Oxytocin Neurotransmission in the A1-area of the Brainstem Induces Hormonal Vasopressin Release in Rats

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Key words: A1 noradrenergic neurons, immunocytochemistry, brainstem infusion, plasma vasopressin, antagonists.

Abstract

To investigate the role of the oxytocin innervation of the caudal ventrolateral medulla, immunocytochemical techniques were used to demonstrate the presence of oxytocin fibres and terminals in close apposition to noradrenergic neurons of the A1-area.

Subsequently, in freely moving animals fitted with an indwelling jugular venous catheter and a bilaterally implanted chronic cannula in the A1-area, it was examined whether infusions of oxytocin in this area were able to influence hormonal vasopressin release. It appeared that nanomolar (50–500 nM) concentrations of oxytocin induce a fourfold rise in plasma vasopressin values. The specificity of this effect was established with control infusions of Ringer, vasopressin, and the addition of an antagonist to oxytocin. It was not possible to demonstrate a major role for oxytocin in the A1-area in the release of hormonal vasopressin occurring during haemorrhage.

These data permit us to conclude that oxytocin acts on presumably noradrenergic neurons of the A1-area leading to the release of vasopressin into the peripheral circulation. The circumstances under which oxytocin is released in this area remain to be established.

Introduction

It has become more and more evident that many of the peptides which are synthetized in the hypothalamus for release as hormones from the median eminence or neurohypophysis are also present in extrahypothalamic fibre systems. For the neurohypophysial hormones vasopressin (VP) and oxytocin (OT) this concept was recognized early: immunocytochemical techniques (Buijs, 1978; De Vries and Buijs, 1983; Sofroniew and Wondl, 1978; Swanson, 1977) revealed that central pathways containing VP and OT immunoreactivity arise, for example, from parvocellular (pc) neurons in the paraventricular hypothalamic nucleus (PVN). These peptidergic fibres innervate several brain regions which are known to project to the supraoptic nucleus (SON) and PVN (Buijs, 1983; Silverman et al., 1981; Tribollet and Dreyfuss, 1981; Tribollet et al., 1985). This information raises the possibility that central OT and VP participate in the regulation of these hypothalamic nuclei.

In the brain stem, the OT fibres predominately innervate the dorsomedial and caudal ventrolateral medulla (CVLM) (Buijs, 1978; Sofroniew and Schrell, 1981; Sladek and Sladek, 1983) at sites containing the cell bodies of the A1 and A2 noradrenergic (NA) neurons which project directly to the SON and PVN (Sawchenko and Swanson, 1982). To assess a possible interaction between OT fibres and A1 NA neurons, we immunocytochemically stained alternate sections for OT and NA to evaluate the localization of OT fibres relative to the NA neurons.

The functional nature of the OT innervation of these NA neurons may be inferred from the anatomical, electrophysiological, and physiological studies performed on the A1-area. Tracing studies revealed that the NA neurons of the A1-area have direct projections onto the magnocellular VP-containing cell bodies in the PVN and SON (Sawchenko and Swanson, 1982). In addition, electrical stimulation in the A1-area was shown to give a rise in firing frequency of these same neurons, apparently mediated by its NA projections (Day et al., 1984). These data suggested that A1 NA neurons have a stimulatory input on hormonal VP release, a concept later established by means of an injection of bicuculline in this area which resulted in a rise of plasma VP levels (Sved et al., 1985). In view of this known function of the A1-area physiological experiments were carried out to investigate whether infusions of OT in this brain region were able to influence VP release into the circulation.

Recent studies on the CVLM have demonstrated that this area is an integral part of the baroreflex-pathway through which the VP release is mediated in response to a decrease of central blood
pressure (Blessing and Willoughby, 1985; Ciriello and Caverson, 1984a,b). The question was raised whether, under circumstances of hypovolaemia, OT release in the A1-area is essential for this hormonal VP release. Therefore, it was investigated by means of an OT antagonist whether inhibition of OT neuro-transmission in the A1-area could lead to a blockade or delay of the VP release which occurs after haemorrhage.

