JOURNAL OF EXPERIMENTAL ZOOLOGY 284:407–413 (1999)

Natriuretic Peptide Binding Sites in the Brain of the Atlantic Hagfish, *Myxine glutinosa*

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ABSTRACT We have previously used immunohistochemistry to show that the brain of the hagfish, *Myxine glutinosa,* contains a rich distribution of natriuretic peptide-immunoreactive elements with the densest distribution occurring in the telencephalon and the diencephalon. In this study, the distribution of ^{125}I-rat ANP and ^{125}I-porcine CNP binding sites was determined in the brain of *M. glutinosa.* The binding pattern of ^{125}I-rat ANP and ^{125}I-porcine CNP showed similarities; however, some differences were observed in the olfactory bulb and the caudal brain regions. Specific ^{125}I-rat ANP and ^{125}I-porcine CNP binding was observed in the olfactory bulb, outer layers of the pallium, and in regions of the diencephalon. Very little specific binding was observed in the habenula and the primordium hippocampi. In the diencephalon, a distinct zone of specific ^{125}I-rANP binding separated a region of moderate binding in the lateral regions of the diencephalon from the thalamic and hypothalamic nuclei. Moderate levels of specific ^{125}I-rANP binding were observed in the mesencephalon and medulla oblongata; little or no ^{125}I-porcine CNP binding was observed in these regions. The data, in combination with previous immunohistochemical studies, show that the natriuretic peptide system of the hagfish brain is well-developed and suggest that natriuretic peptides have a long evolutionary history as neurotransmitters and/or neuromodulators in the vertebrate brain. *J. Exp. Zool. 284:407–413, 1999.*

In mammals, natriuretic peptides (NPs) are a family of peptide hormones which occur in the brain and periphery and are strongly implicated in the reduction of hypervolemia. To date, four types of mammalian NP have been isolated: atrial NP (ANP), brain natriuretic peptide (BNP), C-type natriuretic peptide (CNP), and urodilatin (Yandle, '94). The effects of NPs are mediated by two genetically distinct types of natriuretic peptide receptors (NPR-A and NPR-B) which possess an intracellular guanylate cyclase (GC) domain; binding of NPs to the extracellular domain stimulates the conversion of guanosine triphosphate to cGMP (Yandle, '94). NPR-A is primarily activated by atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP), whereas NPR-B is primarily activated by C-type natriuretic peptide (CNP). A third NPR, called NPR-C, is a unique receptor that binds all NPs with high affinity but does not have an intracellular GC domain. It is generally considered that the NPR-C function as a mechanism for clearing NPs from the circulation and tissues by internalization, but it may also function through a non-cGMP signal transduction pathway (Anand-Srivastava and Trachte, '93; Yandle, '94).

The distribution of NPs in the central nervous system of mammals has been determined with immunohistochemistry (IHC) and mRNA expression (e.g., see Langub et al., '95). The presence of NPs in the diencephalon strongly supports a role for NPs in neuroendocrine regulation of osmotic and cardiovascular homeostasis (Gutkowska et al., '97). However, NPs are extensively expressed in other areas of the brain, which indicates a role for NPs in a range of brain functions (Langub et al., '95). The distribution of NPs and the NPR in the mammalian brain overlap to some extent (ANP, Quirion et al., '84; BNP, Konrad et al., '90; CNP, Brown and Zuo, '93).

In spite of various NPs having been isolated from either the heart or brain of non-mammalian vertebrates, the function of NPs in lower vertebrates has yet to be unequivocally determined. However, linkage to the control of ion and fluid homeostasis is emerging (Evans and Takei, '92;

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Received 17 June 1998; Accepted 6 January 1999.

Takei and Balment, '93; Hagiwara et al., '95). Most studies in lower vertebrates have focussed on the role of cardiac NPs, but, as in mammals, NPs are widely distributed in the brain of representative species of cyclostome (Donald et al., '92), elasmobranch (Donald et al., '92), and teleost fish (Donald and Evans, '92) suggesting that the peptides function as neuromodulators and/or neurotransmitters, in addition to their effects in the periphery (Evans and Takei, '92). Few studies have examined the distribution of NPR in the brains of lower vertebrates, and to date, the distribution of NPR in the brain has only been examined in two amphibians (*Xenopus laevis,* Kloas and Hanke, '92; *Rana ridibunda*, Tong et al., '89) and the lungfish, *Protopterus annectens* (Vallarino et al., '96).

