Mechanism of cardiovascular effects of nociceptin microinjected into the nucleus tractus solitarius of the rat

Vineet C. Chitravanshi and Hreday N. Sapru
Department of Neurological Surgery, University of Medicine and Dentistry of New Jersey-New Jersey Medical School, Newark, New Jersey

Submitted 8 November 2004; accepted in final form 13 January 2005

Chitravanshi, Vineet C., and Hreday N. Sapru. Mechanism of cardiovascular effects of nociceptin microinjected into the nucleus tractus solitarius of the rat. Am J Physiol Regul Integr Comp Physiol 288: R1553–R1562, 2005. First published January 20, 2005; doi:10.1152/ajpregu.00762.2004.—Microinjections (100 nl) of 0.15, 0.31, 0.62, and 1.25 mmol/l of nociceptin into the medial nucleus tractus solitarius (mNTS) elicited decreases in mean arterial pressure (11 ± 1.8, 20 ± 2.1, 21.5 ± 3.1, and 15.5 ± 1.9 mmHg, respectively) and heart rate (14 ± 2.7, 29 ± 5.5, 39 ± 5.2, and 17.5 ± 3.1 beats/min, respectively). Because maximal responses were elicited by microinjections of 0.62 mmol/l nociceptin, this concentration was used for other experiments. Repeated microinjections of nociceptin (0.62 mmol/l) into the mNTS, at 20-min intervals, did not elicit tachyphylaxis. Bradycardia induced by microinjections of nociceptin into the mNTS was abolished by bilateral vagotomy. The decreases in mean arterial pressure and heart rate elicited by nociceptin into the mNTS were blocked by prior microinjections of the specific ORL1-receptor antagonist [N-Phe1]-nociceptin-(1–13)-NH2 (9 mmol/l). Microinjections of the ORL1-receptor antagonist alone did not elicit a response. Prior combined microinjections of GABAa and GABAb receptor antagonists (2 mmol/l gabazine and 100 mmol/l 2-hydroxyaclofen, respectively) into the mNTS blocked the responses to microinjections of nociceptin at the same site. Prior microinjections of ionotropic glutamate receptor antagonists (2 mmol/l NBQX and 5 mmol/l d-AP7) also blocked responses to nociceptin microinjections into the mNTS. These results were confirmed by direct neuronal recordings. It was concluded that 1) nociceptin inhibits GABAergic neurons in the mNTS, 2) GABAergic neurons may normally inhibit the release of glutamate from the terminals of peripheral afferents in the mNTS, and 3) inhibition of GABAergic neurons by nociceptin results in an increase in the release of glutamate in the mNTS, which in turn elicits depressor and bradycardic responses via activation of ionotropic glutamate receptors on secondary mNTS neurons.

bradycardia: depressor responses; opioid peptides

THE PRESENCE OF A NOVEL G-protein-coupled receptor [opioid receptor-like receptor (ORL1) or OP2 receptor] throughout the central nervous system has been repeatedly demonstrated (1, 3, 6, 12, 18, 20–22). There is a high sequence similarity of this receptor with other opioid receptors (e.g., μ, δ, and κ-receptors) (3, 6, 21, 36). Nociceptin (orphanin FQ) (4, 25, 26), a heptadecapeptide, has been demonstrated to be an endogenous ligand of ORL1 receptor because of its high and selective affinity for this receptor and a very poor affinity for μ-, δ-, and κ-opioid receptors. One notable difference between other opioid receptor agonists (e.g., endomorphins, enkephalins, and dynorphin A) and nociceptin is that naloxone blocks the effects of these opioid receptor agonists but not those of nociceptin (4, 5, 13, 16, 29).

The presence of an endogenous ligand for ORL1 receptors (nociceptin or orphanin FQ) has been demonstrated throughout the central nervous system, including the nucleus tractus solitarius (NTS) (10, 23, 35). There are very few reports in which the cardiovascular effects of ORL1 receptor activation by nociceptin in the medial nucleus tractus solitarius (mNTS) have been studied (19). Preliminary results of the present study were presented at a symposium held in Orlando, Florida; the proceedings of this symposium have been published (28). The present investigation was undertaken to carry out a systematic study on the cardiovascular effects of microinjections of nociceptin into the mNTS.

MATERIALS AND METHODS

General procedures. Experiments were done in 103 adult male decerebrate Wistar rats (Charles River Laboratories) weighing 300–350 g. All animals were housed under controlled conditions with a 12:12-h light-dark cycle. Food and water were available to the animals ad libitum. The experimental procedures were performed in accordance with Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (publication no. 80-23, revised 1996). All protocols were approved by the Institutional Animal Care and Use Committee at the University of Medicine and Dentistry of New Jersey, New Jersey Medical School (Newark, NJ).

General procedures used in this study, including the decerebration, microinjection, extracellular neuronal recording, and histological procedures, have been described in our previous publications (9, 16, 34). The trachea, one of the femoral veins, and arteries were cannulated; the animals were artificially ventilated; and blood pressure (BP), heart rate (HR), and rectal temperature were monitored by standard techniques.

