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Regional distribution of putative vasopressin receptors in rat brain and pituitary by quantitative autoradiography

(neuropeptides/peptide neuroregulator/proteolytic inhibition)

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ABSTRACT **Quantitative light microscopic autoradiography was used to map and characterize the distribution of [^{3}H]arginine vasopressin ([^{3}H]AVP) binding sites in the rat brain. HPLC analysis for possible degradation of AVP during binding indicated that addition of specific peptidase inhibitors prevented metabolism of AVP. Binding sites for [^{3}H]AVP were observed in the hypothalamus and pituitary as well as in brain regions where AVP may act as a neuroregulator. Within the hypothalamus, dense AVP binding sites were seen in the suprachiasmatic, supraoptic, and paraventricular nuclei. High specific binding was also apparent in the median eminence tubero-infundibular region and in the posterior lobe of the pituitary. [^{3}H]AVP labeling at possible neuroregulatory sites was observed in the hippocampus, lateral septum, superficial cortex, cerebellum, nucleus tractus solitarious, adenohypophysis, and spinal cord.**

Vasopressin, a neuropeptide with well-characterized peripheral endocrine functions, has been shown to significantly enhance memory processes (1–4). Evidence for central nervous system (CNS) localization of arginine vasopressin (AVP) was first documented by immunohistochemical (5–7) and radioimmunological findings (8–10). These initial studies showed AVP to be present in cell bodies and nerve terminals within the hypothalamus while present only synaptically in extrahypothalamic areas. Very recent studies, however, have described AVP immunoreactive cell bodies outside the hypothalamus, in the bed nucleus of the stria terminalis (11, 12).

Evidence for a neuroregulatory effect of AVP has come from electrophysiological, pharmacological, and behavioral studies. Neuronal depolarization following exposure to AVP has been observed in hypothalamic (13, 14), hippocampal (15), and identified invertebrate neurons (16). Vasopressin has also been shown to increase catecholamine turnover (17, 18) as well as to enhance catecholamine activation of adenylate cyclase (19, 20). Increased memory for learned behaviors, following treatment with AVP, has been observed in rodents (1, 2), primates (3), and humans (4).

Our work has focused on the hypothesis that AVP acts as a neuroregulator in the CNS. To confirm this postulate, it is essential to demonstrate the existence of specific AVP binding sites in the CNS. Our initial report of specific AVP binding sites in brain (21, 22), which has been observed and characterized in several other laboratories (23–27), led us to undertake a more detailed investigation. Thus, we now report a comprehensive quantitative autoradiographic analysis of the regional distribution of putative AVP receptors in the rat brain and pituitary.

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MATERIALS AND METHODS

High-Pressure Liquid Chromatography (HPLC) Analysis. To determine if intact [^{3}H]AVP or a metabolite was binding to the receptor, HPLC analysis of extracted [^{3}H]AVP that had been incubated with synaptosomes from whole brain was performed. Synaptosomes were prepared from whole rat brains including pituitaries following the method of Gray and Whittaker (28). The incubation buffer, peptidase concentrations, and incubation conditions were the same as those described below for autoradiographic studies. [^{3}H]-AVP (10 nM) or AVP (100 μM) was incubated with 2 mg of tissue per ml for 60 min at 4°C in the presence or absence of peptidase inhibitors. Following incubation all samples were boiled (15 min) to cease enzymatic activity and then placed on ice for 30 min. Control samples containing peptidase inhibitors and [^{3}H]AVP or AVP but without tissue underwent the same procedures as experimental samples. Samples were then centrifuged at 1500 × *g* for 15 min and aliquots of the supernatant were frozen at −28°C until HPLC analysis.

HPLC separation and detection of AVP and possible metabolites followed the method described by Davis *et al.* (29). The HPLC system consisted of two model 6000 A pumps and a model 680 solvent programmer (Waters Associates). AVP and its metabolites were detected at 210 nm by using a Perkin–Elmer model LC-65T variable wavelength detector coupled to an Axxiom model 301-99 Datasaver and a Hewlett–Packard model 3390A recording integrator. Peptide fragments were separated on a Beckman Ultrasphere ODS 5-μm column using a linear gradient of acetonitrile against 0.1 M NaH_2PO_4 buffer (pH 2.2) from 15% to 30% over 40 min. The flow rate was maintained at 2.0 ml/min and the column temperature was 40°C. Fractions from [^{3}H]AVP samples were collected at 30-sec intervals. Radioactivity in 50-μl effluent aliquots was counted by liquid scintillation spectrophotometry following the addition of scintillation cocktail. Using radioactivity, the HPLC assay has a detection limit of <1 ng onto the column.