Materials and Methods

Male Wistar rats (TNO Zeist, The Netherlands; 300–400 g) were used in all studies. They were kept in a temperature-controlled environment (20.22 °C) on a 12 h light, 12 h dark schedule (07.00 h lights on). Food and water were available ad libitum.

Seven rats were anaesthetized with pentobarbitral (Nembutal: 1 ml/kg, intraperitoneally) and perfused intracardially with 50 ml saline followed by 500 ml of 5% glutaraldehyde-1% paraformaldehyde in 0.1 M phosphate buffer, at pH 4 (Merck Chemicals). The brains were dissected and put overnight in 0.05 M Tris buffer containing 1% Na₂S₂O₃ and 25% sucrose, at pH 7.6. The brains were frozen in liquid nitrogen, and 5-10 μm sections were made on a cryostat. The sections were mounted on chrom-alum coated glass slides and alternately stained for OT and NA.

Prior to NA staining, a treatment was carried out in which the cryostat sections were kept in 0.1% NaBH₄ dissolved in 0.05 M Tris buffer containing 0.9% NaCl (Tris-NaCl) for five minutes. Thereafter they were thoroughly washed in 0.05 Tris buffer containing 1% Na₂S₂O₃ (Tris-Na₂S₂O₃).

The alternate cryostat sections were incubated with anti-OT (O-1-V) preadsorbed to AVP diluted 1:400 in Tris-NaCl containing 0.5% Triton X-100 (Tris-NaCl-Triton) and with anti NA (W4) diluted 1:1000 in Tris-Na₂S₂O₃-Triton, during one night. Subsequently the sections were stained as described previously (Buijs, 1978).

For the physiological experiments a total of 60 animals of 290-320 g were anaesthetized with Hypnorm (Diprhe, The Netherlands; 0.6 ml/kg, subcutaneously) and placed in a David Kopf stereotax. A 25 gauge stainless steel tube (length 16 mm, o.d. 0.5 mm, i.d. 0.3 mm) was lowered into each A1-area according to the following co-ordinates: 3.5 mm caudal to lambda; 2.0 mm lateral to the midline and from this point 10.0 mm below the skull surface. Incisor bar: +5.0 mm. These cannulas were fixed into place with three stainless steel screws and self-cure acrylic dental cement (Hommedica Int., Ltd, UK). Removable stainless steel stylets were inserted to seal the cannulas.

After a recovery period of one week, in a second operation a catheter was inserted into the right external jugular vein according to the method of Steffens (1969).

Experiments were started at least one week after the last operation. In a volume of 200 ml Ringer-solution OT (10, 100, 200, and 1000 pg) (both Sigma Chemicals) was infused into the A1-area for 2 min. The infusion was accomplished using a Carnegie CMA 100 microinjection pump that was attached to two 30 gauge stainless steel tubes (o.d. 0.28 mm) via two PP10 polyethylene tubes (Portex Ltd, UK). These tubes were inserted into the cannulas together with an adjustment of a PP.100 tube to the venous catheter, after which the animal was allowed to adapt in the cage for 30 min. 3 min before, and 2, 5, and 10 min after the start of the infusion, blood samples were taken, the volume of each (1 ml) immediately being replaced by heparinized donor blood. The blood samples were collected in heparinized tubes placed in ice, centrifuged within 15 min after the experiment, and stored at -20 °C until VP- or OT-RIA according to Dogterom et al. (1977). In addition, infusions of Ringer-solution, a combination of 100 pg OT and 100 pg of the OT antagonist d(CH₂)₃[Tyr(Me)₂]OVT (MEOV) and of 100 pg VP were made in order to investigate the specificity of the OT effect in this in vivo situation. Furthermore, solutions of 1 ng OT together with 100 pg of the VP antagonist d(CH₂)₃[Tyr(Me)₂]AVP (MEAV) (the antagonists were kindly provided by Dr M. Manning from Toledo, USA) and of a 100 pg OT combined with 100 pg VP were infused in order to investigate a possible binding of OT onto VP receptors when administered at high concentrations.

To investigate the effects of OT and VP infusion on blood pressure some of the rats were additionally anaesthetized with an intravenous infusion of urethane (BDH, The Netherlands; 1.5 g/kg body weight, 25% solution), and the artery ilica externa was catheterized with a PP.50 tube. Subsequently, this tube was connected to a Harvard BPI transducer and a Servogor recorder to register the arterial blood pressure before, during, and after the infusions.

