THIRST AND SODIUM APPETITE IN MICE: ANGIOTENSIN, BRAIN Fos, BLOOD PLASMA HORMONES, AND FLUID INTAKE

By

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Questions regarding the role of angiotensin (Ang) were examined in mice. In contrast to rats, CD-1 male mice failed to drink water or sodium after exogenous Ang injection. Fos-IR in these mice was prevalent in the subfornical organ (SFO) and there were no differences in Fos-IR among different strains or gender of mice. Like rats, mice drank both sodium and water after furosemide treatment. Mice given furosemide were also hypovolemic (increased plasma protein and hematocrit ratios) and had increased plasma renin activity (PRA). Fos-IR in these mice was similar to that seen after exogenous Ang treatment. Whereas rats drink both sodium and water after polyethylene glycol treatment, mice did not show a clear sodium appetite. However, these mice were hypovolemic, had increased PRA, had modestly increased aldosterone, and drank water. Fos-IR was prevalent in almost all brain regions examined after this treatment. Thus, our results revealed several differences between rats and mice with regard to responses after procedures that mimic the renin-angiotensin-system (RAS).

CHAPTER 1
INTRODUCTION

Intracellular and Extracellular Fluid Compartments

Water and minerals are in constant motion thoughout the body's various compartments. Imbalances occur as water or minerals are lost from the compartments. These imbalances initiate a variety of autonomic and behavioral responses to maintain normal homeostasis. Body fluid compartments are divided into two general categories: intracellular fluid (ICF) or fluid within cells; and extracellular fluid (ECF) or fluid between cells. The ECF includes intravascular (blood plasma) and interstitial components. While the ICF contains more potassium and chloride, the ECF contains more sodium [1, 8].

In general, total body water accounts for 60% body weight of humans. Forty percent is devoted to the ICF and 20% to the ECF. Of the ECF, 15% is interstitial fluid (ISF) and 5% is blood plasma [1]. So, for a 200-pound person, blood plasma alone weighs nearly 10 pounds. In their steady state, cells are in osmotic equilibrium with the ISF that surrounds them [1]. In clinical calculations with humans, a 60:40:20:5 ratio is used to determine amounts of fluid lost from each compartment after dehydration, as well as how fluids used in replenishment distribute themselves among the compartments after a steady state has been reached [1].

Osmotic Thirst

A common form of intracellular fluid loss results from an increase in tonicity of the ECF. In the laboratory, this is seen after injection of a hypertonic solution. In nature,

this is most commonly seen after ingestion of a salty food or drink. An increase in the tonicity of the ECF draws fluid from within cells (intracellular dehydration) to the extracellular matrix by osmosis, diluting the ECF and causing an increase in osmotic pressure in specific areas of the body. This increase in osmotic pressure activates osmoreceptors in the brain and the gut, which in turn is thought to cause many responses, including the release of the hormone vasopressin, or antidiuretic hormone (ADH) [1]. ADH stimulates reabsorption of water within the nephons of the kidney and decreases output of urine [1, 8]. The drinking of water after intracellular dehydration is known as osmotic thirst.

It is important to note that water is the correcting agent since salts are impermeable to cell membranes without help from channels or transporters. When there is decreased ECF tonicity (loss of sodium, or hyponatremia) water moves into cells. In cases of hypernatremia (increased ECF tonicity), water moves out of cells to the ECF, resulting in cellular dehydration.

Hypovolemic Thirst

A common form of ECF fluid loss results from simply a decrease in the volume of ECF, or hypovolemia. Hypovolemia is the result of many stimuli, including excessive exercise, respiration, sweating, and hemorrhage [1]. In the laboratory, hypovolemia may be stimulated by blood loss or by other measures that diminish blood flow such as sequestering the ECF by injection of a colloid subcutaneously or by interfering with venous return by ligation of the vena cava [8]. In many cases, there is no net change in tonicity since both water and minerals (salts) are lost. Thus, in addition to thirst, hypovolemia may also result in sodium appetite.

A loss of ECF volume causes release of renin from the kidney and possibly the brain; and eventually causes the formation of angiotensin II (Ang) though a cascade of events known as the renin-angiotensin system (RAS) [1, 6, 8, 9, 26, 27]. It is thought that procedures that elicit hypovolemia stimulate the renal RAS more than procedures that stimulate Ang release directly [6, 8]. In short, while direct injection of Ang mimics only the end result of RAS stimulation, hypovolemia stimulates all components of the RAS, including renin release. In addition to physiological responses, drinking of water (called thirst) is seen in addition to the ingestion of sodium (called sodium appetite) after hypovolemia. This drinking behavior is known as hypovolemic thirst.

The thesholds, or degree of stimuli needed to produce a particular response, are different for osmotic and hypovolemic thirst. While hypovolemic thirst requires an 8 to 10% reduction in plasma volume, osmotic thirst can occur after only a 1 to 2% decrease in cellular water [1, 8]. This is because variations in blood plasma levels are buffered by fluid from the interstitial compartment within the ECF, allowing for some fluctuations to occur [1, 8]. While dehydration from either the intra- or extra-cellular compartment alone is sufficient for eliciting drinking, the response is further increased by dehydration from both compartments [1, 8]. In other words, the increase in drinking is greater than the combined increase seen after dehydration from the compartments alone.

Thirst and Sodium Appetite

Fitzsimons (1998) noted some facts when analyzing thirst in animals. Thirst, in the most common definition, is a sensation resulting from the need for water, and is relieved by drinking [6]. However, a drink of water on a crisp morning is drinking without a particular need, and so is not—in a strict interpretation—thirst [6]. When we speak of drinking behavior after certain stimuli, then, we probably mean an appetite for

water, although in the laboratory it is generally assumed that there is a need involved [6]. Furthermore, laboratory animals, like humans, are not perfect in their drinking behavior [6]. They may drink too much water or consume too much sodium, overcompensating for its loss. In those cases, further responses take place to correct the imbalance, like urination or re-absorption of water or sodium in the kidney.

Unlike the term thirst, the term sodium appetite includes both the need and preference for sodium [6]. More so than with thirst, sodium appetite is increased by previous experience in many animals including rats [21]. Learning mechanisms associate sodium with being pleasurable (as in sodium-replete animals), and eventually sodium can be desired without a particular need (called sodium preference). In many cases, though, both the need and the preference operate, collectively called sodium appetite [6].

Generation of Angiotensin and the Renin-Angiotensin System (RAS)

Fitzsimons (1998) has dated the discovery of what is now known as the renin-angiotensin-system (RAS) to the late 19th century [6]. Researchers noticed that saline extracts of renal cortex caused rises in blood pressure when injected into anesthetized rabbits [6]. While the medullary extracts were inactive, the pressor response of the cortical extracts was astonishingly resilient; and the extracts retained the pressor response at high temperatures [6]. Thus, a substance existed in the renal cortex that caused this pressor response. They named the substance renin and suggested that renin had a role in vascular resistance and cardiac hypertrophy and certain renal diseases [6]. However, the researchers did not suggest how renin caused increased blood pressure, or whether its secretion was causative or correlated with hypertension. It wasn't until nearly 40 years later that the nature of this substance was characterized. It was found that renin (an

enzyme) acts on a substrate (now known as angiotensinogen) in the blood plasma to yield angiotensin—a peptide capable of vasoconstriction [6].

Renal renin comes from the juxtaglomerular apparatus of the nephon—lying between the glomerulus and the distal tubule [8, 15]. Active renin is an acid protease with specificity for a single peptide bond in the angiotensinogen molecule [8, 15]. The angiotensinogen gene has been very well conserved over time, upholding its importance across species [8, 15]. The angiotensinogen molecule that is present in the liver is then cleaved by renin to form angiotensin I, which is transported through the bloodstream [8, 15]. Ang I is then cleaved by Angiotensin Converting Enzyme (ACE) to form Ang II. This cascade of events, from the conversion of angiotensinogen by renin to form Ang II, is known as the classical pathway, although it is now believed that many Ang II generating systems exist in several tissues that may not involve the classical pathway [9, 22].

There is now evidence supporting a theory that states that there are many tissue-based Ang II generating systems, called tissue RAS, that exist in several tissues or organs, including the brain, heart, gonads, adrenals, and pancreas [9, 22]. Further, ACE is thought to exist in several places including the pulmonary vasculature, organs such as the heart, pancreas, gonads, or even within cells [9, 22]. These tissue-based RAS systems may involve key components of the classical RAS (Ang II, angiotensinogen, and receptors) while other factors (renin and ACE) may have limited or varying roles [9, 22]. Thus, these tissue-based RAS systems may not involve the cascade of events that use renin to form ANG II, but rather, generate Ang II from alternate means.

These tissue-based RAS systems may act as a paracrine/ autocrine component that can operate independently of the endocrine/ hormonal (circulating) RAS [9, 22]. It is not known; however, whether tissue-based RAS potentiate systemic or local functions [9, 22]. Locally, it is thought that tissue RAS systems may play a role in many tissue-specific diseases, such as cardiac hypertrophy, tissue-specific inflammatory disease, ischemia, and cancers [9, 22].

Characteristics of Angiotensin

Angiotensin is a powerful inducer of thirst and sodium appetite. It has been said that Ang injected into the limbic structures of the brain can stimulate drinking so rapid that in 15 minutes the animal has exceeded normal unstimulated drinking amounts normally seen in a 24 hour period [6]. The increase in drinking is dependent upon the dose of Ang.

There are angiotensinergic nerve endings in the central nervous system, particularly in the anteroventral third ventricle (AV3V), that are sites for the delivery or production of Ang peptides [6]. These areas include the organum vasculosum of the lamina terminalis (OVLT), median preoptic nucleus (MnPO), subfornical organ (SFO), supraoptic nucleus (SON), paraventricular nucleus (PVN), central nucleus of the amygdala, and the brain stem nuclei [6]. It is thought that Ang may be delivered to these sites in two different ways: Ang may act as a neurotransmitter with fast and direct signaling on low affinity receptor sites; or Ang may leak into the extracellular space and act on high affinity receptor sites from a distance [6, 8]. The complexity of the many Ang peptides may give us insight on how both of these hypotheses may be true [1, 6, 8].

In addition to the ability of Ang to stimulate a pressor response and drinking, renal-derived Ang is also synergisitic with the release of aldosterone in cases of sodium

deficiency. Aldosterone is released from the adrenal cortex and stimulates the reabsorption of sodium and water in the distal tubule of the kidney [6]. Thus, in addition to Ang, aldosterone also contributes to sodium appetite. Aldosterone release conserves sodium by increasing the number of sodium pumps in the basolateral membranes of epithelial cells located in the tubular lumen [6. 8]. The uptake of the free sodium in the tubular lumen is also stimulated by aldosterone, in which the expression of sodium channels is increased [6]. While Ang release is a significant regulator of aldosterone release, it is not the only one since aldosterone is also stimulated by increases in blood levels of potassium [6].

Experimental Procedures That Elicit Thirst and Sodium Appetite

Exogenous Angiotensin

Exogenous Ang injection is thought to elicit both thirst and sodium appetite in rats by mimicking the responses subsequent to RAS stimulation, without involving hypovolemia. In short, direct injection of Ang in rats stimulates increased drinking of sodium and water, Fos-IR in many brain areas, and increased aldosterone production. However, since there is no hypovolemia, exogenous Ang does not induce RAS stimulation or endogenous Ang production.

Furosemide

Furosemide is called a loop diuretic, acting on the Loop of Henle within the nephon to inhibit reabsorption of sodium and increase excretion of sodium and water [6]. After furosemide treatment, the buildup of sodium in the kidney causes the influx of water and increased excretion of both sodium and water [6]. Hypovolemia is induced since there is a loss of ECF, but there is generally not hypotension [6]. Thus, the renal RAS is stimulated at the initial stages of the classical pathway. In laboratory animals,

furosemide has been widely used to stimulate acute sodium depletion more easily than procedures that restrict dietary sodium. These latter procedures often take longer.

Furosemide is also under the pharmacological name of Lasix or water pills. Lasix has been used for decades in the treatment of hypertension in humans, and its efficacy has recently been upheld despite some of the recently developed medications that are more costly.

Polyethylene Glycol

Polyethylene glycol (PEG) traps ECF in an edema located near the site of injection [6]. PEG has a very high molecular weight and is therefore viscous when made into suspension form. Further, PEG is a colloid that has hydrophobic and hydrophilic properties, capable of trapping fluid and sequestering fluids at its hydrophobic site. PEG traps the ECF in an isotonic state and does not preferentially trap either water or salts. Further, it traps ECF without a change in blood pressure. Physiological mechanisms are able to keep blood pressure in check since the loss of volume is gradual and slow [6].

