Activation of δ-Opioid Receptors Reduces Excitatory Input to Putative Gustatory Cells Within the Nucleus of the Solitary Tract

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Zhu M, Cho YK, Li C-S. Activation of δ-opioid receptors reduces excitatory input to putative gustatory cells within the nucleus of the solitary tract. J Neurophysiol 101: 258–268, 2009. First published November 19, 2008; doi:10.1152/jn.90648.2008. The rostral nucleus of the solitary tract (NST) is the first central relay in the gustatory pathway and plays a key role in processing and modulation of gustatory information. Here, we investigated the effects of opioid receptor agonists and antagonists on synaptic responses of the gustatory parabrachial nuclei (PbN)-projecting neurons in the rostral NST to electrical stimulation of the solitary tract (ST) using whole cell recordings in the hamster brain stem slices. ST-evoked excitatory postsynaptic currents (EPSCs) were significantly reduced by met-enkephalin (MetE) in a concentration-dependent fashion and this effect was eliminated by naltrexone hydrochloride, a nonselective opioid receptor antagonist. Bath application of naltrindole hydrochloride, a selective δ-opioid receptor antagonist, mimicked the effect of MetE-Arg6-Gly7-Leu8-immunoreactive cells (Murakami et al. 1996). This effect was eliminated by naltrexone hydrochloride, a nonselective opioid receptor antagonist. Bath application of naltrindole hydrochloride, a selective δ-opioid receptor antagonist, eliminated MetE-induced reduction of EPSCs, whereas CTOP, a selective μ-opioid receptor antagonist, had no effect, indicating that δ-opioid receptors are involved in the reduction of ST-evoked EPSCs induced by MetE. SNC80, a selective δ-opioid receptor agonist, mimicked the effect of MetE. The SNC80-induced reduction of ST-evoked EPSCs was eliminated by 7-benzylidenenaltrexone, a selective δ-opioid receptor antagonist but not by naltrexone mesylate, a selective δ-opioid receptor antagonist, indicating that δ-opioid receptors mediate the reduction of ST-evoked EPSCs induced by SNC80. Single-cell reverse transcriptase–polymerase chain reaction analysis revealed the presence of δ-opioid receptor mRNA in cells that responded to SNC80 with a reduction in ST-evoked EPSCs. Moreover, Western blot analysis demonstrated the presence of 40-kDa δ-opioid receptor proteins in the rostral NST tissue. These results suggest that postsynaptic δ-opioid receptors are involved in opioid-induced reduction of ST-evoked EPSCs of PbN-projecting rostral NST cells.

INTRODUCTION

The rostral portion of the nucleus of the solitary tract (NST) in the medulla is the first central taste relay that receives gustatory information from the tongue and oral cavity via the facial (VIIth) and glossopharyngeal (IXth) nerves (Contreras et al. 1982; Hamilton and Norgren 1984; Norgren and Leonard 1971; Whitehead and Frank 1983). Neurons in the rostral NST send axonal projections to the medial regions of the parabrachial nuclei (PbN) in the pons with an ipsilateral predominance (Halsell et al. 1996; Travers 1988; Whitehead 1990; Williams et al. 1996). In the hamster, 80% of the NST cells that respond to taste stimulation of the anterior tongue send axons to the gustatory PbN (Cho et al. 2002). NST neurons also receive descending projections from forebrain structures that are related to gustatory or ingestive behavior. In addition, cells in the NST reciprocally communicate with the contralateral and caudal NST, premotor nuclei, or reticular formation in the brain stem (Beckman and Whitehead 1991; Halsell et al. 1996; van der Kooy et al. 1984; Whitehead et al. 2000).

The presence of glutamate, substance P (SP), γ-aminobutyric acid (GABA), and opioids was identified (Davis 1993; Davis and Kream 1993; Kalia et al. 1985; Maley 1996; Maley and Panment 1988; Sweazey 1996) and their involvement in synaptic transmission was demonstrated in the rat and hamster rostral NST (Davis and Smith 1997; King et al. 1993; Li and Smith 1997; Liu et al. 1993; Smith and Li 1998; Wang and Bradley 1995; 1993). Opioids are peptides that are known to regulate food intake and modulate palatability of taste (Kelley et al. 2002; Levine et al. 1985; Morley et al. 1983; Parker et al. 1992; Rideout and Parker 1996). Recent studies have begun to elucidate a role for opioids in the modulation of taste responses and feeding behavior within the gustatory region of the NST. Met-enkephalin (MetE), a nonselective opioid receptor agonist, blocked taste responses of the cells in the NST when microinjected into the vicinity of the recorded cells (Li et al. 2003). Microinjection of naltrexone, a nonselective opioid receptor antagonist, into the rostral NST blocked feeding induced by neuropeptide Y (NPY) injection into the paraventricular nucleus (PVN) in the rat (Kotz et al. 1995, 2000). The involvement of opioids in the rostral NST in modulation of taste responses and feeding behavior was further supported by immunohistochemical studies that have shown the presence of MetE-Arg6-Gly7-Leu8-immunoreactive cells (Murakami et al. 1987) or opioid receptors in the rat rostral NST (Lynch et al. 1985; Mansour et al. 1994a; Nomura et al. 1996). In the hamster, MetE-immunoreactivity was detected in the terminals and neural somata in the rostral pole of the NST (Davis and Kream 1993) and μ-opioid receptors were identified in the incoming fiber terminals of the solitary tract (ST) and the neuropil within the rostral NST, whereas δ-opioid receptors were expressed on the neural somata of the rostral NST (Li et al. 2003).

