Immunocytochemically stained binding sites for oxytocin and α -melanocyte-stimulating hormone in rat brain following ventricular administration

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Binding sites for oxytocin (OXT) and α -melanocyte-stimulating hormone (α -MSH) in brain of homozygous Brattleboro rats were immunocytochemically visualized after ventricular administration of the peptides by Accurel implants. Two patterns were found: (1) 'ring type' staining in perineuronal structures was observed in CA1 and CA3 areas of ventral hippocampus and in subiculum for OXT implanted brains and a very weak staining in striatum for α -MSH-implanted brains; (2) cytoplasmic staining of intracellular binding sites was observed in the bed nucleus of the stria terminalis (BST) in brains with OXT implants and in the anterodorsal thalamic nucleus (AD) and postcingulate cortex in brains with α -MSH implants. These localizations are different from those described for vasopressin binding sites in the same rat strain.

Oxytocin (OXT) is produced by the magnocellular neurons of the supraoptic and paraventricular nuclei²⁶. In addition to its peripheral endocrine functions, OXT is involved as a neurotransmitter or neuromodulator in regulating central processes such as maternal behavior, avoidance behavior and blood pressure⁵. Recent light-microscopic autoradiographic studies reported high affinity binding sites for OXT in various regions of the rat brain^{3,6,10,31}.

α -MSH is synthesized predominantly in the intermediate lobe of the pituitary, but also by neurons in the arcuate nucleus of the hypothalamus and in the dorsolateral hypothalamus³² which send axonal projections to many brain areas^{9,30,33}. α -MSH-like compounds have been found to be present throughout the nervous system of the rat^{27,28}, whereas binding sites for α -MSH have been described only in two studies using autoradiography^{12,19}.

Recently, we introduced a new immunocytochemical technique for the localization of vasopressin (VP) binding sites in rat kidney and brain^{21,22}. This technique yields a better resolution than the autoradiographic procedures and is based upon in vivo administration of the ligand, using Accurel polymer

tubing for continuous release of the peptide. In the present study, the same procedure was applied to localize OXT and α -MSH binding sites.

Eighteen male Brattleboro (*di/di*) rats, homozygous for diabetes insipidus, 3 months of age, were obtained from Central Animal Supply House, TNO (Zeist, The Netherlands). Since these VP-deficient rats were used in a previous study to avoid cross-reactive immunostaining of the antisera used with endogenous VP, they were also used in the present study to compare the various localizations of binding sites for the different peptides. Continuous release of OXT or α -MSH into the cerebrospinal fluid (CSF) was obtained by the miniaturized controlled-drug-delivery 'Accurel' technique^{2,13} in a way similar to that described in our study on VP binding sites²². Unilateral Accurel implants in the lateral ventricle contained either 15 μ g OXT ($n = 5$), α -MSH ($n = 5$), or water (controls, $n = 5$). The following preparations of peptides were used: oxytocin (Sigma, St. Louis, grade X, 540 IU/mg); α -MSH (WB 1138 K1, kindly donated by Dr. H. Greven, Organon, The Netherlands). In addition to the control group that was implanted with water-filled tubing, non-implanted

rats ($n = 3$) were used. Two weeks later, rats were perfused intracardially with 2.5% glutaraldehyde and 1.0% paraformaldehyde and the brains were prepared for immunocytochemical staining. Transverse free-floating vibratome sections were used as previously described for VP implants²² using 1:1000 dilutions of antibodies directed against OXT (0-1-V, 4.4.75) or α -MSH (4394, 23.4.75). No loss of anti-OXT or anti- α -MSH binding capacity was observed, even after 12 months of storage of the brains in fixative. Specificity of the staining in vivo bound OXT and α -MSH was performed by (1) control staining

with preimmune serum (Ducky 1:1000) or by (2) staining with OXT-antiserum preadsorbed with VP²⁰⁻²².

The well-known staining pattern of OXT cells in the supraoptic and paraventricular nuclei²⁶ and OXT fiber staining in the medial septum and median eminence⁴ were present in all the examined brains, as was the staining of α -MSH neurons previously described in the arcuate nucleus^{9,30,32,33}. This staining of endogenous OXT or α -MSH is not discussed in further detail. Only additional staining to that seen in untreated controls was regarded as indicating bind-