Statistical analyses. The means and SE were calculated for maximum changes in mean arterial pressure (MAP), HR, or neuronal firing, in response to microinjections or microapplications of nociceptin or L-glutamate. Comparisons of changes in MAP, HR, or neuronal firing elicited by different concentrations of the agonists were made by using a one-way ANOVA combined with the Tukey-Kramer test for post hoc analyses of intergroup comparisons. Comparisons of the maximum changes in MAP, HR, or neuronal firing elicited by nociceptin before and after the administration of ORL1-receptor antagonists were made by using paired t-test. In all cases, the differences were considered significant at P < 0.05.

Drugs and chemicals. The following drugs and chemicals were used: [N-Phe1]-nociceptin-(1–13)-NH2 (ORL1-receptor antagonist) (5); nociceptin (ORL1-receptor agonist); endomorphin-2 (μ-opioid receptor agonist) (16); d-(-)-2-amino-7-phosphono-heptanoic acid [d-AP7; N-methyl-d-aspartate (NMDA)-receptor antagonist] (9, 34);

http://www.ajpregu.org
0363-6119/05 $8.00 Copyright © 2005 the American Physiological Society
R1553
l-glutamate monosodium; 2,3-dioxo-6-nitro-1,2,3,4-tetrahydro-benzof[1]quinoxaline-7-sulfonamide disodium (NBQX disodium salt; non-NMDA-receptor antagonist) (9, 34); gabazine bromide (GABA<sub>G</sub>-receptor antagonist) (16); 2-hydroxysaclofen (GABA<sub>B</sub>-receptor antagonist); decamethonium; and isoflurane. All of the solutions for the microinjections were freshly prepared in artificial cerebrospinal fluid (aCSF; pH 7.4) (16). The control solutions for microinjections (100 nl) consisted of aCSF (pH 7.4). Where applicable, the concentration of drugs injected into the mNTS refers to their salts. All drugs were obtained from Sigma (St. Louis, MO) except the ORL1-receptor antagonist; decamethonium; and isoflurane. All of the solutions for the microinjections of l-glutamate and other drugs was 100 nl. Only the sites from which l-glutamate elicited depressor and bradycardic responses were chosen for further studies. Microinjections of aCSF at these sites did not elicit any response. The interval between the microinjections of L-glutamate and other agents was at least 5 min. Nociceptin in different concentrations (0.15, 0.31, 0.62, and 1.25 mmol/l) was microinjected into the mNTS. These concentrations of nociceptin elicited decreases in MAP (11 ± 1.8, 20 ± 2.1, 21.5 ± 3.1, and 15.5 ± 1.9 mmHg, respectively; Fig. 1A) and HR (14 ± 2.7, 29 ± 5.5, 39 ± 5.2, and 17.5 ± 3.1 beats/min, respectively; Fig. 1B) (n = 20). Responses to the concentration of 1.25 mmol/l were smaller than those obtained at 0.62 mmol/l. Similar concentration-response curves have been reported for microinjections of other peptides into the NTS (7, 16). The onset of these responses ranged between 7.6 ± 1.3 and 12.9 ± 1.2 s. The duration of these responses ranged between 4.7 ± 0.7 and 8.6 ± 0.7 min. Maximal responses were elicited by a 0.62 mmol/l concentration. Therefore, this concentration was selected for other studies in this paper. The concentration (0.62 mmol/l) of nociceptin that elicited maximal depressor and bradycardic responses in the mNTS elicited no responses when injected intravenously.

To exclude tachyphylaxis of nociceptin-induced responses, a maximally effective concentration of nociceptin was microinjected into the mNTS at least three times, at 20-min intervals. In this group of rats (n = 5), the decreases in MAP in response to three consecutive microinjections of nociceptin (0.62 mmol/l) were 18.6 ± 3.1, 16.3 ± 2.2, and 19 ± 3.9 mmHg, respectively, and decreases in HR were 32.5 ± 3.1, 36.7 ± 4.2, and 30 ± 9.1 beats/min, respectively. These results indicated that no tachyphylaxis of responses occurred with repeated microinjections of nociceptin.

