Effect on Vasopressin Release of Microinjection of Cholinergic Agonists into the Rat Supraoptic Nucleus (43500)

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Abstract. **It is likely that central cholinergic pathways to the paraventricular and supraoptic nuclei participate in the control of vasopressin release. We have shown previously that this is due, in part, to activation of muscarinic, but not nicotinic, receptors in the paraventricular nucleus. There is, however, reason to believe that this cholinergic effect in the supraoptic nucleus may be the result of activation of nicotinic receptors. To test this possibility, we have studied in conscious unrestrained rats the effect of microinjection of muscarinic and nicotinic agonists into the supraoptic nucleus on vasopressin release, mean arterial blood pressure, and heart rate. Under ether anesthesia, a stainless steel guide cannula was placed in the supraoptic nucleus 5-7 days before the experiment, and femoral, arterial, and venous catheters were implanted 1 day before the experiment. Microinjection of nicotine into the supraoptic nucleus at doses of 1 and 10 μg resulted in transient increases in the plasma vasopressin concentration that were 7-fold and 11-fold greater, respectively, than control values at 3 min. There were also small transient increases in mean arterial blood pressure, but heart rate was unchanged. The microinjection of 2 and 20 ng of oxotremorine, a muscarinic agonist, into the supraoptic nucleus had no effect on the plasma vasopressin concentration, mean arterial blood pressure, or heart rate. These doses of oxotremorine were previously shown to have potent stimulatory effects on vasopressin release when microinjected into the paraventricular nucleus. These findings suggest that the central cholinergic stimulation of vasopressin release is due, in part, to activation of muscarinic receptors in the paraventricular nucleus and nicotinic receptors in the supraoptic nucleus.**
[P.S.E.B.M. 1992, Vol 201]

Early work by Pickford and her associates (1–4) strongly suggested the involvement of cholinergic mechanisms in the central neural control of vasopressin release. Since then, considerable evidence has accumulated to indicate that acetylcholine can act centrally to stimulate vasopressin release (5). The issue, however, of whether this action of acetylcholine is due to activation of muscarinic or nicotinic receptors is controversial. On the one hand, we have found (6) that the stimulation of vasopressin secretion by the intracerebroventricular injection of carbachol in the conscious rat was blocked by the intracerebroventricular administration of the muscarinic antagonist, atropine, but not by the nicotinic antagonist, hexamethonium. These findings are consistent with a number of earlier observations in which antidiuresis was used as an index of vasopressin release (7–12). We subsequently demonstrated (12) that microinjection of oxotremorine, a muscarinic agonist, into the paraventricular nucleus stimulated vasopressin release, whereas nicotine had little or no effect. On the other hand, there is evidence that suggests that activation of nicotinic receptors in the supraoptic nucleus (SON) can also increase vasopressin release (13–17). In the present report, we have examined further the role of supraoptic

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Received February 20, 1992. [P.S.E.B.M. 1992, Vol 201]
Accepted June 5, 1992.

0037-9727/92/2012-0208$3.00/0
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muscarinic and nicotinic receptors in the control of vasopressin release by comparing the effects of microinjection of oxotremorine and nicotine into the SON on the plasma vasopressin concentration in conscious male rats.

Materials and Methods

The experiments were carried out in male Sprague-Dawley rats (Harlan Sprague-Dawley, Inc., Indianapolis, IN) weighing 270–350 g. They were housed in individual cages in a room with a 12:12 hr light:dark cycle at 22°C, with free access to water and food (Purina rat chow; Ralston Purina Co., St. Louis, MO).

