum current required to evoke an AP. The AP voltage threshold ($V_{\text{threshold}}$) was defined as the first point of the rising phase of the spike at which the change in voltage exceeded 50 mV/ms. Latency was the duration from the starting of the current injection to the starting of AP at $V_{\text{threshold}}$. The spike amplitude was quantified as the difference between the $V_{\text{threshold}}$ and the peak voltage. The duration of the AP was measured at the threshold voltage. The spike width was measured at 1/2 of the total spike amplitude (measured from the $V_{\text{threshold}}$ level). The changes in the resting membrane potential (RMP), $V_{\text{threshold}}$, AP amplitude, rheobase and spike number were always selected to estimate the influence of different drugs to the recorded cells.

In all cases, 0.2% biocytin (Sigma-Aldrich, St. Louis, MO, USA) was introduced into the recording solution to identify the morphological properties of the recorded neurons. After recording, the slices were immediately fixed in 4% paraformaldehyde in 0.1 M PB for 4 h at RT and subsequently stored in 30% sucrose solution overnight at 4°C. The sections were then transferred to 0.01 M PBS containing 1% Triton X-100 (PBS-TX) and stored at 4°C for 24 h. After thoroughly washing with PBS, the tissue was incubated with rabbit antiserum against TMR (1:200, A637, Invitrogen, Eugene, Oregon, USA) diluted in PBS-NDS overnight at RT, followed with Alexa594-donkey anti-rabbit IgG (1:500, A21207, Invitrogen) for 6 h at RT. Finally, the sections were incubated with FITC-Avidin (1:1,000) diluted in PBS-X for 3 h at RT. The immunofluorescence-labeled sections were then observed with a confocal microscope (FV-1000) under the appropriate filters for Alexa594 and FITC.

Drug preparation

AP-5 and CNQX were purchased from Abcam (Cambridge, MA, USA). EM2, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂ (CTOP), guanosine 5'-[β-thio]diphosphate trilithium salt (GDP-β-S), and strychnine were purchased from Sigma-Aldrich. All drugs were prepared as stock solutions according to the manufacturer's instructions and were stored frozen at -20°C. Before each experiment, the drug stock solutions were added to the normal-aCSF solution to obtain the experimental concentrations.

Statistical Analysis

The paired *t* test was used for comparisons between paired data. The cumulative probabilities of the inter-event intervals and the amplitudes of the sEPSC and sIPSC were compared using Kolmogorov-Smirnov tests. Two-way repeated measures ANOVA was used for comparing spike numbers of PNs following EM2 application. One-way repeated measures ANOVA was used for comparisons among multi groups to check the roles of MOR. In all cases, *P* < 0.05 was considered to be significantly different.

Results

Contacts between EM2-ir terminals and spinoparabrachial PNs in lamina I of the SDH

Because our aim was to label the maximum number of PNs in lamina I, we used relatively large injections of FG that extended into adjacent regions including the medial parabrachial area and Kölliker-Fuse nuclei [41, 42]. In all 3 cases where 5 rats received FG injection, the FG injection sites included the whole LPB, with variable spread into surrounding areas (Fig. 1a, b). Many FG-labeled neurons were observed bilaterally throughout the SDH with dominance on the side contralateral to the FG injection, in lamina I, the lateral spinal nucleus (LSN), and the deeper laminae of the SDH (laminae III and V) (Fig. 1c, d). EM2-ir fibers and terminals were densely located in lamina I and outer part of lamina II (Ilo), less in inner part of lamina II (Ili), laminae III, and IV (Fig. 2a). Some EM2-ir terminals were found to be closely connected with FG-labeled PNs in lamina I (Fig. 2b-h). The quantitative data were assessed for the presence of contacts from EM2-ir fibers and terminals. The proportion of PNs contacted by EM2-ir fibers and terminals among all FG-labeled PNs in lamina I was 51.4% (145 neurons among 282 neurons). Among the 145 FG-labeled neurons, we selected 64 neurons to examine and observed that all of them had been contacted with EM2-ir fibers and terminals on their dendrites, and in most cases, also on their neuronal cell bodies (Fig. 2f). The density of these contacts on combined somatic and dendritic plasma membrane varied from 0.78 to 9.66/100 μm^2 (mean 2.91 ± 0.21 SEM).

