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AUTORADIOGRAPHIC LOCALIZATION AND CHARACTERIZATION OF NATRIURETIC PEPTIDE BINDING SITES IN THE RAT CENTRAL NERVOUS SYSTEM

by

Edyta M. Wróbel-Konrad M.D. (Medical Academy, Szczecin, Poland)

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

© Edyta M. Wróbel-Konrad, March 1992

Department of Medicine Division of Experimental Medicine McGill University, Montréal, Québec



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PEPTIDE BINDING SITES IN RAT CNS

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To my parents, Teresa and Florian

To Janusz

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ABSTRACT

Atrial natriuretic factor (ANF), brain natriuretic peptide (BNP) and Ctype natriuretic peptide (CNP) are members of a family of natriuretic peptides that may be involved, among other functions, in the maintenance of proper fluid and electrolyte balance and blood pressure control, as both circulating hormones and neuropeptides. The studies presented in this thesis were undertaken primarily to provide a deeper insight into the mechanisms of action of natriuretic peptides on the central nervous system (CNS). Using autoradiographic and cross-linking techniques, the precise cellular localization, specificity, and subtypes of natriuretic peptide binding sites in selected areas of the rat CNS were established. ANF binding sites are evident primarily on the basolateral plasmalemma of the epithelial cells in the choroid plexus and on plasmalemma of axons, dendrites, and astrocytes in the area postrema. This cellular localization is consistent with the notion that circulating natriuretic peptides may exerts their effects via a CNS locus. Furthermore, evidence is provided that ANF binding sites are not only specific to ANF, but also to BNP, whereas CNP exhibits a distinct receptor selectivity. Finally, these studies provide a regional localization of three subtypes of natriuretic peptide receptor. In contrast to binding sites with characteristics of natriuretic peptide receptors A and B, which are detected in a number of CNS areas, natriuretic peptide receptor C is detected in only a few CNS structures. These studies are expected to contribute to a better understanding of the mechanism of natriuretic peptides action on the CNS.

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RÉSUMÉ

Le facteur natriurétique des oreillettes (ANF), le peptide natriurétique du cerveau (BNP) ainsi que le peptide natriurétique de type C (CNP) sont membres d'une famille de peptides natriurétiques homologues qui sont probablement impliqués en tant qu'hormones et neuropeptides en plus de leurs autres fonctions dans le maintien de l'homéostase hydro-électrolytique et le contrôle de la tension artérielle. Les études présentées dans cette thèse ont été entreprises d'abord afin d'approfondir nos connaissances sur le mécanisme d'action des peptides natriurétiques sur le système nerveux central (SNC). Nous avons établi avec précision la localisation cellulaire, la spécificité et les sous-types des sites de liaison des peptides natriurétiques dans certaines régions spécifiques de SNC du rat en utilisant l'autoradiographie et le marquage par réticulation. Les sites de liaison de l'ANF son: présents surtout sur les membranes plasmatiques basolatérales des cellules épithéliales des plexus choroides et sur les membranes plasmatiques des axones, dendrites et des astrocytes de l'area postrema. Cette localisation cellulaire est en accord avec l'hypothèse que les peptides natriurétiques peuvent exercer leurs effets via une région du SNC. De plus, nous démontrons que les sites de liaison ne sont pas spécifiques à l'ANF mais aussi au BNP tandis que le CNP montre une sélectivité distincte. Finalement ces études indiquent que les trois sous-types des récepteurs des peptides natriurétiques possèdent une localisation régionale différente. Contrairement aux sites de liaisons ayant des caractéristiques des sous-types A et B des récepteurs des peptides natriurétiques qui ont été détectés dans plusieurs régions du SNC, le sous-type C n'a pu être détecté que dans quelques structures anatomiques du SNC. Nous pensons que ces travaux contribuent à améliorer nos connaissances sur le mécanisme d'action des peptides natriurétiques dans le SNC.

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I am extremely indebted to the late Dr. Marc Cantin, my first thesis supervisor, for introducing me to the world of science and showing me the joy of medical research. I am particularly grateful for his tactful advice, caring attitude and generosity, and for his appreciation of my work.

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ABBREVIATIONS

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ACTH:	adenocorticotropic hormone
ANF:	atrial natriuretic factor
ANG II:	angiotensin II
ATP:	adenosine triphosphate
AP:	area postrema
AZG:	adrenal zona glomerulosa
B _{max} :	maximal binding capacity
BNP:	brain natriuretic peptide
cAMP:	3',5'-cyclic adenosine monophosphate
cDNA:	complementary deoxyribonucleic acid
cGMP:	3', 5'-cyclic guanosine monophosphate
ChP:	choroid plexus
CNP:	C-type natriuretic peptide
CNS:	central nervous system
cpm:	counts per minute
CSF:	cerebrospinal fluid
CV:	cardiovascular
CVOs:	circumventricular organs
dpm:	disintegration per minute
EM:	elecron-microscopic
fmol:	femtomole
GFR:	glomerular filtration rate
HD:	half distance (a distance from a line radioactive source
	that contains 50% of grains)
HPLC:	high-performance liquid chromatography

ic:	intracarotid
icv:	intracerebroventricular
IC ₅₀ :	unlabeled ligand concentration at which the maximum
	binding of labeled ligand is displaced by 50%.
K _d :	equilibrium dissociation constant
k _f :	filtration coefficient
kDa:	kilodalton
LM:	light-microscopic
mRNA:	messenger ribonucleic acid
NPR-A:	natriuretic peptide receptor A
NPR-B:	natriuretic peptide receptor B
NPR-C:	natriuretic peptide receptor C
NTS:	nucleus of the solitary tract
OB:	olfactory bulb
PA:	pia-arachnoid
PVN:	paraventricular nucleus
SD:	Sprague-Dawley rats
SFO:	subfornical organ
SHR:	spontaneously hypertensive rats

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PREFACE

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The work presented in this thesis consists of five chapters, out of which three contain published (or soon to be published) material, as allowed by the Faculty of Graduate Studies, McGill University, in the Guidelines Concerning Thesis Preparation:

"... The candidate has the option, <u>subject to the approval of the</u> <u>Department</u>, of including as part of the thesis the text, or duplicated published text, of an original paper, or papers.

- Manuscript-type thesis must still conform to all other requirements explained in Guidelines Concerning Thesis Preparation.

- Additional material (procedural and design data as well as description of equipment) must be provided in sufficient detail (e.g., in appendices) to allow clear and precise judgement to be made of the importance and originality of the research reported.

- The thesis should be more than a mere collection of manuscripts published or to be published. I<u>t must include a general abstract, a full</u> <u>introduction and literature review and a final overall conclusion</u>. Connecting texts which provide logical bridges between different manuscripts are usually desirable in the interest of cohesion.

- It is acceptable for theses to include, as chapters, authentic copies of papers already published, provided these are duplicated clearly and bound as an integral part of the thesis. <u>In such instances, connecting texts are mandatory</u> and supplementary explanatory material is always necessary.

- Photographs or other materials which do not duplicate well must be included in their original form.

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- While the inclusion of the manuscripts co-authored by the candidate is acceptable, the candidate is required to make an explicit statement in the thesis of who contributed to such work and to what extent, and supervisors must attest to the accuracy of the claims at the Ph.D. Oral Defense. Since the task of the Examiners is made more difficult in these cases, it is in the candidate's interest to make the responsibilities of authors perfectly clear."

As required by the Guidelines, Chapter 1 of the thesis is a General Introduction of the subject matter. Chapters 2 through 4 comprise scientific articles published or accepted for publication in peer review journals. An outline of research goals precedes each chapter. Each article is complete in itself with its own Abstract, Introduction, Materials and Methods, Results, and Discussion. The publications included in the chapters are as follows:

Chapter 2:

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- Konrad, E. M., Bianchi, C., Thibault, G., Garcia, R., Pelletier, S., Genest, J., and Cantin, M. Localization and characterization of binding sites for circulating and cerebroventricular atrial natriuretic factor in rat choroid plexus. Neuroendocrinology, 51: 304-314, 1990.
- Konrad, E. M., Thibault, G., and Schiffrin, E. L. Atrial natriuretic factor binding sites in rat area postrema. Autoradiographic study. Am. J. Physiol., (in press).

Chapter 3:

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 Konrad, E. M., Thibault, G., Pelletier, S., Genest, J., and Cantin, M. Brain natriuretic peptide binding sites in rats. In vitro autoradiographic study. Am. J. Physiol., 259: E246-E255, 1990.

Chapter 4:

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- Konrad, E. M., Thibault, G., Cantin, M., and Schiffrin E. L. Atrial natriuretic factor receptor subtypes in the rat central nervous system. Hypertension, 17: 1144-1151, 1991.
- 5) Konrad, E. M., Thibault, and Schiffrin E. L. Autoradiographic visualization of the natriuretic peptide receptor B in rat tissues. Reg. Pept., 39: 177-189, 1992.

Except for papers 1 and 3, the text of manuscripts has been reproduced unchanged from the published version except for few minor modifications executed for the sake of cohesion. In the case of paper 1, additional experiments on possible degradation *in vivo* were necessary. Similarly, a change in presentation of data was required. In the case of paper 3, additional competition binding and affinity cross-linking experiments were needed to reinforce the conclusions reached. Consequently, changes and additions to the papers 1 and 3 were introduced.

Apart from my thesis supervisors, the late Dr. Marc Cantin and Dr. Ernesto L. Schiffrin, roles of the co-authors in the above listed publications were as follows: Dr. Césario Bianchi, a former Ph.D. student in our laboratory, taught me the techniques of *in vivo* and *in vitro* autoradiography. Dr. Gaétan Thibault, a current director of Dr. Cantin's laboratory, provided, in the majority of cases, iodinated peptides. Suzanne Pelletier, a summer student in our laboratory, performed quantification of ¹²⁵I-BNP binding sites in somatic tissues. Drs. Jacques Genest and Raul Garcia provided suitable guidelines and were recognized in publications as members of the Hypertension Research Group in the Clinical Research Institute of Montreal. Research facilities within the framework of this group made the execution this work possible. Finally, Chapter 5 contains a brief general discussion of the thesis as a whole, with suggestions for future research, as well as the claims to originality.

For the sake of style, throughout the thesis, the terms "binding sites" and "receptors" were used interchangeably despite the difference in meaning of these terms. Actually, autoradiography, the technique primarily used in this work, permits visualization of binding sites which may or may not correspond to true receptors.

The papers included in this thesis are arranged by topic and not by chronology. At the time experiments were performed for paper 1 (Chapter 2.A) and paper 3 (Chapter 3.A), only two natriuretic peptide receptors were known: the natriuretic peptide receptor A (NPR-A) and C (NPR-C). At the time of experimentation for paper 4 (Chapter 4.A), the natriuretic peptide receptor B (NPR-B) had also been described but it was impossible to study it by affinity binding techniques because of the lack of a specific ligand. NPR-A was often referred to, in these papers, as guanylate cyclase-containing receptor, whereas NPR-C was frequently referred to as guanylate cyclase-free receptor. The term atrial natriuretic factor binding sites (or receptors) is supposed to encompass primarily both NPR-A and NPR-C.

At the time of experimentation for paper 2 (Chapter 2.B) and paper 5 (Chapter 4.B), three natriuretic peptide receptors were already known, NPR-A, NPR-B and NPR-C, and it was possible to distinguish them using binding techniques. The term guanylate cyclase-containing natriuretic peptide receptors, if used in these papers, denotes both NPR-A and NPR-B.

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CHAPTER 1

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GENERAL INTRODUCTION

This chapter presents the background of the thesis. It is subdivided into two major sections. The first section (1.A) is a literature review on natriuretic peptides, their receptors and biological functions. The second section (1.B) deals with methods utilized in receptor mapping. Major emphasis was placed on in vivo and in vitro autoradiography, the two methods primarily used in the experimental work included in this thesis.

1.A FAMILY OF NATRIURETIC PEPTIDES: REVIEW OF LITERATURE

It has been known for a quarter of century that cardiac atria contain specific granules resembling secretory granules of endocrine tissues. Their function was largely unknown before the crucial observation made in 1981 by de Bold, that intravenous administration of atrial homogenate produces a rapid and potent diuresis and natriuresis accompanied by a fall in blood pressure. This discovery generated great interest leading rapidly to identification and purification of atrial natriuretic factor. Since then, numerous research laboratories around the world have contributed to an explosion of information on the molecular biology, biochemistry, physiology and, pathophysiology of this hormone and its receptors. This exciting and rapidly changing field of research resulted thereafter in identification of a whole family of natriuretic peptides which participates in the regulation of blood pressure, fluid volume and other body functions acting as cardiac hormones and neuropeptides.

1.A.1 Classification of natriuretic peptides

Several homologous polypeptides participating in homeostatic balance of water, electrolytes, and blood pressure constitute a family of natriuretic peptides [120,157]. The first peptide of this family to be described was atrial natriuretic factor (ANF). Members of this natriuretic peptide family share many common features. They all have a very characteristic core structure consisting of a 17-amino acid loop formed by an intramolecular disulfide linkage (Figure 1.A.1) which is required for the elevation of 3', 5'-cyclic guanosine monophosphate (cGMP) [157]. They all elicit natriuretic, diuretic and vasorelaxant responses in chick and rat bioassay systems, although their relative potencies are different [157]. These natriuretic peptides can be further

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divided into three subfamilies: type A, B and C, based on their genetic and amino acid homology with their prototypes, ANF, brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP), respectively (Figure 1.A.1).



Figure 1.A.1 Amino acid sequence and schematic representation of porcine ANF, BNP, and CNP. Identical residues among these three peptides are shaded (A) or identified by open circles (B). (Based on Sudoh et al., Biochem. Biophys. Res. Comm., 168: 863-870, 1990.)

1.A.1.1 Type A natriuretic peptides

Atrial natriuretic factor is the best-known member of this subfamily. It has been previously termed cardionatrin, cardiodilatin, atriopeptin, or auriculin.

1.A.1.1.1 Atrial ANF

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ANF was originally isolated from atrial tissue [43,164]. A single copy of the ANF gene has been identified in all mammalian species examined to date [6,137,173]. In all species the gene has a similar structural organization with three exons separated by two introns. Transcription of the ANF gene results in mRNA that encodes prepro-ANF [138]. This ANF precursor of 152 amino acids contains a 24-amino acid signal sequence at the N-terminal, which is cleaved off during its entry into the rough endoplasmic reticulum. The resulting 126amino acid pro-ANF peptide (also highly conserved among many species [105]) is a predominant storage form of ANF in atrial granules [92]. Upon appropriate signal for hormone release, ANF is cleaved into two fragments: a 98-amino acid-long N-terminal and a 28-amino acid C-terminal, the biologically active hormone ANF-(Ser99-Tyr126) [43]. The maturating cleavage is either directly associated with exocytosis of the granular content or takes place in the environment directly adjacent to the atrial cells within the extracellular space [166].

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The mature ANF sequence is characteristically highly conserved across species. There is a simple replacement of methionine at position 110 which is present in ANF of humans [137], dogs [105] and cows [173] to isoleucine which is present in rats [138], mice [137] and rabbits [105]. As all natriuretic peptides, ANF contains a characteristic 17-amino acid disulfide link between Cys105 and Cys121 which is essential for the full expression of ANF biological activity [23]. Asn122, Ser123 and Phe124 residues are also crucial whereas N-terminal amino acids of ANF are less important as determined in various bioassays [165]. Besides the 28-amino acid bioactive ANF, a variety of shorter ANF forms have been isolated but they are presently considered to be simply hydrolytic artefacts generated during the purification procedure [62].

ANF has short half-life in the circulation, ranging from less than 1 min to 4.5 min depending on species [13,78]. Tissues particularly active in clearance of ANF from the circulation include kidneys, intestines, and muscle and sex organs [78]. Several possible mechanisms for the removal of atrial peptides from the circulation have been reported. They include binding of atrial peptides to the so-called "clearance receptors" (known also as C-receptors or natriuretic peptide receptors C), followed by internalization and degradation by specific intracellular proteolytic enzymes [103]. Other mechanisms include degradation by various membrane-bound endo- and carboxypeptidases with different cleavage characteristics isolated from various tissues [33,56,65,66,107,151,168].

1.A.1.1.2 Extra-atrial ANF

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Apart from cardiac atria which is the major site of ANF synthesis, ANF production has been documented, although in much lower quantities, in both neural and non-neural tissues. Evidence for the local ANF synthesis has been based on either the detection of high molecular weight ANF prohormone, or mRNA for ANF, or both.

The heart ventricles appear to be next abundant source of ANF, expressing the ANF gene at the level of about 1% of that in the atria [98]. In contrast to atria, ventricles are believed to release ANF constitutively, contributing substantially, due to their large mass, to circulating ANF levels [14]. Other non-neural tissues where local ANF synthesis was shown include vascular wall of arteries [49]) and of veins [7]), pulmonary tissue [89], the gastrointestinal tract [176]), the reproductive tract [55], lymphoid tissue [174], the anterior pituitary [99], and the parotid gland [175].

The peripheral and central nervous system also appear to produce ANF locally. ANF has been detected in several autonomic ganglia [32] as well as in the eye [153]. Production of ANF has been demonstrated in adrenal chromaffin cells [108]. In the brain, the hypothalamus was found to contain the highest concentration of mRNA for ANF (which constitutes less than 2% of that found in cardiac atria [50,99,147]) and ANF itself [71,93]. Other brain regions where the presence of both ANF transcripts [50,147] and peptides [71,93,94,145] was detected include the cerebral cortex, cerebellum, thalamus, septum, hippocampus, midbrain, spinal cord, olfactory bulb, pons, and medulla. Additionally, the presence of ANF immunoreactivity has been traced to individual ANF-containing neuronal cell bodies and fibres by immunocytochemistry on brain sections (Figure 1.A.2) [127,145]. ANF-positive neurons have a widespread distribution from telencephalon to rhombencephalon. However, the largest collection of immunoreactive (ir) ANF neurons was localized in the hypothalamus, most notably in the anterior

tip of the third ventricle, a region implicated in the blood pressure regulation as well as fluid and electrolyte balance.



Figure 1.A.2 Schematic diagram of a rat brain in horizontal projection, illustrating the major groups of ANF immunoreactive neurons and fibers, along with some of their connections. ABM, nucleus ambiguous; ARC, arcuate nucleus; AVPV, anteroventral periventricular nucleus; BST, bed nucleus of the stria terminalis; CE, central nucleus of the amygdala; IP, interpeduncular nucleus, LHA, lateral hypothalamic area; ME, median eminence; MM, medial mammillary nucleus; NST, nucleus of the solitary tract; PB, parabrachial nucleus; PVp, posterior periventricular nucleus; PCT, pedunculopontine nucleus; PVH, paraventricular nucleus of the hypothalamus; SC, suprachiasmatic nucleus; SM, supramammillary nucleus; TLD, laterodorsal tegmental nucleus; Illv, third ventricle.(Based on Standaert et al., Frontiers in Neuroendocrinology, vol. 10, edited by L. Martini and W.F. Ganong, Raven Press, New York 1988.)

Hypothalamic ANF gene transcripts have identical structure to their atrial counterparts [50]. Interestingly, despite an identical pro-ANF sequence, the major molecular form of ANF in the brain differs from that in atria and consists of a high molecular weight form (pro-ANF) and two low molecular weight forms, such as ANF-(102-126) and ANF-(103-126) [52,144]. The differences in the stored and secreted forms of these peptides must arise from post-translational modifications of pro-ANF.

1.A.1.1.3 Urodilatin

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In addition to atrial and extra-atrial ANF, a peptide termed urodilatin is classified as one of the type A natriuretic peptides. Urodilatin was isolated from urine as a 32-amino acid peptide with identical sequence to that of ANF but with a 4-amino acid extension [39,135]. Urodilatin represents probably an atypical processing of pro-ANF which appears to be synthesized in renal cultured cells and secreted in a constitutive manner [119]. In contrast to ANF, urodilatin appears to be resistant to proteolytic degradation by enzymes residing on kidney membranes[47] and is believed to mediate, in a paracrine fashion, renal tubular actions previously ascribed to circulating ANF [35,53].

1.A.1.2 Type B natriuretic peptides

BNP, a prototype of this subfamily, was initially isolated from the porcine brain [155] and subsequently from the heart [123]. The biosynthetic pathway of BNP is similar to that of ANF. Transcription of a single BNP gene [139] results in mRNA that encodes a precursor molecule containing a hydrophobic signal sequence. In contrast to ANF, sequence homology among BNP precursors is remarkably low and homologous sequences are confined to the signal peptide and the C-terminal bioactive unit [74]. Furthermore, in contrast to cDNA encoding ANF, BNP cDNA contains clustered AT sequences in the 3'untranslated region [74] which are known to destabilize mRNA in the cell [142]. This suggests a differential regulation of these two natriuretic peptides at the level of transcription [74]. Subsequent production of prohormones and active peptides is also species-dependent. In the pig, three mature forms of BNP have been found in plasma (26, 29, and 32-amino acid-long) and two forms in the brain (26 and 32-amino acids long) [1,155]. In humans, mature BNP contains 32 amino acids [156], in bovine 35 amino acids [100], and in rats 45 amino acids [2]. It is interesting to note that the rat BNP has been simultaneously identified by 3 independent groups of investigators and has been termed iso-ANF [44], cardiac natriuretic peptide [68], or rat BNP [2,74]. In addition to dissimilar size, BNP molecules exhibit across species lower sequence homology than in the case of ANF. Within the 17-amino acid loop structure only 59% of amino acid residues are invariant. Greater homology exists between ANF and BNP molecules in a species than between BNP molecules from different species.

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> In the porcine brain, BNP is present in 10 times higher concentration than ANF [171,172]. In the rat brain, BNP mRNA was detected in concentration 2-7 times lower than ANF mRNA [29]. BNP was found there only in small amounts [58]. BNP-immunoreactive neurons are differentially distributed when compared to the distribution of ANF-containing neurons [128]. The highest concentrations of irBNP were detected in the brainstem, striatum, spinal cord, and hypothalamus [171]. In the heart, where BNP is actually much more abundant than in the CNS [2,123,172], atria contain 100 and 3 times higher concentration of BNP and BNP mRNA than ventricles, respectively [172,]. However, there is more ANF than BNP in atria (200-fold) and ventricle (5-fold) [29,172]. Interestingly, taking into account the difference in size of ventricles and atria, cardiac ventricles appear to be the most prominent site of BNP synthesis and secretion [29,104]. Low levels of irBNP was also found in plasma, where BNP consists about 5% of plasma irANF [2,58]. The BNP molecule and its prohormone have been demonstrated in bovine adrenal chromaffin cells [100,101]. In these cells the 35-amino acid peptide isolated was termed aldosterone secretion inhibitory factor (ASIF) and was proposed to act as a local modulator [100,101]. Evidence for local BNP synthesis has been also provided for tissues such as lung and aorta [29]. BNPimmunoreactivity was found in nerves of stomach and jejunum [140], as well as in several peripheral tissues [172] but local synthesis in these structures has not been as yet documented.

1.A.1.3 Type C natriuretic peptides

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CNP, the most recently identified natriuretic peptide, belongs to the type C natriuretic peptide family. Like BNP, it was originally isolated from porcine brain [157], where it is found in concentration slightly higher than that of irBNP and about 10 times higher than that of irANF [172]. The biosynthetic CNP pathway is slightly different from that of ANF [75,162,163]. The CNP precursor, consisting of 126-amino acid residues, exhibits remarkably high homology across species (higher than between ANF precursors) [75]. Cleavage of the first 23-amino acid signal peptide results in production of a 103-amino acid residue peptide, pro-CNP. Further processing of the pro-hormone generates one of two biologically active peptides: CNP-22 (a 22-amino acid form) or CNP-52 (an N-terminal elongated 52-amino acid residue sequence which is the major CNP form in the porcine brain [91,172]). CNP exhibits very high sequence homology across mammalian species [75,163] and seems to be the most conserved peptide among the three natriuretic peptide families. The CNP-22 appears to be actually identical in the human, rat, and pig [163]. As well, CNP shows a high sequence homology to ANF and BNP, especially in its 17-amino acid residue ring structure, although it lacks the C-terminal extension from the ring (Figure 1.A.1) that is commonly found in ANF and BNP [157]. The characteristic absence of the C-terminal residues in CNP does not reflect post-translational processing but results from a unique gene transcript [75,162,163]. Interestingly, the 3'-untranslated region of CNP cDNA contains 4 sets of ATTTA sequence [75], which was found also in a very clustered form in cDNA of BNP but not ANF [74]. Since these nucleotide sequences are known to destabilize mRNA in the cell [142], expression of the three natriuretic peptides may be regulated through different mechanisms [74].

CNP-immunoreactivity is widely distributed in the brain with high concentrations in the hypothalamus, cerebellum, midbrain, medulla, thalamus, and septum [77,172]. CNP-like immunoreactivity has also been detected in some other areas of the nervous system and its related structures

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such as the spinal cord [172], adrenal medulla [172], adrenal cultured chromaffin cells [8], or anterior and neurointermediate pituitary gland [77]. However, CNP synthesis has been documented to date only in the brain [75] and adrenal chromaffin cells [8]. In addition, small amounts of irCNP have been found in some non-neural tissues including the kidney, colon, and ileum [77]. CNP has not been found in plasma in significant amounts [77]. The predominant location of CNP in structures derived from the neuroectoderm as well as the failure to find this peptide in significant amounts in the heart and plasma [77] has led some investigators to think that, in contrast to ANF and BNP which are mainly cardiac peptides, CNP acts primarily as a neurotransmitter or neuromodulator in the central nervous system [76,77].

1.A.2 Classification of natriuretic peptide receptors

Biological effects of natriuretic peptides are presumably initiated by binding to their specific receptors. The distribution and binding characteristics of these receptors (K_d in picomolar or low nanomolar range [152]) have been intensely studied in various tissues mostly with the use of ¹²⁵I-ANF as a radioligand. These binding sites have been detected in somatic tissues of various species, particularly in the kidney [12,16,21,121], blood vessels [130,131], adrenals [12,21,30,130], intestine [12], pancreas [20], liver [12], lung [12], gonads [112], mammary gland [113], platelets [132], and placenta [57]. ANF binding sites also have been detected in many discrete areas of the central and peripheral nervous system, most apparently in the olfactory bulb, area postrema, subfornical organ, median preoptic nucleus, interpeduncular nucleus, nucleus of the solitary tract, median eminence, choroid plexus, ependyma, hypophysis, eye, and autonomic ganglia [51,54,117].

Subtypes of the natriuretic peptide receptor have been intensely studied using various techniques including affinity binding techniques, receptor purification and affinity cross-linking followed by electrophoresis, or molecular cloning techniques.

رچه خې Affinity binding studies initially did not resolve various classes of the natriuretic peptide receptor. That was most probably because natriuretic peptide receptor subtypes that bind ANF have similar apparent affinities for ANF, the first known and used ligand for this receptor [83, 178]. With the discovery of natriuretic peptide receptor C (NPR-C)-selective peptides [15,88,129] discrimination between different subtypes of the natriuretic peptide receptor was possible by affinity binding techniques and was widely utilized in a number of studies involving many tissues. Using an affinity binding technique, differences in natriuretic peptide receptor subtypes between normal and diseased strains of animals, such as Wistar-Kyoto rats and spontaneously hypertensive rats (SHR), were detected [17,18].

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Receptor purification and cross-linking studies combined with electrophoresis explored the structural properties of the natriuretic peptide receptor and resulted in the demonstration of receptor subunits with a mass of 65-70 kDa [160,170], 120-140 kDa [160], and 180 kDa [141].

Molecular cloning techniques identified three natriuretic peptide receptors each derived from a different gene [26,46,136]. Thus, existence of at least three subtypes of the natriuretic peptide receptor was fully documented: the natriuretic peptide receptors A and B (NPR-A and NPR-B, both corresponding to 120-140 kDa receptors) as well as the NPR-C (a dimer of 65-70 kDa subunits). Interestingly, these receptors are extremely well conserved across species exhibiting overall homology ranging from 93% to 97% [110,116].

1.A.2.1 Natriuretic peptide receptor A

The NPR-A is a membrane-bound polypeptide with a single transmembrane domain [26] which may exist in the cell membrane (at least in the bovine adrenal cortex) as a tetramer composed of four 140 kDa disulfide-linked molecules [64]. This receptor (alone or together with the NPR-B, see below) has been also designated R_1 or guanylate cyclase-containing receptor. Other designations include ANF-A or the B receptor (for biologically active

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receptor, since it is presumed that this receptor mediates biological actions of ANF).



Figure 1.A.3 Hormone specificity for natriuretic peptide receptors. A schematic representation of the ability of the human natriuretic peptides to specifically activate the guanylate cyclase of human NPR-A or NPR-B or bind to human NPR-C. The lines connect the receptors with their preferred ligands. TM, transmembrane region. (Based on Koller et al., Science, 252: 120-123, 1991.)

The NPR-A contains an extracellular ligand-binding domain, a single transmembrane domain and two intracellular domains: a protein kinase homology domain and a guanylate cyclase catalytic domain (Figure 1.A.3). Binding to the extracellular domain results in activation of guanylate cyclase, which increases intracellular cGMP levels. The protein kinase-like domain acts as a negative regulatory element [27] and probably contains a binding site for ATP [82] which is critical for signal transduction [28]. A suggested model for signal transduction through the NPR-A resembles in a way the signal transduction proposed for receptors coupled to the adenylate cyclase system [27,28,82]. It assumes that a hormone which binds to the extracellular domain initiates conformational changes in the intracellular domain which result in promotion of ATP binding to, or in the vicinity of, the protein kinase domain. This, on one hand, may produce allosteric changes which lead to an activation of the particulate guanylate cyclase and generation of cGMP, and, on the other

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hand, may promote dissociation of the hormone from its binding site at the extracellular domain.

The receptor NPR-A/guanylate cyclase responds preferentially to ANF [76,158]. BNP is also effective at stimulating that receptor but is slightly less potent [136]. CNP has been reported either not to stimulate cGMP production at this receptor site [76] or to generate insignificant increases [158]. Short ring-deleted or linear ANF analogues do not stimulate this receptor even at pathophysiological concentrations [15,88].

Expression of the gene for the NPR-A was documented in the lung, kidney, adrenals, duodenum, colon, placenta, vasa vasorum, liver, brain, and cardiac atrium and ventricle [60,136,180]. Differences in tissue concentration of this receptor among species were evident.