Five to 7 days before an experiment, the rats were anesthetized with ether and, using a stereotaxic frame (David Kopf, Tajunga, CA), a 26-gauge stainless steel guide cannula was inserted unilaterally into the SON with the following coordinates: 1.0 mm posterior to bregma, 1.7 mm lateral to the midline, and 8.9 mm below the level of the skull. The cannula, sealed with a 33-gauge obturator, was secured to the skull with two jeweler's screws, and the screws and the area around the cannula were covered with dental cement. The day before the experiment, the left femoral artery was catheterized with PE-50 tubing, and the left femoral vein was catheterized with Tygon microbore tubing (i.d. 0.02 in, o.d. 0.04 in, wall thickness 0.01 in). The catheters, filled with heparinized saline (100 units/ml), were exteriorized and secured at the back of the neck. The rats were kept in individual cages and given food and water *ad libitum*. To ensure adequate water intake after surgery, an additional 20 ml of 5% dextrose was also provided in a separate bottle.

On the day of the experiment, the rats were placed in individual opaque plastic boxes that measured 12 × 14 × 25 cm and that allowed the rats to move backward and forward and to turn around. The arterial catheter was connected to a Statham P23ID strain gauge (Statham, Oxnard, CA) for recording mean arterial blood pressure (MABP) on a Gould Brush Mark 2600S recorder (Gould, Cleveland, OH). Heart rate was recorded from the arterial pulse with a Gould Biotach. The rats were allowed to become accustomed to their environment for 20 to 30 min. A 3-ml blood sample was then taken from the arterial catheter into a plastic syringe moistened with heparin (1000 units/ml). This and all subsequent blood samples were replaced by the simultaneous intravenous injection of an equal volume of heparinized blood obtained from ether-anesthetized rats by decapitation on the morning of the experiment and warmed to 37°C before use. Eight minutes later, the obturator of the guide cannula in the brain was removed, and a 33-gauge stainless steel injector that extended 0.5 mm beyond the tip of the guide cannula was inserted. Ten minutes after the initial blood sample (zero time), 0.1 μl of artificial cerebrospinal fluid (ACSF) alone or containing either oxotremorine at doses of 2 ng and 20 ng or nicotine at doses of 1.0 μg and 10 μg (Sigma Chemical Co., St. Louis, MO) was injected. Additional blood samples, each 2.5 ml in volume, were taken 3 and 15 min after the microinjection. In an extensive series of previously conducted control experiments, we found that this blood-sampling procedure does not affect the plasma vasopressin concentration, MABP, or heart rate. Each rat was used in only one experiment. To verify the position of the injection site in the brain, the rats were anesthetized with methohexital sodium at the conclusion of the experiment, and the head was perfused transcardially with 10% phosphate-buffered formalin. The brains were removed and stored in 10% phosphate-buffered formalin until they were frozen, sectioned, and stained with cresyl violet. The brain sections were examined under a light microscope.

In an additional series of experiments, we determined the effect of the intracerebroventricular administration of oxotremorine on MABP, heart rate, and the plasma vasopressin concentration. The rats were prepared as described above, except a 23-gauge stainless steel cannula was inserted into a lateral cerebral ventricle, instead of the SON, with the following coordinates: 1.0 mm posterior to bregma, 1.0 mm lateral to midline, and 5.0 mm below the level of the skill. Oxotremorine (500 ng) or the ACSF vehicle (10 μl) was injected at 0 min with a 30-gauge stainless steel injector that extended 0.5 mm beyond the tip of the guide cannula. Blood samples were taken at −10 min, 3 min, and 15 min.

Blood samples were kept on ice until they were centrifuged at 1000*g* for 30 min at 4°C. One milliliter of plasma was placed in a plastic tube containing 0.1 ml of EDTA (4%) and stored at −40°C until the subsequent extraction of vasopressin. Vasopressin was extracted from plasma with Sep-Pak C_{18} cartridges (Waters Associates, Milford, MA), and measured by radioimmunoassay, as described previously (18). The vasopressin standard was the U.S.P. Posterior Pituitary Reference Standard (The U.S. Pharmacopoeial Convention, Rockville, MD). Recovery of vasopressin from rat plasma averaged 75.6 ± 1.8%. Intra- and interassay coefficients of variation were 7.6 ± 1.2% and 15.3%, respectively. Hematocrit was determined by a microcapillary method. The plasma concentrations of sodium and potassium were measured by flame photometry (Corning M480 flame photometer; Corning, Medford, MA), and plasma osmolality was measured by freezing point depression (Advanced Systems micro-osmometer 3MO; Advanced Systems, Needham, MA).