Quantitative Autoradiographic Studies. Male Sprague–Dawley rats (250–350 g, University of Arizona breeding colony) were killed by decapitation and the brains were removed immediately and placed on ice. Each brain was prepared for microtome sectioning by coating the brain with plastic embedding medium and freezing it onto a microtome chuck by immersion in 2-methylbutane cooled with liquid nitrogen. Preparation of brain sections for light microscopic autoradiography followed the method of Wamsley and Palacios (30). Ten-micron coronal or sagittal sections were cut on a cryostat microtome and thaw mounted on chrome alum/gelatin-coated slides. Slide mounted sections were stored desiccated overnight at 0°C.

Abbreviations: AVP, arginine vasopressin; CNS, central nervous system.

Prior to the labeling of brain sections with [^{3}H]AVP (51.0 Ci/mmol, New England Nuclear; 1 Ci = 37 GBq) serial sections were thawed and preincubated at room temperature in 20 mM Tricine (pH 7.4) containing 15% ethanol for 30 min. [^{3}H]AVP binding to paired brain sections was carried out in buffer containing 20 mM Tricine (pH 7.4) at 4°C, 0.2% bovine serum albumin, 40 μg of bacitracin per ml, 10 μg of aprotinin per ml, and 10 μM amastatin. A concentration of 5 nM [^{3}H]AVP was used to label AVP receptors, while various concentrations of unlabeled AVP (1 μM, 10 μM, 200 μM, or 1 mM; Bachem, Torrance CA) were used to estimate AVP-inhibitable binding. Incubations were carried out at 4°C for 60 min. The binding reaction was terminated by two 30-sec rinses in ice-cold 50 mM Tris·HCl buffer (pH 7.4) followed by a final rinse (1 sec) in distilled water. This rinse procedure was found to yield the best signal-to-noise ratio of specific to nonspecific [^{3}H]AVP binding. Slide mounted sections were then dried under a stream of cold dry air and stored desiccated overnight at 0°C.

Autoradiographs were prepared by exposing slide mounted sections to ^{3}H-sensitive film (LKB-Ultrofilm) in x-ray cassettes. Films were developed following exposure for 30 days. Photographic and densitometric analyses were performed on a Leitz Orthoplan microscope equipped with a DADS model 560 computer (Stahl Research Laboratories, Rochester, NY) interfaced with a MPV compact microphotometry system. Measurements of optical density were calculated in film areas equivalent to 150 or 250 μm^2. Femtomoles of [^{3}H]AVP bound per mg of tissue was calculated by comparing the autoradiographic grain density generated over each area of tissue with that produced by 10-μm thick [^{3}H]thymidine-labeled standards of brain tissue prepared as described (30). Specific binding ranged from 30% to 75% of total binding depending upon the brain region and the concentration of AVP used to inhibit [^{3}H]AVP binding.

RESULTS

Data from HPLC analyses showed that in the presence of several peptidase inhibitors there was no detectable degradation of unlabeled AVP or [^{3}H]AVP (Fig. 1 *A* and *B*). In contrast, there was marked breakdown of [^{3}H]AVP in the absence of peptidase inhibitors with or without tissue (Fig. 1 *C* and *D*).

Autoradiographic visualization and quantification of [^{3}H]AVP binding sites showed a regional distribution of receptors in the hypothalamo-hypophysial, limbic, and motor systems (Fig. 2 and Table 1). AVP binding sites were also observed in the suprachiasmatic nucleus (Table 1), cerebral cortex, anterior pituitary, nuclei involved with autonomic nervous system function, and the spinal cord (Fig. 2 and Table 1).