Hypertonic Saline

Hypertonic saline evokes drinking of water in many animals by inducing cellular dehydration (osmotic thirst) as previously described. Hypertonic saline increases the tonicity of the ECF and leads to increased osmosis of water to the ECF. The renal RAS is not stimulated since there is no hypovolemia or decreased blood flow to the kidney. Thus, renal-derived Ang is thought to have a limited role in osmotic thirst [1, 6, 8].

On the other hand, vasopressin, which inhibits diuresis, is thought to be the major contributor to drinking after hypertonic saline treatment [6, 12, 23, 24]. Vasopressin is synthesized by the magnocellular neurosecretory cells (MNCs) of the SON and PVN.

These nuclei project their axon terminals to the posterior pituitary, where vasopressin is released into the bloodstream [6, 12].

Other Treatments

Angiotensin Coverting Enzyme Inhibitors (ACEIs)

Of recent concern has been the use of ACE inhibitors, like captopril. These block the endogenous production of Ang II from Ang I. In short, if an ACE inhibitor were given to an animal that previously received systemic Ang I, drinking would not occur [6]. However, an ACE inhibitor given to an animal that previously received Ang II would still drink [6]. In short, the use of ACE inhibitors has been vital in studies that examine the role of the RAS.

Beta-Adrenergics

Another treatment of concern is the use of beta-adrenergics such as isoproterenol (isoprenaline). Isoproterenol is a nonselective beta-adrenergic agonist that releases renal renin by acting on the juxtaglomerular apparatus both directly and indirectly [6]. Thus, in simple terms, isoproterenol artificially activates the RAS by increasing endogenous rennin [6]. This treatment has also been vital in understanding the nature of the RAS. By using ACEIs coupled with beta-adrenergics, the relative roles of the brain and peripheral RAS can be examined.

CHAPTER 2
LITERATURE REVIEW

Exogenous Administration of Angiotensin in the Rat

Fitzsimons first noted that intravenous administration of Ang II stimulates both drinking and a pressor response in rats. This study, providing a link between the ability of Ang II to increase both drinking and blood pressure, has paved the way for future studies that have examined other connections involving the nervous, vascular, endocrine, and behavioral responses to dehydration.

The drinking effect of Ang administration is most robust after intracranial administration [6]. This is because Ang can act directly on "drinking centers" in the brain to stimulate drinking [6]. When Ang is administered though a cannula directly into the brain in such a way that it is not disturbed during the infusion, a rat will stop whatever it is doing and head straight for the water, usually in less than a minute [6]. The animal may drink too much water, and will then develop an appetite for sodium [6].

When Ang is administered systemically, drinking is not as robust as that seen after intracranial administration. It has been suggested that the amount of peripheral Ang II present in the body needed to elicit a drinking response is similar to that seen after 48 hours of water deprivation [6]. Thus, large doses of systemically-administered Ang were required in to elicit a drinking response. The large dose requirement may be explained by the pressor response of Ang, in which vasoconstriction temporarily hides the fact that the vessels contain less volume in cases of hypovolemia. In fact, after chonic subcutaneous injection of Ang (2.8 μg/h) in both Fischer 344 and Sprague-Dawley rats, a 32-mm Hg

increase in systolic blood pressure was found, while controls given no treatment did not show a reliable increase in systolic blood pressure [2]. A weaker pressor response after Ang administration is also seen in cases where the peptide is delivered to the brain [6, 8, 26, 32].

Because of this pressor response, it was hotly debated whether the doses of Ang needed to elicit drinking were physiological (able to be achieved unstimulated) or pharmacological [6]. However, when measures are taken to negate the pressor response of systemically administered Ang, the drinking response is robust using physiological doses of Ang [1, 6]. For example, after surgical removal of cardiopulmonary and arterial baroreceptors (which detect blood pressure) in dogs after Ang II, there is an increase in drinking of water [1, 8]. Despite the fact that both peripheral and central Ang elicit a pressor response, the reason the drinking response in rats is more robust after central administration is because the peptide is in closer proximity to the limbic structures of the brain that control drinking. It is unclear how the pressor response after Ang administration may contribute to sodium appetite.

Among its other activities, Ang also stimulates the release of aldosterone, which is released from the adrenal gland and stimulates renal sodium conservation as well as drinking of sodium [6]. The synergy of aldosterone and Ang II has been well documented [5, 6, 8, 11, 20, 21, 27-30, 32]. One study suggested that increases in aldosterone stimulate increased sensitivity of Ang receptors [11]. Whether Ang alone can stimulate sodium appetite (without increases in aldosterone) is less clear, although sodium deficient, adrenalectomized rats have clearly showed a sodium appetite [27]. However, some complexity with these studies can easily be imagined. Completely

removing contributions from lingering aldosterone after removal of the adrenals may be extremely difficult. While both Ang and aldosterone are believed to be involved in sodium appetite, neither hormone is absolutely necessary to produce it [6]. Sodium appetite remains a highly examined behavior and it is unclear what other mechanisms may be involved.

After 100 μg/kg Ang II (subcutaneous), rats of two strains drank about 4-5 mL of water and about 7-8 mL of 0.15 M NaCl in 1 hour [2]. Control rats injected with 0.15 M NaCl drank only trace amounts of water and about 0-2 mL of 0.15 M NaCl [2]. Thus, exogenous Ang clearly elicits both thirst and sodium appetite in rats.

In addition to the behavioral drinking effects of peripheral Ang in rats, brain activation is also increased. After chonic intravenous Ang at dipsogenic doses for 6 hours, Fos-IR was maintained in the SFO, OVLT, MnPO, SON, and the paraventricular hypothalamus (PVH) [10, 17, 18]. Furthermore, lesions of the SFO abolished brain activation in the SON and PVH, suggesting that the SFO has neural connections to these areas and that they have similarly structured Ang receptors [18, 25].

Strain differences have been noted in drinking induced by exogenous Ang administration [2]. Fisher 344 rats were shown to drink less water than Sprague-Dawley rats after central or peripheral administration of another angiotensin peptide, Ang III [2]. Further, salt appetite was induced by chonic, peripheral Ang II administration in Sprague-Dawley rats but not in Fischer 344 rats [2]. While the two strains had similar plasma renin activity (PRA) in response to isoproterenol, they differed in blood pressure responses [2].

Gender differences have also been noted [6]. In males, a renin-like enzyme is present in much higher concentration than in females [6]. Furthermore, in the non-pregnant state, the drinking of female rats shows cyclical variation with the different phases of the menstrual and estrous cycles, with water intakes higher after ovulation [6]. Strain and gender differences are important to note, as they may give us insight into the genetic nature of the RAS as well as how to configure genetic knockouts for future research.

Acute Sodium Depletion by Furosemide in the Rat

In a series of experiments, Rowland and colleagues (1996) aimed to find a relationship between sodium appetite and brain activation after furosemide. These results were compared to results found after increases in brain renin [16]. After furosemide (10 mg/kg, subcutaneous, two injections 2 hours apart) and a 24-hour period of fluid restriction, rats showed increased water and sodium (0.3 M NaCl) intakes [16]. There was a slight preference for sodium, since "sodium fractions," or measures that indicate the degree of sodium appetite, were about 0.64 (as determined by amount 0.3 M NaCl ingested (mL) divided by the total amount of water (mL) and 0.3 M NaCl (mL) ingested). If rats were allowed to drink water after furosemide injection but before the drinking test, they preferred sodium to an even greater extent (salt fraction of 0.91) [16]. In rats pre-exposed to a sodium free diet and given furosemide and restricted from sodium and water for 24 hours after injection, intakes of 0.3 M NaCl and water were about 16 mL and 7.5 mL, respectively [16]. Thus, intakes were significant in both cases but there was a larger preference for sodium. In rats pre-exposed to a regular chow diet, intakes of sodium and water were similar (around 14 mL and 13 mL, respectively) [16].

Brain activation in rats given a treatment similar to that in the previous behavioral study—those that received furosemide without access to water and pre-exposed to a sodium-free diet—revealed significant Fos-IR [16]. This was especially apparent in the SFO [16]. Furthermore, plasma renin activity (PRA; about 14 ng Ang I/mL/h), and plasma protein levels (about 9 g/dL) were significant ($P < 0.05$) in these rats while percent hematocrit (about 55%) was elevated but not significant [16]. In rats pre-exposed to a normal chow diet, similar results were found with slightly lower responses in each parameter, presumably because sodium and water are present in the normal chow diet [16].

While furosemide stimulated circulating Ang and the RAS, it was also found that infusion of renin into the brain caused Fos-IR in the brain and increased sodium and water intakes, although there was a larger preference for water than for sodium after this treatment than with furosemide [16]. Furthermore, the drinking response occurred more quickly, having a latency of less than 30 minutes, compared to the 24 hour latency period examined after furosemide [16]. Thus, the drinking response to furosemide may take many hours to develop.

While the study discussed above involves multiple injections of furosemide, physiological responses after a single injection of furosemide (10 mg/kg, subcutaneous) have also been examined. This study expanded on previous studies that did not analyze how responses to furosemide change over time intervals after injection. Examining the effect of time enabled the researchers to more clearly analyze the development of thirst and sodium appetite, as well as the contributions from the RAS and aldosterone. The results are summarized in very rough estimates without standard deviations or

significance values in Table 2-1 [20]. All rats were given a low sodium diet for 48 h immediately before analyses, with injections taking place during this time [20].

Table 2-1. Responses in rats after furosemide

H after furo	Amount 0.3 M NaCl / water Ingested (mL)	Aldo (pg/mL)	PRA (ng Ang I/ mL/h)	% Hematocrit	Plasma Protein (g/dL)	Fos-IR in SFO (# cells)
3	4 / 0.4	550	34	50.5	9.3	225
12	6 / 0.8	1200	13	52.5	9.1	150
24	10 / 4.6	1100	10	50.0	8.8	210

Taken together, these data indicate that after furosemide and sodium restriction, there is increased activation of the RAS, thirst, and sodium appetite after 3 hours [20]. After 12 hours, there is even greater thirst and sodium appetite with an increased contribution from aldosterone despite declining contributions from the RAS [20]. By 24 hours, there are even further increases in thirst and sodium appetite despite a decline in all other measures [20]. This suggests that the physiological responses to furosemide injection may hit a peak prior to the behavioral manifestation of these responses [20].

A similar study analyzed the contributions of Ang and aldosterone after furosemide using a similar procedure to that discussed above with the exception that mice were pre-exposed to a high-sodium diet rather than a low-sodium diet. Thus, increases in PRA and aldosterone were much slower onset. It was shown that PRA shows a steep increase after 8 hours of sodium restriction, is maintained at similar levels until 48 hours, then shows a return to near 8 hour levels after 120 hours [11]. In contrast, aldosterone responses were found to be more gradual [11]. The response was significant after 8 hours, but continued to increase after 8, 16, 24, and 48 hours [11]. By 120 hours, aldosterone levels were near 24 hour levels [11]. In short, Ang and aldosterone were shown to have different response

curves to sodium deficit in rats. Whereas PRA showed a steep increase, increases in aldsterone were more gradual.

Hypovolemia by Polyethylene Glycol in the Rat

Unlike furosemide injection, in which renal derived circulating Ang II is believed to have a role in the vascular, neural, and behavioral responses to hypovolemia, renal-derived Ang II after PEG injection may have a different role. It is suggested that peripheral Ang II produced after PEG injection somehow activates the cerebral RAS to produce its own Ang II of brain origin [23, 24, 27]. In other words, it is thought that thirst and sodium appetite induced by PEG injection has much less to do with renal renin secretion as with cerebral renin secretion, even though renal secretion starts the process. It has also been suggested that many types of receptors are able to detect changes in blood volume (not necessarily blood pressure) and may have an influence on PEG-induced drinking independent of the circulating RAS [24, 27]. While the role of the RAS remains uncertain after PEG treatment, it is nevertheless true that PEG stimulates gradual hypovolemia very well in rats, and elicits both thirst and sodium appetite.

As in the case with furosemide, differences in diet prior to injection have been found to alter the responses seen after injection. In rats given a sodium-rich diet prior to PEG injection, rats first drank water followed by saline and then a combination of both fluids [27]. For rats given a sodium deficient diet, they first drank saline, followed by water, and then a combination of the two [27]. Further, the onset of sodium appetite was quicker in rats given a sodium deficient diet (less than an hour) as compared to rats that were given a normal diet (about 5 hours) [27]. Thus, it is important to note when analyzing the responses to PEG that the diet prior to injection is taken into account.

In rats given a normal chow diet, intakes of water and 0.5 M NaCl 7 hours after PEG (30% w/v) injection were about 12 mL and 1.3 mL, respectively [27]. Twenty-four hours after injection, intakes were about 46 mL and 15 mL, respectively [27]. In a similar test involving smaller time frames, the same researcher gave rats either a two-bottle test involving both 0.5 M NaCl and water or either of the fluids alone [27]. The results are summarized in Table 2-2 [27].