Data are presented as means ± SE. Statistical analyses were performed with one- and two-way analyses of variance for repeated measures, with a subsequent Newman-Keuls test when appropriate.

Results

There were no differences in the initial values of the plasma vasopressin concentration (Fig. 1), MABP and heart rate (Table I), hematocrit, plasma osmolality, the plasma concentrations of sodium and potassium, or body weight among the groups of rats treated with the ACSF vehicle, nicotine, or oxotremorine. The microinjection of the ACSF vehicle (0.1 μl) for nicotine and oxotremorine into the SON had no effect on the plasma vasopressin concentration (Fig. 1). There was, however, a small increase in MABP 3 min after microinjection (Fig. 2; $P < 0.01$), although heart rate was unaffected (Fig. 3).

The microinjection of nicotine into the SON at

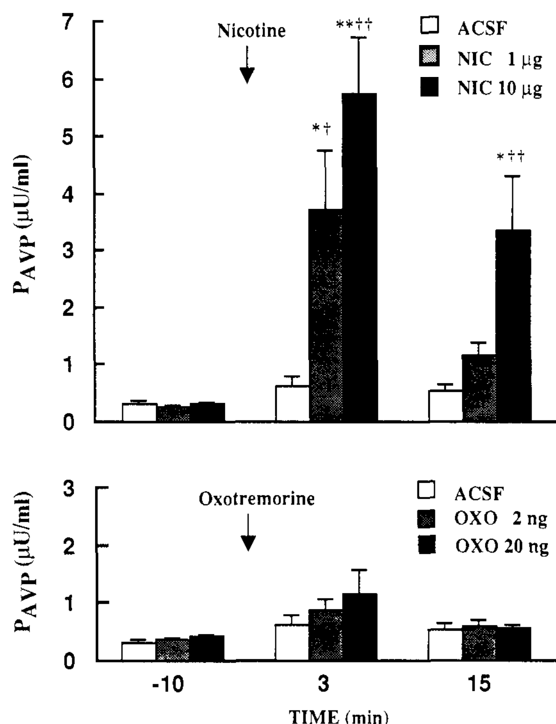

Figure 1. Effect of microinjection of nicotine (top panel), oxotremorine (bottom panel), or the ACSF vehicle into the SON on the plasma vasopressin concentration (P_{AVP}). For comparison purposes, the data from the same ACSF group are shown in both panels. *,** $P < 0.05$, 0.01 with respect to preinjection values. †,†† $P < 0.05$, 0.01 with respect to the ACSF vehicle.

Table I. Initial Values for Hemodynamic Variables[a]

Group	MABP (mm Hg)	Heart rate (beats/min)
ACSF (6)	107 ± 2.1	381 ± 5.4
Oxotremorine		
2 ng (6)	107 ± 1.8	405 ± 7.2
20 ng (5)	108 ± 1.4	391 ± 8.3
Nicotine		
1 μg (6)	105 ± 2.4	397 ± 8.8
10 μg (6)	110 ± 1.5	389 ± 8.5

[a] Values are mean ± SE. Numbers in parentheses indicate number of animals.

