Neural and Behavioral Correlates of Motivation in Sodium Deplete Animals

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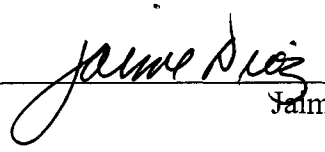

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Abstract

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Sodium is critical for survival and is maintained in strict homeostatic balance. Sodium depletion, by natural or experimental means, results in sodium appetite; a strong, specific drive characterized by the avid consumption of sodium-containing solutions at concentrations much higher than those accepted by sodium-replete animals. Research using experimental models of sodium appetite has demonstrated a role for brain regions including the subfornical organ and the supraoptic nucleus, and the hormones aldosterone and angiotensin, in the regulation of sodium balance. However, little research has focused on the role of the brain's motivation circuitry in the response to sodium appetite. Because sodium-deplete animals are highly motivated for sodium-containing solutions, and the effects of the drive state and reward experience can be studied separately, this model appears to be ideal for the study of reward motivation. The present work is aimed at characterizing sodium appetite as a model of natural reward. These studies use immunohistochemical and pharmacological measures to examine the role of the mesolimbic circuitry, specifically the nucleus accumbens, as well as dopamine and opiate receptors, in response to sodium depletion and expression of sodium appetite. Behavioral measures were also used to examine sodium appetite expression in a number of

experimental conditions. The results of this work indicate that under normal conditions, sodium appetite is not a strong activator of the nucleus accumbens, nor does its expression require activation of dopamine receptors. However, under sham-drinking conditions, sodium appetite dramatically activates the nucleus accumbens and depends on dopamine receptor activation. This work also supports the hypothesis that sodium appetite is a highly specific motivation. Sodium depletion has no effect on the response for another reward. The present results also suggest that sodium appetite expression may be driven by the increased hedonic value of sodium solutions, as opiate receptor blockade reliably decreases NaCl intake. Overall, these findings contribute to the characterization of sodium appetite as a reward model. And while it differs from many other models, the unique properties of sham-drinking during sodium appetite expression revealed in this work suggest a valuable model to study natural reward motivation.

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DEDICATION

To my husband, Howard Voorhies. This is a small measure of my infinite gratitude for your support. Thank you for your patience, encouragement, and seeing me through.

CHAPTER 1
GENERAL INTRODUCTION

An extensive body of research has focused on the neuroanatomical, pharmacological and behavioral responses to a variety of motivating stimuli. This research has revealed the neural systems and specific brain regions involved in mediating the response to motivation, the roles of numerous neurotransmitters in this response, and the characteristic behaviors that constitute a motivated response. Research has also identified specific properties of numerous motivating stimuli, how they activate the motivation circuitry and the resulting behaviors produced.

This previous research supports the hypothesis that the mesolimbic dopamine (DA) system is a critical neural substrate mediating response for rewarding stimuli (reviewed in Kelley & Berridge, 2002). Numerous models of reward have implicated the anatomical and neurochemical systems of this circuit in the response to such motivators. Drugs such as amphetamine and cocaine (Crombag et al., 2002; Hedou et al., 2002; Vanderschuren et al., 2002; Hope et al., 1992; Graybiel et al., 1990), as well as food (Park & Carr, 1998), copulation and receptive partners (Bradley & Meisel, 2001; Wenkstern et al., 1993), and interaction with offspring (Ferris et al., 2005; Champagne et al., 2004) have all been shown to activate the mesolimbic reward circuit, including the nucleus accumbens (NAc). Sensitizing regimens of both drug administration and sodium depletion have also been shown to alter neuronal morphology in the NAc (Li et al., 2003; Roitman et al., 2002; Robinson & Kolb, 1999, 1997). Studies examining neurochemical activity in the mesolimbic DA system have identified the response properties of several receptor types involved in response to motivating stimuli, including specific DA (Beninger & Miller, 1998) and opiate (Schad et al., 1996; Jones & Holtzman, 1992; Portoghese et al., 1988) receptors.

Although a great deal of research on the response for reward has supported mesolimbic DA system involvement in motivation, the role of this circuit in response to sodium appetite is less clear. The present studies are intended to contribute to an expanded understanding of the role of the motivation circuit and its associated

neurochemical systems in the response to sodium appetite. This, in turn, may contribute to a more detailed understanding of the characteristics of motivation for natural rewards.

Reward motivation

A "reward" is an event or experience that will increase the likelihood of a behavior. A reward typically satisfies an internal drive, and is sought out and consumed in a free-choice setting (Figlewicz, 2003). Other authors specify that the reward experience also involves both liking and wanting. Berridge (1996) defines liking as deriving pleasure and palatability, whereas wanting is defined as having an appetite and the disposition to consume the reward. While a reward will reinforce a behavior by satisfying an internal drive, its hedonic value will fluctuate with the level of physical drive (Kelley & Berridge, 2002). Thus, a reward is only deemed palatable when an appetite is present. As wanting decreases, so too does liking. This phenomenon, known as alliesthesia (Cabanac, 1979, 1971), is particularly relevant in the study of natural rewards, for which organisms have a negative feedback circuit that controls this appetite/palatability dichotomy, and in turn, the consumatory behavior itself.

Natural rewards, defined as those substances or experiences that the organism finds innately palatable and needs for the survival of itself or its species, result in enhanced fitness. Examples of natural rewards include food, water, sucrose, copulation and essential minerals, such as sodium. Artificial rewards are drugs or artificially induced neural stimulation that also produce reinforcing sensations. These rewards are considered artificial because they are not necessary for the survival of the animal, but appear to utilize the same neural circuitry to produce a pleasurable sensation.

The behavioral response to a reward is measured in terms of reward consumption. This may involve a behavioral response as simple as licking a spout or eating from a food bowl, or may require a more complex response, such as lever-pressing or climbing over a barrier. Reward motivation is measured in terms of how much work an animal will do for the reward.

Both natural and artificial rewards have been shown to activate the mesolimbic dopamine system. Increased dopamine release is found in the nucleus accumbens (NAc)

in response to feeding (Taber & Fibiger, 1997; Richardson & Gratton, 1996), sucrose ingestion (Hajnal & Norgren, 2001) and sexual activity (Pfaus et al., 1995), as well as to cocaine and amphetamine administration (Koob, 1992) and electrical stimulation of the media forebrain bundle (Nakahara et al., 1992). It is likely that this system evolved to respond to natural rewards in order to increase the likelihood of behaviors that benefit reproductive fitness. However, as extensive research on the neural response to drugs of abuse has shown, this system is involved in responding to artificial rewards as well (Kelley & Berridge, 2002).