Although the effects of opioid agonists and antagonists on synaptic transmission in the rat caudal NST are well documented (Appleyard et al. 2005; Glatzzer and Smith 2005; Rhim and Miller 1994; Rhim et al. 1993), whether opioids are involved in synaptic transmission within the rostral NST has not been examined yet in vitro. In the present study we used the
hamster brain stem slice preparation to investigate whether opioid receptors are involved in modulation of synaptic transmission between ST fiber terminals and rostral NST cells that project to the ipsilateral gustatory PbN. To identify rostral NST neurons that project to the ipsilateral gustatory PbN, fluorescent latex microspheres (FLMs) were microinjected into the gustatory PbN using electrophysiological guidance 1 wk prior to the slice preparation. We also tested whether opioid receptor proteins are present in rostral NST tissue using Western blot analysis. In a subset of NST neurons, the presence of δ-opioid receptor mRNA was confirmed using single-cell reverse transcriptase–polymerase chain reaction (RT-PCR) analysis from the cytoplasm harvested from single PbN-projecting rostral NST cells in which ST-evoked excitatory postsynaptic currents (EPSCs) were reduced by an opioid receptor agonist.

METHODS

Animals

All experimental procedures were performed in accordance with the guidelines on the use and care of laboratory animals set by the National Institutes of Health and approved by the Institutional Animal Care and Use Committees of the University of Tennessee Health Science Center and Southern Illinois University at Carbondale. Young Syrian golden hamsters (Mesocricetus auratus, 1–2 mo old) of either sex were used. Animals were housed in plastic cages at 23 ± 2°C and a humidity (relative humidity = 55 ± 15%) controlled room with a 12-h light/12-h dark cycle.

Materials

FLMs were obtained from Lumafluor (Naples, FL). TRIzol reagent and ultra agarose were purchased from Invitrogen Life Technologies (Carlsbad, CA). 7-Benzylidenedenatrexone (BNTX), D-Pen-Cys-Tyr-D-Trp-Om-Thr-Pen-Thr-NH2 (CTOP), naltrexone hydrochloride (NTX), (EPSCs) were reduced by an opioid receptor agonist.

NST cells in which ST-evoked excitatory postsynaptic currents (EPSCs) were reduced by an opioid receptor agonist.

Materials

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Retrograde labeling of rostral NST cells that project to the gustatory PbN

Microinjections of FLMs into the gustatory portion of the PbN were performed using electrophysiological guidance. Animals were anesthetized with pentobarbital sodium (50 mg/kg, administered intraperitoneally [ip]). Then, each hamster was mounted in a stereotaxic instrument (Narishige SR-6N) using a hand-made nontraumatic head holder (Erickson 1966). The snout was angled downward 27° from the horizontal to straighten the brain stem and minimize brain movement associated with breathing. Body temperature was maintained at 37 ± 1°C with an electric heating pad. A sagittal skin incision was made along the midline overlying the posterior skull and a 2- to 3-mm-diameter hole was made on one side of the occipital bone to access the PbN.

The gustatory PbN was located by physiological guidance with a combined recording/injecting glass pipette assembly, which consisted of a recording micropipette (tip diameter = 1–2 μm) glued to an injecting micropipette (tip diameter = 30 μm). The tip of the recording micropipette extended 1 mm beyond the tip of the injecting pipette. The injecting pipette was loaded with 0.2 μl of FLMs. The recording micropipette (resistance = 5–7 MΩ) was filled with 2% (wt/vol) solution of Chicago Sky Blue dye (Sigma, St. Louis, MO) in 0.5 M sodium acetate. The mean coordinates for the PbN recordings were 3.4 ± 0.09 (SD) mm anterior to the posterior edge of the occipital bone at midline and 1.3 ± 0.06 mm lateral to the midline. The recording/injecting micropipette assembly was initially positioned about 3.4 mm anterior to the posterior edge of the occipital bone and 1.3 mm lateral to the midline and lowered slowly to search for the taste-responsive neurons. The taste-responsive neurons were initially identified by a change in neural activity associated with the application of an electrical shock (≤40 μA, 500-ms duration) to the anterior tongue and their identity was confirmed by testing their response to chemical stimulation of the anterior tongue. Taste stimuli presented to the anterior tongue were 0.032 M sucrose, 0.032 M sodium chloride (NaCl), 0.032 M quinine hydrochloride (QHCl), and 0.0032 M citric acid. After a taste-responsive neuron was isolated in the PbN, the recording/injecting pipette assembly was further advanced 1 mm to place the tip of the injecting pipette to where the PbN neuron was recorded. At this position, a volume of 0.2 μl FLMs was pressure-injected into the PbN. The electrode assembly was kept in position for ≥15 min prior to its withdrawal from the brain stem. After FLMs injection, the surgical area was cleaned using physiological saline, and the muscle covering the skull and the overlying skin was sutured separately. The surgical area was cleaned again with physiological saline and 70% alcohol. Breathing and body temperature were monitored for 6 h, then the animal was returned to the animal facility and checked daily for signs of pain, distress, and infection for 1 wk. Animals were kept for 1–3 wk before being killed for brain stem slice preparations.