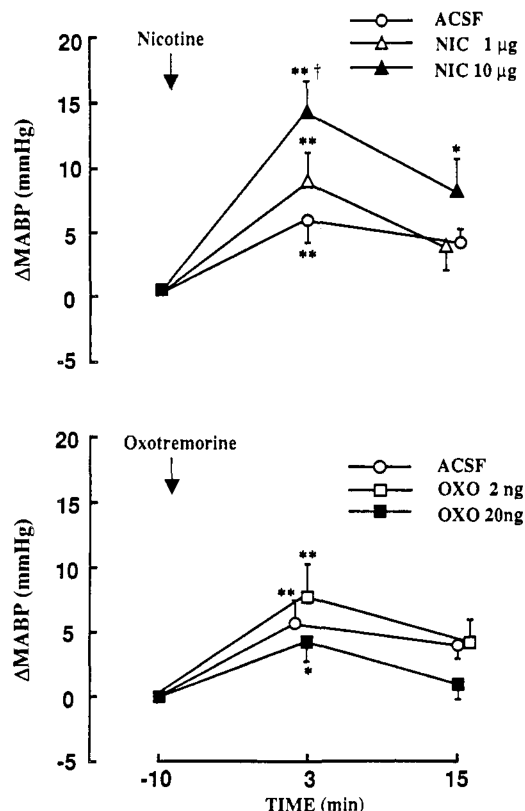

Figure 2. Changes in mean arterial blood pressure (ΔMABP) in response to microinjection of nicotine (top panel), oxotremorine (bottom panel), or the ACSF vehicle into the SON. For comparison purposes, the data from the same ACSF group are shown in both panels. Initial values for MABP are shown in Table I. *,** $P < 0.05$, 0.01 with respect to preinjection values. † $P < 0.05$ with respect to the ACSF vehicle.

doses of 1 and 10 μg resulted in dose-dependent increases ($P < 0.05$ and 0.01, respectively) in plasma vasopressin levels at 34 min that were 7-fold and 11-fold greater, respectively, than the concentration in the vehicle-treated rats (Fig. 1). Fifteen minutes after microinjection, the plasma vasopressin concentration was still elevated ($P < 0.05$) in the rats given the higher dose of nicotine, but had returned to a level that was not significantly different from the control value in the rats given the lower dose of nicotine. In a previous study (12), the microinjection of nicotine at these doses into the paraventricular nucleus (PVN) was without effect on the plasma vasopressin concentration. On the other hand, the microinjection of the muscarinic agonist oxotremorine into the SON at doses of 2 and 20 ng, doses which we previously (12) found to be highly effective in stimulating vasopressin release when injected into the PVN, failed to affect plasma vasopressin levels (Fig. 1).

The microinjection of either nicotine or oxotremorine into the SON resulted in transient increases in MABP (Fig. 2; $P < 0.01$), but only the response to the higher dose of nicotine at 3 min was significantly greater

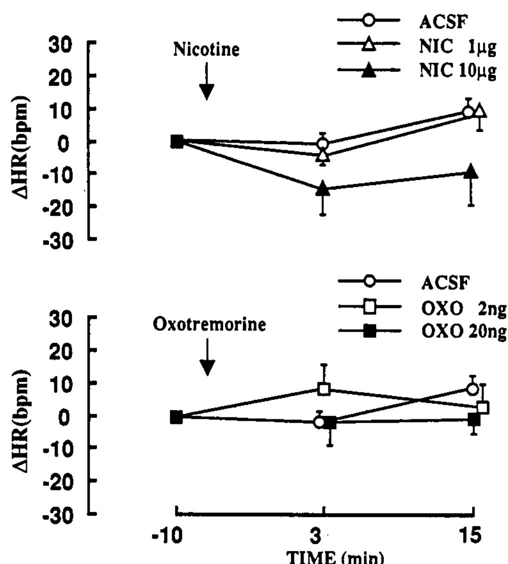

Figure 3. Changes in heart rate (ΔHR) in response to microinjection of nicotine (top panel), oxotremorine (bottom panel), or the ACSF vehicle into the SON. For comparison purposes, the data from the same ACSF group are shown in both panels. Initial values for HR are shown in Table I.

than the response to ACSF alone ($P < 0.05$). Neither drug had a statistically significant effect on heart rate (Fig. 3), although there was a tendency for heart rate to decrease after the administration of the higher dose of nicotine.