While it has been shown repeatedly that the mesolimbic DA system, and NAc cells in particular, are activated by both natural and artificial rewards (Vanderschuren et al., 2002; Bradley & Meisel, 2001; Park & Carr, 1998; Hope et al., 1992; Graybiel et al., 1990), work by Carelli et al. (2003, 2002, 2000) has suggested that different cells respond to natural and drug rewards. This work used extracellular recording in the NAc in response to cocaine administration or a natural reinforcer (water or food) following an operant response. It was shown that NAc cells exhibit different, nonoverlapping firing patterns in response to the drug or natural reward (Carelli et al., 2002, 2000). Although a very small percentage of cells responded to both water and cocaine, most cells responded to only one or the other. This work is provocative in that it both confirms the activation of the NAc by natural and artificial rewards and challenges the assumption that the same cells respond to all rewards. Carelli (2002) suggests that these findings may indicate the presence of neuronal ensembles that have different functional properties and patterns of connectivity throughout the brain. This possibility is supported by the extensive afferent and efferent connections that make the NAc a central part of a large system controlling motivated behavior. It is conceivable that input from neural regions specific to the nature of the reward would activate distinct NAc cell populations, producing the variations in cellular response seen by Carelli et al. Unfortunately, these studies did not identify the neurochemical phenotype of neurons responding to either cocaine or water. In light of this, it is possible that the cells responding for each reward are neurochemically distinct.

This, combined with the likely differences in connectivity of these cells, might help to explain the differences seen in this set of studies.

Sodium depletion and salt appetite

Because sodium is essential for physiological functioning, it is under strict homeostatic control. Sodium depletion produces a powerful motivation to correct this imbalance, which is expressed by robust ingestion of salt (Denton, 1982; Richter, 1956). Experimental sodium depletion can be induced by administration of a natriuretic agent, such as furosemide. This causes a rapid loss of sodium in the urine, which leads to elevations in levels of plasma aldosterone and angiotensin (Rowland & Morian, 1999; Speilman & Davis, 1974). Aldosterone is known to provoke immediate sodium chloride (NaCl) intake through its interaction with membrane-associated ligand-gated ion channels, in addition to inducing gene expression which results in the production of angiotensin II (ANG II; Fluharty & Sakai, 1995). ANG II plays a critical role in maintaining extracellular fluid concentration, and increases in its levels elicit substantial intake of both NaCl and water (Bryant et al., 1980; Avrith & Fitzsimmons, 1980). The increased levels of these hormones work synergistically as the endocrinological mechanism responsible for sodium appetite, and produce its characteristic immediate and prolonged NaCl intake.

Sodium depletion elicits a strong sodium appetite, which is characterized by the avid ingestion of salt at concentrations much higher than those tolerated by an animal in sodium balance (Berridge et al., 1984). Further, this appetite is characterized by a high degree of specificity for sodium-containing salt (Nachman, 1963a). Sodium-deplete rats show a remarkable ability to discriminate between sodium salts and nonsodium salts, with the exception of lithium chloride (LiCl), which is not discriminated from NaCl (Nachman, 1963b). This strong, adaptive, innate motivational response provides an excellent model of a natural reward system, in which both the drive state and reward consumption can be carefully manipulated. Further, avid salt seeking by a sodium-deplete animal has striking parallels to drug seeking in addiction. Like an animal in an operant chamber working for access to cocaine, a sodium-deplete animal will perform

instrumental responses to obtain access to salt (Clark & Bernstein, 2006; Quartermain et al., 1967).

Sham drinking

Under normal conditions, a sodium-deplete animal will consume NaCl solution until its sodium appetite has been satiated. However, if a gastric cannula is used to empty the contents of the stomach, preventing ingested solution from being absorbed by the stomach, the animal will continue to drink vigorously (Roitman et al., 1997; Frankmann et al., 1996). This enhanced ingestive behavior, along with the elimination of post-ingestive effects of the NaCl solution, provides a model in which the effects of taste and post-ingestive sodium repletion on the activation of the neural reward system can be studied separately. Further, unlike other models of natural reward that include a negative feedback pathway to regulate the motivated behavior, sham drinking has no such negative control. This suggests that mechanisms underlying sham drinking may more closely resemble those responsible for the motivation for drug rewards, making this a unique model to study natural reward motivation.

Several studies have used sham drinking to examine the role of orosensory effects of different substances on ingestion. Sucrose has been shown repeatedly to initiate and maintain sham drinking, despite the lack of a nutritive outcome from such ingestion (Hajnal et al., 2004; Weingarten and Kulikovsky, 1989; Nissenbaum and Sclafani, 1987). Similar effects have been seen in rats sham drinking polysaccharide (Polycose; Nissenbaum & Sclafani, 1987) and glucose (Sclafani & Nissenbaum, 1985) solutions. Interestingly, however, rats that are not thirsty do not show this enhanced ingestive response when sham drinking non-nutritive saccharin solution (Sclafani & Nissenbaum, 1985). This suggests a mechanism for nutrient recognition independent of post-ingestive absorption. This idea is supported by findings that sodium-deplete animals will sham feed significantly greater volumes of salt solutions than real drinking animals (Roitman et al., 1997; Frankmann et al., 1996). Further, this phenomenon occurs independent of learned associations between the taste and post-ingestive effects of the salt solution. Animals sham drink significantly more than real drinking animals during their first

exposure to a solution (Roitman et al., 1997; Weingarten & Kulikovsky, 1989). And one of the present studies demonstrated that rats will continue to sham-drink equivalent volumes of NaCl solution over repeated sessions. Sham-drinking, sodium-deplete animals are undeterred by a taste that is unpalatable to non-deplete animals. This further supports a role for taste recognition of nutrients without post-ingestive reinforcement.

