OXYTOCIN LOCALIZATION AND FUNCTION IN THE A₁ NORADRENERGIC CELL GROUP: ULTRASTRUCTURAL AND ELECTROPHYSIOLOGICAL STUDIES

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Abstract—Antibodies to oxytocin and noradrenalin were utilized in an immunocytochemical study of the caudal ventrolateral medulla of the rat brainstem. Noradrenalin was visualized by using antibodies to noradrenalin and by means of a silver–gold intensification of diaminobenzidine, whereas oxytocin could be demonstrated in the same section by using the diaminobenzidine precipitate as a marker. At the light microscopic level, oxytocin fibers were densely distributed around the A₁ cell bodies. At the ultrastructural level, oxytocin-containing fibers were seen to terminate synaptically onto noradrenalin-containing neurons. Previous studies have shown that electrical stimulation of A₁ neurons selectively activates vasopressin-secreting neurons in the supraoptic nucleus. Therefore, separate electrophysiological studies were set up, in which we observed that oxytocin infusions (100–200 pg) into the A₁ area enhanced the activity of 16 out of 19 putative vasopressin-secreting neurons and elicited no response from any of 10 oxytocin-secreting neurons.

This finding suggests that some of the parvicellular neurons in the paraventricular nucleus of the hypothalamus, from which the A₁ neurons derive their oxytocin innervation, can activate the A₁ cell group via this peptidergic neurotransmitter. One of the consequences of A₁ neuronal activation is enhanced firing of hypothalamic supraoptic (and paraventricular) vasopressin-secreting neurons, and a consequent rise in plasma vasopressin.

The paraventricular nucleus of the hypothalamus (PVN) consists of both magnocellular and parvicellular subdivisions, each with its specific projections.^{35,37} Magnocellular PVN neurons (PVNmc), together with the magnocellular cells in the supraoptic nucleus (SON), synthesize either vasopressin or oxytocin and find their target site mainly in the neurohypophysis, where these peptidergic hormones are released into the systemic circulation. Hormone release occurs in proportion to the pattern and level of activity of these neurons, features that in turn are partly determined by their synaptic innervation.²⁴ Of relevance here is a catecholaminergic input derived from the noradrenergic A₁ cell group in the caudal ventrolateral medulla^{9,29,30,38} which is particularly prominent around vasopressin-positive neurons.^{19,36} It is notable that stimulation of the A₁ area of the brainstem selectively excites putative vasopressin-secreting neur-

ons in SON and PVNmc^{10,11} and releases vasopressin into the systemic circulation.⁴ Since exogenously applied noradrenalin induces an A₁-mediated increase in excitability from SON vasopressin-secreting neurons^{12,22} and prompts the release of vasopressin *in vitro*^{2,3} and *in vivo*,⁴² it would seem that the A₁ noradrenergic neurons play a facilitating role in the release of vasopressin into the general circulation.

Neurons in at least one of the parvicellular divisions of the PVN (PVNpc) project to areas of the brainstem and spinal cord that regulate autonomic outflow.^{16,18,29,31,35,37} For some of these descending PVN projections the identity of the transmitter may be suspected on the basis of immunocytochemical data.^{29,33} One candidate is oxytocin, which is present in PVNpc neurons and in axon terminals in specific areas of the brainstem.^{6,41} The observation of a dense plexus of oxytocin-positive fibers in the A₁ (and A₂) areas suggests that oxytocin might be able to influence the excitability of noradrenergic cell bodies.^{7,32}

In the present study, anatomical and electrophysiological approaches were utilized to evaluate the distribution and function of oxytocin in the caudal ventrolateral medulla (VLM) area. We used antibodies to noradrenalin and oxytocin in a double labeling method to determine at the ultrastructural level whether oxytocin-immunoreactive axons termi-

Abbreviations: DAB, diaminobenzidine; NTS, nucleus of the solitary tract; PAP, peroxidase–antiperoxidase; PVN, paraventricular nucleus of the hypothalamus; PVNmc, paraventricular nucleus magnocellular part; PVNpc, paraventricular nucleus parvocellular part; SON, supraoptic nucleus; TBD, Tris buffer containing 1% Na₂S₂O₅, pH 7.4; TBS, Tris buffer + 0.9% NaCl, pH 7.4; TBSG, TBS + 0.2% gelatin, pH 7.5; VLM, ventrolateral medulla.