Table 2-2. Drinking responses after PEG injection in rats

Bottle Test (mL)	Time after PEG (hs)	Water Intake (mL)	0.5 M NaCl Intake
Water Only	2	14	
	4	18	
	6	22	
Saline Only	2		4
	4		7
	6		10
Water and Saline	2	24	4
	4	32	9
	6	37	12

It can be seen from these data that PEG has the ability to stimulate both thirst and sodium appetite in the rat. Though not specifically addressed in this thesis, it is nevertheless interesting to note that subsequent administration of captopril, which inhibits the peripheral formation of Ang II from Ang I, increases drinking in each case and follows a similar pattern to the table shown above [27]. This may suggest that excessive Ang I is delivered to the brain, where conversion to Ang II is not inhibited since it is protected from captopril by the blood-brain-barrier and may therefore contribute to thirst and sodium appetite [27]. Thus, PEG-induced drinking may be partially or completely independent of the circulating RAS [27, 14].

In rats, plasma protein concentrations and percent hematocrit after PEG were similar to those found after furosemide, although brain activation was different and there

was not hyponatremia [7]. Like furosemide, rats given PEG were indeed made hypovolemic, as indicated by plasma protein (about 9.1 g/dL) and percent hematocrit (about 53%) 3 hours after injection [7]. These rats were pre-exposed to a sodium-free diet, and when pre-exposed to a normal chow diet, these measures were slightly lower although not significantly [7]. Whereas furosemide stimulated brain activation in the OVLT and SFO in rats, PEG stimulated activation in the SON and PVN, with weaker activation in the OVLT and SFO [7].

In short, it seems clear that PEG effectively stimulates the RAS and normotensive hypovolemia in rats, as indicated by increased Fos-IR, drinking of water and sodium, increased percent hematocrit and plasma protein levels, as well as increased PRA and aldosterone levels. The possible routes and roles of Ang II and the RAS after PEG and furosemide; however, may differ.

Cellular Dehydration in the Rat

In contrast to the previously described treatments, hypertonic saline administration leads to cellular dehydration and drinking without directly involving the circulating RAS, hypovolemia, or sodium depletion. Rather, increased osmosis of water from cells is detected directly by specific osmoreceptors in the gut and brain, and triggers the release of oxytocin and vasopressin from the posterior pituitary [1, 6, 8]. These hormones stimulate sodium excretion and renal water conservation and are correlated with increases in Fos-IR in structures with osmoresponsive neurons within the magnocellular hypothalamus and AV3V regions, as well as forebrain and hindbrain structures that mediate reflex, endocrine, and behavioral responses to osmoregulation [1, 6, 8].

In rats injected subcutaneously with 2.0 M NaCl, Fos-IR was significantly increased in all areas as compared to controls injected with isotonic saline (0.15 M NaCl) [12]. Fos-IR counts in five representative areas are shown in Table 2-3 [12].

Table 2-3. Fos-IR in rats after subcutaneous hypertonic saline injection

Brain area	Number of Fos-positive cells (M ± SE)
SFO	106 ± 22
OVLT	138 ± 21
PVN	978 ± 40
SON	78 ± 6
AP	137 ± 9

This Fos-IR data is similar to other routes of administration of hypertonic saline (intravenous or intraperitoneal) in rats, but differs from Fos-IR after furosemide or PEG since counts are higher in the SON and PVN, while they are decreased in other areas [13].

Exogenous Administration of Angiotensin in the Mouse

Like rats, injection of Ang into the third ventricle of the brain (intracerebroventricular) increases intake of sodium and water in mice [4]. After chonic infusion of Ang, mice ingested sodium that was two-to-three times their body sodium content per day, and water intake was 40-60% body weight per day [4]. Thus, centrally administered Ang clearly had some effect on the neural systems that contribute to thirst and sodium appetite in mice.

Unlike rats, however, intakes of food, water, and sodium solutions after systemic Ang II (intravenous or subcutaneous) were insignificant in mice, raising the possibility that mice are somehow more refractory to these treatments [4, 14]. The researchers measured intakes of sodium solution (0.2 M NaCl), water, and food after these three infusion sites, as seen in Table 2-4 [4].

Table 2-4. Methods used to analyze Ang injection in mice

Mode of Injection	Dose and Method	Time of Analysis
Intraperitoneal (ip)	direct injection; 10 μg/ mouse (n = 5) and 10 μg/ 10 g body wt. (n = 5)	after two and 6 hours
Subcutaneous (sc)	miniosmotic pump; 10 μg/ day for 6 or 7 days	at a specific time of day for 6 or 7 preinfusion (control) days, infusion days, and post-infusion days.
Intracerebroventricular (icv)	miniosmotic pump; 10 μg/ day for 5 days, then switched to artificial CSF for 5 days.	at a specific time of day for 5 preinfusion (control) days, infusion days, and artificial CSF infusion days.

After subcutaneous infusion of Ang, intakes varied between 0.5-1.1 mL of 0.3 M NaCl per day, as well as 2.2-4.0 mL of water per day [4]. The intakes during the infusion days did not differ from pre- or post-infusion days [4]. Thus, while central administration of Ang clearly had an effect on drinking in mice, systemic administration did not [4].

Like the previous study, drinking of water was not found after subcutaneous injection of Ang II at varying doses (200, 600, and 1200 μg/kg, sc)—doses 2 to 12 times higher than doses found in rats to elicit a drinking response [14]. In a 2 hour water-drinking test, mice drank about 0.2 mL after the 200 μg/kg dose and about 0.4 mL after the 600 and 1200 μg/kg doses [14]. Despite the lack of a drinking response after subcutaneous Ang II, mice did drink after hypertonic saline (about 0.9 mL), simple water deprivation (about 1.0 mL), and a high dose of isoproterenol (about 0.5 mL) [14].

In a different study using mice, water deprivation (which is thought to induce endogenous Ang production) was found to activate regions of the brain previously shown in rats to be associated with angiotensinergic nerve endings [31]. After 24 and 48 hours of water deprivation, Fos-IR was increased in areas similar to those activated in rats. However, Fos-IR after peripheral Ang II administration has not been studied in mice.

Acute Sodium Depletion by Furosemide in the Mouse

The drinking response after sodium depletion by furosemide has also been examined in mice. Prior to a 24 hour depletion period, mice were injected with furosemide (1.2 mg in 0.12 mL in normal saline, intraperitoneally) [33]. Afterwards, mice were allowed access to 0.3 M NaCl, water, and food for both 2 and 6 hour intervals [33]. After 2 hours, mice ingested 0.9 mL sodium, 0.4 mL water, and 0.4 g food [33]. After 6 hours, mice ingested 1.1 mL sodium, 0.6 mL water, and 1.1 g food [33].

Rowland & Fregly (1988) reported comparable intakes of sodium, though sodium was presented in concentration that was half that of Weisinger and colleagues (1990). After 2 hours of drinking time, mice given furosemide (1 mg/20 g weight) drank 1.4 ± 0.3 mL of 0.15 M NaCl on one test and 1.6 ± 0.2 mL on another test administered 1 week later [14]. While rats meet or exceed urinary sodium output [16], mice did not [15]. Also unlike rats, mice did not significantly increase sodium intakes on a subsequent test, in which rats nearly double their intakes [14, 21].

A hot topic for debate with regard to drinking in mice after furosemide are the contributions from the renal and brain RAS. In rats, administration of captopril at low and high doses decreases sodium appetite after furosemide by decreasing peripheral Ang II [16]. In rats this decrease is prevented by systemic administration of Ang II at doses that alone do not increase sodium appetite [16]. In mice, only high doses of captopril decrease sodium appetite caused by furosemide, and this decrease, as in rats, is prevented by systemic Ang II [33]. However, after a low dose of captopril in sodium deficient mice, there is an increase in sodium appetite, presumably because there is both an excess of peripheral Ang I that is able to enter the brain as well as an increase in number or affinity of central Ang II receptors, although this is not wholly known [33]. Likewise, it

may be the case that the number or affinity of Ang II receptors decreases in mice pre-exposed to a high-sodium diet. Furthermore, captopril does not increase sodium appetite in sodium-replete mice, indicating that without a need for sodium, there is not sufficient RAS stimulation to increase blood Ang I and be converted to Ang II inside the brain [33]. In spite of these analyses, studies that have analyzed RAS stimulation after furosemide in mice have failed to analyze Fos-IR, aldosterone contributions to drinking, and the extent to which furosemide actually causes hypovolemia in mice.

Hypovolemia by Polyethylene Glycol in the Mouse

Intakes of 0.15 M NaCl and water were studied in mice that received PEG injection (30% w/v, subcutaneous) [14]. In one group of mice, mice were injected and 2 hours later given a water drinking test for 6 hours [14]. The mice drank about 1.1 ± 0.3 mL, and most of the drinking occurred after only 2 hours [14]. In another group of mice, mice were pre-exposed to separate bottles of 0.15 M NaCl, then injected, and after 2 hours were given a two-bottle choice test of 0.15 M NaCl and water [14]. Intakes of sodium solution and water in this group of mice were 1.5 ± 0.4 mL and 0.7 ± 0.2 mL, respectively [14]. Thus, there is a quick, robust drinking of water and 0.15 M NaCl after PEG treatment in mice.

Curiously, after blood was collected in these mice and analyzed for percent hematocrit, no significant increases were found as compared to sham-injected controls (about 47.5% in each case) [14]. Thus, there is still some question as to how well PEG stimulates hypovolemia and the RAS in mice despite a robust drinking response [14].

Cellular Dehydration in the Mouse

Significant drinking of water (0.9 ± 0.2 mL/2 h) has been reported after hypertonic saline in mice [14]. These intakes were similar to those seen after 24 hours of simple

water deprivation [14]. Fos-IR in mice after hypertonic saline; however, has not been examined.

Objectives

One goal of these studies is to confirm or refute two hypotheses that may account for the fact that mice do not drink after peripherally administered Ang II. One hypothesis is that peripherally administered Ang does not have an effect on the mouse brain. However, if it is found that Ang does significantly increase Fos-IR, a second hypothesis is that some other inhibitory mechanisms must exist that prevent drinking. Our second goal is to further elucidate how mice respond to various treatments that mimic various components of the RAS. These analyses will take into account analyses of Fos-IR, drinking, hypovolemia, as well as aldosterone and PRA. These analyses may help explain why mice do not drink after exogenous Ang. Comparisons between the two species (rats and mice) will be made to further clarify differences that may exist.

CHAPTER 3
GENERAL PROCEDURES

Animals and Housing

Male and female mice primarily of the CD-1 strain were used, with the exception of a single study involving peripheral angiotensin administration, in which DBA and C- 57 strains were also used. Mice were between the ages of 3 and 9 months of age at the time of the experiment. Mice were housed separately in polycarbonate tub cages (13 x 10 x 10 cm) with Sani-chips (Teklad-Harlan) bedding. Cages were stored in a room with a temperature of 23 ± 1° C and with a 12:12 light:dark cycle. Purina laboratory chow pellets (#5001) and tap water were available ad libitum, except between injection and analysis. For several days leading up to the experiment, mice were handled to minimize confounding stress responses during and after injection, since the release of adrenocorticotropic hormone (ACTH), which is associated with stress, has been found to increase sodium appetite in mice [3]. After injection, mice were placed in a new cage with fresh bedding in order to limit ingestion of residual water or urine. A new environment was not thought to significantly increase stress since identical brands of tub cages and bedding were used in all experiments. In addition, all procedures were performed in the same room. After injection, food and water were restricted. Food and water restriction was an important step in the procedure, since a sodium-free diet (commonly used in the laboratory in a powder form) may be confounding since little is known about its effect on drinking behavior in mice. In addition, there is likelihood of spillage.

Drinking Studies

After injection, graduated burets—fitted with stoppers and spouts—of either distilled water, saline (of varying concentration), or both were introduced to the cages after the appropriate delay. Spouts were fitted between the bars of the cage on the top of the lid, and careful measures were taken to limit dripping. In tests that involved a choice test (two tubes introduced, one each of distilled water and saline), colored tape was placed on the tubes to identify them. The placement of the tubes was also taken into account—distilled water spouts were placed on the right side of half of the cages and on the left side of the other half. All drinking tests were performed between the times of 0800 and 1500.

Fos-Immunoreactivity (Fos-IR) and Brain Activation

Over the last 10 years, the use of Fos-IR has been central to the investigation of brain activation with regard to fluid balance research. The use of Fos-IR is particularly useful since, in contrast to earlier methods, provides for single-cell neuron resolution [13]. Basically, when neurons in the brain are stimulated, immediate early genes (IEG's) such as the Fos gene undergo rapid transcription and translation to produce protein products, which are detectable with appropriate antibodies. Antibodies bind to the protein products and also adhere to a stain, which is detected under the microscope in the cell body of neurons. In this way, staining of protein products of the IEG Fos gene allow for quantitative mapping of brain activation.

