Oxytocin Pathways Mediate the Cardiovascular and Behavioral Responses to Substance P in the Rat Brain

Tanja Maier, Wen-Jie Dai, Tamás Csikós, Gustav F. Jirikowski, Thomas Unger, Juraj Culman

Abstract—Stimulation of brain periventricular and hypothalamic substance P receptors induces a pressor response and tachycardia associated with mesenteric and renal vasoconstriction and hindlimb vasodilation resembling thus the classical defense reaction. This cardiovascular response is brought about by the activation of the sympathoadrenal system and is accompanied by grooming behavior. To address the role of oxytocinergic pathways in the brain in the mediation of these responses, we investigated the effects of central pretreatment of rats with oxytocin antisense, mixed base, and sense oligodeoxynucleotides on mean arterial pressure, heart rate, and grooming behavior induced by intracerebroventricular injections of substance P (50 pmol). Central pretreatment of conscious rats with the oxytocin antisense oligodeoxynucleotide (intracerebroventricular injections, 8 and 4 hours before administration of substance P) attenuated the mean arterial pressure (by 55%) and heart rate responses (by 50%) as well as grooming behavior induced by the peptide. A complete recovery of all substance P-induced responses was observed 28 hours after antisense oligodeoxynucleotide pretreatment. Intracerebroventricular pretreatment of rats with mixed base and sense oligodeoxynucleotides did not affect the cardiovascular and behavioral responses to substance P. The signal for oxytocin mRNA in the paraventricular nucleus was reduced only in rats pretreated with the antisense oligodeoxynucleotide. These results demonstrate that oxytocin neurons in the paraventricular nucleus, which innervate the cardiovascular centers in the hindbrain and the spinal cord, mediate the increases in blood pressure and heart rate induced by stimulation of substance P receptors in the forebrain. These neurons may also transmit signals, which are generated by substance P in the hypothalamus and are responsible for the sympathoadrenal activation in response to stress (Hypertension. 1998;31[part 2]:480-486.)

Key Words: substance P ■ blood pressure ■ heart rate ■ behavior ■ oxytocin ■ brain

Numerous studies have posited a link between stress, sympathoadrenal activation, and the pathogenesis of arterial hypertension. The sympathoadrenal outflow and blood pressure are principally controlled by complex interactions between areas localized in the lower brain stem, the hypothalamus, and the intermediolateral column of the spinal cord. A number of transmitters and neuromodulators in the brain participate in the regulation of autonomic functions. Substance P (SP), a natural ligand for NK receptors, acts in the forebrain as a potent activator of the sympathoadrenal system. Stimulation of periventricular SP receptors or direct microinjections of the peptide into various hypothalamic regions induce an elevation of arterial blood pressure, tachycardia, increase in cardiac output, vasodilation in skeletal muscles, and vasoconstriction in the splanchnic area and in the kidney. The central cardiovascular responses produced by SP are brought about by sympathoadrenal stimulation as evidenced by increases in efferent splanchnic and renal nerve activities and elevated concentrations of plasma noradrenaline and adrenaline. The cardiovascular response is associated with a release of oxytocin into the circulation and extensive grooming behavior. The whole response pattern is consistent with the integrated response of rodents to noxious stimuli and stress.

Numerous lines of evidence have suggested that the cardiovascular, behavioral, and endocrine responses induced by stimulation of forebrain SP receptors are generated in the hypothalamus. However, the question as to which neurotransmitter systems and neural circuits mediate these responses has not yet been answered. The paraventricular nucleus (PVN) of the hypothalamus represents an important site for the integration of autonomic, visceral, neuroendocrine, and behavioral responses, including those to stress. A variety of neuroactive substances, including oxytocin and vasopressin, were found in the paraventriculospinal tract, which projects directly to the intermediolateral column at the thoracolumbar level of the spinal cord. Recent studies have suggested that oxytocin pathways originating in the PVN and innervating the cardiovascular centers in the lower brain stem and sympathetic preganglionic neurons in the spinal cord mediate the tachycardic response to stress. Because the cardiovascular responses induced by stimulation of SP receptors in the forebrain and by stress are virtually identical, we hypothesize that brain oxytocin pathways originating in the PVN may also mediate the cardiovascular response produced by SP.