Brain stem slice preparation

After the hamster had fully recovered from FLM injection surgery, the animal was anesthetized by chloral hydrate (400 mg/kg, ip) and decapitated. The brain stem was then rapidly removed and trimmed in ice-cold oxygenated (95% O2:5% CO2) artificial cerebrospinal fluid (aCSF) of the following composition (in mM): 124 NaCl, 3 KCl, 1.3 CaCl2, 1.3 MgSO4, 10 glucose, 5 BES, and 25 NaHCO3 (pH 7.27, 300 mOsm). The brain stem was then mounted on a specimen-mounting block, immersed into 4°C oxygenated sucrose-aCSF in the specimen tray, and sectioned into 350-μm-thick coronal slices from 1.6–2.9 mm anterior to the obex using a Vibratome 3000 sectioning system (Vibratome, St. Louis, MO). The sucrose-aCSF had the following composition (in mM): 230 sucrose, 26 NaHCO3, 2.5 KCl, 10 glucose, 0.5 CaCl2, 10 MgCl2, and 0.25 Na2HPO4. The brain stem slices were transferred to a holding chamber and incubated for ≥1 h in 30°C oxygenated normal aCSF and then kept in a holding bath at room temperature (25°C) until used for recording. For recordings, a single slice was transferred to a recording chamber (RC-26GLP, Warner Instruments, Hamden, CT) on a fixed-stage upright epifluorescent microscope (BX51WI, Olympus Optical, Tokyo, Japan), and continuously perfused at a rate of 1.5 ml/min with oxygenated normal aCSF. In the recording chamber, the slices were maintained at 30 ± 0.2°C using an Automatic Temperature Controller (TC-324B, Warner Instruments).

Electrophysiological recording

Conventional voltage-clamp recordings were performed using the whole cell configuration. FLMs containing rostral NST neurons,
which were retrogradely labeled from the gustatory PbN, were visualized with near-infrared differential interference contrast optics and a ×40 water-immersion objective (Olympus optical, Tokyo). Recordings were made from these PbN-projecting neurons in the rostral NST for ≤6 h under these conditions. Recording pipettes were pulled (P-97 Flaming/Brown micropipette puller; Sutter Instrument, Novato, CA) from standard-wall borosilicate glass with filament (OD = 1.5 mm; Sutter Instrument). The patch electrodes had a resistance of 6–9 MΩ and were filled with a solution of the following composition (in mM): 125 K-glucuronate, 2 MgCl₂, 1 NaCl, 10 glucose, 10 HEPES-K, 0.2 EGTA, 2 K₂-ATP, and 0.2 GTP (pH 7.3, 290 mOsm). A color CCD video camera (HV-D30, Hitachi Kosuka Electric, Akita, Japan) was used to capture the image of the recorded neuron and displayed on a color monitor (TM-H1750C, JVC. Yokohama, Japan). The image of the recorded cell was digitized through a digital converter (PX-AV100U, Plextor America, Fremont, CA), captured using Presto Video Works (NewSoft America, Fremont, CA), and stored in the computer.

Recordings were obtained using a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA). Analog signals were low-pass Bessel filtered at 2 kHz, digitized at 5 kHz through a Digidata 1322A interface (Molecular Devices), and stored in a computer using Clampex 9.2 software (Molecular Devices). The membrane potential was held at −70 mV. EPSCs of FLMs-labeled rostral NST cells were induced following ST stimulation. Electrical stimulation (PG4000A digital stimulator, Cygnus Technology, Delaware Water Gap, PA) was delivered to the ST through an isolated current source (SIU91, Cygnus Technology). The electrode was constructed from a pair of parallel stainless steel (SS) wires (50 μm, A-M Systems, Everett, WA), which was insulated except for the tips. Rectangular pulses (100 μs, 40–200 μA) were delivered to the ST at 0.2 Hz. The stimulating electrode was positioned to lie radial to the NST-recording sites, within the ST. Drugs were applied to the slices by switching the normal aCSF to an aCSF containing various drugs at specific concentrations. Data analysis was performed using Clampfit 9.2 software (Molecular Devices). The criteria for successful recording included the electrical resistance of the seal between the outside surface of the recording pipette and the criterion for successful recording included the electrical resistance of 9–10 MΩ, and all reported voltage-clamp measurement were not corrected for this potential.

**Extraction of total RNA and RT-PCR**

In all, 10 hamsters were used for RNA extraction. Brain stem slices containing rostral NST were sectioned as described earlier from animals without FLM injection. The rostral NST was obtained by punching the brain stem slices using a 15 gauge SS tubing (ID = 1.5 mm). The tissue was then homogenized in ice-cold TRIZol reagent (100 mg fresh tissue/1 ml) using a glass homogenizer. After homogenization, the tissue was incubated at room temperature for 10 min, lysed in TRIZol reagent, and RNA was extracted as described previously (Zhu et al. 1998, 2000).