Previously, we have used IHC to show an extensive distribution of NP-immunoreactive (IR) structures in the brain of the hagfish, *Myxine glutinosa* (Donald et al., '92). These data demonstrated that the presence of NPs in the brain is a very primitive vertebrate characteristic, since it is hypothesised that hagfish branched from the vertebrate line over 500 million years ago (Forey and Janvier, '93). In the present study, the distribution of NP binding sites in the brain of *M. glutinosa* has been determined using autoradiography to correlate the distribution of immunoreactive NP with putative sites of action. Both ^{125}I-rat ANP and ^{125}I-porcine CNP were used as ligands since homologous *M. glutinosa* peptides have not been isolated and sequenced.

MATERIALS AND METHODS

Animals

Atlantic hagfish *Myxine glutinosa* (25–50 g) of either sex were trapped in the Gulf of Maine by collectors at the Huntsman Marine Laboratory (St. Johns, NB, Canada) and maintained in a recirculating sea water (12–15°C) aquarium. Four specimens were used for the study.

Autoradiography

For autoradiography, brains were freeze-mounted in Tissue Tek (Miles Inc., Elkhart, IN) in a microtome cryostat (Minotome, IEC, Needham Heights, MA), and 20 μm frontal sections were cut and mounted on gelatin-chromium aluminium coated slides and dried overnight under vacuum at 4°C. The sections were used immediately, or were stored in sealed boxes at –20°C until used.