In recent years, antisense oligonucleotides specifically designed to interact with mRNA coding for peptides or their...
receptors have been used increasingly to study physiological functions of various regulatory peptides in freely moving conscious animals. This experimental approach offers the possibility of blocking specific gene expression without multiple, nonspecific side effects. In the present study, experiments were carried out to evaluate the role of oxytocin pathways in the mediation of SP responses by testing the effects of an intracerebroventricular (ICV) pretreatment of rats with an antisense oligodeoxynucleotide directed against the mRNA for oxytocin gene expression on the cardiovascular and behavioral responses induced by SP in the forebrain.

**Materials and Methods**

**Animals and Surgery**

Male Wistar rats weighing 300 to 350 g were obtained from Charles River (Sulzfeld, Germany). Rats were housed on a 12/12-hour light/dark cycle with free access to food and water.

**Surgical Methods**

For ICV injections, indwelling polyethylene cannulae (PP 20, LHD, Heidelberg, Germany) were implanted under chloral hydrate anesthesia (400 mg/kg, IP) into the left lateral ventricle 7 to 10 days before the experiment. The stereotaxic coordinates were 1.3 mm lateral to the midsline, 0.6 mm posterior to the bregma, and 6 mm vertical from the skull surface. Five days after surgery, angiotensin II (25 pmol ICV) was injected. Only those rats that responded by immediate drinking were included in further experiments. Two days before the experiment, rats were anesthetized again and a polyethylene catheter (PP 50, LHD) filled with heparinized saline was inserted through one femoral artery into the abdominal aorta and connected to a pressure processor (Gould Inc, Valley View, Ohio) coupled to a Gould Brush recorder (Gould Series 2400, Gould Inc.). The analog output signals of MAP and HR from the Gould Brush pressure computer were digitized and then processed using a computer program. This program permits sampling of hemodynamics data from experimental animals directly onto a hard disk of the computer and subsequent analysis with an interactive graphic program. The hemodynamic data are sampled, compressed, and stored continuously in real time during the entire experiment. The analysis of MAP and HR changes was performed by a computer program as described recently. Both parameters are expressed as area under the curve (MAP=mm Hg x mm, HR=beats/min x mm). The value represents the sum of MAP or HR changes integrated in time, i.e., one number quantitatively describes the whole MAP or HR response.

**Experimental Protocols**

Experiments were conducted 48 hours after the implantation of the femoral catheter. Rats received two ICV injections at 4-hour intervals with vehicle (saline), the behavioral responses represent the spontaneous behavior over a 20-minute period after vehicle injection. Twenty-four hours after the first SP injection, rats received the second ICV injection of vehicle or oligodeoxynucleotides was given between 8 and 9 AM and the second between 12 AM and 1 PM, exactly 4 hours after the first one. Four hours after the second ICV injection, SP (50 pmol ICV) was injected, and the cardiovascular and behavioral responses were recorded over a 20-minute period. Control rats (n=9) received 5 μL saline without any pretreatment. The ICV injection of saline was used to determine the cardiovascular effects induced by vehicle (saline), the behavioral responses represent the spontaneous behavioral activity over a 20-minute period after vehicle injection. Twenty-four hours after the first SP injection, rats received the second injection of SP (50 pmol ICV) to test the reversibility of any changes in the responses induced by treatment of rats with oligodeoxynucleotides on the previous day.

