

Characterization and distribution of receptors for the atrial natriuretic peptides in mammalian brain

(brain-heart peptides/receptor autoradiography/blood pressure/water loading)

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Communicated by Irvine H. Page, August 19, 1985

ABSTRACT Both rat ^{125}I -labeled atrial natriuretic polypeptide [^{125}I -ANP or atrial natriuretic factor fragment ANF-(99-126)] and human ^{125}I -ANP [^{125}I - α -ANP or human ANF-(99-126)] bind with high specificity and affinity ($K_d = 20\text{--}80\text{ pM}$) to an apparent single class of sites in guinea pig brain. The ligand selectivity pattern demonstrates that ANF-(101-126) > ANF-(99-126) > ANF-(103-125) > ANF-(103-123) on ^{125}I - α -ANP binding sites. [International nomenclature starting at the end of the signal peptide of the recently sequenced prepropeptide is used; thus, ANF-(101-126) corresponds to the earlier designation ANF-(8-33), ANF-(103-123) to rat atriopeptin I, and ANF-(103-125) to rat atriopeptin II.] Similar results have been reported in peripheral tissues, which indicate that central and peripheral ANP binding sites have fairly similar structural requirements. *In vitro* receptor autoradiography shows that in the guinea pig brain, ^{125}I -ANP binding sites are highly concentrated in the external plexiform layer of the olfactory bulb, subfornical organ, various thalamic nuclei, medial geniculate nucleus, and cerebellum. Lower densities are found in the central nucleus of the amygdala, dentate gyrus, hippocampus, and area postrema. Most remaining regions contain much lower densities of sites. In rat brain, ^{125}I -ANP binding sites are differentially distributed, with high densities in the subfornical organ, area postrema, and linings of ventricles but low densities in the thalamus and cerebellum. In monkey brain, ^{125}I -ANP binding sites are concentrated in the cerebellum. The presence of high densities of ^{125}I -ANP binding sites in various brain regions strongly suggests the existence of a family of brain-heart peptides, in analogy to the well-known brain-gut peptides. Moreover, the extensive distribution of ^{125}I -ANP binding sites in mammalian brain suggests that the possible roles of ANP/ANF-like peptides in brain are not restricted to the central regulation of cardiovascular parameters.

Various atrial natriuretic peptides have been isolated recently from secretory-like granules present in the myocytes of mammalian atria. These include the atrial natriuretic factors (ANF) (1-8), atrial natriuretic polypeptides (ANP) (9, 10), auriculins (9), atriopeptins (11-13), cardionatrin (14-16), and cardiodilatin (5, 17). As revealed by cDNA analysis, all of these peptides are likely to be derived from a multi-hormone precursor (18-25). Strong sequence homology between rat and human ANP demonstrates the conservation of amino acid composition during mammalian evolution (1-17). These various peptides modulate blood-pressure homeostasis, aldosterone production, and extracellular fluid volume (for review, see refs. 7 and 26) by acting as a circulating hormone stimulated by volume loading (27). Recently, the presence of ANP or ANF-like immunoreactivity (28, 29) in rat brain has been demonstrated. We also provided preliminary evidence for the existence of ANF binding sites in rat

brain, using an autoradiographic technique (30). However, essential saturation analysis and ligand selectivity pattern were not obtained because of the low density of sites in most regions of the rat brain.

Here we report on the characterization and differential autoradiographic distribution of ANP receptor binding sites in various mammalian brains. In the guinea pig brain, ^{125}I -labeled ANP (^{125}I -ANP) binds with high affinity (K_d between 0.02 and 0.08 nM) to receptor sites that are mainly concentrated in the olfactory bulb, subfornical organ, paraventricular, paratenial, paracentral, and centrolateral nuclei of the thalamus, hippocampus, medial geniculate nucleus, and lobules 9 and 10 of the cerebellum. The high density of ANP binding sites in these various regions suggests the possible existence of a family of brain-heart peptides that could act as neurotransmitters/neuromodulators, in analogy to the well-known brain-gut peptides.

