Interactions of Angiotensin II and Atrial Natriuretic Peptide in the Brain: Fish to Rodent (44045)

SARA M. GALLI AND M. IAN PHILLIPS[1]
Department of Physiology, College of Medicine, University of Florida, Gainesville, Florida 32610-0274

Abstract. **The brain peptides atrial natriuretic peptide (ANP) and angiotensin II (AngII) have antagonistic actions centrally that have evolved to play an important role in maintaining the homeostasis of fluid volume and electrolytes. This paper discusses the possible evolution of these functions as viewed through studies on fish and rats. In the euryhaline teleost fish, the major form of ANP is CNP. CNP is important for the adaptation of fish in sea water to water with lower salinity (50% SW). The concentration of CNP in the hypothalamus (HTS-CNP) of toadfish is significantly increased when the fish moves from SW to 50% SW. Interestingly, the plasma CNP concentrations of these fish go in the opposite direction to the brain CNP. Plasma CNP is reduced 24 hrs and 10 days after the fish has been in 50% SW. We postulate that the increased hypothalamic CNP is correlated with an increase in hypothalamic dopamine turnover. Dopamine is the main inhibitory factor for the release of prolactin. In seawater-adapted fish, prolactin plasma levels are low. In 50% SW, the levels are increased. Extracts of fish-brain CNP caused an increase in urinary sodium and volume, indicating the natriuretic action of CNP. In contrast to CNP, hypothalamic AngII was decreased during adaptation to 50% SW. Thus, CNP and AngII in the brain have opposite actions in fish which aid in their survival in adapting to different salinities. This implies that the hormones evolved as sodium/osmoregulatory peptides, not volume-regulatory peptides. In moving from an aquatic to a terrestrial environment, ANP further evolved in mammals as a volume-regulating hormone while retaining its sodium-regulating properties. In the brain the antagonism between the peptides is apparent in many actions, but when volume is changed both peptides are increased. This is in contrast to plasma ANP and plasma AngII, which have opposite actions during hemorrhage. Plasma AngII is increased and plasma AnP is decreased after blood volume loss. Brain ANP counteracts the thirst-inducing and volume-restoring roles of brain AngII in mammals. By these actions, the brain peptides play significant roles in maintaining volume and electrolyte homeostasis.** [P.S.E.B.M. 1996, Vol 213]

Two peptides, atrial natriuretic peptide (ANP) and angiotensin II (Ang II), play vital roles in maintaining fluid balance, which is essential to life. Deviation from homeostatic balance induces the release of ANP or Ang II in opposite directions. ANP is the principal hormone released in response to volume expansion of blood, hypervolemia, by stretching of the atria, but also by stimulation of α-adrenergic receptors and acetylcholine receptors and changes in vagal tone (1–3). Ang II is the principal hormone of hypovolemia, responding whenever blood volume contracts (4). ANP opposes volume expansion by increasing diuresis and natriuresis, and lowering blood pressure through vasodilation of blood vessels in mammals (1, 5) and in fish (6–9). ANP receptors are present in the vasculature and in the renal tubules of human, rat, and fish kidney (10–14). Ang II, on the other hand, stimulates AT_1 angiotensin receptors in the smooth muscle of blood vessels, which causes va-

[1] To whom requests for reprints should be addressed at Department of Physiology, College of Medicine, University of Florida, Gainesville, FL 32610-0274.
This manuscript was supported by a National Institutes of Health MERIT Award (HL27334).

0037-9727/96/2132-0128$10.50/0

Table I. Antagonistic Effects of ANP and Ang II in the Brain

ANP	Ang II
↓ MAP, ↓ HR	↑ MAP, ↑ HR
↓ Renal SNA	↑ Renal SNA
(−) Ang II–induced ↑ BP	↓ Brain ANP
(−) Ang II effects on drinking	↑ Drinking
↓ Brain DA turnover	↑ Brain DA turnover
↑ PRL secretion	↓ PRL secretion
↓ GH secretion	
↓ ACTH secretion	↑ ACTH secretion
↓ LH secretion	↑ LH secretion
↓ ADH secretion	↑ ADH secretion

soconstriction and eventually hypertension (15). In mammals, Ang II in blood can cause anti-natriuresis by directly stimulating proximal tubular reabsorption of sodium (16) and indirectly by decreasing glomerular filtration rate (17). In fish, angiotensins are present in plasma, brain, kidney, and rectal gland (18, 19). Although the presence of the renin-angiotensin system in teleost from marine and fresh water environments and its influence in maintaining blood pressure and blood volume has been shown (20), there is no clear evidence that plasma angiotensin helps to retain sodium in fish in hypo-osmotic media (21, 22). The involvement of angiotensin in compensatory drinking in teleost fish has been well supported (23, 24). Angiotensin promotes drinking in several species of euryhaline teleost including killifish (*Fundulus heteroclitus*) (25), eel (*Anguilla Japonica*) (26), and flounder (24). Interestingly, angiotensin was more potent to induce drinking in sea water–adapted fish than in fresh water–adapted fish (23, 24).

ANP and Ang II, while initially considered to be only blood-borne hormones, have both been demonstrated to be synthesized independently in the brain (27–29). The effects of ANP and Ang II in the brain are also opposite in their effects (Table I). To understand more fully these opposed physiological actions of brain ANP and angiotensin, we need to consider the question: How did the function of these peptides evolve?

The comparative approach offers some insight into the evolution of physiology, and we will briefly review some experiments on ANP and angiotensin in the brain and periphery of fish as they move from sea water to fresh water, and vice versa, and how the roles of ANP and angiotensin in fluid balance may have evolved from marine life to terrestrial life. This paper is limited to experiments in teleost fish and rodents.

ANP in Fish

In sea water, teleost fish must drink or die of dehydration. Because of the high sodium content in sea water, they are compelled to lose sodium at a rate that prevents osmotic extraction of water from cells. To do this, fish in sea water must drink sea water constantly and excrete high amounts of sodium while retaining as much water as possible. Much of the salt that is present is absorbed across the gut wall, and water follows this solute by osmosis. The excess of salt in teleost fish is excreted by specialized cells called "chloride cells" present in the gills. In fresh water, fish are faced with the opposite problem. They must stop drinking or drown. They must conserve as much sodium as possible and get rid of water. The osmotic imbalance between extracellular fluid and intracellular fluid in fresh water fish has to be guarded against too much water entering the cells. Generally in sea water teleosts, sodium levels in the plasma are high (150–180 mEq/l), whereas in fresh-water teleosts the sodium levels in plasma are 110–130 mEq/l. Many species of fish can tolerate a change from sea water to a lower salinity and maintain plasma volume and electrolytes. Some fish, the euryhaline fish, regularly move between sea water and fresh water. These species have selective osmoregulatory mechanisms that are not the same in all species but all of which involve the participation of such tissues as gills, kidney, gut, and skin which are involved in the excretion and conservation of water and salts under a variety of hormones such as neurohypophysial hormones, prolactin, growth hormones, corticotropin-releasing hormones, adrenocorticoids, catecholamines, and the peptide hormones ANP and angiotensin. The question we asked is, Do plasma and brain ANP levels change when teleost fish move from sea water to a lower-salinity sea water and vice versa? To study this, three species of euryhaline fish were used (toadfish, *Opsanus tau;* mullet, *Mugil*