A second set of experiments was conducted to evaluate the effects of oligodeoxynucleotides on oxytocin mRNA levels in the PVN. The protocol was similar to that described above. Rats (n=6 per group) underwent ICV pretreatment with vehicle (saline) or oligodeoxynucleotides. Four hours after the second ICV injection, three rats in each group were deeply anesthetized and intracardially perfused with PBS followed by 4% paraformaldehyde solution. The remaining rats were perfused on the next day, 28 hours after the second ICV injection. The fixed brains were removed, postfixed overnight in 4% paraformaldehyde, and then incubated for 72 hours in 30% sucrose at 4°C for cryopre-
TABLE 1. Resting Mean Arterial Pressure and Heart Rate Values 24 Hours Before, 4 and 28 Hours after Intracerebroventricular Treatment of Rats With Vehicle, Antisense, Mixed-Base, and Sense Oligodeoxynucleotides

<table>
<thead>
<tr>
<th>Group</th>
<th>MAP (mm Hg)</th>
<th>HR (beats/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h Before</td>
<td>4 h After</td>
</tr>
<tr>
<td>Vehicle</td>
<td>93±2</td>
<td>96±2</td>
</tr>
<tr>
<td>Antisense ODN</td>
<td>96±2</td>
<td>105±3*</td>
</tr>
<tr>
<td>Mixed base ODN</td>
<td>97±2</td>
<td>96±3</td>
</tr>
<tr>
<td>Sense ODN</td>
<td>96±3</td>
<td>100±2</td>
</tr>
</tbody>
</table>

Values represent the means±SEM of (n) rats (vehicle n=9, antisense oligodeoxynucleotide [ODN] n=10, mixed base ODN n=9, sense ODN n=12).

*P<0.05, statistical comparison to the corresponding value recorded 24 hours before ICV treatment, calculated with ANOVA followed by Duncan test.

Cryostat-cut coronal sections were processed for in situ hybridization (see above).

These experimental protocols have been approved by the State Governmental Committee for Ethical Use of Animals.

**Chemicals**

SP was purchased from Bachem Biochemica GmbH (Heidelberg, Germany). SP was dissolved in isotonic saline. The stock solution of the peptide (500 pmol/1 μL) was divided in aliquots and stored at -20°C for no longer than 3 weeks. On the day of the experiment, the stock solution was further diluted with saline to obtain the desired concentration of SP (50 pmol/1 μL).

**Statistical Analysis of Data**

All values are expressed as means±SEM. Data were subjected to one-way analysis of variance (ANOVA). Duncan test was used as a follow-up test to analyze differences between groups. A significance level of P<0.05 was accepted.

**Results**

Pretreatment of rats with the sense oligodeoxynucleotide increased the resting MAP slightly but significantly (Table 1). Fig 1 shows representative profiles of MAP and HR changes induced by ICV injection of SP 4 hours after pretreatment of rats with vehicle or the antisense oligodeoxynucleotide oligomer. Pretreatment of rats with the oxytocin antisense significantly reduced the MAP and HR responses to SP. The effects of ICV pretreatment with vehicle or oligodeoxynucleotides on MAP and HR responses to ICV administration of SP expressed as area under the curve are shown in Fig 2. ICV injection of SP induced marked increases in MAP and HR in the vehicle-treated group. Pretreatment with the antisense oligodeoxynucleotide significantly reduced both responses (MAP by 55%, HR by 58%) to values that did not significantly differ any more from values obtained in controls injected with saline. MAP and HR responses in rats pretreated with mixed base and sense oligodeoxynucleotides did not differ significantly from responses obtained in vehicle-pretreated, SP-injected rats, although the MAP response tended to be reduced somewhat in rats pretreated with the sense oligodeoxynucleotide. The HR responses to SP were slightly attenuated in rats pretreated with both sense and mixed-base oligodeoxynucleotides. Treatment of rats with the antisense oligodeoxynucleotide also attenuated the maximal increases in MAP and HR induced by ICV administration of SP (Table 2).

ICV treatment with oligodeoxynucleotides did not affect the resting MAP and HR values 28 hours afterward. A slight, significant increase in the resting MAP was observed only in rats pretreated with vehicle (Table 1). Pressor and HR responses to ICV injection of SP 24 hours after the first SP injection were similar in rats pretreated with vehicle and oligodeoxynucleotide, indicating that the antisense oligodeoxynucleotide-induced inhibition of the cardiovascular responses to SP was temporary and completely reversible (Table 3).