Fig. 1. Immunocytochemical staining of in vivo bound OXT or α -MSH in vibratome sections (50 μ m) of Brattleboro *dd/di* rat brain. V, ventricle; PVA, paraventricular thalamic nucleus anterior; sm, stria medularis; AD, anterodorsal thalamic nucleus. The broken line in 1c indicates the midline. Perineuronal staining of binding sites for OXT, surrounding CA1 pyramidal cells of ventral hippocampus (a) and cytoplasmic staining in cell bodies of the bed nucleus of the stria terminalis (b) after ventricular administration of OXT. Cytoplasmic binding sites for α -MSH were observed in the anterodorsal nucleus of the thalamus (c) and postcingulate cortex (d) after α -MSH administration. Calibrations: 10 μ m for (a,d) and insert in (b); 25 μ m for (b) and insert in (c); 100 μ m for (c).

ing sites. Binding sites were revealed bilaterally in various brain regions in two distinct patterns: (1) in perineuronal structures and (2) within cell bodies. Perineuronal OXT binding sites surrounded the pyramidal cells of CA1 and CA3 areas in ventral hippocampus (Fig. 1a) and in subiculum. For α -MSH, a very weak perineuronal staining was observed in striatum. Cytoplasmic binding sites for OXT were present in the bed nucleus of the stria terminalis (BST) in both lateral and medial parts (Fig. 1b). Intracellular binding sites for α -MSH were found in anterodorsal thalamic nucleus (AD) (Fig. 1c) and postcingulate cortex (Fig. 1d). No such staining patterns were seen either in the control brains implanted with empty tubings or in brains with OXT or α -MSH implants stained with control sera.

The OXT binding sites localization agrees with autoradiographic and biochemical data concerning the ventral hippocampus, subiculum and BST^{1,6,10,31}. The distribution pattern is also partly congruent with innervation sites for OXT, concerning the hippocampus and BST^{4,23} as has also been reported for autoradiographic localization of OXT binding sites. OXT affects maternal behavior¹⁸ and the presence of binding sites for OXT in the BST agrees with reported involvement of this brain region in maternal behavior¹⁷. The distribution of OXT binding sites was different from that of VP²² or α -MSH, which seems to rule out the possibility of non-specific binding of these peptides in the brain. In contrast to binding observed in brains with VP implants, no binding sites for OXT or α -MSH could be demonstrated e.g., in the lateral septum or the dentate gyrus of the hippocampus. Brainstem areas such as nucleus of the solitary tract and amygdala, which had been reported to contain high density of autoradiographically visualized VP binding sites^{6-8, 31} did not show binding sites staining of either OXT or α -MSH in the present study. Staining of OXT binding sites in ventral hippocampus and subiculum was more intense than VP binding sites²², which agrees with electrophysiological findings on OXT being more effective than VP with regard to increase in spontaneous activity of hippocampal neurons^{14,15,29}.

The very weak staining of α -MSH binding sites in striatum agrees with autoradiographic studies^{12,19} and with the presence of α -MSH-like compounds and low density of fibers^{11,27,28,30}. The very clear cytoplas-

mic staining of α -MSH binding sites in AD and in postcingulate cortex has not been described before. α -MSH was reported to be present in the thalamic dorsomedial nuclear group, ventromedial nuclear group, habenular nucleus and cingulate cortex^{9,30}. In addition, high density of α -MSH had been described in the paraventricular nucleus of the thalamus (PVA)¹¹. The present study strongly stresses the need to investigate in particular the involvement of the AD in α -MSH effects.

The concept of using immunohistochemistry to detect peptide binding sites is neither new nor without controversy²⁵. Estrogen as well as luteinizing hormone-releasing hormone receptors have been detected intracellularly in pituitary gonadotrophs and VP receptors were visualized in the kidney^{24,25}. Although there is some controversy concerning the question whether a peptide bound to a receptor can still be recognized by its antibody, all our controls show that this indeed is the case for the neuropeptides studied. Moreover, the high resolution of the immunocytochemical procedure enabled us to show two types of localizations for the peptidergic binding sites. Perineuronal staining probably represents postsynaptic binding sites on cell membranes. Whether the perineuronal binding sites are indeed related to synapses, still remains to be established by immunoelectron microscopy. The cytoplasmic binding sites for OXT and α -MSH are a new finding and may represent intracellular peptidergic receptor sites. Internalized ligand-receptor complexes have been suggested, e.g., for rat renal and urinary bladder VP receptors¹⁶ but have not yet been described with respect to the brain. On the other hand, the possibility that the intracellular staining represents newly synthesized receptor molecules cannot be excluded.

Concluding that the specific binding sites for OXT and α -MSH that we have detected are true physiological receptors is premature. However, since OXT and α -MSH binding sites stained by the present procedure are present in regions known to mediate central effects of the peptides and agree well with autoradiographic data, this might well be the case.

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