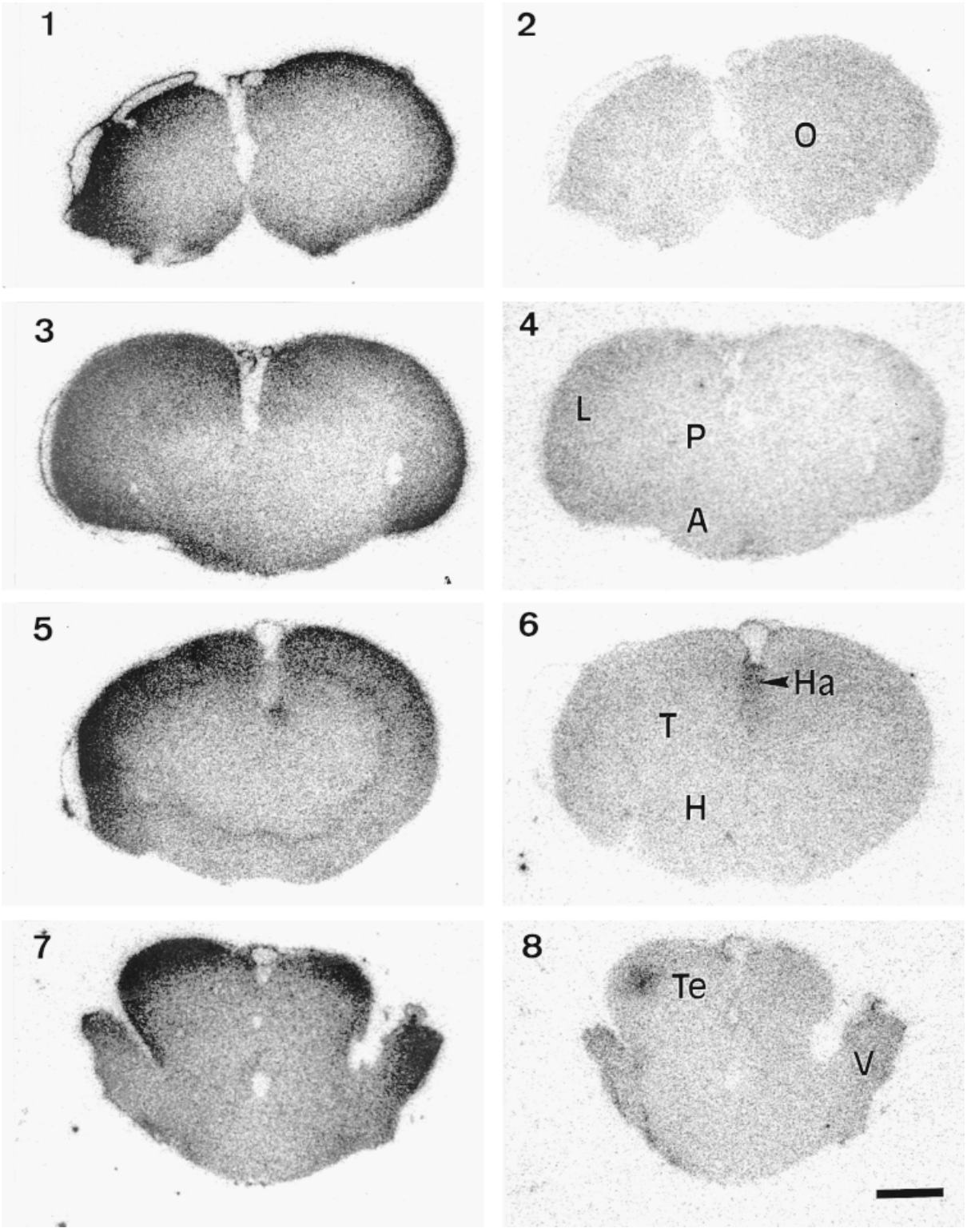
Preincubation of brain sections was performed at room temperature (22–24°C) for 15 min in 50 mM tris-(hydroxymethyl)aminomethane (Tris) HCl buffer (pH 7.4), 50 mM NaCl, 5 mM $MgCl_2$, 0.1% bovine serum albumin (BSA), and 0.05% bacitracin. This was followed by incubation for 60 min in the same buffer supplemented with 4 μg/ml of leupeptin, 2 μg/ml chymostatin, 2 μg/ml pepstatin, 10^{-6} M phenylmethylsulfonylfluoride (PMFS), and either rat ^{125}I-ANP (termed ^{125}I-rANP here) or porcine ^{125}I-Tyr^0-CNP (termed ^{125}I-pCNP here). Displacement of specific binding for ^{125}I-rANP was examined in adjacent sections in the presence of 1 μM rat 3-28 ANP (rANP). Displacement of specific binding for ^{125}I-pCNP was examined in consecutive sections in the presence of 1 μM porcine CNP. After incubation the slides were washed (2 × 10 min at 4°C) in preincubation buffer, fixed for 20 min in 4% formaldehyde in 0.1 M phosphate buffer (pH 7.4, 4°C), washed in 0.1 M phosphate buffer (pH 7.4, 4°C), and then in distilled water (1 min) and dehydrated in alcohol and dried overnight at 60°C. The sections were then apposed to Hyperfilm-βmax for 5–21 days (depending on specific activity) at room temperature. The film was developed in Kodak GBX developer for 4 min, washed in water for 2 min, and fixed in Kodak GBX fixer for 5 min. X-ray films were photographed on a white light-box using a Wild dissecting microscope and Wild Leitz MPS 46 Photoautomat camera. Brain anatomy was determined according to the descriptions of Jensen ('30).

Materials

Rat (3-[^{125}I]*iodotyrosol*^{28}) atrial natriuretic peptide (1800–2000 Ci/mmol) and Hyperfilm-βmax were purchased from Amersham (Arlington Heights, IL). Porcine ^{125}I-Tyr^0-CNP (1400–1600 Ci/mmol) was obtained from Peninsula (Belmont, CA). Rat ANP (3-28), porcine CNP, and C-ANF (rat des[Gln^{18}, Ser^{19}, Gly^{20}, Leu^{21}, Gly^{22}]ANP-(4-23)-NH_2) were obtained from Bachem (Torrance, CA). All other chemicals were reagent grade and were purchased from Sigma (St. Louis, MO).