Cytoplasmic harvesting of the recorded neurons and reverse transcription of δ₁-opioid receptors were performed. For these recordings, the glass patch pipettes were washed once in ethanol and three times in distilled water and were then autoclaved for 30 min. After drying, these pipettes were kept in a sealed box under vacuum until used for recordings. At the end of the voltage-clamp recordings, the intracellular content of the cell was carefully aspirated into the recording pipette by applying negative pressure to the recording pipette while maintaining the tight seal. The content of the pipette was then expelled into the RT-PCR tube by breaking off the tip of the recording pipette and adjusted to a volume of 8 μl. RT-PCR was performed using a reverse transcription system and PCR master mix (Promega). The RT was performed at 25°C for 10 min followed by 42°C for 30 min, 95°C for 5 min, and then incubated at 4°C for 10 min. The first PCR was performed at 95°C for 5 min, followed by 35 cycles at 95°C for 45 s, 63°C for 60 s, and 72°C for 60 s. The final extension of the first PCR was achieved at 72°C for 10 min, then 4°C for 30 min. The second PCR was performed at 95°C for 5 min, followed by 35 cycles at 95°C for 45 s, 63°C for 60 s, and 72°C for 60 s. The final extension of the second PCR was achieved at 72°C for 10 min and then incubated at 4°C overnight in a Mastercycler (Eppendorf, Germany). The PCR products were electrophoresed on a 2% agarose/TBE gel containing (in mM): 90 Tris-HCl, 90 boric acid, and 2 Na₂EDTA, and visualized with ethidium bromide (EB). In all situations, exclusion of either RNA or AMV reverse transcriptase resulted in no visible band following gel electrophoresis.

**Preparation of total protein**

In all, 40 hamsters were used in protein preparation. The brain stem slices containing the rostral NST or rostral NST tissue punched out from the brain stem slices was homogenized in normal aCSF, containing 1 mM EDTA. Samples were centrifuged at 800 × g for 20 min at 4°C. Then, the supernatant was centrifuged at 8,000 × g for 20 min at 4°C, and the supernatant was transferred to fresh centrifuge tubes. The supernatant was centrifuged again at 18,000 × g for 30 min at 4°C. The pellets of the membrane fraction were collected and dissolved in 0.9% sodium chloride solution. Then, they were stored at −20°C and used within 1 mo. The concentration of collected proteins was measured by Advanced Protein Assay (Fluka) using an image station (Kodak, IS2000R) at wavelength of 595 nm at room temperature.

**Western blot analysis**

Detection of δ-opioid receptor proteins was performed by Western blot analysis using a rabbit polyclonal antibody to the δ-opioid receptor. Two separate analyses were performed: one analysis used the tissue derived from brain stem slices that contained rostral NST and the other used the tissue derived specifically from rostral NST obtained by punching brain stem slices with 1.5 mm SS tubing. All procedures were described in detail in previous studies (Zhu et al. 1998, 2000).

**Statistical analysis**

Two-sample comparisons were performed using a Student’s t-test, whereas multiple comparisons were made using a one-way ANOVA followed by the Student–Newman–Keuls method. Numerical data are expressed as means ± SE.

**RESULTS**

**Retrograde labeling of the rostral NST neurons from the gustatory PbN**

The FLM-labeled cells in the rostral NST were visualized using an epifluorescent microscope. The fluorescent beads made PbN-projecting NST neurons easily identifiable for recordings (Fig. 1). Following microinjection of FLMs into the gustatory PbN, retrogradely labeled cell bodies were found throughout the rostral NST, including the rostral central and
measured at a holding potential of normal aCSF. The jitters ranged between 10 and 97 membrane current noise level. The latencies varied between the time at which the differentiated signal trace exceeded the measured as the time between the onset of ST stimulation and mean jitter of 59.3

\[ \text{mean jitter} = 59.3 \pm 16.7 \] 

\[ \text{to} \quad 97 \] 

\[ \text{as possible from the recording area.} \] 

\[ \text{(Hamilton and Norgren 1984; Whitehead and Frank 1983).} \]

from the tongue and oral cavity is carried by this structure gustatory PbN. We tested whether these neurons could be NST neurons that were retrogradely labeled by FLMs from the NST neurons peduncle (not shown).

\[ \text{neuron identified as a PbN-projecting neuron (arrow) because it was retrogradely labeled with fluorescent latex microspheres (FLMs) following microinjection of FLMs into the gustatory PbN.} \]

\[ \text{high-power photograph of a coronal section through the rostral central subdivision of the NST showing retrograde labeling of the NST cells following microinjection of FLMs into the gustatory PbN.} \]

The photograph was taken at the end of the recording of the cell. Scale bar = 10 \( \mu \text{m} \) in A and B and 20 \( \mu \text{m} \) in C.

rostral lateral subdivisions, which is the area of the NST receiving its predominant gustatory input from the VIIth nerve (Whitehead 1988; Whitehead and Frank 1983). Histological examination of the FLM injection sites revealed that the injections were centered in the medial PbN, at the level of the middle of the mesencephalic trigeminal nucleus (Me5), where the locus coeruleus (LC) is most evident in its anteroposterior extent. The FLMs did not spread over the surrounding structures such as the LC, the Me5, or the superior cerebellar peduncle (not shown).