Ultrastructural features of EM2-ir terminals and the synaptic connections between EM2-ir terminals and spinoparabrachial PNs in the superficial laminae of the SDH

WGA-HRP-labeled spinoparabrachial PNs in lamina I of the SDH were detected based on the presence of highly electron-dense clumps of crystalline material and the occasional presence of amorphous puncta in the cytoplasm and large dendrites (Fig. 3a). Under electron microscope, EM2-ir axonal terminals in the SDH were identified by the presence of homogeneously distributed fine, granular, electron-dense DAB reaction products. Large dense-coated granular vesicles (LDGV), which usually contain neuropeptides and are located in the peripheral region of the axon terminals, were found in most of the detected EM2-ir terminals (Fig. 3b-d). Consistent with the light microscope observations, the EM2-ir axonal terminals, typically filled with synaptic vesicles, containing DAB reaction products formed synaptic connections with the dendritic profiles containing WGA-HRP-labeled products (Fig. 3c-d). Most synapses were asymmetrical (288 among 319, 90.28%) ones (Fig. 4c), but symmetrical synapses were also found (31 among 319, 9.72%) (Fig. 3d).

Inhibitory effects of EM2 on the excitatory synaptic transmission of PNs in lamina I through presynaptic mechanisms

The morphological results suggested that EM2 might directly and/or indirectly regulate the activity of PNs in lamina I of the SDH. To confirm the functional involvement of EM2, we performed whole cell patch clamp recordings on PNs in lamina I that were retrogradely labeled with TMR (Fig. 4a). Biocytin was introduced into the intracellular solution to visualize the morphological characteristics of the recorded PNs, which were labeled with TMR (Fig. 4b-f). These results further confirmed that the recorded neurons were PNs in lamina I of the SDH. The electrophysiological properties of these PNs were subsequently recorded and analyzed.

Fig. 1. Retrogradely labeled neurons projecting from spinal cord to the parabrachial nucleus. a, b: FG was injected into the LPB on the left side (a). The cytoarchitectonic areas involved in the injection site were confirmed by Nissl staining of the same section (b). Neurons in lamina I, III, IV, and LSN that were presumably projection neurons were labeled with FG (c and d). I-IV: lamina I to lamina IV; 4V, fourth ventricle; DF: dorsal funiculus; DH: dorsal horn; LPB: lateral parabrachial nucleus; LSN: lateral spinal nucleus; MPB, medial parabrachial nucleus; scp, superior cerebellar peduncle. Scale bars = 200 μ m (a and b), 100 μ m (in c), and 50 μ m (in d).

Fig. 2. Immunofluorescent histochemical double-staining showing the connections between EM2-ir terminals (a, c, d, and f-h, green) and FG labeled spinoparabrachial projection neurons (b, d, and e-h, red) in lamina I. The rectangle areas in a-c are enlarged in d-f, respectively. The merged 3D images in f-h revealed close contacts between EM2-ir terminals (green) and one FG-labeled cell body (red). The arrowheads in d and f showed EM2-ir terminals connecting to the projection neurons. I-IV: lamina I to lamina IV. Scale bars = 100 μ m (in a-c) and 8 μ m (in d and e).

Fig. 3. The ultrastructural features and synaptic connections between EM2-ir terminals and dendrites of the WGA-HRP retrogradely labeled neurons in lamina I of the spinal dorsal horn after injecting WGA-HRP into the contralateral parabrachial nucleus. a: Dendritic profiles (Den) of a lamina I projection neuron contained TMB histochemical reaction products (HRP) of the transported WGA-HRP made asymmetrical synapse (solid arrowheads) with an unlabeled axon terminal (Ax). b: EM2-ir terminals (Ax) formed a symmetrical synapse (hollow arrowheads) synapse with an unlabeled dendritic process (Den). EM2-ir terminals (Ax in c and d) contained DAB reaction products formed asymmetric (solid arrowheads) (c) or symmetric (hollow arrowheads) (d) synapses with WGA-HRP-labeled dendritic profiles (HRP). Note that the EM2-ir products are principally identified in large dense-coated granular vesicles (LDGV) within axon terminals in the superficial laminae of the SDH. Scale bars = 500 nm (a-d).