The highest density of [^{3}H]AVP binding was seen in the hypothalamo-neurohypophysial system, with the posterior

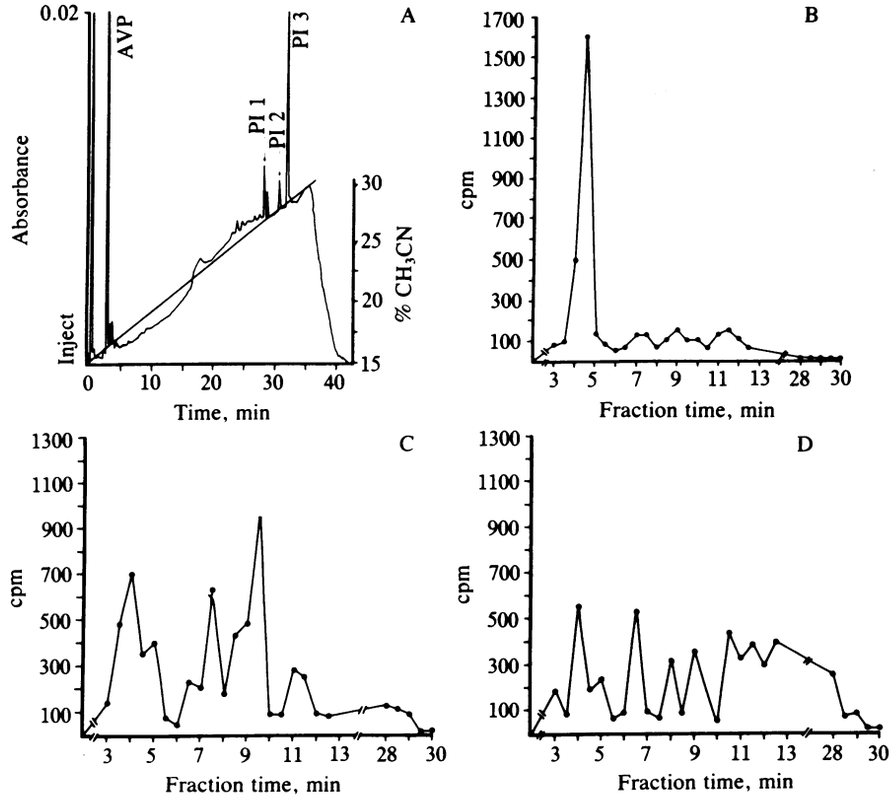

FIG. 1. (*A*) HPLC chromatogram of AVP incubated with protease inhibitors. The chromatogram shows separation of AVP and peptidase inhibitors, amastatin, aprotinin, and bacitracin (PI 1, PI 2, and PI 3). Peptides were separated on a Beckman Ultrasphere ODS 5-μm column (25 cm × 4 mm) using a linear gradient of acetonitrile (15–30%) against 0.1 M phosphate buffer delivered at 2.0 ml/min for 40 min at 40°C. Detection was UV at 210 nm, set at 0.02 absorbance unit full scale. Sample injected (10 μl) was equivalent to 1 μg of AVP. (*B*) HPLC radiochromatogram of [^{3}H]AVP incubated with tissue and protease inhibitors. The radiochromatograph shows the elution pattern of radioactivity associated with [^{3}H]AVP. Radioactive peak corresponds to exact retention time of unlabeled AVP. Sample injected (100 μl) was equivalent to 2 ng of [^{3}H]AVP. (*C*) HPLC radiochromatogram of [^{3}H]AVP incubated without tissue or protease inhibitors. The radiochromatograph shows marked degradation of [^{3}H]AVP incubated for 60 min at 4°C in the absence of both tissue and peptidase inhibitors. (*D*) HPLC radiochromatogram of [^{3}H]AVP incubated with tissue but without peptidase inhibitors. The radiochromatograph shows the elution pattern of radioactivity associated with [^{3}H]AVP incubated in the presence of tissue but in the absence of peptidase inhibitors. Again, significant enzymatic metabolism of the intact peptide occurred in the absence of peptidase inhibitors.