1.A.2.2 Natriuretic peptide receptor B

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The NPR-B is structurally similar to the NPR-A [22,136] (Figure 1.A.3). It exhibits considerable high sequence homology in the extracellular and particularly in the intracellular domains with the NPR-A. The extracellular domain of the NPR-B has 44% amino acid identity with the NPR-A. However one stretch of 28 residues included in this domain has a 79% sequence identity and is possibly important for the recognition of natriuretic peptides. The protein kinase domain of the NPR-B is identical in 63% of residues to the respective domain of the NPR-A, whereas the guanylate cyclase domain, with 88% identical residues, constitutes the most conserved sequence between the NPR-A and NPR-B.

The NPR-B also generates cGMP in response to occupancy by biologically active natriuretic peptides. Nonetheless, the binding selectivity pattern of this receptor dramatically differs from that of NPR-A. The NPR-B is activated most efficiently by CNP, the putative physiological ligand of this receptor [76]. However, it also slightly responds to stimulation by BNP and ANF [22,76,158]. Expression of the NPR-B gene has been documented in the same organs as the NPR-A gene (as discussed in Section 1.A.2.1). However, the concentrations of mRNA for NPR-A and NPR-B detected were different [136].

1.A.2.3 Natriuretic peptide receptor C

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The NPR-C, designated also R₂, ANF-C, C-receptor guanylate cyclase-free natriuretic peptide receptor, silent or clearatice receptor, exists on the cell membrane predominantly as a homodimer of 60-70 kDa subunits [170]. It was the first natriuretic peptide receptor cloned [46]. This receptor substantially differs from the NPR-A and NPR-B (Figure 1.A.3). It consists of a large extracellular domain (probably containing attached carbohydrates), a single transmembrane domain, and a small (37 amino acids) intracellular domain. The extracellular domain shares 30-33% of identical residues with the NPR-A and NPR-B. There is a region in this domain that is more conserved (~70% homology with the NPR-A and NPR-B) which might be important for natriuretic peptide recognition. The transmembrane domain is not highly conserved among all the natriuretic peptide receptors [136]. The protein kinase and guanylate cyclase homology regions identified in the cellular domain of the NPR-A and NPR-C.

The NPR-C, in contrast to the both guanylate cyclase-containing receptors, does not impose stringent constraints on ligand structure for binding. It binds ANF, BNP and CNP as well as a range of truncated, ring-deleted and linear ANF analogues with high affinity [15,46,76]. These ANF analogues have proven to be very useful in ligand binding studies for discrimination between guanylate cyclase-containing and guanylate cyclase-free natriuretic peptide receptors [15]. NPR-C, which is often more abundant than NPR-A and NPR-B [15,88], has been found to be expressed in several mammalian tissues including kidney, adrenals, heart, placenta, as well as smooth muscle and endothelial cells [46,60,116,180].

In contrast to the guanylate cyclase-containing receptors, which have an established role in mediation of most biological actions of the natriuretic

peptides, the role of the NPR-C still remains controversial. Early studies did not demonstrate an association of this receptor with any second messenger system but showed its involvement in the internalization and metabolic clearance of natriuretic peptides [88,103]. The NPR-C has been postulated to function as a buffer to prevent sudden and large changes in blood pressure when ANF (or BNP) are secreted from the heart [88]. More recently, however, various data suggest that it signals through G proteins inhibiting adenylate cyclase [4,34] or activating the phosphoinositide pathway [59]. There is increasing evidence that the NPR-C may mediate biological effects [67,86].

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1.A.3 Major biological actions of natriuretic peptides

Biological actions of natriuretic peptides have been most extensively studied for ANF and only recently for BNP and CNP. Many of the actions exhibited by natriuretic peptides are related to the maintenance of body fluid, electrolyte, and cardiovascular homeostasis and tend to promote decrease of blood pressure and extracellular volume. Unfortunately, most of the information on the biological actions of natriuretic peptides is based on experiments which rely on observation of the effects of stimulation of the natriuretic peptide receptor system by applying exogenous natriuretic peptides to the mammalian body or to other bioassay systems. That type of experiments does not allow, however, to draw direct conclusions as to the possible physiological significance of endogenous natriuretic peptides. Exact delineation of physiological responses of natriuretic peptides was hampered by lack of good specific pharmacological antagonists. Attempts have been made to circumvent this obstacle by using ANF antibodies or surgical atrial appendectomy. However, local synthesis of ANF often made these approaches difficult to interpret. To date, animals deficient in ANF have not been produced. Still, great deal of information was derived from studies on transgenic mice overexpressing ANF.

1.A.3.1 Renal actions

At the level of the kidney ANF promotes excretion of water and sodium [184]. After a bolus injection the onset of action is rapid (within 5 min) and is relatively short-lived (2 to 30 min). However, the mechanisms underlying these functions have not been fully elucidated. They appear unique and likely represent the composite result of multiple sites of action including hemodynamic and epithelial effects.

ANF, depending on its circulating levels, can enhance glomerular filtration rate (GFR) by increasing both glomerular capillary hydrostatic pressure and the filtration coefficient (k_f) [84]. The increase in glomerular hydrostatic pressure is attributed to a combination of efferent vasoconstriction and afferent vasodilation, or solely to vasoconstriction of post-glomerular vessels. The mechanism whereby ANF increases k_f is not known but, since high affinity ANF binding sites are present on mesangial cells, it is conceivable that ANF alters k_f by regulating the ultrafiltration surface area [84]. Although controversies exist it is assumed that in glomeruli cGMP mediates the actions of ANF [9]. Due to increase in GFR, the filtered load of sodium increases but the peritubular oncotic pressure generated by the increased filtration fraction also increases, thus enhancing absolute proximal reabsorption of sodium. Still, a modest increment in salt and water delivery out of proximal tubule is noted. In the cortical collecting duct ANF decreases the water reabsorpting effect of vasopressin which may account for some of the diuresis observed during administration of ANF [184]. In the medullary collecting duct ANF was shown to inhibit directly reabsorption of luminal sodium entry into collecting duct cells by regulating the amiloride-sensitive sodium channel [185]. This action is mediated by cGMP. Additionally, at the level of the medulla, ANF has been shown to dilate vasa recta which results in elevation in papillary blood flow [72]. This, in turn, affects papillary interstitium properties such as hypertonicity and pressure, which subsequently result in secretion of sodium from interstitium into the tubular fluid and contributes to an increased

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tubular fluid sodium concentration. Thus, the combined effects of enhanced GFR and diminished medullary tubular sodium and water reabsorption [that can be additionally magnified by ANF-caused inhibition of aldosterone, vasopressin, and angiotensin II (ANG II) secretion or action] seem to lead to the striking natriuresis and diuresis observed after administration of ANF. However, transgenic mice, whose irANF levels were increased ~10-fold, essentially maintain normal fluid and electrolyte homeostasis, which argues against a major physiological role of ANF in water and sodium balance [40]. These mice, however, also show a ~20 to 25 mm Hg reduction in blood pressure compared with their normotensive siblings [149]. It is conceivable that in these mice a reduction in renal perfusion pressure might antagonize a direct natriuretic action of ANF on the kidney. In support of this concept is the observation that after acute hypervolemia, perfusion pressure increases in these mice and allows the full renal expression of high ANF levels.

1.A.3.2 Cardiovascular actions

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ANF can lower blood pressure in both normal and hypertensive subjects [167]. At the level of vasculature ANF relaxes isolated vascular smooth muscle segments *in vitro* [38,48], an effect which appears to be direct and involves activation of particulate guanylate cyclase [181]. Preparations from larger vessels, such as aortic and renal arteries, are in general more sensitive to the peptide than those from smaller vessels [38] where ANF has little or no effect [109]. The vasorelaxant actions are more prominent in the presence of vasoconstrictors such as ANG II or noradrenaline [73]. However, *in vivo* the acute hypotensive response to ANF appears to be due predominantly to a decreased cardiac output resulting from a reduced venous return to the heart and consequently a reduced stroke volume [111]. The reduced venous return results from ANF-altered extracellular fluid distribution which favours interstitium over plasma (reduced net capillary absorption) [169]. The initial fall in cardiac output gives way with time to a decrease in total peripheral resistance accounting for a continued hypotensive effect [111]. Studies on

transgenic mice overproducing ANF showed that the animals were significantly hypotensive (~20 to 25 mm Hg below controls) [149], suggesting that ANF may play a major role in the maintenance of normal blood pressure.

Interestingly, ANF-evoked decrease in cardiac output and arterial blood pressure is accompanied by a decrease or no change in heart rate rather than by the expected baroreceptor-mediated reflex tachycardia [3]. Therefore, it has been proposed that neural reflexes are impaired by ANF. ANF has been reported to influence arterial chemoreceptor and/or baroreceptor reflexes, which in turn modulate sympathetic outflow to vasculature as well as to other organs including the heart [61,133]. It is conceivable that one of the sites where systemic ANF may influence these reflexes is the CNS with its circumventricular organs which lack the normal blood-brain barrier, possess a high density of ANF receptors, and show abundant connections with CNS centers modulating vasomotor and baroreflex information.

1.A.3.3 Hormonal actions

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ANF has been shown to have various modulatory effects on hormonal systems including the renin-angiotensin-aldosterone system, vasopressin, anterior pituitary hormones, and other hormones.

ANF inhibits renin release from juxtaglomerular cells [81]. The mechanism of this action correlates well with increases in cGMP and is undermined by the cGMP-inhibitor, methylene blue [81], suggesting that the inhibition occurs by a cGMP-mediated pathway. Indeed, administration of ANF antibodies results in increased plasma renin activity in rats [96].

ANF also inhibits basal and stimulated (by ACTH, potassium, or ANG II) release of aldosterone by the adrenal zona glomerulosa [23,24], primarily at the early steps of steroidogenesis (formation of pregnenolone) and to a lesser extent at the later steps (conversion to aldosterone) [19]. In contrast, alterations in cGMP may not account for this inhibition, because the addition of a cGMP derivative does not seem to mimic the action of ANF [36]. The effect is, therefore, expected to be mediated through receptors which are utilizing a

different, not cGMP-mediated, signal transduction pathway, or through an as yet undiscovered receptor population. The mechanism by which ANF inhibits aldosterone secretion may involve inhibition of adrenal calcium channels [25,90]. Interestingly, however, in ANF-transgenic mice a 2-fold increase of aldosterone level and a marked hyperplasia of the adrenal zona glomerulosa was observed [150]. Conceivably, chronic hypotension evoked by ANF may induce a secondary hyperaldosteronism even in the presence of increased levels of circulating ANF. In addition, ANF and BNP have been shown to inhibit secretion of other adrenal hormones including 19-OH androstenedione and cortisol [97]. Furthermore, ANF has been demonstrated to enhance secretion of a testicular hormone, such as testosterone [45].

ANF has also been reported to decrease the release of several pituitary hormones. For example, it inhibits vasopressin [124,115], luteinizing hormone [125], ACTH [41], and prolactin [126] release. Despite controversial reports the site of ANF action probably lies at the level of the hypothalamus in the case of vasopressin, prolactin and luteinizing hormone [124,125,126] and at the level of the pituitary in the case of ACTH [41].

1.A.3.4 Central nervous system actions

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The widespread CNS distribution of ANF-containing neuronal cell bodies and fibres as well as ANF receptors combined with the finding that ANF is also synthesized [147] and secreted in the CNS [161] implies a role for ANF in the modulation of CNS activity. Indeed, ANF and BNP have been demonstrated to have an effect on neuronal excitability in several nuclei of the hypothalamus, thalamus and brain stem [37,106,146,182]. The ability of ANF to modify CNS neuronal excitability supports the view that natriuretic peptides may act as neuromodulators or neurotransmitters within the CNS.

Stimulation of brain ANF receptors by intracerebroventricular (icv) administration of ANF produce several CNS-mediated actions which complement those produced by systemic ANF. Using this approach, ANF has been shown to inhibit water intake stimulated by ANG II or by dehydration [5], while its antibodies have been demonstrated to produce an opposite effect [69] suggesting a physiological antidipsogenic role of the brain pool of ANF. Furthermore, ANF has been shown to blunt salt appetite in salt-depleted animals with an exaggerated urge to consume salt water over tap water [42]. Moreover, centrally administered ANF produced a fall in blood pressure in normal rats after sinoaortic denervation [134] and in SHR [85]. It attenuated the increase in blood pressure produced by icv ANG II and potentiated the central depressor effect of saralasin, an ANG II receptor blocker [143]. Interestingly, SHR have been shown to have a reduced number and affinity of ANF binding sites in areas rich in ANG II receptors, such as the subfornical organ [122], which additionally supports the view that brain ANF interacts with the central renin-angiotensin system. As mentioned before (Section 1.A.3.3), icv ANF has a profound effect (with a site of action at the level of the CNS) on secretion of certain pituitary hormones including vasopressin [115,124], luteinizing hormone [125], and prolactin [126]. The effect of ANF on vasopressin production is additionally supported by the demonstration of a decreased specific vasopressin immunoreactivity of neurons in paraventricular and supraoptic nuclei as well as in varicose fibers in the hypothalamo-hypophyseal tract [87]. Reports documenting that icv ANF produces a significant increase in urinary volume may also be consistent with the concept that ANF inhibits vasopressin secretion [42]. Additionally, ANF has been shown to inhibit production of the eye aqueous humor and cerebrospinal fluid [148]. The mechanism of ANF action in the brain is not clear but it may be in part mediated in some CNS regions via the dopaminergic system, because icv ANF preferentially decreased dopamine levels in the septum and hypothalamus [95].

1.B AUTORADIOGRAPHY AND OTHER RECEPTOR-MAPPING TECHNIQUES

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There are various approaches which make possible localization of hormone-specific binding sites. Some of them provide visualization and mapping of receptors within an anatomical framework. The fundamental advantages of these latter approaches are increased anatomical resolution and greatly enhanced sensitivity of measurement when compared with biochemical methods (even if they are combined with microdissection techniques). Thus, receptor mapping will be useful in any situation which requires receptor measurement in small regions or where the overall quantities of the receptors are low. Receptor visualization provides a powerful insight into the mechanism of hormone, neurotransmitter, and drug action because the mapping helps to identify those tissue elements which are directly affected by drug administration or by changes in levels of hormones or neurotransmitters. Receptor mapping indicates regions of importance for further studies. Since receptors may change in a variety of disorders, and it is likely that receptor changes may mediate or may be a primary cause of some of these disorders, receptor mapping can be a powerful tool in exploring pathology.

In the field of receptor mapping autoradiography has emerged as a frontline tool. There are other, very good techniques used in this area which are briefly discussed in this work, such as positron emission tomography, immunocytochemistry, techniques based on the measurement of receptormediated changes in second messenger levels or detecting mRNA for receptors.

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1.B.1 Receptor autoradiography

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Receptor autoradiography encompasses various approaches that enable visualization of receptor binding sites in relatively intact tissues labeled in vivo or in vitro with a receptor-specific radioligand. Before any radioligand is chosen for visualization of receptors, it is necessary to have an adequate guide to its usefulness. All criteria for true receptor binding should be satisfied, e.g., dose-dependency, saturability, high affinity, recognition specificity. Since the density of receptors for most peptides is low, to ensure sufficient sensitivity of the method the ligand should be labeled with high specific activity. Therefore, ¹²⁵I is often used as a tracer since it allows much higher specific activity than e.g., tritium, the second most often utilized tracer. However, it is not always possible to use iodinated peptides for receptor binding studies, particularly if iodine itself or the iodination procedure changes significantly the binding properties of the ligand molecule. Additionally, in contrast to tritium, the short half-life of ¹²⁵I does not ensure long-term availability. The ligand used should be pure and, preferably, chemically stable and resistant to enzymatic degradation. If not, conditions should be created that minimize degradation (e.g., short time of exposure, use of proteolytic inhibitors, low incubation temperature).

The physiologically effective ligand for a receptor to be studied is the first choice for localizing the latter but in the majority of cases one is faced with several limitations. These ligands are more sensitive to enzymatic degradation than for example their analogues with D-amino acids [114]. They often bind to more than one subtype of a given receptor and are unable to discriminate between receptor subtypes. Hence, a number of subtype-selective analogues have been developed and used for this purpose. Furthermore, the binding of physiologically effective ligands is reversible and may pose a problem during tissue preparation for generation of autoradiograms. If significant diffusion of a ligand from the receptor occurs, there will be a serious loss of anatomical resolution and perhaps a misleading distortion of the image obtained by

autoradiography. To circumvent this problem, irreversibly binding ligands (e.g., nitrogen mustard for the muscarinic acetylcholine receptor) or pseudoirreversibly binding ligands (e.g., α -neurotoxins) are used [10]. Since the majority of available ligands bind in a reversible manner, tissue preparation for obtaining the image of receptor distribution must be done in a way that takes into account the diffusible nature of the signal.

1.B.1.1 In vivo labeling of receptors

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In this procedure binding sites are labeled in intact living tissues by systematic administration of small amounts of receptor-specific radioligand. The ligand is carried to all tissues by the blood stream and binds to its receptor and to non-receptor binding sites. The ligand bound to non-receptor sites is then removed, at least in part, by whole-body perfusion with Ringer's solution. The high affinity of the ligand for the receptor causes its retention on the receptor. However, some of the receptor-bound ligand may be lost. A demonstration of specific receptor labeling is an extremely important factor. It is done with control experiments in which the same small amount of radiolabeled ligand is injected along with large quantities of unlabeled ligand with affinity for the same receptor. In these control experiments the quantities given in excess should block the binding of the small doses of the radioligand. Specific binding is then found as the difference in bound radioligand between the experimental preparation (which shows total binding) and control preparation (which shows nonspecific bindings).

After labeling, autoradiograms are generated to provide information on the distribution of receptor binding sites at microscopic level. Since most often reversible ligands are used, autoradiograms should be produced by methods which minimize diffusion of the ligand from a binding site. One way is to use fixatives, preferably administered by perfusion immediately following administration of the Ringer's solution. These are believed to leave the protein ligand attached to its binding site without translocation which could occur during the following steps for tissue processing for electron- or light-

microscopic examination, such as fixation by immersion, dehydration, infiltration, embedding, sectioning, coating with photographic emulsion, and exposure. This method is excellent for electron-microscopic visualization of receptor binding sites and has led to mapping of receptor distribution of various peptide hormones (including ANF) in a number of tissues [11,12,179].

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There are other approaches minimizing the diffusion of ligands, especially important for non-peptide ligands, like steroid hormones, that cannot be cross-linked by fixatives. These approaches involve special processing of frozen, unfixed tissue labeled in vivo (or in vitro). This procedure, first introduced by Stumpf and Roth [154], utilizes thaw-mounting or dry-mounting of cryostat-cut sections to emulsion-coated slides in the dark [80]. In the dry-mount method the tissue is not allowed to thaw and the dry photographic emulsion is pressed onto the frozen section and exposed in approximately -80°C. Technically easier, but less reliable (since some risk of translocation may be incurred), is to mount thawing sections on the emulsion-coated slide and expose at 2°C. The important feature of this overall procedure is that the mounted tissue sections are not exposed to an aqueous environment where the ligand could diffuse away from the receptor binding site. On the other hand, while fixation gives an excellently preserved cell and tissue structure for viewing in electron and light microscope, the "dry" techniques do not offer such good tissue morphology, because they utilize frozen sections.

In vivo labeling of receptor binding sites respects the anatomical, histological, and cytological integrity of the tissue and, therefore, is a priori preferable when compared with *in vitro* techniques. Nevertheless, *in vivo* labeling has serious limitations. It must tackle the existence of the blood-brain barrier, which prevents entry of most ligands into the mammalian CNS except at the level of circumventricular organs. Furthermore, there could be changes in the regional blood flow after administration of a vasoactive ligand and, as a result, the ligand may not be distributed to all cells. Successful application of

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the technique depends on the existence of high affinity ligands that are resistant to metabolism in a living organism. Also, the binding conditions that are so critically important to control the specificity of labeling cannot be modified or controlled very easily *in vivo*. *In vivo* labeling is expensive since an entire animal must be loaded with radioactive ligand even if only small portions of tissue are actually needed in the experiment.

1.B.1.2 In vitro labeling of receptors

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In this technique, first introduced by Young and Kuhar in 1979 [183], frozen (sometimes lightly fixed by perfusion) tissue sections are thaw-mounted onto microscope slides and incubated with radiolabeled ligand in vitro [63]. The incubation is preceded by a preincubation step in which endogenous ligands are expected to dissociate from their receptors and leave the sites unoccupied for the subsequent radioligand binding. Following the receptor binding sites labeling the slide-mounted tissue sections are washed in order to decrease the amount of nonspecific absorption of the radioligand and improve the ratio of specific to nonspecific binding. After that, tissue is dried as rapidly as possible in cold air. Alternatively, the labeled and washed tissue sections are exposed to glutaraldehyde in order to irreversibly bind the ligand to its binding site and then rinsed, dehydrated in alcohol, and dried (Figure 1.B.1). Once the slidemounted tissue sections have been prepared in such a way that selective receptor labeling has occurred, autoradiograms can be generated. This is done by apposition of the labeled slide-mounted tissue to emulsion-coated coverslips or to films sensitive to radioisotopic radiation. Section-bearing slides could be also coated with molten emulsion. The required exposure time varies for each receptor system and is directly related to the specific activity of the ligand used and to the total number of receptors present in the area of interest. After exposure and development, receptor binding sites are visualized either as grain density in photographic emulsion or optical density on autoradiographic film.

CHAPTER 1



Figure 1.B.1 Quantitative receptor autoradiography.

The emulsion-coated slide method offers the possibility of cellular localization of receptor binding sites under a light microscope. The emulsioncoated coverslip technique is also claimed to be suitable for the above but it cannot provide high resolution, because the silver grains and the tissue sections are at different focal levels under the microscope. The

autoradiographic images produced by these two techniques are examined under dark-field or bright-field illumination. The dark-field illumination is usually employed to scan the slides, because the grains are readily visible and small quantities of them are more easily detected. The tissue structure, however, is not well visualized. The bright-field illumination shows the tissue morphology (after histological staining), but at the same time much higher number of grains must be present to be demonstrable.

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The film-based procedure is easy to perform and may be readily analyzed by computerized techniques, but it provides reduced anatomical resolution when compared with the resolution obtained with the nuclear emulsion. It provides information as to the regional but not the cellular distribution of receptor binding sites. This stems from the fact that in this method the recording film and the radiation source (slide-mounted sections) are processed separately, viewed independently and thereafter compared or realigned.

In general, one of the great advantages of autoradiography is that it is quantitative both when in vivo [11] or in vitro labeling [63] of receptor binding sites are employed. However, grain density in nuclear emulsion and optical density in film are not always linearly related to the radioactivity content of the tissue. Hence, if quantification is required, standards should be included in an autoradiographic experiment in order to construct calibration curves relating grain or optical densities to radioactivity. Tissue radioactivity content may be then converted to binding site levels knowing the specific activity of the radioligand. The radioactivity in the standards should be embedded in material similar to the experimental material, especially if low energy tracers, like tritium, are used. This is because lower-energy β -emission is absorbed by molecules of the surrounding material (depending on its density) before this emission has had the opportunity of interacting with the emulsion. Radioactive standards can be purchased commercially or can be prepared by the investigator by mixing a known amount of radioactivity in tissue paste. For quantification or semi-quantification of the experimental data (and standards) visualized as grain density, a manual (but also automatic) count of autoradiographic grains in a unit area is normally employed using a microscope equipped with a calibrated grid. These manual approaches can be very laborious. As far as quantification or semi-quantification of data (and standards) visualized as film optical density is concerned, computer-assisted image analysis systems are often used allowing collection of data from complex images in a short time and conversion of optical density to tissue radioactivity content or to binding site levels. The main advantage of the filmbased autoradiography is that many slides can be exposed at the same time. Addition lly, in contrast to techniques involving molten nuclear emulsion, the film-based procedure may provide autoradiographic data from a single specimen more than once. This is important in situations when a tissue contains regions that have high levels of receptors and other regions with low levels of receptors, and it is necessary to expose the same tissue for several different periods of time. Furthermore, the film-based autoradiography technique allows pharmacological characterization of receptor binding sites to an extent similar to biochemical methods, providing at the same time a greater sensitivity and anatomical resolution.

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The *in vitro* labeling procedure has many advantages over *in vivo* labeling. Since receptor binding site labeling is performed with single tissue sections mounted on slides, it is possible to examine different receptors in different adjacent sections. Similarly, it is possible to label tissues under a wide variety of conditions e.g., examine the effects of various ions or nucleotides on ligand binding. Possible degradation of ligands can be readily controlled by addition of proteolytic inhibitors or lowering of the incubation temperature. Ligands that will not cross the blood-brain barrier *in vivo* can be used in this *in vitro* technique. It is possible to carry out studies with human postmortem tissue.

In vitro autoradiography has been an important advance in the field of receptor mapping but it still has significant limitations. Although ligands with

a lower affinity than that which is necessary for *in vivo* autoradiography can be used, low affinity sites are still not easily identified. This is because the dissociation rate of these low affinity ligands may be rapid enough that diffusion of these ligands away from low affinity receptor binding sites occurs during the washing necessary after the incubation step. Furthermore, preparation of the tissue, which involves dissection and freezing, may produce morphological defects in the tissue. An important disadvantage of *in vitro* autoradiography employing frozen tissue is that it lacks the ultrastructural resolution necessary for the fine localization of receptor binding sites. However, for irreversible ligands it is possible to process *in vitro*-labeled and fixed tissues for electron-microscopic autoradiography [79].

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1.B.2 Positron emission tomography

Positron emission tomography (PET) utilizes the unique properties of the radiation generated when positrons, produced by radioactive decay, are annihilated by interacting with electrons. In this in vivo technique, a receptor-specific ligand labeled with a radioactive isotope, that decays by emitting a positron, is injected into an animal or human. The emitted positrons combine almost immediately with electrons. The two are mutually annihilated emitting two γ -rays at approximately 180° from each other, which pass through the tissue and are detected outside of the subject by a circular array of detectors. The annihilation radiation can be differentiated from the background. The signals sensed by the detectors are transformed by a computer which rapidly reconstructs the spatial distribution of the radioactivity, corrects for the attenuation or absorption of annihilation radiation by the object being imaged, and displays the results on a screen as a two- or even threedimensional image. Moreover, these computer-processed images can be rotated on a screen. The resolution of detection systems has steadily improved and is now in the range from less than 1.0 to 1.4 cm for most tomographs. The ultimate resolution is limited by the fact that the positron, emitted by radioactive marker of the receptor ligand, travels a distance from 1 to 6 mm before the annihilation event occurs [118].

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Although this area of research is just at its beginning a considerable number of papers documenting the distribution of receptors obtained by PET scanning have been published. These studies have focused mostly on neurotransmitter receptors, especially of the dopaminergic and serotoninergic systems [159,177] of baboon and human brains.

The great advantage of the PET is that it is a non-invasive method, whereby receptor binding sites can be quantitatively and spatially detected in humans and animals, and the image can be obtained in minutes. This provides a much larger population of receptors available for measurement than it is possible with postmortem tissue. Since alterations in receptor concentration may be involved in some neuropsychiatric disorders, the possibility of evaluating receptor concentration *in vivo* in humans before, during, and after onset of symptoms or drug treatment provides a very unique opportunity to assess the importance of receptors in these disorders. An obstacle in implementing a PET method is that it requires numerous skilled personnel and complicated expensive equipment, such as a dedicated cyclotron and facilities for rapid synthesis of receptor-specific radioligands. Another disadvantage is that PET scans are of much lower resolution when compared to microscopic approaches.

1.B.3 Immunohistochemistry

The availability of antibodies for receptors provides a possibility of receptor localization using immunocytochemistry. This technique relies upon specific recognition of an antigen (receptor) present in a tissue by an antibody, making detection and subcellular localization of receptors possible even in the absence of suitable ligands. The method can be conducted either with polyvalent antisera or with monoclonal antibodies. It enables detection of receptor molecules occupied or unoccupied by receptor ligands. The antibodies may be used to localize receptors at the light-microscopic level using fluorescent or enzymatic markers such as peroxidase. For electron microscopy enzymatic or electron-dense labels, such as ferritin or colloidal gold, are commonly employed. With immunocytochemistry a number of receptors have been localized, including receptors for ANF [70].

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Immunocytochemical methods have many advantages in receptor visualization. They are faster than autoradiography, because exposure and emulsion development are not required. Cells containing receptors are clearly delineated which is not the case with autoradiography. Ultrastructural techniques with antibodies have better electromicroscopic resolution than with autoradiography. Sensitivity of the method is in general very high but depends on conditions of fixation and tissue processing. Although efforts have been made to quantify immunohistochemical approaches, they do not seem to be as readily quantitative as autoradiography.

1.B.4 Receptor-mediated changes in second messenger activity

Receptors are coupled to various second messenger systems and can act e.g., by stimulating or inhibiting the formation of cAMP or cGMP, stimulating the hydrolysis of phosphatidylinositol, and/or altering the flux of various ions. Thus, instead of measuring the receptor levels themselves it is possible to detect them by measurement of receptor-mediated changes in second messenger levels, an approach which is normally more sensitive than the former. Such an approach provides a good index of not only the binding site but also of the interaction between the binding site and its effector complex, thus delivering information on the whole functional receptor system. Changes in the second messenger system due to a receptor stimulation by a specific ligand can be detected on tissue slides using immunocytochemistry with antibodies directed against e.g., cGMP [31]. Thus, the measurement of receptor-mediated changes in second messenger activity could be one of the techniques used for receptor visualization within its anatomical framework.

1.B.5 In situ hybridization

In situ hybridization enables detection and quantification of specific nucleic acid sequences in individual cells in the context of surrounding tissues. It involves the hybridization of a nucleic acid probe with a specific nucleic acid target within a tissue section. Since genes for many receptors (or its subunits) have been cloned and molecular probes to detect mRNA for various receptors have become available, this method can be employed to study the distribution of mRNA for receptors within their anatomical framework. In other words, it can be used to study the synthetic capability for the receptors in individual cells. This method has been successfully employed to study a number of receptors, including the receptor subtypes for natriuretic peptides [180].

In situ hybridization may complement very well receptor autoradiography or immunocytochemistry. The combined use of these techniques provides a cross-check of their specificity. Additionally, since mRNA levels may indicate the functional state of cells and in many cases do not correlate very well with their respective protein levels, combination of these methods can give new insights into the synthesis rates of receptors as well as the turnover of the receptor macromolecules.

As in the case of receptor autoradiography, precise cellular localization and great sensitivity are two main advantages of *in situ* hybridization. However, this method is not without limitations. The *in situ* cDNA-mRNA hybridization method may not be sensitive enough to detect a specific mRNA that is present at sub-threshold levels of detection. This would be of even greater concern if the mRNA is present within discrete cells scattered over a large tissue area. Similarly, there is a possibility of false labeling by the *in situ* technique. This would be particularly true in situations wherein different cell populations make different mRNAs from the same primary transcript [102].