\[ \text{FIG. 1. Identification of parabrachial nuclei (PbN)–projecting rostral nucleus of the solitary tract (NST) neurons.} \]

\[ \text{A: photomicrograph of a rostral NST neuron identified as a PbN-projecting neuron (arrow) because it was retrogradely labeled with fluorescent latex microspheres (FLMs) following microinjection of FLMs into the gustatory PbN.} \]

\[ \text{B: high-power photograph of a coronal section through the rostral central subdivision of the NST showing retrograde labeling of the NST cells following microinjection of FLMs into the gustatory PbN.} \]

\[ \text{The photograph was taken at the end of the recording of the cell. Scale bar = 10} \quad \mu \text{m} \]

\[ \text{in A and B and 20} \quad \mu \text{m in C.} \]

\[ \text{ST-stimulation–evoked EPSCs in PbN-projecting rostral NST neurons} \]

Whole cell voltage-clamp recordings were made from rostral NST neurons that were retrogradely labeled by FLMs from the gustatory PbN. We tested whether these neurons could be activated by stimulation of the ST, since gustatory information from the tongue and oral cavity is carried by this structure (Hamilton and Norgren 1984; Whitehead and Frank 1983). Care was taken to avoid direct stimulation of the recorded cells by positioning the stimulating electrode on the visible ST as far as possible from the recording area.

The peak amplitude of the ST-evoked EPSCs ranged from -16.7 to -39.1 pA, with a mean of -31.7 ± 6.3 pA (n = 48) measured at a holding potential of -70 mV. Latencies were measured as the time between the onset of ST stimulation and the time at which the differentiated signal trace exceeded the membrane current noise level. The latencies varied between 1.6 and 6.1 ms with a mean latency of 3.12 ± 0.16 ms in normal aCSF. The jitters ranged between 10 and 97 μs with a mean jitter of 59.3 ± 5.1 μs. The relatively low variation in the latencies indicates that FLM-labeled cells receive monosynaptic inputs from ST fiber terminals (Doyle and Andre-}
The effect of MetE on ST-evoked EPSCs was mediated by δ- or μ-opioid receptors. Application of 50 μM NTD, a selective nonpeptide δ-opioid receptor antagonist, almost eliminated the reduction of ST-evoked EPSCs produced by 10 μM MetE (P < 0.001, MetE vs. MetE + NTD groups, n = 7). There was no difference between control and MetE + NTD groups (P = 0.97). EPSCs returned to control level after washing the slices using normal media.

In addition, ST-evoked EPSCs were significantly reduced by bath application of 25 μM SNC80, a highly selective δ-opioid receptor agonist (22.0 ± 1.4%, P < 0.001, n = 29; Fig. 5).
application of 25 \( \mu \)M SNC80 that was not affected by the presence of 10 \( \mu \)M NTB, was completely eliminated by subsequent bath application of 1 \( \mu \)M BNTX \( (P < 0.001, n = 11; \text{Fig. 6}, B \) and C). These results demonstrate that the SNC80-induced reduction of ST-evoked EPSCs was \( \delta_1 \)- but not \( \delta_2 \)-opioid receptor mediated.

Expression of \( \delta \)-opioid receptor proteins in the rostral NST

Our electrophysiological experiments demonstrated that \( \delta \)-opioid receptors mediate SNC80-induced reduction of ST-evoked EPSCs. To scrutinize the hypothesis that \( \delta \)-opioid receptor proteins are present in the rostral NST tissue, Western blot analyses were performed. The tissue used for Western blot analyses derived from brain stem slices that contained rostral NST or rostral NST tissue that was obtained by punching the brain stem slices with 1.5-mm-diameter SS tubing. Consistent with our electrophysiological results, the expression of \( \delta \)-opioid receptor proteins was confirmed with Western blotting. Representative Western immunoblot data are shown in Fig. 7A. Immunoblotting with rabbit polyclonal \( \delta \)-opioid receptor antibodies demonstrated that the corresponding \( \delta \)-opioid receptor protein migrated as a nearly 40-kDa protein in both the brain stem slice tissue that contained the rostral NST (Fig. 7A, I) and in the tissue obtained from the rostral portion of the hamster NST (Fig. 7A, 2).

Detection of \( \delta \)-opioid receptor mRNA in Pbn-projecting single rostral NST cells in which ST-evoked EPSCs were reduced by SNC80

Results of the present electrophysiological and Western blot analyses indicate that \( \delta \)-opioid receptors are involved in the reduction of ST-evoked EPSCs. Furthermore, the paired-pulse stimulation experiment suggests that MetE modulates EPSCs magnitude of the reduction of the ST-evoked EPSCs by SNC80 was similar to that produced by MetE \( (22.0 \pm 1.4 \) vs. \( 24.2 \pm 3.5\% ) \). Also, the effect of 25 \( \mu \)M SNC80 was completely eliminated in the presence of 50 \( \mu \)M NTD \( (P < 0.001, n = 9, \text{Fig. 5}) \). These results further suggest that the MetE-induced reduction of ST-evoked EPSCs was \( \delta \)-opioid receptor mediated.