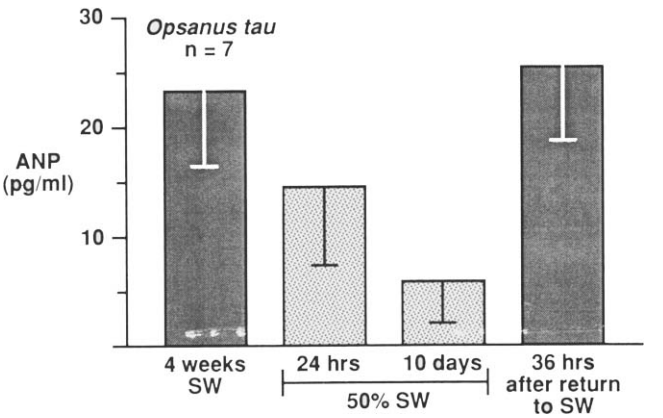

Figure 1. Plasma ANP response during transition from sea water → 50% SW → SW. A group of seven adult toadfish (*O. tau*), 350–750 g body wt) was maintained in a large running SW aquarium for 4 weeks, after which they were transferred to a 50% SW aquarium. Blood samples (600–800 μl) were taken by caudal puncture of the dorsal aorta at 24 hr and 10 days of adaptation. On Day 11, fish were transferred back to the SW aquarium and remained there for 36 hr, after which a last blood sample was taken. ANP values are expressed as pg/ml, average ± SD. (From Ref. 31, with permission.)

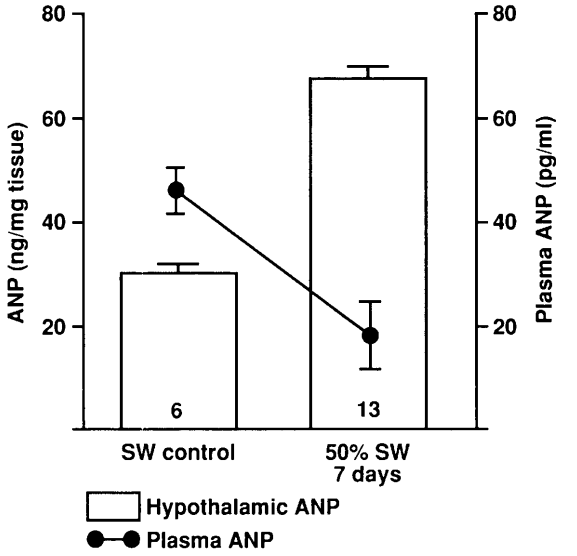

Figure 2. Brain ANP response in the adaptation of toadfish to 50% SW for 7 days. Nineteen adult toadfish (350–650 g body wt) were maintained in SW for 2 weeks and divided into two groups. The SW control (n = 6) remained in SW for a week, after which the hypothalamus was taken for control SW values. The second group (n = 13), while in SW was subjected to blood sampling as described earlier. The fish were then transferred to 50% SW for 7 days. On Day 7, blood samples were taken and after spinal cord transection the brain was removed to dissect out the hypothalamus. Plasma and tissues were processed for ANP RIA, plasma ANP (pg/ml), and tissue ANP (ng/ml) expressed as average ± SDM. (From Ref. 31, with permission.)

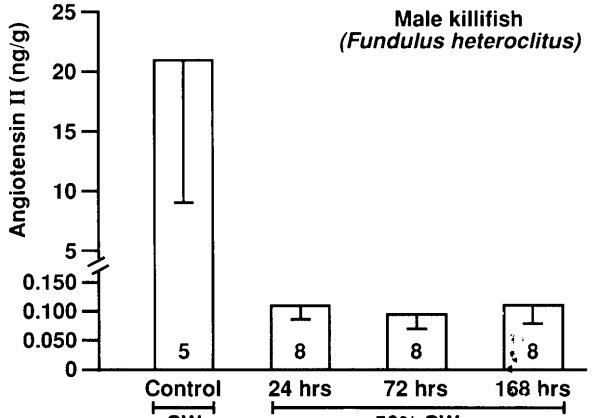

Figure 3. Response by the hypothalamus Ang II of killifish during adaptation to 50% SW. A group of 87 adult male killifish (*F. heteroclitus*) were adapted to SW for 2 weeks, after which a number of fish (n = 72) were transferred into a large 50% SW aquarium, where they were maintained for 24, 72, and 168 hr. After each of these periods, the HTS was removed and processed for Ang II RIA. Each of the Ang II measurements (n = 8) represent a pool of n = 3 HTS. The SW control group remained in SW for 2 weeks (n = 15) after which the HTS was dissected out, pooled in groups of n = 3, and processed for Ang II RIA. (From Ref. 31, with permission.)

cephalus; and killifish, *F. heteroclitus*). The fish were maintained in sea water (SW) for 2–4 weeks and then transferred to 50% SW for 24 hr or 10 days, after which they were transferred back to SW for 36 hr. In each group there were 4–10 fish. Blood samples (600–1000 μl) were taken from each group by caudal-aortic puncture. The brains were removed and dissected into blocks for the measurement of ANP concentration. ANP was extracted, purified, and measured by radioimmunoassay using an antibody developed to ANP 1–28 (30).

The results of ANP levels in plasma of toadfish are shown in Figure 1. Plasma ANP levels from fish with 4 weeks in sea water were 23 ± 7 pg/ml. Twenty-four hours after transfer to 50% SW, the ANP levels decreased to 15 ± 7 pg/ml, and after 10 days in 50% SW ANP levels were reduced to 6 ± 3 pg/ml. When these fish were returned to sea water and allowed to adapt for 36 hr, their levels of ANP had risen back to 26 ± 5 pg/ml. Thus, in this group of fish the results clearly showed that a mild hypo-osmotic environmental stimulus, such as moving *O. tau* from 100% to 50% SW, caused a significant reduction in plasma ANP that was fully reversible upon the fish's return to SW.

ANP and Ang II in Fish Brain

Levels of ANP in brain of fish were measured to establish baselines for experiments on transferring the fish through different salinities. Killifish, for example, were taken from SW (in summer), the brain removed and dissected. The highest levels of ANP in these species were in the hypothalamus and measurable amounts were found in the telencephalon, mesencephalon, brain stem, and spinal cord. Very low concentrations were measured in the pituitary. Similarly, Ang II in the fish brain was also highest in the hypothalamus. To study the response of brain and plasma ANP response during changes in salinity, toad fish were transferred from SW to 50% SW for 7 days (killifish would not provide sufficient blood volume for hormone studies). In SW, plasma ANP levels were 46 ± 2 pg/ml, and after 7 days in 50% SW they had significantly decreased, to 19 ± 3 pg/ml ($P < 0.01$). ANP concentrations in the hypothalamus from the same fish (*O. tau*) showed the opposite effect (Fig. 2). In SW, hypothalamic levels were 28 ± 2 ng/mg tissue. In 50% SW for 7 days, the hypothalamic ANP levels rose to 68 ± 4 ng/mg tissue ($P < 0.01$). Thus, although the plasma ANP levels decrease as the fish adapt to lower salinity, the ANP concentrations in the brain increase. In contrast, Ang II levels in the hypothalamus of fish during adaptation to 50% SW are significantly decreased. In control fish (killifish) in SW, levels of hypothalamic levels of Ang II were 21 ± 10 ng/g tissue. After 24, 72, and 168 hr in 50% SW, the levels were reduced greatly to <0.120 ng/g tissue (Fig. 3). The results show opposite effects of Ang II and ANP in the brains of euryhaline fish as they adapt to different environmental salinities. These changes, mainly in the hypothalamic tissue, indicate that the two peptides, ANP and Ang II, in fish brains evolved as neuroendocrine hormones,