For the final experiment a 10 min infusion of 500 pg oxytocin antagonist MEOV in 500 ml Ringer or vehicle alone was performed during which (from the start of the infusion) 3 ml/kg body weight was withdrawn 5 times for VP-RIA every 2 min. A final 1 ml blood sample was taken at the end of the infusion after which the total amount of removed blood was returned to the animal by means of donor blood.

After each experiment each animal was given a recovery period of at least 3 days before undergoing the next experiment. At the end of the experiments the animals were decapitated, and the brains were frozen and sectioned on a cryostat to analyse the position of the cannulas.

Results

Light microscopical observation showed the presence of OT fibres in the region of the NA neurons of the A1-area. Careful analysis of sequential 5-10 μm thick sections of this area alternatively stained for NA or OT revealed the presence of OT fibres in close contact with NA positive neurons (Fig. 1).

Effects on plasma VP- and OT-levels of OT-, VP-, and vehicle-infusions into the A1-area

Data from animals in which the cannulas were placed incorrectly were excluded from statistical analysis; only results from animals in which the cannula-tips were within the A1-area were used (Fig. 2).

Experiments in which control blood samples revealed basal plasma VP levels above 5 pg/ml (3% of the cases) were not taken into account so as to prevent the effect of OT infusion being confused with other factors (such as stress, hypotension, or hyperosmolarity) apparently influencing VP release at that very moment. Nevertheless, the few data thus omitted confirmed the trend of the results and would not have influenced the results significantly if they had been included.

OT into the A1-area produced an increase in plasma VP levels when infused within a narrow range of concentrations. After 2 min 100 and 200 pg OT gave a fourfold rise of VP in the plasma which slowly declined afterwards but was still elevated at 10 min.
Infusion of 10 pg OT induced only modest increases in a few rats while 1 ng had no effect at all on plasma VP levels (Fig. 3). Cannulas placed just outside the A1-area resulted in no visible change in plasma VP levels (data not shown). No changes were detected in plasma levels of OT after administration of 100 pg OT in the A1 area. Infusion of Ringer-solution (Fig. 3) and 100 pg VP (Fig. 4) did not affect VP values in the plasma. Figure 4 also illustrates that the effect of 100 pg OT could be markedly attenuated by simultaneous infusion of an equally large amount of the OT antagonist MEOV.

To investigate whether the lack of effect of 1 ng OT is attributable to binding of this peptide to VP receptors possibly present in the A1 area, we infused this amount of OT in combination with 100 pg of the VP antagonist MEAV. From Figure 4 it can be concluded that occupation of the VP receptors by means of the VP antagonist does not result in reappearance of the rise in plasma VP.

Since the VP antagonist MEAV is also able to bind to OT receptors (Mühlthaler et al., 1982, 1983), we added 100 pg MEAV to 100 pg OT so as to exclude the possibility that the result of the former experiment was due to this interaction. It appeared that we were indeed dealing with binding of the VP antagonist to OT receptors in the A1-area: the stimulation of hormonal VP release by 100 pg OT was completely blocked by addition of 100 pg MEAV to the infusion solute (Fig. 4).

In view of the unspecificity of the VP antagonist MEAV in this experiment, we finally infused OT and VP together (both 100 pg) to see whether activation of VP receptors had an opposite effect on VP release compared to OT receptor stimulation. The preliminary data (n = 3) indicated that VP counteracts the effect
Fig. 4. Changes in plasma VP levels after A1-infusion of 100 pg VP and the combinations of: 100 pg OT + 100 pg MEAV 1000 pg OT + 100 pg MEAV 100 pg OT + 100 pg MEAV

* Significantly different from plasma VP levels seen after Ringer infusion (p<0.05). Wilcoxon text for related samples, corrected for ties.

### Discussion

The present results show that infusion of OT in the A1-area of the brainstem induces an elevation of the VP values in the plasma, levels of OT remaining unaffected. The specificity of this effect was indicated by the fact that VP infusion in the A1 area did not result in any changes in hormonal VP release, while the OT antagonist MEAV appeared to be effective in blocking the response to 100 pg OT. Since the localization of the cannula had to be very accurate in order to obtain a response, it can be concluded that diffusion of this peptide cannot account for the described effect of OT and that it is specific for the A1-area.