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# **CHAPTER 2**

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# CELLULAR LOCALIZATION OF ATRIAL NATRIURETIC FACTOR BINDING SITES IN THE CNS

Binding sites for atrial natriuretic factor have already been demonstrated in a number of mammalian tissues. However, most of the techniques employed to study distribution of these binding sites give excellent information on their regional localization but lack the anatomical resolution to address the question as to their precise cellular localization in highly organized intact tissues, particularly in the central nervous system. Consequently, the cells containing ANF binding sites i.e., the target cells for ANF action, have been poorly studied in the central nervous system.

The studies presented in this chapter were performed in an attempt to localize ANF binding sites in individual cells of the choroid plexus and area postrema, two central nervous system structures which play a prominent role in the central regulation of blood pressure as well as water and electrolyte balance. Availability of selective ligands also allowed to determine the subtype of ANF receptors present in each tissue. The information on the type of cells containing ANF receptors as well as the type of the ANF receptor in these tissues may provide a clue as to the possible involvement of these central nervous system structures in volume and blood pressure regulating effects of circulating ANF.

# 2.A LOCALIZATION AND CHARACTERIZATION OF ATRIAL NATRIURETIC FACTOR BINDING SITES IN THE RAT CHOROID PLEXUS

This section is devoted to the choroid plexus, one of the central nervous system structures with large numbers of ANF binding sites, lacking the normal blood-brain barrier and, therefore, accessible to intravascularly administered <sup>125</sup>I-ANF. ANF binding sites in the choroid plexus were characterized and the proportion of guanylate cyclase-containing to guanylate cyclase-free ANF receptor subtypes in this tissue was estimated by quantitative autoradiography in vitro. Secondly, the ultrastructural localization of ANF binding sites in this tissue was visualized by electron-microscopic autoradiography. Finally, the penetration of ANF from the blood to the cerebrospinal fluid was studied and the nature of the choroid plexus-bound material was analyzed by high-performance liquid chromatography.

As mentioned before, ANF binding sites were studied in this work using <sup>125</sup>I-ANF as a radioligand. Since this peptide demonstrates an apparent high affinity for both NPR-A and NPR-C (referred to, in this work, as guanylate cyclase-containing and guanylate cyclase-free natriuretic peptide receptors, respectively), these studies allowed visualizing the localization of both receptor subtypes. NPR-B was not known at the time of experimentation for this paper, and thus was not investigated.

# 2.A.1 Abstract

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In vitro autoradiographic studies showed that high-affinity atrial natriuretic factor (ANF) binding sites are present on the rat choroid plexus ( $K_{d}$ , 83.8 pM,  $B_{max}$ , 22.9 fmol/mg protein). Guanylate cyclase-containing receptors represent approximately 30% and guanylate cyclase-free ANF receptors represent 70% of the total ANF receptors present in this tissue. To provide

detailed cellular localization of these binding sites, the technique of light- and electron-microscopic autoradiography was applied using <sup>125</sup>I-ANF-(Ser99-Tyr126) as an *in vivo* ligand. Light-microscopic autoradiography demonstrated that ANF binds specifically to the choroid plexus. Electron-microscopic autoradiography showed that silver grains were localized primarily on epithelial cells of the choroid plexus (99%) and marginally on endothelial and pial cells (1%). In choroidal epithelial cells, ultrastructural analysis of silver grain distribution revealed that, at 2 min after intracarotid <sup>125</sup>I-ANF injection, the basolateral plasmalemma was the most distinctly labeled organelle (the highest ratio of % real grains/% random hypothetical grains ). Intracellular compartments such as cytoplasmic vesicles, Golgi apparatus, and lysosomes were labeled distinctly as well (although lower number of grains was associated with these organelles) suggesting a possible internalization of ANF binding sites. HPLC analysis disclosed that 2 minutes after injection 95.3% of the choroid plexus-bound <sup>125</sup>I-ANF was eluted from the reverse-phase HPLC column at the elution time corresponding to the intact peptide. These results indicate that ANF binding sites are present on the choroidal epithelium, primarily at the basolateral plasmalemma. Cellular localization of these sites may relate to their possible involvement in the central regulation of salt and water homeostasis as well as of cardiovascular functions of circulating ANF.

# 2.A.2 Introduction

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The choroid plexus (ChP) is a structure located in all four cerebral contricles, specialized for solute and solvent transport between blood and cerebrospinal fluid (CSF) compartments [5]. It is a major site of CSF production. The ChP is a relatively simple tissue composed of a secretory epithelium surrounding a vascular connective tissue core [9,14]. The apical surface of epithelial cells, with numerous microvilli, faces the CSF, while the basal surface of these cells, with abundant infoldings, faces the perivascular space. The ChP possesses fenestrated capillaries but most of the substances passing out of the vasculature are prevented from entering the CSF because

the apical ends of epithelial cells are joined by "leaky" tight junctions. The tight junctions constitute the blood-CSF barrier at the level of the ChP [14].

Several lines of evidence suggest that atrial natriuretic factor (ANF) influences the function of the ChP. ANF binding sites have been detected in the ChP by autoradiography *in vitro* [29,32] and by light-microscopic (LM) autoradiography *in vivo* [2], and they have been characterized by quantitative autoradiography *in vitro* [18,19,31]. It has been demonstrated that in the ChP ANF stimulates guanylate cyclase activity [10,35] and increases cyclic GMP (cGMP) levels [37]. ANF significantly decreases the rate of CSF formation when injected into the cerebral ventricles [35].

Although ANF receptors have been localized in choroidal epithelial cells in culture (by demonstration of stimulation of guanylate cyclase by ANF) [35], the precise cellular localization of the ANF binding sites has never been demonstrated in the intact ChP. We have approached this problem, in the present studies, by applying the technique of electron-microscopic (EM) autoradiography using <sup>125</sup>I-ANF-(Ser99-Tyr126) as an *in vivo* ligand. In the course of these studies we also characterized the ANF binding sites in the ChP and estimated the proportion of guanylate cyclase-containing to guanylate cyclase-free ANF receptor subtypes in this tissue by quantitative autoradiography *in vitro*. We studied also the penetration of ANF from the blood to the CSF and analyzed the nature of the ChP-bound material by highperformance liquid chromatography (HPLC).

# 2.A.3 Materials and Methods

### 2.A.3.1 Iodination of ANF

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Radioiodination of ANF-(Ser99-Tyr126) (Bio-Mega Inc., Laval, Quebec, Canada) was done by the lactoperoxidase method with <sup>125</sup>I-sodium as described earlier [24]. Purification of the monoiodinated form was achieved by HPLC. The specific activity was ~2,400 dpm/fmol (1,100 Ci/mmol).

### 2.A.3.2 Autoradiography in vitro

Preparation of autoradiograms and <sup>125</sup>I-standards, as well as microdensitometric procedures used in our laboratory, have been previously described in detail earlier [3]. Saturation curves were analyzed using EBDA [20] followed by LIGAND [23] computer programs to obtain estimates of the B<sub>max</sub> (maximum binding capacity) and K<sub>d</sub> (dissociation equilibrium constant) of ANF binding sites in the ChP from lateral ventricles. Competition binding curves for ANF-(Ser99-Tyr126) and C-ANF [des-(Gln116-Gly120)ANF-(Arg102-Cys121)-NH<sub>2</sub>] were analyzed simultaneously using ALLFIT computer program [7] to estimate the proportion of guanylate cyclase-containing to guanylate cyclase-free ANF receptor subtypes.

### 2.A.3.3 Autoradiography in vivo

# 2.A.3.3.1 Injection of <sup>125</sup>I-ANF and processing of tissue

Sprague-Dawley rats (~40 g body weight) received, under pentobarbital anesthesia, a single bolus intracarotid injection (tip of the catheter in a cephalad direction) of 41 x 10<sup>6</sup> cpm (22.8 pmol, total binding) <sup>125</sup>I-ANF dissolved in 90 µl of phosphate buffer (0.1 M, pH 7.4). To estimate the nonspecific binding, the same quantity of <sup>125</sup>I-ANF was mixed with an excess of unlabeled ANF (16.7 nmol) and injected in the same volume, in the same manner. In the second experiment of this type, the amount of <sup>125</sup>I-ANF was increased to 90 x  $10^6$  cpm (~50 pmol) and the quantity of unlabeled hormone was changed to 20 nmol. At 2 min after injection, all rats were sacrificed by intracardiac perfusion of 100 ml Ringer-Locke fluid for 1 min followed by 200-250 ml of ice-cold 2% glutaraldehyde buffered with 0.1 M cacodylate (pH 7.4) for 15 min. The brains were then isolated, cut into 3 pieces, fixed by immersion in the same fixative for 18 h at 4°C, and washed 3 times in cacodylate buffer (0.1 M, pH 7.4) containing 2% sucrose. The fixed brain pieces were then sliced (100  $\mu$ m) using the Lancer vibratome. The ChP from lateral ventricles with surrounding tissue was dissected from the slices, postfixed for 1 h in 2% osmium tetroxide buffered with cacodylate (0.1 M, pH 7.4), dehydrated in

ethanol, and embedded in Araldite (JBEM Services, Montreal, Quebec, Canada).

#### 2.A.3.3.2 Preparation of autoradiograms

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دو<del>ن</del>ي. موجي Semithin sections (1  $\mu$ m) as well as thin sections (70 nm) were made from the same tissue blocks in all experiments. Semithin sections were cut with a glass knife using a Reichert OmU2 microtome. They were then coated with diluted Ilford K<sub>5</sub> Nuclear emulsion (Ilford Ltd., UK) and exposed for 1 month in sealed light-tight boxes with desiccant at 4°C. The preparation was then developed with undiluted Kodak D19 developer for 4 min at 18°C, rinsed in distilled water for ~15 sec at 18°C, fixed in 23% sodium sulfate for 5 min at 18°C, and washed in distilled water for 5 min at 37-40°C. Finally, the semithin sections were stained with 1% toluidine blue.

Thin sections were cut with a diamond knife using a Reichert-Jung ultramicrotome, placed on pre-cleaned slides coated with 2% parlodion in amyl acetate, stained with 5% uranyl acetate (10 min) followed by Reynold's lead citrate (3 min), and then covered with a thin carbon film. They were then coated with a monolayer of Ilford  $L_4$  emulsion (Ilford Ltd.) for EM autoradiography, exposed for 2 months in sealed light-tight boxes with desiccant at 4°C, and developed with the Agfa Gevaert physical developer for compact grains [13]. The sections were placed on EM grids (Mecalab, Ltd., Montreal, Quebec, Canada) and scanned in a Jeol EM (JEM-100CX). Photographs were taken randomly, without taking into account the number of silver grains present on the sections. Each micrograph was printed at a final magnification of x 25,935 to obtain 8 x 10 inch prints.

#### 2.A.3.3.3 Analysis of light- and electron-microscopic autoradiograms

LM autoradiograms were evaluated in a Zeiss light microscope (at a magnification of 1,000) to estimate the proportion of specific binding in each experiment. For this purpose, silver grains per unit square area (144  $\mu$ m<sup>2</sup>) were counted over the ChP from total binding and nonspecific binding preparations, from each experiment separately. Specific binding represented

the difference in grain densities between the total and nonspecific images. Between (1) and 300 squares per animal were assessed in this manner. Unpaired Student's t test was used to determine statistical significance of observed difference in grain counts.

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Analysis of EM autoradiograms has been performed according to the procedure described by Kent and Williams [12] and Williams [40]. A 50 % probability circle which was used in this analysis had a diameter of 272 nm, equal to 3.4 x the half distance (HD) [13,40]. The HD (a measure of resolution in EM autoradiography, defined as a distance from a line radioactive source that contains 50% of grains) related to <sup>125</sup>I and to our technical conditions was considered to be 80 nm [13]. A chi-square ( $\chi^2$ ) test was applied (in which real and hypothetical random grain distributions were compared) to exclude the possibility that the grains present on the autoradiograms were random.

Additionally, the line source analysis [33] was performed to detect a possible accumulation of silver grains on the basolateral plasmalemma of epithelial cells of the ChP. In this analysis, distributions of silver grains around this plasmalemma was ascertained by measuring the shortest distance from the center of each silver grain to the nearest plasmalemma, using HD as a unit.

### 2.A.3.4 Penetration of ANF through blood-CSF barrier

Steel catheters were fixed in the cisterna magna of anesthetized Sprague-Dawley rats (~40 g body weight, n=6). Two days later, all rats received an intracarotid injection  $\oplus$  ~41 x 10<sup>6</sup> cpm (~22.8 pmol) <sup>125</sup>I-ANF, as above. At 2 min after injection, samples of the CSF were collected (for ~1 min) into preweighed tubes. Subsequently, the amount of removed CSF was determined by weighing and the radioactive content was evaluated in a LKB gamma counter.

### 2.A.3.5 Degradation studies in vivo

Sprague-Dawley rats (~40 g body weight, n=6) received an intracarotid injection of  $^{125}$ I-ANF (41 x 10<sup>6</sup> cpm,  $^{-22.8}$  pmol) and were processed as

described earlier. The only difference was that the tissues were not fixed but after applying intracardiac Ringer-Locke solution the animals were decapitated, the ChP from lateral ventricles was quickly isolated, placed in 1 ml of 1 N acetic acid (4°C) containing 0.5% Nonidet P-40 and the following inhibitors: 0.1 mM pepstatin, 0.1 mM leupeptin, 2.5 mΜ phenylmethylsulfonyl fluoride, 100 kallikrein inhibiting units/ml aprotinin and 5  $\mu$ M phosphoramidon. Immediately after each sample was boiled for 5 min, homogenized using a polytron, and centrifuged. Supernatant of each sample was applied to a C<sub>18</sub> Sep-Pak cartridge (Waters Associates, Milford, Massachusetts, USA) which had been previously activated with 10 ml of ethanol. The unbound to the cartridge material was washed with water. The bound material was then eluted with 86% ethanol containing 4% acetic acid and evaporated in a Speed-Vac. The proportion of unbound and bound Sep-Pak material was evaluated in a LKB gamma counter. For HPLC analysis, lyophilized samples were dissolved in 0.1% trifluoroacetic acid and loaded on a  $C_{18}$  µ-Bondapak column. The material was eluted at 1 ml/min with a linear gradient (1%/min) of 15-55% acetonitrile in 0.1% trifluoroacetic acid and passed through a FLO-ONE/Beta radioactive flow detector (Radiomatic Instruments) coupled to HPLC.

Control samples (<sup>125</sup>I-ANF with or without perfused and isolated ChP) were processed in a similar fashion and were aimed at detecting a possible degradation taking place during processing steps *in vitro*.

# 2.A.4 Results

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### 2.A.4.1 Autoradiography in vitro

The results obtained from the saturation experiment are depicted in Figures 2.A.1 and 2.A.2. They demonstrate the presence of one class of saturable, high-affinity (K<sub>d</sub>, 83.8 pM) and low-density (B<sub>max</sub>, 22.9 fmol/mg of protein) binding sites for ANF in the rat ChP from lateral ventricles. The results obtained from the competition curves are shown in Figure 2.A.3. C-ANF [which binds at pathophysiological concentrations only to the guanylate



**Figure 2.A.1** Autoradiograms of representative coronal sections of the rat brain showing binding sites (white dots) for <sup>125</sup>I-ANF. This picture was taken directly from the X-ray film apposed to labeled cryostat sections (20  $\mu$ m) for 43 h. Arrows point to the ChP. A: Section incubated with 260 pM <sup>125</sup>I-ANF (total binding). B: Section incubated with 260 pM <sup>125</sup>I-ANF and 100 nM unlabeled ANF (nonspecific binding).



**Figure 2.A.2** Saturation curve of <sup>125</sup>I-ANF binding sites in the rat ChP. Total binding (•); nonspecific binding (o). Each point represents a mean value of data derived from duplicate 20  $\mu$ m cryostat sections. K<sub>d</sub>, 63.8 pM, B<sub>max</sub>, 22.9 fmol/mg protein.

cyclase-free natriuretic peptide receptor subtype (NPR-C)] was able to displace 70% of <sup>125</sup>I-ANF specific binding in the ChP indicating that the ratio of guanylate cyclase-containing to guanylate cyclase-free ANF receptor subtypes

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in the ChP is approximately 3:7.  $IC_{50}$  (the concentration of unlabeled ligand at which the maximum binding of labeled ligand is displaced by 50%) from this study was in the picomolar range (574 pM) for ANF and in the low nanomolar range (2.46 nM) for C-ANF.

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**Figure 2.A.3** Competition of ANF-(Ser99-Tyr126) and C-ANF [des-(Gln116-Gly120)ANF-(Arg102-Cys121)-NH<sub>2</sub>] for binding of <sup>125</sup>I-ANF to rat ChP cryostat sections. Binding of <sup>125</sup>I-ANF (50 pM) in the presence of various concentrations of unlabeled ANF ( $\bullet$ ). Binding of <sup>125</sup>I-ANF (50 pM) in the presence of various concentrations of unlabeled C-ANF ( $\bullet$ ). 30% and 70% represent the estimated proportion of guanylate cyclase-containing to guanylate cyclase-free ANF receptor subtypes, respectively. IC<sub>50</sub>: ANF, 574 pM; C-ANF, 2,460 pM.

## 2.A.4.2 Autoradiography following in vivo <sup>125</sup>I-ANF injection

#### 2.A.4.2.1 Light-microscopic autoradiography: Displacement analysis

LM autoradiograms allowed an estimation of the proportion of specific binding and a view of the localization of ANF binding sites but did not permit detailed morphologic analysis. Autoradiographic reaction produced by <sup>125</sup>I-ANF alone was significantly reduced (an average of 73%) by concomitant administration of an excess of unlabeled ANF, as determined by grain counting on LM autoradiograms (Table 2.A.1, Figure 2.A.4).

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**Figure 2.A.4** Light-microscopic autoradiograms showing that in the ChP of rats receiving intracarotid injection of <sup>125</sup>I-ANF-(Ser99-Tyr126) plus an excess of unlabeled ANF-(Ser99-Tyr126) the autoradiographic reaction was consistently reduced. A: intracarotid injection of 90 x  $10^{6}$  cpm (~50 pmol) <sup>125</sup>I-ANF. B: intracarotid injection of 90 x  $10^{6}$  cpm (~50 pmol) <sup>125</sup>I-ANF. B: intracarotid injection of 90 x  $10^{6}$  cpm (~50 pmol) <sup>125</sup>I-ANF. B: intracarotid injection of 90 x  $10^{6}$  cpm (~50 pmol) <sup>125</sup>I-ANF. B: intracarotid injection of 90 x  $10^{6}$  cpm (~50 pmol) <sup>125</sup>I-ANF plus 20.0 nmol unlabeled ANF.

#### 2.A.4.2.2 Electron-microscopic autoradiography

Analysis of the ultrastructural autoradiograms showed that silver grains were primarily localized on epithelial cells of the ChP (99.1%). Only a minimal proportion of grains was seen over pial cells (0.7%) and endothelial cells (0.2%) (Table 2.A.2). Since only a marginal percentage of the total silver grains was localized on endothelial and pial cells of the ChP, the ultracellular analysis of grain distribution was performed only on epithelial cells. When the latter cells were examined more closely, and the number of grains present on them was considered as 100%, 18.4% of grains were located at or near (within a 50% probability circle) the basolateral membrane, 52.5% of grains were found inside of the cells and 29.1% of grains were present at or near the apical cell surface (Table 2.A.3). The real silver grain distribution pattern on cellular compartments of epithelial cells was compared with a random one using the

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Table 2.A.1Inhibition of 125I-ANF-(Ser99-Tyr126) binding in the ChPby an excess of unlabeled ANF.

| Amount of<br><sup>125</sup> I-ANF injected<br>cpm (pmol) | Amount of<br>ANF injected<br>µg (nmol) | Grain concentration**<br>grain/square<br>mean ± SE | Inhibition<br>% |
|----------------------------------------------------------|----------------------------------------|----------------------------------------------------|-----------------|
|                                                          | ·                                      |                                                    |                 |
| 41 x 10° (22.8)                                          | -                                      | $10.02 \pm 0.82^*$                                 | 78.2            |
| 41 × 10 <sup>6</sup> (22.8)                              | 50 (16.7)                              | 2.18 ± 0.21*                                       | /0.2            |
|                                                          |                                        |                                                    |                 |
| 90 x 10 <sup>6</sup> (50.0)                              | _                                      | 12.04 ± 1.59*                                      | 67.9            |
| 90 × 10 <sup>6</sup> (50.0)                              | 60 (20.0)                              | 3.87 ± 0.51*                                       | 07.9            |
|                                                          |                                        |                                                    |                 |

\* p < 0.001, statistically significant by unpaired Student's t test.

\*\* The grains were counted in 100-300 squares (144  $\mu$ m<sup>2</sup>) at a magnification of x 1,000.

 $\chi^2$  test, and it was found that the real grain distribution is not random (p<0.001). A value of % *real grains/% random grain* ) higher than 1 was considered to represent a distinct labeling of the structure. Structures distinctly labeled (Table 2.A.3) included the basolateral plasmaleinma (Figure 2.A.5, panels A,B, and C), cytoplasmic vesicles (Figure 2.A.5, panels C and D), Golgi apparatus (Figure 2.A.5, panels E and F), lysosomes (Figure 2.A.5 G), and the apical plasmalemma (Figure 2.A.5 H). As shown in Table 2.A.3, the basolateral plasmalemma was the most distinctly labeled organelle (the highest ratio of % *real grains/% random grains* ). Similarly, silver grain distribution around the basolateral plasmalemma in the line-source analysis (peak over that

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| Type of cell      | Number of<br>of grains | %<br>of grains |
|-------------------|------------------------|----------------|
| Endothelial cells | 1                      | 0.2            |
| Pial cells        | 3                      | 0.7            |
| Epithelial cells  | 423                    | 99.1           |
| Total             | 427                    | 100.0          |

Table 2.A.2Localization of silver grains over choroid plexus cells at2 min after intracarotid administration of 125I-ANF. Ultrastructural study.

plasmalemma, Figure 2.A.6) further confirmed that silver grains were particularly concentrated over this structure. The ratio % *real grains/% random grains* for the remainder of analyzed cellular compartments (cytoplasm, nuclei, rough endoplasmic reticulum, mitochondria) was below 1 and, therefore, grains located there were classified as random (Table 2.A.3).

### 2.A.4.3 Penetration of ANF through the blood-CSF barrier

Evaluation of the distribution of silver grains over choroidal epithelial cells at 2 min after intracarotid injection of <sup>125</sup>I-ANF showed that 29.1% of grains were localized at the apical cell surface (Table 2.A.3). We next asked whether ANF reached that surface from outside the epithelial cells (by overcoming the blood-CSF barrier and reaching the CSF) or from inside the cells. If the first possibility takes place then <sup>125</sup>I-ANF, after intracarotid injection, should be present in sufficient quantities in the CSF to produce the

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**Figure 2.A.5** Electron-microscopic autoradiograms showing silver grain distribution over rat choroidal epithelial cells at 2 min following intracarotid injection of 90 x 10<sup>6</sup> cpm (~50 pmol) <sup>125</sup>I-ANF-(Ser99-Tyr126). Silver grains are pointed by arrows. PVS, perivascular space; Bl, basal infoldings; MV, microvilli; TJ, tight junctions; CV, cytoplasmic vesicles; G, Golgi apparatus; L, lysosomes; M, mitochondria.

observed autoradiographic reaction at the apical cell surface or/and silver grains should be found on EM autoradiograms on microvilli in the vicinity of apical tight junctions. The present studies show that penetration to the CSF was not substantial, since after intracarotid injection of 41 x 10<sup>6</sup> cpm (~22.8 pmol) <sup>125</sup>I-ANF only 26.8  $\pm$  13.8 cpm per µl of the CSF was removed from the cisterna magna (Table 2.A.4). However, 18% of grains present on microvilli were found in the vicinity of apical tight junctions. Therefore, a possibility

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| Structure                            | Real<br>grains<br>% | Random<br>grains<br>% | %Real grains<br><br>%Random grains |
|--------------------------------------|---------------------|-----------------------|------------------------------------|
| Basolateral plasmalemma              | 18.4                | 11.8                  | 1.56                               |
| apparatus and lysosomes<br>Cytoplasm | 14.4<br>14.0        | 10.1<br>17.9          | 1.43<br>0.78                       |
| Rough endoplasmic<br>reticulum       | 4.7                 | 11.5                  | 0.41                               |
| Mitochondria                         | 12.3                | 12.5                  | 0.98                               |
| Nucleus<br>Apical plasmalemma        | 7.1<br>29.1         | 11.6<br>24.6          | 0.61<br>1.18                       |
|                                      |                     | <b></b>               |                                    |
| Number of grains counted             | 423                 | 1710                  |                                    |

Table 2.A.3Distribution of real and random hypothetical grainsin autoradiograms of <sup>125</sup>I-ANF-labeled ChP.

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that <sup>125</sup>I-ANF actually overcame the blood-CSF barrier at the time of our experiment without reaching the CSF itself cannot be excluded.

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# Silver grains around basolateral plasmalemma

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**Figure 2.A.6** Silver grain density distribution around the basolateral plasmalemma of epithelial cells in the ChP of rats injected with <sup>125</sup>I-ANF (90 x 10<sup>6</sup> cpm, ~50 pmol). For each silver grain, the shortest distance from its center to the nearest plasmalemma was determined, expressed in HD units (see Materials and Methods) and plotted on the diagram. Grains located up to 10 HD from the plasmalemma were analyzed.

### 2.A.4.4 Degradation studies in vivo

HPLC analysis revealed that 95.3% of the total radioactive material bound to the ChP at 2 min after <sup>125</sup>I-ANF intracarotid administration was eluted from reverse-phase HPLC column at the position corresponding to the intact peptide (Figure 2.A.7).

Table 2.A.4 Amount of radioactivity in the cerebrospinal fluid at2 min after intracarotid injection of <sup>125</sup>I-ANF-(Ser99-Tyr126) (n=6).

| Amount of <sup>125</sup> I-ANF | Radioactivity in the |
|--------------------------------|----------------------|
| injected                       | cerebrospinal fluid  |
| cpm (pmol)                     | cpm/µl               |
| 41 × 10 <sup>6</sup> (22.8)    | 26.8 ± 13.8          |

# 2.A.5 Discussion

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The presence of an autoradiographic reaction over choroidal cells which is consistently reduced by concomitant exposure to an excess of unlabeled ANF suggests that *in vitro* as well as *in vivo* autoradiographic studies localized binding sites for ANF on the ChP. Saturation curves performed by *in vitro* autoradiography showed that they represent a single class of saturable binding sites. In this respect the present work confirms *in vitro* autoradiographic studies conducted by other groups [18,30,31]. However, competition binding analysis using <sup>125</sup>I-ANF as a radioligand whereas ANF and C-ANF as displacing ligands disclosed two types of binding sites in a ratio approximately 3:7. Since affinities of these binding sites for ANF are not different [15,38] they are not recognizable on the basis of affinity. Therefore, saturation curves showed only a single class of sites in spite of the heterogeneity of these receptors.

Previous data [2] reported the presence of ANF binding sites in the ChP on epithelial cells and capillary endothelium, as analyzed by *in vivo* autoradiography at the LM level. In the present studies, although grains were

also seen in those two locations on LM autoradiograms, ultrastructural analysis revealed only minimal percentage of the total number of grains to be present on endothelial (and pial) cells of the ChP. Since EM autoradiography provides better resolution when compared with that of LM autoradiography, we concluded that ANF binding sites in the ChP are present primarily on epithelial cells and marginally on endothelial cells and pial cells. With respect to guanylate cyclase-containing ANF receptors, this observation is in agreement with studies of Steardo and Nathanson [35], who have found mini-



**Figure 2.A.7** Assessment of a possible degradation of <sup>125</sup>I-ANF-(Ser99-Tyr126) in the ChP *in vivo*. <sup>125</sup>I-ANF (41 x 10<sup>6</sup> cpm) was administered by intracarotid injection to rats. Two min after injection rats were sacrificed and choroid plexuses were isolated, washed, placed in 1 N acetic acid containing Nodinet-P40 and proteolytic inhibitors, and then boiled. <sup>125</sup>I-ANF was extracted using C<sub>18</sub> Sep-Pak cartridges and analyzed on a reverse-phase HPLC column.

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mai activity of ANF-stimulated guanylate cyclase in membranes from vascular cell-enriched ChP preparations when compared to ANF-stimulated guanylate cyclase in membranes from epithelial cell-enriched ChP preparations.

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The present studies indicate that the principal site of ANF binding in the ChP is the basolateral plasmalemma of epithelial cells. The results of our studies cannot exclude the possibility that ANF after binding to basolateral portions of plasmalemma might be, at least in part, rapidly internalized by the choroidal epithelium. This is inferred from autoradiographic evidence at the EM level, in which the ratio of % real grains/% random grains was assessed for cell structures known to be involved in the internalization process. At 2 min after intracarotid injection of <sup>125</sup>I-ANF, cytoplasmic vesicles, Golgi apparatus and lysosomes together were already significantly labeled. Similarly fast internalization of <sup>125</sup>I-ANF has been reported in other cell types, such as pancreatic acinar cells [4] and adrenal medullary cells [21], as determined by EM autoradiography. Furthermore, in cultured endothelial [11] and smooth muscle cells [27] as well as in whole isolated perfused kidney [28] lysosomotropic weak bases, such as NH<sub>4</sub>Cl or chloroquine, inhibited very rapidly (within minutes) an endocytotic/degradative process. These lysosomotropic agents are believed to diffuse into cells in their unprotonated form and accumulate in acidic intracellular compartments, such as endosomes and/or lysosomes, where they become protonated and raise intravesicular pH [6]. There is also a large body of evidence indicating that weak bases inhibit the movement of receptors in cells [39]. The possible internalization of ANF by the choroidal epithelial cells is in agreement with the finding that as much as 70% of the total ANF receptors present in the ChP are NPR-C which were previously proposed to be involved in the internalization and degradation of ANF [1,17]. However, more precise studies are necessary to finally resolve the issue of internalization of ANF binding sites in the ChP.

HPLC analysis revealed that 95.3% of the total ChP-bound material represented intact ANF. This was in spite of the presence in the ChP of the

ANF-metabolizing enzyme, endopeptidase 24.11 [8], and despite a large proportion of NPR-C. The co-existence of ANF binding sites and ANF membrane degradation sites is not unique for the ChP; it is also noted in the kidney glomeruli [34] and adrenal zona glomerulosa cells [36].

Studies on penetration of the blood-CSF barrier by ANF in rats revealed that ANF only marginally crosses that barrier to reach CSF. This finding agrees with comparable investigations performed on rabbits [16]. Thus, a possibility cannot be excluded that <sup>125</sup>I-ANF which labeled the apical plasmalemma has been transported to that membrane by an intracellular route. However, out of silver grains present on microvilli, 18% were observed in the vicinity of apical tight junctions, suggesting that some <sup>125</sup>I-ANF might have actually overcome the blood-CSF barrier without reaching CSF.