Fig. 1. Two serial transverse sections (thickness 30 μm in the A₁ area of the rat brain stem). Scale bar = 100 μm . The section in (A) is stained for noradrenalin and shows noradrenalin-containing cell bodies (arrows) and fibers in the A₁ area. The consecutive section (B) is stained for oxytocin and shows, in the same area, a dense innervation with oxytocin-containing fibers in the same region as where noradrenergic cell bodies are located (arrows), suggesting a putative interaction between oxytocin and noradrenergic neurons. Note that oxytocin fibers, too, are present in the region where no noradrenergic neurons were found in the previous section.

not hampered when a glutaraldehyde fixative of pH 4 was used. Unfortunately, the need to use the detergent Triton X-100 in order to visualize oxytocin fibers

disrupted the quality of the ultrastructural morphology. The fixation at pH 4 yielded slightly inferior results with respect to ultrastructural preservation

Fig. 2. Oxytocin-containing terminals that can be recognized by the dark DAB-stained dense core vesicles (arrows), making a synaptic contact with a noradrenalin-containing cell body (A, B) or dendrite (C; arrows). (A) Not only the labeled oxytocin dense core vesicles, but also unstained non-oxytocin ones (thick arrows). The noradrenalin labeling is characterized by the presence of silver-gold deposits spread over the DAB precipitate in the noradrenergic neuron. (C) An oxytocin-containing axon (arrows) running close to a noradrenergic dendrite and forming a synaptic contact at the site of the axonal bouton (double white arrows). Scale bars = 0.5 μ m.

Fig. 3. Oxytocin-containing terminal adjacent to a noradrenergic dendrite, but forming a synaptic contact with an unlabeled neuron opposite the noradrenergic dendrite (arrow). Scale bar = 0.5 μ m.

Fig. 4. Noradrenalin-containing terminal forming a synaptic contact with a noradrenalin-containing dendrite in the A₁ area of the brainstem. The noradrenalin staining can be recognized by the presence of gold-silver deposit on the DAB precipitate (arrows). Scale bar = 0.5 μ m.

as compared with the fixation at pH 7. However, this was more than compensated for by a higher frequency in the detection of oxytocin-positive terminals.

In control sections that were processed without the use of oxytocin antiserum and were consequently subjected to a second DAB staining, noradrenergic neurons were always marked through gold labeling. No DAB-positive structures could be observed without silver-gold precipitate. Within the DAB-positive structure, however, the gold labeling varied from light to very dense, usually depending on the depth at which the positively stained structures were found (Figs 2 and 3). Noradrenalin-containing axons were frequently found to be in contact with noradrenalin-containing cell bodies or dendrites throughout the entire A₁ area (Fig. 4).

Oxytocin was found only in the surface layers of the tissue section, while the noradrenalin staining penetrated to greater depths. The oxytocin terminals were usually characterized by DAB deposits over dense core vesicles with a diameter of approximately 100 nm (Figs 2 and 3). From rostrally to caudally in the A₁ area, oxytocin fibers were found to be in synaptic contact with noradrenalin-positive neurons. Oxytocin-positive terminals were found to form synaptic junctions both with noradrenergic dendrites and with noradrenalin-stained cell bodies. Here, too, the presence of a dense gold precipitate in the noradrenergic neuron often prevented the detection of

synaptic specializations. In order to obtain an impression of the frequency at which oxytocin forms (synaptic) contacts with noradrenalin-positive neurons as compared with non-labeled neurons, the frequency of such synaptic structures was counted in a number of sections. Thus, it was found that most of the oxytocin-positive terminals were in apposition to noradrenalin-positive profiles, while the majority of the oxytocin terminals seen on cell bodies contacted a noradrenalin-positive cell body (Table 1). In addition, oxytocin terminals were mostly detected on noradrenalin-containing dendrites. In one case an oxytocin-containing terminal in apposition to a noradrenalin-containing neuron formed a synaptic contact with an unlabeled structure at the opposite site (Fig. 3). More frequently, however, oxytocin-containing axons were found to run along a noradrenalin-containing dendrite forming a synaptic contact at the site of the swelling (Fig. 2A-C).