RESULTS

Concentration response. Resting MAP and HR results in the decerebrate rats were 103.1 ± 4.6 mmHg and 451.3 ± 4.8 beats/min, respectively. In each rat, the mNTS on either side was selected at random for microinjections. Four- or five-barreled micropipettes were used for microinjections; one barrel contained aCSF, the second barrel contained l-glutamate, and the remaining barrels contained two or three concentrations of nociceptin selected at random. The mNTS site was identified by microinjections of 5 mmol/l l-glutamate; depressor (51.3 ± 3.6 mmHg) and bradycardic (88.8 ± 13.2 beats/min) responses were elicited. The microinjection volume for l-glutamate and other drugs was 100 nl. Only the sites from which l-glutamate elicited depressor and bradycardic responses were chosen for further studies. Microinjections of aCSF at these sites did not elicit any response. The interval between the microinjections of l-glutamate and other agents was at least 5 min. Nociceptin in different concentrations (0.15, 0.31, 0.62, and 1.25 mmol/l) was microinjected into the mNTS. These concentrations of nociceptin elicited decreases in MAP (11 ± 1.8, 20 ± 2.1, 21.5 ± 3.1, and 15.5 ± 1.9 mmHg, respectively; Fig. 1A) and HR (14 ± 2.7, 29 ± 5.5, 39 ± 5.2, and 17.5 ± 3.1 beats/min, respectively; Fig. 1B) (n = 20). Responses to the concentration of 1.25 mmol/l were smaller than those obtained at 0.62 mmol/l. Similar concentration-response curves have been reported for microinjections of other peptides into the NTS (7, 16). The onset of these responses ranged between 7.6 ± 1.3 and 12.9 ± 1.2 s. The duration of these responses ranged between 4.7 ± 0.7 and 8.6 ± 0.7 min. Maximal responses were elicited by a 0.62 mmol/l concentration. Therefore, this concentration was selected for other studies in this paper. The concentration (0.62 mmol/l) of nociceptin that elicited maximal depressor and bradycardic responses in the mNTS elicited no responses when injected intravenously.

To exclude tachyphylaxis of nociceptin-induced responses, a maximally effective concentration of nociceptin was microinjected into the mNTS at least three times, at 20-min intervals. In this group of rats (n = 5), the decreases in MAP in response to three consecutive microinjections of nociceptin (0.62 mmol/l) were 18.6 ± 3.1, 16.3 ± 2.2, and 19 ± 3.9 mmHg, respectively, and decreases in HR were 32.5 ± 3.1, 36.7 ± 4.2, and 30 ± 9.1 beats/min, respectively. These results indicated that no tachyphylaxis of responses occurred with repeated microinjections of nociceptin.

In another group of rats (n = 5), the effect of microinjections (100 nl) of nociceptin (0.62 mmol/l) into more rostral areas of the NTS (1–1.5 mm rostral to the calamus scriptorius, 1.2–1.5 mm lateral to the midline; and 0.5–0.7 mm ventral to the dorsal medullary surface) elicited smaller depressor and bradycardic responses or no response at all. Moreover, microinjections of the same concentrations of nociceptin into areas adjacent to the NTS, including the cuneate nucleus (0.5 mm rostral to the calamus scriptorius, 1.2 mm lateral to the midline, and 0.5 mm deep from the dorsal medullary surface), elicited no cardiovascular responses, indicating that spread of nociceptin to adjacent regions outside NTS was not responsible for its depressor and bradycardic responses. In these rats, microinjections of nociceptin into the mNTS elicited usual decreases in MAP (20 ± 3.8 mmHg) and HR (31 ± 8.9 beats/min).

Blockade of nociceptin-induced responses. In another group of rats (n = 8), the resting values for MAP and HR were 113.2 ± 4.4 mmHg and 408.9 ± 5.6 beats/min, respectively. Microinjections of l-glutamate (5 mmol/l) into the mNTS elicited decreases in MAP (75 ± 2.7 mmHg) and HR (160 ± 32.4 beats/min) (Fig. 2A; typical tracing). After an interval of 5 min, microinjection of nociceptin (0.62 mmol/l) at the same site elicited decreases in MAP (30.6 ± 4.6 mmHg) and HR (31 ± 8.6 beats/min).

Blockade of nociceptin-induced responses. In another group of rats (n = 8), the resting values for MAP and HR were 113.2 ± 4.4 mmHg and 408.9 ± 5.6 beats/min, respectively. Microinjections of l-glutamate (5 mmol/l) into the mNTS elicited decreases in MAP (75 ± 2.7 mmHg) and HR (160 ± 32.4 beats/min) (Fig. 2A; typical tracing). After an interval of 5 min, microinjection of nociceptin (0.62 mmol/l) at the same site elicited decreases in MAP (30.6 ± 4.6 mmHg) and HR (31 ± 8.6 beats/min) (Fig. 2A). Twenty minutes after recovery of the responses, another opioid peptide, endomorphin-2 (0.2 mmol/l), was microinjected at the same site; depressor (28.1 ± 4.9 mmHg) and bradycardic (37 ± 2 beats/min) responses were elicited (Fig. 2C). Selection of this dose of endomorphin-2 was based on our earlier report (16) in which it was found to be the maximally effective concentration. Twenty minutes after the microinjection of endomorphin-2, the ORL1-receptor antagonist [N-Phe<sup>1</sup>]-nociceptin-(1–13)-NH<sub>2</sub> (9 mmol/l) was microinjected into the same site. No significant...
changes were seen in MAP and HR (Fig. 2D); the MAP values before and after the microinjection of the ORL1-receptor antagonist were 97.5 ± 4.2 and 96.3 ± 4.1 mmHg, respectively (P > 0.05), and the HR values before and after the microinjection of the ORL1-receptor antagonist were 436.9 ± 5.9 and 435.6 ± 6.2 beats/min (P > 0.05), respectively. Two minutes after the microinjection of the ORL1-receptor antagonist, nociceptin was again microinjected into the same site. The responses to microinjection of nociceptin were completely blocked by prior microinjection of the ORL1-receptor antagonist (Fig. 2E); the blocking effect of the ORL1-receptor antagonist lasted for 60–90 min. The lack of responses to nociceptin was not due to tachyphylaxis because, as mentioned earlier, repeated microinjections of nociceptin did not elicit tachyphylaxis if the interval between injections was at least 20 min. The specificity of the ORL1-receptor antagonist was indicated by the observation that this antagonist did not alter the depressor and bradycardic responses to microinjections of endomorphin-2 (Fig. 2F). The decreases in MAP to microinjections of 0.2 mmol/l endomorphin-2 before and after the microinjection of the ORL1-receptor antagonist were 28.1 ± 4.9 and 21.9 ± 3.8 mmHg, respectively (P > 0.05), and the decreases in HR before and after the microinjection of the antagonist were 37 ± 2 and 30 ± 5.4 beats/min, respectively (P > 0.05).