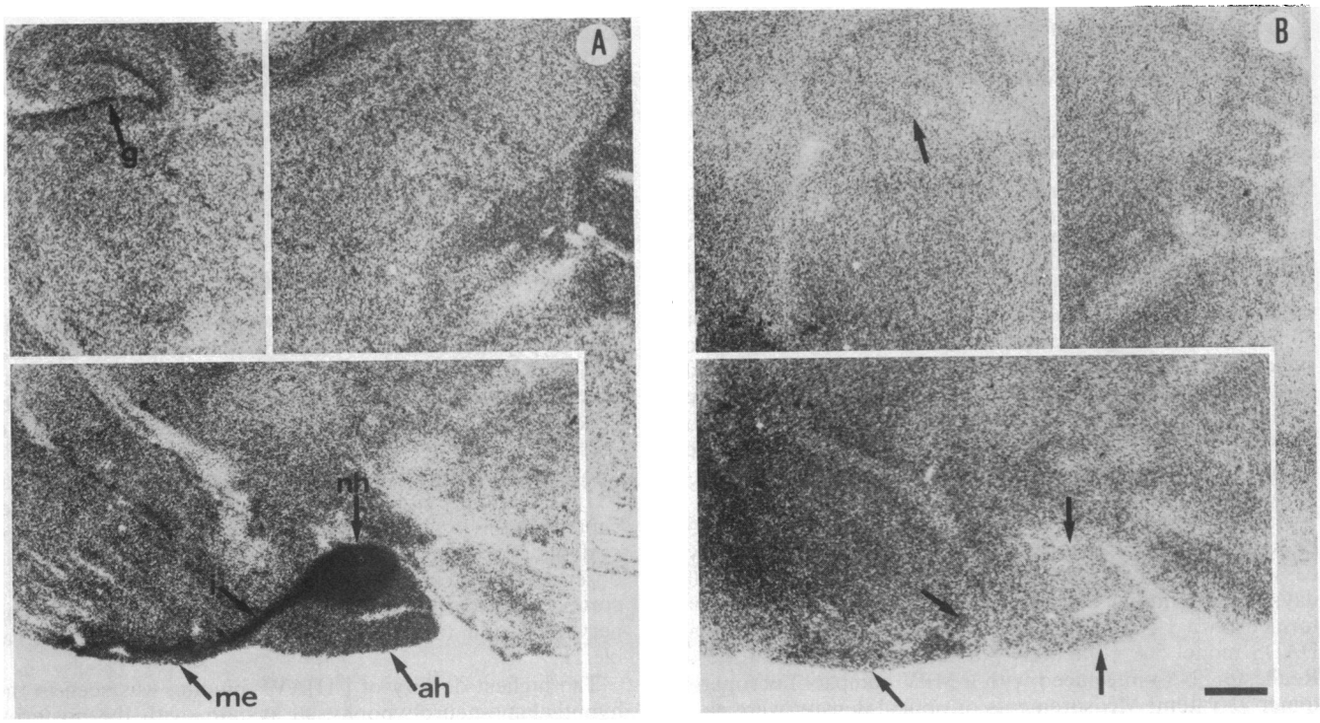

FIG. 2. Autoradiographic localization of receptor sites for [^{3}H]AVP. (*A*) Photomicrograph showing density and distribution of autoradiographic grains (black dots against a white background) as they appeared on the ^{3}H-sensitive film placed over a sagittal section, lateral 0.9 mm (31) of rat brain labeled with 5 nM [^{3}H]AVP. A dense accumulation of grains, representing [^{3}H]AVP binding, can be seen in regions corresponding to the neurohypophysis (nh), infundibular stalk (i), and median eminence (me). A relatively lower grain density appeared in the adenohypophysis (ah) and a markedly discrete labeling appeared in the granule cell layer of the dentate gyrus (g). (*B*) This autoradiogram was generated by a section adjacent to the one shown in *A*. The tissue was labeled by using the same conditions, except for the addition of 1 mM unlabeled AVP. Thus, the autoradiographic grains present in regions seen in *A* and not observed here represent areas of AVP-inhibitable binding. Note marked displacement of binding in areas cited above. (Bar = 500 μm.)

pituitary and median eminence being among the most densely labeled (Fig. 2 and Table 1, 10 μM AVP). Also densely labeled were the supraoptic and paraventricular nuclei (Table 1, 1 μM AVP) and the suprachiasmatic nuclei (Table 1, 200 μM AVP).

Within the limbic system, vasopressin binding sites observed in the hippocampus were localized to the CA 1 region, with labeling seen in the stratum oriens, stratum radiatum, and stratum lacunosum moleculare. Also labeled in the hippocampus were the molecular layer (Table 1, 1 μM AVP) and the granule cell layer (Fig. 2*A*) of the dentate gyrus. [^{3}H]AVP binding was observed in the lateral septum as well (Table 1, 10 μM AVP).

In other brain regions where AVP may act as a neuroregulator, the parietal cortical layers I–IV (Table 1, 1 μM and 10 μM AVP) and caudate-putamen (Table 1, 10 μM AVP) contained a relatively moderate density of [^{3}H]AVP binding sites. Within the cerebellum, [^{3}H]AVP binding was inhibited only with 1 mM AVP. Other areas showing AVP-inhibitable [^{3}H]AVP binding included the nucleus tractus solitarious (Table 1, 10 μM AVP) in the brainstem and the ventral and dorsal horns of the spinal cord (Table 1, 200 μM AVP).