The presence of ANF binding sites on the ChP epithelium, a structure which plays a major role in the regulation of CSF volume as well as CSF levels of sodium, potassium, chloride, bicarbonate, and pH, suggest that circulating ANF may regulate its function. It has already been demonstrated that ANF stimulates the production of cGMP in rat ChP explants [37] and activates guanylate cyclase in rat ChP epithelial cell-enriched preparations [35]. Furthermore, ANF modulated permeability of ions through apical tight junctions of choroidal epithelial cells [25]. Moreover, infusion of ANF into cerebral ventricles inhibited the production of CSF by ~35% as detected by ventricular-cisternal perfusion in rabbits [35]. Thus, the above findings suggest that the ANF binding sites localized in the present study may have a functional significance. This functional role is perhaps not limited to regulation of CSF production and its composition but it may also relate indirectly to blood pressure regulation. Recent studies suggest that modest elevations in CSF production may produce significant increases in intracranial pressure [22]. Because small increases in intracranial pressure may produce sustained increases in systemic pressure [26], mechanisms that participate in the regulation of CSF balance may also influence control of blood pressure.

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In summary, we have shown in the present study that saturable ANF binding sites of high affinity ( $K_d$ , 83.8 pM) and low density ( $B_{max}$ , 22.9 fmol/mg protein) are present in the ChP, primarily on the basolateral plasmalemma of epithelial cells. Both guanylate cyclase-containing and guanylate cyclase-free ANF receptor subtypes are present in the ChP and their ratio in this tissue is approximately 3:7. Internalization of ANF receptors in choroidal epithelial cells cannot be excluded. Cellular localization of these binding sites suggests their possible involvement in the regulation of CSF balance as well as in central blood pressure control exerted by circulating ANF.

# 2.A.6 Acknowledgements

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# 2.B ATRIAL NATRIURETIC FACTOR BINDING SITES IN RAT AREA POSTREMA: AUTORADIOGRAPHIC STUDY

This section is devoted to the area postrema, one of the brain structures harbouring a high number of ANF binding sites, lacking the normal bloodbrain barrier and, therefore, accessible to intravascularly administered <sup>125</sup>I-ANF. Firstly, the presence of natriuretic peptide receptor subtypes in the area postrema was established by in vitro autoradiography. Secondly, the type of receptor interacting with ANF was identified. Finally, its cellular localization was demonstrated by electron-microscopic autoradiography.

As stated in the "Preface", at the time the experiments for this paper were performed, three natriuretic peptide receptors were already known and it was possible to distinguish them by affinity binding techniques. NPR-A and NPR-B were referred to as guanylate cyclase-containing natriuretic peptide receptors whereas NPR-C was referred to as guanylate cyclase-free natriuretic peptide receptor. The term ANF binding sites should encompass primarily both NPR-A and NPR-C.

### 2.B.1 Abstract

The area postrema is a brainstem circumventricular organ implicated, among other functions, in central cardiovascular regulation and body fluid homeostasis. Competition binding analysis performed by quantitative *in vitro* autoradiography demonstrated specific, high-affinity (K<sub>d</sub>, 0.32  $\pm$  0.11 nM), lowcapacity (B<sub>max</sub>, 57.5  $\pm$  10.9 fmol/mg protein) atrial natriuretic factor (ANF) binding sites in the AP. C-ANF [des-(Gln116-Gly120)ANF-(Arg102-Cys121)-NH<sub>2</sub>] and ANF-(Phe106-Ile113)-NH<sub>2</sub> (two ligands endowed with selectivity for the natriuretic peptide receptor C), as well as C-type natriuretic peptide (CNP)

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did not compete noticeably at pathophysiological concentrations for <sup>125</sup>I-ANF binding. <sup>125</sup>I-[Tyr<sup>o</sup>]-CNP bound to the area postrema to a much lower extent than <sup>125</sup>I-ANF. Electron-microscopic autoradiography *in vivo* disclosed that <sup>125</sup>I-ANF was preferentially bound to axon, dendrite, and astrocyte plasmalemma. These studies demonstrate that the area postrema contains natriuretic peptide binding sites with pharmacological characteristics of the natriuretic peptide receptors A and B but not of the natriuretic peptide receptor C. In the area postrema ANF interacts with those sites resembling natriuretic peptide receptor A. Cellular localization of these binding sites may relate to their possible involvement in the centrally-mediated salt and water regulation and/or cardiovascular effects of circulating ANF.

# 2.B.2 Introduction

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The area postrema (AP) of the rat is a wedge-shaped midline structure situated on the dorsal surface of the medulla oblongata, protruding into the fourth ventricle and overlying the nucleus of the solitary tract (NTS) [20]. It belongs to a group of specialized brain regions called circumventricular organs (CVOs) with a unique relationship to both blood supply and cerebrospinal fluid [5,20]. The AP, like other CVOs, is principally composed of neuronal and astroglial cells which are penetrated by an abundance of fenestrated capillaries with vast perivascular spaces, allowing the transfer of macromolecules between the blood and interstitium. The lack of a blood-brain barrier may be important for neurohemal interactions since peptides and other blood constituents, which do not have access to the central nervous system (CNS) elsewhere, may be sensed by receptors located in the AP. The AP has a relatively large complement of receptors for numerous neuroactive compounds, including those for atrial natriuretic factor (ANF) [19,20]. Anatomic tracing studies have revealed efferent neural projections from the AP through which effects mediated by the receptors may be elicited. These studies have shown that neurons included in the AP send processes to the brainstem and pons structures, such as the NTS, dorsal motor nucleus of the

vagus, parabrachial nucleus, nucleus ambiguous, and ventrolateral medulla [structures potentially involved in cardiovascular (CV) regulation] [20]. Electrical stimulation of the AP has been shown to increase glucose metabolism not only in the above mentioned nuclei but also in magnocellular and parvocellular subdivisions of the hypothalamic paraventricular nucleus, supraoptic and suprachiasmatic nuclei, as well as median eminence [12]. Major afferent input to the AP is provided by neurons in the paraventricular and dorsomedial hypothalamic nuclei, whereas the minor input comes from the NTS and parabrachial nucleus [20]. An neurons contain considerable quantities of noradrenaline, dopamine, and serotonin [5]. In addition, a variety of neuropeptides [27], including ANF [32], have been identified by immunocytochemistry in nerve fibers of this tissue. The AP has been proposed to play a role in several physiological processes, including regulation of the sleep cycle, control of emesis, and food intake [20]. There is a compelling evidence of its participation in central CV control [9] as well as central regulation of water and sodium homeostasis [14].

In the current investigations, we attempted to characterize pharmacologically ANF binding sites in the AP and describe their cellular distribution therein. To approach this, we employed autoradiographic techniques: 1) competition analysis performed by autoradiography *in vitro* [to evaluate the presence of natriuretic peptide receptors A (NPR-A), B (NPR-B), and C (NPR-C) in the AP and to establish which of them interact with ANF]; and 2) electron-microscopic (EM) autoradiography using <sup>125</sup>I-ANF as an *in vivo* ligand combined with a refined multistep analysis of autoradiograms (to study cellular localization of ANF binding sites).

# 2.B.3 Materials and Methods

## 2.B.3.1 Ligand iodination

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Rat ANF-(Ser99-Tyr126) (Bio-Mega Inc., Laval, Quebec, Canada) was radioiodinated with Na<sup>125</sup>I by the lactoperoxidase method, as previously des-

cribed [26]. The monoiodinated form of ANF was purified by highperformance liquid chromatography (HPLC). The specific activity of <sup>125</sup>I-ANF was ~2,400 dpm/fmol (1,100 Ci/mmoi).

Rat <sup>125</sup>I-[Tyr<sup>o</sup>]-C-type natriuretic peptide (Gly82-Cys103) (<sup>125</sup>I-[Tyr<sup>o</sup>]-CNP) was purchased from Peninsula Laboratories Inc., (Peninsula Laboratories Inc., Belmont, California, USA). The specific activity of the product was ~2,000 dpm/fmol (900 Ci/mmol).

### 2.B.3.2 Quantitative in vitro autoradiography

#### 2.B.3.2.1 Preparation of autoradiograms

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Adult Sprague-Dawley rats (~250 g body weight) were sacrificed by decapitation, and their brains rapidly removed, frozen, and sectioned (20  $\mu$ m) in a cryostat. These consecutive tissue sections were preincubated at room temperature for 10 min in 50 mM Tris-HCl buffer (pH 7.5) containing 0.5% bovine serum albumin. They were then incubated for 60-90 min (time long enough to reach equilibrium) in a similar buffer but supplemented with appropriate concentrations of <sup>125</sup>I-ANF or <sup>125</sup>I-[Tyr<sup>o</sup>]-CNP and competing unlabeled peptides (see below), as well as 5  $\mu$ M MnCl<sub>2</sub> and the following inhibitors in order to assure an effective protection against proteases present in the brain tissue: 1  $\mu$ M phenylmethylsulfonyl fluoride, 200 kallikrein inhibiting units/ml aprotinin and 0.05% bacitracin.

For <sup>125</sup>I-ANF competition analysis, the incubation buffer contained 100-200 pM <sup>125</sup>I-ANF and concentrations ranging from 1 pM to 10  $\mu$ M of either unlabeled peptides: ANF-(Ser99-Tyr126), C-ANF [des-(Gln116-Gly120)ANF-(Arg102-Cys121)-NH<sub>2</sub>], ANF-(Phe106-Ile113)-NH<sub>2</sub> (all from Bio-Mega Inc.), or CNP-(Gly82-Cys103) (from Peninsula Laboratories Inc.). To test the specificity of <sup>125</sup>I-ANF binding, a set of sections was incubated in the presence of 100 pM <sup>125</sup>I-ANF with 1  $\mu$ M of one of the following peptides: angiotensin II (ANG II), glucagon, substance P, or calcitonin. Following incubation, the slides were washed (4°C, 2 x 10 min, time allowing for a high signal-to-noise ratio) in 50 mM Tris-HCl (pH 7.5) containing 0.5% bovine serum albumin, fixed in 2% glutaraldehyde (pH 7.5, 4°C, 15 min), washed in 0.1 M phosphate buffer (pH 7.5, 4°C, 5 min) and in distilled water (30 sec), dehydrated in alcohol, and dried overnight at 60°C. Subsequently, the sections along with <sup>125</sup>I-standards (see below) were apposed to X-ray films for 2-3 days at room temperature to produce autoradiograms.

To investigate the presence of the NPR-B in the AP, consecutive brain sections were exposed in the incubation buffer to 130 pM  $^{125}$ I-[Tyr<sup>o</sup>]-CNP and 1  $\mu$ M C-ANF in the presence or absence of 1  $\mu$ M CNP. Following incubation, the slides were washed (2 x 10 min, 4°C) in 50 mM Tris-HCl (pH 7.5) containing 0.5% bovine serum albumin, air-dried in a cold chamber (-5°C) to maximize the chance of getting the strongest signal, and apposed to X-ray film for 24 days at room temperature.

#### 2.B.3.2.2 Determination of radioligand stability

Before and after exposure of the sections, samples of the incubation buffer were collected to assess the degree of radioligand degradation during the incubation period. Degradation was determined by reverse-phase HPLC [26]. The intact radioligand contained in the buffer after incubation was expressed as a percentage of the intact radioligand present in the buffer before exposure of the sections.

#### 2.B.3.2.3 Analysis of autoradiograms

Prior to microdensitometric analysis, the radioligand-labeled sections were stained with cresyl violet and coverslipped with Permount. They were subsequently compared to their respective autoradiograms to facilitate the anatomical identification of structures in autoradiographic images. These autoradiograms were then analyzed with a computerized microdensitometer consisting of a Sun 386i workstation, a video camera (Hitachi, KP-130U) equipped with a Nikon objective (micro-Nikkor 55 mm 1:2.8), a Matrox (MVP-AT) video processor, and an Electrohome video monitor (38-DO5IMA-YU). The microdensitometer electronically converted black and white

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autoradiographic images on Kodak film negatives to color-coded ones. Such enhanced images allow to perceive and measure subtle differences of optical density in originally monochrome negatives. Areas of interest were selected for analysis by positioning windows on autoradiographic images with a joystick coupled to the monitor. Readings of optical densities from the autoradiographic images of tissues were preceded by acquisition of optical densities of standards. The following two standards were used: commercially available <sup>125</sup>I-microscale (Amersham, Arlington Heights, Illinois, USA) and a standard prepared from brain homogenates containing known amount of <sup>125</sup>I and proteins (17). An equation describing the linear segment of the sigmoidal standard curve was employed to convert optical densities to disintegrations per minute (dpm) per mg of standard (commercial microscale) or per mg of protein (tissue homogenate standard). Given the specific activity of the radioligand, dpm was then transformed to femtomoles (fmol), generating fmol/mg of standard (commercial microscale standard) or fmol/mg of protein (tissue homogenate standard).

The resultant competition binding curves (derived from 5 animals) were analyzed with the EBDA [23] and the LIGAND computer programs [25] to obtain estimates of the  $K_d$  (dissociation equilibrium constant) and the  $B_{max}$  (maximum binding capacity).

### 2.B.3.3 Autoradiography in vivo

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## 2.B.3.3.1 Injection of <sup>125</sup>I-ANF and processing of tissue

Under pentobarbital anesthesia, Sprague-Dawley rats (~40 g body weight) received a single intracarotid (tip of the catheter in a cephalad direction) bolus injection of 55 x  $10^6$  dpm (22.8 pmol) <sup>125</sup>I-ANF-(Ser99-Tyr126) dissolved in 90 µl of phosphate buffer (0.1 M, pH 7.4). To estimate nonspecific binding, the same quantity of <sup>125</sup>I-ANF was mixed with an excess of unlabeled ANF (16.7 nmol) and injected in the same volume in the same manner. In a second experiment of this type, the amount of <sup>125</sup>I-ANF was increased to 120 x  $10^6$  dpm (~50 pmol) and the quantity of unlabeled hormone was changed to 20 nmol. At 2 min

after injection, all rats were sacrificed by intracardiac perfusion of 100 ml Ringer-Locke fluid for 1 min (to wash out unbound ligands) followed by 200-250 ml of ice-cold 2% glutaraldehyde buffered with 0.1 M cacodylate (pH 7.4) for 15 min (to fix irreversibly radioligand to its binding sites). The brains were then isolated, cut into 3 pieces, fixed by immersion in the same fixative for 18 h at 4°C, and washed 3 times in cacodylate buffer (0.1 M, pH 7.4) containing 2% sucrose. The fixed brain pieces were then sliced (100  $\mu$ m) with a Lancer vibratome. The AP with surrounding tissue was dissected from the slices, postfixed for 1 h in 2% osmium tetroxide buffered with cacodylate (0.1 M, pH 7.4), dehydrated in ethanol, and embedded in Araldite (JBEM Services, Montreal, Quebec, Canada).

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#### **2.B.3.3.2** Preparation of light and electron-microscopic autoradiograms

Semithin sections (1  $\mu$ m) were cut with a glass knife using a Reichert OmU2 microtome. They were coated with diluted Ilford K<sub>5</sub> nuclear emulsion (Ilford Ltd., U.K.) and exposed for 2 months in sealed light-tight boxes with desiccant at 4°C. The preparation was then developed with undiluted Kodak D19 developer for 4 min at 18°C, rinsed in distilled water for ~15 sec at 18°C, fixed in 23% sodium sulfate for 5 min at 18°C, and washed in distilled water for 5 min at 37-40°C. Finally, the semithin sections were stained with 1% toluidine blue.

Thin sections (70 nm) were cut (from the same tissue blocks as the semithin sections) with a diamond knife using a Reichert-Jung ultramicrotome, placed on pre-cleaned slides coated with 2% parlodion in amyl acetate, stained with 5% uranyl acetate (10 min) followed by Reynold's lead citrate (3 min), and covered with a thin carbon film. They were then coated with a monolayer of Ilford L<sub>4</sub> emulsion (Ilford Ltd.), exposed for 6 months in sealed light-tight boxes with desiccant at 4°C, and developed with the Agfa Gevaert physical developer for compact grains (18). The sections were placed on EM grids (Mecalab Ltd. Montreal, Quebec, Canada) and scanned in a Jeol electron microscope (JEM-100CX) at an initial magnification of x 9,535. For the analysis of silver grain distribution, photographs were obtained taking into account the presence of silver grains on the autoradiograms. To determine the hypothetical random grain distribution (and at the same time, to evaluate the area occupied by a cell structure), photographs were taken randomly from regions of the autoradiograms previously used for silver grain analysis. Each micrograph was printed at a final magnification of x 25,935 to obtain 8 x 10 inch prints.

#### 2.B.3.3.3 Analysis of light-microscopic autoradiograms

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Light-microscopic (LM) autoradiograms were evaluated in a Zeiss light microscope (at a magnification of x 1,000) to estimate the proportion of specific binding in each experiment. For this purpose, silver grains per unit square area (144  $\mu$ m<sup>2</sup>) were counted over the AP from total binding and nonspecific binding preparations, from each experiment separately. Specific binding represented the difference in grain densities between the total and nonspecific images. At least 300 squares per animal were assessed in this manner.

#### 2.B.3.3.4 Analysis of electron-microscopic autoradiograms

Cells or cell elements present in the AP were recognized on the EM autoradiograms and classified as neuronal cell bodies, dendrites, axons, astrocytes, fibroblasts, macrophages, or endothelial cells, using morphologic criteria [20,28]. Brief characterization of the elements which may pose identification problems on electron micrographs follows. Neuronal cell bodies of the AP tend to be small ranging from about 8 to 15 µm in diameter and have few proximal dendrites. An ovoid aucleus containing a single nucleolus has deep invaginations of the nuclear membrane and is surrounded by only a thin rim of cytoplasm. Cytoplasmic components include Nissl substance (rough endoplasmic reticulum), abundant juxtanuclear Golgi bodies, and an average number of other cell organelles. Dendrites have somewhat irregular contours with protuberances and spinous processes. They branch by bifurcating at relatively acute angles. Collateral branches tend to be thinner than the parent stem. Dendrites generate arborization rather close to and often

enclosing the neuronal cell body. The most prominent organelles present in dendrites are parallel microtubules, long mitochondria, and tubules of smooth endoplasmic reticulum. Dendrites also contain Nissl bodies (which become progressively smaller with increasing distance from the cell center), elongated Golgi apparatus, often clustered ribosomes, and small numbers of neurofilaments. Axons have rather regular contours and show frequent varicosities only in the terminal parts of their arborization. The varicosities contain small clear-cored vesicles and occasionally large dense-cored vesicles. In the AP axons are usually unmyelinated. They often tend to branch at right angles and their branches have the same caliber as the parent stem. Axons usually generate their full arborization at some distance away from the cell body. They lack Nissl bodies, Golgi apparatus, and free ribosomes, although the latter can appear in the initial segment. They contain mitochondria, many neurofilaments (which become progressively less abundant with increasing distance from the cell center), few microtubules, smooth endoplasmic reticulum, and multivesicular bodies. Astrocytes of the AP are fairly small in diameter, averaging about 10 µm. They have very pleomorphic nuclei, often with large central nucleolus. Astrocytes have many extensively branched processes which envelope neuronal elements within the AP.

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The distribution of real grains was ascertained by allocating and tabulating each structure beneath the center of each silver grain (direct scoring

method) or structure(s) included in the 50% probability circle (method of Williams). Cellular components were classified as "single structures" if they wholly bore a 50% probability circle. If the circle encompassed more than one cellular elements, they were tabulated as "apposed structures". Random grain distribution (simultaneously a measure of the area occupied by a cellular structure) was determined by overlaying a transparent screen displaying an array of points (direct scoring method) or an array of 50% probability circles (method of Williams). A ratio of the real to the random grain distribution was calculated to determine the relative degree to which the label was concentrated by each structure. A value of % *real grains/% random grains* higher than 1 pointed at distinct labeling of the structure. A  $\chi^2$  test (in which real and random grain distributions were compared) was applied to exclude the possibility that the silver grains present on the autoradiograms were random.

Line source analysis was performed to determine whether silver grains were localized on neuronal or astrocytal plasmalemma. In this analysis, distributions of silver grains around the plasmalemma of astrocytes, dendrites, axons, and neuronal cell bodies were ascertained by measuring the shortest distance from the center of each silver grain to the nearest plasmalemma of each kind, using HD as a unit.

# 2.B.4 Results

#### 2.B.4.1 Quantitative autoradiography in vitro

### 2.B.4.1.1 Radioligand stability

Degradation of the radioligand during the incubation period was negligible since after incubation around 98% of <sup>125</sup>I-ANF (Figure 2.B.1) and 97% of <sup>125</sup>I-[Tyr<sup>o</sup>]-CNP were eluted from the reverse-phase HPLC column at the elution time corresponding to the intact peptides.

#### 2.B.4.1.2 Competition analysis

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Competition binding studies demonstrated that in the AP  $^{125}$ I-ANF interacted with a single class of binding sites (slope factor, 1.0 ± 0.2) in a dose-

dependent manner (Figure 2.B.2). These sites had high affinity (K<sub>d</sub>, 0.32 ± 0.11 nM) and low maximal binding capacity (B<sub>max</sub>, 57.5 ± 10.9 fmol/mg protein) which corresponded well with the affinity and capacity of binding sites previously identified in this tissue by saturation binding experiments [19]. C-ANF and ANF-(106-113)-NH<sub>2</sub> (two ANF analogues endowed with selectivity for the guanylate cyclase-free NPR-C [6,22]), as well as CNP (a peptide predominantly binding to the NPR-B and NPR-C [15]) did not compete for <sup>125</sup>I-ANF binding or competed only at a very high concentration (Figures 2.B.2 and 2.B.3). This suggests that the NPR-C is not present in the AP, at least in quantities detectable by our assay. As expected, a variety of unrelated peptides, including glucagon, ANG II, substance P, and calcitonin at a concentration of 1  $\mu$ M, did not compete for <sup>125</sup>I-ANF binding, demonstrating a specificity of ANF interaction (data not shown).

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**Figure 2.B.1** Assessment of possible degradation of <sup>125</sup>I-ANF-(Ser99-Tyr126) during 90-min incubation period: Reverse-phase HPLC of a representative experiment. The material from incubation medium before (left) and after (right) the incubation was loaded on  $C_{18}$  µ-Bondapak column, eluted at 1 ml/min with a linear gradient of 15-55% acetonitrile in 0.1% trifluoroacetic acid, and passed through a FLO-ONE/Beta radioactive flow detector coupled to HPLC.

To ascertain the possible presence of the guanylate cyclase-containing NPR-B in the AP, an interaction of this tissue with <sup>125</sup>I-[Tyr<sup>o</sup>]-CNP in the presence of an excess of C-ANF was examined. As illustrated on Figure 2.B.3, <sup>125</sup>I-[Tyr<sup>o</sup>]-CNP bound to the AP (although to a much lower extent than <sup>125</sup>I-ANF), suggesting the presence of the NPR-B in this tissue. Preliminary saturation binding experiments revealed that the  $B_{max}$  of NPR-B in the AP is approximately 5 to 10 fmol/mg protein.

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Figure 2.B.2 Competition of ANF, C-ANF, ANF-(106-113)-NH<sub>2</sub>, and CNP for binding of <sup>125</sup>I-ANF to rat area postrema cryostat sections. Each point represents a mean value of data derived from 5 animals. ANF:  $K_d$ , 0.32 ± 0.11 nM;  $B_{max}$ , 57.5 ± 10.7 fmol/mg protein.
CHAPTER 2



**Figure 2.B.3** Autoradiograms of rat coronal brain sections at the level of the area postrema (pointed by arrows) labeled *in vitro* with <sup>125</sup>I-ANF or <sup>125</sup>I-[Tyr<sup>o</sup>]-CNP in the presence or absence of unlabeled peptides. Optical densities (mean  $\pm$  SE of 10 determinations) of the area postrema are as follows: <sup>125</sup>I-ANF, 81.1  $\pm$  0.7; <sup>125</sup>I-ANF + ANF, 16,5  $\pm$  0.01; <sup>125</sup>I-ANF + ANF-(106-113)-NH<sub>2</sub>, 84.1  $\pm$  1.5; <sup>125</sup>I-ANF + CNP, 75.6  $\pm$  1.3; <sup>125</sup>I-[Tyr<sup>o</sup>]-CNP + C-ANF, 28.0  $\pm$  0.1; <sup>125</sup>I-[Tyr<sup>o</sup>]-[Tyr<sup>o</sup>]-CNP + C-ANF, 28.0  $\pm$  0.1; <sup>125</sup>I-[Tyr<sup>o</sup>]-[Tyr<sup>o</sup>]-[Tyr<sup>o</sup>]-[Tyr<sup>o</sup>]-[Tyr<sup>o</sup>]-[Tyr<sup>o</sup>]-[Tyr<sup>o</sup>]-[Tyr<sup>o</sup>]-[Tyr<sup>o</sup>]-[Tyr<sup>o</sup>]-[Tyr<sup>o</sup>]-[Tyr<sup>o</sup>]-[Tyr<sup>o</sup>]

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ср-Св From the above it seems that both guanylate cyclase-containing receptors (NPR-A > NPR-B) are present in the AP, but the NPR-C cannot be detected. Since CNP did not displace <sup>125</sup>I-ANF binding in the AP, it appears that ANF binds appreciably to the NPR-A but not to the NPR-B.

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# 2.B.4.2 Light-microscopic autoradiography after in vivo injection of <sup>125</sup>I-ANF

LM autoradiograms allowed an estimation of the proportion of specific binding and a view of the localization of ANF binding sites, but did not permit detailed morphologic analysis.



**Figure 2.B.4** Light-microscopic autoradiograms demonstrating the intensity of autoradiographic reactions over the area postrema from rats injected with  $^{125}$ I-ANF (120 x 10<sup>6</sup> dpm, ~50 pmol) (A) and rats co-injected with an excess (20.0 nmol) of unlabeled ANF (B). x 1,000.

Figure 2.B.4 and Table 2.B.1 illustrate differences in the density of silver grains on LM autoradiograms of the AP derived from experimental (exposed to <sup>125</sup>I-ANF alone) and control (exposed simultaneously to <sup>125</sup>I-ANF and an excess of unlabeled ANF) animals, demonstrating the specificity of <sup>125</sup>I-ANF

uptake. The autoradiographic reaction produced by <sup>125</sup>I-ANF injected *in vivo* into experimental animals (Figure 2.B.4 A), was reduced by 61-83% when unlabeled ANF was concomitantly injected, as seen on autoradiograms derived from control animals (Figure 2.B.4 B, Table 2.B.1).

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Table 2.B.1Inhibition of 125I-ANF-(Ser99-Tyr126)binding to the areapostrema by an excess of ANF. Light-microscopic autoradiography in vivo.

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| Amount of<br><sup>125</sup> I-ANF injected<br>dpm (pmol) | Amount of<br>ANF injected<br>μg (nmol) | Grain concentration<br>grain/unit area<br>mean ± SE | Inhibition<br>% |
|----------------------------------------------------------|----------------------------------------|-----------------------------------------------------|-----------------|
| 55 x 10 <sup>6</sup> (22.8)                              | <b>__</b>                              | 1.96 ± 0.16                                         | <u>۲</u>        |
| 55 × 10 <sup>6</sup> (22.8)                              | 50.0 (16.7)                            | $0.76 \pm 0.06$                                     | 0176            |
| 120 x 10 <sup>6</sup> (50.0)                             | -                                      | 2.92 ± 0.26                                         | 83%             |
| 120 x 10 <sup>6</sup> (50.0)                             | 60.0 (20.0)                            | $0.51 \pm 0.04$                                     | 00 70           |

One or two animals were used for each concentration of the injected peptides. The grains were counted in 300-400 unit areas ( $144 \mu m^2$ ) per peptide concentration at a magnification of x 1,000.

# 2.B.4.3 Electron-microscopic autoradiography after in vivo injection of <sup>125</sup>I-ANF

As observed at the LM level (Table 2.B.1), autoradiograms derived from control animals which received an *in vivo* injection of  $120 \times 10^6$  dpm of <sup>125</sup>I-ANF showed a relatively low level of non-displaceable grains (17%). Additionally, a considerable number of these grains may have represented a background related to the LM autoradiography, since silver grains were very

rarely observed on ultrastructural autoradiograms derived from the same control animals' tissue blocks. For this reason, the distribution and concentration of silver grains in AP cells were analyzed on ultrastructural autoradiograms of the AP from experimental animals injected with  $120 \times 10^6$  dpm of <sup>125</sup>I-ANF alone.



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**Figure 2.B.5** Electron-microscopic autoradiograms of the area postrema from rats injected with  $^{125}$ I-ANF (120 x 10<sup>6</sup> dpm, ~50 pmol). Arrows point to silver grains. Abbreviations: Den, dendrite; Ax, axon; NCB, neuronal cell body; As, astrocyte. x 25,935.

As shown on Figure 2.B.5 and Table 2.B.2, grains were localized on nonneuronal (58.8 %) and neuronal (42.2%) cells. Their distribution pattern was not random, as revealed by the  $\chi^2$  test (p < 0.001).

Although our pilot study (direct scoring method) revealed very weak, close to random concentrations of silver grains over whole astrocytes (Table 2.B.2), more refined analysis using a 50% probability circle (Table 2.B.3) disclosed that the astrocyte surface was preferentially labeled rather than their interior.

| Table 2.B.2 | Distribution   | of real | and   | random   | grains | in a | autoradiograms | of | <sup>125</sup> ]- |
|-------------|----------------|---------|-------|----------|--------|------|----------------|----|-------------------|
| ANF-labele  | d area postrem | a. Dire | ct sc | oring me | thod.  |      |                |    |                   |

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| Structure                  | Real<br>grains<br>% | Random<br>grains<br>% | % Real grains<br><br>%Random grains |  |  |
|----------------------------|---------------------|-----------------------|-------------------------------------|--|--|
|                            | ~~~~~~              |                       |                                     |  |  |
| Neuronal_elements:         |                     |                       |                                     |  |  |
| Neuronal cell bodies       | 12.2                | 30.1                  | 0.41                                |  |  |
| Axons                      | 9.9                 | 3.4                   | 2.91                                |  |  |
| Dendrites                  | 20.1                | 12.4                  | 1.62                                |  |  |
| Non-neuronal elements:     |                     |                       |                                     |  |  |
| Astrocytes                 | 52.7                | 49.3                  | 1.07                                |  |  |
| Endothelial cells          | 1.9                 | 1.9                   | 1.00                                |  |  |
| Other cells                |                     |                       |                                     |  |  |
| (fibroblasts, macrophages) | 3.2                 | 2.9                   | 1.10                                |  |  |
|                            | <b></b>             |                       |                                     |  |  |
| Number of grains counted   | 433                 | 3325                  |                                     |  |  |

The distribution of real grains was ascertained by scoring and tabulating structures underlying the center of each silver grain, expressed as a percentage of the total number of silver grains analyzed. The random grain distribution (expressed as a percentage of the total number of points analyzed) was determined by overlaying a transparent screen displaying an array of points on randomly taken EM pictures of the area postrema and tabulating underlying structures. Keal and random grain distributions were compared to establish the relative degree to which the label was concentrated by each structure. A % *real grains*/% *random grains* value higher than 1 pointed to distinct labeling of the structure. The  $\chi^2$  test demonstrated significant differences (p<0.001) between the real and random grain distribution patterns, indicating that silver grain distribution was not random.