Nociceptin-induced bradycardic responses: role of vagus. The decreases in MAP and HR before and after ipsilateral vagotomy were 25.7 ± 7.6 and 19 ± 1.9 mmHg and 40 ± 4.5 and 22 ± 3.7 beats/min, respectively (n = 5). Thus ipsilateral vagotomy did not exert a statistically significant effect (P > 0.05) on nociceptin-induced depressor and bradycardic responses. After ipsilateral vagotomy, a stabilization period of 15 min was allowed, and then the second vagus was sectioned. Bilateral vagotomy increased MAP from a resting value of 107.1 ± 2.9 to 119.6 ± 3.3 mmHg and HR from a resting value of 435.8 ± 9.8 to 470.8 ± 8.8 beats/min. The increase in MAP after bilateral vagotomy may be explained by the increase in HR. The decrease in MAP after bilateral vagotomy was 15 ± 2.2 mmHg; thus bilateral vagotomy did not significantly (P > 0.05) alter the depressor responses to nociceptin. The decrease in HR was almost completely abolished by bilateral vagotomy (Fig. 3).

Effect of GABA-receptor blockade on nociceptin-induced responses. A tracing of the effect of GABA-receptor blockade on nociceptin responses is shown in Fig. 4. The mNTS was identified by microinjections of L-glutamate (Fig. 4A). Within 5 min, nociceptin (0.62 mmol/l) was microinjected at the same site; the usual decreases in MAP and HR were elicited (Fig. 4B). The group data for the nociceptin-induced decreases in MAP and HR were 23.3 ± 4.0 mmHg and 33.3 ± 10.2 beats/min (n = 6). After an interval of 20 min, gabazine (2 mmol/l) and 2-hydroxysaclofen (100 mmol/l) were microinjected, sequentially within an interval of 2 min, into the same mNTS site; decreases in MAP (group data, 13.3 ± 2.5 mmHg) lasting for 2–3 min and HR (group data, 39.2 ± 3 beats/min) lasting for more than 60 min were observed (Fig. 4C). Five minutes after the recovery of MAP, 0.62 mmol/l nociceptin was again microinjected at the same site. Nociceptin-induced decreases in MAP (group data, 5 ± 4.7 mmHg) and HR (group data, 3.3 ± 3.1 beats/min) were significantly blocked (P < 0.05) after the microinjections of the GABA-receptor blockers (Fig. 4D). However, the responses to L-glutamate (5 mmol/l), microinjected within 7 min of the microinjections of the GABA receptor blockers, were not attenuated (Fig. 4E). L-Glutamate-induced decreases in MAP before and after the microinjection of GABA-receptor blockers (group data, 37.5 ± 4.6 and 29.1 ± 3 mmHg, respectively) were not statistically different (P > 0.05). However, as mentioned earlier, microinjections of GABA receptor blockers into the mNTS exerted a long-lasting fall in baseline HR. Therefore, L-glutamate-induced decreases in HR before (group data 55.8 ± 8.2 beats/min) and after (group data, 31.7 ± 4 beats/min) the microinjection of GABA-receptor blockers were significantly different (P < 0.05) (Fig. 4E).
Effects of nociceptin on neuronal activity. The results obtained in the microinjection studies were confirmed by direct neuronal recordings. In all neuronal recording experiments, different agents were applied directly to the neuron using a picospritzer, and the volume applied was always 5 nl. Each agent was applied to the neuron only when its firing returned to basal levels after the previous manipulation or drug application. The concentrations of different agents were as follows: 5 mmol/l L-glutamate, 0.62 mmol/l nociceptin, 9 mmol/l [N-Phe1]-nociceptin-(1–13)-NH2 (ORL1-receptor antagonist), 5 mmol/l D-AP7 (NMDA-receptor antagonist), 2 mmol/l NBQX and 5 mmol/l D-AP7 do not alter the responses to carbachol (16).