Neural circuitry of reward motivation

A great deal of research has identified the neural regions involved in the mediation of response to numerous rewards. This research has focused on the mesolimbic dopamine system, which is often called the "final common reward pathway" (Spanagel & Weiss, 1999). This system is comprised primarily of the dopamine neurons of the ventral tegmental area (VTA) which project to the shell and core subregions of the NAc and to the medial prefrontal cortex (mPFC). The mPFC, in turn, sends glutamatergic projections to the NAc and VTA. The NAc does not project directly to the PFC, but it does send both GABA- and dynorphin-containing projections to the VTA (Pierce & Kalivas, 1997). In addition, several other structures send input to the mesolimbic system. The NAc shell receives glutamatergic input from the amygdala (Wright et al., 1996; Robinson & Beart, 1988). And both the core and shell receive glutamatergic projections from the hippocampus and thalamus (Zahm, 2000; Walaas & Fonnum, 1979). It has been shown that these glutamatergic afferents synapse on the same medium spiny neurons that receive synaptic input from dopaminergic projections, which suggests that dopamine and glutamate interact in their influence on NAc functioning (Pierce & Kalivas, 1997). The VTA receives glutamatergic inputs from the amygdala (Pierce & Kalivas, 1997) and GABA-ergic inputs from the ventral pallidum (Kalivas et al., 1993), while it sends dopaminergic input back to the ventral pallidum (Klitenick et al., 1992). In addition to its dopaminergic input from the VTA, the mPFC also receives glutamatergic input from the medial dorsal thalamus, which receives GABA-ergic input from the ventral pallidum (Pierce & Kalivas, 1997). Because the NAc also has reciprocal GABA-ergic connections with the ventral pallidum (Churchill & Kalivas, 1994), its input to the medial dorsal thalamus allows for interconnection between

the regions of the mesolimbic reward system. The convergence of these inputs in the NAc allows it to act as a common pathway between limbic structures and motor outputs that produce a behavioral response for the reward.

Upstream of this final common pathway and its associated brain regions, other brain regions respond for specific rewards. For example, the lateral hypothalamus is a region central to mediating energy balance. As such, it is critically involved in hunger, and the response for food rewards (Williams et al., 2000). However, the lateral hypothalamus does not have as large a role in the response for other non-food rewards. Similarly, certain regions are specifically involved in sodium appetite. The supraoptic nucleus, subfornical organ, organum vasculosum laminae terminalis and paraventricular hypothalamic nuclei are central to the expression of sodium appetite following sodium depletion (Fitts et al., 2004; Morris et al., 2002; Thunhorst et al., 1998; Rowland et al., 1996). While the role of these regions in sodium appetite is well-understood, a downstream role for the NAc is less well-understood.

Measurement of neuronal activation

Examination of immediate early gene (IEG) expression is one method commonly used to assess neuronal activation in response to experimental manipulation. Fos, the protein product of the IEG c-*fos*, can be visualized using immunohistochemical techniques and is considered a marker of activation. Structures that express increased levels of Fos following experimental manipulation are believed to be involved in the mediation of response to the manipulation. Several studies report increased expression of Fos immunoreactivity (Fos-IR) in structures associated with the mesolimbic DA system in response to various rewards. Fos-IR has been reported in the NAc (Vanderschuren et al., 2002; Hope et al., 1992; Graybiel et al., 1990), caudate-putamen (Vanderschuren et al., 2002; Moratalla et al., 1996; Graybiel et al., 1990), medial prefrontal cortex (Hedou et al., 2002), central nucleus of the amygdala (Day et al., 2001), and substantia nigra (Jaber et al., 1995) following administration of either amphetamine or cocaine. Research on motivation for natural rewards such as sexual experience and food has suggested that many of the same brain areas implicated in the response to drug administration are

activated by these rewards. Studies examining the neural circuitry activated by sexual experience have found increased Fos-IR in the NAc following exposure to a sexually receptive partner (Lopez & Ettenberg, 2002) as well as to copulation (Bradley & Meisel, 2001). Fos-IR is also increased in the NAc in response to expectations of a food reward (Park & Carr, 1998).

In addition to changes in gene expression, other manipulations have been used to determine the role of mesolimbic nuclei in the response for reward. It has been shown that lesioning the NAc prevents the development of conditioned responses to amphetamine and other drugs (Olmstead & Franklin, 1996). Other research has shown that inactivation of the NAc using tetrodotoxin eliminates behavioral responding for a drug reward (Grimm & See, 2000). Studies in which rewarding drugs are infused directly into mesolimbic nuclei have shown that the NAc is critical to self-administration behaviors and the VTA supports development of conditioned responses to a variety of drugs (reviewed in McBride et al., 1999). Further, lesion studies have also supported the evidence for NAc involvement in response to natural rewards, as NAc lesions prevent responses typically conditioned by sucrose (Everitt et al., 1991).

Neurotransmitter systems involved in motivation

Like the research on the activation of neural circuitry, a great deal of research exists on the neurotransmitter systems involved in response to drugs and other artificial reinforcers. This research has implicated both the DA and opiate receptor systems in the neural and behavioral response to drug administration. Extensive research has demonstrated that drugs such as amphetamine and cocaine, act on the DA system. Cocaine interrupts the actions of the DA transporter, blocking the reuptake of DA by the presynaptic terminal (Koob, 1992; Ritz et al., 1987), while amphetamine acts directly to stimulate DA release in addition to blocking DA reuptake (Jaber et al., 1995; Mackler & Eberwine, 1991; Wise & Bozarth, 1985). Both of these drugs result in increased DA in the synapses and prolonged activation of the mesolimbic DA system. Increased DA release in the mesolimbic system is also correlated with a number of natural rewards. Like drug administration, sexual experience (Pfaus et al., 1995; Wenkstern et al., 1993)

and ingestion of palatable substances (Bassareo et al., 2002) similarly induce the release of DA in the NAc. Additionally, blockade of the DA system has been associated with attenuation of the incentive value of rewards (Wise, 1985).

Research has indicated that DA D1 receptors are critical to the neuronal and behavioral response to drug administration. Studies have shown that blocking the D1 receptors attenuates reward-seeking behaviors (Hunt & McGregor, 2002; Beninger & Miller, 1998). Further, D1 receptor activation is necessary for gene expression induced by cocaine or amphetamine (Zhang et al., 2002; Moratalla et al., 1996; Young et al., 1991), as well as lateral hypothalamic stimulation (Hunt & McGregor, 2002). In contrast, the relationship between behavioral responding and neuronal activation following D2 receptor blockade is less direct. Although D2 antagonists elicit gene expression in the mesolimbic DA circuit (Hunt & McGregor, 2002; Dilts et al., 1993), these antagonists attenuate reward seeking and response to drug administration (Crombag et al., 2002; Adams et al., 2001; Chausmer & Katz, 2001; Roitman et al., 1997; Ranaldi & Beninger, 1993). D1 receptors appear to have a larger role in the incentive properties of natural rewards and influence the motivation to obtain rewards, while D2 receptors play a greater role in the motor function associated with obtaining rewards (reviewed in Beninger & Miller, 1998). It appears that activation of both D1 and D2 receptors is necessary for the behavioral response to rewards; however the relationship between specific receptor activation and behavioral outcomes is unclear.