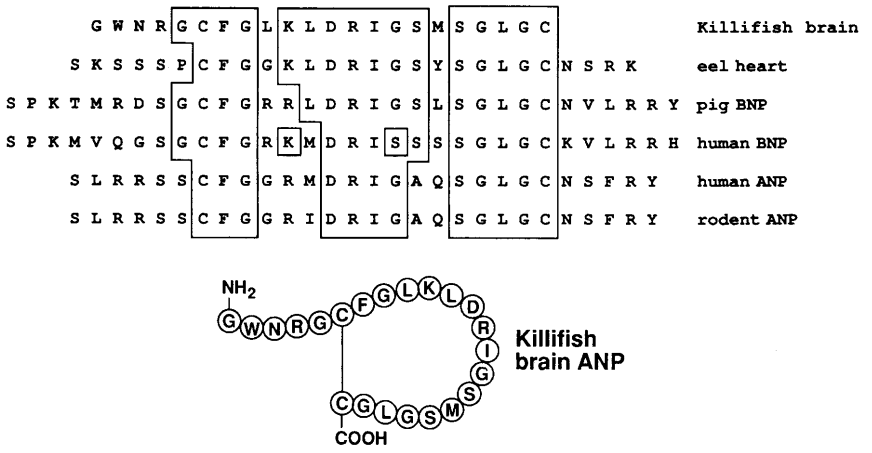

Figure 4. The amino acid sequence of the killifish (*F. heteroclitus*) brain ANP. The sequences of the peptides are compared with other ANP peptides (top). The residues in these other peptides that are identical to those in *F. heteroclitus* are boxed. The one-letter abbreviations for the amino acids are used. The killifish brain ANP has a structure (bottom) identical to mammalian CNP. (Based on Ref. 32.)

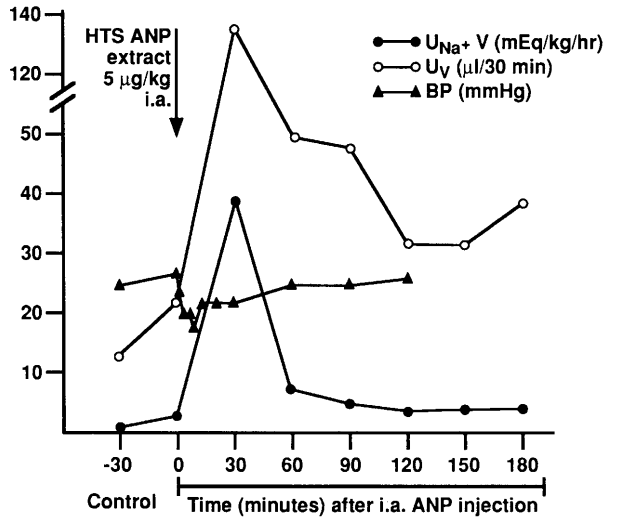

Figure 5. The biological effect of fish brain CNP extracts. A group (n = 3) of adult toadfish kept in SW were subjected to implant of plastic catheters into the dorsal aorta, mesenteric vein, and ureters. After 24 hr of recovery in SW control values of blood pressure and urinary volume excretion were recorded. Homologous hypothalamic ANP extracts (5 μg/kg fish) was administered and the blood pressure and urinary excretion was recorded every 30 min for the following 180 hr. Values are the average of n = 3. (From Ref. 31, with permission.)

involved in the regulation of osmotic balance. The opposite effect of ANP in the brain versus the plasma, indicates the different roles of ANP peripherally and centrally in adapting to the variations of saline environment, but the peptides from both sources are acting through their respective mechanisms to achieve the common result of maintaining body fluid volume and plasma electrolytes in changing conditions.

The Sequence of ANP in Fish Brain

Measurements of ANP were carried out with our radioimmunoassay, based on the use of an antibody to α-human ANP (1–28 amino acids). To study the structure of ANP in fish, Price *et al.* (32) used our antibody to identify extracts of killifish brain and sequenced the amino acids recognized by the antibody. The finding was that fish brain ANP has a high homology with ANP in eel, chicken, frog, rat, pig, dog, and human. However, the sequence is the same as mammalian C-type ANP (CNP). CNP contains the same conserved loop as ANP but is missing the carboxyl terminal tail attached to the cystine ring (Fig. 4). In human and rodent ANP 1–28, there is a tail of Asn-Ser-Phe-Arg-Tyr. In human and rodent BNP there is a 5–amino

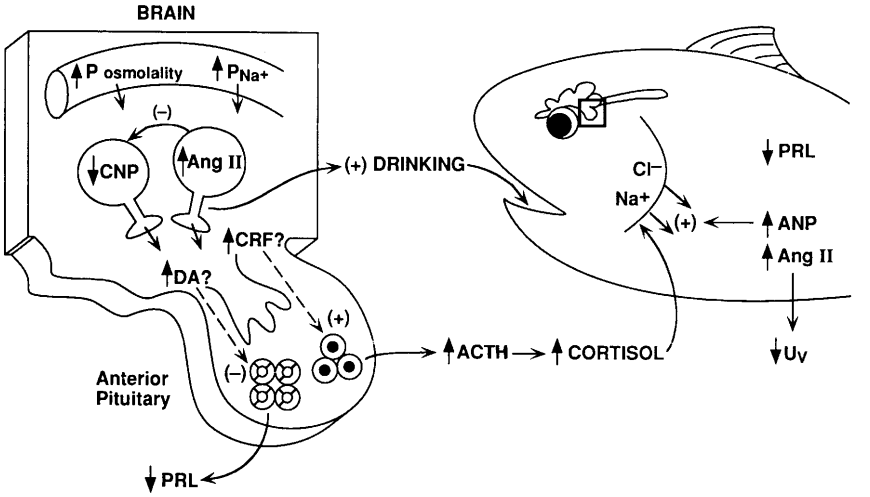

Figure 6. Interactions of brain ANP and Ang II in fish in sea water. The Ang II increases drinking and inhibits ANP. The reduction in ANP increases dopamine (DA) which inhibits prolactin (PRL) and increases indirect cortisol. The effects are increased loss of Na^+ and conservation of water.