OT gives hormonal VP-release in quite a narrow range of concentrations: while administration of 100 and 200 pg results in a fourfold rise, no significant changes can be detected after infusion of 10 pg and 1 ng, albeit that with 10 pg several plasma values rise well above the detection level.

These data demonstrate that with this technique of administration only nanomolar concentrations of OT are able to induce changes in peripheral VP release. They indicate that in order to determine the functions of OT in the central nervous system the choice of the concentration of OT can be a very critical condition. This was already recognized by several authors who applied OT and VP in the brain and demonstrated a decrease in effect or an appearance of non-specific changes when higher doses of peptide were administered (Albers et al., 1986; Riphagen and Pittman, 1986). In contrast to these studies, others, which exposed the dorsal motor nucleus of vagus to OT, showed similar effects with concentrations ranging from $10^{-5}$ to $10^{-7}$ M (Raggensbass et al., 1987; Rogers and Hermann, 1987). It must be noted, however, that the use of different administration-techniques and volumes may define what concentration of the peptide is reached at the receptor-level.

Since it has been described that stimulation of VP receptors has an effect opposite to that of OT receptors in behavioural test situations (Bohus et al., 1978), the lack of effect with 1 ng OT in this physiological experiment could probably be attributed to the binding of OT to VP receptors when it is administered in this concentration. Therefore, it was investigated whether the addition of the VP antagonist MEAV to 1 ng OT, in order to block the VP receptor, could result in a regaining of the rise in plasma VP. No rise in VP was found, however. As could be expected (Audiger and Barberis, 1985; Mühlethaler et al., 1983; Manning and Sawyer, 1983), MEAV appeared to be unsuitable for investigation of this issue: this antagonist turned out to block OT as well as VP receptors, as can also be concluded from the absence of a plasma VP rise after infusion of 100 pg OT together with 100 pg MEAV.

As a consequence it is very difficult to get a clear picture of what will happen when higher doses of OT are infused in the A1-area. Preliminary results from infusions of OT and VP together indicate, however, that binding of VP to the VP receptor is able to inhibit the effect of OT. Therefore, an interaction of a high dose of OT with the VP receptor is presumably the cause of the observed lack of effect in this situation. In addition, these results indicate that in vivo OT is also able to bind to VP receptors as has been demonstrated for VP and OT receptors (Audiger and Barberis, 1985; Mühlethaler et al., 1983).

Light-microscopical observations indicated that a dense network of OT fibres is present in the NA A1-area and that these OT fibres possibly contact NA neurons. Also, in the present experiment it was demonstrated that OT fibres are indeed located in close apposition to NA-containing cell bodies, which is in agreement with previous observations (Sladek and Sladek, 1983). A simultaneous study at the electron microscopical level shows that there are indeed synaptic contacts between OT and NA-immunoreactive structures in the A1-area (Buijs et al., in preparation). In vitro studies indicated that it is possible to induce a Ca$^{2+}$ dependent release of this peptide in areas where these OT fibres terminate synaptically by means of potassium depolarization (Buijs and Van Heerikhuize, 1982). Electrophysiological studies showed that OT application in the brain stem of the rat has an excitatory influence on the largest part of the neurons (Charpak et al., 1984; Raggensbass et al., 1987). Moreover, electrical stimu-
lation of this area in the brain stem resulted in an increase in firing of VP neurons in the SON, an effect which disappeared after lesioning with 6 hydroxydopamine (Day et al., 1984; Day and Renaud, 1984). Along with the necessity of precise placement of the cannula in the A1-area to obtain an effect, all these observations indicate that the present results can be ascribed to simulation of neurotransmitter effects on A1 NA neurons.