Effects of nociceptin on neuronal activity. The effects of combined microinjections of these antagonists into the mNTS at the ionotropic glutamate receptors has also been demonstrated. For example, combined microinjections of 2 mmol/l NBQX and 5 mmol/l D-AP7 significantly attenuated the responses to carbachol (16).
NBQX (non-NMDA-receptor antagonist), and 0.5 mmol/l carbachol. An intravenous bolus injection of phenylephrine (3 μg/kg) was used to test whether the mNTS neuron was involved in baroreceptor function.

In one group of rats (n = 10), basal firing rate of the neurons (36 neurons) was 10.7 ± 1.3 spikes/s. An intravenous bolus injection of phenylephrine increased MAP (34.6 ± 3 mmHg) as well as the neuronal firing [31.3 ± 3.4 spikes/s (P < 0.05)], and this excitation lasted for 2.6 ± 0.4 s. Direct application of L-glutamate increased the neuronal firing to 32.6 ± 3 spikes/s (P < 0.05), and this excitation lasted for 0.6 ± 0.2 s. Application of aCSF did not elicit any responses, indicating that pressure applications alone were not responsible for the changes in neuronal firing. Direct application of nociceptin also increased the firing of these neurons to 26.2 ± 2.7 spikes/s (P < 0.05), and this excitation lasted for 0.3 ± 0.05 s. Application of the ORL1-receptor antagonist did not alter the neuronal firing compared with the basal firing rate; the firing rates of these neurons before and after the application of ORL1-receptor antagonist were 12.1 ± 1.9 and 11.8 ± 1.8 spikes/s, respectively (P > 0.5). Within 2–3 s, nociceptin was again applied onto these neurons; the ORL1-receptor antagonist blocked the excitatory effect of nociceptin. Nociceptin-induced increases in the neuronal firing rates, before and after the application of the ORL1-receptor antagonist, were 26.2 ± 2.7 and 0.2 ± 0.3 spikes/s, respectively (P < 0.05). However, the ORL1-receptor antagonist did not alter the responses to direct application of L-glutamate; L-glutamate-induced increases in firing rate of the neurons before and after the application of the ORL1-receptor antagonist remained unchanged (32.6 ± 3 spikes/s). After an interval of 40–50 s, application of nociceptin (0.62 mmol/l, 5 nl) onto these neurons again elicited increase in the firing rate (12 ± 3.2 spikes/s) after the ORL1 receptor blockade. Typical tracings of these neuronal recording experiments are shown in Fig. 6.

In another group of rats (n = 10), basal rate of firing of mNTS neurons (35 neurons) was 14.9 ± 1.6 spikes/s. An intravenous bolus injection of phenylephrine increased MAP (34.1 ± 4.5 mmHg) and firing rate of these neurons (34.4 ± 3.5 spikes/s) (P < 0.05), and this excitation lasted for 1.5 ± 0.2 s. Direct application of L-glutamate increased the neuronal firing to 46.5 ± 4.2 spikes/s (P < 0.05), and this excitation lasted for 0.8 ± 0.2 s. Nociceptin increased the firing of the neurons to 27.7 ± 2.3 spikes/s (P < 0.05). NBQX and D-AP7 were sequentially applied (within an interval of 2–3 s) onto these neurons; the basal firing rate of these neurons was reduced by these applications from 14.9 ± 1.6 to 4.8 ± 1 spikes/s. After the firing was reduced (10–15 s after the application of glutamate-receptor antagonists), nociceptin was again applied to the neurons. Combined applications of NBQX and D-AP7 blocked the excitatory effect of nociceptin; the increases in the firing rate of these neurons induced by nociceptin, before and after the application of NBQX and D-AP7, were 27.7 ± 2.3 and 0.7 ± 0.4 spikes/s, respectively (P < 0.01). NBQX and D-AP7 did not significantly attenuate the responses to L-glutamate. The firing rates induced by L-glutamate onto these neurons before and after the application of NBQX and D-AP7 were 46.5 ± 4.2 and 36.1 ± 4.3 spikes/s, respectively (P > 0.05). After an interval of 40–50 s, application of nociceptin onto these neurons again elicited an increase in the firing (8.3 ± 1 spikes/s) after the ionotropic glutamate receptor blockade. Typical tracings of these experiments are shown in Fig. 7.

The specificities of NBQX and D-AP7 as ionotropic glutamate-receptor blockers were tested in another group of rats (n = 5). The basal firing of these neurons (25 neurons) was 20.8 ± 3.3 spikes/s. Intravenous bolus of phenylephrine increased MAP (40 ± 10 mmHg) and the neuronal firing rate to a maximum of 39 ± 3.5 spikes/s within 2 s (P < 0.05). Direct application of carbachol increased the neuronal firing to a maximum of 44.3 ± 4.5 spikes/s within 0.2 s; the neuronal firing returned to the basal level within 0.7 ± 0.2 s. Two to three seconds later, application of aCSF did not alter the basal firing rate of these neurons. Combined application of NBQX and D-AP7, 2–3 s after carbachol, decreased the basal firing rate to a maximum of 5.4 ± 0.7 spikes/s within 10–15 s and returned to basal levels within 40–50 s. Application of carbachol, 2–3 s after the firing was reduced due to applications of NBQX and D-AP7, continued to elicit excitation of the neuron; carbachol-induced increases in the firing rate before and after the application of glutamate receptor antagonists were 44.3 ± 4.5 and 44 ± 4.7 spikes/s, respectively (P > 0.05). Thus NBQX and D-AP7 did not alter the effects of carbachol on mNTS neurons. Typical tracings of this experiment are shown in Fig. 8.