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| Structure                       | Real   | Random | % Real grains   |  |  |
|---------------------------------|--------|--------|-----------------|--|--|
|                                 | grains | grains |                 |  |  |
|                                 | %      | %      | % Random grains |  |  |
|                                 |        |        |                 |  |  |
| Single structures:              |        |        |                 |  |  |
| Neurons                         |        |        |                 |  |  |
| Cell bodies                     | 10.4   | 29.4   | 0.35            |  |  |
| Axons                           | 6.9    | 2.8    | 2.46            |  |  |
| Dendrites                       | 12.9   | 9.4    | 1.37            |  |  |
| Astrocytes                      | 20.8   | 28.6   | 0.73            |  |  |
| Endothelial cells               | 1.9    | 1.9    | 1.00            |  |  |
| Other cells                     |        |        |                 |  |  |
| (fibroblasts, macrophages)      | 3.2    | 2.9    | 1.10            |  |  |
| Apposed structures:             |        |        |                 |  |  |
| Neuronal cell bodies/Astrocytes | 2.5    | 1.3    | 1.92            |  |  |
| Axons/Astrocytes                | 5.8    | 1.6    | 3.63            |  |  |
| Dendrites/Astrocytes            | 13.4   | 6.1    | 2.20            |  |  |
| Astrocytes/Astrocytes           | 22.2   | 16.0   | 1.39            |  |  |
| Number of grains counted        | 433    | 3325   |                 |  |  |

Table 2.B.3Distribution of real and random grains in autoradiograms of 125I-ANF-labeled area postrema. 50% probability circle method.

The distribution of real grains was ascertained by means of a silver grain count performed on EM autoradiograms derived from the area postrema which was exposed to <sup>125</sup>I-ANF. Silver grains were allotted to underlying cellular elements using a 50% probability circle. The random grain distribution was determined by overlaying a transparent screen displaying an array of 50% probability circles on randomly taken EM pictures of the area postrema and tabulating underlying structures. The ratio: % *real grains/% random grains >* 1 pointed to distinct concentration of the label by a structure. The  $\chi^2$  test demonstrated that the rea! and random grain distribution patterns were significantly different (p<0.001), indicating a non-random distribution of silver grains.

Similarly, the shape of silver grain distribution around the astrocyte cell membrane in the line source analysis (peak over the plasmalemma with more than 50% of grains found within a distance of 1 HD from the membrane, Figure 2.B.6 D) further confirmed that silver grains were particularly concentrated over astrocyte plasmalemma.



**Figure 2.B.** Silver grain density distribution on either side of the plasmalemma of dendrites (A), axons (B), neuronal cell bodies (C), and astrocytes (D) in the area postrema of rats injected with  $^{125}$ I-ANF (120 x 10<sup>6</sup> dpm, ~50 pmol). For each silver grain, the shortest distance from its center to the nearest plasmalemma of each kind was determined, expressed in HD units (see Materials and Methods), and plotted on the diagram. Negative values indicate distances inside the structure whereas positive values refer to distances outside the structure. Grains located up to 10 HD from the plasmalemma were analyzed.

In contrast, analysis of silver grain concentration over neuronal cell bodies did not reveal any distinct labeling of the whole structure (Table 2.B.2) or of the interior of the cell (Table 2.B.3) (% real grains/% random grains < 1). Furthermore, line-source analysis failed to disclose silver grain condensation on the neuronal cell body plasmalemma (Figure 2.B.6 C). From comparison of the silver grain distribution around astrocyte and neuronal cell body plasmalemma (Figure 2.B.6), one can presume that grains in the vicinity of neuronal cell body/astrocyte contact (Table 2.B.3) originated from <sup>125</sup>I-ANFlabeled binding sites located on astrocyte plasmalemma itself rather than from the adjoining neuronal cell body membranes. If any binding sites are present over neuronal cell body plasmalemma, their number must have been below the detection limit of our method. The only indication in our studies that a small number of ANF binding sites may be present on neuronal cell body membranes is the observation that there is a small increase in the % real grains/% random grains value for neuronal cell bodies/astrocytes (1.92) when compared to astrocytes/astrocytes (1.39) (Table 2.B.3).

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Silver grains were particularly concentrated (% real grains/% random grains >1) over axons and dendrites (Figure 2.B.5, panels A, B, and C) as demonstrated by both the direct scoring (Table 2.B.2) and 50% probability circle (Table 2.B.3) methods. Line source analysis (Figure 2.B.6, panels A and B) as well as 50% probability circle (Table 2.B.3) methods pointed to their membranes as possible sources of radiation. It seems that silver grains were not associated with synaptic junctions since synaptic differentiations were not included within the 50% probability circle in the case of 96% of axonic and dendritic junctional grains. The silver grains present on dendrite/astrocyte and axon/astrocyte interfaces (Table 2.B.3) may have equally originated from astrocytes and not necessarily from dendrites and axons, especially since the following observations, we believe that ANF binding sites are indeed present over AP axon and dendrite plasmalemma: 1) the silver grain distribution around dendrite and axon plasmalemma had a peak over these membranes

(Figure 2.B.6, panels A and B); 2) there was an increase in the % *real grains/% random grains* ratio for dendrites/astrocytes (2.20) and for axons/astrocytes (3.63) in comparison with astrocytes/astrocytes (1.39) (Table 2.B.3); and 3) whole dendrites and axons were significantly labeled (Table 2.B.2).

Silver grains were detected also on fibroblasts and macrophages as well as on endothelial cells (Table 2.B.2). Unfortunately, because of low total number of grains found over those cells and low signal-to-noise ratio (% *real grains*/% *random grains* = 1.1 and 1.0, respectively) it seems that this method can not give conclusive information regarding the presence of ANF binding sites on these cells.

## 2.B.5 Discussion

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It has already been demonstrated that quantitative autoradiography *in vitro* with computerized densitometry is a powerful technique which allows the detection and pharmacologic characterization of ANF binding sites in cryostat sections of many tissues [34]. With this method using <sup>125</sup>I-[Tyr<sup>0</sup>]-CNP as a radioligand we were also able to visualize binding sites with pharmacological characteristics of the NPR-B in locations which have recently been reported to contain NPR-B transcripts by in situ hybridization [37]. EM autoradiography with administered *in vivo* <sup>125</sup>I-ligands combined with multistage analysis of autoradiograms [31] has been shown to be a potent method capable of localizing binding sites of various peptides [4,24,31], including those of ANF [24], in cellular and subcellular components. Using these two techniques, we have attempted in the present study to pharmacologically characterize and ultrastructurally localize binding sites interacting with ANF in the AP.

The results of competition studies performed by quantitative autoradiography *in vitro* demonstrate that in the AP ANF interacts with binding sites which have pharmacological characteristics resembling those of the NPR-A. This high-affinity interaction is not inhibited by CNP and peptides known to be NPR-C-selective, nor by unrelated peptides. The observation that <sup>125</sup>I-[Tyr<sup>o</sup>]-CNP binds to the AP while unlabeled CNP does not substantially displace <sup>125</sup>I-ANF binding in this tissue, suggests that a certain amount of NPR-B is present in the AP but ANF does not noticeably interact with it. In this respect our results confirm previously reported studies performed on NPR-A, NPR-B, or NPR-C-expressing cell cultures which showed that ANF binds preferentially to the NPR-A and NPR-C [15]. The presence of the NPR-B in the AP implies an existence of a natural ligand for the receptor in this tissue or circulation. Although this has not as yet been demonstrated, significant amounts of immunoreactive CNP have been detected in the whole medulla oblongata [16]. The miniature size of the AP does not allow confirmation of our observations regarding the presence of the NPR-A and NPR-B by measurements of ANF- or CNP-induced cGMP production in this tissue. However, upon exposure to ANF, the cGMP concentration was increased 13fold over control values, in slices from the medulla oblongata which included the AP, implying the presence of the guanylate cyclase-containing NPR-A in these brain preparations [35].

The major contribution of the present study is the localization of ANF binding sites in cellular elements of the intact AP. The tracer <sup>125</sup>I, which was used in the study, is a radiolabel of choice for ultrastructural analysis [4,30]. Given the very small size of the AP, we could not perform experiments to ensure that at 2 min after intracarotid injection of <sup>125</sup>I-ANF the AP-bound radioligand actually did represent intact ANF. Similar studies, however, performed under identical conditions on choroid plexus (structure large enough to quickly dissect at 2 min postinjection) revealed only small percentage (4.75%) of degraded radioligand (Konrad, unpublished data) in spite of the presence of the ANF-metabolizing enzyme endopetidase 24.11 [7] and despite large proportion of NPR-C [17] which are presumably involved in the degradation process [1]. Therefore, it is not likely that major radioligand degradation takes place in the AP at 2 min after injection.

Our study allows us to conclude that ANF binding sites with pharmacologic characteristics of the NPR-A are present on AP astrocytes. They do not seem to be internalized, at least up to 2 min after injection of the radioligand. These binding sites may indeed represent functional receptors since ANF-induced stimulation of cGMP has already been reported in glial cell cultures [11]. Thus ANF, as other active compounds whose receptors reside on these cells [36] is expected to regulate activity of glial cells. For example, it may be important in maintaining around neurons the ionic composition of extracellular fluid, a function which has its well-known counterpart in other tissues such as the kidney [39], intestine [29], and choroid plexus [33].

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Our demonstration of ANF binding sites with characteristics resembling those of the NPR-A on neuronal elements is in agreement with previous investigations: ANF-induced cGMP stimulation has been reported in neuronal cells in culture [10], and the effect of ANF on neuronal excitability has been detected in several brain regions, including the brainstem [8].

The ultrastructural localization of ANF binding sites can suggest a mode of ANF action. At the level of the dendrite, it may enhance or depress the excitability of responsive neuronal cells. At the level of axons, ANF may modulate the release of neurotransmitters or other neuroactive compounds. Furthermore, the observation that only a small proportion of silver grains was actually associated with synaptic junctions implies that ANF is likely to act primarily on non-differentiated membrane targets in the rat AP. The extrasynaptic localization of receptors has been noted in the case of other neuroactive compounds e.g., opioids [13].

From our studies it is impossible to deduce whether the dendrites and axons containing ANF binding sites are members of the CNS circuits involved in CV or water and sodium regulation, or other circuits in which the AP is implicated such as regulation of the sleep cycle, control of emesis and food intake [20]. The latter three possibilities, however plausible, are less likely since to date no reports have appeared suggesting the ANF involvement in such actions. As far as CV regulation is concerned, to date published reports indicate that the brain pool of ANF participates in CV regulation: ANF applied to the fourth ventricle [21], or microinjected into the NTS or other brainstem nuclei [8], elicits a significant decline in mean arterial pressure, whereas ANF administered into lateral ventricles attenuates ANG II-induced pressor response [38]. There is a possibility that the circulating pool of ANF, apart from other means of blood pressure regulation, may also modulate blood pressure through a CNS locus via CVOs which harbor ANF binding sites e.g., the subfornical organ, organum vasculosum of the lamina terminalis, and AP [19]. Extensive neural projections from the AP to the brain structures potentially involved in CV regulation are in agreement with the concept of a putative involvement of the AP in blood pressure lowering effect of ANF. As far as sait and water homeostasis is concerned, intracerebroventricular injections of ANF have been shown to inhibit saline preference in salt-depleted rats [3], whereas intracerebroventricular or intravenous ANF administration has diminished dehydration-induced water intake in unrestrained rats [2]. Circulating ANF may modulate these actions through CNS locus via ANF receptors located in the above mentioned CVOs including the AP.

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In summary, in the above experiments we have demonstrated that the AP harbours natriuretic peptide binding sites displaying pharmacological characteristics of the guanylate cyclase-containing NPR-A and NPR-B, but not of the guanylate cyclase-free NPR-C. In the AP, ANF interacts with the binding sites resembling the NPR-A. These sites are primarily localized over the plasmalemma of astrocytes, dendrites, and axons. The presence of ANF binding sites with characteristics of a functional ANF receptor over neuronal elements of the AP lends support to the idea that the AP may participate in mediation of the ANF-induced decline in blood pressure and/or salt and water intake by relaying information regarding circulating ANF status to the central neural autonomic circuits.

## 2.B.6 Acknowledgments

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## **CHAPTER 3**

## SPECIFICITY OF NATRIURETIC PEPTIDE RECEPTORS

The discovery of new members of the natriuretic peptide family, such as BNP and CNP, necessitated the determination of the relationship between these peptides and natriuretic peptide receptors. Thus, the specificity of natriuretic peptide receptors deserved investigation and was studied in this work with special emphasis placed on the effect the newly discovered peptides could have on these receptors.

Chapter 3.A aims at establishing the relationship between ANF binding sites and BNP. Studies on the effect of CNP on natriuretic peptide receptor subtypes are included in Chapters 2.B and 4.B. Studies on the impact of unrelated peptides on these receptors are described in Chapters 2.B, 3.A, 4.A, and 4.B.

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## 3.A BRAIN NATRIURETIC PEPTIDE BINDING SITES IN RATS

With the discovery of BNP, a novel natriuretic peptide homologous to ANF, exhibiting a spectrum of pharmacological actions similar to that of ANF, an interesting question arose: Does BNP exert its effects by acting on the same receptors as ANF? The presented work is an attempt to answer this question.

At the time BNP binding sites distribution and saturation experiments were performed, porcine BNP was the only known member of the new family of brain natriuretic peptides. Later, iso-ANF, i.e., rat BNP, was discovered. Despite structural differences, porcine and rat BNP and ANF seem to bind to the same binding sites.

As mentioned before, at the time of experimentation for this paper, two natriuretic peptide receptors were described: NPR-A (guanylate cyclasecontaining natriuretic peptide receptor) and NPR-C (guanylate cyclase-free natriuretic peptide receptor). The second guanylate cyclase-containing natriuretic peptide receptor, NPR-B, was not known at the time the experiments for this work were performed.

## 3.A.1 Abstract

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Brain natriuretic factor (BNP) is a recently discovered member of the family of natriuretic peptides highly homologous to atrial natriuretic factor (ANF). Quantitative *in vitro* autoradiography with a computerized microdensitometer demonstrated that the distribution of BNP binding sites is similar to the known distribution pattern of ANF binding sites in rat tissues. Analysis of saturation and competition curves disclosed that the maximal binding capacity for porcine BNP-(Asp81-Tyr106) and rat ANF-(Ser99-Tyr126) is similar within the plexiform layer of the olfactory bulb, choroid plexus, and

adrenal zona glomerulosa. Examination of the competition curves of porcine BNP-(Asp81-Tyr106), rat ANF-(Ser99-Tyr126), rat iso-ANF-(Asn64-Phe95) (rat BNP), and C-ANF (a ligand known to be selective for the guanylate cyclase-free natriuretic peptide receptor) for the binding of <sup>125</sup>I-BNP-(Asp81-Tyr106) and <sup>125</sup>I-ANF-(Ser99-Tyr126) revealed that ANF and iso-ANF fully displaced <sup>125</sup>I-BNP binding and, conversely, BNP and iso-ANF completely displaced <sup>125</sup>I-ANF binding in these tissues, whereas C-ANF partially displaced <sup>125</sup>I-BNP and <sup>125</sup>I-ANF binding. Angiotensin II, insulin, glucagon, and substance P had no influence on <sup>125</sup>I-BNP binding in the above tissues. These results support the view that peptides belonging to BNP and ANF families share the same binding sites in rats.

## **3.A.2 Introduction**

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Brain natriuretic peptide (BNP) is a new member of the family of natriuretic peptides with a remarkably high sequence homology to atrial natriuretic factor (ANF) [42]. It was initially isolated from the porcine brain [42] and then identified in the porcine and human heart and plasma [34,45,46], canine brain [18], and bovine chromaffin cells [29]. cDNA encoding the precursor for porcine [32] and human [43] BNP has been cloned and sequenced. Immunoreactive (ir) BNP distribution in the porcine brain is different from that of irANF, as determined by radioimmunoassay [18] and immunocytochemistry [35]. There is, however, accumulating evidence that ANF and BNP share the same receptors. BNP has been shown to bind to the same binding sites as ANF in some tissues, including the adrenal cortex [15], renal glomeruli [30], heart endocardium [30], and vascular smooth muscle cells [17,40]. Like ANF, BNP stimulates guanosine 3', 5'-cyclic monophosphate (CGMP) production in adrenocortical [15], endothelial [30,40], and smooth muscle cells [17,40] as well as in fibroblasts [40], in a non-additive manner with ANF [17]. Many biological activities have already been shown to be common to these peptides. In vivo studies on the rat [42] and investigations of adrenocortical cells in culture [15] have revealed that porcine BNP possesses a

pharmacological spectrum (including diuretic, natriuretic, hypotensive, smooth muscle relaxant [42], and steroidogenesis inhibitory [15] responses) similar to that of ANF. Administration of porcine BNP in rat cerebral ventricles inhibits angiotensin II (ANG II)- and dehydration-induced water intake [12] and suppresses ANG II-induced pressor responses [39] as well as basal and ANG II-induced vasopressin secretion in a manner similar to ANF [48].

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Recently, three independent groups of investigators reported the existence of a novel peptide in the rat heart, structurally more related to BNP than to ANF, and designated iso-ANF [11], rat BNP [1,20], or cardiac natriuretic peptide [19]. cDNA encoding the precursor for this peptide has been cloned and sequenced [20]. This peptide appears to be present in the heart (at a relatively high concentration), as well as in the lung, kidney, adrenal gland, brain, and other rat tissues (at lower concentration) [2,16,38].

The purpose of the present study was to determine whether BNP binds to the same population of brain binding sites as ANF and to provide further evidence that BNP and ANF families share the same receptors in peripheral tissues. For this purpose, we examined the distribution and characteristics of BNP binding sites in rat tissues using autoradiographic and affinity crosslinking techniques. In this communication BNP denotes porcine BNP, whereas ANF and iso-ANF denote rat ANF and BNP, respectively.

## 3.A.3 Materials and Methods

#### 3.A.3.1 Iodination of BNP and ANF

Radioiodination of porcine BNP-(Asp81-Tyr106) and rat ANF-(Ser99-Tyr126) (Bio-Mega, Laval, Quebec, Canada) was performed by the lactoperoxidase method with <sup>125</sup>I-sodium as described earlier [27]. Purification of the monoiodinated forms of ANF and BNP was achieved by highperformance liquid chromatography (HPLC). The specific activity of <sup>125</sup>I-ANF and <sup>125</sup>I-BNP was ~2,400 dpm/fmol (1,100  $\mu$ Ci/nmol). Radioiodinated ligands were used for experiments within 2-3 days after the iodination.

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#### **3.A.3.2 Preparation of autoradiograms**

Sprague-Dawley rats weighing 200-250 g were killed by decapitation. Their tissues were dissected and immediately freeze-mounted on cryostat chucks in a microtome cryostat at  $-35^{\circ}$ C. Consecutive tissue cryostat sections (20 µm) were mounted on 1% gelatin-0.05% chromium potassium sulfate-coated microscope slides, dried under vacuum at 4°C for 2 h, and stored in sealed boxes with desiccant at  $-70^{\circ}$ C until used.

Preincubation of the tissue sections was carried out at room temperature for 10 min in 50 mM Tris-HCl buffer (pH 7.5) containing 0.5% bovine serum albumin. This was followed by incubation for 90 min in the same buffer supplemented with appropriate concentrations (see below) of <sup>125</sup>I-BNP or <sup>125</sup>I-ANF, BNP-(Asp81-Tyr106) or ANF-(Ser99-Tyr126) or iso-ANF-(Asn64-Phe95), or des-(Gln116-Gly120)ANF-(Asp102-Cys121)-NH<sub>2</sub> (C-ANF, a ligand known to be selective for the guanylate cyclase-free natriuretic peptide receptor; [22]), 1  $\mu$ M phosphoramidon, 1  $\mu$ M leupeptin, 1  $\mu$ M pepstatin, 100  $\mu$ M EDTA, 1  $\mu$ M phenylmethylsulfonyl fluoride, 200 kallikrein inhibiting units/ml aprotinin, 5  $\mu$ M MnCl<sub>2</sub> and 0.05% bacitracin. For analysis of the distribution of binding sites the incubation buffer contained 280 pM (for brain sections) and 100 pM (for other tissue sections) <sup>125</sup>I-BNP. Nonspecific binding was defined as the binding of <sup>125</sup>I-BNP in the presence of 0.5 µM unlabeled BNP. To test the specificity of binding, some sections (olfactory bulb, choroid plexus, adrenals, kidney) were incubated in the presence of 1  $\mu$ M of one of the following peptides: ANG II, glucagon, substance P, or insulin. For saturation analysis, concentrations of <sup>125</sup>I-BNP ranging from 14 to 1,000 pM were employed. Nonspecific binding was estimated in parallel incubations in the presence of 0.5 µM unlabeled BNP. For competition analysis, the incubation buffer contained 140 pM <sup>125</sup>I-BNP or 50 pM <sup>125</sup>I-ANF and either unlabeled BNP, ANF, iso-ANF, or C-ANF in concentrations from 1 pM to 1  $\mu$ M. After incubation, the slides were washed (2 x 10 min,  $4^{\circ}$ C) in 50 mM Tris-HCl (pH 7.5) containing 0.5% bovine serum albumin, fixed (15-20 min) in 2% glutaraldehyde (pH 7.5, 4°C), washed (5 min) in 0.1 M phosphate buffer (pH 7.5, 4°C) and then in

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distilled water (1 min), dehydrated in alcohol, and dried overnight at 60°C. Subsequently, the sections, along with <sup>125</sup>I-standards were apposed to X-ray films from 0.5 to 6 days at room temperature. The films were developed in undiluted Kodak D19 developer for 4 min at 20°C, washed in water for 1 min, and fixed in Kodak Ektaflo fixer (dilution 1:3) for 4 min. Two types of <sup>125</sup>I-standards were used, a commercially available <sup>125</sup>I-microscale (Amersham, Arlington Heights, Illinois, USA) and standards prepared from brain or kidney homogenates as described earlier [5].

# 3.A.3.3 Assessment of the radioligand depletion and degradation

Before and after exposure of the sections, samples of the incubation buffer were collected to appreciate the degree of radioligand depletion and degradation during the 90-min incubation period. The radioligand depletion was measured by a gamma counter and defined as the difference of radioactivity content in the incubation medium before and after exposure of the sections. The radioligand degradation was assessed by reverse-phase HPLC and expressed as a difference of the intact radioligand content in the incubation buffer before and after exposure of the sections.

#### 3.A.3.4 Analysis of autoradiograms

Autoradiograms were analyzed with a computerized microdensitometer [5]. To facilitate anatomical identification, they were compared with the <sup>125</sup>I-BNP labeled sections which were subsequently stained with luxol fast blue and cresyl violet for myelin and nerve cell visualization (brain sections) or with cresyl violet alone (remaining tissue sections).

Saturation curves (2 separate experiments, 1 animal per experiment) and competition curves of BNP for <sup>125</sup>I-BNP binding, as well as competition curves of ANF for <sup>125</sup>I-ANF binding, were first analyzed with the EBDA computer program [25) and then with LIGAND (a nonlinear, least-square, curve-fitting computer program [26]) to determine the number of classes of BNP binding

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sites, the maximal binding capacity  $(B_{max})$ , and the equilibrium dissociation constant  $(K_d)$  of the binding sites.

Competition binding curves (3 separate experiments, 1 animal per experiment) were analyzed with the ALLFIT computer program, based on a four-parameter logistic equation [10], to obtain estimates of the degree of displacement of one ligand bound by another and to compute the  $IC_{50}$  (unlabeled ligand concentration at which the maximum binding of labeled ligand is displaced by 50%). Experiments involving <sup>125</sup>I-BNP and <sup>125</sup>I-ANF have been performed on the same day each time.

#### 3.A.3.5 Preparation of membranes

The olfactory bulbs (OB) of nine Sprague-Dawley rats were dissected, cleaned from surrounding highly vascularized membranes, and homogenized with a hand homogenizer in a buffer containing 20 mM NaHCO<sub>3</sub>, 2 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 1  $\mu$ M aprotinin, 1  $\mu$ M leupeptin, 1  $\mu$ M phosphoramidon and 0.1  $\mu$ M pepstatin A. The homogenate was centrifuged at 1,000 g for 10 min. The pellet was discarded and the supernatant was centrifuged at 25,000g for 20 min. The resulting pellet was washed twice with the homogenization buffer and resuspended in 200  $\mu$ l of a buffer containing 50 mM Tris-HCl, 250 mM sucrose, 0.1 mM EDTA and 1 mM MgCl<sub>2</sub> (pH 7.4). Aliquots were taken for protein determination [6] and for the binding assay. All membranes were kept frozen at -70°C until they were used.

#### 3.A.3.6 Affinity cross-linking and sodium dodecyl sulfatepolyacrylamide gel electrophoresis

Hundred-microgram protein aliquots of the OB membranes were incubated for 60 min at room temperature in a binding buffer containing 550 pM  $^{125}$ I-ANF and 10 nM or 1  $\mu$ M unlabeled ANF, BNP, iso-ANF, or C-ANF. Subsequently, the membranes were treated in 0.5 mM disuccimidyl suberate dissolved in dimethylsulfoxide for 15 min at room temperature. The reaction was stopped by addition of ammonium acetate to a final concentration of 50 mM. Samples then were denatured with 2.5% sodium dodecyl sulfate

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containing 2%  $\beta$ -mercaptoethanol, boiled for 5 min, and resolved on 8% polyacrylamide gel [36] along with molecular mass standards (Pharmacia LKB, Uppsala, Sweden). The gel was stained in Coomassie R-250, dried, and exposed for 15 days to Kodak X-Omat RP film (Eastman Kodak Co., San Diego, California, USA) at -70°C.

## 3.A.4 Results

#### 3.A.4.1 Radioligand depletion and degradation

Radioligand depletion was <15%. Degradation of the radioligands during the incubation period was also negligible (after incubation >90% <sup>125</sup>I-BNP and >98% <sup>125</sup>I-ANF eluted from the reverse-phase HPLC column at the elution time corresponding to intact ligands; Figure 3.A.1).

#### 3.A.4.2 <sup>125</sup>I-standard

Comparison of the commercially available <sup>125</sup>I-standard and standards prepared from brain or kidney homogenates for the same optical densities demonstrated a ratio of 1:23 of the commercial standard (fmol/mg polymer) to the tissue homogenate standards (fmol/mg protein). This allowed us to use the commercial standard and to express the binding data in fmol/mg protein, referring to the tissue homogenate standards. Given the average protein content (18  $\mu$ g) and the mean surface area (19.6 mm<sup>2</sup>) of homogenate standard sections, 1.09 mm<sup>2</sup> of the kidney homogenate standard (20  $\mu$ m thickness) contained 1  $\mu$ g of protein.

## 3.A.4.3 Specificity of <sup>125</sup>I-BNP binding

<sup>125</sup>I-BNP binding in the plexiform layer of the OB, choroid plexus (ChP), adrenal zona glomerulosa (AZG), and renal glomeruli was displaced by BNP, ANF, and iso-ANF but was unaffected by unrelated peptides, including ANG II, insulin, glucagon, and substance P at the concentration of 1  $\mu$ M (data not shown).



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**Figure 3.A.1** Assessment of <sup>125</sup>I-BNP-(Asp81-Tyr106) and <sup>125</sup>I-ANF-(Ser99-Tyr126) degradation during 90-min incubation period; reverse-phase HPLC of representative experiments. The material was loaded on  $C_{18}$  µ-Bondapak column, eluted at 1 ml/min with a linear gradient of 15-55% acetonitrile in 0.1% trifluoroacetic acid, and passed through a FLO-ONE/Beta radioactive flow detector coupled to HPLC. A,C: radioligand in the incubation medium before exposure of sections. B,D: radioligand in the incubation medium after exposure of sections. BNP, brain natriuretic peptide; ANF atrial natriuretic factor; cpm, counts/min.

#### 3.A.4.4 Distribution of BNP binding sites in peripheral tissues

The distribution and relative concentration of <sup>125</sup>I-BNP binding sites in rat peripheral tissues are illustrated in Table 3.A.1.

Among endocrine glands, BNP binding sites were observed in the adrenal gland and hypophysis. High-density binding sites occurred in the AZG (Figure 3.A.2, panels A and B), whereas moderate- to low-density receptors were seen in the adrenal medulla and inner zones of the adrenal cortex (Figure 3.A.2, panels A and B). In the hypophysis, BNP binding sites were confined to both the anterior and posterior lobes, where small concentrations of receptors were noted.

Table 3.A.1 Relative concentrations of porcine <sup>125</sup>I-BNP-(Asp81-Tyr106) binding sites in the rat peripheral tiscues determined by quantitative autoradiography.