MATERIALS AND METHODS

In preliminary experiments, we observed that the guinea pig cerebellum and thalamus/hypothalamus area were enriched in ^{125}I -ANP binding sites. In other regions such as the striatum, cortex, and brain stem, it was not possible to perform appropriate receptor binding studies (saturation, ligand selectivity pattern) because of the low densities of sites in these areas. Thus, we used those first two brain regions to further characterize those sites. Male guinea pigs (500 g) were decapitated, and the thalamus/hypothalamus area and the cerebellum were rapidly dissected out on ice before homogenization in 10 vol of 150 mM Tris-HCl buffer (pH 7.4 at 4°C) containing 120 mM NaCl and 5 mM KCl using a Brinkmann polytron at setting 6 for 20 sec. Homogenates were then centrifuged for 10 min at 49,000 \times g. Supernatants were discarded, and pellets were resuspended in 50 mM Tris-HCl buffer (pH 7.4 at 4°C) containing 300 mM KCl and 10 mM Na_2EDTA before incubation on ice for 30 min with gentle agitation. After centrifugation as above, pellets were resuspended in 20 vol of 50 mM Tris-HCl buffer (pH 7.4 at 4°C) and recentrifuged at 49,000 \times g for 15 min. Final pellets were washed twice with 50 mM Tris-HCl buffer (pH 7.4 at 4°C) before resuspension in approximately 60 vol of the same buffer. Aliquots were taken for protein determination.

For binding assays, 200 μl of membrane suspension were incubated for 60 min at 25°C with 400 μl of 50 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl, 5 mM MnCl_2 , 40 μg of bacitracin (Sigma) per ml, 0.5% bovine serum albumin (Sigma), and various concentrations (for saturation experiments) or 0.05 nM (for displacement experiments) of the 3- ^{125}I iodotyrosyl-126 derivative of rat ANP (^{125}I -ANP) or of human α -ANP (^{125}I - α -ANP) (2000 Ci/mmol, Amersham International; 1 Ci = 37 GBq) and various ANP-related

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Abbreviations: ANF, atrial natriuretic factor(s); ANP, atrial natriuretic polypeptide(s).

peptides or other drugs as indicated. Incubations were terminated by rapid filtration under reduced pressure and three 5-ml rinses with cold incubation buffer through Whatman GF/C filters presoaked for 2–3 hr in 0.1% polyethyleneimine (PEI) to reduce binding to filters. Specific binding was calculated as the difference in radioactivity bound in the presence and absence of 1.0 μ M ANF-(101–126). Binding of ligands to filters was quantitated by counting filters in a LKB γ counter with 75–80% efficiency.

For receptor autoradiography, rat, guinea pig, and monkey brain sections were prepared as described before (30, 31). Briefly, small animals were killed by decapitation, and the brains were rapidly isolated and snap-frozen in 2-methylbutane (Kodak) at -40°C , mounted on cryostat chucks, and cut into 20- μm -thick sections at -14°C . Male vervet monkeys were killed by an overdose of anesthetic agents, and their brains were rapidly isolated and processed as described above for smaller animals. Sections were thaw-mounted near the edge of precleaned gelatin-coated slides and then were stored at -80°C until used. Frozen slide-mounted brain sections were preincubated for 15 min in 50 mM Tris-HCl buffer (pH 7.4 at 25°C) containing 0.1% PEI to reduce binding of either rat or human ^{125}I -ANP to gelatin-coated slides. Sections were then incubated for 60 min under the conditions described above for membrane binding assays. At the end of the incubation, slides were placed in racks and transferred sequentially through four rinses (1 min in each) of cold incubation buffer and finally were dipped in distilled water to wash out salts. Incubated slides were then rapidly dried and juxtaposed tightly against tritium-sensitive film (Ultrofilm, LKB) and stored at room temperature for 8–10 days before development as described (31), with underlying tissue sections counterstained to facilitate the identification of brain microregions. The international nomenclature starting at the end of the signal peptide (amino acid positions 1–128 for the rat and 1–126 for man) has been used to number peptide fragments used in our assays (7). According to this nomenclature, ^{125}I -ANP and ^{125}I - α -ANP correspond to ANF-(99–126), and earlier designations ANF-(8–33) and rat atriopeptins I and II correspond respectively to ANF-(101–126), ANF-(103–123), and ANF-(103–125).