Figs. 1–8. Photomicrographs of *M. glutinosa* brain sections exposed to X-ray film showing the distribution of ^{125}I-rANP binding sites. Specific binding can be determined by comparison of total ^{125}I-rANP binding (Figs. 1, 3, 5, 7) and non-specific binding (Figs. 2, 4, 6, 8); non-specific binding is determined by incubation of sections with ^{125}I-rANP plus 1 μM unlabelled rANP. All figures are frontal sections and only Figs. 2, 4, 6, and 8 (non-specific binding) are labelled. Scale bar on Fig. 8 is 500 μm and is the same for each figure.

Figs. 1 and 2. Section through the olfactory bulb. Specific ^{125}I-rANP is observed in the outer regions of the olfactory bulb (O).



Figs. 3 and 4. Section through the hemisphere. Specific ^{125}I-rANP binding is observed in the pallial layers (L) and ventral region of the hemisphere (area basilis telencephali, A), but little specific ^{125}I-rANP is present in the primordium hippocampi (P).

Figs. 5 and 6. Section through the diencephalon. Specific ^{125}I-rANP was observed in the lateral regions of the diencephalon, but little specific binding was present in the region of the thalamic (T) and hypothalamic (H) nuclei. Little specific binding was observed in the habenula (Ha).

Figs. 7 and 8. Section through the mesencephalon. Specific ^{125}I-rANP binding was observed in the dorsal region of the tectum (Te) and laterally in the hindbrain in the region of the trigeminal nuclei (V).

RESULTS

Specific binding was determined as the difference in binding between sections incubated with either 200 pM ^{125}I-rANP or ^{125}I-pCNP alone and adjacent sections incubated with radiolabelled ligand and 1 μM of unlabelled ligand.

A dense distribution of ^{125}I-rANP binding sites was present in the olfactory bulb and pallial region of the telencephalon, particularly in the outer layers (Figs. 1–4). Further caudal, dense binding was again observed in the lateral pallial layers and moderate levels of binding were observed in the basal region of the telencephalon; very little specific binding was observed in the habenula and the primordium hippocampi (Figs. 3, 4). In the diencephalon, a distinct zone of specific ^{125}I-rANP binding separated a region of moderate binding in the lateral regions of the diencephalon from the thalamic and hypothalamic nuclei; low levels of specific ^{125}I-rANP binding were observed in the thalamic and hypothalamic regions of the diencephalon (Figs. 5, 6). Little specific binding was observed in the habenula (Figs. 5, 6). In the mesencephalon and medulla oblongata, moderate to dense levels of ^{125}I-rANP binding were observed in tectum and the region of the trigeminal nuclei respectively (Figs. 7, 8).