The cardiovascular response to SP was associated with increased locomotion and intense grooming behavior. FW and HG represent the most prominent behavioral variables ob-

Figure 1. Representative profiles of MAP and HR responses to ICV injection of vehicle (A), substance P (50 pmol) after ICV pretreatment with vehicle (B), and substance P (50 pmol) after ICV pretreatment with the oxytocin antisense oligodeoxynucleotide (C). The time point of the ICV injection of vehicle or substance P is indicated by the arrow.
values obtained in the remanng groups at the level P < 0.1 and AHR in antisense-treated rats differs significantly from the AMAP and AHR Group (ICV) AMAP

<table>
<thead>
<tr>
<th>Group (ICV pretreatment)</th>
<th>Vehicle</th>
<th>Antisense ODN</th>
<th>Mixsed base ODN</th>
<th>Sense ODN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injection</td>
<td>SP</td>
<td>SP</td>
<td>SP</td>
<td>SP</td>
</tr>
<tr>
<td>n</td>
<td>9</td>
<td>7</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>MAP (AUC) (mm Hg)</td>
<td>105±14</td>
<td>119±15</td>
<td>104±11</td>
<td>88±16</td>
</tr>
<tr>
<td>(AUC) (beats/min)</td>
<td>768±60</td>
<td>553±68</td>
<td>500±87</td>
<td>662±97</td>
</tr>
</tbody>
</table>

Values represent the means±SEM of (n) rats. Mean arterial pressure (MAP) and heart rate (HR) are expressed as area under the curve (AUC) (MAP-mm Hg x min, HR-beats/min x min). AUC was calculated for a period of 20 minutes starting at the time point of the ICV injection of substance P or saline (control rats). Values represent means±SEM. *P < 0.05, **P < 0.01, ***P < 0.001. Statistical comparisons were calculated with ANOVA followed by Duncan test.

The considerable potency of SP in the forebrain to activate the sympathoadrenal system points to a role of this peptide in eliciting cardiovascular response to stress. Indeed, we have recently demonstrated in rats that an inhibition of central Nk1 receptors attenuates the pressor, HR, and behavioral responses to noxious stimuli. The hypothalamus, especially the PVN, has been proposed as a site of the generation of these responses. The key role of the PVN neurons for the regulation of autonomic functions has been well established. Neurons in the PVN stained for oxytocin and vasopressin project directly to the intermedialateral column of the spinal cord and to the dorsal vagal complex. Oxytocin-stained neurons are more frequent, outnumbering AVP-stained neurons by a ratio of 3 to 1. It seems that oxytocin neurons in the PVN can substantially contribute to control of sympathoadrenal activity through direct action on sympathetic preganglionic neurons.

Recently, antisense oligodeoxynucleotides have been used in vivo experiments to study the physiological functions of neuropeptides and their receptors in the brain. Here, we demonstrate that specific targeting of oxytocin neurons with the antisense oligodeoxynucleotide directed against the mRNA for oxytocin reduced the pressor response and tachycardia as well as the behavioral response induced by central injection of SP. The effect of the antisense oligodeoxynucleotide treatment on these responses was only transient.
### TABLE 4. Face Washing/Head Scratching (FW) and Hindquarter Grooming/Biting (HG) Induced by Substance P (50 pmol) Injected Intracerebroventricularly 4 Hours After Pretreatment With Vehicle, Antisense, Mixed Base, and Sense Oligodeoxynucleotides