The membrane capacitance (C_m) was 78.92 ± 6.31 pF. The membrane resistance (R_m) was 339.0 ± 19.65 M Ω , and the access resistance (R_a) was 22.1 ± 1.49 M Ω . To investigate whether EM2 could change the intrinsic properties of TMR-labeled PNs, the single action potentials (APs) at the threshold and firing patterns (Fig. 5), we use EM2 (3 μ M) bath application into the ACSF following our previous studies [20, 31]. No changes were found in the firing pattern test, where EM2 did not change the spike number in most of the recorded

Fig. 4. Identification of the patch clamp recorded projection neurons by immunofluorescent histochemical double-staining in lamina I. The projection neuron was labeled with TMR injected into the parabrachial nucleus (a and d in red) and recorded indicating by filled biocytin (b, c, e, and f in green). The merged images (c and f) showed that the biocytin-filled neuron was the TMR-containing neurons. I-IV: lamina I to lamina IV. Scale bars = 200 μ m (in a-c) and 40 μ m (in d-f).

Fig. 5. The effects of EM2 on the intrinsic properties of TMR-labeled spinoparabrachial projection neurons in lamina I of the spinal dorsal horn. a: A superimposed sample traces showing action potentials (APs) evoked by the same current injections (15 ms, 40 pA), before (black) and after (red) EM2 (3 μ M) bath application. The resting membrane potential (RMP), spike amplitude, threshold potential (TP), and rheobase before and after EM2 application were summarized in (b). There is no significant difference between before and after EM2 application in these properties. c: A superimposed sample trace showing that the number of APs evoked by the same current injection (400 ms, 20 pA) did not change with EM2 application (baseline, 9 AP; EM2, 9 AP). d: Averaged AP trains induced by step current injections (400 ms, 0-80 pA) showing that there were no significant changes in the number of APs before and after EM2 application (n=15, $P=0.16$, two-way repeated measures ANOVA).

properties. c: A superimposed sample trace showing that the number of APs evoked by the same current injection (400 ms, 20 pA) did not change with EM2 application (baseline, 9 AP; EM2, 9 AP). d: Averaged AP trains induced by step current injections (400 ms, 0-80 pA) showing that there were no significant changes in the number of APs before and after EM2 application (n=15, $P=0.16$, two-way repeated measures ANOVA).

TMR-labeled PNs (n = 15, from 8 rats, $F_{(1,70)}=1.64$, $P=0.16$, two-way repeated measures ANOVA). There were no significant differences in most of the parameters, such as the spike amplitude (before: 66.07 ± 3.21 mV; after: 63.42 ± 3.81 mV, $P=0.19$), half width (before: 1.21 ± 0.08 ms; after: 1.26 ± 0.12 ms, $P=0.38$), rise (before: 0.32 ± 0.04 ms; after: 0.32 ± 0.03 ms, $P=0.87$) and decay (before: 0.62 ± 0.05 ms; after: 0.65 ± 0.09 ms, $P=0.54$) times (Table 1). Moreover, it was also observed that the resting membrane potential (RMP) (before: 60.57 ± 2.09 mV; after: 60.61 ± 2.70 mV, $P=0.96$), threshold of AP (before: TP, 39.67 ± 2.06 mV; after: 39.38 ± 1.79 mV, $P=0.79$) and the rheobase (before: 17.0 ± 0.86 pA; after: 15.3 ± 1.64 pA, $P=0.21$) were unchanged. The above results indicated that the intrinsic properties of the LPB-projecting neurons were not changed after EM2 bath application.