Since there are two \( \delta \)-opioid receptor subtypes (\( \delta_1 \) and \( \delta_2 \)), we examined which subtype mediates the reduction of ST-evoked EPSCs. We tested the effect of BNTX, a highly selective \( \delta_1 \)-opioid receptor antagonist, on the reduction of ST-evoked EPSCs induced by SNC80. Bath application of 1 \( \mu \)M BNTX prevented the reduction of ST-evoked EPSCs \( (P = 0.001, n = 8; \text{Fig. 6A}) \). We also assessed the influence of NTB, a highly selective \( \delta_2 \)-opioid receptor antagonist, on the reduction of ST-evoked EPSCs induced by SNC80. Contrary to the effect of BNTX, 10 \( \mu \)M NTB had a minimal effect on SNC80-induced reduction of ST-evoked EPSCs \( (P = 0.001, n = 8; \text{Fig. 6C}) \). Furthermore, \( \delta \)-opioid receptor-mediated reduction of ST-evoked EPSCs, induced by bath...
reduction of the ST-evoked EPSCs produced by SNC80, whereas 1
were significantly reduced by application of 25
ST-evoked EPSCs
SNC80. However, subsequent administration of 1
bath after the SNC80 effect was stabilized did not influence the effect of

$^{(P/H11021}$

$^{(n/SNC80 vs. control, n=9)}$

The reduction of the ST-evoked EPSCs induced by the
presence of 25 $\mu$M SNC80 was completely eliminated by bath application of 1 $\mu$M 7-benzylideneanthrexone (BNTX) ($P<0.01$, SNC80 vs. SNC80 + BNTX, $n=9$). B: representative time course of the effect of the administration of 25 $\mu$M SNC80 on ST-evoked EPSCs. Bath application of 25 $\mu$M SNC80 reduced ST-evoked EPSCs. Adding 10 $\mu$M naltriben mesylate (NTB) to the bath after the SNC80 effect was stabilized did not influence the effect of SNC80. However, subsequent administration of 1 $\mu$M BNTX completely eliminated the effect of SNC80 on ST-evoked EPSCs. C: bar graph showing the group data corresponding to the time course in Fig. 5B. ST-evoked EPSCs were significantly reduced by application of 25 $\mu$M SNC80 ($P<0.001$, SNC80 vs. control, $n=20$). Supplementary administration of 10 $\mu$M NTD ($P<0.001$, SNC80 + NTD vs. control groups, $n=11$) did not affect the reduction of the ST-evoked EPSCs produced by SNC80, whereas 1 $\mu$M BNTX ($P<0.006$, SNC80 vs. SNC80 + NTD + BNTX groups, $n=9$) almost completely eliminated the effect of SNC80 on ST-evoked EPSCs.

FIG. 6. Effects of antagonization of specific $\delta$-opioid receptor subtypes on SNC80-induced reduction of ST-evoked EPSCs of PbN-projecting rostral NST neurons. A: bar graph showing the ST-evoked EPSCs were significantly reduced by application of 25 $\mu$M SNC80 (24.3 $\pm$ 1.7%, $P<0.001$, SNC80 vs. control, $n=9$). The reduction of the ST-evoked EPSCs induced by the presence of 25 $\mu$M SNC80 was completely eliminated by bath application of 1 $\mu$M 7-benzylideneanthrexone (BNTX) ($P<0.01$, SNC80 vs. SNC80 + BNTX, $n=9$), B: representative time course of the effect of the administration of 25 $\mu$M SNC80 on ST-evoked EPSCs. Bath application of 25 $\mu$M SNC80 reduced ST-evoked EPSCs. Adding 10 $\mu$M naltriben mesylate (NTB) to the bath after the SNC80 effect was stabilized did not influence the effect of SNC80. However, subsequent administration of 1 $\mu$M BNTX completely eliminated the effect of SNC80 on ST-evoked EPSCs. C: bar graph showing the group data corresponding to the time course in Fig. 5B. ST-evoked EPSCs were significantly reduced by application of 25 $\mu$M SNC80 ($P<0.001$, SNC80 vs. control, $n=20$). Supplementary administration of 10 $\mu$M NTD ($P<0.001$, SNC80 + NTD vs. control groups, $n=11$) did not affect the reduction of the ST-evoked EPSCs produced by SNC80, whereas 1 $\mu$M BNTX ($P<0.006$, SNC80 vs. SNC80 + NTD + BNTX groups, $n=9$) almost completely eliminated the effect of SNC80 on ST-evoked EPSCs.

FIG. 7. Detection of $\delta$-opioid receptor protein from the rostral NST tissue and $\delta$-receptor protein mRNA from single PbN-projecting rostral NST cells. A: representative Western blot data showing the presence of $\delta$-opioid receptor protein in tissue derived from brain stem slices containing the rostral NST (1) and in rostral NST tissue (2). The migration of a 40-kDa receptor protein was detected using rabbit polyclonal antibody (1:1,000) to $\delta$-opioid receptor. Experiments were repeated at least three times. B: current traces from a PbN-projecting rostral NST neuron showing that 25 $\mu$M SNC80 reduced the ST-evoked EPSC and that the SNC80 effect was blocked in the presence of 50 $\mu$M NTD (bottom left traces). C: after the recording, the neuron was prepared for single-cell RT-PCR. Ethidyum bromide–stained agarose gel showing the RT-PCR DNA product that corresponds to $\delta$-opioid receptor mRNA. The left lane is a 100-bp DNA ladder. Lane 1 shows $\delta$-opioid receptor mRNA from brain stem slices that contained the rostral NST (131 bp, 35 cycles), and lane 2 shows $\delta$-opioid receptor mRNA from the cell shown in Fig. 7B (131 bp, 70 cycles). Experiments were repeated at least three times.