After the appropriate delay (1 h after Ang injection, 3 and 24 h after furosemide, 2 and 6 h after polyethylene glycol and 1 h after hypertonic saline), mice were anesthetized with sodium pentobarbital (200 mg/kg, intraperitoneal) and perfused intracardially using 4% paraformaldehyde and saline by gravity-drip infusion. Brains were then removed and

placed into paraformaldehyde solution overnight to be fixed. On the next day, brains were sliced coronally 75 μm thick using a vibratome. The slices were washed with sodium borohydride, which destroys peroxidases that limit the binding of antibodies. A primary antibody specific for the Fos gene (SC-52; Santa Cruz Biotechnology, 1: 20,000) was then added to the floating sections. After incubating in a cold room (4 ° C) for 2 days, secondary antibody (Zymed Labs, Inc.) was added. After rinsing the slices with potassium phosphate buffer solution, ABC stain (Vector Labs) was added to the slices and were then rinsed. After that, 3,3 diaminobenzidine (Sigma) was added to the slices. Slices were placed atop a shaker for about 10 minutes, or until gray-black and were again rinsed and placed from rostral to caudal on gelatin-coated slides for viewing under a microscope fitted with a camera for projection onto a television monitor.

Using a template, the numbers of Fos-positive cells were counted within each area of interest. Two observers counted, with the numbers averaged in each case. Only dark black cells were considered positively stimulated. For each area, the section with the most number of Fos-positive cells was used in statistical analyses. Specifically, seven brain areas of the OVLT were analyzed (Figure 3-1) [6, pp. 632]: subfornical organ (SFO), median preoptic nucleus (MnPO), anterior and posterior paraventricular nucleus (PVN), supraoptic nucleus (SON), basal striatum terminalis, lateral division BSTLD, not shown), area postrema (AP).

Blood Sampling and Hormonal Assays

For some of the studies involving furosemide and polyethylene glycol treatments, blood was collected for analysis. In these animals, mice were sedated by inhalation of isoflurane vapor (Aerrane, Henry Schein Inc., Melville NY) while trapped in a closed

container for not more than 10 seconds. The appropriate amount of anesthesia was determined by pinching the mouse-tail and feet with aggressive pressure. If flinching was found, animals were placed back into the container for a few more seconds.

Blood was collected using a capillary tube placed behind the eye, which punctures a retro-orbital artery that supplies blood to the eye. Blood was dripped into two 50 μl plastic vials containing ice-chilled EDTA. One was used for determination of plasma renin activity (PRA), while the other was used for determination of aldosterone.

The capillary tube that was used to collect the blood was immediately placed into a centrifuge and spun at 50,000 revolutions per minute for about 5 minutes. The capillary tubes were then placed into a hematocrit ratio reader, which uses geometric ratios based on the height of the hematocrit-plasma line compared to the height of the entire blood sample (Clay-Adams) within the capillary tube.

After that, blood was extracted from the capillary tube onto a clean side by connecting plastic tubing to the capillary tube and blowing lightly with the mouth. The slide was placed into a hand refractometer (Atago) to determine plasma protein concentration. The refractometer works by using the principle that plasma protein refracts light. The higher the protein content, the more light is refracted and the darker the field of view inside the refractometer.

The two centrifuge tubes filled with blood were kept over ice until chill-centrifuged to separate plasma from hematocrit. Plasma was extracted from the samples and was frozen at –60 °C until analyzed by radioimmunoassay. Concentrations of angiotensin I (called PRA, or plasma renin activity) was determined using a kit (Nen Life Science Products), as was aldosterone content (Diagnostic Products Corporation).

More specifically, the kits used an excess of radioactive tracer that binds to a ligand and either Ang I or aldosterone. The more Ang I or aldosterone present in the blood sample, more ligand bound to the hormone and less ligand was available to bind to the radioactive tracer, resulting in decreased radioactivity (as indicated by a gamma ray counter). In short, percent of the sample bound to ligand was inversely proportional to the amount of hormone present in the blood sample. A standard curve was constructed to determine how the ligand and tracer bind. Based on this curve, percentages of the sample that bound to the standard tracers were converted to Ang I values, given that specific percentages correspond to specific Ang I levels in the blood.

Analysis of Data and Statistics

Data were analyzed using SPSS version 11.0 software for Windows. Groups of data were compared using t-tests or one-way ANOVA with post-hoc (Duncan) tests. Significance value was set at $P < 0.05$.

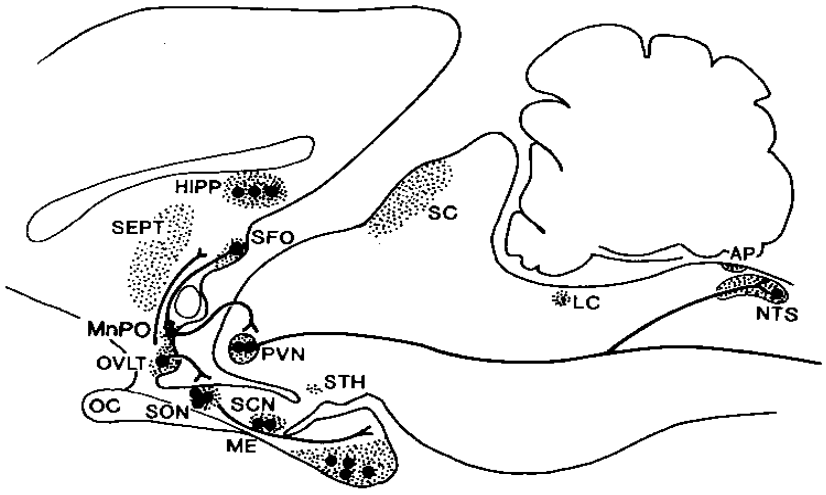


Figure 3-1. Locations of representative brain regions used in Fos-IR

CHAPTER 4
EXPERIMENT-SPECIFIC PROCEDURES

Exogenous Angiotensin Administration

Fos-Immunoreactivity

Strain and dose

Two males and two females of each of the CD-1, DBA, and C-57 strains were used to analyze the effect of strain of mice on Fos-IR. One male and one female mouse within each strain received a high (1000 μg/kg, sc) dose of Ang II while the other male and the other female within each strain received a low (200 μg/kg, sc) dose of Ang II. After injection, mice were placed into new individual cages without food or water for 1 hour. After that, brains were removed for Fos-IR.

Gender

In addition to the mice used above, which includes two males and two females of the CD-1, DBA, and C-57 strains, five additional CD-1 males and 5 additional CD-1 females were used. These additional mice were all given a low (200 μg/kg, sc) dose of Ang II. Furthermore, six CD-1 male mice were injected with water (1 mg/kg, sc) and used as controls. Thus, a total of twenty-eight mice were used. After injection, mice were placed into new individual cages without food or water for 1 hour. After that, brains were removed for Fos-IR.

Drinking

Twelve CD-1 male mice were injected with Ang II (200 μg/kg, sc) and four with water (1 mg/kg, sc). After injection, mice were placed into new individual cages without

food or water for 1 hour. After that, separate burets of water and hypertonic saline (0.45 M NaCl) were introduced to the cages. Water burets were placed on the right side of half of the cages and on the left side of the other half. Intakes were recorded after the next hour.

Acute Sodium Depletion by Furosemide

Fos-Immunoreactivity and PRA

Twenty-four CD-1 male mice were used. Mice were separated into two groups of twelve. In each group, seven mice were injected with furosemide (40 mg/kg, sc) and five were injected with water (4 mg/kg, sc). One group was placed into new individual cages where food and water was restricted for 3 hours. The other group was placed into new individual cages where food and water was restricted for 24 hours. After that, blood was collected from the ice to determine PRA. After that, brains were removed for Fos-IR.

Percent Hematocrit, Plasma Protein, and Aldosterone

The same twenty-four CD-1 mice used for determination of Fos-IR and PRA were used for determination of percent hematocrit, plasma protein, and aldosterone. Twelve of the mice were females and twelve were males. In six males and six females, blood was collected 3 hours after injection. Likewise, in the other six males and six females, blood was collected 24 hours after injection. During the time between injection and blood collection, mice were placed into new individual cages without food or water. Within the 3 and 24 h groups, six were injected with furosemide (40 mg/kg, sc) and six were injected with water (4 mL/kg, sc).

Drinking

Twenty-four CD-1 male mice were separated into four groups—those that received a furosemide (40 mg/kg, sc) or water (4 mg/kg, sc) injection and given a drinking test

either 3 or 24 hours after injection. There were six mice in each group. In the time interval between injection and the drinking test, mice were placed into new individual cages without food or water. After that, a single buret of isotonic saline (0.15 M NaCl) was introduced to each cage. After the next hour, intakes were recorded.

Twelve additional CD-1 male mice were used in a separate drinking test. Six were injected with furosemide (40 mg/kg, sc) and six were injected with water (4 mg/kg, sc). Mice were placed into new individual cages without food or water for 24 hours. After that, separate burets of 0.45 M NaCl and water were introduced to each cage. This test, involving hypertonic saline rather than isotonic saline, was designed to be a better indicator of a true sodium appetite. If mice drink hypertonic saline, it is more accurately assumed that the mice need it. Water and 0.45 M NaCl were placed on the right side of half of the cages and on the left side in the other half. After the next hour, intakes were recorded.

Hypovolemia by Polyethylene Glycol

Percent Hematocrit, Plasma Protein, Aldosterone, PRA, and Fos-IR

Ten CD-1 male mice were injected with PEG (30 % w/v) and six were given a sham injection. After that, mice were placed into new individual cages without food or water for 2 hours. Then, blood was collected for determination of percent hematocrit, plasma protein, and aldosterone.

Six additional CD-1 male mice were injected with PEG (30 % w/v) and two additional mice were given a sham injection. Afterwards, mice were placed into new individual cages without food or water for 2 hours. Then, blood was collected for determination of PRA. After that, brains were removed for Fos-IR.

Six different CD-1 male mice were injected with PEG (30 % w/v) and another six were given a sham injection. After injection, mice were placed into new individual cages without food or water for 6 hours. After that, blood was collected for determination of percent hematocrit, plasma protein, and aldosterone. Eight of these mice—the six that received a PEG injection and two of the sham injected controls chosen at random—subsequently had their brains removed for Fos-IR.

Drinking

Five CD-1 male mice were injected with PEG (30% w/v) and another two CD-1 male mice were given a sham injection (by inserting a needle and then withdrawing it with no fluid injected). Mice were placed into a new cage without food or water for 2 hours. After that, burets of 0.45 M NaCl and water were introduced to each mouse cage, with the water and 0.45 M NaCl on the right side of half of the cages and on the left side of the other half. Intakes were recorded at each hour for the next 2 hours. The same mice were used 1 week later using a similar injecting procedure but a different drinking test. A single buret of water was introduced to each cage 2 hours after injection. Intakes were recorded after the next hour of drinking time.

Six different CD-1 male mice were injected with PEG (30% w/v) and another six CD-1 male mice were given a sham injection. Mice were placed into a new cage without food or water for 6 hours. After that, burets of water were introduced to each mouse cage. Intakes were recorded at each hour for the next 2 hours. After drinking, these mice had blood withdrawn for analysis of percent hematocrit and plasma protein.

Intracellular Dehydration by Hypertonic Saline

Only Fos-IR was examined in mice with regard to intracellular dehydration. Eight CD-1 male mice were injected with 1.0 M hypertonic saline (10 mL/kg, ip). After

injection, mice were placed into new individual cages without food or water for 1 hour. After that, brains were removed for Fos-IR.

CHAPTER 5
RESULTS

Peripheral Angiotensin Administration

Brain Activation

Results (Figure 4-1A) showed no significant differences among strains in any brain area. Fos-IR was not dose-dependent, since there were no significant differences in Fos-IR between the 200 and 1000 μg/ kg doses of Ang II (Figure 4-1B). Further, Ang II significantly increased Fos-IR, as compared to controls, in the male SFO, PVNp, MnPO, BSTLD, and SON, as well as in the female SFO, MnPO, and BSTLD (Figure 4-1C). When comparing males and females, males had significantly higher activation in only the SON (Figure 4-1C). Since gender, strain and dose differences were largely inapparent, male mice of the CD-1 strain were used for the remaining experiments.

Drinking

There were no differences in sodium (0.45 M NaCl) (0.08 ±0.02 mL, 0.03 ± 0.03 mL) or water (0.07 ± 0.02 mL, 0.13 ± 0.06 mL) intakes between mice injected with Ang II and controls injected with water, respectively (Figure 4-1D).