**Histology.** The mNTS sites, where nociceptin elicited excitatory effects by direct neuronal applications or microinjections, were marked with India ink in 36 rats. A typical microinjection site, marked with 30 nl of India ink, is shown in Fig. 8.
In neuronal recording studies, the sites were marked with a smaller volume of India ink (5 nl). Composite diagrams of the sites marked after microinjections or neuronal recordings are shown in Fig. 9, B and C. In these diagrams, each spot represents either a site of microinjection or a neuronal recording. In Fig. 9, B and C, only 27 spots are visible because other spots overlapped. The sites marked with ink were located in mNTS, 0.5–0.6 mm rostral to the calamus scriptorius, 0.5–0.6 mm lateral to the midline, and 0.5–0.6 mm deep from the dorsal medullary surface.

DISCUSSION

The main finding of this study was that microinjections of nociceptin into the mNTS of the rat elicited depressor and bradycardic responses that were mediated via ionotropic glutamate receptors. Local distortion of brain tissue or any other nonspecific effects were not responsible for these responses because microinjections of aCSF into the mNTS did not elicit any response. Concentrations of nociceptin microinjections into the mNTS that elicited depressor and bradycardic responses did not elicit a response when injected intravenously,

---

**Fig. 6. Effects of nociceptin on mNTS neuron.**

**A:** Basal firing rate (15 spikes/s) of one mNTS neuron. **B:** Bolus injection of 3 μg/kg iv phenylephrine (PE) increased MAP (40 mmHg) and neuronal firing (95 spikes/s), which lasted for 2–3 s. **C:** When neuronal firing recovered to basal levels, direct application of L-glutamate (5 mmol/l, 5 nl) increased the neuronal firing (70 spikes/s). **D:** 2–3 s after the recovery of firing, application of artificial cerebrospinal fluid (aCSF) did not alter basal rate of neuronal firing. **E:** Subsequent application of nociceptin (0.62 mmol/l, 5 nl) increased the firing rate (60 spikes/s). **F:** 2–3 s after the recovery of firing, application of aCSF did not alter basal rate of neuronal firing. **G:** After the neuronal firing was reduced, direct application of nociceptin (0.62 mmol/l, 5 nl) failed to excite the neuron (firing rate was 5 spikes/s compared with 60 spikes/s in E). **H:** Excitatory response (18 spikes/s) to direct application of nociceptin (0.62 mmol/l, 5 nl) recovered (72%) within 1 min. Firing rates mentioned in B, C, E, and H reflect the peak increase. In each panel (except B), arrows indicate ejection of different agents on the mNTS neurons. In B, arrow indicates intravenous injection of PE.

**Fig. 7. Effect of nociceptin on mNTS neuron is abolished by prior blockade of ionotropic glutamate receptors.**

**A:** Basal firing rate (5 spikes/s) of one mNTS neuron. **B:** Bolus injection of 3 μg/kg iv PE increased MAP (40 mmHg) and neuronal firing (36 spikes/s), which lasted for 2–3 s. **C:** When the neuronal firing recovered to basal level, direct application of L-glutamate (5 mmol/l, 5 nl) increased the neuronal firing (18 spikes/s). **D:** 2–3 s after the recovery of firing, application of aCSF did not alter basal rate of neuronal firing. **E:** 2–3 s later, application of nociceptin (0.62 mmol/l, 5 nl) increased the firing of the neuro (18 spikes/s). **F:** 2–3 s after the recovery of firing, application of NBQX (2 mmol/l, 5 nl) and D-AP7 (5 mmol/l, 5 nl) (interval between the 2 injections was 2–3 s) decreased the neuronal firing within 10–15 s (2 spikes/s; compare with A). **G:** After the neuronal firing was reduced, direct application of nociceptin (0.62 mmol/l, 5 nl) failed to excite the neuron (firing rate was 5 spikes/s compared with 18 spikes/s in E). **H:** Excitatory response (13 spikes/s) to direct application of nociceptin (0.62 mmol/l, 5 nl) recovered (72%) within 1 min. Firing rates mentioned in B, C, E, and H reflect the peak increase. In each panel (except B), arrows indicate ejection of different agents on the mNTS neurons. In B, arrow indicates intravenous injection of PE.
indicating that leakage, if any, of nociceptin from the microinjection site into the peripheral circulation was not responsible for the observed responses. The sites at which nociceptin elicited depressor and bradycardic responses were restricted to mNTS.

