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ARGININE-VASOPRESSIN BINDING SITES IN RAT BRAIN: A QUANTITATIVE AUTORADIOGRAPHIC STUDY

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Specific binding sites for arginine-vasopressin (AVP) were detected in rat brain after incubation of tissue sections with [^{3}H]AVP. AVP and two selective AVP antagonists are capable of displacing [^{3}H]AVP with an IC_{50} in the 10^{-8}–10^{-7} molar range, while oxytocin and $ACTH_{4-10}$ were much less effective. The neuroanatomical distribution of [^{3}H]AVP-labeled sites was studied with autoradiography utilizing tritium-sensitive LKB film and computerized densitometry for quantitative analyses of the film images. The highest density of [^{3}H]AVP binding sites was observed in hippocampal regions, the lateral septum, olfactory and amygdaloid nuclei, and the nucleus tractus solitarii (NTS) of the brainstem.

Arginine-vasopressin (AVP) influences memory processes [4, 6]. This action of AVP takes place in certain discrete limbic-midbrain regions [7]. AVP also acts on biochemical and electrophysiological processes in these areas. It was found that the peptide affects indices for catecholamine turnover in the hippocampal dentate gyrus, dorso-lateral septum and nucleus tractus solitarii (NTS) [7, 12], and alters the firing pattern of neurons in the lateral septum [5]. Immunocytochemical studies have demonstrated that these limbic-midbrain regions are innervated by AVP-containing neurons [3]. Recent advances in autoradiographic methods introduced by Kuhar and coworkers [8] have made it possible to study receptor distribution in vitro in brain sections. A modification using tritium-sensitive sheet film has been introduced [9]. Based on this method preliminary reports have appeared showing that autoradiography of [^{3}H]AVP binding sites is feasable [2, 10, 13]. In the present

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study we used densitometry for quantitative evaluation for film images obtained by in vitro autoradiography of [^{3}H]AVP-labeled binding sites in rat brain.

Twelve male Wistar rats of our own breeding and two male Brattleboro rats homozygous for diabetes insipidus (Central Breeding Laboratories, TNO, Zeist, The Netherlands) weighing 150–200 g at the time of sacrifice were used for the experiments. Light in the animal house was on from 05.00 to 19.00 h and the animals were sacrificed between 09.00 and 10.00 h by decapitation. The brains were rapidly removed from the skull and frozen in dry ice. Tissue preparation and autoradiographic procedures were performed according to previously published methodology [9]. For autoradiographic localization 32 μm sections were preincubated for 20 min at room temperature in a 0.17 M Tris buffer, pH 7.6, containing 4 mM $CaCl_2$. After drying, 10 nM [^{3}H]AVP (NEN, 42.1 Ci/mmol) in a total volume of 300 μl buffer (0.1 M Tris, 0.1% BSA, 0.5 mg/ml bacitracin, 10 mM $MgCl_2$) was applied to the sections and they were incubated for 30 min at room temperature followed by 2 $\times$ 4 min washes in Tris 0.17 M at 4°C and a short dip in distilled water to remove buffer salts. In adjacent sections 1 μM unlabeled AVP was included to correct for non-specific binding. Slides were dried at 60°C and opposed to LKB ultrofilm for 1 month. Eight tissue standards containing known amounts of tritium-labeled leucine per mg protein were exposed alongside the samples. Following standard developing and fixing procedures the autoradiograms were examined with an IBAS-2 image analysis system (Kontron, München, F.R.G.). The images of the autoradiograms of both one section and the set of tissue standards were simultaneously registered by a videocamera equipped with a Plumbicon tube. The image is digitalized and stored in one of the image memories (resolution 512 $\times$ 512 pixels). The areas to be measured, including the background, were delimited interactively. After subtraction of the background value, the determined optical density values were expressed as values in fmol/mg protein by recalculation on the basis of the optical density data each time obtained from the set of tissue standards.

Optimal conditions of the binding were determined on sections which were removed for scintillation counting. The primary goal was to examine the competition of endogenous AVP for the externally applied [^{3}H]AVP. Two methods were employed to circumvent this potential source of errors. Firstly, a pre-incubation of the tissue section for 20 min at room temperature in the presence of Ca^{2+} and absence of peptidase or protease inhibitors to promote release and degradation of endogenous AVP. Secondly, the use of Brattleboro rats homozygous for diabetes insipidus, who lack endogenous AVP. While pre-incubation had no effects on displaceable binding on brain sections of Brattleboro rats, which exhibited about 32% specific binding, in normal rats preincubation (20 min) increased specific binding from 4% to more than 30% of total binding (hippocampal coronal level). We concluded that a 20 min pre-incubation is sufficient for removal of endogenous AVP and this interval was adopted in further experiments.