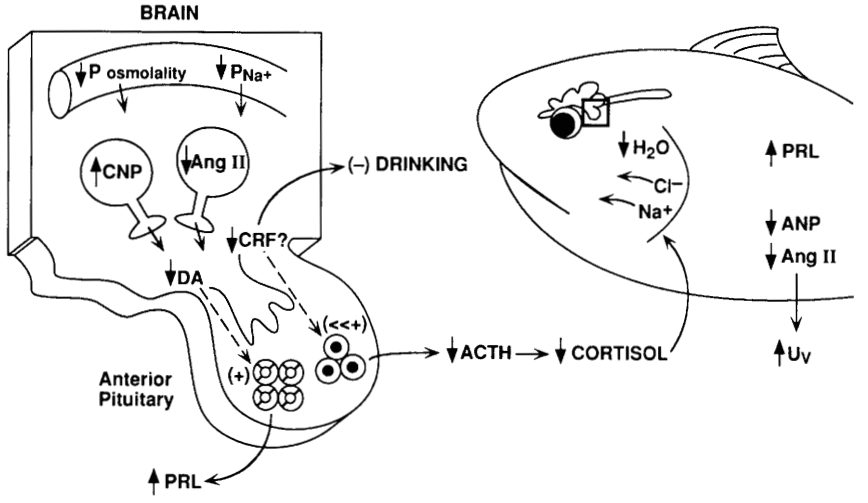

Figure 7. Interactions of brain ANP and Ang II in fish in fresh water. The ANP levels increase and inhibit dopamine (DA) which releases prolactin (PRL) from the pituitary into the plasma and acts on gills to reduce permeability to water. The decrease Ang II inhibits drinking and cortisol release. The net effect is conservation of Na and protection from water lowering osmotic pressure.

acid tail with a different sequence. The finding of CNP in fish brain could suggest that the brain CNP may have evolved earlier than the ANP molecule, or that it was derived from pre-pro ANP but particularly useful for brain mechanisms involved in adaption to and survival in changing salinities. The amino acids in the fish-brain CNP sequence are the same as those in the human and rodent CNP. The role played by this sequence in the physiological actions of natriuretic peptides is not clear, but evidence from rat studies suggests that CNP is the major brain peptide of the ANP family and acts on specific CNP receptors on neurons (33–35). To test the physiological effects of the fish CNP, toadfish were cannulated in the mesenteric vein and after 24 hr of recovery in SW, CNP extracted from toadfish hypothalamus (5 ng/kg) was injected iv. Blood pressure was measured *via* catheters in the dorsal aorta (Fig. 5). Within a few seconds after injection, blood pressure fell rapidly and slowly recovered after 90 min. Urinary sodium excretion rose rapidly, peaked at 30 min, and recovered over the next 180 min. Similarly, urinary volume is also dramatically increased by a single injection of hypothalamic CNP extract. Thus, the CNP is biologically active in fish, suggesting it had a physiological role as far back as the teleost fish. In the toadfish, we also find that CNP causes vasodilation and hypotension, and directly affects kidney tubular sodium reabsorption. It is interesting to note that the toadfish is an aglomerular fish; therefore, the observed increase in sodium and urinary volume excretion must be in response to the direct action of CNP on the renal tubules. In the toad fish the administration of homologous heart extract also caused a significant natriuretic response (36).

The central and peripheral role of CNP in teleost fish in sea water appears to be significant in their survival and to have evolved to protect loss of sodium in low-salinity environments and to allow the fish in SW to get rid of excess plasma sodium (37, 38). The postulated mechanisms involving CNP and Ang II in the brain are shown in Figure 6 for fish in sea water and in Figure 7 for fish in fresh water. In the plasma, ANP decreases as fish adapt to low salinity; in SW the ANP level is higher, which leads to the excretion of sodium. In the brain, CNP is decreased in SW and higher in low salinity. The significance of the brain CNP in adaptation to low environmental salinity may be related to the control of dopamine-prolactin levels. In the rat, it has been shown that intracerebroventrical injection of ANP decreases the level of dopamine in the hypothalamus (39). In our studies, toadfish also show a decrease in hypothalamic dopamine turnover during adaptation from sea water to 50% SW. This finding suggests that, in fish, one of the main mechanisms of CNP is the indirect control of prolactin release from the pituitary gland. Prolactin is one of the main anterior pituitary hormones and allows the fish to survive when transferred from sea water to fresh water (40, 41). On the other hand, a high dopamine level in the hypothalamus would inhibit prolactin release and lower plasma prolactin. This is characteristic of teleost fish in sea water, where plasma prolactin is low and brain CNP is relatively low. In sea water we also noted the fish brain Ang II was high compared with levels in fish in 50% SW. We hypothesize that in fish brain there is an interaction between Ang II and corticotropin-releasing hormone (CRH), as has been shown for mammals (42), by which brain Ang II stimulates or facilitates the release of ACTH. This enables Ang II in fish to play a role in maintaining the high plasma cortisol levels observed in SW fish. Cortisol is the main mineralocorticoid hormone in teleost fish and is known to be involved in gill extrusion of sodium and chloride in sea water fish (43). In addition, the high hypothalamic Ang II levels found in SW-adapted fish would stimulate drinking behavior by the fish, which is one of the basic

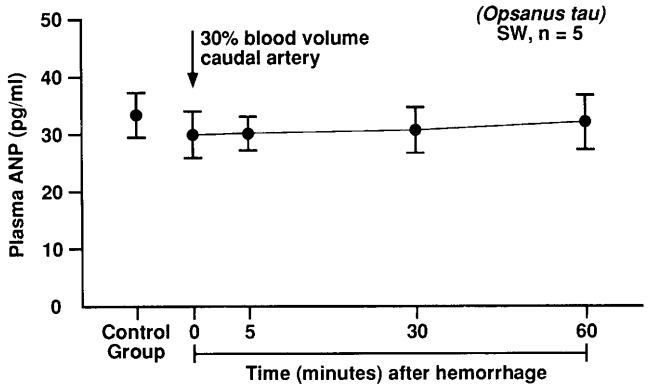

Figure 8. Effect of hemorrhage on plasma ANP in toadfish. A group of 10 toadfish (380–650 g body wt) was maintained in SW for 2 weeks. On Day 15, they were divided into two groups: control (n = 5) and hemorrhage group (n = 5). In the latter, blood samples (500–600 μl) were taken by puncture of the dorsal aorta at 0, 5, 30, and 60 min after withdrawal of 30% of total blood volume. Only one sample of blood was taken from the control fish. Plasma ANP was extracted and measured by RIA and expressed in pg/ml of plasma as average ± SD. (From Ref. 31, with permission.)

adaptive mechanisms for the fish survival in SW. On the other hand, when in sea water the lower CNP levels in the fish brain present less opposition to Ang II stimulation of drinking and inhibit ACTH release (44). The same fish in fresh water (Fig. 7) show an increase in brain CNP, which we hypothesize is related to an inhibitory effect on dopamine. The reduced dopamine would increase prolactin secretion. In fresh water fish, prolactin is known to reduce osmotic water permeability of the gills (45). In addition, the teleost fish in fresh water has lower brain Ang II (Fig. 7), and this would reduce the brain Ang II–CRF interactions, leading to less ACTH release and less cortisol, and therefore less sodium extrusion by the gills. Reduced brain Ang II would account for the reduced water intake of fish in fresh water. Thus, the yin-yang balance of brain CNP and brain Ang II in fish in fresh water may have evolved early in the evolution of vertebrates in order to decrease total sodium excretion, and to inhibit drinking. In our studies, fish in fresh water, show plasma ANP levels significantly lower than in sea water, in keeping with the need of fish to preserve sodium in plasma (37). Another euryhaline specie, *Gila atraria*, showed lower plasma ANF immunoreactivity in fresh water adapted fish than in salt water adapted fish (46). In sea water, plasma high concentration of ANP may facilitate sodium extrusion by the gills (47) and inhibit sodium reabsorption by the fish kidney.