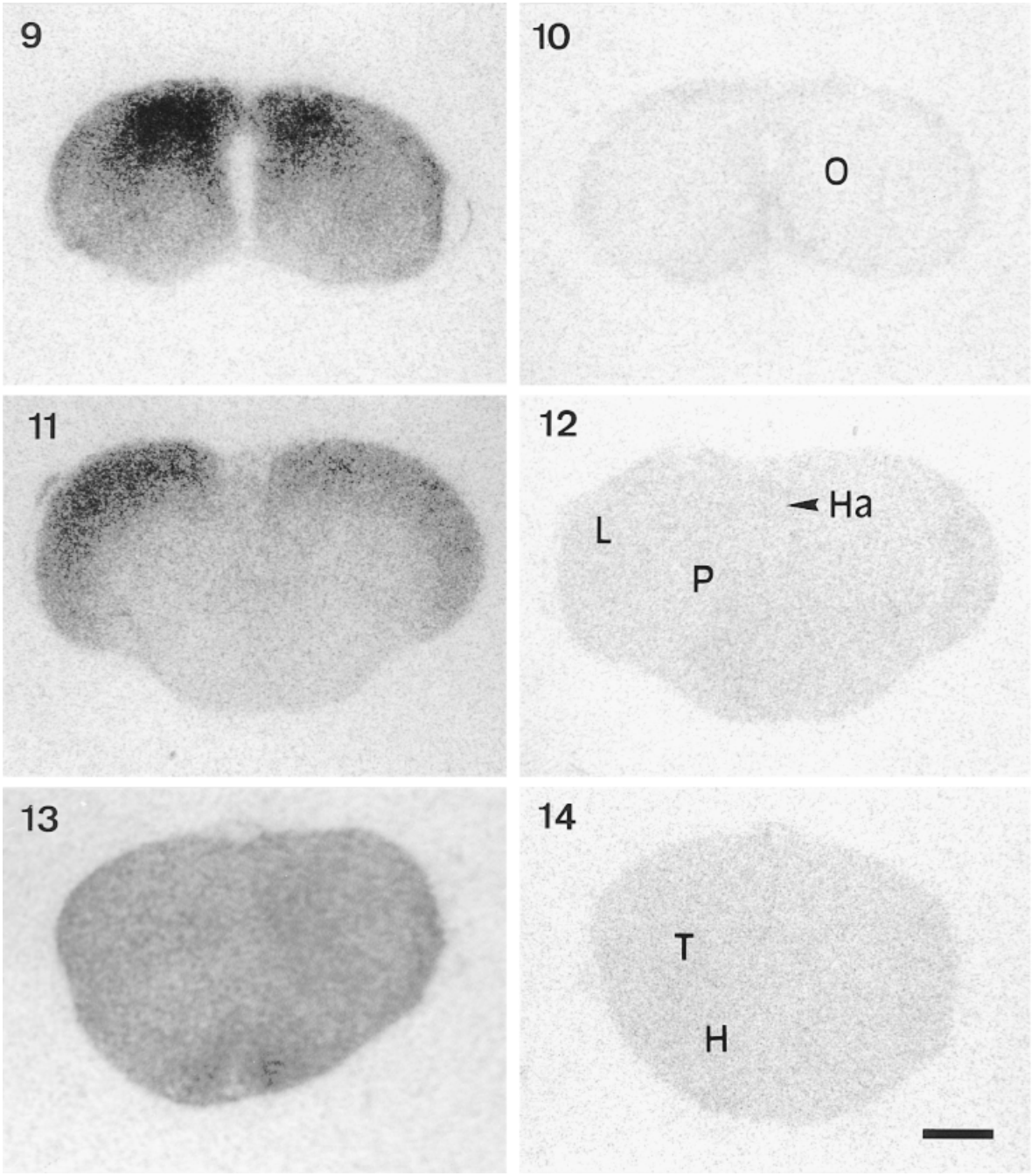
In contrast to ^{125}I-rANP binding, ^{125}I-pCNP binding occurred predominantly in the olfactory bulb, telencephalon, and diencephalon. Specific ^{125}I-pCNP binding was present in the olfactory bulb (Figs. 9, 10), and ^{125}I-pCNP binding in central region of the bulb was denser than ^{125}I-rANP (c.f. Figs. 1 and 9). Specific ^{125}I-pCNP binding was present in the outer pallial layers of the telencephalon, but little if any specific binding was present in the primordium hippocampus and the ventral regions of the telencephalon (Figs. 11, 12). More caudally, diffuse specific binding was present in the thalamic and hypothalamic regions of the diencephalon, with a zone of denser binding being present in the area of the hypothalamus (Figs. 13, 14). Little if any specific ^{125}I-pCNP binding was observed in the mesencephalon and medulla oblongata.

A comparison of the distribution of ^{125}I-rANP and ^{125}I-pCNP binding sites with the location of natriuretic peptide-immunoreactive (NP-IR) structures (Donald et al., '92) in the brain of the hagfish, *Myxine glutinosa*, is shown in Table 1.