by a postsynaptic mechanism. To further demonstrate the involvment of postsynaptic $\delta$-opioid receptors in the reduction of ST-evoked EPSCs, we performed RT-PCR analysis to check for the presence of $\delta$-opioid receptor mRNA in rostral NST tissue and in the cytoplasm harvested from single rostral NST cells in which ST-evoked EPSCs were reduced by SNC80 and the reduction was eliminated by NTD (Fig. 7B). Using oligonucleotide primers for the mouse $\delta_1$-opioid receptor gene we detected $\delta_1$-opioid receptor mRNA in tissue harvested from the rostral NST (Fig. 7C, 1) as well as in a single PbN-projecting rostral NST cell in which the ST-evoked EPSCs were reduced by SNC80 and the SNC80 effect was eliminated by NTD (Fig. 7C, 2). The molecular weight bands (Fig. 7C, ladder) shown corresponded to 131 bp as predicted. Therefore Western immunoblots and single-cell RT-PCR data were consistent with the results of the whole cell recording experiments that showed a role of $\delta$-opioid receptors in the reduction of ST-evoked EPSCs induced by SNC80 in PbN-projecting rostral NST neurons.

DISCUSSION

In the present study, we have provided evidence that postsynaptic $\delta_1$-opioid receptors are involved in the opiate-induced reduction of ST-evoked EPSCs in gustatory PbN-projecting rostral NST cells. Such evidence was obtained by means of whole cell recordings in brain stem slices, Western blot analysis using rostral NST tissue, and single-cell RT-PCR analysis using the cells that responded to SNC80.

Retrogradely labeled neurons from the ipsilateral gustatory PbN in the rostral NST

The VIIth and IXth cranial nerves that carry taste informa-
tion terminate topographically within the rostral NST via the
ST in a rostral-to-caudal sequence (Hamilton and Norgren
1984; Whitehead 1986; Whitehead and Frank 1983). Second-
order gustatory neurons in the rostral NST

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Involvement of opioid receptors in the reduction of ST-evoked EPSCs in cells that project to the gustatory PbN

In the present study, we showed that bath application of MetE, a μ- and δ-opioid receptor agonist significantly reduced ST-evoked EPSCs of the PbN-projecting rostral NST cells. Furthermore, the MetE effect on EPSCs was completely eliminated by NTX, a broad-spectrum opioid receptor antagonist. These results indicate that opioid receptors mediate inhibition of synaptic transmission between the ST fiber terminals and rostral NST cells that project to the gustatory PbN. In addition, the jitters of evoked EPSCs varied by <100 μs following ST stimulation, indicating monosynaptic connections of ST terminals with the recorded cells. NST cells that respond with <100 μs jitter in response to ST stimulation are believed to be monosynaptically connected to ST terminals (Andresen et al. 2001; Doyle and Andresen 2001). It has been reported in the rat that brain regions that particularly express opioids and opioid receptors include nuclei associated with taste and/or reward system, such as the nucleus of accumbens, bed nucleus of the stria terminalis, central nucleus of the amygdala (CeA), lateral hypothalamus (LH), PbN, and NST (Fallon and Leslie 1986; Mansour et al. 1987, 1994a; McLean et al. 1987; Simantov et al. 1977; Van Ree et al. 2000). In the rat rostral NST, opioids and opioid receptors were detected (Lynch et al. 1985; Mansour et al. 1994b; Murakami et al. 1987). In the hamster, MetE-like immunoreactivity has been shown in both the neuronal somata and fiber terminals of the rostral NST (Davis and Kream 1993). The distribution of μ- and δ-opioid receptors was distinct from each other within the rostral NST of the hamster; μ-opioid receptor immunostaining was restricted to the neuropil of the NST and to peripheral afferent fibers that enter the NST from the ST, whereas numerous δ-opioid receptor immunoreactive cell bodies were observed throughout the rostrocaudal extent of the NST (Li et al. 2003). These observations support our results that opioid receptors mediate inhibition of synaptic transmission between the ST fiber terminals and putative gustatory cells within the NST.

Although MetE has stronger affinity for δ-opioid receptors than for μ-receptors, it is necessary to clarify whether the reduction of the ST-evoked EPSCs induced by MetE was mediated by δ- or μ-opioid receptors (Raynor et al. 1994). The elimination of MetE-induced reduction of ST-evoked EPSCs by NTD but not by CTP, suggests that δ-opioid receptors mediate the MetE effect. Furthermore, the results that SNC80 but not DAMGO (data not shown) reduce ST-evoked EPSCs also support the involvement of δ-opioid receptors in the mediation of opioid-peptide–induced reduction of ST-evoked EPSCs.