The hypothesis above places ANP in fish as a sodium- or osmotic-regulating hormone, but in humans it is a major volume-regulating hormone. The effect of hemorrhage is to reduce drastically extracellular volume, without generally affecting osmotic levels. Therefore, it would be predicted that if ANP in fish is

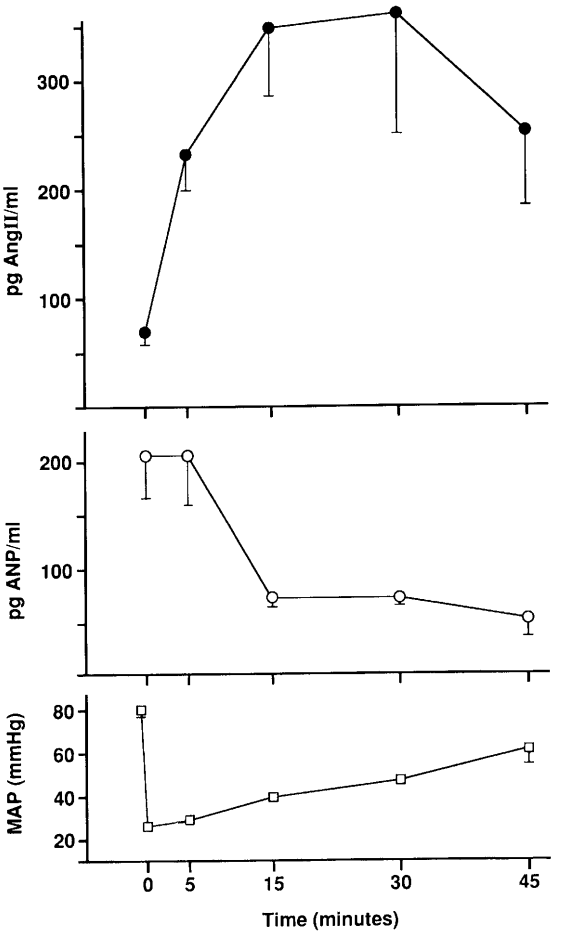

Figure 9. Response to hemorrhage in rats. Ang II and ANP response to hemorrhage (33% blood volume). (top) Plasma Ang II. (middle) Plasma ANP (note 5-min delay before response of a decreased level). (bottom) Mean arterial blood pressure (MAP) showing an immediate fall in pressure and gradual recovery.

a volume-regulating hormone, it would respond to hemorrhage by increasing plasma volume. If, however, ANP is not involved as a blood-volume hormone in fish, but serves principally as a sodium-regulating hormone, it would not be affected by hemorrhage. To test this, five toadfish maintained in sea water were hemorrhaged (30% blood volume from the caudal artery). Plasma ANP was measured before this and 5, 30, and 60 min after hemorrhage. No significant changes in plasma ANP levels were noted at any of the post-hemorrhage times (Fig. 8). Therefore, it appeared that in marine fish, plasma ANP does not respond to a significant decrease in extracellular volume. We conclude that plasma ANP in fish evolved principally as a sodium-regulating hormone. The role of ANP in brain, which we now know is CNP, probably evolved as a neuroendocrine hormone indirectly controlling prolactin levels and opposing the central actions of brain Ang II to regulate water and sodium content of the extracellular fluids. The balance among Ang II, ANP, and brain CNP effectively regulates internal hydromineral

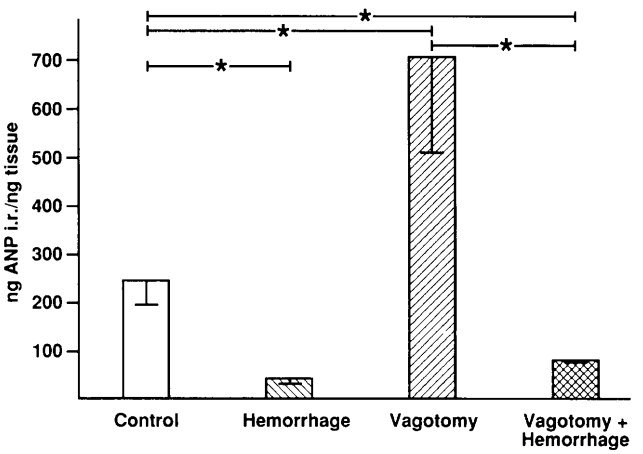

Figure 10. Effect of hemorrhage on plasma ANP. Groups of seven rats were hemorrhaged 33% and the effect of bilateral vagotomy tested. Heart rate did not significantly change and central venous pressures were reduced after vagotomy. The increase in ANP after vagotomy was significant ($P < 0.01$) and after hemorrhage ($P < 0.05$).

homeostasis and enables fish to move between different salinities without dehydrating or drowning.

ANP in Mammals

When vertebrates evolved from aquatic to terrestrial animals, ANP continued to be a regulator of sodium but has also evolved a function in the maintenance of volume. This is illustrated in Figure 9, showing the plasma levels of ANP and Ang II in rats after hemorrhage. Hemorrhage (33% blood volume) produces a rapid rise in Ang II for several hours. In contrast, ANP within 5 min after hemorrhage rapidly declines from control levels. The fall in plasma ANP levels may be interpreted as removing active opposition to the vasoconstrictor effects of Ang II. The ANP levels remain decreased for longer than 45 min. During this time there is some recovery of the blood pressure. Without ANP to oppose its actions, Ang II can increase the blood pressure, and as blood pressure increases the plasma Ang II levels start to return to baseline. The mechanism of the Ang II response to hemorrhage is well known. Reduced pressure stimulates kidney renin release, which activates the renin-angiotensin system, producing more Ang II in the plasma. The response of ANP to hemorrhage is less well studied (48, 49). A decline in pressure would not necessarily reduce stretch on the atria, which is a major stimulus of ANP release. The decline in blood pressure would increase vagal tone to the heart and increase heart rate *via* the baroreflex. To test the effects of the baroreflex on ANP release, vagotomy was produced in rats and the ANP levels measured in hemorrhage and nonhemorrhaged groups (3). The results (Fig. 10) show that vagotomy alone produced a highly significant increase in plasma ANP release. The increased level of ANP, following vagotomy, implies that there is a tonic influence by the vagus on ANP release from the heart. Whether this is an afferent or efferent action needs to be further explored. Hemorrhage, the data suggests, induces increased tonic (vagal) inhibition of ANP release by stimulating a reflex to the loss in pressure. However, this reflex is not the only factor, since vagotomy before hemorrhage did not completely prevent the reduction in plasma ANP levels.