The NA projections of the A1-area to the magnocellular VP neurons in the PVN and SON are involved in relaying cardiovascular information, which is carried to the brain at the nucleus tractus solitarius (NTS) via the ninth and tenth nerve; stimulation of these nerves appeared to affect more than half of the A1 neurons projecting to the hypothalamic neurosecretory cells (Ciriello and Caverson, 1984a,b). Furthermore, inhibition of neuronal function in the region of the A1-area resulted in abolishment of the rise in plasma VP in response to haemorrhage, indicating that the A1 area is essential for the baroreceptor-initiated hormonal VP release (Blessing and Willoughby, 1985).

The stimulation of VP release by OT infusion in the A1 area found here raised the question whether under circumstances of activation of the A1-area, for instance during haemorrhage, at least part of this activation might be due to OT neurotransmission. Therefore an attempt was made to inhibit OT neurotransmission with the aid of the OT antagonist MEOV and blood volume was subsequently reduced to activate the baroreflex pathway. This experimental procedure did not result in a delay of a rise of plasma VP, suggesting that in the A1-area OT does not play a major role in the stimulation of VP release under these circumstances. However, although the MEOV antagonist is able to block the effect of exogenously applied OT (Fig. 4), it is not certain whether this antagonist will also be able to block the effect of endogenously released OT. Yet, Rogers and Hermann demonstrated the effectiveness of an OT antagonist similar to the one used in our study by showing inhibition of the effect of PVN stimulation on gastric and cardiovascular parameters when this antagonist was simultaneously injected in the dorsal motor nucleus of the vagus (Rogers and Hermann, 1986, 1987). Consequently, these results suggest that, under normal circumstances, OT neurotransmission will not influence the baroreflex-release of VP. Most likely a direct projection from the NTS to the A1-area (Ciriello and Caverson, 1986; Ross et al., 1985), instead of the pCPVN being interconnected, will be far more important for the initiation of this VP release. Nevertheless, it is likely that OT neurotransmission, initiated by the right physiological conditions, will be able to increase the release of peripheral VP considerably.

It remains a matter of future investigation under which physiological conditions OT derived from the pCPVN is released in the A1-area. Since it has been suggested recently that the ventral noradrenergic bundle is implicated in the stimulation of the release of the corticotrophin releasing factor (CRF) (Guillaume et al., 1987), maybe, along with the stimulation of peripheral VP release, OT may enhance CRF release as well. Consequently, OT in the A1-area might be implicated in the control of ACTH release. Since pCPVN neurons projecting to the spinal cord provide axon collaterals to the dorsal vagus complex (Swanson and Kuyper, 1980) it seems likely that OT neurons sending their projections to the A1-area may also innervate portions of these brain regions. This organization would provide an anatomical basis for the simultaneous release of OT in the A1 area and other regions in the brain stem, leading to an integrated physiological response.

The only way to get a better understanding of the significance of OT in the brain stem may be to investigate what parameters are changed by OT application to these other sites, in order to acquire knowledge of the physiological consequences of stimulation of the OT neurons in the pCPVN. This, in turn, may give rise to speculations concerning the events that influence the excitability of those OT neurons.

Acknowledgements

The authors wish to thank Dr Maurice Manning (Dept. Bioch. Med. College Ohio, Toledo, USA) for his generous gift of vasopressin and oxytocin antagonists, Professors D. F. Swaab and L. P. Renaud for their stimulating discussions, and Mr A. Jansen for correcting the English text and secretarial assistance. This investigation was supported by the European Community twinning contract No. ST2 00271.

Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Meaning</th>
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<tr>
<td>AVP</td>
<td>arginine vasopressin</td>
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<td>CRF</td>
<td>corticotropin releasing factor</td>
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<td>CVLM</td>
<td>caudal ventrolateral medulla</td>
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<td>MEAV</td>
<td>vasopressin antagonist</td>
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<td>MEOV</td>
<td>oxytocin antagonist</td>
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<td>NA</td>
<td>noradrenalin</td>
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<td>OT</td>
<td>oxytocin</td>
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<td>pCPVN</td>
<td>paraventricular neurons of the paraventricular nucleus of the hypothalamus</td>
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<td>PVN</td>
<td>paraventricular nucleus of the hypothalamus</td>
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<td>RIA</td>
<td>radioimmunoassay</td>
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<td>SON</td>
<td>supraoptic nucleus</td>
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<td>VP</td>
<td>vasopressin</td>
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References