In addition to the data on DA receptor involvement in reward motivation, there is evidence to support the involvement of opiate receptors in the mesolimbic circuitry involved in responding for reward. Behavioral studies have shown that amphetamine-induced increases in locomotor activity (Schad et al., 1995; Hooks et al., 1992; Andrews & Holzman, 1987), responding for an operant reinforcer (Schaefer & Michael, 1990; Franklin & Robertson, 1982; Harris & Snell, 1980; Holtzman, 1974) and development of conditioned place preference (Trujillo et al., 1991) are all attenuated by administration of opiate receptor antagonists naloxone or naltrexone. Opiate receptors also play a critical role in the incentive properties of natural rewards. Opiate receptor agonists enhance taste

palatability (Doyle et al., 1993) and opiate receptor antagonists reduce preference for highly palatable substances (Cooper, 1983; Apfelbaum & Mandenoff, 1981; LeMagnen et al., 1980). In more specific studies, injections of μ-opiate receptor agonists into the NAc resulted in increased intake of a high-fat diet (Zhang & Kelley, 2000), saccharin and salt (Zhang and Kelley, 2002). Studies examining κ-opiate receptors have indicated that κ receptor agonists also induce food intake (Gosnell & Levine, 1996), while κ-opiate antagonists reduce food intake by attenuating hedonic value (Leventhal et al., 1995; Carr et al., 1993). Thus, it appears that many aspects of the system mediating response to drug stimuli are also involved in the response to natural rewards. Though the mechanisms are likely not identical, the similarities suggest a reward substrate common to all rewarding stimuli.

Although research has identified sodium appetite as a strong natural motivator, and has characterized many properties of this motivation model, a number of questions remain unanswered. The present studies are aimed at extending what is known about sodium appetite, including the anatomical and pharmacological substrates underlying response for a salt reward, and the resultant behaviors. These studies examine the effects of sodium depletion and sodium appetite expression on the nucleus accumbens region of the mesolimbic motivation circuit, roles for dopamine and opiate receptors in mediating sodium appetite expression, and the effects of sodium depletion on general reward motivation. It is the aim of these studies to further characterize a sodium appetite model of motivation.

CHAPTER 2
LACK OF ACTIVATION OF THE NUCLEUS ACCUMBENS BY SODIUM APPETITE INDUCTION

Introduction

Sodium appetite is a behavioral response to a sodium deficit. Expression of this appetite is characterized by consumption of NaCl at volumes and concentrations much higher than those freely consumed by sodium-replete animals. The expression of a sodium appetite is adaptive, innate and shows a high degree of specificity for sodium-containing solutions (Stellar, 1993; Denton, 1982; Richter, 1956). Because sodium is critical for physiological functioning, its balance in the body is meticulously maintained by homeostatic mechanisms. These mechanisms work to produce a robust sodium appetite in response to disruption of physiological sodium balance (Richter, 1956). The hormones angiotensin and aldosterone play a key role in the response to sodium depletion and induction of a sodium appetite. Levels of these hormones are elevated in the blood following experimental sodium depletion, and blocking the effects of these hormones has been shown to attenuate expression of sodium appetite in deplete animals (Sakai, 1986; Speilman, 1974). Further, exogenous administration of these hormones can provoke a need-free sodium appetite (Prakash & Norgren, 1991). Brain regions responsive to disruption of fluid and sodium balance have been studied extensively and include the subfornical organ (SFO), organum vasculosum laminae terminalis (OVLT), paraventricular hypothalamic nuclei (PVN) and supraoptic nucleus (SON). Evidence for involvement in sodium appetite includes disruption by lesions of the SFO and OVLT (Fitts et al., 2004; Morris et al., 2002) and strong neuronal activation, as indicated by increased cFos expression, in the SFO, OVLT, SON and PVN (Thunhorst et al., 1998; Rowland et al., 1996). The degree to which induction of sodium appetite leads to activation of general motivational circuitry (e.g. nucleus accumbens) is less clear.

The present studies examined patterns of neuronal activation in the nucleus accumbens (NAc) following induction of sodium appetite, using immunohistochemistry to label Fos.

Materials and Methods

Subjects

Adult, male Long-Evans rats (Charles-Rivers Laboratories) were housed individually in stainless steel wire cages on a 12-hour light-dark schedule. Rodent chow (Teklad, Madison, WI) and water were available *ad libidum* except as noted. All animals were accustomed to handling prior to beginning experimental procedures.

Study 2a

Previous studies have reported that sodium depletion using furosemide increases cFos expression in the SFO and OVLT (Rowland & Morian, 1999; Thunhorst et al., 1998; Rowland et al., 1996). However, it is unclear what effects this depletion protocol has on mesolimbic regions. In order to explore the possibility that induction of the strong sodium appetite produced by furosemide also activates the motivation circuit, study 2a examined Fos-IR in the NAc 24 hours following administration of furosemide or vehicle.

Sodium appetite was induced using a procedure modified from Wolf (1982), in which the diuretic furosemide is used to produce acute sodium depletion. On the day of depletion, food and water were removed from the home cages. Animals were randomly assigned to either sodium-deplete or non-deplete control groups. Animals in the sodium-deplete groups were given a subcutaneous injection of furosemide (10mg/kg, s.c.). Animals in the control group received an injection of an equivalent volume of isotonic saline. Following injections, animals were placed in a clean cage identical to their home cage and returned to the same location on the cage rack. Three hours after injections, animals were weighed, and depleted animals given sodium-free chow (MP Biomedicals, Aurora, OH) and distilled water (dH_2O). Control animals were given regular chow and tap water. 24 hours following depletion and control injections, subjects were anesthetized with pentobarbital and transcardially perfused with phosphate buffered saline, followed by 4% paraformaldehyde.

Study 2b

Administration of the hormones aldosterone and angiotensin II has been shown to produce a need-free sodium appetite, and to provoke increased Fos-IR in the SFO and

SON (Rowland et al., 1996; Rowland & Morian, 1999). In order to examine the possibility that the appetite produced by these hormones may also activate neural motivation circuitry, independent of a sodium deficit, study 2b looked at Fos-IR in the NAc following administration of aldosterone, angiotensin II, a combination of both, or vehicle injections.