RESULTS

Characterization of ^{125}I -ANP Binding Sites. Both ^{125}I -ANP and ^{125}I - α -ANP bound with high affinity to an apparently single class of saturable sites in guinea pig membrane preparations. The respective affinity of the two ligands for their binding sites in cerebellum and thalamus/hypothalamus area were similar and in the picomolar range (Table 1). Membrane preparations derived from other areas such as the cortex, striatum, and brain stem were devoid of significant densities of sites that would permit adequate saturation experiments to be performed.

Table 2 shows the relative potency of various ANP-related peptides in displacing ^{125}I - α -ANP binding in guinea pig brain membranes. ANF-(101–126), the cyclic polypeptide derived from the large cardiocalin prohormone, was the most potent analogue tested, whereas ANF-(99–126) was 5–20 times less potent and ANF-(103–125) was much weaker (Table 2). ANF-(103–123) was almost inactive (Table 2). Other neuropeptides and drugs were not able to compete for ^{125}I - α -ANP binding sites (Table 2).

Autoradiographic Distribution of ^{125}I -ANP Binding Sites. The autoradiographic visualization of ^{125}I -ANP and ^{125}I - α -ANP binding sites showed that both ligands bind to sites that are identically distributed in brain. This suggests that both peptide homologues bind to the same family of sites in brain. Also the distribution of ^{125}I -ANP receptors in mammalian brain was unique and quite distinct from those of other peptide receptors

Table 1. Comparative receptor binding properties of rat ^{125}I -ANP and human ^{125}I - α -ANP in guinea pig brain membrane preparations

Ligand	Brain region			
	Thalamus/ hypothalamus		Cerebellum	
	K_d , nM	B_{max} , fmol/ mg of protein	K_d , nM	B_{max} , fmol/ mg of protein
Rat ^{125}I -ANP	0.02	4.0	0.06	4.7
Human ^{125}I - α -ANP	0.04	6.2	0.08	4.5

Brain membranes were prepared as described and then incubated for 60 min in 50 mM Tris-HCl, pH 7.4 at 25°C /150 mM NaCl/5 mM MnCl_2 /40 μg of bacitracin per ml/0.5% bovine serum albumin containing radioactive ligands at concentrations between 0.002 and 1.0 nM. Incubations are terminated by rapid filtration under reduced pressure through GF/C filters that had been presoaked in 0.1% PEI to reduce binding to filters. Specific binding was defined as the amount of ligands bound in the absence and presence of 1.0 μM ANF-(101–126). Values represent the mean of two determinations, each in duplicate, that varied <15%. According to the international nomenclature, rat ^{125}I -ANP corresponds to rat ANF-(99–126) and human ^{125}I - α -ANP corresponds to human ANF-(99–126) (see ref. 7.).

previously visualized (for example, see refs. 32–35). In the guinea pig brain, high densities of ^{125}I -ANP binding sites were present in the external plexiform layer of the olfactory bulb, subfornical organ (Fig. 1A), paraventricular, paratenial, paracentral, and centrolateral nuclei of the thalamus (Fig. 1A–C), medial geniculate nucleus (Fig. 1D), and cerebellum, especially lobules 9 and 10 (Fig. 1E and F). Moderate-to-high densities of sites were found in the rostral portion of the central nucleus of the amygdala (Fig. 1A), dorsal portion of the nucleus of the lateral olfactory tract (Fig. 1A), granular cell layer of the dentate gyrus (Fig. 1C and D), pyramidal cell layer of the hippocampus (Fig. 1B–D), the surrounding of the interpeduncular nucleus (Fig. 1D), and area postrema. In the hippocampus, the highest density of sites was observed in the CA₃ area followed by the CA₂ and the CA₁ areas (Fig. 1B–D). Low-to-moderate concentrations of sites were present in the lateral septum, tractus diagonalis, and periventricular nucleus