DISCUSSION

The vertebrate NP system is a complex peptidergic system comprised of peripheral and central peptides and receptors which are implicated in the coordinated control of salt and water balance (Takei and Balment, '93; Gutkowska et al., '97). A number of studies have described a broad distribution of NP and NPR in the mammalian brain, including the key osmoregulatory centres, and there is emerging evidence of their involvement in the control of salt and fluid balance (Gutkowska et al., '97). In contrast, the role of NPs in the brain of non-mammalian vertebrates is unknown. Anatomical studies in lower vertebrates have shown that NPs have a broad distribution in the brain of cyclostomes [Rienecke, '87; Donald et al., '92), elasmobranchs (Vallarino et al., '90; Donald et al., '92), teleosts (Donald and Evans, '92), dipnoans (Vallarino et al., '96), and amphibians (Feuilloley, '93; Netchitailo, '87). However, NP binding sites have only been demonstrated in dipnoans (Vallarino et al., '90) and amphibians (Tong et al., '89; Kloas and Hanke, '92). The general conclusion from these studies is that NPs and NP-binding sites are located in brain regions such as the diencephalon and the hypophysis which, by homology with the mammalian brain, are important in the central control of osmoregulation. It is, therefore, interesting to examine the brain NP system of hagfishes, since their requirement for osmoregulation is reduced because they are isoosmotic with seawater (Evans, '93).

Previous IHC studies have shown an extensive distribution of NP-IR in the brain of *M. glutinosa,* using an antibody that cross-reacts with ANP and CNP (Donald et al., '92). Immunoreactive-perikarya and fibers were observed in the pallium, the primordium hippocampi, the pars ventralis thalami, pars dorsalis thalami, the nucleus diffusus hypothalami, the nucleus profundus, the nucleus tuberculi posteriosis, and the nucleus ventralis tegmenti. The current study has demonstrated for the first time the presence of ANP- and CNP-binding sites in the brain of *M. glutinosa* and provides further evidence that the NP system of the agnathan brain is well developed. Thus, the evolution of brain NPs and their receptors occurred early in the vertebrate radiation and probably before the migration of early vertebrates into fresh water. The distribution of ^{125}I-rANP and ^{125}I-pCNP binding sites in the brain of *M. glutinosa* was similar, with the most abundant binding occurring in the telencephalon and diencephalon. The major difference occurred in the caudal regions of the brain where little ^{125}I-pCNP binding was observed in contrast to a moderate distribu-



Figs. 9–14. Photomicrographs of *M. glutinosa* brain sections exposed to X-ray film showing the distribution of ^{125}I-pCNP binding sites. Specific binding can be determined by comparison of total ^{125}I-pCNP binding (Figs. 9, 11, 13) and non-specific binding (Figs. 10, 12, 14); non-specific binding is determined by incubation of sections with ^{125}I-pCNP plus 1 μM unlabelled pCNP. All figures are frontal sections and only Figs. 10, 12, and 14 (non-specific binding) are labelled. Scale bar on Fig. 14 is 500 μm and is the same for each figure.

Figs. 9 and 10. Section through the olfactory bulb (O). Specific ^{125}I-pCNP is observed in the olfactory bulb (O) with the densest binding in dorsal regions.

Figs. 11 and 12. Section through the hemisphere. Specific ^{125}I-pCNP binding is observed in the pallial layers (L), but little specific ^{125}I-pCNP is present in the primordium hippocampi (P) and the habenula (Ha).

Figs. 13 and 14. Section through the caudal diencephalon. Moderate levels of specific ^{125}I-pCNP were present in the thalamic (T) and hypothalamic zones (H) of the diencephalon, with a zone of denser binding being present in the area of the hypothalamus (H).

TABLE 1. A comparison of the distribution of ^{125}I-rANP and ^{125}I-pCNP binding sites with the location of natriuretic peptide-immunoreactive (NP-IR) structures (Donald et al., '92) in the brain of the hagfish, Myxine glutinosa[1]

Brain region	^{125}I-rANP Binding	^{125}I-pCNP Binding	NP-IR
Olfactory bulbs	++ lateral	++ lateral and central	+, Δ
Telencephalon: pallium	++ outer layers	++ outer layers	+, Δ
Primordium hippocampi	–	–	++, Δ
Habenula	–	–	+, Δ
Thalamus	+	+	++, Δ
Hypothalamus	+	++	++, Δ
Mesencephalon: tectum	++	–	+
Mesencephalon: ventral	+	–	++, Δ
Medullary horns	++	–	+
Medulla	+	–	+