Acute Sodium Depletion by Furosemide

Brain Activation

In both the 3 (45 ± 14 cells) and 24 (49 ± 8 cells) h groups, furosemide increased ($P < 0.01$) Fos-IR in the SFO (see Figure 4-2). In the 3 h group, Fos-IR increased ($P < 0.01$) in the BSTLD (48 ± 6 cells), while in the 24 h group Fos-IR increased ($P < 0.01$) in the MnPO (57 ± 8 cells) (see Figure 4-2). Two of the mice in the furosemide group analyzed

after 3 hours were omitted from analyses due to bad perfusions prior to Fos-IR that resulted in indistinguishable data.

Plasma Renin Activity and Hypovolemic Measures

After 3 (12.1 ± 3.1 ng Ang I/mL/h) and 24 hours (18.6 ± 4.8 ng Ang I/mL/h), PRA was significantly ($P < 0.05$) increased in mice injected with furosemide as compared to the corresponding controls (Figure 4-3A). Plasma protein was also increased ($P < 0.001$) in the 3 and 24 h groups (8.5 ± 0.20 g/dL and 8.6 ± 0.5 g/dL, respectively) as was percent hematocrit ($P < 0.05$) (51 ± 1 % and 52 ± 2 %, respectively) (Figure 4-3B and 4-3C). Levels of plasma protein and percent hematocrit in control mice were 7.2 ± 0.1 g/dL, 46 ± 3 % and 7.3 ± 0.2 g/dL, 49 ± 1 % for the 3 and 24 h groups, respectively (Figure 4-3B and 4-3C).

Aldosterone Levels

Three ($P < 0.01$) and 24 hours ($P < 0.001$) after furosemide injection, levels of aldosterone were significantly increased as compared to corresponding water-injected control mice (Figure 4-4).

Drinking

Three hours after furosemide injection, mice injected with furosemide showed an appetite for isotonic saline since they drank more ($P < 0.01$) 0.15 M NaCl than controls (Figure 4-5A). However, after 24 hours, there was not a significant difference in the amount of isotonic saline drank between mice injected with furosemide and control mice.

In a more stringent and reliable drinking test, two burets—one of hypertonic (0.45 M NaCl) saline and water—were introduced to each mouse cage rather than a single buret of isotonic saline. The hypertonic saline used in this drinking test was meant to better indicate a true sodium appetite than with the use of isotonic saline. Mice injected

with furosemide drank more ($P < 0.05$) 0.45 M NaCl but not water ($p = 0.84$) than controls (Figure 4-5B).

Hypovolemia by Polyethylene Glycol Administration

Hypovolemic Measures

Percent hematocrit and plasma protein were significantly increased ($P < 0.05$) in mice injected with PEG (49.5 ± 3.5 %, 8.3 ± 0.8 g/dL) as compared to controls (44.7 ± 1.6 %, 7.3 ± 0.1 g/dL, respectively, see Figure 4-6B and 4-6C) 2 hours after injection. After 6 hours, these increases were even more robust (Figure 4-6B and 4-6C). Mice injected with PEG had a percent hematocrit of 59.2 ± 8.2 %, while in controls this was 46.5 ± 1.5 % ($P < 0.01$). Plasma protein levels were (10.4 ± 2.1 g/dL) in mice injected with PEG and (7.4 ± 0.4 g/dL) in controls. Noticeably, plasma protein and percent hematocrit increased between 2 and 6 hours. Qualitatively, some of the mice had very viscous blood as it was sampled, indicating extreme hypovolemia.

Aldosterone Levels

After 2 hours, aldosterone levels were significantly increased ($P < 0.05$) in mice injected with PEG (1007 ± 226 pg/mL) as compared to controls (291 ± 73 pg/mL) (Figure 4-6A). After 6 hours, aldosterone levels were not significantly increased in mice injected with PEG (1987 ± 1007 pg/mL) than controls (396 ± 34 pg/mL), and large standard deviations should be noted (Figure 4-6A). In the group of mice analyzed for aldosterone content 6 hours after PEG injection, there were some outliers. Whereas most individual aldosterone levels ranged from 900 to 2000 pg/mL, the aldosterone content for one mouse was just over 4000 pg/mL, and in two others was fewer than 500 pg/mL.

Plasma Renin Activity

PRA was increased ($P < 0.05$) in mice injected with PEG (19.3 ± 6.1 ng Ang I/mL/h) as compared to controls (4.8 ± 0.6 ng Ang I/mL/h) (Figure 4-7). Results of the plasma renin activity for mice analyzed after 6 hours was not examined, but it is assumed that there is a further increase.

Brain Activation

Six hours after injection, mice injected with PEG had significantly ($P < 0.05$) increased Fos-IR in five of the seven areas analyzed (Figure 4-8 and Figure 4-9) as compared to sham injected controls. Brain activation in the two remaining areas was near significant ($p = 0.08$), with somewhat larger standard deviations precluding significance in these areas. However, there were no outliers.

Drinking

Two hours after PEG injection, mice did not drink more hypertonic saline (0.45 M NaCl) or water than sham-injected controls after either 1 or 2 hours of drinking time (Figure 4-10). Again, there were large standard deviations among mice injected with PEG; however, there were no outliers. One week later, a subsequent water-only drinking test was performed. Two hours after PEG injection, mice injected with PEG drank significantly more ($P < 0.05$) water (0.7 ± 0.3 mL) than controls (0.3 ± 0.2 mL) after 2 hours of drinking time (Figure 4-10).

Six hours after injection, a similar 2 hour water-only drinking test was given to mice injected with PEG or given a sham injection. Intakes were recorded after both 1 and 2 hours of drinking. After 1 hour of drinking, intake of water (0.8 ± 0.5) was similar to that seen after 2 hours of drinking 2 hours after PEG injection (0.7 ± 0.3 mL) and was significantly increased ($P < 0.01$) over the corresponding controls (Figures 4-10 and

4-11). After 2 hours of drinking, intake (1.2 ± 0.6 mL) was significantly increased ($P < 0.01$) over the corresponding controls (0.3 ± 0.3 mL) and was only slightly increased over that seen after 1 hour of drinking (Figure 4-11).

A separate experiment analyzed percent hematocrit and plasma protein after drinking of water to find out if drinking of water decreases these measures. Percent hematocrit and plasma protein both decreased after drinking to near baseline levels seen in animals given a similar procedure but not allowed to drink (Figure 4-11). Percent hematocrit dropped from 59.2 ± 8 % to 51 ± 7% while plasma protein dropped from 10.4 ± 2.1 g/dL to 8.6 ± 1.6 g/dL (Figure 4-11). However, large standard deviations precluded significance. Further, there were no outliers in this group of mice.

Intracellular Dehydration by Hypertonic Saline (Control Animals)

Brain activation after this procedure was largely similar to that seen in mice injected with Ang (200 μg/ kg, sc), with the exception that Fos-IR was increased in the SON (about 75 cells versus about 39 cells, respectively) and decreased in the SFO (about 27 cells versus about 75 cells, respectively).

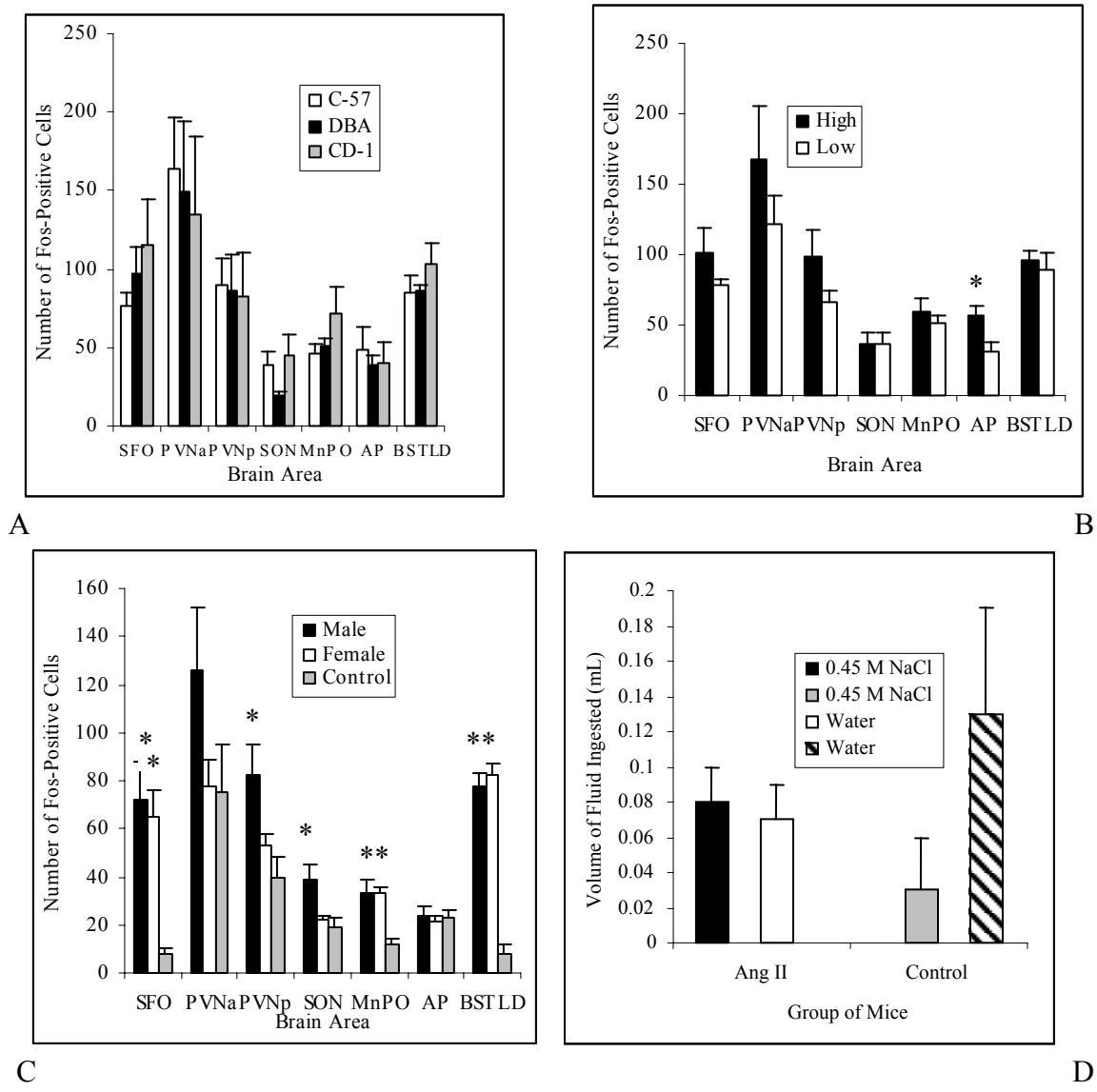


Figure 4-1. Fos-IR in various groups of mice and drinking among CD-1 male mice after subcutaneous Ang II. A) There were no apparent strain differences in Fos-IR (shown as M ± SD) among C-57, DBA, and CD-1 mice (males and females) in any brain area. B) Significant differences (*P < 0.05) in Fos-IR existed only in the AP when using a high dose (1000 µg/kg) as compared to a low dose (200 µg/kg) of Ang II. C) Ang II (200 µg/kg) elicited significant (*P < 0.05) Fos-IR in many areas of the male and female mouse brain as compared to controls injected with water. D) There was not significant drinking of either water or 0.45 M NaCl immediately after subcutaneous Ang II (200 µg/kg). Intakes were recorded after 1 hour of drinking time.

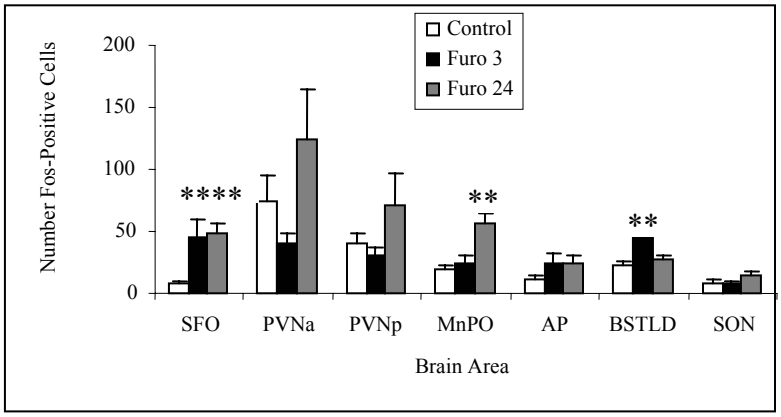


Figure 4-2. Fos-IR 3 and 24 h after furosemide. Compared to controls, Fos-IR (shown as M ± SE) was significantly (**P < 0.01) increased in the SFO and BSTLD 3 h after furosemide (40 mg/kg, sc) and in the SFO and MnPO 24 h after furosemide.