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| Tissue             | <sup>125</sup> I-BNP specific binding |  |  |
|--------------------|---------------------------------------|--|--|
| Adrenal gland      |                                       |  |  |
| Zona glomerulosa   | $24.2 \pm 0.4$                        |  |  |
| Zona fasciculata   | $10.2 \pm 0.9$                        |  |  |
| Zona reticularis   | $8.0 \pm 1.0$                         |  |  |
| Medulla            | $14.6 \pm 0.8$                        |  |  |
| Pituitary gland    |                                       |  |  |
| Anterior lobe      | $3.1 \pm 0.4$                         |  |  |
| Posterior lobe     | $4.2 \pm 0.4$                         |  |  |
| Kidney             |                                       |  |  |
| Glomerulus         | $10.5 \pm 0.4$                        |  |  |
| Outer medulla      | $2.6 \pm 0.3$                         |  |  |
| Inner medulla      | $4.2 \pm 1.1$                         |  |  |
| Urethra            | $8.4 \pm 1.4$                         |  |  |
| Uterus             |                                       |  |  |
| Muscular layer     | 5.3 ±1.0                              |  |  |
| Ovaries            |                                       |  |  |
| Medulla            | $3.2 \pm 0.5$                         |  |  |
| Corpus luteum      | $3.3 \pm 0.5$                         |  |  |
| Testis             |                                       |  |  |
| Interstitium       | $1.0 \pm 0.3$                         |  |  |
| Heart              |                                       |  |  |
| Endocardium        | $4.1 \pm 0.8$                         |  |  |
| Aorta              | $7.2 \pm 0.4$                         |  |  |
| Vena cava superior | $3.2 \pm 0.4$                         |  |  |
| Vena cava inferior | $2.8 \pm 0.8$                         |  |  |
| Spleen             |                                       |  |  |
| Red pulp           | $3.4 \pm 0.7$                         |  |  |
| White pulp         | $6.5 \pm 0.4$                         |  |  |

#### Table 3.A.1 (cont.)

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| Tissue         | <sup>125</sup> I-BNP specific binding |  |  |
|----------------|---------------------------------------|--|--|
| <br>Thymus     |                                       |  |  |
| Cortex         | $5.6 \pm 0.4$                         |  |  |
| Medulla        | $6.1 \pm 0.3$                         |  |  |
| Lung           |                                       |  |  |
| Parenchyma     | $48.0 \pm 4.5$                        |  |  |
| Stomach        |                                       |  |  |
| Pars cardiaca  |                                       |  |  |
| Muscular layer | $8.5 \pm 0.5$                         |  |  |
| Pars pylorica  |                                       |  |  |
| Mucosal layer  | $10.5 \pm 0.9$                        |  |  |
| Muscular layer | $9.6 \pm 1.6$                         |  |  |
| Duodenum       |                                       |  |  |
| Mucosal layer  | $11.2 \pm 0.5$                        |  |  |
| Muscular layer | $8.2 \pm 0.6$                         |  |  |
| Jejunum        |                                       |  |  |
| Mucosal layer  | $10.8 \pm 1.8$                        |  |  |
| Muscular layer | $2.8 \pm 0.9$                         |  |  |
| Ileum          |                                       |  |  |
| Muscular layer | $11.8 \pm 2.8$                        |  |  |
| Colon          |                                       |  |  |
| Mucosal layer  | $1.8 \pm 0.3$                         |  |  |
| Muscular layer | $9.4 \pm 0.3$                         |  |  |
| Rectum         |                                       |  |  |
| Mucosal layer  | 2.8 ±1.4                              |  |  |
| Muscular layer | $3.1 \pm 0.5$                         |  |  |
| Liver          | $24.0 \pm 2.1$                        |  |  |

Values are means  $\pm$  SE in fmol/mg protein.

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**Figure 3.A.2** Computer-enhanced autoradiograms of rat tissue cryostat sections (20  $\mu$ m) labeled *in vitro* with 140 pM <sup>125</sup>I-BNP-(Asp81-Tyr106) alone (total binding; A,C,E,G,I,K,M,O) or with 0.5  $\mu$ M unlabeled BNP-(Asp81-Tyr106) (nonspecific binding; B,D,F,G,J,L,N,P). Increasing densities of BNP binding sites are indicated by different colors in the following order: dark blue < light blue < green < yellow < red. A,B: adrenal gland and kidney. C,D: lung and liver. E,F: olfactory bulb. G,H: coronal section through the rat brain at the level of the subfornical organ. I,J: coronal section through the rat brain at the level of the median eminence. K,L: coronal section through the rat brain at the level of the interpeduncular nucleus. O,P: coronal section through the rat brain at the level of the interpeduncular nucleus. O,P: coronal section through the rat brain at the level of the olfactory bulb; SFO, subfornical organ; CHP, choroid plexu; PA, pia/arachnoid; MHN, medial habenular nucleus; IPN, interpeduncular nucleus; AP, area postrema.

In the urogenital system, the kidney was the most predominantly labeled organ (Figure 3.A.2, panels A and B). A moderate concentration of specific BNP binding sites was evident in the glomerulus, whereas the inner and outer medulla displayed low concentrations. In other organs of the urogenital system, low numbers of receptors were detected in the muscular layer of the uterus and the urethra, in the medulla and corpus luteum of the ovary, and in the interstitium of the testis (Table 3.A.1).

In the gastrointestinal system, the muscular and mucosal layers displayed negligible to moderate concentrations of BNP binding sites virtually along the entire digestive tract. Other organs associated with the gastrointestinal system showed high (liver, Figure 3.A.2, panels C and D) or negligible (pancreas) populations of receptors.

In the cardiovascular system, a low concentration of BNP binding sites was detected in the aorta and veins, including the superior and inferior vena cava. The endocardium was weakly labeled as well.

In the immune system, a moderate concentration of binding sites was present in the cortex and medulla of the thymus and in the white and red pulp of the spleen.

Among other peripheral tissues examined, the lung parenchyma (Figure 3.A.2, panels C and D) showed high numbers and the parotid glands showed negligible numbers of BNP receptors.

## 3.A.4.5 Distribution of BNP binding sites in the brain

The distribution and relative concentration of BNP binding sites in the brain are depicted in Table 3.A.2.

Preliminary studies have shown that to reliably measure BNP binding sites in the brain, the concentration of <sup>125</sup>I-BNP in the incubation medium has to be increased (when compared with the <sup>125</sup>I-BNP concentration used to investigate binding sites in peripheral tissues). Because in both cases (i.e., to determinate the distribution of peripheral and central BNP binding sites) nonsaturable concentrations of <sup>125</sup>I-BNP were employed, the increased <sup>125</sup>I-BNP concentration in the incubation medium resulted in an augmentation of specific binding sites detected. Therefore, the values from Table 3.A.1 could not be compared with those of Table 3.A.2.

 
 Table 3.A.2
 Relative concentrations of porcine <sup>125</sup>I-BNP-(Asp81-Tyr106) binding sites in selected regions of the
rat central nervous system.'

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|                   |                                                                                              | Binding sites, fmol/mg protein                                                                               |                            |                 |                                     |
|-------------------|----------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------|----------------------------|-----------------|-------------------------------------|
|                   | 1-10                                                                                         | 10-20                                                                                                        | 20-30                      | 30-40           | > 40                                |
| Telencephalon     | Parietal cortex<br>Striatum                                                                  | Hippocampal<br>formation                                                                                     | Lateral olfactory<br>tract |                 | Olfactory bulb<br>(plexiform layer) |
| Diencephalon      | Corpus callosum<br>Suprachiasmatic n.<br>Periventricular<br>hypothalamic n.<br>Mammillary n. | Mediał preoptic n.<br>Median preoptic n.<br>Paraventricular n.<br>Medial habenular n.<br>Anterior commissure |                            |                 |                                     |
| Mesencephalon     |                                                                                              | Substantia nigra<br>Periaqueductal                                                                           | Interpeduncular n.         |                 |                                     |
| Rhombencephalon   | Locus coeruleus<br>Dorsal parabrach. n.<br>Superior colliculi                                | Dorsal raphe n.<br>Pontine nuclei<br>Parvocellular<br>reticular n.                                           |                            |                 |                                     |
|                   | Nucleus of the<br>solitary tract<br>Hypoglossal n.<br>Medullary reticular n.                 |                                                                                                              |                            |                 |                                     |
| Circumventricular | 5                                                                                            | Median eminence                                                                                              | Subfornical organ          | Area postrema   | Pia-arachnoid                       |
| organs            |                                                                                              | (4th ventricle)                                                                                              | (lateral ventricle)        | (3rd ventricle) |                                     |
| Other tissues     |                                                                                              | Pineal gland                                                                                                 |                            |                 |                                     |

CHAPTER 3

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The plexiform layer of the OB (Figure 3.A.2, panels E and F) and piaarachnoid (Figure 3.A.2, panels I and J) displayed the highest BNP binding site concentrations in the brain. The exact meningeal layer bearing BNP receptors could not be resolved using this technique. The ChP possessed a high density of BNP binding sites (Figure 3.A.2, panels I,J,K, and L). The concentration of BNP receptors appeared to vary within different parts of this tissue; ChP from the 3rd ventricle had the highest, whereas ChP from the 4th ventricle showed the lowest concentration of BNP binding sites. In other circumventricular organs, including the area postrema (Figure 3.A.2, panels O and P), subfornical organ (Figure 3.A.2, panels G and H), and median eminence, moderate to high numbers of BNP binding sites were evident. The interpeduncular nucleus (Figure 3.A.2, panels M and N) and medial habenular nucleus (Figure 3.A.2, panels K and L) displayed the highest level of BNP receptors in the mesencephalon and diencephalon, respectively. Other areas, where a distinct concentration of specific BNP binding sites was measured, are listed in Table 3.A.1.

#### **3.A.4.6** Characterization of BNP binding sites

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The plexiform layer of the OB, the ChP, and the AZG were chosen as representative tissues to study the characteristics of BNP binding sites in the rat body because 1) they showed large populations of BNP receptors; 2) they are relatively large tissues, giving a sufficient number of sections for saturation and competition experiments; and 3) they represent different compartments of the body (the adrenal gland is a peripheral tissue, the OB a brain tissue, and the ChP a circumventricular organ).

In all tissues studied, the saturation curves and selected competition curves, analyzed according to a model of <sup>125</sup>I-BNP binding to one or two independent classes of receptors, either did not resolve more than one class of binding sites or the curves fitted better to a single site model system. Analysis of the saturation curves showed that BNP binding sites in the areas examined were saturable, of high affinity, and of low capacity (Figure 3.A.3). The

competition curves disclosed that the  $B_{max}$  for BNP and the  $B_{max}$  for ANF were similar within each tissue examined (data not shown).

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Figure 3.A.3 Saturation binding curves of <sup>125</sup>I-BNP-(Asp81-Tyr106) in rat tissue cryostat sections (20  $\mu$ m). Each point represents the mean of 18-20 measurements from a representative experiment. A: plexiform layer of the olfactory bulb (OB): K<sub>d</sub>, 323 pM; B<sub>max</sub>, 51 fmol/mg protein. B: choroid plexus (ChP) from the 3rd ventricle: K<sub>d</sub>, 363 pM; B<sub>max</sub>, 40 fmol/mg protein. C: adrenal zona glomerulosa (AZG): K<sub>d</sub>, 213 pM; B<sub>max</sub>, 72 fmol/mg protein.

Analysis of the competition curves of BNP, ANF, iso-ANF, and C-ANF for <sup>125</sup>I-BNP and <sup>125</sup>I-ANF binding revealed that not only BNP but also ANF and iso-ANF completely displaced <sup>125</sup>I-BNP binding (Figure 3.A.4), and, conversely, not only ANF but also BNP and iso-ANF fully displaced <sup>125</sup>I-ANF binding (Figure 3.A.5) in the plexiform layer of the OB, in the ChP from the third ventricle, and in the AZG. ANF was consistently the most potent peptide in displacing <sup>125</sup>I-ANF and <sup>125</sup>I-BNP binding in all tissues examined (Figures 3.A.4 and 3.A.5). BNP and iso-ANF competed for both radioligands' binding with generally similar potency (Figures 3.A.4 and 3.A.5). All the unlabeled peptides were more effective (at average 8 times lower IC<sub>50</sub>) in displacing <sup>125</sup>I-BNP than <sup>125</sup>I-ANF binding.

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Figure 3.A.4 Competition binding curves of porcine BNP-(Asp81-Tyr106), rat ANF-(Ser99-Tyr126), rat iso-ANF-(Asn64-Phe95), and des-(Gln116-Gly120)ANF-(Asp102-Cys121)-NH<sub>2</sub> (C-ANF) for binding <sup>125</sup>I-BNP (140 pM) in rat tissue cryostat sections. Each point represents the mean of 16-20 measurements from 3 separate experiments. A: plexiform layer of the olfactory bulb (OB). IC<sub>50</sub>: ANF, 0.09 nM; BNP, 0.44 nM; iso-ANF, 0.40 nM; C-ANF, not determined. B: choroid plexus from the 3rd ventricle (ChP). IC<sub>50</sub>: ANF, 0.08 nM; BNP, 0.25 nM; iso-ANF, 0.37 nM; C-ANF, 0.80 nM. C: adrenal zona glomerulosa (AZG). IC<sub>50</sub>: ANF, 0.05 nM; BNP, 0.10 nM; iso-ANF, 0.32 nM; C-ANF, not determined.

#### 3.A.4.7 Affinity cross-linking studies

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To verify that BNP and iso-ANF displace <sup>125</sup>I-ANF from the same receptor as ANF, affinity cross-linking of <sup>125</sup>I-ANF to OB membranes was performed. OBs were chosen because of their relatively high density of ANF binding sites. As shown on Figure 3.A.6, <sup>125</sup>I-ANF was covalently incorporated into a band with an apparent molecular mass of approximately 120 kDa (lane 1), which presumably corresponds to the guanylate cyclase-containing natriuretic pepticle receptor. The presence of increasing concentrations of unlabeled ANF-(Ser99-Tyr126) in the binding assay prior to cross-linking.resulted in a progres-



**Figure 3.A.5** Competition binding curves of porcine BNP-(Asp81-Tyr106), rat ANF-(Ser99-Tyr126), rat iso-ANF-(Asn64-Phe95), and des-(Gln116-Gly120)ANF-(Asp102-Cys121)-NH<sub>2</sub> (C-ANF) for binding <sup>125</sup>I-ANF (50 pM) in rat tissue cryostat sections. Each point represents the mean of 16-20 measurements from 3 separate experiments. A: plexiform layer of the olfactory bulb (OB). IC<sub>50</sub>: ANF, 0.11 nM; BNP, 2.39 nM; iso-ANF, 4.03 nM; C-ANF, not determined. B: choroid plexus from the 3rd ventricle (ChP): IC<sub>50</sub>: ANF, 0.15 nM; BNP, 1.27 nM; iso-ANF, 0.52 nM; C-ANF, 9.97 nM. C: adrenal zona glomerulosa (AZG). IC<sub>50</sub>: ANF, 0.25 nM; BNP, 2.85 nM; iso-ANF, 0.52 nM; C-ANF, not determined.

sive decrease in the intensity of that band (Figure 3.A.6, lanes 2 and 3). BNP (Figure 3.A.6, lanes 4 and 5) and iso-ANF (Figure 3.A.6, lanes 6 and 7) also displaced completely <sup>125</sup>I-ANF binding. C-ANF at the concentration of 1  $\mu$ M decreased the intensity of the 120 kDa band only slightly (Figure 3.A.6, lane 9).



**Figure 3.A.6** Autoradiogram of sodium dodecyl sulfate-polyacrylamide gel electrophoresis of rat olfactory bulb membrane cross-linked under reducing conditions to <sup>125</sup>I-ANF in the absence (lane 1) or presence of either unlabeled ANF (1  $\mu$ M, lane 2; 10 nM, lane 3), BNP (10 nM, lane 4; 1  $\mu$ M; lane 5), iso-ANF (10 nM, lane 6; 1  $\mu$ M, lane 7), or C-ANF (10 nM, lane 8; 1  $\mu$ M, lane 9).

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## 3.A.5 Discussion

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Comparison of the distribution of BNP binding sites in rat tissues with the earlier reported localization of ANF receptors [4-6,13,23,31,33,41] reveals interesting close parallels. Virtually all structures where BNP binding sites were detected in the present study had earlier been demonstrated to contain ANF receptors. The only exceptions were the locus coeruleus, periventricular hypothalamic nucleus, and medial preoptic nucleus among the central nervous system structures, as well as the urethra and stomach among the peripheral structures. In our study, a low concentration of BNP binding sites was demonstrated in the above areas, whereas the presence of ANF receptors was not reported or not investigated at all. The striking parallelism in the anatomical distribution of binding sites for these two peptides suggests that they may bind to the same population of receptors.

Wherever possible we compared the relative concentration of binding sites for BNP and ANF in many tissues. In the brain, the sequence of tissues with decreasing population of BNP binding sites was very similar to that of ANF receptors. In particular, this was apparent when comparisons were made with the very detailed study of Gibson et al. [13], who evaluated the distribution of ANF binding sites in the brain, using the same technique and animal species as that we employed to determine the distribution of BNP receptors. Such a strong correlation in the relative numbers of binding sites for these two peptides further supports the hypothesis that they share the same receptor population. However, some differences in the relative concentrations of binding sites for both peptides were observed when peripheral tissues were examined. Still, these discrepancies may simply be due to the different techniques (in vivo vs. in vitro autoradiography) or animal species (guinea pig vs. rat) used to evaluate the distribution of binding sites for ANF and BNP. However, these discrepancies may also be due to a possible differential selectivity of natriuretic peptide receptor subpopulations for one peptide or the other.
Consistent with the hypothesis of common binding sites is the observation that in the saturation experiments, the ChP and AZG exhibited a  $B_{max}$  for BNP comparable to that previously reported for ANF [24,44,47]. However, the lower  $B_{max}$  for ANF in the OB, reported by Glembotski et al. [14], as compared to the  $B_{max}$  for BNP recorded in the present study, may be due to the fact that these investigators took into consideration the whole OB and not only its plexiform layer (as we did), which contains the highest concentration of ANF binding sites in this structure. Therefore, it is possible that the  $B_{max}$  for ANF and BNP in the plexiform layer of the OB are similar as well. Moreover, as calculated from appropriate competition curves, the maximal density of binding sites for ANF and for BNP appeared to be alike within each tissue studied, which also supports this hypothesis.

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 Furthermore, the results obtained from the binding displacement experiments likewise suggest that peptides belonging to BNP and ANF families utilize the same binding sites, because specific <sup>125</sup>I-BNP binding was fully displaced not only by BNP but also by ANF and iso-ANF, and, conversely, specific <sup>125</sup>I-ANF binding was completely displaced not only by ANF but also by BNP and iso-ANF in the OB, ChP, and AZG. Affinity cross-linking studies further confirmed this notion.

The total displacement of one peptide by the other indicates that BNP does not bind selectively to one of the subclasses of the natriuretic peptide receptor in the tissues examined. This notion is also supported by the demonstration that BNP binds to guanylate cyclase-containing as well as to guanylate cyclase-free natriuretic peptide receptor subtypes. The former was revealed by our affinity cross-linking studies and by stimulation of cGMP formation by BNP in smooth muscle cells [17,40], endothelial cells [30,40], fibroblasts [40], and adrenocortical cells [15] in a non-additive manner with ANF [17]. The latter was evident by partial displacement of <sup>125</sup>I-BNP binding by C-ANF in the ChP, a tissue which predominantly contains the guanylate cyclase-free natriuretic peptide receptors. However, the competition (and

saturation) binding studies suggest that natriuretic peptide receptor subtypes have slightly lower affinity for BNP than for ANF.

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The hypothesis of common binding sites for ANF and BNP explains the common biological responses to exogenously-applied ANF, BNP, or iso-ANF. When administered intravascularly into the rat body, porcine or rat BNP elicit a pharmacological spectrum (including diuretic, natriuretic and hypotensive responses) that is similar to that of ANF [11,42]. Moreover, studies on bovine adrenocortical cells have revealed an impact resembling the suppressive action of ANF on steroidogenesis [15]. Furthermore, exposure of rat cerebral ventricles to porcine BNP inhibits ANG II- and dehydration-induced water intake [12], ANG II-induced pressor responses [39], as well as basal and ANG II-induced vasopressin secretion [48] in a manner similar to that caused by ANF.

However, the hypothesis of common binding sites for peptides of ANF and BNP families does not answer the question whether these receptors physiologically respond to both or to only one of these endogenously-released peptides. In this respect, the work of Saper et al. [35], in which the differential distribution of irBNP in the rat brain was reported, supports both possibilities. The distribution of irBNP partially overlapped that of irANF, which suggests a joint action of ANF and BNP on the receptors. In other regions of the brain, a complementary distribution of these two peptides was noted (including structures with ANF/BNP binding sites and irBNP but little or no irANF), which in turn suggests a sole action of one of the peptides on the neighboring receptors. As far as the peripheral ANF/BNP binding sites are concerned, it seems that they may respond not only to locally produced ANF but also to in situ produced BNP, since local production of rat and bovine BNP has been documented [9,28]. They may also respond not only to circulating ANF but also to circulating BNP, since the presence of BNP in rat, porcine, and human plasma has been reported [16,34,46]. Whether both families of peptides are secreted from the heart in response to the same stimuli remains to be elucidated.

Wherever possible, a comparison of the distribution of BNP-containing neurons, reported by Saper et al. [35], with the localization of brain BNP binding sites was performed and revealed that in many of the examined brain areas there was a correlation between the distribution of BNP neurons and BNP receptors. For example, both irBNP and BNP binding sites were found in the following brain structures: the cerebral cortex, hippocampus, olfactory bulb, subfornical organ, paraventricular nucleus, mammillary nucleus, raphe nuclei, locus coeruleus, nucleus of the solitary tract, and area postrema. However, as in the case of other neuropeptides, a mismatch between distribution of BNP neurons and receptors was observed as well, e.g., in the hypoglossal and periventricular hypothalamic nuclei. Several interpretations have been proposed to justify the phenomenon of receptor-neuropeptide mismatch [21]. An interesting one says that brain is an endocrine organ where neuropeptides may be synthesized at one locus and released from another one, while acting elsewhere in the brain; the communication residing in the specificity of receptors rather than in physical juxtaposition.

The existence of two distinct families of peptides capable of binding to the same population of receptors is expected to have physiological and/or perhaps evolutionary significance, which as yet has not been explained. It may contribute to a fine tuning of biological functions regulated by these peptides, because their expression may each be subject to different forms of control [20], resulting in their differential tissue distribution [35]. Additionally, the heterogeneity of guanylate cyclase-containing natriuretic peptide receptors (the NPR-A and NPR-B receptors) [8] together with a differential selectivity of receptors for one peptide or the other [8,37], and the apparent tissue-specific expression of these receptors [8] suggest a further multifactorial control of biological responses elicited by these two peptide families.

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# 3.A.6 Acknowledgments

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# **CHAPTER 4**

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# REGIONAL LOCALIZATION OF NATRIURETIC PEPTIDE RECEPTOR SUBTYPES

Regional localization of ANF binding sites has already been studied in a number of mammalian tissues by autoradiography and other techniques. Expression of the natriuretic peptide receptor subtypes, NPR-A, B and C, has also been recently studied by Northern Blot analysis. This technique, however, despite providing an excellent and reliable information on the expression of natriuretic peptide receptor subtypes, gives insufficient anatomical resolution. Consequently, little is known about the regional and cellular distribution of natriuretic peptide receptor subtypes. Information on the localization of receptor subtypes is critical for understanding possible differential roles members of the natriuretic peptide family may have in regulation of physiological functions of each tissue.

Availability of iodinated putative physiological agonists of NPR-A (ANF) and NPR-B (CNP), as well as availability of selective ligands for NPR-C [C-ANF or ANF-(106-113)-NH<sub>2</sub>)] enabled studies on the regional distribution of these natriuretic peptide receptor subtypes in several tissues, mostly central nervous system, using in vitro autoradiographic approach. In vivo autoradiography, which involves circulation of the radioligand in blood, was not suitable for this kind of investigation, because of the presence of the bloodbrain barrier which is tight (with the exception of the circumventricular organs) for natriuretic peptides.

# 4.A. ATRIAL NATRIURETIC FACTOR RECEPTOR SUBTYPES IN THE RAT CENTRAL NERVOUS SYSTEM

This section provides information on the presence, anatomical location, and relative proportion of subtypes of natriuretic peptide binding sites, possessing pharmacological characteristics of NPR-A and NPR-C, in central nervous system structures. In vitro autoradiographic and affinity cross-linking techniques were used to study this subject. Although the NPR-B was already cloned and sequenced at the time of experimentation for this paper, it was not studied due to lack of a selective ligand for this receptor at that time.

### 4.A.1 Abstract

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In this study we investigated the presence and anatomical location of atrial natriuretic factor (ANF) receptor subtypes in the rat central nervous system using in vitro autoradiographic and cross-linking techniques. <sup>125</sup>I-ANF-(Ser99-Tyr126) served as a labeled ligand, whereas ANF-(Ser99-Tyr126) and two peptides endowed with selectivity for the natriuretic peptide receptor C - namely, C-ANF [des-(Gln116-Gly120) ANF-(Asp102-Cys121)-NH<sub>2</sub>] and (Phe106-Ile113)-NH<sub>2</sub> - were used as displacing agents. Distribution studies revealed the presence of specific ANF binding sites in a number of central nervous system areas examined. C-ANF (at 1  $\mu$ M) competed for <sup>125</sup>I-ANF binding to a much lower extent than ANF in many of those structures, whereas ANF-(106-113)-NH<sub>2</sub> (at 1  $\mu$ M) did not have a significant effect on the radioligand binding except in the choroid plexus, pia-arachnoid, and olfactory bulb. Analysis of the competition curves revealed that in the choroid plexus, pia-arachnoid, olfactory bulb, subfornical organ, area postrema, and habenular nucleus, ANF interacts with its binding sites with high affinity (IC<sub>50</sub>, 0.46-0.77 nM). In contrast, C-ANF and ANF-(106-113)-NH<sub>2</sub> competed for <sup>125</sup>I-ANF

binding with high potency (IC<sub>50</sub>, 2-16 nM) in the choroid plexus and piaarachnoid only, where they were able to displace 60-70 % of the radioligand binding. <sup>125</sup>I-ANF cross-linking to olfactory bulb membranes resolved a specific 120-kDa band corresponding to the high molecular weight receptor but did not disclose a specifically labeled band corresponding to the low molecular mass receptor. Therefore, these studies revealed the presence of ANF binding sites with the characteristics of the natriuretic peptide receptor C in the choroid plexus and pia-arachnoid but not in other central nervous system structures.

### 4.A.2 Introduction

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Atrial natriuretic factor (ANF) is a circulating hormone and neuropeptide involved, among other functions, in the regulation of blood pressure and fluid and electrolyte homeostasis [1,2]. The biological effects of this peptide are presumably initiated by ANF binding to its specific receptors. To date, three types of plasma membrane natriuretic peptide receptors have been defined by molecular cloning techniques: natriuretic peptide receptor A (NPR-A), B (NPR-B), and C (NPR-C) [3-5]. The NPR-A and NPR-B contain both an natriuretic peptide binding site and a region with guanylate cyclase activity within a single protein molecule [3,4] and exhibit an apparent molecular mass of 120-140 kDa [1,2]. These two receptor subtypes impose stringent constraints on ligand structure for binding and stimulation of cGMP [6]. The NPR-C is a non-guanylate cyclase-containing receptor [5], existing on the cell membrane predominantly as a homodimer of 60-70 kDa subunits [1,2,5,7]. This receptor subtype binds native ANF as well as a range of truncated, ring-deleted and linear ANF analogues with high affinity [6]. One of the first synthetic ligands showing selectivity for the NPR-C was C-ANF [des-(Gln116-Gly120)ANF-(Asp102-Cys121)-NH<sub>2</sub>] [3]. More recently, a number of linear peptides corresponding to various fragments of the ANF sequence have been shown to retain nanomolar affinities for NPR-C but not for guanylate cyclase-containing receptors, thus being able to discriminate between guanylate cyclase-containing and non-guanylate cyclase-containing natriuretic peptide receptor subtypes [6].

In fact, linear peptides as short as 7 to 10 amino acids in length, encompassing residues 109-113 of rat ANF, are endowed with selective NPR-C binding abilities [6].

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Linkage of the NPR-C to a second messenger system or systems is a matter of debate. Early studies did not demonstrate an association of this receptor with any second messenger system but showed its involvement in a sequestration and metabolic clearance of ANF [3]. Recently, however, the NPR-C was demonstrated to be coupled to the adenylate cyclase/cAMP system (through G<sub>i</sub> proteins) [9], and to phosphoinositide turnover (also through G regulatory proteins) [10]. In addition, other reports have suggested a possible indirect coupling of this receptor to the guanylate cyclase/cGMP system [11-13]. Whether these mechanisms of signal transduction are interrelated is as yet unknown.

Binding and affinity cross-linking studies have shown that the ratio of guanylate cyclase-containing to guanylate cyclase-free ANF receptors varies extensively from tissue to tissue [13,14]. In peripheral tissues, the NPR-C usually constitutes the majority of total ANF binding sites [6,15]. However, only fragmentary information is available on this receptor in the central nervous system (CNS) [9,16,17]. We therefore conducted *in vitro* autoradiographic and cross-linking studies to explore the presence and anatomical location of NPR-C in the brain and related structures, as well as the relative proportion of NPR-C to all ANF specific binding sites in a given tissue.

# 4.A.3 Materials and Methods

#### 4.A.3.1 Radioiodination of atrial natriuretic factor

Iodination of rat ANF-(Ser99-Tyr126) (Bio-Mega Inc., Laval, Canada) was performed by the lactoperoxidase method with <sup>125</sup>I-sodium [18]. Purification of the monoiodinated form of ANF was achieved by high-performance liquid chromatography. The specific activity of <sup>125</sup>I-ANF was ~2,400 dpm/fmol (1,100 Ci/mmol).

#### 4.A.3.2 In vitro autoradiography

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The method of *in vitro* autoradiography has been described in detail elsewhere [19,20]. Briefly, adult Sprague-Dawley rats were killed by decapitation, and their brains were removed rapidly and frozen and sectioned in a cryostat. The cryostat tissue sections then were preincubated at room temperature for 10 min in 50 mM Tris-HCl buffer (pH 7.5) containing 0.5% bovine serum albumin and were incubated for 90 min in the same buffer supplemented with 100 pM <sup>125</sup>I-ANF and the appropriate concentrations of unlabeled ligands (see below), as well as 1  $\mu$ M phosphoramidon, 1  $\mu$ M leupeptin, 1 µM pepstatin A, 100 µM EDTA, 1 µM phenylmethylsulfonyl fluoride, 200 kallikrein inhibiting units/ml aprotinin, 5 µM MnCl<sub>2</sub>, and 0.05% bacitracin. For analysis of the distribution of binding sites, the incubation buffer contained 1 µM of either unlabeled ANF-(Ser99-Tyr126), C-ANF, or ANF-(106-113)-NH<sub>2</sub> [ANF-(Phe106-Ile113)-NH<sub>2</sub>, also known as rANF-(8-15)-NH<sub>2</sub>] (all peptides from Bio-Mega Inc.). To test the specificity of <sup>125</sup>I-ANF binding, a set of sections was incubated in the presence of 1  $\mu$ M angiotensin II, glucagon, or substance P. For competition analysis, the incubation buffer contained either unlabeled ANF, C-ANF or ANF-(106-113)-NH<sub>2</sub> in concentrations ranging from 1 pM to 10  $\mu$ M. After incubation, the slides were washed (2 times, 10 min each, 4°C) in 50 mM Tris-HCl (pH 7.5) containing 0.5% bovine serum albumin, were fixed (15 min) in 2% glutaraldehyde (pH 7.5, 4°C), were washed (5 min) in 0.1 M phosphate buffer (pH 7.5, 4°C) and in distilled water, and were dehydrated in alcohol and dried overnight at 60°C. Subsequently, the sections along with <sup>125</sup>I-standards were apposed to X-ray films for 2-3 days at room temperature.