<table>
<thead>
<tr>
<th>Group (ICV pretreatment)</th>
<th>ICV Injection</th>
<th>n</th>
<th>FW</th>
<th>HG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls Saline</td>
<td>9</td>
<td></td>
<td>1.2±0.7</td>
<td>1.5±0.5</td>
</tr>
<tr>
<td>Vehicle SP</td>
<td>9</td>
<td></td>
<td>8.1±1.7</td>
<td>7.8±1.3</td>
</tr>
<tr>
<td>Antisense ODN SP</td>
<td>10</td>
<td></td>
<td>2.7±1.0**††</td>
<td>1.4±0.7††</td>
</tr>
<tr>
<td>Mixed base ODN SP</td>
<td>9</td>
<td></td>
<td>7.0±1.3</td>
<td>5.1±1.9</td>
</tr>
<tr>
<td>Sense ODN SP</td>
<td>12</td>
<td></td>
<td>7.3±1.4</td>
<td>6.5±1.6</td>
</tr>
</tbody>
</table>

*Values represent the frequency of individual behavioral manifestations for 20 minutes and are indicated by the means±SEM of (n) rats. The values in all groups except for the antisense oligodeoxynucleotide (ODN)-treated group differ significantly from controls (significance not shown).

**P<.05 vs vehicle-treated group.
†P<.05 vs mixed-base ODN-treated group.
‡P<.05 vs sense ODN-treated group; statistical comparisons calculated with ANOVA followed by Duncan test.

because the inhibition was no longer observed when SP was injected 28 hours after the nucleotide treatment.

In most of the in vivo studies, antisense oligodeoxynucleotides were administered using multiple injections or infusions over several days, but very rapid effects after a single antisense oligodeoxynucleotide injection could also be demonstrated. The efficiency of the two consecutive ICV injections of the antisense probe used in the present study to affect the function of oxytocin neurons in the PVN was controlled by an in situ hybridization analysis for the oxytocin mRNA signal in the PVN. It is assumed that after crossing the cell membrane, antisense oligonucleotides hybridize to the target mRNAs, forming a substrate for nucleases. Correspondingly, in the present study, ICV treatment of rats with the antisense oligodeoxynucleotide reduced the oxytocin mRNA signal in the PVN, as revealed by in situ hybridization analysis. These findings indicate that the dose of the oxytocin antisense oligodeoxynucleotide was sufficient to temporarily alter the production of oxytocin, which might be associated with modification of neuronal activity and excitability.

The antisense probe used in the present study contained a G-tetrad. Although antisense experiments in vitro have shown that probes containing repeated G-sequences also induce toxic effects, eg, by blocking the DNA-polymerase II, such effects have not been observed in vivo so far. The oxytocin antisense probe used in the present study is unlikely to have exerted toxic actions because of the time course and complete reversibility of the effects of the antisense treatment on the SP-induced cardiovascular and behavioral responses. Oxytocin antisense probes to various different sequences of the oxytocin start coding region, with or without repeated G-sequences, were used in previous studies. None of these probes showed any sequence-independent effects.

The finding of the reduced pressor and HR responses to SP in rats pretreated with the antisense oligodeoxynucleotide indicates that oxytocin neurons in the PVN, which project to the spinal cord, participate in the activation of the sympathoadrenal system in response to stimulation of forebrain NK1 receptors. We assume that the SP-induced activation of neuronal circuits in the hypothalamus stimulates oxytocin neurons in the PVN, which results in an increased release of oxytocin from nerve terminals localized on sympathetic preganglionic neurons in the intermediolateral column of the spinal cord. In general, oxytocin has been reported to excite spinal sympathetic neurons. In the rat, intrathecally administered oxytocin at the thoracic level increased HR, and oxytocin injected at the lumbar level induced a rise in blood pressure.

The mechanisms by which the antisense nucleotide reduces the neuronal activity of oxytocin neurons in the PVN and, consequently, the release of oxytocin from nerve terminals are not known. One of the proposed mechanisms of the antisense oligodeoxynucleotide actions is the reduction of oxytocin synthesis in the neuronal perikarya. Oxytocin synthesized in the PVN is transported to the thoracic spinal cord via a fast component of axonal transport and may thus reach oxytocin terminals localized in the upper thoracic spinal cord within 6 hours. Because 8 hours elapsed between the first antisense probe application and the recording of the cardiovascular

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![Figure 3](https://example.com/figure3.png)