Detection of δ-opioid receptor proteins in rostral NST and δ-opioid receptor mRNA in single cells that respond to opioids

Our electrophysiological experiments demonstrated that δ-opioid receptors mediate the reduction of ST-evoked EPSCs induced by SNC80. Western blot analysis using rabbit polyclonal anti-δ-opioid receptor antibodies revealed the presence of δ-opioid receptor proteins in the rostral NST tissue. This further supports our electrophysiological results, indicating that δ-opioid receptors mediate SNC80-induced reduction of ST-evoked EPSCs in putative gustatory cells within the NST. The examination of the paired-pulse depression ratio during MetE application showed that the EPSCs evoked by both the conditioning and test pulses were reduced to the same extent. Since the ratio of the second EPSC to the first one remained unchanged, this indicates that the MetE-induced modulation of ST-evoked EPSCs occurred by a postsynaptic mechanism. This result is consistent with our previous immunohistochemical study. The characteristics of the distribution of the δ- and μ-opioid receptor immunostaining in the hamster rostral NST (Li et al. 2003) showed that MetE and SNC80 might act on postsynaptic δ-opioid receptors. To further provide direct evidence for the presence of postsynaptic δ-opioid receptors, single-cell RT-PCR was performed using the cytosolic contents harvested from single cells whose SNC80-induced reduction of ST-evoked EPSCs was eliminated by NTD. We detected RT-PCR DNA products that correspond to the δ1-receptor mRNA on EB-stained gels from both rostral NST
tissue and from the cytosol of single cells that have shown SNC80 response. The presence of δ-receptor mRNA inside SNC80-responding cells provides direct evidence that SNC80 acted, at least in part, on postsynaptic δ-opioid receptors and led the reduction of ST-evoked EPSCs. Additional experiments are warranted to elucidate whether activation of postsynaptic δ-opioid receptors reduce ST-evoked EPSCs via modulation of the kinetic properties of iGluR receptors or via a decrease in input resistance, which may lead to a shunting of EPSCs.

Physiological implications

Endogenous opioids are involved in many physiological modulations. The roles of endogenous opioids or agonists of opioid receptors include regulation of alcohol and/or food intake (Bodnar 2004; Morley et al. 1983; Yeomans and Gray 2002). Opioids are found in various brain regions, including the regions involved in feeding behavior (Harrison and Grandy 2000; LaMotte et al. 1978; Mansour et al. 1987; Van Ree et al. 2000). Administration of opioid receptor agonists or antagonists into these brain structures modulates ingestive behavior: the type of such modulation and the effective sites of opioid injection are often diverse, depending on which opioid subtype is activated (for review see Bodnar 2004). For example, microinjection of opioid agonists into the LH (Stanley et al. 1988), CeA (Giraudo et al. 1998a,b), ventral tegmental area (Bakshi and Kelley 1993; Giraudo et al. 1998a), or lateral PbN (Nicklous and Simansky 2003; Wilson et al. 2003) induced feeding behavior, and the opioid receptor antagonist NTX blocked opioid-induced increase of food intake in the rat. Taken together with the diverse distribution of endogenous opioids and opioid receptors within the neural feeding circuitry, it was suggested that opioids regulate feeding behavior in multiple feeding systems by a different mechanism (Glass et al. 1999).

Opioid-mediated modulation of feeding is likely to occur, in part, through a hedonic shift in the palatability of taste of food, since opiates influence the palatability of both hedonic and aversive taste (Appleyard et al. 2005). For example, morphine increases, whereas NTX reduces, sweetness preference in rodents (Calcagnetti and Reid 1983; Czirr and Reid 1986; Rideout and Parker 1996; Siviy and Reid 1983). Further, morphine reduced the aversive hedonic properties of QHCl solution and NTX diminished the positive hedonic properties of sucrose solution in the rat (Parker et al. 1992).

The rostral NST is a possible brain region in which opioid-mediated gustatory and metabolic integration takes place to regulate ingestive behavior (Glass et al. 1999). The NST is an important taste-relay station with reciprocal neural connections to various forebrain structures involved in feeding regulations including the LH, CeA, and PVN (van der Kooy et al. 1984; Whitehead et al. 2000). Opioids were shown to increase food intake when microinjected into the rostral NST in the rat and this increase was blocked by NTX injection into the CeA and vice versa, suggesting that the opioid–opioid pathway between the rostral NST and the CeA modulates feeding behavior (Giraudo et al. 1998b). Examination of the rostral NST showed that some forebrain gustatory nuclei, such as CeA, send efferent projections to the NST and make synaptic contact with cells that express opioid receptors (Pickel and Colago 1999). Kotz et al. (1995) reported that administration of NTX into the rostral NST resulted in complete blockade of increased feeding induced by NPY injection into the PVN. These findings suggest that the NST may play a critical role in the control of food intake triggered by opioid mechanisms in various forebrain areas.

The results of the current study are in agreement with our previous findings. Previously, we reported in an in vivo recording experiment that MetE modulates taste responses of a subset of neurons in the hamster NST and there was no indication that a cell type with a particular chemosensitive profile was susceptible (Li et al. 2003). Along with the present study, this suggests that the rostral NST is indeed a feasible brain area in which opioids interact with taste processing and that such opioid-mediated modulation may occur, at least in part, through a postsynaptic δ-opioid receptor mechanism.

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