Animals were randomly assigned to injection groups. Food and water were removed from the cage, and animals were given a subcutaneous injection of either aldosterone (0.1 mg/kg), angiotensin II (0.2 mg/kg), a combination of aldosterone and angiotensin II (same doses as above) or isotonic saline (1 ml/kg). These doses were based on previous research that demonstrated production of sodium appetite (Rowland et al., 2003; Ventura et al., 2001). Following injections, animals were placed in a clean cage identical to their home cage and returned to the same location on the cage rack. Two hours after injections, animals were anesthetized and perfused as described above.

Immunohistochemistry

Immunohistochemical labeling was used to stain the protein product cFos, which is expressed as a black nuclear stain. Following perfusion, the brains were removed and post-fixed for 24 hours. Serial 50μm coronal sections were cut on a vibratome. Sections containing the NAc, SON and SFO were selected for immunolabeling. Slices were immunostained for cFos using an affinity purified goat polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Briefly, free-floating sections were rinsed (3x, PBS), incubated for 30 minutes in 0.3% H_2O_2 in 10mM PBS to inactivate endogenous peroxidase, rinsed (2x, PBS), and incubated 1 hour in 2% rabbit serum, 0.1% albumin, 0.4% Triton X-100 in PBS. Slices were then transferred without rinsing to the primary antibody solution (dilution 1:20,000). After a 48-hour incubation at 4°C, slices were rinsed (2x, PBS, then 1x, PBS with 2% rabbit serum) and processed using the standard ABC method (Vector Laboratories, Burlingame, CA). Slices were transferred to biotinylated rabbit anti-goat antibody for 1 hour, rinsed (3x, PBS), transferred to avidin-biotin complex for 1 hour, rinsed (1x, PBS, then 2x PB1X), and developed with diaminobenzadine (DAB) substrate (2 minutes). Slices were rinsed (10x, PB1X) and

mounted on gel-coated slides, dehydrated, and cover-slipped with Permount. Sections from animals representing each treatment group were immunostained simultaneously to prevent interassay variability.

Stereological Quantification

A computerized image analysis system (Neurolucida, MicroBrightfield; Colcester, VT) was used for Fos quantification. All tissue samples were coded before analysis to assure that the rater was blind to the experimental condition. The core and shell regions of the NAc, the SFO and the SON were located using the rat brain atlas of Paxinos and Watson (1986). Two representative samples of both SFO and SON were identified and outlined for each subject, and all labeled nuclei within the border of each region were quantified. Labeled nuclei per region were averaged for each subject. Using the optical dissector method (Adkins et al., 2004), sample areas were selected throughout the extent of the core and shell subregions for quantification of Fos-IR nuclei. An unbiased counting frame was superimposed over each sample to delineate the precise sample area to be quantified. Twenty-four samples were taken from the NAc of each subject (four samples per core and four per shell, from each of three brain slices per animal). The volume of the sample frame was calculated by multiplying the length by the width of the frame by depth of the section. Within each sample volume, clearly labeled neurons were quantified. All labeled neurons were totaled for the shell and core of each subject and cell density was calculated as the total number of labeled cells divided by the total sample volume for each subject.

Statistical analysis

The SPSS statistical program was used to analyze Fos-IR data for all groups in these studies. In study 2a the two groups were compared using a t-test. The groups in study 2b were compared using ANOVA to determine significant differences in Fos-IR between the groups. Planned comparisons were performed to determine the level of significance for between-group differences.

Results

Study 2a

In order to examine activation of the NAc by the induction of a sodium appetite, Fos-IR was examined in the NAc of sodium-deplete (Deplete, n=6) or control (Control, n=6) animals 24 hours after furosemide injections. Numerous prior studies in this lab and others have shown that at this time point, a strong sodium appetite is exhibited if rats are offered a NaCl solution (e.g. intakes of 0.3M NaCl range from 8ml to 18ml). As this study was designed to look at effects of depletion (induction of the sodium appetite), animals in this study were not given access to NaCl.

As shown in figure 2.1, the results of this study indicate that sodium depletion alone does not increase Fos-IR in the NAc. Statistical analysis with t-tests showed no significant differences in Fos-IR in the NAc shell ($t_{10} = 1.25$, $p = .24$) and core ($t_{10} = 1.13$, $p = .29$) of the Deplete and Control groups. (Tissue from another set of animals not involved in this study was assayed simultaneously and significant differences in Fos-IR were found in the experimental condition. This validates the histology presented here, and supports the reliability of the present findings.)

Study 2b

As in study 2a, animals in study 2b were not given access to NaCl solution. Fos-IR was examined in the NAc two hours following administration of aldosterone (n=6), angiotensin II (n=6), a combination of the hormones (n=5) or isotonic saline (n=6). The results of study 2b revealed no effect of hormone administration on Fos-IR in the NAc shell ($F_3 = 0.243$, $p = 0.87$) or core ($F_3 = 0.459$, $p = 0.71$, Figure 2.2).

To ascertain the efficacy of the hormone doses used, Fos-IR was also examined in the SFO and SON, which have previously been shown to be activated by these hormones. These results confirmed that, relative to control animals, angiotensin II, with and without aldosterone, significantly increased Fos-IR in the SFO (angiotensin II: $t_{19} = 5.16$, $p < 0.0001$; angiotensin II and aldosterone: $t_{19} = 5.74$, $p < 0.0001$) and SON (angiotensin II: $t_{18} = 4.75$, $p < 0.0001$; angiotensin II and aldosterone: $t_{18} = 2.05$, $p < 0.05$; Figure 2.3).

Discussion

The present studies demonstrate that sodium appetite induction, either by furosemide-induced sodium depletion or hormone administration, is not associated with significant elevations in Fos-IR in the NAc. The results of study 2a indicate that a physiological sodium deficit does not activate the neurons of the NAc. Although sodium depletion has been associated with activation of the SFO and SON (Thunhorst et al., 1998; Rowland et al., 1996), the present study did not find significant activation of the NAc, a region in the mesolimbic motivation circuitry. Study 2b also failed to support a role for the NAc in the induction of sodium appetite. The results of this study indicated that need-free motivation to ingest NaCl, likewise, does not rely on NAc activation.