**Figure 3.** Effects of ICV treatment with vehicle (A), antisense (B), mixed-base (C), and sense oligodeoxynucleotides (D) on oxytocin mRNA hybridization signal in the PVN. Rats were killed 4 hours after the second ICV injection of vehicle or the respective oligodeoxynucleotide.
responses to SP, a rapid inhibition of oxytocin synthesis in the PVN and reduced amounts of the peptide being transported to the spinal cord might decrease the oxytocin content and consequently the release of the peptide from nerve terminals. This, however, may not be true, because the large storage capacity of nerve terminals for oxytocin provides for an adequate release of the peptide when its synthesis is altered. Morris et al. recently reported a rapid increase in brain stem oxytocin content in response to antisense oligomer infusion into the PVN. It has been hypothesized that the increased levels of oxytocin in the brain stem, which represents a site of the termination of oxytocin fibers deriving from the PVN, may be related to a reduction in oxytocin release, which would result in an accumulation of the peptide. The idea is indirectly supported by recent data demonstrating a reduced neuronal excitability of oxytocin neurons after antisense oligodeoxynucleotide treatment. In view of these findings, we suppose that central treatment with the antisense nucleotide reduces the excitability of oxytocin neurons projecting into the spinal cord and also the release of oxytocin from the nerve terminals localized on sympathetic preganglionic neurons.

Moms et al. recently demonstrated that infusion of antisense oligodeoxynucleotide in the PVN prevents the tachycardia but not the pressor response induced by staker stress. Centrally administered vasopressin-oxytocin antagonists or lesions of the PVN also prevented increases in HR induced by stress. Several lines of evidence indicate that SP belongs to the neurotransmitter substances in the brain which generate stress-induced pressor responses and tachycardia. It seems that SP and other neurotransmitters or neuromodulators in the forebrain, most probably in hypothalamic neural circuits, activate oxytocin neurons in the PVN that increase the sympathetic drive, resulting in tachycardia. Although our present data demonstrate that central oxytocin pathways are crucial in mediating the SP-induced pressor responses, neurotransmitter systems other than oxytocin probably mediate increases in blood pressure in response to stress.

It has been reported that oxytocin antisense oligomer injected into the PVN did not affect basal levels of oxytocin in plasma or resting cardiovascular parameters. In contrast to these findings, we observed that treatment of rats with the oxytocin antisense oligomer slightly increased the resting MAP. Oxytocin fibers deriving from the PVN densely innervate the nucleus tractus solitarius and the dorsal motor nucleus of the vagus nerve, which represent the key areas in the maintenance of normal and reflex control of blood pressure. It is, therefore, conceivable to assume that the increased resting MAP might result from a modified release of oxytocin in these areas as a consequence of altered neuronal excitability of oxytocin neurons after antisense oligodeoxynucleotide treatment.

Treatment of rats with the antisense probe attenuated grooming behavior in response to SP. Excessive grooming and skin biting are the most characteristic behavioral manifestation elicited by stimulation of central NK receptors. This behavioral response pattern represents an integral part of the reaction of rodents to noxious stimuli and stress. We recently reported that the behavioral response to a noxious stimulus can be prevented by inhibition of central NK receptors. It seems likely that SP acting on NK receptors, localized most probably in the hypothalamic neural circuitry, activates oxytocin neurons in the PVN which then initiate and mediate the behavioral effects. Although oxytocin administered ICV has been reported to induce an intense grooming behavior, it remains to be established in which brain areas the release of oxytocin evokes grooming behavior in the rat.

Our current studies indicate that central oxytocin pathways projecting to the hindbrain and the spinal cord mediate SP-induced sympathoadrenal activation. In view of previous findings demonstrating that SP in the brain represents a potent activator of the sympathoadrenal system and participates in the generation of the cardiovascular responses to stress, the present data contribute to our understanding of the mechanisms leading to the sympathoadrenal inhibition observed after lesions of the PVN and may also help to explain why lesions of this area prevent the development of spontaneous hypertension.

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