DISCUSSION

The autoradiographic evidence presented in this study and in earlier work (21–24) demonstrates the existence of [^{3}H]AVP binding sites, most likely representing AVP receptors. These data are consistent with the hypothesis that vasopressin may influence memory processes through receptors within the CNS. In support of this hypothesis is the autoradiographic localization of [^{3}H]AVP binding sites within the hippocampus, a structure known to be involved with memory function. Moreover, we observed specific [^{3}H]AVP binding within the cerebral cortex, which further suggests receptors for modulation of complex psychological processes.

Comparing the density of [^{3}H]AVP binding sites associated with the hypothalamo-neurohypophysial system with that in other brain regions, a clear distinction emerges. The density of AVP binding sites is considerably greater within the hypothalamo-neurohypophysial system than in other brain regions. Binding sites within the nuclei that synthesize AVP, the median eminence, and the posterior pituitary may be of two types, neurophysin binding sites and/or membrane-bound receptors. First, [^{3}H]AVP could be binding to, as we have suggested previously (21, 22), the neurophysin for AVP, pressorphysin. To test this possibility, we undertook an autoradiographic study in homozygous and heterozygous Brattleboro rats. The homozygous Brattleboro rat has an absolute genetic deficiency in brain and is unable to synthesize AVP or its precursor, pressorphysin (42), whereas the heterozygous Brattleboro rat has a partial deficiency. Thus, if the binding we observed is associated with pressorphysin, [^{3}H]AVP binding should be present in the heterozygous but not the homozygous Brattleboro rat. However, we found dense [^{3}H]AVP binding sites in the supraoptic nucleus, median eminence, and neurohypophysis of both heterozygous and homozygous Brattleboro rats (unpublished data). These results indicate that [^{3}H]AVP is not binding to pressorphysin in the homozygous animal. It is still possible that AVP is binding to the neurophysin for oxytocin. However, the optimal pH for oxytocin and vasopressin binding to their respective neurophysins is 5.5 (32), whereas our binding experiments were carried out at pH 7.4.

Vasopressin binding in the neurohypophysis and median eminence, while unlikely to be to pressorphysin, may be to

Table 1. Inhibition of [^{3}H]AVP binding by unlabeled AVP in rat brain: Regional distribution and quantitative analysis

Region	AVP bound, fmol/mg of tissue			
	1 μM AVP	10 μM AVP	200 μM AVP	1 mM AVP
Pituitary				
Neurohypophysis		285 ± 16	287 ± 21	406 ± 41
Adenohypophysis		13 ± 1	99 ± 12	132 ± 8
Hypothalamus				
Paraventricular nucleus	162 ± 9			
Supraoptic nucleus	213 ± 14			
Suprachiasmatic nucleus			190 ± 7	
Median eminence		285 ± 29		396 ± 38
Parietal cortex				
I–IV	35 ± 2	55 ± 2	58 ± 4	105 ± 7
V		24 ± 1		61 ± 5
VI		20 ± 1		37 ± 2
Hippocampus				
Stratum oriens		22 ± 1	65 ± 4	95 ± 5
Stratum radiatum		14 ± 1		100 ± 3
Stratum lacunosum molecular		11 ± 1		70 ± 2
Dentate gyrus				
Hilus		00 ± 0		51 ± 2
Molecular layer	35 ± 2	29 ± 1	33 ± 3	105 ± 3
Lateral septum		14 ± 9		
Caudate putamen		43 ± 2		85 ± 5
Cerebellum				
Granule cell layer		00 ± 0		20 ± 1
Molecular layer		00 ± 0		29 ± 2
Nucleus tractus solitarious		20 ± 1		37 ± 1
Spinal cord				
Dorsal horn			78 ± 3	
Ventral horn			77 ± 5	

Values represent fmol bound per mg of tissue, mean ± SEM, based on total binding minus non-inhibitable binding in the presence of varying concentrations of unlabeled AVP (1 μM–1 mM). Femtomoles of [^{3}H]AVP bound per mg of tissue was calculated by comparing the autoradiographic grain density generated over each area of tissue with that produced by 10-μm thick ^{3}H standards of brain tissue. Optical density readings were made from individual tissue areas as well as from the standards by using a window setting that encompassed an area equivalent to 150 or 250 μm^2 of tissue.