ANP in the Brain

The presence of ANP in mammalian brain has been shown by numerous studies. One of the earliest was the demonstration of immunoreactive ANP-like material in areas of the hypothalamus that have become known as "cardioregulatory nuclei" (29). These same sites demonstrated very high levels of binding to ^{125}I-ANP (50). However, this would not have ruled out the possibility of ANP in the blood reaching these sites, either through circumventricular organs which have no blood-brain barrier (BBB) or in some way crossing the BBB. By demonstrating that there were high ANP levels in neurons and glia, we were able to establish that ANP is synthesized in the brain in the absence of plasma ANP (30, 51). The ANP that exists in the brain is of three kinds. There is the human ANP 1–28 amino acid sequence which is found in the atrial cardiomyocytes and adrenal glands, as well as in the hypothalamus. BNP 1–32, which was misnamed because it was first found in the brain but is actually more concentrated in cardiomyocytes of the ventricles (52). Thirdly, CNP 1-22 which was found in mammalian brain (33) and, as we showed above, in fish brain (32). CNP is characterized by a lack of a carboxyl post-cystine tail sequence plus variations in nine other residues. The exact roles of each of these three peptides in the brain have not been differentiated. Our antibody cross-reacts with both CNP and ANP, and in our measurements in brain tissue we did not separate the two brain peptides. Others have shown CNP mRNA to be predominant in the brain (33). Therefore, it is likely that (as in fish brain) we are measuring mainly CNP in the rodent brain.

The brain has specific receptors for ANP and there are at least three subtypes, GC-A, GC-B, and ANP-C. CNP specifically binds to the GC-B receptor which is coupled to a membrane guanylate cyclase and triggers cGMP (52). Sumners and Tang (53) showed that CNP was the most potent ANP in stimulating cGMP in neurons in culture, whereas ANP and BNP stimulated cGMP in glial cells. Thus, the CNP peptide appears to be the significant ANP for neural action. When ANP or BNP are injected they may cross-react with GC-B receptors but the endogenous effects are probably CNP mediated. One area to be explored is that of ANP and Ang II interactions. Are they in the same cells,

and do they involve common pathways? Ang II receptors are G protein seven transmembrane receptors of two main types, AT_1 and AT_2. The AT_1 in neurons inhibits cGMP (35).

In contrast to the effects of hemorrhage on plasma ANP in rats, hemorrhage increases ANP in the hypothalamus. Vagotomy has no effect on ANP concentration in the hypothalamus, alone or in the presence of hemorrhage. Unlike ANP, the Ang II response to hemorrhage is an increase in both plasma Ang II and hypothalamic Ang II. Therefore, separating the brain and plasma response of Ang II is more subtle. We have to consider the possibility of high plasma Ang II levels contaminating brain Ang II concentrations. However, the rise in brain Ang II during hemorrhage seems to be independent of the rise in plasma Ang II (54). In the hypothalamus, Ang II levels are significantly increased to a maximum of 33% hemorrhage, whereas plasma levels continue to rise linearly with increased hemorrhage. In the brainstem, there is no effect of hemorrhage on brain Ang II despite high plasma levels. Therefore, the changes in brain Ang II cannot be simply due to excess Ang II passing through the blood-brain barrier. We interpret the increase in hypothalamic Ang II as a response to the fall in blood pressure. Increased hypothalamic Ang II would release vasopressin, which is itself a vasoconstrictor (55). Also, it would inhibit the baroreflex (56), preventing slowing of heart and activating an increase of sympathetic output to stimulate vasoconstriction. These factors, in addition to the high levels of plasma Ang II leading to further vasoconstriction, tend to work together to restore the blood pressure and conserve the body fluids. However, the increase in hypothalamic ANP during hemorrhage is unexpected. Since most of the actions of central ANP oppose the actions of central Ang II, a lower level of ANP in the hypothalamus would have been predicted. In the fish study we had also noted that brain CNP increased when peripheral ANP decreased. We had interpreted that effect as actually facilitating the physiological consequences of reduced peripheral ANP during adaptation to fresh water. In this condition, we found increased ANP in the hypothalamus of fish, associated with decreased dopamine, which allowed prolactin release from the pituitary and plasma prolactin making gill epithelia less permeable to water, thus preserving sodium and plasma volume. In the hemorrhage situation, we suggest that the increased brain CNP or ANP in the rodent also reflects a facilitation of the role of plasma ANP in hemorrhage—that is, it produces indirect effects which permit Ang II to be unopposed, and therefore more effective in restoring volume and blood pressure. Exactly what those effects are and whether they are related to dopamine turnover or even to prolactin release have not yet been investigated in rats.

ANP and Angiotensin Effects on Thirst

One of the clearly demonstrated actions of Ang II in the mammalian brain is the induction of thirst for water and salt (55, 57). It has been shown that the dipsogenic effects of Ang II in the brain are opposed by prior infusion of ANP into the brain (58, 59). One of the problems in studying the normal physiology of ANP has been the lack of specific antagonists. Thus, when ANP is injected into the brain it is not certain if this models ANP from cardiac sources or brain sources. McCann and colleagues used antibodies to neutralize the effects of ANP (60, 61). A new approach that we have started using for specific Ang II synthesis and receptor inhibition is the use of antisense inhibition. Antisense oligonucleotides (AS-ODN) are short (15- to 18-mer) DNA base sequences designed to inhibit specific mRNA by blocking its transcription or translation. Antisense oligos are specific in inhibiting peptides and effective *in vivo* (62–64). We have designed an antisense oligonucleotide to inhibit ANP mRNA and ANP synthesis. Based on the sequence for ANP mRNA (65), we constructed a 15-mer oligodeoxynucleotide to hybridize in the antisense direction to sequences upstream from the AUG initiation site of AT_1 receptor mRNA. To test the ANP AS-ODN, we used Ang II induced drinking in the rat as a sequencing test. As ANP inhibits central Ang II drinking effects, it was predicted that an AS-ODN to ANP would elicit an increase in Ang II–induced drinking. Rats were prepared with implanted cannulae and tested for drinking response to 50 ng Ang II injected into the lateral ventricles. This produces a drinking response of 7 ± 2 ml/10 min. Another group of rats were pretreated with antisense oligonucleotides iv, injected in a single dose (50 μg/2 μl). Twenty-four hours later, the rats were tested with a 5-ng iv injection of Ang II. Their drinking levels increased significantly (100%) by 14 ± 1 ml/10 min, compared with control water intakes to the same dose of Ang II. Thus, ANP antisense oligonucleotides can be used as specific antagonists for ANP *in vivo*. The results indicate that endogenous ANP in the brain was inhibited by the antisense and its action inhibited the counteractive effects of endogenous ANP on Ang II–induced dipsogenic effects. The use of ANP antisense may help resolve unanswered questions about the normal physiology of ANP centrally and peripherally. Many of the previous studies have involved injecting ANP in doses that may or may not be physiologically relevant. By inhibiting the specific mRNA for ANP, the true physiological role of central and peripheral ANP can be revealed.

Summary and Conclusions

1. In the evolution of the physiological role of ANP, the peptide probably originated as a sodium-

regulating hormone for survival in sea water and adapting to changing saline environments.

2. In moving from an aquatic to a terrestrial environment, ANP additionally evolved in mammals as a hormone to counteract volume expansion while retaining its sodium-regulating properties.