We also compared the effects of EM2 on the excitatory input (sEPSC) and inhibitory input (sIPSC) to the TMR-labeled PNs. In the presence of EM2, the frequency of the sEPSC was inhibited by 40.5% (baseline, 1.64 ± 0.19 Hz; EM2, 0.97 ± 0.11 Hz. $t = 4.80$, $P < 0.001$, $n = 12$ from 6 rats, paired t -test) (Fig. 6a-c). There were no considerable changes in the sEPSC's amplitude after EM2 application on these PNs (baseline, 23.01 ± 2.04 pA; EM2, 22.70 ± 2.03 pA. $t = 0.63$, $P = 0.54$, paired t -test). In contrast, EM2 did not inhibit the frequency and amplitude of the sIPSC on TMR-labeled PNs (frequency: baseline, 0.46 ± 0.06 Hz; EM2, 0.47 ± 0.08 ; $t = 0.21$, $P = 0.84$; amplitude: baseline, 20.46 ± 1.39 pA; EM2, 21.30 ± 1.35 pA; $t = 1.29$, $P = 0.24$. $n = 9$, paired t -test) (Fig. 6d-f). The above results show that EM2 has an inhibitory effect on the frequency of sEPSCs but no effects on the amplitude of sEPSCs or the frequency and amplitude of sIPSCs of the PNs, suggesting that EM2 inhibit the excitatory synaptic transmission of the PNs in lamina I through presynaptic mechanisms.

Table 1. Effects of EM2 bath application on the intrinsic properties of projection neurons in the lamina I of SDH. Values are means $\pm$ SEM, n is the number of neurons. RMP, resting membrane potential. Baseline *versus* EM2: there is no significant difference in all these rows, paired t -test

	Projection Neuron in Lamina I (n=15)	
	Baseline	EM2
RMP (mV)	-60.57 ± 2.09	-60.61 ± 2.70
Rheobase (pA)	17.0 ± 0.86	15.3 ± 1.64
$V_{\text{threshold}}$ (mV)	-39.67 ± 2.06	-39.38 ± 1.79
Latency (ms)	7.31 ± 1.09	9.13 ± 0.22
Peak Amplitude (mV)	66.07 ± 3.21	63.42 ± 3.81
Time of Peak (ms)	40.53 ± 1.24	41.65 ± 0.71
Area (mV·ms)	-272.97 ± 31.51	-235.38 ± 39.24
Rise Time (ms)	0.32 ± 0.04	0.32 ± 0.03
Rise Slope (mV/ms)	99.76 ± 9.85	92.49 ± 12.53
Decay time (ms)	0.62 ± 0.05	0.65 ± 0.09
Decay Slope (mV/ms)	-55.60 ± 5.38	-52.32 ± 6.75

The selective MOR antagonist CTOP blocks the inhibitory effects of EM2

EM2 is an endogenous ligand for MOR with high affinity and selectivity. We investigated the effects of the selective MOR antagonist CTOP on EM2-induced suppression of sEPSCs. Five of the above PNs, which were from 3 rats, were chosen for testing after the washout of EM2, in which EM2 clearly decreased the frequency of sEPSCs. Although 7 other neurons also decreased the frequency of sEPSCs, the inhibitory effects of EM2 were not totally disappeared due to the long-term effects of EM2. The activity of the PNs labeled with the tracer TMR may be another possible reason for this. In the presence of CTOP, the inhibitory effects of EM2 on the frequency of sEPSCs were completely blocked, and CTOP alone had no effects on the frequency of sEPSCs (baseline, 1.59 ± 0.10 Hz; CTOP, 1.60 ± 0.10 Hz; CTOP + EM2, 1.59 ± 0.09 Hz. $F = 0.21$, $P = 0.82$, $n = 5$, one-way repeated measures ANOVA). Meanwhile, the amplitude of sEPSCs was not changed by EM2 in the presence of CTOP (baseline, 23.8 ± 2.24 pA; CTOP, 23.2 ± 3.15 pA; CTOP + EM2, 24.6 ± 2.57 pA. $F = 0.34$, $P = 0.72$, $n = 5$, one-way repeated measures ANOVA) (Fig. 7). These results indicate that EM2 inhibit the presynaptic excitatory synaptic transmission of the PNs through binding to MOR.