The depressor and bradycardic responses to microinjections of nociceptin into the mNTS were mediated via ORL1 opioid receptors because a specific antagonist for ORL1 opioid receptors (5) abolished these responses. Microinjections of the ORL1-receptor antagonist by itself did not elicit any response, suggesting that ORL1 opioid receptors in the mNTS are not endogenously active in the rat. Endogenous ORL1 opioid receptors may get activated in a yet unidentified situation and exert modulatory influences on cardiovascular regulation. In the concentrations used, the ORL1-receptor antagonist did not alter basal rate of neuronal firing (34 spikes/s). Firing rates mentioned in B, C, and F reflect the peak increase. In each panel (except B), arrows indicate ejection of different agents on the mNTS neurons. In B, arrow indicates intravenous injection of PE.

As mentioned earlier, the depressor and bradycardic responses elicited by microinjections of nociceptin were abolished after the blockade of ionotropic glutamate receptors in the mNTS. We have reported earlier similar observations with another opioid-receptor agonist, endomorphin-2 (16). The specificity of the ionotropic glutamate-receptor antagonists (NBQX and D-AP7) was indicated by the observation that these antagonists did not alter the responses to microinjections of another unrelated agonist, carbachol, into the mNTS (16). Unilateral blockade of ionotropic glutamate receptors by unilateral microinjections of kynurenic acid into the NTS did not elicit a significant increase in baseline BP (11). Perhaps compensatory mechanisms in the contralateral NTS prevent the baseline BP from rising. Consistent with this explanation are the reports that bilateral blockade of glutamate receptors in the NTS are necessary to increase the baseline BP (24). Furthermore, blockade of ionotropic as well as metabotropic glutamate receptors in the NTS may be necessary to elevate baseline BP (11). We have previously shown that the concentrations of D-AP7 and NBQX used in this study are sufficient to block the responses to ionotropic glutamate-receptor agonists NMDA and AMPA, respectively (9, 34). However, in our study, blockade of ionotropic glutamate receptors alone was not sufficient to block the effects of exogenously injected glutamate. This observation is in agreement with other earlier reports. For example, Pawloski-Dahm and Gordon (24) and Talman et al. (33) reported that concentrations of kynurenic acid (that blocked NMDA and kainate receptors) did not attenuate the responses to exogenously microinjected L-glutamate into the mNTS, whereas the effects of endogenously released glutamate (e.g., by aortic nerve stimulation) were abolished. It has been reported that ionotropic glutamate receptors may be located within the glutamatergic synapse, whereas the metabotropic glutamate receptors may be located in the perisynaptic region (16, 30). Therefore, it has been hypothesized that this anatomic arrangement of glutamate receptors makes ionotropic glutamate receptors readily accessible to glutamate released endogenously in the synapse and blockade of these receptors is more likely to abolish the effects of endogenously released glutamate (16). On the other hand,
exogenously microinjected glutamate may reach metabotropic receptors in the perisynaptic region as well as ionotropic receptors within the synapse. Therefore, blockade of ionotropic glutamate receptors alone is not sufficient to abolish the effects of exogenous glutamate; blockade of both ionotropic and metabotropic receptors may be necessary to abolish the response to exogenously injected glutamate into the NTS (11).

The blockade of GABA_A and GABA_B receptors in the mNTS, by gabazine and 2-hydroxysaclofen, respectively, also abolished the depressor and bradycardic responses to microinjections of nociceptin into the mNTS. In the experiments in which ionotropic glutamate-receptor and GABA-receptor antagonists blocked nociceptin-induced responses, tachyphylaxis was not responsible for the lack of responses to nociceptin because the interval between the two microinjections of nociceptin was >20 min. The concentrations of gabazine and 2-hydroxysaclofen used in this study have been shown to block specifically GABA_A and GABA_B receptors, respectively (16).

Neuronal recording experiments confirmed our results using the microinjection technique. In these experiments, the involvement of the neuron under investigation in cardiovascular function was confirmed by excitation of the neuron when systemic blood pressure was temporarily increased to stimulate baroreceptors by an intravenous bolus injection of phenylephrine. Excitation of the neuron by direct application of L-glutamate indicated that the activity was recorded from a neuron rather than from a fiber of passage. Excitation of neurons by nociceptin was not due to any nonspecific effects of pressure applications because application of aCSF did not elicit any changes in the neuronal firing.

Excitatory effects of nociceptin on the mNTS neurons were prevented by prior application of the ORL1-receptor antagonist, indicating that the responses were indeed mediated via ORL1 opioid receptors. Application of the ORL1-receptor antagonist by itself did not elicit any response, suggesting that ORL1 opioid receptors on the mNTS neurons involved in cardiovascular regulation are not endogenously active in the rat.