### 4.A.3.3 Microdensitometry and <sup>125</sup>I-standards

Autoradiograms were analyzed with a computerized microdensitometer [20]. Optical density values were converted into dpm/mg of polymer and fmol/mg of protein using two types of <sup>125</sup>I-standards: a commercially available <sup>125</sup>I-microscale (Amersham, Arlington Heights, Illinois, USA) and standards

prepared from brain homogenate as follows: Four Sprague-Dawley rat brains were homogenized with a polytron (speed 10, 2 min). Aliquots of the brain mash were placed in microfuge tubes (diameter 5 mm; Beckman Instruments Inc., Fullerton, California, USA) supplemented with 20  $\mu$ l of <sup>125</sup>I-ANF in increasing concentrations (ranging from  $0.78 \times 10^6$  to  $50 \times 10^6$  dpm), were mixed carefully by vortexing for 5 min, were frozen on dry ice, and were sectioned in a cryostat (20  $\mu$ m). The amount of radioactivity was measured in a gamma counter (Pharmacia LKB Biotechnology, Uppsala, Sweden), whereas the protein content per section was assessed by the method of Bradford [21]. To facilitate anatomical identification of brain structures on autoradiograms, the <sup>125</sup>I-ANF-labeled sections were stained with cresyl violet after autoradiography and subsequently were compared to their respective autoradiograms.

#### 4.A.3.4 Analysis of binding data

Results from the binding distribution studies are presented as means  $\pm$  SEM from 2 to 5 experiments (<sup>125</sup>I-ANF binding displaced, expressed in fmol/mg protein; Table 4.A.1) or as means  $\pm$  SE from 9 to 16 readings from one representative experiment (<sup>125</sup>I-ANF binding bound, given as dpm/mg polymer standard; Figure 4.A.1) (one animal per experiment). Statistical differences between means were analyzed by the Student's t test. Differences were considered significant at p<0.05. Competition binding curves (1 to 3 separate experiments, 1 to 3 animals per experiment) were analyzed with the ALLFIT computer program, based on a four-parameter logistic equation [22], to obtain estimates of the degree of displacement of bound <sup>125</sup>I-ANF by unlabeled ligands and to compute the unlabeled ligand concentration at which the maximum binding of labeled ligand is displaced by 50% (IC<sub>50</sub>).

#### 4.A.3.5 Preparation of membranes

The olfactory bulbs (OBs) of 10 Sprague-Dawley rats were dissected, cleaned from surrounding highly vascularized membranes, and homogenized with a hand homogenizer in a buffer containing 20 mM NaHCO<sub>3</sub>, 2 mM EDTA, 10  $\mu$ M phenylmethylsulfonyl fluoride, 1  $\mu$ M aprotinin, 1  $\mu$ M

leupeptin, 1  $\mu$ M phosphoramidon and 100 nM pepstatin A. The homogenate was centrifuged at 1,000 g for 10 min. The pellet was discarded, and the supernatant was centrifuged at 25,000g for 20 min. The resulting pellet was washed twice with the homogenization buffer and resuspended in 200  $\mu$ l of a buffer containing 50 mM Tris-HCl, 250 mM sucrose, 0.1 mM EDTA, and 1 mM MgCl<sub>2</sub> (pH 7.4). Aliquots were taken for protein determination [21] and for the binding assay. All membranes were kept frozen at -70°C until they were used.

#### 4.A.3.6 Affinity cross-linking and sodium dodecy' sulfatepolyacrylamide gel electrophoresis

Fifty-microgram protein aliquots of the OB membranes were incubated for 60 min at room temperature in a binding buffer containing 55 pM <sup>125</sup>I-ANF and increasing concentrations (0-1  $\mu$ M) of unlabeled ANF, C-ANF or ANF-(106-113)-NH<sub>2</sub>. Subsequently, the membranes were treated in 0.5 mM of disuccimidyl suberate dissolved in dimethylsulfoxide for 15 min at room temperature. The reaction was stopped by addition of ammonium acetate to a final concentration of 50 mM. Samples were then denatured with 2.5% sodium dodecyl sulfate containing 2% β-mercaptoethanol, boiled for 5 min, and resolved on 8% polyacrylamide gel [23] along with the molecular mass standards (Pharmacia LKB). The gel was stained in Coomassie R-250, dried and exposed for 5-14 days to Kodak X-Omat RP film (Eastman Kodak Co., San Diego, California, USA) at -70°C. The relative density of the bands on the resulting autoradiograms was assessed by densitometry [20].

### 4.A.4 Results

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#### **4.A.4.1** Distribution binding studies

As shown in Table 4.A.1 and Figure 4.A.1, specific ANF binding sites were widely, but distinctly, distributed in the rat CNS. The highest concentration of ANF binding sites occurred in the pia-arachnoid (PA), external plexiform layer



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**Figure 4.A.1** Autoradiograms of rat coronal brain sections at the level of the olfactory bulb (panels A-E), median eminence (panels F-J) and area postrema (panels K-O), labeled *in vitro* with 100 pM  $^{125}$ I-atrial natriuretic factor (ANF) in the absence or presence of 1 µM unlabeled ligands. epl, external plexiform layer of the olfactory bulb; chp, choroid plexus; pa, pia-arachnoid; ap, area postrema. Results are mean ± SEM (9 to 16 readings) of  $^{125}$ I-ANF bound, expressed in dpm/mg polymer standard. Panels A, F, and K ( $^{125}$ I-ANF): epl, 1847 ± 14; chp, 1704 ± 10; ap, 1913 ± 11; pa, 1670 ± 20 - 2217 ± 25 dpm/mg standard; panels B, G, and L ( $^{125}$ I-ANF + ANF): epl, 847 ± 5; chp, 870 ± 4; ap, 804 ± 6; pa, 789 ± 16 - 864 ± 18 dpm/mg standard; panels C, H, and M ( $^{125}$ I-ANF + C-ANF): epl, 1463 ± 9; chp, 1123 ± 6; ap, 1683 ± 7; pa, 1187 ± 21 - 1258 ± 45 dpm/mg standard; panels D, I, and N [ $^{125}$ I-ANF + ANF-(106-113)-NH<sub>2</sub>]: epl, 1723 ± 16; chp, 1240 ± 10; ap, 1704 ± 8; pa, 1166 ± 22 - 1201 ± 27 dpm/mg standard; panels E and O ( $^{125}$ I-ANF + angiotensin II): epl, 1906 ± 10; ap, 2030 ± 7; pa, 2212 ± 19 - 2256 ± 34 dpm/mg standard; panel J ( $^{125}$ I-ANF + glucagon): chp, 1644 ± 7; pa, 1623 ± 13 dpm/mg standard.

Table 4.A.1 Displacement of <sup>125</sup>I-ANF binding by ANF, C-ANF, and ANF-(106-113)-NH<sub>2</sub> in the rat central nervous system structures, determined by quantitative autoradiography in vitro, expressed in fmol/mg protein.

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| Tissue                                             | <sup>125</sup> I-ANF     | <sup>125</sup> I-ANF           | <sup>125</sup> I-ANF |
|----------------------------------------------------|--------------------------|--------------------------------|----------------------|
|                                                    | + ANF                    | + C-ANF                        | +ANF-(106-113)-NH    |
| RHINENCEPHALON AN                                  | ND TELENCEP              | HALON                          |                      |
| Olfactory bulb                                     |                          |                                | ۰.                   |
| (external plexiform laver)                         | $9.6 \pm 1.4$            | $3.6 \pm 0.7$                  | $1.1 \pm 0.2$        |
| Parietal cortex                                    | $1.8 \pm 0.6$            | $0.7 \pm 0.7$                  | NS                   |
| Globus pallidus                                    | $1.5 \pm 0.9$            | $1.3 \pm 0.2$                  | NS                   |
| Corpus callosum                                    | 5.2 ±1.8                 | $2.5\pm0.6$                    | NS                   |
|                                                    |                          |                                |                      |
| Anterior wall of the                               |                          |                                |                      |
| third ventricle                                    | 32+04                    | 21+06                          | NS                   |
| Median preoptic nucleus                            | $3.2 \pm 0.4$            | $0.5 \pm 0.5$                  | NS                   |
| Paraventricular nucleus                            | $0.0 \pm 0.4$            | $0.5 \pm 0.3$                  | NS                   |
| Supreoptic puclous                                 | $0.9 \pm 0.4$<br>27 + 03 | $0.5 \pm 0.5$                  | NS                   |
| Habopular nuclous                                  | $43 \pm 16$              | $1.5 \pm 0.0$<br>$1.6 \pm 1.3$ | NS                   |
|                                                    |                          | 1.0 ± 1.0                      |                      |
| MESENCEPHALON AN                                   | D RHOMBENG               | CEPHALON                       |                      |
| Interpeduncular nucleus<br>Nucleus of the solitary | $6.1 \pm 1.2$            | $1.4 \pm 0.1$                  | NS                   |
| tract (medial subnucleus)                          | $1.2 \pm 0.6$            | $0.3 \pm 0.3$                  | NS                   |
| CIRCUMVENTRICUI A                                  | R ORGANS AN              | VD OTHER STR                   | UCTURES              |
| Subfornical organ                                  | 5.9+1.1                  | 2.3+0.9                        | NS                   |
| Area postrema                                      | $11.5 \pm 1.4$           | $2.0 \pm 0.0$<br>$2.1 \pm 1.0$ | 1.0 + 1.0            |
| Choroid plexus                                     | $7.0 \pm 1.1$            | $5.2 \pm 1.0$                  | $3.7 \pm 0.3$        |
| Pia-arachnoid                                      | $10.2 \pm 2.8$           | $7.0 \pm 2.1$                  | $6.4 \pm 2.0$        |
| Destanion situations                               | 24491                    | $0.4 \pm 0.3$                  | NIS                  |

The extent of displacement of <sup>125</sup>I-ANF binding by an unlabeled ligand was calculated as a difference in <sup>125</sup>I-ANF binding to a given structure in the absence and presence of 1  $\mu$ M of unlabeled ligand. Values are presented as means ± SEM (fmol/mg protein) from 2 to 5 experiments. NS, nonsignificant displacement of <sup>125</sup>I-ANF binding (by the Student's t test, p<0.05).

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of the OB, area postrema (AP), choroid plexus (ChP), interpeduncular nucleus, habenular nucleus, and subfornical organ (SFO).

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C-ANF at a concentration of 1  $\mu$ M had a smaller but significant effect on <sup>125</sup>I-ANF binding in the CNS structures examined (Table 4.A.1, Figure 4.A.1, panels C, H, and M). A relatively high displacement occurred (approximately 70%) in two richly vascularized tissues: the ChP (Figure 4.A.1 H) and PA (Figure 4.A.1, panels C, H, and M). A considerable C-ANF-induced inhibition of <sup>125</sup>I-ANF binding was also detected in brain structures located beyond the blood-brain barrier, including the OB (Figure 4.A.1 C), globus pallidus, supraoptic and paraventricular hypothalamic nuclei. They were also seen in the anterior wall of the third ventricle, SFO, and AP.

In contrast, ANF-(106-113)-NH<sub>2</sub> (another ligand selective for the NPR-C) at a concentration of 1  $\mu$ M displaced <sup>125</sup>I-ANF binding extensively (approximately 60-70%) in the PA (Figure 4.A.1, panels D, I, and N) and ChP (Figure 4.A.1 I), minimally in the OB (Figure 4.A.1 D) and AP (Figure 4.A.1 N), but not elsewhere in the CNS structures examined (Table 4.A.1).

Unrelated peptides (at a concentration of 1  $\mu$ M) generally did not affect <sup>125</sup>I-ANF binding (Figure 4.A.1, panels E, J, and O); however, in a few experiments a nonspecific displacement occurred in some tissues (e.g., the median preoptic nucleus, SFO).

#### 4.A.4.2 Competition binding studies

To investigate the characteristics of ANF, C-ANF, and ANF-(106-113)-NH<sub>2</sub> interactions with <sup>125</sup>I-ANF binding and to estimate the ratio of NPR-C to all ANF binding sites in CNS structures, competition binding studies wi h ANF, C-ANF, and ANF-(106-113)-NH<sub>2</sub> were performed (Figure 4.A.2). In these experiments we investigated CNS structures endowed with relatively high numbers of ANF binding sites and large enough to provide a sufficient number of consecutive sections. Analysis of competition curves revealed that ANF displaced <sup>125</sup>I-ANF binding with an IC<sub>50</sub> in subnanomolar range in all structures examined: the ChP, 0.55 nM; PA, 0.77 nM; SFO, 0.46 nM; AP, 0.58 nM; external plexiform layer of the OB, 0.70 nM; and the habenular nucleus, 0.54 nM (Figure 4.A.2).



**Figure 4.A.2** Competition of atrial natriuretic factor (ANF), C-Al $(\hat{v}_{1})$  and ANF-(106-113)-NH<sub>2</sub> for the binding of <sup>125</sup>I-ANF (100 pM) to rat tissue cryostat sections.

In contrast, C-ANF competed with an IC<sub>50</sub> in nanomolar concentrations only in the ChP and PA (IC<sub>50</sub>, 2.4 and 16.1 nM, respectively), and was able to displace approximately 70% of <sup>125</sup>I-ANF binding to these structures (Figure 4.A.2, panels A and B). In other tissues examined, C-ANF displaced <sup>125</sup>I-ANF binding but only at very high concentrations (IC<sub>50</sub> >1  $\mu$ M) (Figure 4.A.2, panels C-F). This displacement did not have the characteristics of interaction with

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NPR-C and probably represented the competition for <sup>125</sup>I-ANF at the guanylate cyclase-containing natriuretic peptide receptor sites.

ANF-(106-113)-NH<sub>2</sub> competed for <sup>125</sup>I-ANF binding in the ChP and PA with high potency (IC<sub>50</sub>, 4.2 and 7.8 nM, respectively) (Figure 4.A.2, panels A and B), comparable to that of C-ANF, being able to displace in these structures approximately 60-70% of <sup>125</sup>I-ANF binding. In other tissues, in contrast to C-ANF, ANF-(106-113)-NH<sub>2</sub> did not displace or only minimally displaced the binding of <sup>125</sup>I-ANF, even at concentrations as high as 10  $\mu$ M (Figure 4.A.2, panels C-F). This suggests that ANF-(106-113)-NH<sub>2</sub> has a better selectivity factor in comparison to C-ANF, which renders the peptide more suitable for discriminating between the guanylate cyclase-containing and non-guanylate cyclase-containing receptor subtypes, as well as for assessing their relative proportions in target tissues.

#### 4.A.4.3 Affinity cross-linking studies

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To ascertain the molecular mass of the ANF receptor present in the brain and to identify the receptor from which C-ANF displaced <sup>125</sup>I-ANF binding in competitive binding studies, affinity cross-linking of <sup>125</sup>I-ANF to OB membranes was performed. OBs were chosen because of their relatively high density of ANF binding sites. As shown on Figure 4.A.3, <sup>125</sup>I-ANF was covalently incorporated into a band with an apparent molecular mass of approximately 120 kDa. The presence of increasing concentrations of unlabeled ANF-(Ser99-Tyr126) in the binding assay before cross-linking resulted in a progressive decrease in the intensity of that band (Figure 4.A.3, lanes 2-4), demonstrating the specificity of the interaction with <sup>125</sup>I-ANF. C-ANF did not inhibit binding of <sup>125</sup>I-ANF to that band over concentrations ranging from 10 pM to 100 nM (lanes 5-7) but did clearly decrease the intensity (approximately 20%) of that band at 1  $\mu$ M (lane 8). In contrasi, ANF-(106-113)-NH<sub>2</sub> had a negligible effect on <sup>125</sup>I-ANF binding at this site (lanes 9-11).



**Figure 4.A.3** Autoradiogram of sodium dodecyl sulfate-polyacrylamide gel electrophoresis of rat olfactory bulb membranes cross-linked under reducing conditions to <sup>125</sup>I-atrial natriuretic factor (ANF) in the absence (lane 1) or presence of either unlabeled ANF (10 pM, 1 nM, 100 nM; lanes 2-4), C-ANF (100 pM, 10 nM, 100 nM, 1  $\mu$ M; lanes 5-8), or ANF-(106-113)-NH<sub>2</sub> (10 nM, 100 nM, 1  $\mu$ M; lanes 9-11).

In addition, on the top of the gel, between 170 to 212 kDa molecular size markers, a very prominent band was observed whose intensity also was decreased on addition of increasing concentrations of unlabeled ANF. That band, however, seemed to represent merely an unresolved portion of the receptors, because it did not appear on autoradiograms (exposed to equivalent intensity of 120 kDa band) when a lesser quantity of the cross-linked membranes was applied to the gel (data not shown). Finally, a faint band migrating with an apparent molecular mass of 67 kDa also was identified but was considered to be nonspecific, because labeling of this site was not

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diminished by the addition of increasing concentrations of the unlabeled competitive peptides.

### 4.A.5 Discussion

It already has been demonstrated [24,25] (and reconfirmed in this study) that ANF binding sites are present in the brain. In the current examination, we investigated the presence and anatomical location of NPR-C in the rat CNS using in vitro autoradiographic and affinity cross-linking techniques. The fact that C-ANF, a selective ligand for the NPR-C [3], displaced (in our distribution experiments) <sup>125</sup>I-ANF binding from a number of brain structures at a concentration of 1  $\mu$ M led us to think that the NPR-C could be expressed in the brain. To further investigate this possibility, we performed competition binding experiments on a number of CNS structures that were large enough to provide a sufficient number of sections for full displacement analysis and that possessed a relatively high concentration of ANF receptors. In addition, we used another ligand selective for the NPR-C, namely, ANF-(106-113)-NH<sub>2</sub>, whose amino acid sequence encompasses the structural feature responsible for recognition of the NPR-C [6]. This peptide retains a high affinity (K<sub>i</sub>, 2-15 nM) for the NPR-C in cultured vascular smooth muscle cells and isolated perfused kidney [26], and inhibits adenylate cyclase activity and cAMP levels in vascular smooth muscle cells from rat aorta with a potency similar to that of C-ANF and ANF [9]. Analysis of the competition curves revealed, however, that C-ANF inhibition of <sup>125</sup>I-ANF binding in CNS structures, with the exception of the PA and ChP, had the characteristics of interaction with guanylate cyclasecontaining natriuretic peptide receptors rather than NPR-C (IC<sub>50</sub>>1  $\mu$ M). Affinity cross-linking studies with the OB membranes provided compelling evidence that the C-ANF-induced inhibition of <sup>125</sup>I-ANF binding in the OB, observed in the autoradiographic studies, was indeed due to its interaction (at high concentrations only) at the 120-kDa receptor sites and not due to its effect on the NPR-C. However, the linear ANF analogue ANF-(106-113)-NH<sub>2</sub> has been shown to possess a better selectivity factor than C-ANF, thus making this

ligand more suitable for discriminating between guanylate cyclase-containing and guanylate cyclase-free ANF receptor subtypes, as well as for assessing their ratio in target structures. The high selectivity for the NPR-C displayed by ANF-(106-113)-NH<sub>2</sub> analogue was particularly useful in those CNS structures in which neither cross-linking nor full competition studies could be performed. Therefore, considering the effect of ANF-(106-113)-NH<sub>2</sub> on <sup>i25</sup>I-ANF binding as a sensor of the NPR-C presence in CNS structures, this investigation demonstrated the existence of ANF binding displaying the characteristics of the NPR-C in the ChP and PA, but not in the other CNS structures examined. The slight but significant ANF-(106-113)-NH2-induced displacement of <sup>125</sup>I-ANF binding in the OB detected in the distribution studies may not represent the displacement from NPR-C, because the existence of a specific band that would correspond to the low molecular weight receptor has not been detected by our and other [27] cross-linking studies. Similarly, the effect of ANF-(106-113)-NH2 on <sup>125</sup>I-ANF binding in the AP has not been consistent in two experiments performed, and hence not much credibility can be given to this finding.

Our demonstration of the presence of NPR-C in the PA and ChP is in agreement with previous investigations [16,17,28]. The failure to detect receptors of this kind elsewhere in the brain also is consistent with earlier autoradiographic studies [16]. However, those investigations did not detect the NPR-C in any CNS structures examined with the exception of the PA. Interestingly, binding studies performed on membranes from cultured glial cell derived from the rat diencephalon [29] and neocortex [30] (note that glial cells constitute up to 50% of the total mass of the brain) showed a 95% displacement of <sup>125</sup>I-ANF binding by C-ANF with an IC<sub>50</sub> in subnanomolar concentrations [29,30]. Similarly, affinity cross-linking studies, carried out on membranes from rat cultured glial cell, demonstrated the presence of bands corresponding to the low (and high) molecular weight ANF receptors [29]. These findings, however, must be interpreted very cautiously, because it has been shown that expression of several receptors (including the ANF receptor

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[31]) can be dramatically influenced by cell culture conditions or by the early developmental stage of cultured cells. In this respect, autoradiographic studies performed on intact animal tissues are superior to those carried out on cultured cells, because they more closely resemble the *in vivo* conditions.

The abundance of NPR-C in highly vascularized tissues located outside the blood-brain barrier, such as the PA and ChP, is consistent with the proposed functional role of the NPR-C in clearance and metabolism of circulating ANF [3]. The absence of NPR-C in the brain beyond the blood-brain barrier would suggest that these receptors do not have a similar role in the inactivation of brain-released ANF. However, the failure to identify NPR-C by in vitro autoradiographic techniques in a majority of CNS structures does not necessarily indicate a lack of expression of this receptor in those tissues. This may be due to occupancy of NPR-C with endogenous ANF before labeling of the receptor with iodinated ligand, or to the sensitivity limit of the method. The first possibility, however plausible, is rather unlikely, because before exposure to radioligand the tissue sections were preincubated at room temperature in a peptidase inhibitor-free buffer, and during this step the endogenous peptides would be expected to dissociate from their receptors. With respect to the second possibility, although the *in vitro* autoradiographic technique provides several-fold greater sensitivity and better anatomical resolution than conventional membrane binding studies [32], receptors present at very low concentration may still remain undetectable. In fact, assuming that the NPR-C is coupled to adenylate cyclase through G<sub>i</sub> proteins [9], its presence has been shown in the rat striatum and anterior pituitary by the demonstration of inhibition of both the adenylate cyclase activity and cAMP levels produced by C-ANF, ANF-(106-113)-NH<sub>2</sub>, and other truncated and linear ANF analogues [9]. Whether the receptors reside in the vascular bed of the brain or beyond the blood-brain barrier was not shown by these studies. Other highly sensitive techniques that allow simultaneous morphological identification of ANF receptor-bearing cells are needed to address this question and to resolve present discrepancies.

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# 4.A.6 Acknowledgments

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# 4.B AUTORADIOGRAPHIC VISUALIZATION OF THE NATRIURETIC PEPTIDE RECEPTOR B IN RAT TISSUES

In this section, regional localization of natriuretic peptide binding sites with pharmacological characteristics of the NPR-B was investigated using in vitro autoradiographic techniques. The recent discovery of CNP as a selective putative physiological ligand for the NPR-B as well as availability of CNP in a iodinated form made this study possible.

### 4.B.1 Abstract

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> Natriuretic peptide receptor B (NPR-B) was visualized in rat tissues by in vitro autoradiography, using its putative physiological agonist C-type natriuretic peptide (CNP). In initial studies, we determined that atrial natriuretic factor (ANF) is not a suitable ligand for labeling the NPR-B: in tissues reported to contain NPR-B transcripts, CNP did not inhibit <sup>125</sup>I-ANF binding except to NPR-C sites. Therefore, to visualize the NPR-B we used <sup>125</sup>I[Tyr<sup>o</sup>]-CNP as a radioligand with an excess of NPR-C-blocking peptide: C-ANF. With this approach we detected the highest number of NPR-B-like sites in the pars intermedia of the pituitary gland. A large number of these sites were present in pituitary neural and anterior lobes, area postrema, adrenal medulla, and cortex. A moderate NPR-B population was observed in the subfornical organ, plexiform layer of the olfactory bulb, and kidney. Low concentrations of NPR-B were noted in the cerebellum and cerebrum but not in the choroid plexus and pia-arachnoid. Saturation experiments performed on cerebellum sections revealed a very low concentration ( $B_{max}$ , 4.8 fmol/mg protein) of high-affinity (K<sub>d</sub>, 1.2 nM) NPR-B-like sites. This study is the first

demonstration of <sup>125</sup>I[Tyr<sup>o</sup>]-CNP binding sites with characteristics of the NPR-B in intact tissues.

### **4.B.2** Introduction

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Several homologous polypeptides participating in salt and water homeostasis as well as blood pressure regulation constitute the family of natriuretic peptides [1,2]. Atrial natriuretic factor (ANF) was the first described peptide of this family. The natriuretic peptides can be further divided into 3 subfamilies, each derived from a distinct gene: type A, B and C [3]. ANF and brain natriuretic peptide (BNP) are the most intensely studied representatives of type A and B natriuretic peptides, respectively. Both are circulating hormones released primarily from cardiac atria [1] or ventricles [4], respectively. They are expressed also in several extracardiac tissues (including the brain) [2,5-7], where they probably function in a paracrine manner. C-type natriuretic peptide (CNP), the newest member of the natriuretic peptide family, represents the type C subfamily. Unlike ANF and BNP, it has been detected primarily in tissues derived from neurectoderm, such as the central nervous system [8] and adrenal chromaffin cells [9]. Small amounts of CNPlike immunoreactivity have been found in the kidney, colon, and ileum [8]. Members of the natriuretic peptide family share many common features. They all have a core structure consisting of a 17-amino acid loop formed by a disulfide linkage which is required for the elevation of cyclic GMP (cGMP) [3]. They all elicit natriuretic, diuretic, and vasorelaxant responses in chick and rat bioassay systems, although their relative potencies are different [3].

To date, at least 3 natriuretic peptide receptors, each derived from a distinct gene, have been identified: natriuretic peptide receptor A (NPR-A, B (NPR-B), and C (NPR-C). Both NPR-A and NPR-B contain, within a single protein molecule, a natriuretic peptide binding site and guanylate cyclase which directly generates the intracellular second messenger (cGMP) in response to extracellular hormone binding. The NPR-A responds preferentially to ANF and BNP stimulation ([10], Table 4.B.1). CNP has been

reported either not to stimulate cGMP levels at this receptor site [10] or to produce very weakly increases [11]. The NPR-B is activated fairly selectively by CNP ([10], Table 4.B.1) but also responds to stimulation by ANF and BNF [10,11]. The NPR-C, unlike NPR-A and NPR-B, does not contain guanylate cyclase activity and has been variously reported to function as a clearance receptor [12] or signal through G proteins, attenuating cyclic AMP levels [13,14] or stimulating the phosphoinositide pathway [15]. This receptor recognizes ANF, BNP and CNP [10,11] as well as ring-deleted ANF analogues, such as ANF-(106-113)-NH<sub>2</sub> [ANF-(Phe106-Ile113)-NH<sub>2</sub>, also known as rANF-(8-15)-NH<sub>2</sub>] [16] or C-ANF [des-(Gln116-Gly120) ANF-(Asp102-Cys121)-NH<sub>2</sub>] ([12], Table 4.B.1).

 Table 4.B.1
 Natriuretic peptide receptors and their primary ligands.

|                               | <b></b>                       |  |  |
|-------------------------------|-------------------------------|--|--|
| Natriuretic peptide receptors | Ligands                       |  |  |
| NPR-A                         | ANF, BNP                      |  |  |
| NPR-B                         | CNP                           |  |  |
| NPR-C                         | ANF, BNP, CNP, C-ANF,         |  |  |
|                               | ANF-(106-113)-NH <sub>2</sub> |  |  |

Unlike the NPR-B, ANF receptors (which presumably primarily include NPR-A and NPR-C [10]) have been fairly well mapped in the mammalian body by radioligand binding techniques [17,18]. Expression of the NPR-B has been studied to date by detection of NPR-B mRNA, using in situ hybridization

[19], Northern Blot [20], or polymerase chain reaction (PCR) [21] techniques. The recent discovery of an NPR-B-selective peptide, CNP [10], now allows mapping of the NPR-B by radioligand binding techniques. In the present study, we employed receptor autoradiography *in vitro* with <sup>125</sup>I[Tyr<sup>o</sup>]-CNP serving as a radioligand in the presence of an excess of C-ANF. With this approach, we have succeeded in visualizing NPR-B in locations reported to contain NPR-B transcripts by in situ hybridization [19]. This suggests that our technique can be at least as sensitive as the above mentioned method. Moreover, we have pharmacologically characterized <sup>125</sup>I[Tyr<sup>o</sup>]-CNP binding sites in the cerebellum and detected them in some other not yet screened structures.

# 4.B.3 Materials and Methods

#### 4.B.3.1 Ligand iodination

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Rat ANF-(Ser99-Tyr126) (Bio-Mega Inc., Laval, Quebec, Canada) was radioiodinated with <sup>125</sup>I-sodium by the lactoperoxidase method, as described previously [22]. The monoiodinated form of ANF was purified by high-performance liquid chromatography (HPLC). The specific activity of <sup>125</sup>I-ANF was ~2,400 dpm/fmol (1,100 Ci/mmol).

Rat <sup>125</sup>I[Tyr<sup>o</sup>]-CNP-(Gly82-Cys103) was purchased from Peninsula Laboratories Inc. (Belmont, California, USA). Its specific activity was ~2,000 dpm/fmol (900 Ci/mmol).

#### 4.B.3.2 Preparation of autoradiograms

Adult Sprague-Dawley rats (weighing ~250 g) were sacrificed by decapitation, and their organs removed rapidly, frozen and sectioned ( $20 \mu m$ ) in a cryostat. These consecutive tissue sections were mounted on 1% gelatin/0.05% chromium potassium sulfate-coated microscope slides, dried under vacuum over desiccant at 4°C for at least 2 h, and stored in sealed boxes with desiccant at -70°C until used. Prior to assay the slides were brought to room temperature. They were then preincubated at room temperature for 10

min in 50 mM Tris-HCl buffer (pH 7.5) containing 0.5% bovine serum albumin and subsequently incubated for 60 min in a fresh buffer supplemented with appropriate concentrations of <sup>125</sup>I-ANF or <sup>125</sup>I[Tyr<sup>o</sup>]-CNP and competing peptides (see below) as well as  $5 \mu$ M MnCl<sub>2</sub>, 100 mM NaCl,  $1 \mu$ M phosphoramidon,  $1 \mu$ M leupeptin,  $1 \mu$ M pepstatin A, 100  $\mu$ M?EDTA,  $1 \mu$ M phenylmethylsulfonyl fluoride, 200 kallikrein inhibiting units/ml aprotinin, and 0.05% bacitracin.