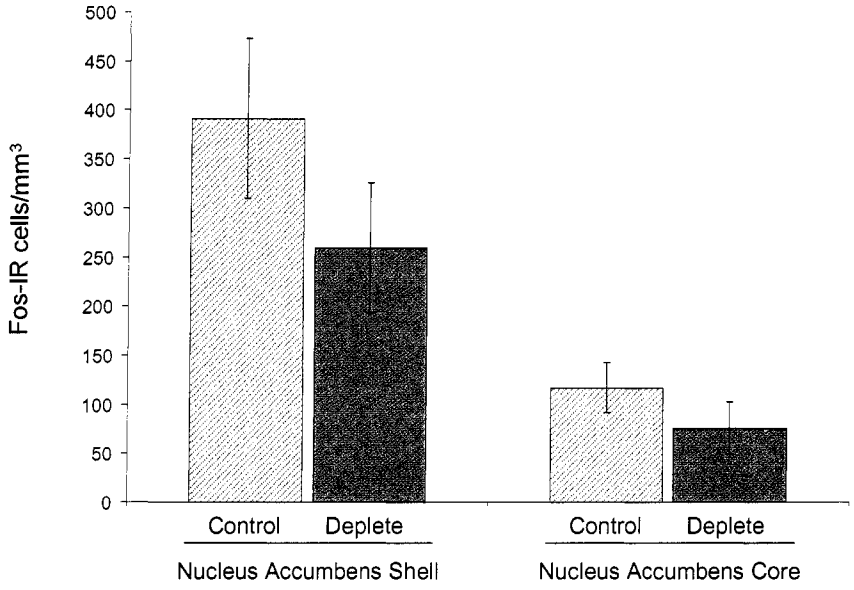
The demonstration of significant increases in Fos-IR in the SFO and SON following angiotensin II administration support the effectiveness of the hormone treatment. This supports the possibility that a normal sodium appetite, which activates the SFO and SON, would not strongly activate the NAc.

The absence of elevations in Fos-IR does not necessarily indicate that no neural activation occurred in the NAc after induction of sodium depletion. Fos-IR is not considered a particularly sensitive index of neuronal activation. Further, brains were analyzed at only a single time point. Therefore, it remains possible that elevations in Fos expression would have been seen at some other time point, or that activation of the NAc by induction of sodium appetite might be detected with a more sensitive measure, such as electrophysiology or voltammetry. Interestingly, strong elevations in Fos expression have been demonstrated in regions such as OVLT and SFO both two and 24 hours following sodium depletion with furosemide (Rowland & Morian, 1999; Thunhorst et al., 1998; Rowland et al., 1996), suggesting that in those regions, at least, elevated Fos is not transient. While it cannot be concluded that sodium appetite has no effect on the NAc, it is likely that any possible effects are subtle and/or of short duration.

Although sodium appetite is a well-documented motivated behavior, the present studies suggest that appetite alone does not rely on activation of neurons in this

motivation-related region. The effects of expression of sodium appetite on the NAc remain to be explored.

A)



B)

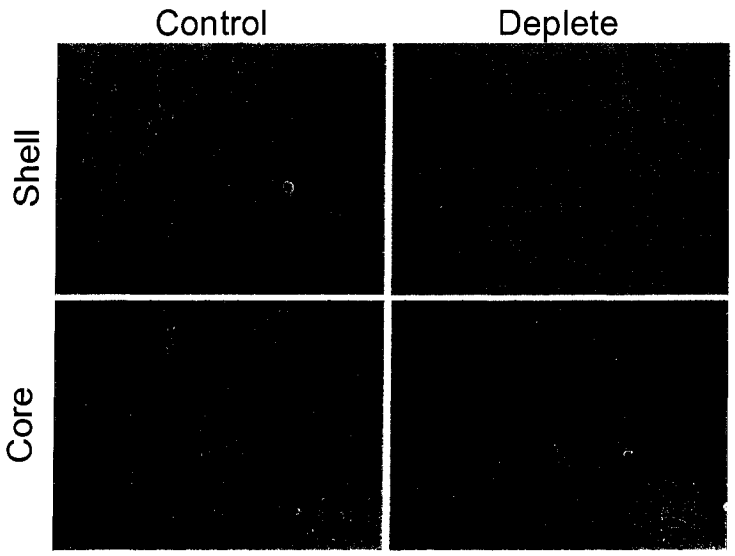
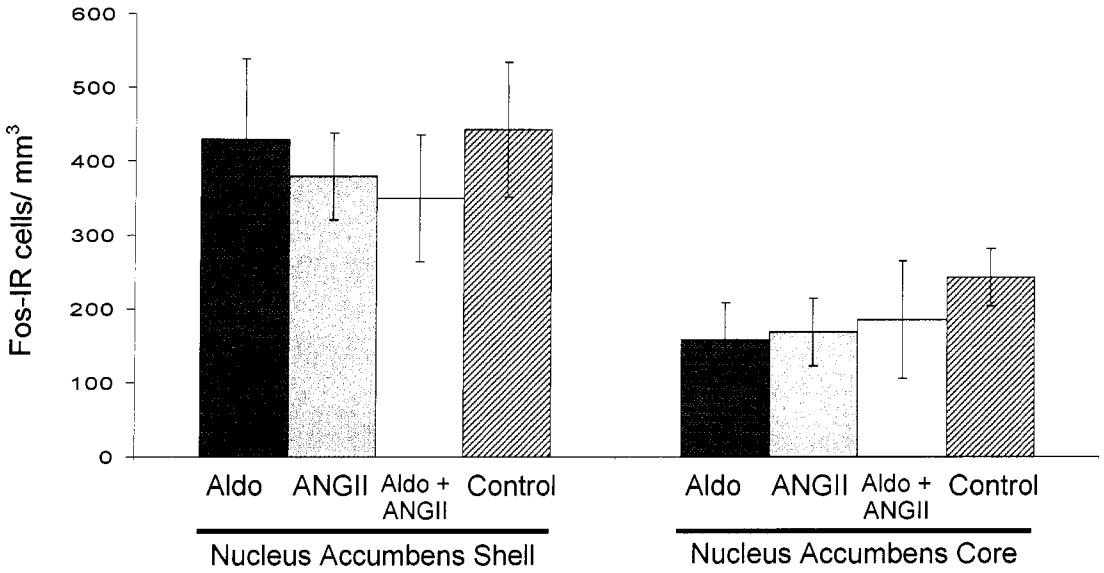


Figure 2.1. A) Fos-IR in the nucleus accumbens shell and core following sodium depletion by furosemide or control injections. There was no effect of sodium depletion on activation of the NAc in this study.
B) Representative micrographs of Fos-IR in the NAc shell and core of deplete and control animals.