The vasopressin and cardiovascular responses to nicotine were specific to the SON; injection of nicotine at sites removed from this structure was ineffective (Fig. 4). The plasma vasopressin concentrations in these latter experiments 3 min after the microinjection of 1 μg and 10 μg of nicotine were 0.42 ± 0.08 and 0.46 ± 0.13 μU/ml, respectively. These values were not significantly different from those obtained with the ACSF vehicle. Oxotremorine at doses of 2 and 20 ng also failed to have a significant effect on plasma vasopressin levels at sites removed from the SON with plasma vasopressin concentrations of 0.40 ± 0.10 and 0.30 ± 0.05 μU/ml, respectively (Fig. 4).

In order to verify the biological effectiveness of the oxotremorine used in these experiments, the drug was given intracerebroventricularly at a dose of 500 ng in a separate series of experiments (Table II). There was a marked sustained ($P < 0.05$) increase in the plasma vasopressin concentration that was accompanied by an increase in MABP ($P < 0.05$) and a reduction in heart rate ($P < 0.05$). The intracerebroventricular injection of the ACSF vehicle (10 μl) had no effect on these variables.

Discussion

In this report, we demonstrated that the activation of nicotinic receptors in the SON resulted in an increased release of vasopressin. On the other hand, microinjection into the SON of oxotremorine, a specific muscarinic agonist equal in potency to acetylcholine but lacking nicotinic activity (19), was without effect on vasopressin release. The doses of nicotine and oxotremorine used in the present experiments are the same as those used in a previous study in which these drugs were microinjected into the PVN. In that study, oxotremorine was highly effective in stimulating vasopressin release; dose of 20 ng, the maximal dose used in the present experiments, caused a 10-fold increase in the plasma vasopressin concentration. Nicotine, however, was essentially inactive.

We cannot rule out the possibility that there are in the SON muscarinic receptors that are much less sensitive than those in the PVN, but our findings are consistent with other evidence that it is activation of nicotinic receptors in the SON, not muscarinic receptors, that stimulates vasopressin release. Thus, microinjection of nicotine into the SON of the anesthetized cat (13) and addition of nicotine to the incubation medium of the organ-cultured hypothalamoneurohypophysial complex, which contains the SON but not the PVN, increased vasopressin release (15, 16). In this latter preparation, the stimulation of vasopressin release by acetylcholine was blocked by nicotinic antagonists, but not by a muscarinic antagonist, and a muscarinic agonist was without effect on vasopressin secretion (16). There is also electrophysiological evidence for nicotinic-responsive neurons in the SON (17, 20–22).

Receptor-binding and immunohistochemical studies also tend to support the presence of nicotinic receptors in the SON. When labeled α-bungarotoxin was used as a probe for nicotinic receptors, significant binding was found in the SON (23–27) in close association with vasopressin magnocellular neurons (26, 27). The significance of these reports is, however, uncertain, since Härfstrand *et al.* (28) and Sharp *et al.* (30) failed to find a correlation between the binding of labeled nicotine and labeled α-bungarotoxin in the SON. Be that as it may, Sharp *et al.* (30) found binding of [^{3}H] nicotine in the neuropil surrounding the SON, and Härfstrand *et al.* (28) observed a low level of binding of [^{3}H]nicotine in the hypothalamus. In addition, Mason (29) reported that a monoclonal antibody directed against the α-subunit of the nicotinic receptor stained magnocellular neurons in the SON.

The findings of Sharp *et al.* (30) and Theodosis and Mason (31), who determined the binding of monoclonal antibodies to choline acetyltransferase, suggest the possibility that the cholinergic input is to interneurons in the perinuclear zone, which then project to vasopressin magnocellular neurons in the SON and PVN. Indeed, we cannot rule out the possibility that, in the present experiments, the microinjected nicotine diffused to sites closely adjacent to the SON to activate nicotinic receptors.