Table 2. Inhibition of specific ^{125}I - α -ANP binding to guinea pig membrane preparations by related peptides

Peptide	Thalamus/ hypothalamus		Cerebellum	
	IC_{50} , nM	Relative potency	IC_{50} , nM	Relative potency
ANF-(101–126)	0.07	100	0.03	100
ANF-(99–126)	0.27	26	0.76	3.9
ANF-(103–125)	17.6	0.4	11.2	0.3
ANF-(103–123)	>1000	<0.01	>1000	<0.01

IC_{50} is the concentration of peptide displacing 50% of specifically bound ^{125}I - α -ANP (0.05 nM). Values represent the mean of two experiments, each performed in triplicate. Incubations were done as described in the text and in the presence of 40 μg of bacitracin per ml. All peptides were obtained from the Institut Armand Frappier, Laval, PQ, Canada. Other peptides and drugs that did not displace binding at 1 μM were angiotensin II, β -endorphin, cholecystokinin-8, substance P, somatostatin, haloperidol, muscimol, atropine, isoproterenol, clonidine, carbachol, and prazosin. In typical experiments, the total binding (mean \pm SEM) was 2271 ± 211 cpm per tube, and the nonspecific binding [in the presence of 1.0 μM ANF-(101–126)] was 834 ± 119 cpm per tube in the cerebellum and 1320 ± 112 cpm (total) and 511 ± 22 cpm (nonspecific) in the thalamus/hypothalamus. Peptide nomenclature is according to the international system based on the sequence of the prepropeptide (7), with residue 1 following the end of the signal peptide.