For <sup>125</sup>I-ANF competition analysis, the incubation buffer contained 200 pM of <sup>125</sup>I-ANF and concentrations ranging from 3.3 pM to 33  $\mu$ M of the unlabeled peptides: ANF-(Ser99-Tyr126), ANF-(Phe106-Ile113)-NH<sub>2</sub> (both from Bio-Mega Inc.), or CNP-(Gly82-Cys103) (from Peninsula Laboratories Inc.). Following incubation, the slides were washed (2 x 10 min, 4°C) in 50 mM Tris-HCl (pH 7.5) containing 0.5% bovine serum albumin, fixed (15 min) in 2% glutaraldehyde (pH 7.5, 4°C), washed (5 min) in 0.1 M phosphate buffer (pH 7.5, 4°C) and distilled water, dehydrated in alcohol, and dried overnight at 60°C. Subsequently, the sections along with <sup>125</sup>I-standards (see below) were apposed to X-ray films for 1-2 days at room temperature to produce autoradiograms.

For analysis of <sup>125</sup>I[Tyr<sup>o</sup>]-CNP binding to NPR-B, consecutive tissue sections were exposed in the incubation buffer to 170 pM (distribution experiments) or to a range from 64 to 2,058 pM (saturation experiments) of <sup>125</sup>I[Tyr<sup>o</sup>]-CNP in the presence of 1  $\mu$ M C-ANF. Nonspecific binding was estimated from parallel incubations with the addition of 1  $\mu$ M unlabeled CNP. Some sections were incubated with 1  $\mu$ M angiotensin II to test the specificity of <sup>125</sup>I[Tyr<sup>o</sup>]-CNP binding. Following incubation, the slides were wished (2 x 10 min, 4°C) in 50 mM Tris-HCl (pH 7.5) containing 0.5% bovine serum albumin, air-dried in a cold chamber (-5°C) to maximize the chance of obtaining the strongest signal, and apposed to X-ray film for 24 days at room temperature to produce autoradiograms.

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#### 4.B.3.3 Determination of radioligand stability

In each experiment, at least 3 samples of the incubation buffer were collected before and after exposure of the sections to assess the degree of a possible radioligand degradation during the incubation period. Degradation was determined by reverse-phase HPLC [22]. The intact radioligand contained in the buffer after incubation was expressed as a percentage of intact radioligand present in the buffer before exposure of the sections.

#### 4.B.3.4 Microdensitometry and <sup>125</sup>I-standards

Prior to microdensitometric analysis, the radioligand-labeled sections were stained with cresyl violet and coverslipped with Permount. They were subsequently compared to their respective autoradiograms to facilitate the anatomical identification of structures in autoradiographic images. These autoradiograms were then analyzed with a computerized microdensitometer. This devise consisted of a Sun 386i workstation, a video camera (Hitachi, KP-130U) equipped with a Nikon objective (micro-Nikkor 55 mm 1:2.8), a Matrox (MVP-AT) video processor, and an Electrohome video monitor (38-DO5IMA-YU). The microdensitometer electronically converted black and white autoradiographic images on Kodak film negatives to color-coded ones. Such enhanced images allow to perceive and measure subtle differences of optical density in originally monochrome negatives. Areas of interest were selected for analysis by positioning windows on autoradiographic images with a joystick coupled to the monitor. Readings of optical densities from the autoradiographic images of tissues were preceded by acquisition of optical densities of standards. The following two standards were used: commercially available <sup>125</sup>I-microscale (Amersham, Arlington Heights, Illinois, USA) and a standard prepared from brain homogenates containing known amount of <sup>125</sup>I and proteins [18]. The latter standard was used for all final calculations whenever binding site concentration was required. An equation describing the linear segment of the sigmoidal standard curve was employed to convert optical densities to disintegrations per minute (dpm) per mg of standard

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(commercial microscale) or per mg of protein (tissue homogenate standard). Given the specific activity of the radioligand, dpm was then transformed to femtomoles (fmol), generating fmol/mg of standard (commercial microscale standard) or fmol/mg of protein (tissue homogenate standard).

#### 4.B.3.5 Analysis of binding data

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Results from the competition binding study were analyzed with the ALLFIT computer program, based on a four-parameter logistic equation [23], to obtain estimates of the degree of displacement and to compute the  $IC_{50}$  of the displacing peptides. The  $IC_{50}$  was subsequently transformed to binding affinity constant of unlabeled ligand (K<sub>i</sub>) using the Cheng-Prusoff equation\* [24]. Competition binding curves were additionally analyzed by the LIGAND computer program [25] preceded by the EBDA program [26] in order to determine if they fit better to a single or two site model system.

Data from saturation studies were analyzed by computer assisted nonlinear regression analysis using the LIGAND computer program [25] preceded by the EBDA program [26] for determination of  $K_d$  (dissociation equilibrium constant) and  $B_{max}$  (maximum binding capacity).

Results from the binding distribution studies were presented as means  $\pm$  SE of 20 determinations i.e., 20 microdensitometric measurements derived from all available sections (2-12) of each area of interest, obtained from a single experimental animal. Statistical differences between total and nonspecific <sup>125</sup>I-[Tyr°]-CNP bindings were analyzed by the Student's t-test. P<0.05 was considered significant. In each analyzed tissue, specific binding was calculated and tabulated in Table 4.B.2 only if the difference between the total and the nonspecific binding was significant.

<sup>\*</sup>  $K_i = IC_{50}/(1 + [L]/K_d)$ , where  $K_i$  is the affinity constant of unlabeled ligand;  $IC_{50}$  is the concentration of the unlabeled ligand at which maximum binding of the labeled ligand is displaced by 50%; L is the concentration of the labeled ligand and  $K_d$  is the dissociation equilibrium constant.

### 4.B.4 Results

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In our initial studies we determined that <sup>125</sup>I-ANF was not a suitable ligand to label the NPR-B. This was based on the observation that CNP did not visibly compete for <sup>125</sup>I-ANF binding, even in tissues (such as the cerebellum and adrenal medulla) which were reported to contain NPR-B transcripts [19], unless at a very high concentration or unless they harboured NPR-C (Figure 4.B.1). Despite the fact that <sup>125</sup>I-ANF binds to NPR-A and NPR-C ([10], Table 4.B.1) its apparent affinity for these two classes of receptors is virtually the same [27,28] and therefore the two existing receptors are unrecognizable on the basis of affinity. In our studies there was no indication of peptides binding to two classes of sites: Hill coefficient was close to 1, the LIGAND program either did not resolve more than one class of binding sites or the curves fitted statistically better to a single-site model system. NPR-C were, therefore, identified in these structures by inhibition of <sup>125</sup>I-ANF binding by ANF-(106-113)-NH<sub>2</sub>, a linear NPR-C-selective ANF analogue which has been shown to discriminate well between the guanylate cyclase-containing and guanylate cyclase-free receptors [16,18,29]. CNP and ANF-(106-113)-NH<sub>2</sub> competed for <sup>125</sup>I-ANF to a similar extent, suggesting that CNP recognizes the NPR-C. Therefore, in the following experiments, to visualize only the NPR-B we used <sup>125</sup>I[Tyr<sup>o</sup>]-CNP as a radioligand under conditions where the NPR-C was blocked. This was achieved by adding an excess of C-ANF, another NPR-Cselective peptide [12], to the incubation medium. The addition of angiotensin II to the incubation buffer did not affect <sup>125</sup>I[Tyr<sup>o</sup>]-CNP binding. Likewise, we determined once more that the inhibitor cocktail present in the incubation buffer significantly prevented degradation of the ligands during incubation. As in other studies performed under these incubation conditions [30], degradation of the radioligands during incubation was negligible since after incubation around 98% of <sup>125</sup>I-ANF and 97% of <sup>125</sup>I[Tyr°]-CNP were eluted from the reverse-phase HPLC column at elution times corresponding to the intact peptides.
**CHAPTER 4** 



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**Figure 4.B.1** Competition of ANF, CNP and ANF-(106-113)-NH<sub>2</sub> for <sup>125</sup>I-ANF (200 pM) binding to rat tissue cryostat sections. A: Plexiform layer of the olfactory bulb; K<sub>i</sub>, ANF 0.2 nM; CNP and ANF-(106-113)-NH<sub>2</sub> not determined. B: Choroid plexus from the third ventricle; K<sub>i</sub>, ANF 0.7 nM; CNP 3.8 nM; ANF-(106-113)-NH<sub>2</sub> 1.5 nM. C: Area postrema; K<sub>i</sub>, ANF 0.4 nM, CNP and ANF-(106-113)-NH<sub>2</sub> not determined. D: Cerebellum; K<sub>i</sub>, ANF 2.0 nM; CNP 6.4 nM; ANF-(106-113)-NH<sub>2</sub> 0.5 nM. E: Adrenal medulla; K<sub>i</sub>, ANF 0.9 nM; CNP and ANF-(106-113)-NH<sub>2</sub> not determined.

Saturation experiments performed on cerebellum sections demonstrated that  $^{125}I[Tyr^o]$ -CNP in the presence of an excess of C-ANF binds to one class of receptors (Hill coefficient, 0.97; linear Scatchard plot) (Figure 4.B.2). These binding sites possessed a high affinity (K<sub>d</sub>, 1.2 nM) and very low binding capacity (B<sub>max</sub>, 4.8 fmol/mg protein). The large amount of nonspecific binding (true nonspecific and C-ANF-displaceable) and the very small proportion of specific binding to NPR-B may raise doubts as to whether indeed there is any

specific binding. However, since the computerized treatment of the data (with LIGAND) is very rigorous and incorporates statistical testing, one may confidently state that although the number of specific binding sites (corresponding to NPR-B) is very low, it is likely that specific binding sites are indeed present.

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Figure 4.B.2 Total, nonspecific and specific binding of  $^{125}I[Tyr^{0}]$ -CNP to rat cerebellum sections in the presence of an excess of C-ANF as a function of radioligand concentration. Each point represents a mean ± SE of 10 measurements. K<sub>d</sub>, 1.2 nM; B<sub>max</sub>, 4.8 fmol/mg protein. Inset: Scatchard plot.

Subsequently, utilizing a similar approach, we visualized NPR-B-like binding sites in several selected tissue sections (Table 4.B.2). As shown in Table 4.B.2 and Figure 4.B.3 A, we detected the highest number of these sites in the pars intermedia of the pituitary gland. High levels of these sites were present in structures derived from the neurectoderm, such as the adrenal medulla (Figure 4.B.3 C), area postrema (Figure 4.B.3 B), and neural lobe of the pituitary (Figure 4.B.3 A). The anterior lobe of the pituitary and the entire adrenal cortex(Figure 4.B.3 C) harboured large numbers of these binding sites as well. A moderate population of NPR-B-like binding sites was observed in the subfornical organ and plexiform layer of the olfactory bulb as well as in the kidney cortex and medulla (Figure 4.B.3 C). None of renal regions was particularly enriched with NPR-B-like sites. Low concentrations of NPR-B were noted in the cerebellum (Figure 4.B.3 B) and cerebral cortex. They were not detected in the choroid plexus and pia-arachnoid.

#### 4.B.5 Discussion

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. شە This communication documents the first demonstration of NPR-B in intact tissues by a ligand binding technique. Although no attempt has been made to provide a detailed map of these receptors in the mammalian body, the data presented in this paper support and further extend the in situ hybridization study recently reported by Wilcox et al. designed to detect mRNA for the NPR-B [19]. Both the studies of Wilcox and our own have detected NPR-B transcripts and NPR-B-like binding sites, respectively, in the pituitary gland, adrenal medulla, and cerebellum. In the pituitary gland, we found the highest concentration of NPR-B-like binding sites in the pars intermedia, whereas lower and comparable concentrations of these sites were present in the anterior and neural lobes. The same pattern was faithfully reflected in the in situ hybridization study where mRNA for the NPR-B was evaluated. The above comparison lends further support to the notion that in the present investigation we have indeer<sup>a</sup> measured <sup>125</sup>I[Tyr<sup>o</sup>]-CNP binding to NPR-B.

## Table 4.B.2Autoradiographic distribution of NPR-B in selectedtissues.

| Structure                        | Specific binding concentration fmol/mg protein |
|----------------------------------|------------------------------------------------|
|                                  | ·                                              |
| Olfactory bulb (plexiform layer) | $1.3 \pm 0.2$                                  |
| Cerebral cortex                  | $0.3 \pm 0.1$                                  |
| Cerebellum                       | $0.7 \pm 0.2$                                  |
| Subfornical organ                | $1.7 \pm 0.1$                                  |
| Area postrema                    | $3.4 \pm 0.2$                                  |
| Choroid plexus                   | ND                                             |
| Pia-arachnoid                    | ND                                             |
| Pituitary gland                  |                                                |
| Neural lobe                      | $3.2 \pm 0.1$                                  |
| Pars intermedia                  | $4.8 \pm 0.5$                                  |
| Anterior lobe                    | $3.0 \pm 0.1$                                  |
| Adrenal medulla                  | $3.4 \pm 0.3$                                  |
| Adrenal cortex                   | $2.9 \pm 0.4$                                  |
| Kidney cortex                    | $1.7 \pm 0.2$                                  |
| Kidney medulla                   | $1.8 \pm 0.1$                                  |

Quantification was performed on autoradiograms of tissue sections incubated with 170 pM  $^{125}I[Tyr^{o}]$ -CNP in the presence of 1  $\mu$ M C-ANF with or without 1  $\mu$ M unlabeled CNP as described in Materials and Methods. Data are expressed as the means  $\pm$  SE of 20 determinations. ND, not detected.

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**Figure 4.B.3** Autoradiograms of adjacent (or next adjacent) rat tissue cryostat sections labeled *in vitro* with <sup>125</sup>I[Tyr<sup>0</sup>]-CNP in the presence of 1  $\mu$ M C-ANF without (column 1, total binding) or with (column 2, nonspecific binding) of 1  $\mu$ M unlabeled CNP. The illustrated autoradiograms are enlargements of the Kodak film negatives. The numbers in parentheses below denote optical densities (mean ± SE of 10 determinations) of the pointed structures on autoradiograms from total and nonspecific binding preparations, respectively. A: Pituitary gland incubated in 170 pM of the radioligand; nl, neural lobe (33.3 ± 0.2, 21.5 ± 0.3); al, anterior lobe (34.3 ± 0.4, 23.0 ± 0.1); pi, pars intermedia (41.9 ± 1.2, 24.1 ± 1.2). B: Brain at the level of area postrema incubated in 130 pM of the radioligand; ap, area postrema (28.7 ± 0.3, 19.2 ± 0.2); cer, cerebellum (23.6 ± 0.1, 21.5 ± 0.01). C: Adrenal gland and kidney incubated in 170 pM of the radioligand; am, adrenal medulla (40.1 ± 1.0, 27.5 ± 0.8); ac, adrenal cortex (48.3 ± 1.0, 37.5 ± 0.1); kc, kidney cortex (26.0 ± 0.5, 19.9 ± 0.3); km, kidney medulla (26.9 ± 0.5, 20.4 ± 0.1).

Comparison of NPR-B distribution with localization of ANF receptors [17,18,31] discloses interesting differences. For example, ANF was found to bind specifically to both anterior and neural lobes of the pituitary gland but, unlike CNP, not to the pars intermedia [32]. Furthermore, a large population of ANF binding sites (majority of them displaying characteristics of the NPR-C) were detected in the choroid plexus and pia-arachnoid [18]. In contrast, <sup>125</sup>I[Tyr<sup>o</sup>]-CNP specific binding to the NPR-B in these tissues was below the detection limit of our method. A largely dissimilar distribution of ANF receptors and NPR-B appears to exist in the rat adrenal gland. In the adrenal cortex, a very high density of ANF binding sites (mostly with the characteristics of NPR-A [30]) was found overlaying the zona glomerulosa whereas a moderate density occurred in the zona fasciculata [31]. This localization is clearly different from the distribution of NPR-B-like binding sites in the adrenal cortex, as detected in our studies. In the adrenal medulla, ANF binding sites were not detected by *in vitro* autoradiographic technique [31], which clearly contrasts with the high number of the NPR-B noted in the current study. Similarly, renal receptors for ANF are distributed in the cortical and medullar tissues, but they are notably concentrated within the glomeruli [17,31], where they produce on autoradiograms a characteristic highly punctate radioligand binding. In contrast, none of the renal structures was particularly enriched with NPR-B-like binding. The distinct localization of NPR-B, which does not parallel the distribution of ANF receptor sites, provides additional evidence supporting our view that in the present study the NPR-B and not NPR-A or NPR-C was studied.

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In the current studies, we detected NPR-B in several central nervous system (CNS) structures, notably in the area postrema, subfornical organ, plexiform layer of the olfactory bulb, cerebellum, and pituitary gland. Unfortunately, the distribution of CNP in discrete nuclei and subregions of the CNS has not been studied in detail to date. This makes it impossible to compare the distribution of the NPR-B and its putative physiological agonist CNP. Nevertheless, radioimmunoassay studies have detected significant

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amounts of immunoreactive CNP in larger CNS areas, including the olfactory bulb, hypothalamus, medulla oblongata, cerebellum, cerebral cortex, and anterior and neurointermediate lobes of the pituitary gland [8]. The predominant location of CNP in structures derived from the neurectoderm as well as the failure to find this peptide in significant amounts in the heart and plasma [8] has led some investigators to think that CNP acts primarily as a neurotransmitter or neuromodulator rather than as a cardiac hormone [8,10].

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Although the present data do not contradict the above mentioned view, the detection of NPR-B-like binding sites in the kidney and adrenal cortex, as reported in the current study, suggests that CNP may also play a role outside the nervous system. In fact, several lines of evidence seem to agree with a diffuse localization of the CNP/NPR-B system in the mammalian body. In the rat, for example, Northern Blot analysis has revealed mRNA for NPR-B in the lung, kidney, and liver [20]. In the human, evidence has been obtained using PCR technique that mRNA for the NPR-B is produced in the kidney [21]. Similarly, cDNA encoding the NPR-B has been detected in human placental and porcine atrium cDNA libraries [33]. Furthermore, CNP stimulates cGMP production more efficiently than ANF in rat vascular smooth muscle cells in culture [34], suggesting the presence of the NPR-B in these cells. Finally, small amounts of CNP-like immunoreactivity have been reported in the kidney, ileum, and colon [8]. All these findings tend to support a role of the CNP/NPR-B system in the regulation of functions of a wide variety of tissues. Whether this system functions only in parallel to other natriuretic peptides [3] or whether it fulfills as yet unknown roles remains to be determined. A recent communication reporting that CNP elicits antimitogenic and antiproliferative actions to a greater extent than ANF suggests that the CNP/NPR-B system may function in growth regulation [35]. This opens a new avenue in natriuretic peptide research. Thus, as shown by our findings and these other recent studies demonstrating varied effects of CNP and wider NPR-B distribution than initially envisaged, the precise functional role of the CNP/NPR-B system remains to be elucidated.

#### 4.B.6 Acknowledgments

We thank Dr. R. Garcia for kindly providing the <sup>125</sup>I-[Tyr<sup>o</sup>]-CNP used in this work, which was supported by a Group Grant from the Medical Research Council of Canada to the Multidisciplinary Hypertension Group. E. M. Konrad is a recipient of a fellowship from the Medical Research Council of Canada.

#### 4.B.7 References

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## **CHAPTER 5**

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### **GENERAL DISCUSSION**

This chapter contains a brief discussion of the thesis, summarizing some aspects of conclusions reached in the presented papers. It includes prospects for future research on natriuretic peptide receptors. The section "Claims to originality" is placed at the end of this chapter.

### 5.A DISCUSSION AND PROSPECTS ON FUTURE RESEARCH ON NATRIURETIC PEPTIDE RECEPTORS

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This work describes the cellular localization of ANF binding sites in selected areas of the CNS. It deals with selectivity of natriuretic peptide receptors. It also provides regional localization of three subtypes of natriuretic peptide receptors. The results obtained in these studies will contribute to a better understanding of the mechanism of natriuretic peptide action in the CNS and will provide directions for further research on natriuretic peptide receptor subtypes.

# 5.A.1 Cellular localization of ANF binding sites in selected structures of rat CNS

The ChP and AP belong to the unique group of brain organs which lack the blood-brain barrier and are in a position to monitor changes in levels of circulating hormones. The ChP, apart from its well known role in water and ion homeostasis by regulation of CSF production, can also participate indirectly in CV regulation [5]. The AP, in addition to its well documented CV effects, participates in water and sodium balance by regulating drinking behavior and natriuresis, and also probably by influencing the production of CSF [43]. In the studies presented in Chapter 2, an attempt was made to localize ANF binding sites in individual cells of ChP and AP, because this was expected to provide some clues as to possible central effects of circulating ANF.

As shown in Chapter 2, ANF binding sites were found to be present on basolateral (blood-facing) plasmalemma of the CSF-producing epithelial cells of the ChP and on plasmalemma of dendrites, axons and astrocytes in the AP. Such a cellular localization is consistent with a possible involvement of ANF binding sites in the regulation of CV and body fluid regulating functions of the ChP and AP. In other words, it is suggested that a part of the effects of circulating ANF may be exerted via CNS loci, such as the AP or ChP. This statement is strengthened by the fact that part (ChP), if not all (AP) of ANF binding sites exhibited pharmacological characteristics of NPR-A which is believed to mediate biological actions of ANF through changes in cGMP levels. In the case of ChP, the presence of guanylate cyclase-containing natriuretic peptide receptors was confirmed by demonstration that ANF activates guanylate cyclase [36] and stimulates the production of cyclic GMP [13] in the rat ChP. In the case of the AP, such a confirmation still awaits further investigations. For now it is only known that brain slices which include the AP respond to ANF stimulation with an increase in cGMP concentration [40], implying the presence of guanylate cyclase-containing receptors (presumably NPR-A) in these brain preparations.

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When localization of ANF binding sites in the ChP and AP was examined at the electron-microscopic level, only distribution of silver grains from the total binding preparation was evaluated. Nonspecific grains were not examined due to their very low number on available set of autoradiograms. This approach, however, reduced in a sense the anatomical power of the technique and precluded identification of possible low numbers of specific ANF binding sites on cells in which a small number of silver grains was noted such as endothelial cells, pial cells, fibroblasts and macrophages. Collecting a larger number of grains from a larger set of autoradiograms and including distribution of nonspecific grains (as is routinely done in studies performed by the group of Beaudet [15,11]) could perhaps have given more conclusive information regarding the presence of specific ANF binding sites on the above cells.

In future research it would be interesting to determine the precise cellular localization of natriuretic peptide receptor subtypes in other CVOs involved in CV and body fluid regulation, such as the subfornical organ or organum vasculosum of the lamina terminalis, to broaden the evidence that circulating natriuretic peptides may act through a CNS locus to exert their functional role.

Recording of changes in CV homeostasis or body fluid balance after injection of natriuretic peptides to the CVOs would serve to confirm this hypothesis.

# 5.A.2 Specificity of natriuretic peptide receptor subtypes

Natriuretic peptide research is a fast moving field. Since the time studies on this thesis were initiated, two more families of natriuretic peptides (in addition to ANF and type A family) have been discovered: type B and type C natriuretic peptide families. The number of subtypes of natriuretic peptide receptors described has also grown, and up to now three have been cloned and sequenced. These new developments have necessitated the determination of the relationship between ANF binding sites and the new natriuretic peptide families which demonstrate a similar spectrum of biological effects [37,38].

It was shown in Chapter 3 that ANF and BNP bind to the same binding sites, suggesting that these sites are not only specific for the ANF family but also for the BNP family. These studies were confirmed repeatedly by other laboratories [2,9,10,23]. Similarly, in many cases a function and/or response of second messengers has been ascribed to binding sites localized in these studies [1,8,13,14,26,32,35,40,45] which demonstrate that these binding sites actually may represent functional receptors. However, physiological significance of natriuretic peptide receptors is in many instances still unclear. For example, the liver and the lung contain large amounts of natriuretic peptide binding sites (Table 3.A.1) and the lung was the organ from which NPR-C was initially isolated, purified and precisely characterized [41]. Nevertheless, the function of these receptors remains obscure. These binding sites may in part correspond to NPR-C present on vascular beds and be important for the removal of natriuretic peptides from circulation [12,21], influencing thereby the endogenous plasma concentration of natriuretic peptides.

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As shown in Sections 2.B and 4.B, CNP displays a distinct selectivity for natriuretic peptide receptors. As ANF and BNP it binds to the NPR-C, but in contrast to these peptides, it does not appreciably interact with binding sites displaying pharmacological characteristics of the NPR-A. Indeed, as demonstrated by Koller et al. [19] and Suga et al. [39], CNP preferentially stimulates NPR-B and not NPR-A, and it is believed to serve as a physiological ligand for NPR-B.

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The identification of at least three natriuretic peptide receptor subtypes and three natriuretic peptide families, each binding to a certain set of natriuretic peptide receptor subtypes, suggests a probable complex regulation of homeostatic balance of body fluids and blood pressure by members of this peptide/receptor system. These multiple receptors and peptides may be used to generate a greater diversity of responses than could be achieved with a single peptide or receptor subtype. This multimember system may contribute to the fine tuning of biological functions regulated by these peptides, because the expression of ANF, BNP, and CNP may each be subject to differential forms of control [17,18], resulting in differential tissue distribution [20,30,42]. Additionally, as demonstrated in this work, each of the natriuretic peptide receptor subtypes also has a distinct topographical distribution. This combined with the differential activation of receptors by one peptide or another, and the possibility that each of these receptors may have additionally different signal transduction pathways, reveals endless possibilities of multifactorial control of biological functions these peptides are responsible for. However, the exact functional role and importance of each peptide (or perhaps also other as yet undiscovered new members of the natriuretic peptide family) in the regulation of biological functions deserve and require further extensive study. These topics will continue to be an important subject for future research.

# 5.A.3 Regional localization of natriuretic peptide receptor subtypes

Information on topographical localization of natriuretic peptide receptor subtypes is critical for our understanding of a possible differential role the members of the natriuretic peptide family may have in the regulation of physiological functions of each tissue. In the studies presented, a mapping of a

regional localization of NPR-A, NPR-B, and NPR-C in several, mostly CNS, tissues was provided. Each of the natriuretic peptide receptor subtypes studied had a distinct distribution. Interestingly, in contrast to binding sites with pharmacological characteristics of NPR-A and NPR-B which were detected in a number of areas, the NPR-C was detected only in a few CNS structures. The natriuretic peptide binding sites localized (at least those with characteristics of NPR-A and NPR-C) most probably represent functional receptors since ANF, on one hand, and ligands selective for NPR-C, on the other, produce changes in the generation of cGMP and cAMP, respectively, in a number of CNS areas [1,13,40]. The fact that natriuretic peptides are produced in the CNS [34], released from neurons [40], that they interact with natriuretic peptide receptor subtypes distinctly distributed in the CNS, that they produce changes in neuronal excitability [4,24,33,44] and generate biological responses [3,4,7,6,16,22,25,27,28,29,31] suggest that natriuretic peptides act in the nervous system as neurotransmitters or neuromodulators. Thus, natriuretic peptides may change the activity of the CNS by operating as neurotransmitters or neuromodulators, and also as circulating hormones by acting at the level of the circumventricular organs. This dual mode of action is not unique for natriuretic peptides and is observed in the case of other peptides, e.g., ANG II, vasopressin, and corticotropin-releasing factor. It may be a mechanism for fine tuning of physiological processes and in particular may function to help maintaining adequate cardiovascular homeostasis as well as water and electrolyte balance. However, the wide distribution of natriuretic peptides and of their receptors beyond areas with putative involvement in CV and body fluid regulation suggest that this system may act to control a variety of other biological functions. Localization of natriuretic peptide receptor subtypes, as presented in this study, should give directions for further research on detailed characterization of such functions.

However, investigations on natriuretic peptide receptors are far from complete. Future research may profitably be directed towards development of antagonists for each natriuretic peptide receptor subtype in order to delineate

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the physiological function of each of these receptors and to determine their importance in the regulation of biological actions. Furthermore, to date controversy still exists as to whether clearance of natriuretic peptides is the sole function of NPR-C or whether this receptor is also actively involved in signal transduction. Further *in vitro* experiments with NPR-C-specific ligands will be necessary to fully elucidate the physiological role of this receptor. The question of whether NPR-C occupancy directly evokes intracellular responses can be addressed by transgenic expression of NPR-C in cultured mammalian cells which do not normally express this receptor. Similarly, studies on possible subtypes of NPR-C and establishing a molecular basis for the various functions ascribed to NPR-C will remain an important subject for continuing research. Finally, it is possible that each of the known receptor subtypes may have additional different signal transduction pathways which are biologically distinct from the natriuretic peptide second messengers presently known. Future research should explore such possibility.

In conclusion, the present work on the precise cellular localization, specificity and subtypes of natriuretic peptide binding sites in the rat CNS provides a deeper insight into the multifactorial mode of action of natriuretic peptides and makes a substantial contribution to the understanding of the mechanism of action of these peptides on the CNS.

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### **5.B CLAIMS TO ORIGINALITY**

The present work describes for the first time:

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- 1. The ultrastructural localization of ANF binding sites (with pharmacological characteristics resembling those of NPR-A and NPR-C) in the intact ChP.
- 2. The ultrastructural localization of ANF binding sites (with pharmacological characteristics resembling those of the NPR-A) on the plasmalemma of astrocytes, dendrites and axons in the AP.
- 3. The presence and regional localization of ANF binding sites (with pharmacological characteristics of NPR-A and NPR-C) in the rat CNS.
- 4. The proportion of guanylate cyclase-containing to guanylate cyclasefree natriuretic peptide receptors (NPR-A to NPR-C) in the ChP, AP, plexiform layer of the olfactory bulb, habenular nucleus, adrenal zona glomerulosa.
- 5. The presence and regional localization of <sup>125</sup>I-CNP binding sites with pharmacological characteristics of NPR-B in intact rat tissues.
- 6. The distribution of BNP binding sites in the rat CNS.
- 7. The characterization of BNP binding sites in the ChP, plexiform layer of the olfactory bulb, and adrenal zona glomerulosa.
- 8. The characterization of CNP binding sites with characteristics of NPR-B in the cerebellum.
- 9. The demonstration that porcine BNP, rat iso-ANF, and rat ANF bind to the same binding sites in the rat.
- 10. The demonstration that  $ANF-(106-113)-NH_2$  is a more suitable peptide for discrimination between NPR-A and NPR-C.

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