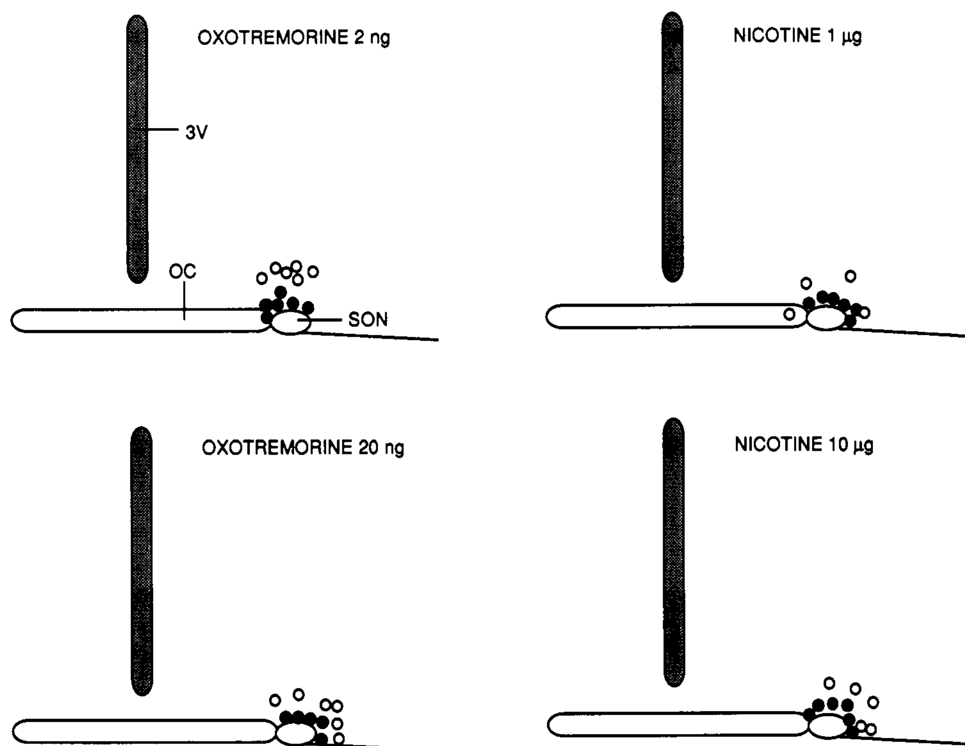

Figure 4. Diagrammatic coronal section of the brain, showing the location of the injector tip in experiments in which oxotremorine (at doses of 10 and 20 ng) and nicotine (at doses of 1 and 10 μg) were microinjected. The filled circles represent experiments in which the injectate was considered to involve the SON. The data from these experiments are presented in Figures 1–3. The open circles are considered misses; there were no significant changes in plasma vasopressin concentrations in these experiments. 3V, third ventricle; OC, optic chiasm.

Our present findings are in disagreement with those of Mori *et al.* (11, 32). They reported that oxotremorine was highly effective in inhibiting a diuresis in the ethanol-anesthetized rat when injected into either the PVN or SON (median effective doses of 76 and 114 ng, respectively); nicotine was also effective in both the PVN and SON, but at much higher doses than oxotremorine (11). Furthermore, the antidiuresis produced by microinjection of oxotremorine into the SON and PVN was prevented by the prior intravenous administration of a vasopressin V_1-V_2 receptor antagonist (32). We cannot explain the differences between these findings and ours other than to point out that these investigators used anesthetized, water-loaded, acutely prepared rats, whereas we used conscious, chronically instrumented rats, and that they did not measure plasma vasopressin concentrations. Perhaps of greater importance, they used a microinjection volume of 1 μl, 10 times the microinjection volume we employed. Thus, the likelihood of spread of the injectate to other structures was greater in the experiments of Mori *et al.* (32) than in the present work. Indeed, they did not report the effects of microinjections located outside the SON and PVN.