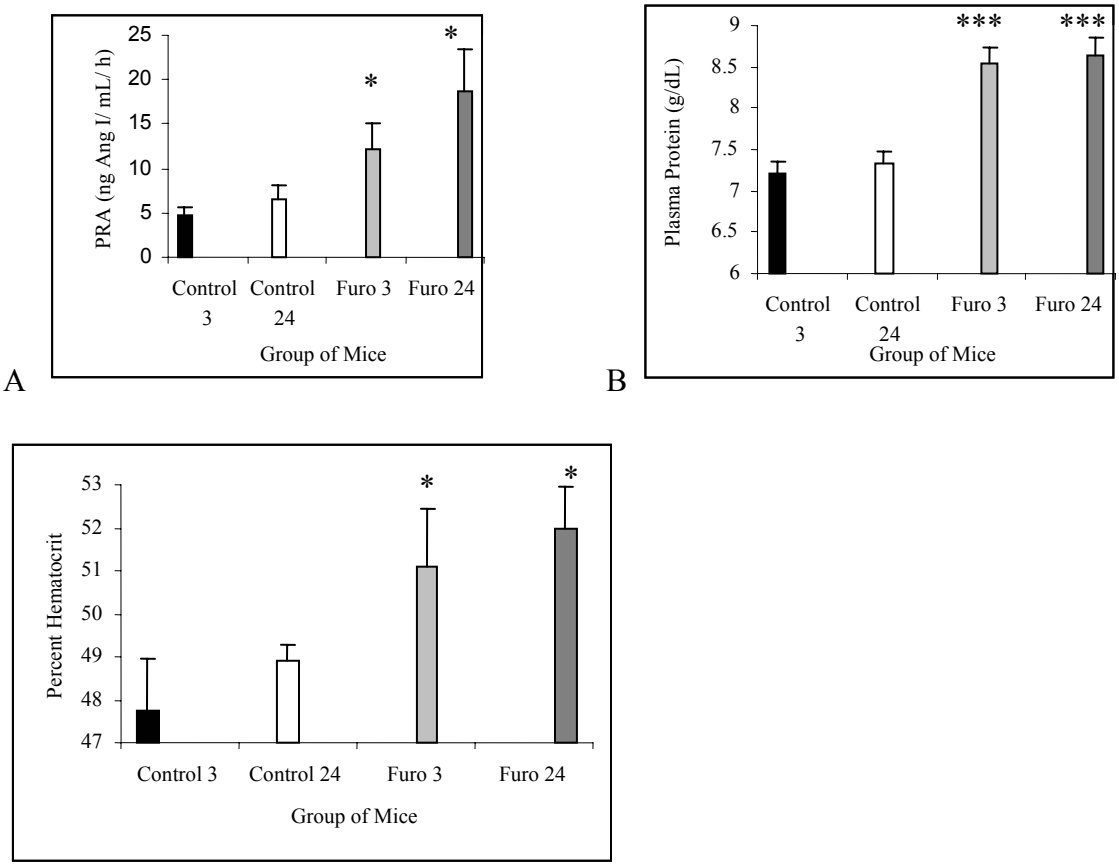


Figure 4-3. Plasma renin activity and hypovolemic measures 3 and 24 h after furosemide. A) PRA (*P < 0.05), B) plasma protein (***P < 0.001), and C) percent hematocrit (*P < 0.05) were significantly increased 3 and 24 h after furosemide (40 mg/kg, sc) as compared to the corresponding controls injected with water. Values are shown as M ± SE.

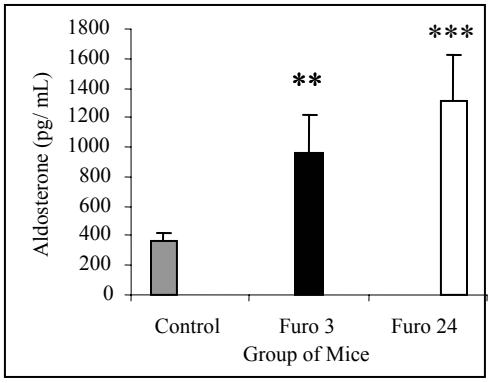


Figure 4-4. Aldosterone levels 3 and 24 h after furosemide. Three hours after furosemide, aldosterone levels were significantly increased over water-injected controls (**P < 0.01), while the response was even more robust (***P < .001) after 24 hours. Values are shown as M ± SE.

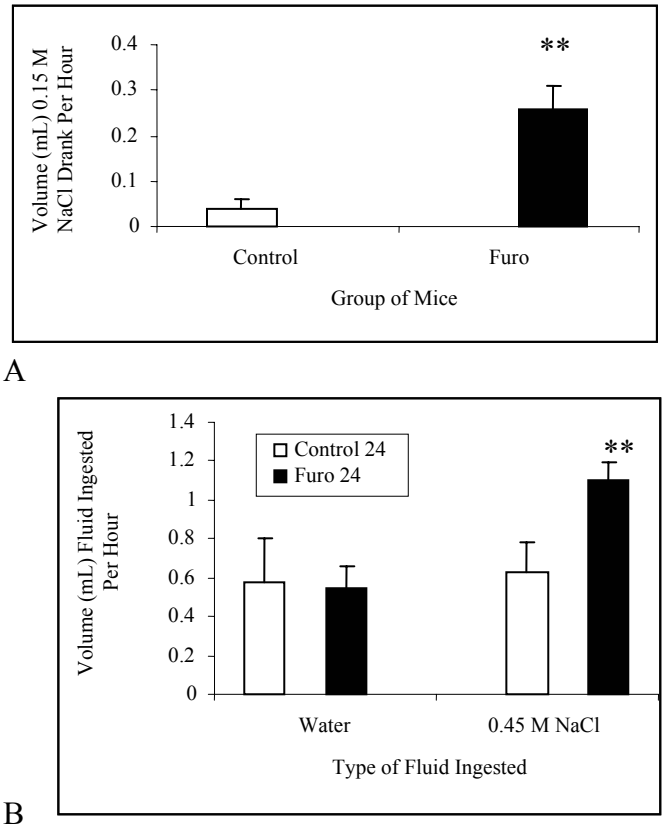


Figure 4-5. Drinking responses 3 and 24 h after furosemide. A) In a sodium-only drinking test given 3 h after furosemide (40 mg/kg, sc), mice drank significantly (**P < 0.01) more isotonic saline (0.15 M NaCl) than did controls. B) In a water and sodium (0.45 M NaCl) drinking test given 24 hours after injection, mice drank more sodium (**P < 0.01) but not more water than controls. Values are shown as M ± SE.

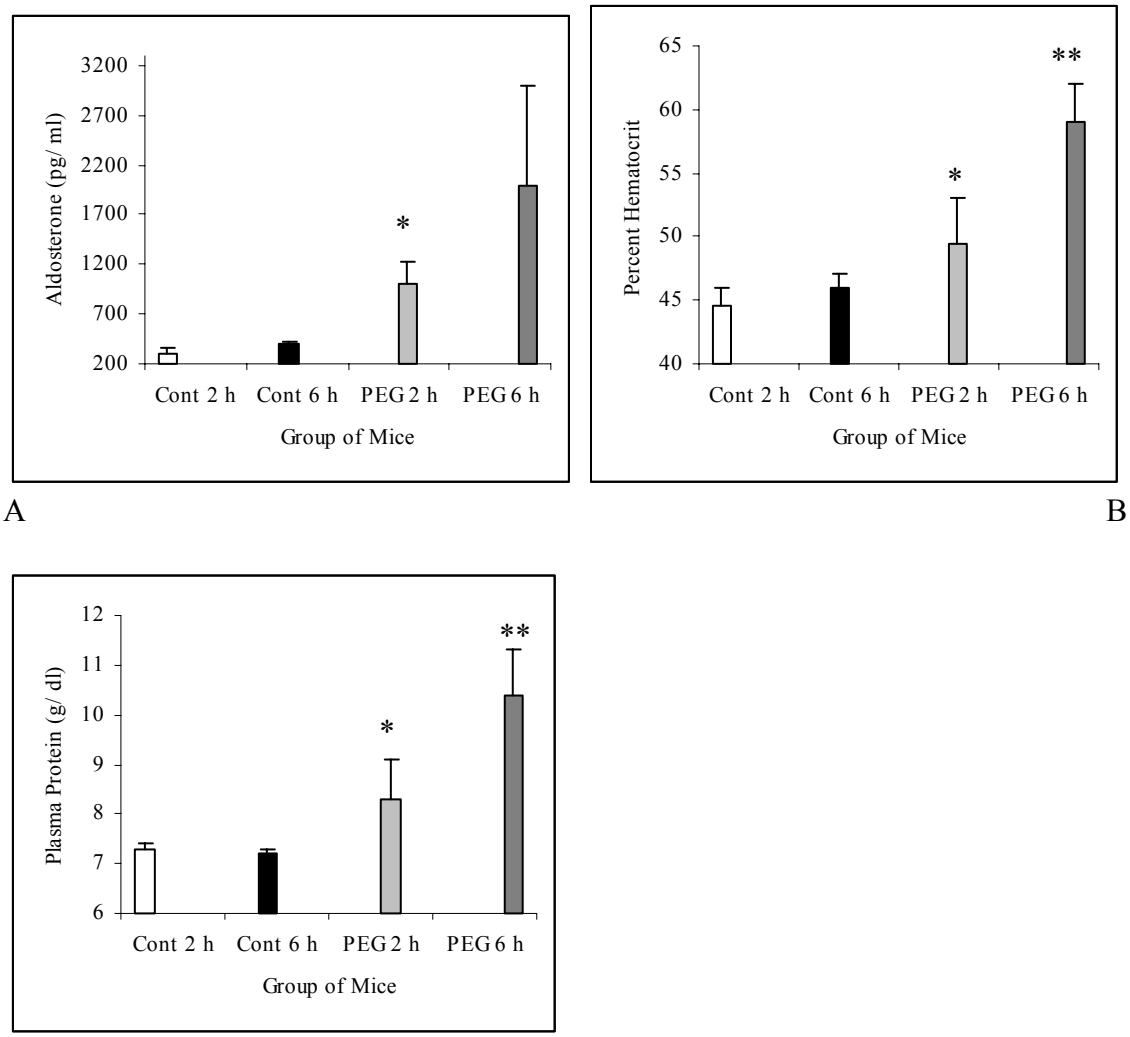


Figure 4-6. Aldosterone, percent hematocrit, and plasma protein 2 and 6 h after polyethylene glycol. A) Aldosterone levels significantly (*$P < 0.05$) increased after 2 hours, but not after 6 hours as compared to corresponding controls. B) Percent hematocrit significantly increased (*$P < 0.05$) after 2 hours and was further increased ($P < 0.01$) after 6 hours, as compared to the corresponding controls. C) Plasma protein levels significantly (*$P < 0.05$) increased after 2 hours with a further increase (**$P < 0.01$) after 6 hours, as compared to the corresponding controls. Values are shown as M ± SE.

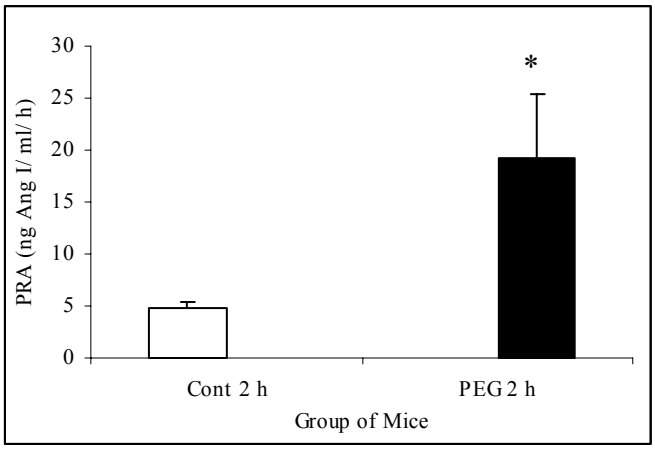


Figure 4-7. Plasma renin activity 2 h after polyethylene glycol. Mice injected with PEG had significantly increased (*P < 0.05) PRA as compared to sham-injected controls. Values are shown as M ± SE.

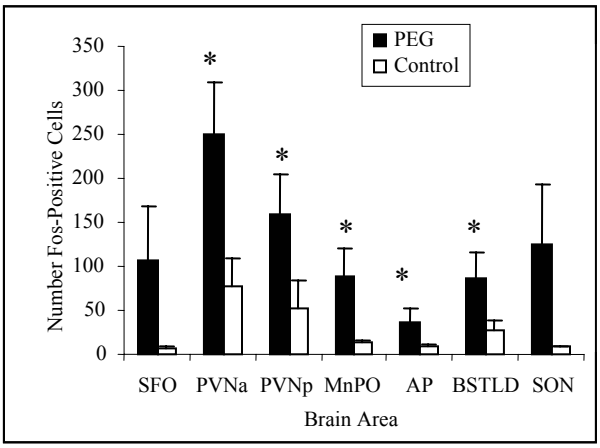


Figure 4-8. Fos-IR 6 h after polyethylene glycol. Fos-IR was significantly (*P < 0.05) increased in five of seven areas analyzed and was near significant (P = 0.08) in the other two areas. Values are shown as M ± SE.