Response of supraoptic nucleus neurons to oxytocin infusions in the A₁ area

Data were obtained from a total of 29 SON neurons. In 11 experiments where histological reconstructions revealed that the tip of the VLM cannula coincided with the location of the A₁ noradrenergic neurons, oxytocin infusions were accompanied by an increase in the activity of 16 out of 19 vasopressin-secreting neurons (Figs 5 and 6) with a peak response of 70–200% enhancement in instantaneous firing

Fig. 6. (A) The upper pair of traces represent arterial pressure tracings (above) and ratemeter records from a continuously active supraoptic neuron designed as vaso-pressin-secreting on the basis of a transient suppression in firing during a metaraminol (Met)-induced rise in arterial pressures. Note that this cell's overall activity is reversibly enhanced during an infusion of oxytocin (100 pg total) into the A₁ area. (B) A similar pair of records from an oxytocin-secreting supraoptic cell during a metaraminol-induced rise in pressure and oxytocin infusion into the A₁ area. Note that the ratemeter record shows no response to either procedure.

catecholamine ring structure by reductants such as Na₂S₂O₅ required the visualization first of noradrenalin and subsequently of oxytocin. Since both antisera employed were raised in rabbits, no combination of antisera during the first incubation could be used. Finally, the best results were achieved by using a modified procedure of Gallyas,^{14,39} the major change being the moment and duration of the application of thioglycolic acid. Since it was found that the use of thioglycolic acid hampered the recognition of oxytocin, it was investigated whether this was the case with antibodies already attached to oxytocin. Thus, it was found that thioglycolic acid treatment after the first or second antibody incubation hardly hampered the recognition of the rabbit anti-oxytocin antibody by the goat anti-rabbit serum or that of the second one by the PAP complex. This may be a useful observation for other double staining procedures, by which the antigens are destroyed by the thioglycolic treatment. However, whether all secondary antibodies can stand this treatment is unclear at present. In addition, it was found that incubation with thioglycolic acid for 4 h at room temperature was sufficient to reduce the non-specific silver-staining.

Although no detergent is needed for the detection of noradrenalin, the disadvantage that Triton X-100 is required to visualize oxytocin⁴¹ decreased the ultrastructural preservation of the tissue. Unfortunately, postembedding staining using gold-labeled secondary antibodies could not be employed using the present antibodies.

Terminals in the A₁ area

A large number of oxytocin-positive profiles could be visualized, about 15% of which were found to terminate synaptically on noradrenalin-positive profiles. The (anecdotal) observation of an oxytocin-containing terminal in apposition to a noradrenalin-containing neuron, but forming a synaptic contact with another unlabeled structure, warrants caution in drawing conclusions regarding the site of termination on the basis of light microscopical observations only. The frequency of synaptic termination in randomly selected sections indicates that most oxytocin-containing fibers will form a synaptic contact within the A₁ area. The largest part of these fibers was found to be in apposition to noradrenalin-positive profiles, thus also indicating that most oxytocin fibers do contact noradrenalin-containing neurons in the A₁ area. For the evaluation of these data one has to take into account that the employed procedure favored the visual detection of oxytocin-containing structures in the middle of other unstained structures while dark oxytocin-positive profiles close to dark noradrenalin-positive profiles could be more easily overlooked. This indicates that the procedure applied slightly overemphasized the number of oxytocin contacts to non-noradrenalin-stained neurons. In addition, since it is evident that in the current immunocytochemical electron microscopic procedure only part of the oxytocin and part of the noradrenalin-containing structures are visualized, it is clear that with the counting of these oxytocin profiles only an impression of the synaptic termination frequency and the postsynaptic structures is obtained.

Functional implications

Given the fact that a synaptic interaction between oxytocin terminals and noradrenergic neurons can be firmly established at the ultrastructural level, the question arises as to the putative functional significance of this finding. It has been demonstrated that the noradrenergic neurons of the A₁ area preferentially project onto vasopressin-containing magnocellular cell bodies in the SON and PVN.^{9,28} Stimulation in the A₁ area resulted in an increase of firing of vasopressin and not oxytocin cells in the magnocellular SON and PVN.^{10,11} In addition, further pharmacological studies indicated that in this pathway noradrenalin serves as an excitatory transmitter on magnocellular vasopressin-containing neurons.¹² The visualization of dense oxytocin innervation in the A₁ area prompted us to investigate a possible action of oxytocin in this circuit. Recently, minute amounts of oxytocin were infused in the A₁ area; in simultaneously withdrawn blood samples an increase of vasopressin and not oxytocin levels could be measured immediately after the oxytocin infusion.¹⁵ According to the present results, it would seem that oxytocin infusions cause activation of A₁ neurons, which then lead to a selective excitation of vaso-

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