A)



B)

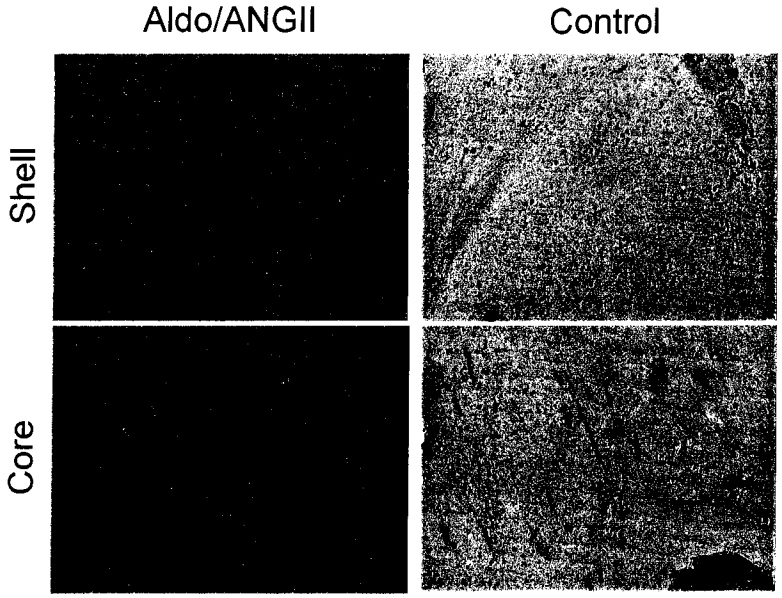
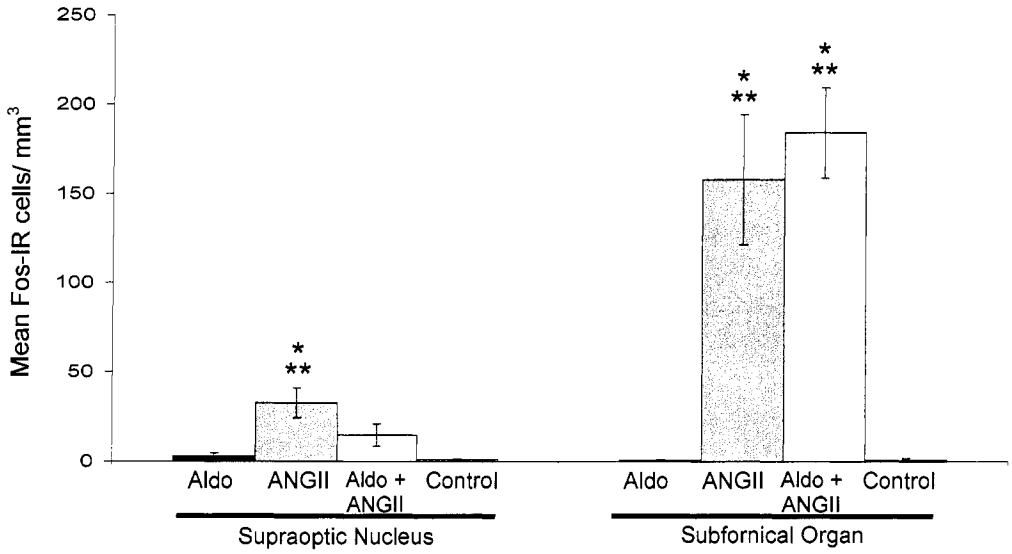


Figure 2.2. A) Fos-IR in the nucleus accumbens shell and core following sodium appetite induction by hormone administration. There was no effect of angiotensin II or aldosterone administration on the shell or the core.
B) Representative micrographs of Fos-IR in the NAc shell and core following administration of angiotensin II and aldosterone or isotonic saline.

A)



B)

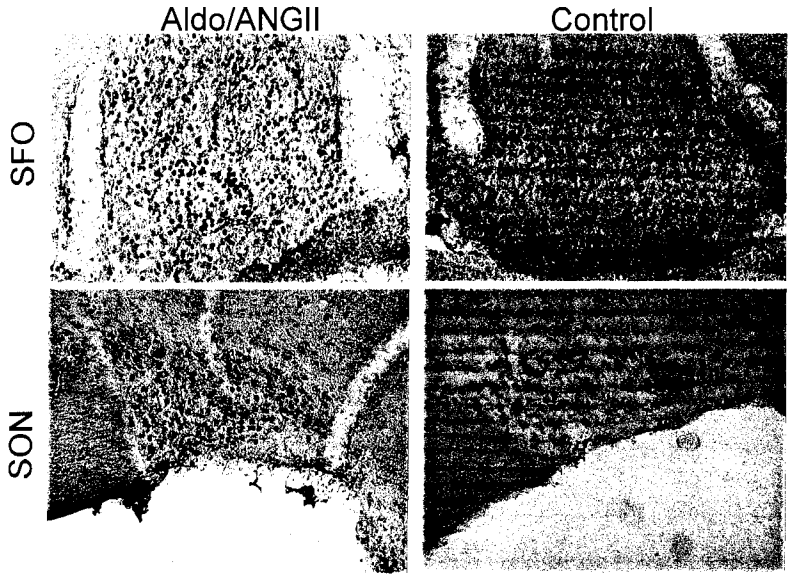


Figure 2.3. A) Fos-IR in the supraoptic nucleus (SON) and subfornical organ (SFO) following sodium appetite induction by hormone administration. There was a significant effect of angiotensin II administration, alone and when combined with aldosterone. $*p < 0.05$ relative to controls, $**p < 0.05$ relative to aldosterone group.
B) Representative micrographs of Fos-IR in the supraoptic nucleus and subfornical organ following administration of angiotensin II and aldosterone or isotonic saline.