Our findings and those of other investigators indicate that stimulation of vasopressin release by cholinergic pathways to the hypothalamus is accomplished by activation of muscarinic receptors in the PVN and nicotinic receptors in the SON. The physiological significance of this difference is only conjectural, but it does suggest that there may be differences in the function of the SON and PVN in vasopressin release. The anatomical evidence is consistent with this possibility. Thus, on the one hand, there are projections from the parvicellular division of the PVN to centers in the brainstem involved in cardiovascular regulation (33), and potent stimuli for vasopressin release are reductions in blood volume and blood pressure (34). However, although there is an effective muscarinic receptor system in the PVN, cholinergic pathways to the anterior hypothalamus have not been implicated in the blood volume-blood pressure control of vasopressin release. On the other hand, there is a heavy projection from the nucleus medianus to vasopressin neurons in the SON, with a lesser projection to the PVN (35). In turn, there is reciprocal innervation between the nucleus medianus and the subfornical organ and the organum vasculosum of the lamina terminalis (35). These structures have been implicated in the osmotic control of vasopressin release (36), and nicotinic receptors in the SON are responsible for the osmotic stimulation of vasopressin release from the organ-cultured hypothalamoneurohypophysial complex (15, 37). Yet, it seems unlikely that, in the intact individual, the PVN does not play an

Table II. Effect of Intracerebroventricular Oxotremorine[a]

Group	MABP (mm Hg)			Heart rate (beats/min)			Plasma vasopressin concentration (μU/ml)		
	−10 min	3 min	15 min	−10 min	3 min	15 min	−10 min	3 min	15 min
ACSF (6)	115 ± 4.1	119 ± 3.4	117 ± 3.3	386 ± 10.8	404 ± 10.3	392 ± 9.7	0.28 ± 0.06	0.24 ± 0.04	0.66 ± 0.07
Oxotremorine (6)	113 ± 3.3	144 ± 1.4[b]	147 ± 3.2[b]	382 ± 8.3	308 ± 10.5[b]	283 ± 10.9[b]	0.45 ± 0.09	42.80 ± 13.70[b]	51.92 ± 21.04[b]

[a] The effect of intracerebroventricular injection of oxotremorine (500 ng) at zero time on MABP, heart rate, and plasma vasopressin concentration. Values are mean ± SE. Numbers in parentheses indicate number of animals.

[b] $P < 0.05$ compared with ACSF group. $P < 0.01$ compared with −10 min.

important role in the osmotic control of vasopressin release. Indeed, the stimulation of vasopressin release by the intracerebroventricular administration of carbachol, which has both muscarinic and nicotinic activity, is completely prevented by pretreatment with a muscarinic antagonist (6). A reasonable speculation at this times is that the PVN may be the site for the integration of osmotic and cardiovascular stimuli for the release of vasopressin. Beyond this, however, it seems premature to attempt to formulate a model for the control of vasopressin release that incorporates the structural and functional differences in the PVN and SON.

The small transient increase in MABP in response to the microinjection of nicotine into the SON may have been due to the concurrent increase in the plasma vasopressin concentration. If some other mechanisms were involved, it is difficult, on the basis of the available information, to state what they might be.

There is evidence that activation of nicotinic receptors within a circumscribed zone on the ventral surface of the brainstem results in vasopressin release (38). In conclusion then, our findings and those of other investigators suggest that the central cholinergic stimulation of vasopressin release involves activation of nicotinic receptors in the SON and the brainstem, and muscarinic receptors in the PVN.

This work was supported by U.S. Public Health Service Grants HL-12990 and HL-19209 from the National Heart, Lung, and Blood Institute of the National Institutes of Health. The authors are grateful for the expert technical assistance of Gregory Festavan.

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