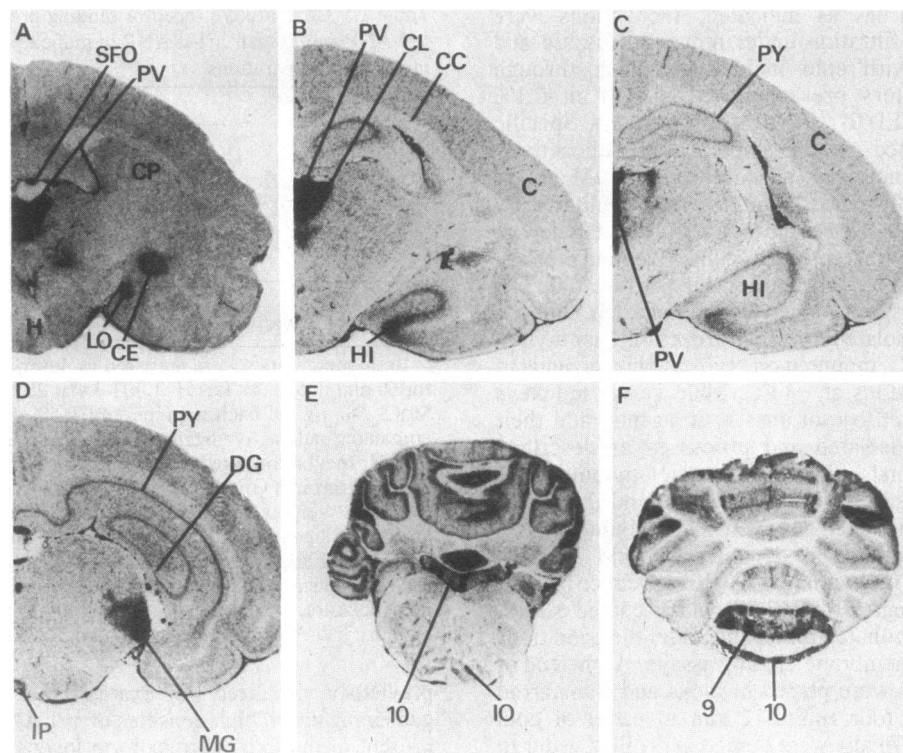


FIG. 1. Photomicrographs of the distribution of ^{125}I -ANP binding sites in guinea pig brain. Brain sections were incubated as described in the presence of $0.05\text{ nM } ^{125}\text{I}$ -ANP. C, cortex; CC, corpus callosum; CE, central nucleus of the amygdala; CL, centrolateral nucleus of the thalamus; CP, caudate-putamen; DG, dentate gyrus; H, hypothalamus; HI, hippocampus; IO, inferior olive; IP, interpeduncular nucleus; MG, medial geniculate nucleus; PV, paraventricular nucleus of the thalamus; PY, pyramidal cell layer of the hippocampus; SFO, subfornical organ; and 9 and 10, lobules 9 and 10 of the cerebellum. For anatomical structures, see ref. 36.

of the hypothalamus. Remaining brain regions such as striatum, cortex, and colliculi contained a low density of sites (Fig. 1). White matter areas such as the corpus callosum were devoid of ^{125}I -ANP binding sites (Fig. 1).

^{125}I -ANP binding sites are differentially distributed in rat brain (Fig. 2). High densities of sites were found in the external plexiform layer of the olfactory bulb (Fig. 2B), subfornical organ (Fig. 2C), nucleus tractus solitarius, and area postrema. Moderate concentrations of sites were seen in the globus pallidus

(Fig. 2A). Low densities of sites were found in most remaining areas, including the thalamus, hippocampal formation, and cerebellum. However, white matter areas such as the linings of the ventricles were enriched in ^{125}I -ANP binding sites (Fig. 2A). Finally, preliminary results showed the presence of high densities of ^{125}I -ANP binding sites in monkey cerebellum (Fig. 3). Other brain regions investigated thus far, such as the cortex, striatum, and mid-brain, were mostly devoid of ^{125}I -ANP binding sites.

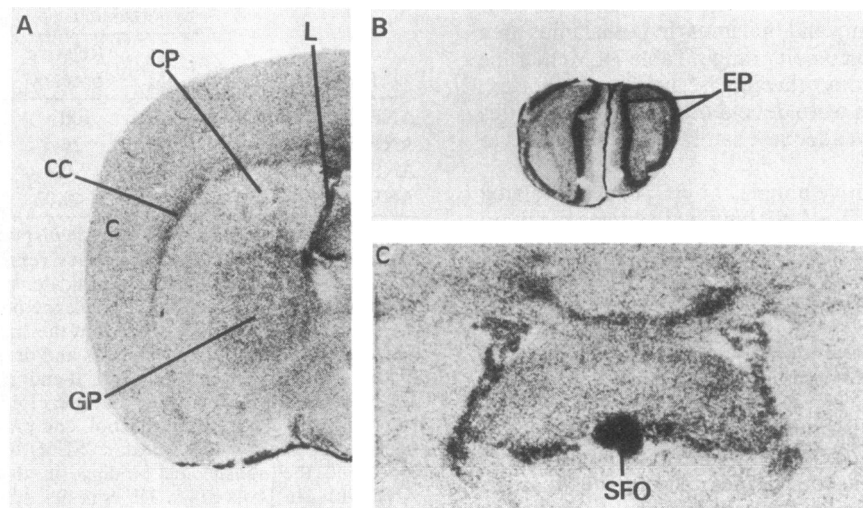


FIG. 2. Photomicrographs of the distribution of ^{125}I -ANP binding sites in rat brain. Brain sections were incubated as described in the presence of $0.05\text{ nM } ^{125}\text{I}$ -ANP. C, cortex; CC, corpus callosum; CP, caudate-putamen; EP, external plexiform layer of the olfactory bulb; GP, globus pallidus; L, linings of ventricule; and SFO, subfornical organ. For anatomical structures, see ref. 37.