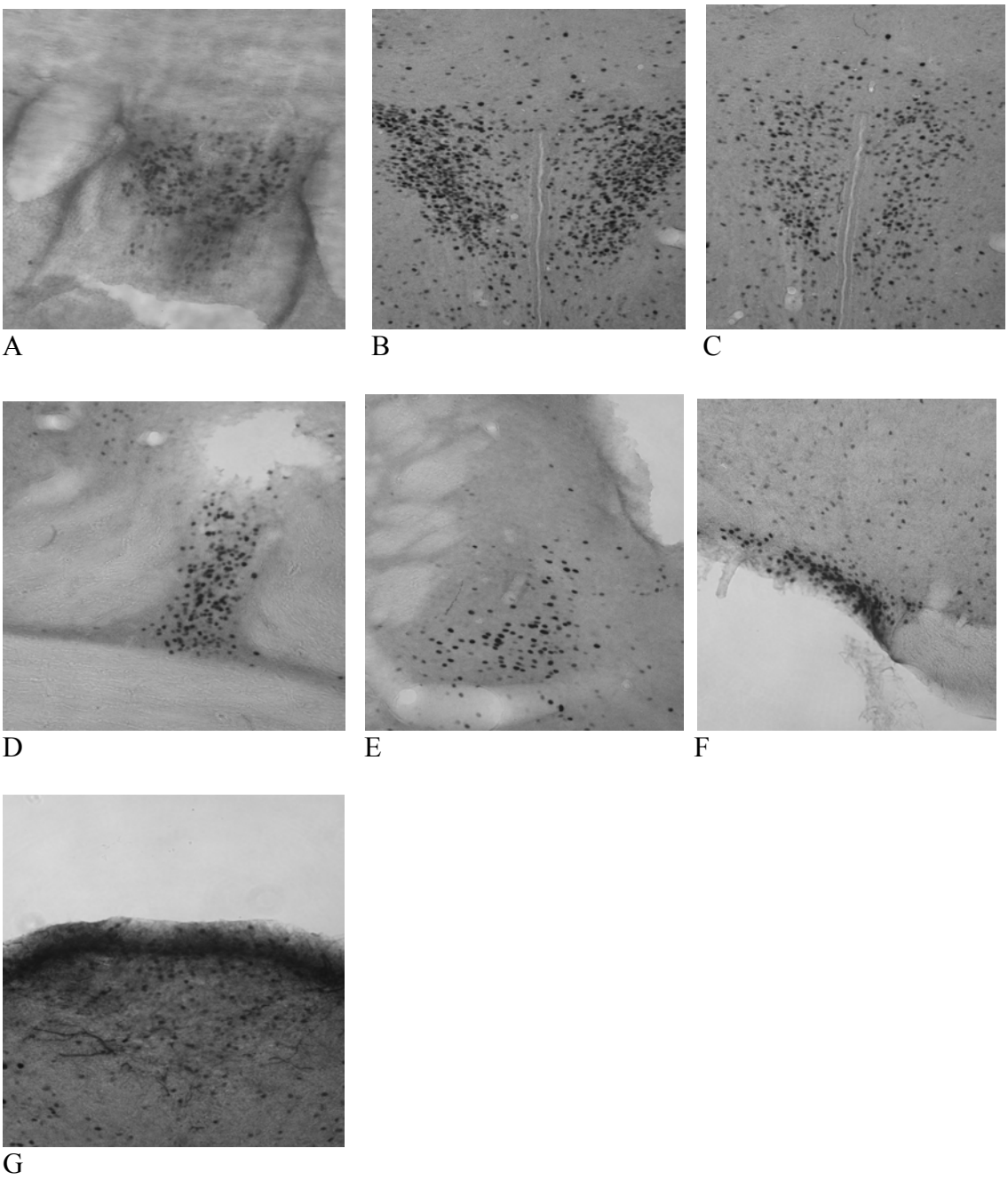
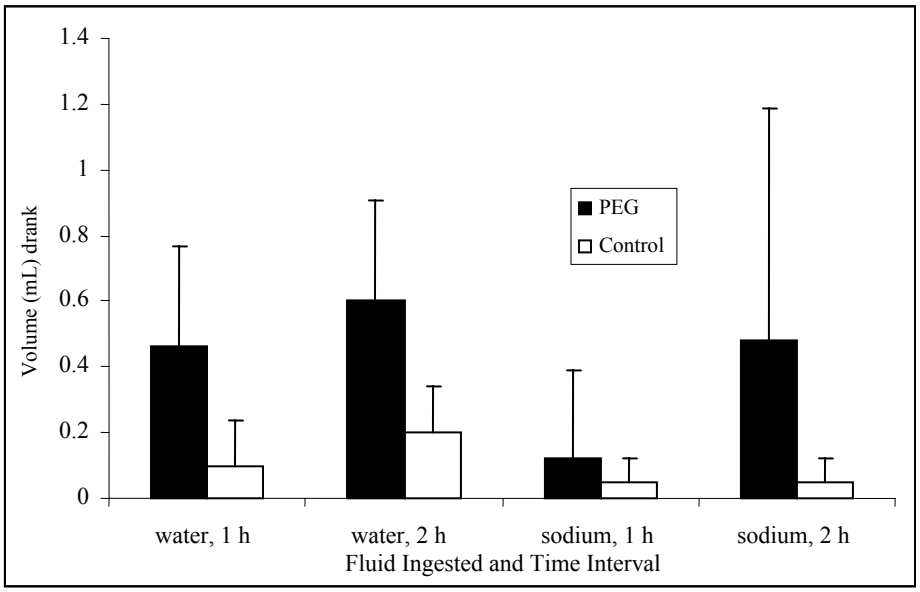


Figure 4-9. Microphotographs showing Fos-IR 6 h after PEG treatment in mice. A) subfornical organ (SFO), B) paraventricular nucleus, anterior (PVNa), C) paraventricular nucleus, posterior (PVNp), D) median preoptic nucleus (MnPO), E) basal striatum terminals, lateral division (BSTLD), F) supraoptic nucleus (SON), G) area postrema (AP).



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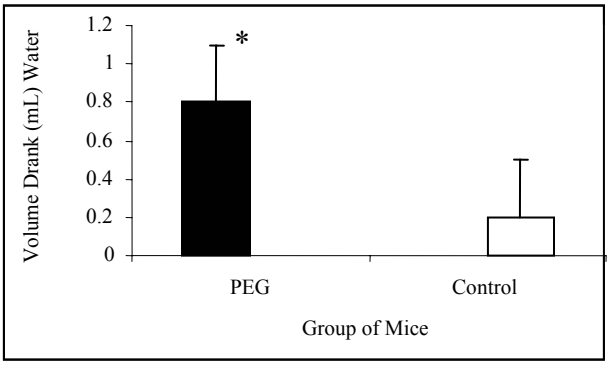
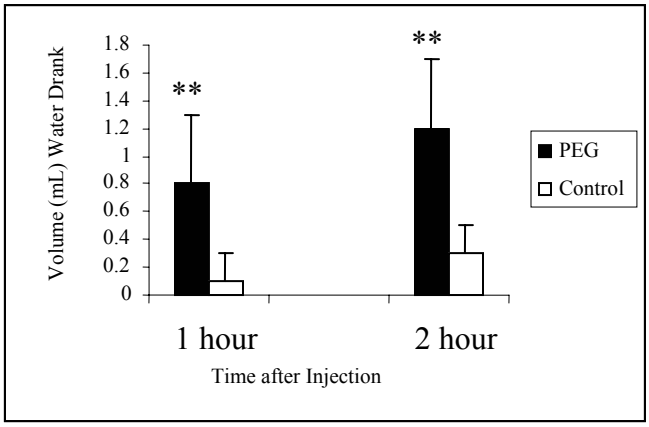


B

Figure 4-10. Drinking responses 2 h after polyethylene glycol. A) In a choice drinking test, mice injected with PEG drank more water after 1 and 2 hours of drinking time, but was not significant due to large standard deviations. Mice injected with PEG also drank an increased amount of sodium (0.45 M NaCl) as compared to controls, but this increase was very small. B) In a water-only drinking test, mice injected with PEG drank significantly (*$P < 0.05$) more water than controls. Values are shown as M ± SD.



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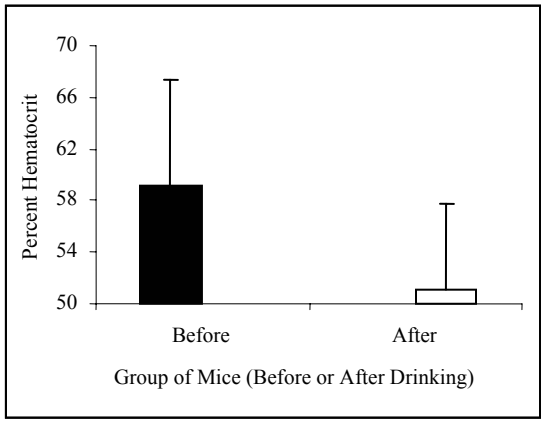

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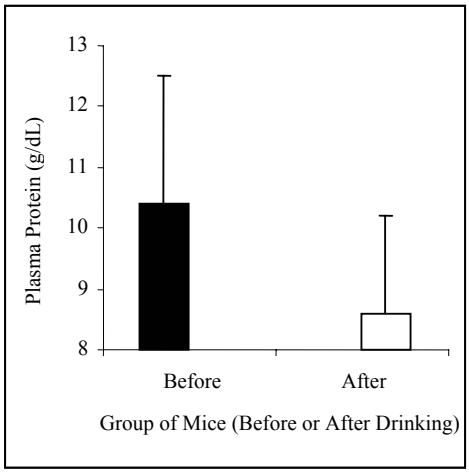

C

Figure 4-11. Drinking 6 hours after polyethylene glycol and its effect on percent hematocrit and plasma protein levels. A) Water intake was significantly (**P < 0.01) increased after 1 and 2 hours of drinking time 6 hours after PEG injection. B) Percent hematocrit and C) plasma protein decreased after the 2 hours of drinking water as compared to controls that did not drink, but these decreases were not significant. Values are shown as M ± SD.

CHAPTER 6
DISCUSSION

The lack of drinking response to peripheral Ang II in mice is particularly curious given that the response is robust in rats. The aim of these experiments was to expand upon results found by Rowland & Fregly (1988) and others that have studied the relative lack of dispsogenicity to peripheral Ang II in mice. Unlike previous studies, these experiments include Fos-IR results, which help to elucidate whether or not Ang actually enters the mouse brain. Many of the experiments included parametric analyses that analyze the effect of time. These analyses may further elucidate some of the questions regarding the lack of dipsogenicity of Ang in mice.

It can be suggested that peripheral Ang II has an effect on the mouse brain, since results showed significant increases in Fos-IR after peripheral Ang II in many brain areas, especially the SFO. From Fos-IR data alone; however, we cannot conclude that Ang II enters the mouse brain, since transcription of the Fos gene can take place though an effect in the brain that could be mediated by Ang action without Ang entering the brain. Further tests may wish to use Ang receptor antagonists and their effect on Fos-IR after Ang II injection in mice to confirm the hypothesis that Ang II entry into the brain increases Fos-IR.

In rats, exogenous Ang elicits Fos-IR in the SFO, AP, SON and PVN [13]; however, Fos-IR in mice was not as robust in the latter structures. Different strains and genders of mice were analyzed since both strain and gender differences have been noted in rats [2]. These parameters; however, were abandoned after preliminary data suggested

that these differences were, for the most part, not initially apparent. Furthermore, since the estrous cycle of the female mice used in our studies were not taken into account, the gender differences that we did find may not be wholly accurate. It was therefore our goal to further elucidate the roles of Ang and of the RAS in response to dehydration in mice by stimulating the RAS endogenously.

In a second set of experiments, excessive sodium loss (natriuresis) was stimulated by injection of furosemide. This experiment differed from that performed by Rowland & Fregly (1988) since: (1) urinary sodium loss was not examined, (2) levels of circulating ANG II and aldosterone as well as brain activation were examined, (3) only one drinking test was performed, (4) food and water were restricted as opposed to giving a sodium-free diet, (5) mice were analyzed after 3 and 24 hours of depletion time. Since urinary output was not analyzed and multiple drinking tests were not performed with the same mice, within-subject analyses were not an issue. Here, the response to sodium deprivation was compared between mice injected with furosemide and analyzed after 3 and 24 hours of depletion time, as well as in controls injected with water.