AVP receptors on blood vessels such as those described by Schiffrin and Genest (33). It is well recognized that the posterior pituitary and median eminence are highly vascularized regions. [^{3}H]AVP binding in the median eminence may be associated with ependymal tanycyte-associated proteins for transport of AVP from the cerebral spinal fluid to portal vessels (34), wherein AVP ultimately influences corticotropin (ACTH) release from the anterior pituitary (35).

Several alternatives exist to explain [^{3}H]AVP binding within the hypothalamus. Intra-hypothalamic binding sites may represent presynaptic autoreceptors or receptors for inter-nuclear communication between the hypothalamic nuclei synthesizing AVP. The existence of interconnecting vasopressin fibers between those nuclei that synthesize vasopressin has been shown by both conventional immunohistochemical methods (5–7) and by the horseradish perioxidase visualization technique (36). Moreover, evidence also exists for intranuclear synapses. Leranth *et al.* (37) found that two-thirds of the synapses within the supraoptic nucleus were of intranuclear origin. Thus, [^{3}H]AVP binding sites observed in the paraventricular nucleus and supraoptic nucleus may be part of a feedback system such as that proposed by Nicoll and Barker (13).

Electrophysiological studies also strongly suggest the existence of AVP receptors within the supraoptic nucleus (14) and in the lateral septum (12). Abe *et al.* (14) have shown a vasopressin-induced depolarization of supraoptic neurons. Somewhat surprisingly, this AVP-induced depolarization appears to be directly linked to an adenylate cyclase system. Within the lateral septum, AVP was shown by De Vries and Buijs to alter the firing pattern of septal neurons (12). Considered with the autoradiographic results, data from immunohistochemical and electrophysiological studies support the contention of hypothalamic [^{3}H]AVP binding sites that represent membrane-bound receptors. However, the relative contributions within the hypothalamo-neurohypophysial system of possible neurophysin binding sites versus AVP receptors for negative feedback, intra-hypothalamic communication, vasculature receptors, or transport binding sites cannot be partitioned at this time.

Data from inhibition of [^{3}H]AVP by varying concentrations of unlabeled AVP suggest the existence of high- and low-affinity binding sites. Several investigators have reported Scatchard analyses consistent with this possibility (25, 27). Lawrence *et al.* (27) reported a nonhomogeneous population of binding sites with K_d values of 0.775 nM and 20.8 nM. These results are consistent with our observations in brain homogenates.

The presence of [^{3}H]AVP binding sites outside the hypothalamo-neurohypophysial system suggests the existence of vasopressin receptors that may be involved in neuroregulation of complex behaviors. This hypothesis is supported by the finding of [^{3}H]AVP binding sites in the hippocampus and cerebral cortex. The discrete distribution of binding sites within the hippocampus and cerebral cortex suggests a precise regulatory effect. Within the hippocampus, Multhaler *et al.* (15) reported that AVP depolarized hippocampal neurons and a very recent study by Versteeg *et al.* (38) showed that AVP inhibits a centrally induced pressor response by modulation of hippocampal mechanisms. Moreover, Church (19)

found that in the hippocampus AVP enhanced norepinephrine-activated cyclic AMP.

Other possible neuroregulatory sites were observed in the anterior pituitary where AVP both stimulates corticotropin secretion and potentiates the effects of corticotropin-releasing factor on ACTH release (35). [^{3}H]AVP binding was also observed in the nucleus tractus solitarious where AVP has been shown to regulate autonomic control of blood pressure (39).

[^{3}H]AVP binding sites were further detected within the motor system. These structures included the caudate-putamen and cerebellum. Within the caudate-putamen, Courtney and Raskind (20) found that AVP enhanced dopamine-activated cyclic AMP, an effect analogous to the AVP enhancement of norepinephrine-activated cyclic AMP in the hippocampus. A cerebellar deficiency in the homozygous Brattleboro rat has been observed by Boer and Swaab (40). This deficiency is corrected by prenatal treatment with AVP (40). It is possible that the low level of cerebellar binding sites we observed represents vestigial receptors for AVP that mediate a prenatal neurotrophic effect similar to the AVP-induced neurotrophism observed by Gruener *et al.* (41) in cultured embryonic neurons.

The regional distribution of putative vasopressin receptors in the CNS suggests a system by which complex but related functions might be acting in concert to modulate adaptive behaviors, like memory. The exact cellular mechanism by which vasopressin can modulate highly complex behaviors is still unknown. We have begun to investigate this question by examining AVP induced responses in cultured neurons (41).

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