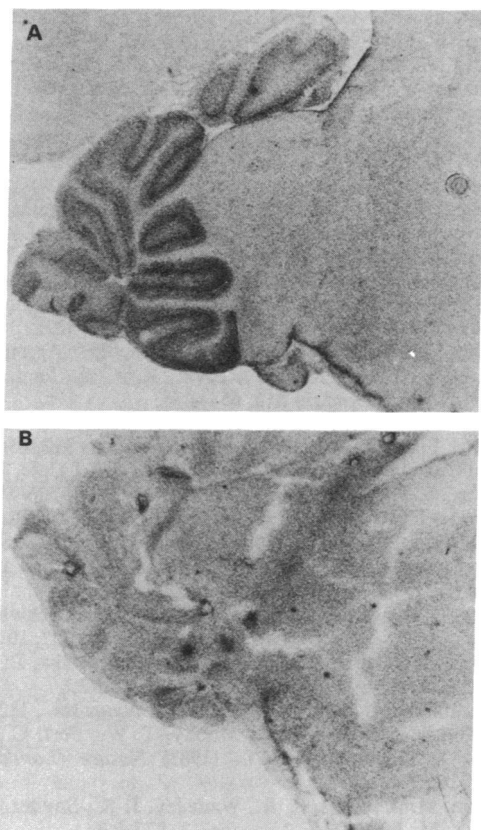


FIG. 3. Photomicrographs of the distribution of ^{125}I -ANP binding sites in monkey cerebellum. Brain sections were incubated as described in the presence of 0.05 nM ^{125}I -ANP (A) and 0.05 nM ^{125}I -ANP/1.0 μM ANF-(101-126) to evaluate the specifically bound ligand.

DISCUSSION

We previously reported the presence of binding sites for ^{125}I -labeled ANF-(101-126), formerly called ^{125}I -labeled ANF-(8-33), in rat brain (30). However, we were unable to characterize these displaceable binding sites because of their limited densities in most areas. Thus, some of the most important criteria for the classification of binding sites as receptors, such as saturability and proper ligand selectivity pattern, were not fulfilled. We now have been able to perform these crucial experiments using guinea pig brain membrane preparations and two other ligands.

Both ligands used in the present study (^{125}I -ANP and ^{125}I - α -ANP) bind with high affinity to an apparently single class of saturable sites in guinea pig cerebellum and the thalamus/hypothalamus area. Similar data have been reported in peripheral tissues such as the bovine adrenal zona glomerulosa (38) and rat kidney (39). However, the density (B_{max}) of sites for ANP-related peptides is tissue-dependent. While low B_{max} values are obtained in both brain regions studied (Table 1), much higher densities of sites have been reported in the bovine adrenal zona glomerulosa (38), for example. The densities of sites labeled by ^{125}I -ANP and ^{125}I - α -ANP are similar in the cerebellum and the thalamus/hypothalamus area, suggesting that these two ligands bind to the same population of receptor sites in these brain regions. This is not surprising because the two peptide sequences are identical except for residue 110, which is methionine in human and isoleucine in rat (7, 10). The ligand selectivity pattern shows that ANF-(101-126) is the most potent competitor against ^{125}I - α -ANP binding, whereas ANF-(99-126) and ANF-(103-125) are weaker and ANF-(103-123) is almost

inactive. These data indicate that the extension of the NH_2 terminus by two amino acids (serine and leucine) in ANF-(99-126) diminishes the potency of ANP/ANF-like peptides as compared to ANF-(101-126). Moreover, the deletion of the two NH_2 -terminal arginine residues and the COOH-terminal tyrosine residue markedly decreases the activity of these peptides in the binding assay as shown by the low potency of ANF-(103-125). Thus, it is likely that these residues are important for the activity of ANP/ANF-like peptides.