Discussion

In the present study, the connections between EM2-ir terminals originating mainly from the primary afferent fibers and PNs projecting to the parabrachial nucleus in lamina I of the SDH and the effects of EM2 on PNs were examined. Terminals containing EM2-ir make synapses with neurons projecting to the parabrachial nucleus, among which most of them are asymmetric synapses. Electrophysiological investigations indicate that EM2 inhibited the excitatory transmission of PNs by presynaptically reducing the frequency of sEPSCs, but not by postsynaptically hyperpolarizing membrane potential. All these results have a facilitation

Fig. 6. The regulatory effects of EM2 on the spontaneous excitatory or inhibitory postsynaptic currents (sEPSC or sIPSC). a, b: Superimposed samples and cumulative fractions results showing that EM2 (3 μ M) bath application decreased the frequency, but not the amplitude, of sEPSCs recorded in TMR-labeled projection neurons in lamina I. The summarized results were plotted in (c), showing that EM2 application significantly reduced the frequency of sEPSCs ($n=12$, $P<0.001$, paired t-test). There was no significant difference in the amplitude between before and after EM2 application ($n=12$, $P=0.54$, paired t-test). d, e: Superimposed samples and cumulative fractions results showing that EM2 bath application did not decrease either the frequency or the amplitude of sIPSCs recorded in the projection neurons. The summarized results were plotted in (f), showing that there was no significant difference in the frequency and amplitude between before and after EM2 application ($n=9$, $P=0.84$ and 0.24 , paired t-test).

Fig. 7. Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-Nh2 (CTOP) blocked the inhibitory effects of EM2 on sEPSCs on the PNs of lamina I. A sample trace of sEPSCs in the baseline, absence and presence of EM2 (3 μ M) together with CTOP (1 μ M) was shown in (a). b: The cumulative distributions of the inter-event intervals (left) and the amplitude (right) of the sEPSCs in the presence of CTOP and EM2 together with CTOP. EM2 did not significantly shift the mean cumulative distribution of the sEPSC frequency or the amplitude ($P>0.05$; Kolmogorov-Smirnov test). These data were obtained from the same neurons in panel (a). c: Bar graph showing the analytical results of five continuous recordings during which different treatments were performed in five PNs. EM2 decreased the frequency of sEP-

SCs in these five PNs. After washing out EM2, the frequency returned to control levels. CTOP had no effects on either the amplitudes or frequency of sEPSCs. However, with perfusion of both CTOP and EM2, EM2 was unable to decrease the amplitudes or frequency of the sEPSCs ($n=5$, $P=0.72$ and 0.82 , one-way repeated measures ANOVA).

effect on our comprehensive and detailed understanding for the mechanisms how opioids produce analgesic effects at the spinal level.

EM2-ir terminals and MOR-ir profiles in the superficial SDH

Base on the the dense aggregation of EM2-ir fibers in the dorsal root and EM2-ir neuronal perikarya in the dorsal root ganglion (DRG) [5, 11], it was hypothesized that EM2 was synthesized in primary sensory neurons in the DRG and then transported to the superficial dorsal horn. Mechanical (deafferentation by dorsal rhizotomy) and chemical (exposure to the primary afferent neurotoxin capsaicin) methods of disrupting spinal primary sensory afferents were used to demonstrate that EM2-ir fibers in the SDH primarily originated from neurons in the DRG [7, 43, 44]. Moreover, some EM2-ir neurons in the bilateral nucleus tractus solitarii (NTS) project to the SDH [7, 43]. Thus, EM2-ir fibers and terminals in the SDH originate from the ipsilateral primary afferents and bilateral descending fibers from NTS.