Nociceptin-induced excitation of the neurons was completely abolished by prior applications of ionotropic glutamate-receptor antagonists (NBQX and D-AP7), indicating that ionotropic glutamate receptors mediated the actions of nociceptin. The firing rate of the neurons in response to direct application of another unrelated agonist, carbachol, was not altered by the application of NBQX and D-AP7, indicating that these ionotropic glutamate-receptor antagonists did not exert any nonspecific effects. Direct application of NBQX and D-AP7 to single mNTS neurons elicited a significant reduction in the firing of these neurons within 10–15 s. Reduction of neuronal firing after the application of NBQX and D-AP7 was expected...
considering that glutamate released from the baroreceptor terminals in the mNTS, in response to increases in systemic BP, excites the mNTS neurons. This observation does not necessarily contradict our observation that unilateral microinjections of NBQX and d-AP7 into the mNTS did not elevate baseline BP because a decrease in firing in one neuron is not sufficient to elicit an increase in baseline BP. In this context, it should be noted that a number of neurons have to be inhibited simultaneously and compensatory effects of contralateral mNTS have to be eliminated (by making lesions or inhibiting neurons in the other mNTS) to elicit an increase in baseline BP.

As noted earlier, nociceptin-induced responses to either microinjections into the mNTS or direct applications to mNTS neurons were abolished by the blockade of ionotropic glutamate or GABA receptors. Consistent with inhibitory effects of nociceptin on neurons (2), it is speculated that GABAergic neurons in the mNTS are hyperpolarized by this opioid peptide, causing “disinhibition” that results in the release of glutamate from nerve terminals in the mNTS and excitation of secondary neurons involved in cardiovascular regulation. Immunohistochemical studies have demonstrated the presence of GABAergic neurons and terminals (17, 32) in the NTS. Detailed studies on the presence of opioid receptors on GABAergic neurons in the mNTS are not available. Therefore, it is speculated that ORL1 receptors may be relatively more numerous on the GABAergic neurons. Normally, the intrinsic GABAergic neurons in the mNTS may inhibit the release of glutamate from their terminals. Thus inhibition of GABAergic neurons by nociceptin-induced hyperpolarization via ORL1 receptors may result in an increase in the neuronal release of glutamate. This may be the reason why nociceptin-induced depressor and bradycardic responses were no longer observed when GABA receptors in the mNTS were previously blocked. Furthermore, blockade of ionotropic receptors by d-AP7 and NBQX also resulted in the abolition of nociceptin-induced depressor and bradycardic responses. Collectively, these observations prompted our hypothesis that nociceptin may elicit disinhibition by exerting an inhibitory effect on adjacent GABAergic neurons and thus enhance the release of glutamate in the mNTS. Other investigators have also reported that excitatory effects of opioids on neurons may be caused by inhibition of GABAergic interneurons (14, 37).

The mechanism of nociceptin-induced bradycardia can also be explained by the disinhibition caused by microinjections of nociceptin into the mNTS. Secondary NTS neurons may be excited via ionotropic glutamate receptors following this disinhibition, and an excitatory projection from the NTS to the nucleus ambiguus may be activated. An increase in the activity of nucleus ambiguous neurons results in an increase in parasympathetic activity to the heart, and bradycardia is elicited (31). Accordingly, bilateral vagotomy abolished the nociceptin-induced bradycardia.

In summary, the results of this investigation show that microinjections of nociceptin into the mNTS elicit depressor and bradycardic responses. These responses were mediated via ionotropic glutamate receptors. Similar observations have been reported by us in response to microinjections of another opioid-receptor agonist, endomorphin-2 (16). This conclusion was based on the observation that the depressor and bradycardic effects of microinjections of nociceptin into the mNTS were completely blocked when the ionotropic glutamate receptors were specifically blocked. The responses to nociceptin were also abolished by GABA-receptor antagonists. It was concluded that nociceptin may inhibit GABAergic neurons in the mNTS. GABAergic neurons may normally inhibit the release of glutamate from the terminals of peripheral afferents in the mNTS. Inhibition of GABAergic neurons may, therefore, result in an increase in the release of glutamate in the mNTS, which, in turn, may elicit depressor and bradycardic responses via activation of NMDA and non-NMDA receptors on the secondary mNTS neurons.

Perspectives

It is well established that in the rat the mNTS is the site where peripheral baroreceptor and cardiopulmonary afferents make their primary synapse (27, 33, 34). The presence of opioid and GABA receptors in the mNTS is also well documented (13, 32). It is hypothesized that opioid peptides may be released in the mNTS during stressful situations, causing a decrease in BP and HR and thus preventing their excessive increases in response to stress. In addition, the release of opioid peptides may modulate cardiovascular reflexes during stressful situations. Indeed Kapusta et al. (15) have demonstrated that, in conscious spontaneously hypertensive rats, acute environmental stimulus of air jet stress produced pressor and tachycardic responses. The pressor responses were prevented whereas tachycardic responses were attenuated by intracerebroventricular injections of nociceptin. Further investigations to test these hypotheses remain to be carried out.

GRANTS

This work was supported by National Heart, Lung, and Blood Institute Grants HL-24347 and HL-076248 awarded to H. N. Sapru.

REFERENCES

11. Foley CM, Mofitt JA, Hay M, and Hassler EM. Glutamate in the nucleus of the solitary tract activates both ionotropic and metabotropic

AJP-Regul Integr Comp Physiol • VOL 288 • JUNE 2005 • www.ajpregu.org