3. In the fish brain, the natriuretic-like peptide is CNP, which has evolved as a neuroendocrine hormone. CNP is involved in the control of prolacting release, *via* dopamine regulation and in the inhibition of thirst mechanism when fish move from sea water into a lower salinity or fresh water environment.

4. In teleost, Ang II brain distribution is similar to rodents. Fish hypothalamic Ang II concentration inversely correlates with the environmental salinity. This supports the role for the hypothalamic Ang II in fish enhanced drinking and mineralocorticoid response while fish adapt to high salinity or sea water.

5. From fish to rodents, ANP in the brain and plasma opposes the actions of Ang II in brain and plasma. The results in rodents, and probably in humans, is that the balance of the two hormones is required to achieve normovolemia.

6. Both brain peptides (CNP and Ang II) are neuroendocrine hormones acting in concert with changes in peripheral ANP or Ang II with opposite actions.

7. ANP counteracts the thirst-inducing and volume expanding roles of brain Ang II in mammals. During volume reduction, ANP plays a permissive role by being inactive allowing Ang II to restore volume. In hemorrhage the effects of peripheral ANP and Ang II are inversely correlated, but in the brain both ANP and Ang II levels are increased specifically in the hypothalamus. However, they may still serve to facilitate the respective roles of peripheral Ang II and ANP.

8. The use of antisense oligonucleotides offers a new approach to selectively inhibit ANP mRNA and thereby reveal the physiological effects of ANP.

1. DeBold AJ, Borenstein AT, Veress AT, Sonnenberg H. A rapid and potent natriuretic response to intravenous atrial myocardial extract in rats. Life Sci **28**:89–94, 1981.
2. Currie MG, Newman WH. Evidence for α-1 adrenergic receptor regulation of atriopeptin release from the isolated heart. BBRC **137**:94–100, 1986.
3. Phillips MI, Kimura B, Wang H, Hoffman WE. Effect of vagotomy on brain and plasma atrial natriuretic peptide during hemorrhage. Am J Physiol **257**:R1393–R1399, 1989.
4. Robertson JI. The renin-angiotensin system and the response to hemorrhage in "The Renin-Angiotensin System". Physiol Therap **2**:71.1–71.3, 1993.
5. Maack T, Kleinert HD. Renal and cardiovascular effects of atrial natriuretic factor. Biochem Pharmacol **35**:2057–2064, 1986.
6. Forssmann WG. Cardiac hormones. I. Review on the morphology, biochemistry, and molecular biology of the endocrine heart. Eur J Clin Invest **16**:439–451, 1986.
7. Evans DH, Chipouras E, Payne JA. Immunoreactive atriopeptin in plasma of fishes: Its potential role in gill hemodynamics. Am J Physiol **257**:R939–R945, 1989.
8. Olson KR, Meisheri KD. Effects of atrial natriuretic factor on isolated arteries and perfused organs of trout. Am J Physiol **25**:R10–R18, 1989.
9. Solomon RJ, Solomon G, Silva P, Epstein FH. The effect of atriopeptin and cardiac extracts on the ventral aorta of *Squalus acathias*. Bull Mt Desert Isl Biol Lab **25**:146–149, 1985.
10. Bianchi C, Gutowska J, Thibault G, Garcia R, Genest J, Cantin M. Radioautographic localization of ^{125}I-atrial natriuretic factor (ANF) in rat tissues. Histochemistry **82**:441–452, 1985.
11. Harris PJ, Thomas D, Morgan TO. Atrial natriuretic peptide inhibits angiotensin-stimulated proximal tubular sodium and water reabsorption. Nature **326**:697–698, 1987.
12. Lynch DR, Braas KM, Snyder SH. Atrial natriuretic factor receptors in rat kidney, adrenal gland, and brain: Autoradiographic localization and fluid balance dependent changes. Proc Natl Acad Sci USA **83**:3357–3361, 1986.
13. Brucksch A, Gröne HJ, Talartschik J, Fuchs E. Binding sites of atrial natriuretic peptide in human renal tissue. Quantification by *in vitro* receptor autoradiography. Klin Wochenschr **66**:303–307, 1988.
14. Kloas W, Flügge G, Fuchs E, Stolte H. Binding sites for atrial natriuretic peptide in the kidney and aorta of the hagfish (*Myxine Glutinosa*). Comp Biochem Physiol **91A**:685–688, 1988.
15. Peach M. Renin-angiotensin system: Biochemistry and mechanisms of action. Physiol Rev **57**:313–370, 1977.
16. Harris PJ, Young JA. Dose-dependent stimulation and inhibition of proximal tubular sodium reabsorption. Plfugers Arch **367**:295–297, 1977.
17. Navar LG, Langford HG. Effects of angiotensin on the renal circulation. In: Page IH, Bumpus FM, Eds. Angiotensin. Heidelberg: Springer-Verlag, pp 455–474, 1974.
18. Galli SM, Qian K. Angiotensin-like immunoreactivity and catecholmaine resonse to captopril in the Nurse shark. Physiologist **34**:26.6, 1991.
19. Takei Y, Hasegawa Y, Watanabe TX, Nakajima K, Hazon N. A novel angiotensin I isolated from an elasmobranch fish. J Endocrinol **139**:381–385, 1993.
20. Nishimura H, Sawyer WH. Vasopressor, diuretic, and natriuretic responses to angiotensins by the American eel, *Anguilla rostrata*. Gen Comp Endocrinol **29**:337–348, 1976.
21. Nishimura H. Comparative endocrinology of renin and angiotensin. In: Johnson JA, Anderson RR, Eds. The Renin-Angiotensin System. New York: Plenum Press, pp 27–29, 1980.
22. Wilson JX. The renin-angiotensin system in nonmammalian vertebrates. Endocr Rev **5**:45–61, 1984.
23. Balment RJ, Carrick S. Endogenous renin-angiotensin system and drinking behavior in flounder. Am J Physiol **248**:R157–R160, 1985.
24. Carrick S, Balment RJ. The renin-angiotensin system and drinking in the euryhaline flounder, *Platichthys flesus*. Gen Comp Endocrinol **51**:423–433, 1983.
25. Malvin RL, Schiff D, Eiger S. Angiotensin and drinking rates in the euryhaline killifish. Am J Physiol **239**:R31–R34, 1980.
26. Takei Y, Hirano T, Kobayashi H. Angiotensin and water intake in the Japanese eel, *Anguilla japonica*. Gen Comp Endocrinol **38**:466–475, 1979.
27. Phillips MI, Speakman EA, Kimura B. Levels of angiotensin and molecular biology of the tissue renin-angiotensin systems. Regul Pept **43**:1–20, 1992.
28. Trolliet MR, Phillips MI. The effect of chronic bilateral nephrectomy on plasma and brain angiotensin. J Hypertens **10**:29–36, 1992.
29. Saper CB, Standaert DG, Currie MG, Schwartz D, Geller DM, Needleman P. Atriopeptide-immunoreactive neurons in the