EMs competed with MOR binding over 1000-fold more potently than either δ -opioid receptor (DOR) or κ -opioid receptor (KOR) [1]. MOR-ir products were found in many small neurons in lamina II and a few in the dorsal part of lamina III. Previous investigations have confirmed that few MOR-ir neuronal cell bodies were observed in lamina I, and no PNs contained MOR [21, 45-49]. In our present study, both the RMP and rheobase were unchanged after applying EM2 to the PNs. These results further demonstrated that there was no expression of MOR on the PNs in lamina I, which is similar to the previous report [50].

Synapses between EM2-ir terminals and PNs in the superficial SDH

Under electron microscope, the EM2-ir reaction products within the confines of large dense-cored vesicles were different from other neurotransmitters, such as glutamate and gamma aminobutyric acid (GABA), which stain the small, round or oval vesicles [6, 12, 22, 51]. Although EM2 is known to exert inhibitory effects in the SDH [23, 31, 50, 52], EM2-ir axon terminals mainly exhibited asymmetric synaptic connections with PNs, and thus it is likely that the synapses are excitatory. In the SDH, periaqueductal gray (PAG) and dorsal raphe nucleus, opioid peptide-ir axon terminals have been reported to mainly establish asymmetric synapses on neurons [6, 12, 22, 53, 54]. In this situation, EMs or opioid peptides produce the inhibitory effects by binding to the presynaptic MOR to modulate the release of presynaptic excitatory neurotransmitters [such as, glutamate, substance P (SP), and calcitonin gene related peptide (CGRP)] from the primary afferents [51, 55, 56]. Thus, EM2 is possible to play a role in regulation as a neuromodulator to limit the duration of excitatory neurotransmitter release.

The effects of EM2 are mediated by the MOR on the membrane. However, the MOR was distributed on the intrasynaptic membrane as well as on the extrasynaptic membrane. The proportion of PNs contacted by EM2-ir boutons among all PNs in lamina I was 51.32%. EM2 may also act other MOR-ir profiles in lamina I or lamina II by volume transmission [60]. The possibility of both synaptic and non-synaptic transmission by opioids has been raised in many reports showing that opioid receptors are present not only in proximity to synapses but also in parasynaptic and extrasynaptic regions [57-59]. The presence of EM2-ir dense-cored vesicles in a non-synaptic region of the neural processes, would be consistent with the concept that EM2 might be involved in modulating neuronal activity by both synaptic and non-synaptic transmissions [1].

Mechanism of spinal analgesia produced by EM2

EM2 binds to spinal MOR, of which 40-70% is located on the central terminals of primary afferent fibers [61-63]. It was observed that binding of opioids to MOR reduces nociceptive signal transmission of lamina I neurons at central A δ - and C-fiber synapses, by mainly inhibiting presynaptic glutamate release [50]. Our electrophysiological evidence that EM2 only decreased the frequency but not the amplitude of the sEPSC strongly indicates that EM2 inhibits the presynaptic glutamate release from the afferent terminals, instead of affecting the post-synaptic glutamate receptors. Since glutamate is the main neurotransmitters mediating the nociceptive information from DRG to spinal cord neurons, EM2 might cause

analgesic effect through this inhibitory effect. The presynaptic inhibition of the glutamate release is also reasonable for explaining that EM2 did not affect the excitability of the projection neurons. However, we cannot totally exclude the possibility that EM2 may also reduce other neurotransmitters' (especially SP) release, which might affect the excitability of the projection neurons. We need to check this possibility in our future works.

In conclusion, the present study provides morphological evidence for the synaptic connections between EM2-ir terminals and spinoparabrachial PNs in lamina I of the SDH. The functional investigation also showed that the inhibitory effects of EM2 on the excitatory synaptic transmission of PNs in lamina I of the SDH through presynaptic mechanisms. These evidences seem to suggest that EM2 might produce analgesic effects in the SDH by directly inhibiting the activities of the spinoparabrachial PNs.

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Disclosure Statement

The authors declare that they have no conflicts of interest with any of the work presented in this manuscript.

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