[1]The distribution of binding sites and NP-IR structures has only been shown for broad areas of the brain. The density of ligand binding and of NP-IR structures has been indicated qualitatively as either (+) or (++), in which (+) represents low to medium density and (++) represents medium to high density; the Δ symbol represents the presence of NP-IR cell bodies in the brain.

tion of ^{125}I-rANP binding. The distribution of NP-IR and NP-binding sites showed concurrence in some areas, but in other areas there was "mismatching." A good correlation was observed in the pallial layers of the telencephalon and the rostral diencephalon, but few binding sites were present in the primordium hippocampi and the caudal regions of the diencephalon and mesencephalon where moderate NP-IR was observed (Donald et al., '92). The "mismatch" between the location of receptors and the corresponding ligand has been reported for other neuropeptide systems in the central nervous system (Kuhar et al., '86).

In earlier studies, we have demonstrated the presence of multiple types of NPR in the peripheral tissues of *M. glutinosa* (Toop et al., '95a,b). The gills, kidney, and aortae bind ^{125}I-rANP, but only the gills showed ^{125}I-pCNP binding. The ^{125}I-rANP and ^{125}I-pCNP binding in the gills was displaced by unlabelled ANP, CNP, and the NPR-C specific ligand C-ANF (Maack, '92). However, in the kidney and aortae, the ^{125}I-rANP binding was only displaced by ANP which suggests that the NPR in these tissues are NPR-A like. Interestingly, both ANP and CNP stimulated guanylate cyclase activity in the kidneys and the gills, with ANP being more potent than CNP. Similarly, in the ventral aortae, rat ANP is more potent than porcine CNP in mediating relaxation of vascular smooth muscle (Evans, '91; Evans et al., '93). Therefore, in the peripheral tissues that have been examined, only the gills appear to possess two types of binding site, a putative NPR-A-like binding site and a binding site similar to NPR-C; the kidneys and aortae appear to contain only the NPR-A-like binding site. In the present study, the observation that ^{125}I-rANP and ^{125}I-pCNP show differences in binding suggest that more than one NPR is present in the brain. If the predominant receptor in the brain was NPR-C-like, both ligands would be expected to bind to this receptor with similar affinity (Maack, '92). A full understanding of the hagfish NP system will only be achieved by molecular characterization of the NPs and receptors.

The presence of a well-developed NP system in the brain and the periphery of *M. glutinosa* is fascinating when considered in the functional context of NPs as fluid volume regulators in higher vertebrates. The fact that hagfishes are osmoconformers and are almost iso-osmotic with the surrounding sea water and presumably do not encounter major ionic and volume perturbations (Evans, '93) raises the question: what is the biological role of NPs as hormones and neuropeptides in hagfishes? Clearly, analysis of the evolutionary history of the biology of NPs in agnathans (and protochordates) is critical in understanding the function of NPs in vertebrates in general. Northcutt ('96) has argued that the brain of hagfishes is highly derived from that of ancestral craniates due to their invasion of the benthic environment in which chemosensory and tactile stimuli are critical for feeding and reproduction. Thus, the hemispheres have become enlarged in comparison to the lampreys (Northcutt, '96). This region of the brain is involved in olfaction and is an important sensory modality in hagfish; the pallial layers of the hagfish brain are believed to receive projections from the olfactory bulbs. The telencephalon of hagfish is heavily invested with NP-IR neurons and NP binding

sites. In addition, previous studies have shown a substantial distribution of NPs and NPR in the telencephalon of lungfish (Vallarino et al., '96), amphibians (Tong et al., '89; Kloas and Hanke, '92), birds (Tavolaro et al., '93), and mammals (see Gutkowska et al., '97). Thus, a role for NPs in olfaction may occur throughout the vertebrates. Interestingly, cGMP, the second messenger in NP signalling, is an important cyclic nucleotide in olfactory signal transduction and is found in high levels in the rat olfactory bulb (Hopkins et al., '96).

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