Compared to controls, drinking of 0.15 M NaCl was significant after 3 hours but not after 24 hours. However, this data may be misleading since it is thought that mice ordinarily do not achieve isotonic balance by ingesting isotonic sodium solution. Rather, mice ordinarily ingest sodium first and then water to obtain an isotonic balance [16]. Thus, a choice test involving hypertonic saline and water was performed. In this more stringent choice test 24 hours after injection, drinking of 0.45 M NaCl was robust, while drinking of water was not. Thus, while furosemide may stimulate a brisk sodium deficiency within an hour or so after injection, the appetite does not come until much

later. After 3 hours, intake of 0.15 M NaCl was below 0.5 mL; however, after 24 hours the intake increased over 3-fold to 1.4 mL. This result is similar to that seen in rats in which it is suggested that it takes furosemide at least 5 hours to elicit sodium appetite [20], even though this is enhanced by pre-exposure to sodium in rats [21] but not in mice [14].

With regard to brain activation after furosemide, the SFO was activated 3 and 24 hours after furosemide. Brain activation was not sustained for both time periods in any other area, although there was activation in the BSTLD after 3 hours and the MnPO after 24 hours. This result is similar to that seen in rats, in which there is a large increase in the SFO but is modest in other areas, even though the response here is about one-fifth the response seen in rats. These results are also similar to that seen in mice after exogenous Ang. This result emphasizes the importance of the SFO in sodium appetite. It is thought that the SFO contains mainly angiotensin type 1 (AT1) receptors and has many connections to other areas in the peri-OVLT region [6]. Further, it lies most dorsal within the anteroventral third ventricle (AV3V) and therefore most vulnerable to the bloodstream [6].

The increase in aldosterone levels in mice both 3 and 24 hours after furosemide are not surprising. In rats, it was found that there is a gradual increase in aldosterone up to 48 hours after injection [20]. In rats, these were found to be 550 pg/mL and 1200 pg/mL after 3 and 24 hours, respectively [20]. In mice, these values were about 970 pg/mL and 1320 pg/mL after 3 and 24 hours, respectively. However, mice require a much larger dose of furosemide than rats (40 mg/kg as opposed to 10 mg/kg) to produce a similar effect.

These studies were able to confirm that furosemide, as in rats, does induce hypovolemia in mice. PRA, plasma protein, and percent hematocrit were significantly increased after 3 hours with further increases seen after 24 hours. These responses; however, differ in part to those found in rats. In rats, all three parameters decreased from 3 to 24 hours [11], while in mice they increased. PRA was also significantly higher in rats as compared to mice after 3 hours (about three-fold), while PRA was lower in rats as compared to mice after 24 hours [11]. Percent hematocrit and plasma protein levels were similar at both time intervals after injection. This suggests that even though hypovolemia and sodium deficiency may be produced within 3 hours, drinking is not, and may require more time to develop.

A normotensive hypovolemia not involving a change in tonicity of the ECF was stimulated by PEG. Brain activation after this procedure was significant in many brain areas 6 hours after injection. The lack of significance in other areas may be attributed to a low number of animals in both the experimental and control groups. Compared with furosemide and Ang II, brain activation was higher after this procedure in all brain areas. This differs in part from that seen in rats. Compared with furosemide, PEG increased Fos-IR in the SON and PVN but not in the SFO and OVLT [7]. In mice, PEG increased Fos-IR in all areas as compared to furosemide. More specifically, in rats, PEG stimulated around 26 Fos-positive cells in the SFO, 26 in the OVLT, 160 in the SON, and 113 in the PVNm [7]. In mice, these values were much higher across the board with the exception of the SON. Fos-IR in mice after hypertonic saline was increased SON and PVH, but not in the SFO or other areas as compared to controls. This is largely similar to the pattern seen in rats.

In rats, PEG stimulates both thirst and sodium appetite [28]. When rats were given access to both 0.5 M NaCl and water, they ingested 24 mL of water and 4 mL of NaCl after 2 hours and 37 mL of water and 12 mL of NaCl after 6 hours [28]. Intakes of water correspond to around 5% of bodyweight after 2 hours and about 10% of bodyweight after 6 hours, with NaCl intake after 6 hours near 4% of bodyweight [28]. In an experiment in which only water was available to rats after PEG injection, rats ingested around 14 mL after 2 hours and 22 mL after 6 hours [28]. Thus, intake of water was decreased when given alone as compared to when water and sodium are given simultaneously.

In mice, these responses are much less robust, especially that of salt intake. In a choice test involving water and 0.45 M NaCl, mice drank around 0.5 mL and 0.6 mL water after 1 and 2 hours, respectively; and around 0.1 mL and 0.5 mL of 0.45 M NaCl after 1 and 2 hours, respectively. These intakes correspond to around 2% of bodyweight in the case of water ingestion and 0.3% in the case of NaCl ingestion—percentages that are much less than that seen in rats.

Since the choice-test did not show a substantial sodium appetite 2 hours after PEG injection in mice, a water-only drinking test was administered to decipher whether drinking of water is robust when administered alone. This was indeed the case, and intakes of water after 2 hours of drinking time (0.8 ± 0.3 mL) were increased ($P < 0.05$) over controls (0.2 ± 0.3 mL).

In a duplicate experiment given 6 hours after injection, intakes of water increased in mice injected with PEG ($P < 0.05$) after both 1 (0.8 ± 0.5 mL) and 2 (1.2 ± 0.5 mL) hours over corresponding controls injected with water (0.1 ± 0.2 mL and 0.3 ± 0.3 mL, respectively). Thus, it was hypothesized that while PEG did not elicit a substantial

sodium appetite, PEG did elicit a strong thirst response. It was hypothesized that drinking of water relieves hypovolemia after PEG in mice. This may have been true, since after 2 hours of drinking water, both percent hematocrit and plasma protein were decreased ($p > 0.05$) as compared to controls not allowed to drink.

The question of whether PEG elicited a strong hypovolemia was answered in analyses of plasma protein and hematocrit. As compared to controls, both of these measures were increased ($P < 0.01$) in mice analyzed 6 hours after PEG. These measures were particularly robust, and were significantly ($P < 0.05$) higher than those seen either 3 or 24 hour after furosemide. Plasma protein levels exceeded 10 g/dL and percent hematocrit was almost 60%.

Aldosterone, thought to be highly correlated with sodium appetite in rats, was not significantly increased in mice 6 hours after PEG (about 2000 ± 1000 pg/mL) as compared to controls (about 400 ± 20 pg/mL) and there were very large standard deviations. The wide range of aldosterone levels in these mice may help explain why, in these experiments, there was not a substantial sodium appetite after PEG.

Since results with PEG found in these studies were, for the most part, highly variable with large standard deviations, it was hypothesized that the dose used (30% w/v) may not have been high enough to induce a universal hypovolemia among the experimental group. In subsequent experiments performed by Rowland & colleagues [18], both 25% and 40% (w/v) doses were used and blood analyses were performed 6 and 24 hours after PEG injection. Six hours after PEG injection, mice drank about 1.0 mL water after receiving the 25% dose and about 2.5 mL after the 40% dose [18]. Thus, the increased dose produced substantially increased drinking of water [18]. Plasma protein,

percent hematocrit, PRA, and aldosterone levels also increased in a dose-dependent way, although increases were not as large as that seen with drinking [15]. When analyzing the correlations between different measures after PEG in mice, significance was found between all physiological measures, and none of the measures were significantly correlated with the amount of fluid ingestion [18].

In the studies presented here, similar correlations could be noted. Because parametric analyses were a large focus of these experiments, within-subject analyses could not be performed for the majority of the mice injected with PEG, since animals were sacrificed after injection. However, in mice injected with furosemide, Fos-IR in the SFO and PRA were found to be significantly correlated (see Figure 5-1).

Taken together, the results presented here suggest that there are many differences between rats and mice with regard to the contribution of Ang in the development of thirst and sodium appetite. As a whole, mice are less responsive to procedures that mimic various components of the RAS than rats. In response to exogenous Ang administration, rats show Fos-IR along the OVLT as well as in the SON and PVH, and there is clear drinking of both water and sodium. In mice, Fos-IR was increased SFO, MnPO, and BSTLD but was less consistent in other areas. Further, there was not robust drinking of either water or sodium.

In response to furosemide, rats show drinking of sodium, Fos-IR that is especially apparent in the SFO, greatly increased PRA after 3 hours with a lesser increase after 24 hours, a sustained increase in plasma protein and percent hematocrit at both time intervals, and an increase in aldosterone after 3 hours with a much sharper increase after

24 hours. Similar patterns of behavior were found in all aspects of the physiological responses in mice, including activation of the SFO at both time intervals.

Whereas rats show both drinking of water and sodium 2 and 6 hours after PEG, mice showed only a thirst for water, despite increased hypovolemia as indicated by percent hematocrit and plasma protein, increased PRA, and increased Fos-IR and aldosterone levels.

Based on these responses, it is suspected that mice are more sensitive to endogenous RAS stimulation by hypovolemia than rats. Both rats and mice show drinking after hypovolemia by PEG and acute sodium depletion by furosemide, but only rats show drinking after exogenous Ang injection. Whereas furosemide and PEG elicit hypovolemia detectable by baroceptors or other cardiopulmonary receptors, exogenous Ang does not elicit hypovolemia. However, these receptors have been suggested to increase drinking directly [18]. This may imply that mice rely on these receptors to a greater degree than rats with regard to drinking behavior. Further research needs to be done to address this issue.

Outlook. The importance of understanding the differences in the role of Ang between rats and mice may be explained by recent research in the field of genetics, which may use genetic knockout mice to determine the significance of a particular gene in the development of disease. Further, there has been a recent push involving research that analyzes gene therapy for hypertension. In this regard, further understanding of the RAS in mice, not just in rats, will prove to be an invaluable tool as targets for gene therapy are introduced.

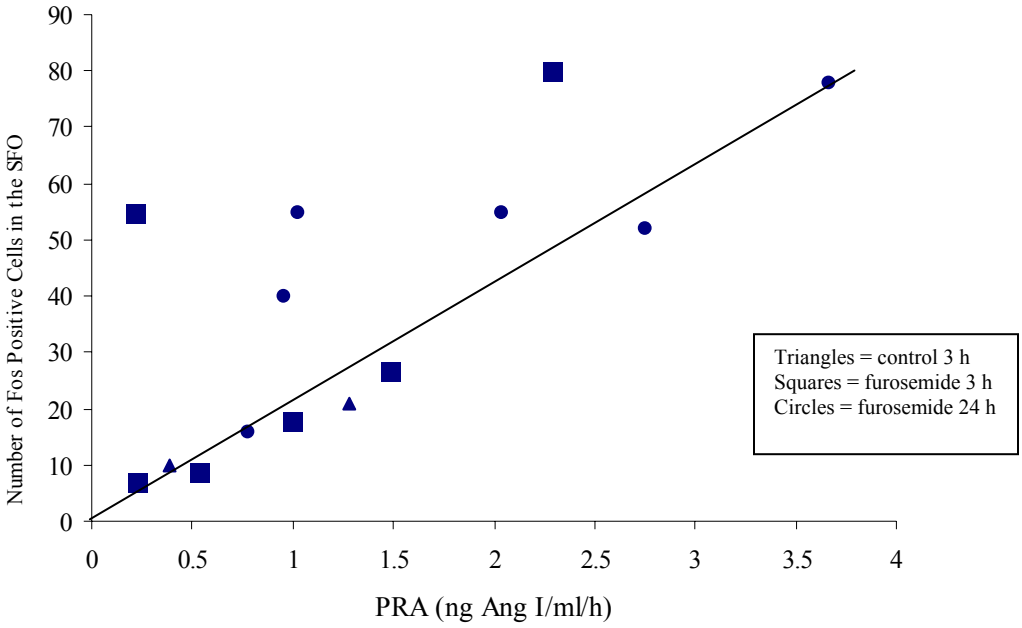

Figure 5-1. Correlation between number of Fos-positive cells (in the SFO) and PRA after furosemide ($r = 0.72$, $P < 0.01$)

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BIOGRAPHICAL SKETCH

Bradley E. Goldstein was born on February 20, 1980 in Dallas, TX. From there, Mr. Goldstein's family was moved to Nashville, TN and then to Pensacola, FL. During high school, Mr. Goldstein attended Pensacola Junior College and the University of West Florida to complete college-level academic requirements before matriculation in a primary undergraduate institution. After high school, Mr. Goldstein attended the University of Virginia (Charlottesville, VA) for his undergraduate work, majoring in Psychology. After graduate studies at the University of Florida, Mr. Goldstein plans to pursue a career in medicine.