For characterization of the binding site specificity related and unrelated peptides

were tested for competition with 10 nM [^{3}H]AVP for its binding sites. Of the compounds tested, AVP and the AVP antagonists [1] dPTyr(Me)-AVP (AAVP^a) and d(CH$_2$)$_5$Tyr(Me)AVP (AAVP^b) exhibited IC_{50}s in the 10^{-7} and 10^{-8} molar range $ACTH_{4-10}$ and oxytocin were competing only in the μmolar range (cf. IC_{50}s of AVP, AAVP^a and AAVP^b: 30, 190 and 60 nM respectively).

Fig. 1 shows examples of the localization of [^{3}H]AVP binding sites in coronal sections of the rat brain. The concentration of binding sites in a number of regions is summarized in Table I. The highest density of specific binding sites was observed in the ventral hippocampus, the CA3 pyramidal cells of the hippocampus, the olfactory nucleus, lateral septum and NTS. A considerable amount of binding sites was also observed in the gyrus dentatus, hippocampal molecular layer, the CA1 and CA2 pyramidal cells, entorhinal cortex, central amygdaloid nucleus, medial geniculate nucleus, and substantia nigra. Striatal, hypothalamic, thalamic and medullary brain regions in general contained low amounts of [^{3}H]AVP-labeled binding sites.

The present study shows that the rat brain contains high affinity binding sites labeled with [^{3}H]AVP. The distribution of binding sites is uneven in the brain with highest density in extrahypothalamic limbic brain regions. The displacement of [^{3}H]AVP by excess unlabeled AVP in regions rich in binding sites was 50–60%, which exceeds very well the 30–40% displacement observed after scintillation counting of the labeled brain sections (this study and refs. 2 and 10). Baskin et al. [2] and Van Leeuwen and Wolters [10] found displaceable binding in the dorsal septal area. In the first report [2], however, preincubation of the brain sections was not reported. This study clearly shows that preincubation is necessary to unmask AVP binding sites. Preincubations were used in the study of Yamamura et al. [13], but occurred in the presence of bacitracin. In our study the use of bacitracin in the preincubation was avoided since it would prevent degradation of endogenous AVP. During the labeling of the binding sites with [^{3}H]AVP, there was however bacitracin in the medium and HPLC analyses showed that there were no conversion products of the peptide detectable.

The highest concentrations of specific [^{3}H]AVP binding were found in hippocampal, septal, amygdaloid and olfactory regions and the NTS. The hippocampal, septal and amygdaloid regions have been demonstrated to respond to microinfusion of AVP and related peptides as measured by indices of behavioral performance [4] and catecholamine neurotransmission [7, 12]. The NTS has a role in blood pressure regulation. Effects of AVP have been described in autonomic regulation of blood pressure [11] and the NTS is densely innervated by AVP fibers [3]. The sites tested at the coronal level of the dorsal hippocampus display high affinity to AVP and two selective AVP antagonists. [^{3}H]AVP is, however, not displaced by a 100-fold excess of oxytocin. It is therefore tempting to speculate that the binding sites visualized, quantified and partially characterized in the present study represent an AVP receptor system.


A
B
C

TABLE I

DENSITY (in fmol/mg protein) OF SPECIFIC BINDING SITES LABELED WITH 10 nM [^{3}H]AVP IN VARIOUS BRAIN NUCLEI

Brain region	Binding (fmol/mg protein)			% Displacement
	Total	Non-specific	Specific	
Ventral hippocampus	308 ± 28	130 ± 7	178	57
CA3	263 ± 16	109 ± 6	152	58
Gyrus dentatus	291 ± 7	208 ± 9	83	28
Dorsal subiculum	187 ± 12	109 ± 9	78	41
Hippocampus molecular layer	194 ± 9	118 ± 7	76	39
CA1, CA2	208 ± 9	144 ± 12	64	30
Lateral septum	248 ± 7	144 ± 12	104	42
Medial septum	196 ± 16	154 ± 9	42	21
Central amygdala	251 ± 19	173 ± 19	78	32
Olfactory nucleus	331 ± 4	177 ± 19	154	46
Nucleus accumbens	210 ± 9	149 ± 9	61	33
Nucleus caudatus	165 ± 21	165	—	—
Parietal cortex	166 ± 9	113 ± 14	54	38
Entorhinal cortex	229 ± 23	142 ± 9	87	38
Substantia nigra	168 ± 4	85 ± 9	83	49
Medial geniculate nucleus	170 ± 5	99 ± 14	71	41
Dorsal raphe nucleus	90 ± 26	52	38	42
Medial raphe nucleus	75	85	—	—
Nucleus tractus solitarii A_1	227 ± 12	87 ± 26	140	61
Nucleus reticularis	85 ± 4	66 ± 7	21	22
Medulla oblongata pars ventralis	135 ± 11	78 ± 14	57	42

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Fig. 1. Distribution of binding sites for [^{3}H]AVP in the rat brain. A: at the septal level A = 8000 μm (König and Klippel), showing labeling of the lateral septum. B: at the hippocampal level A = 2800 μm, showing labeling of the dentate gyrus, ventral hippocampus, CA3, medial geniculate nucleus. C: at the hippocampal level A = 1600 μm showing labeling of the ventral hippocampus and dentate gyrus.

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