brain: Presence in cardiovascular regulatory areas. Science **227**:1047–1049, 1985.
30. Phillips MI, Kimura B, Raizada MK. Measurement of brain peptides: Angiotensin and atrial natriuretic peptide in tissue and cell culture. Methods in Neurosciences **6**:177–206, 1991.
31. Galli SM. Brain CNP-like peptide and osmoregulation in fish. Regul Pept (in press), 1996.
32. Price DA, Doble KE, Lee TD, Galli SM, Dunn BM, Parten B, Evans DH. The sequencing, synthesis, and biological actions of an ANP-like peptide isolated from the brain of the killifish *Fundulus heteroclitus*. Biol Bull **178**:279–285, 1990.
33. Laugub MC, Dolgas CM, Watson RE, Herman JP. The C-type natriuretic peptide receptor is the predominant natriuretic peptide mRNA express in rat hypothalamus. J Neuroendocr **7**:305–309, 1995.
34. Trachte GJ, Kanwal S, Elmquist BJ, Ziegler RJ. C-type natriuretic peptide neuromodulates via "clearance receptors". Am J Physiol **268**:C978–C984, 1995.
35. Sumners C, Myers LM. Angiotensin II decreases cGMP levels in neuronal cultures from rat brain. Am J Physiol **260**:C79–C87, 1991.
36. Lee J, Malvin RL. Natriuretic response to homologous heart extract in aglomerular toadfish. Am J Physiol **252**:R1055–R1058, 1987.
37. Galli SM, Evans DH, Kimura B, Phillips MI. Changes in plasma and brain levels of atrial natriuretic peptide in fish adapting to fresh water and sea water. FASEB J **2**:A524, 1988.
38. Galli SM. Fish adaptation to changes in salinity: A model for understanding the physiological role of vasoactive peptides. Physiologist **33**:A-12, 1990.
39. Nakao K, Katsuura G, Morii N, Itoh H, Shiono S, Yamada T, Sugawara A, Sakamoto M, Saito Y, Eigyo M, Matsushita A, Imura H. Inhibitory effect of centrally administered atrial natriuretic polypeptide on the brain dopaminergic system in rats. Eur J Pharmacol **131**:171–177, 1986.
40. Nicoll CS. Role of prolactin in water and ion balance in vertebrates. In: Jaffe RB, ed. Prolactin. New York: Elsevier, pp 127–166, 1981.
41. Hirano T. The spectrum of prolactin action in teleosts. Developments and directions in progress in clinical and biological research. Comp Endocr. **205**:53–74, 1986.
42. Keller-Wood MK, Stenstrom B, Shinsako J, Phillips MI. Interaction between CRF and Ang II in control of ACTH and adrenal steroids. Am J Physiol **250**:R306–R402, 1986.
43. Hirano T, Mayer-Gostan N. Endocrine control of osmoregulation in fish. In: Gaillard PJ, Boer HH, Eds. Comparative Endocrinology. Amsterdam: Elsevier, pp 209–212, 1978.
44. Fink G, Dow RC, Casley D, Johnston CI, Lim AT, Copolov DL, Bennie J, Carrol S, Dick H. Atrial natriuretic peptide is a physiological inhibitor of ACTH release: Evidence from immunoneutralization *in vivo*. J Endocrinol **131**:R9–R12, 1991.
45. Wendelaer Bonga SE, Flik G, Lowik CWGM, van Eys GJJM. Environmental control of prolactin synthesis in teleost fish. Gen Comp Endocrinol **59**:352–359, 1985.
46. Westenfeldere C, Birch FM, Baranowski RL, Rosenfeld MJ, Shiozawa DK, Kablitz C. Atrial natriuretic factor and salt adaptation in the teleost fish *Gila atraria*. Am J Physiol **255**:F1281–F1286, 1988.
47. Scheide JI, Zadunaisky JA. Effect of atriopeptin II on isolated opercular epithelium of *Fundulus heteroclitus*. Am J Physiol **254**:R27–R32, 1988.
48. Geer PG, Wang BC, Glora-Ginter G, Leadley RJ Jr., Goetz KL. Effect of hemorrhage on plasma atriopeptin levels in conscious dogs. Proc Soc Exp Biol Med **187**:327–334, 1988.
49. Goetz KL. Physiology and pathophysiology of atrial peptides. Am J Physiol **254**:E1–E15, 1988.
50. Castren E, Saavedra JM. Lack of vasopressin increases hypothalamic atrial natriuretic peptide binding sites. Am J Physiol **257**:R168–R173, 1989.
51. Raizada MK, Kimura B, Phillips MI. Immunoreactive atrial natriuretic peptide in neuronal and glial cells of spontaneously hypertensive rat brain. Am J Physiol **258**:C109–C114, 1990.
52. Gardner DG. Molecular biology of natriuretic peptides. Trends Cardiovas Med **4**:159–165, 1994.
53. Sumners C, Tang W. Atrial natriuretic peptide receptor subtypes in rat neuronal and astrocyte glial cultures. Am J Physiol **262**:C1134–C1143, 1992.
54. Phillips MI, Heininger F, Toffolo S. Brain angiotensin in thirst and hemorrhage. Regul Pept (in press), 1996.
55. Phillips MI. Functions of brain angiotensin. *Annu Rev Physiol* **49**:413–435, 1987.
56. Casto R, Phillips MI. Cardiovascular actions of microinjections of angiotensin II in the brainstem of rats. Am J Physiol **2246**:R811–R816, 1984.
57. Fitzsimons JT. The hormonal control of water and sodium intake. In: Martin L, Ganong WF, Eds. Frontiers in Neuroendocrinology. London: Oxford University Press, pp 101–128, 1971.
58. Antunes-Rodriguez J, McCann SM, Rogers LC, Samson WK. Atrial natriuretic factor inhibits dehydration- and angiotensin II–induced water intake in the conscious, unrestrained rat. Med Sci **82**:8720–8723, 1985.
59. Samson WK. Atrial natriuretic factor and the central nervous system. Endocrinol Metab Clin North Am **16**:145–161, 1987.
60. Franci CR, Kozlowski GP, McCann SM. Water intake in rats subjected to hypothalamic immuno-neutralization of angiotensin II, atrial natriuretic peptide, vasopressin or oxytocin. Proc Natl Acad Sci USA **86**:2952–2956, 1989.
61. Franci CR, Anselmo-Franci JA, McCann SM. Opposite effects of central immunoneutralization of angiotensin II or atrial natriuretic peptide on luteinizing hormone release in ovariectomized rats. Neuroendocrinology **51**:683–687, 1990.
62. Wahlstedt D, Pich EM, Koob GM, Yee F. Heilig M. Modulation of anxiety and neuropeptide Y-Y1 receptors by antisense oligodeoxynucleotides. Science **259**:528–531, 1993.
63. Gyurko R, Wielbo D, Phillips MI. Antisense inhibition of AT_1 receptor mRNA and angiotensionogen mRNA in the brain of spontaneously hypertensive rats reduces hypertension of neurogenic origin. Regul Pept **49**:167–174, 1993.
64. Phillips MI, Gyurko R. *In vivo* applications of antisense oligonucleotides for peptide research, "Antisense Issue". Regul Pept **59**:131–141, 1995.
65. Kojima M, Minamino N, Kangawa K, Matuo H. Cloning and sequence analysis of cDNA encoding a precursor for rat brain natriuretic peptide. BBRC **159**:1420–1426, 1989.