Little is known on the relative potency of ANP/ANF-related peptides in other assays. However, as obtained in binding assays, it seems that the deletion of the COOH-terminal tyrosine residue decreases the biological activity of ANP-like peptides in bioassays such as kidney (natriuretic activity) (40-42), rabbit aorta (40, 42), chicken rectum (40, 42), and secretion of aldosterone by the adrenal gland (42). Moreover, Cantin *et al.* (42) have demonstrated recently that the COOH-terminal cleavage of ANF-(101-126) had much more pronounced effect than did NH_2 -terminal cleavage on the biological activities of ANF in various assays. This suggests that the structural requirements of central and peripheral ANP/ANF receptors are similar and that the COOH-terminal portion of the molecule is more important than the NH_2 -terminal end for the binding of ANP/ANF-like peptides to their receptors.

The autoradiographic distribution of ^{125}I -ANP binding sites in brain is species-dependent. For example, while high densities of sites are found in the guinea pig thalamus and cerebellum, low densities are seen in the same structures in the rat brain. The monkey cerebellum is also enriched in ^{125}I -ANP binding sites, while other regions such as the cortex are not. Thus, it would be of interest to determine the distribution of ANP/ANF binding sites in other mammals as well as in lower species.

Little is currently known on the possible relevance of ANP/ANF receptor binding sites in the central nervous system. However, recent studies have demonstrated that injection of ANP-related peptides in the brain markedly affected blood pressure (43), heart rate (43), diuresis (44), and salt appetite (44). Moreover, Samson (45) has just reported that infusion of ANF into the third cerebroventricle of conscious, freely moving rats resulted in a significant inhibition of basal vasopressin release, possibly by acting on hypothalamic structures. Thus, the high densities of ANP binding sites in regions associated with the central regulation of blood pressure and salt and water intake, such as the subfornical organ and the nucleus tractus solitarius, strongly suggest that these sites mediate the central effects of ANP/ANF peptides on those parameters.

Interestingly, the presence of high concentrations of ^{125}I -ANP binding sites in brain regions such as the thalamus, olfactory pathway, amygdala, hippocampus, and cerebellum suggests that putative roles of ANP/ANF-like peptides in the brain are not limited to the central regulation of cardiovascular parameters. Possible effects of ANP/ANF-like peptides in the limbic system should be investigated in relation to the high densities of binding sites in the hippocampus and the amygdala. It is also possible that these peptides could be involved in certain sensory pathways, coordination of movement, and postural control. In that regard, the high densities of sites in the guinea pig and monkey cerebellum is of particular interest. It is well known that the cerebellum is generally devoid of most peptides, except motilin (46). Thus, the presence and possible biological effects of ANP/ANF-like peptides in mammalian cerebellum should be investigated. Moreover, the autoradiographic distribution of ^{125}I -ANP binding sites in mammalian brain strongly suggests that the putative roles of these peptides in the central nervous system is by far more diverse than expected at first.

Finally, the presence of ^{125}I -ANP binding sites in many brain regions strongly suggests the existence of brain ANP-like peptides. Already, two independent groups have reported on the presence of ANP/ANF-like immunoreactive materials in rat brain (28, 29). The exact chemical nature of these materials remains to be determined but Gardner *et al.* (47) have just demonstrated the presence in the rat hypothalamus of an ANF mRNA that is similar to the cardiovascular ANF mRNA. This suggests that brain and heart ANP/ANF peptides are structurally similar.

In summary, highly specific and selective receptor binding sites for ANP-like peptides are present in various regions of the mammalian brain, suggesting that certain of the biological effects of these peptides could be centrally mediated. Moreover, the presence of ANP receptors in the central nervous system strongly suggests the existence of a new family of brain-heart peptides, in analogy to the well-known brain-gut peptides.

We wish to thank Drs. K. G. McFarthing and M. R. Harris (Amersham International, Buckinghamshire, England) for providing rat and human ^{125}I -ANP. R.Q. is a "Chercheur-Boursier" of the Fonds de la Recherche en Santé du Québec. This research was supported by the Medical Research Council of Canada and the Fonds de la Recherche en Santé du Québec.

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