CHAPTER 3
ACTIVATION OF THE NUCLEUS ACCUMBENS BY SODIUM APPETITE EXPRESSION

Introduction

Activation of the nucleus accumbens has been associated with the response for many natural and artificial rewards. Food rewards and reward-predictive environments (Park & Carr, 1998), copulation and receptive partners (Bradley & Meisel, 2001, Wenkstern et al., 1993), interaction with offspring (Ferris et al., 2005; Champagne et al., 2004) and drug administration (Crombag et al., 2002; Hedou et al., 2002; Vanderschuren et al., 2002; Hope et al., 1992; Graybiel et al., 1990) have all been shown to activate the mesolimbic reward circuit, including the NAc. Research has also reported persistent changes found in NAc dendrites following multiple sodium depletions and repletion (Roitman et al., 2002). Further, it has been shown that sodium depletion alters both the palatability of NaCl, making it highly desired (Berridge et al., 1984), as well as the motivation for NaCl, as measured by the increased effort a deplete rat will make to gain access to NaCl (Clark & Bernstein, 2006; Quartermain et al., 1967). These findings suggest that NaCl may be considered a reward to the sodium-deplete animal. And while this suggests that the NAc may be responsive to NaCl, it is currently unclear whether expression of sodium appetite activates the NAc.

Research reported in the previous chapter indicated that induction of sodium appetite, by depletion or hormone administration, is not sufficient to increase Fos expression in the NAc. However, in the previous studies, expression of sodium appetite was not examined, raising the possibility that appetite expression may activate this region. In order to test this hypothesis, study 3a examined Fos-IR in the NAc following expression of sodium appetite in a consummatory model, using an intraoral cannula.

Fos-IR was also examined in the NAc of animals expressing sodium appetite in an appetitive model, using a bottle test to measure NaCl ingestion. To better explore factors contributing to NAc activation during expression of sodium appetite, study 3b was designed to dissociate the NaCl taste stimulation during ingestion from the repletion achieved by absorption of the sodium. To do this, a sham drinking preparation was used.

In this model, a gastric cannula is used to empty the contents of the stomach, preventing ingested NaCl solution from being absorbed. The behavioral effect of this manipulation is dramatic; animals continue to drink vigorously for a long time (Frankmann et al., 1996, Roitman et al., 1997). By minimizing the post-ingestive effects of the NaCl solution, sham-drinking provides a model that dissociates the effects of NaCl taste stimulation from physiological sodium repletion in the activation of the neural reward system. Interestingly, unlike other models of natural reward that include a negative feedback pathway to regulate the motivated behavior, sham drinking has no such negative control. This suggests that mechanisms underlying sham drinking may more closely resemble those controlling the response for drug rewards, making this a valuable model to study reward motivation.

The results of study 3b indicated that sham drinking significantly activates the NAc. Previous studies have demonstrated that sodium depletion is associated with elevated levels of both aldosterone and angiotensin II (Sakai, 1986; Speilman & Davis, 1974) while real drinking during expression of a sodium appetite results in a rapid return of these hormones to normal levels (Tordoff et al., 1991). Because the persistent sodium depletion that results from sham drinking maintains elevated circulating levels of these hormones, it seemed possible that the combination of persistently elevated hormones and NaCl taste might be responsible for the Fos-IR seen in the NAc of sham drinking animals. Neither hormone administration (chapter 1), nor NaCl taste (study 3a), are independently sufficient to increase Fos-IR in the NAc. Further, neither sodium depletion nor need-free sodium appetite significantly activate the NAc. Based on these findings, study 3c examined the possibility that sodium ingestion paired with elevated hormone levels might contribute to increased Fos-IR in the NAc.

Previous studies have shown that sham drinking results in much greater intake compared to real drinking when rats are given unlimited amounts of solution. This has been presumed to be due to the lack of repletion and ongoing motivation to satisfy the sodium appetite. However, motivation caused by the open-cannula state has never been specifically examined. To rule out the possibility that the Fos-IR associated with sham

drinking was caused by an inherently higher behavioral motivation resulting from the sham preparation, study 3d compared the breakpoints of sodium-deplete animals responding for NaCl in an operant task with a progressive ratio reward schedule, in sham- and real-drinking states. Breakpoint is measured as the number of lever presses required to complete the last rewarded trial, and indicates the point at which the reinforcement value of the reward no longer outweighs the cost of the work to obtain it (Reilly, 1999). Because progressive ratio reward schedules require a progressively increasing number of lever presses per trial, the breakpoint is considered an index of motivation for the reward.

Materials and Methods

Subjects

Adult, male Long-Evans rats (Charles-Rivers Laboratories) were housed individually in stainless steel wire cages on a 12-hour light-dark schedule. Rodent chow (Teklad, Madison, WI) and water were available *ad libidum* except as noted. All animals were accustomed to handling prior to beginning experimental procedures.

Study 3a

In order to test the hypothesis that sodium appetite expression activates the NAc, Fos-IR was examined in the NAc of rats two hours following infusion of NaCl solution via intraoral cannula.

Prior to beginning the experiment, all animals were implanted with a unilateral intra-oral (IO) infusion cannulae. Briefly, rats were anesthetized (100 mg/kg Ketamine and 10 mg/kg Xylazine). A 19-gauge stainless steel probe needle was used to insert a length of PE-100 tubing anterolateral to the first maxillary molar, pass it through the cheek, caudal to the eye, and out the scapular region at the back of the head (Schafe & Bernstein, 1996). Side of cannulation was randomized. Following one week of recovery from surgery, animals were habituated to daily IO water infusions while in a cylindrical, plexiglass testing chamber with a solid floor. Animals were habituated for five days prior to depletion.

Following surgery and habituation to the testing chambers, animals were sodium-depleted using the procedure described in chapter 2. Twenty-four hours following

depletion and control injections, animals were placed into the testing chamber, and given a 5ml IO infusion of either 0.5M NaCl or dH_2O over 10 minutes. Ninety minutes following testing, subjects were anesthetized with pentobarbital and transcardially perfused with phosphate buffered saline, followed by 4% paraformaldehyde.

Study 3b

In order to examine the effects of sodium appetite expression in an appetitive model, this study tested animals with NaCl solution presented in bottles. Further, to separate the effects of NaCl taste stimulation and sodium repletion following appetite expression, a sham drinking model was used in this study.

Prior to the start of this experiment, gastric cannulae were implanted using a procedure described by Roitman et al. (1997). Briefly, animals were anesthetized (150 mg/kg chloral hydrate and 34 mg/kg pentobarbital). An incision was made into the peritoneal cavity and the stomach was exposed. A stainless steel cannula was then inserted through a puncture wound into the lumen, along the greater curvature of the stomach, and secured with sutures. The cannula was then exteriorized through the muscle and skin and sutured in place (Figure 3.1). The cannula was kept closed with a stainless steel screw, except where otherwise noted. Following one week of recovery, animals were accustomed to having their cannulae opened, stomachs rinsed and then cannulae closed. Two hours after rinsing, animals were placed in a cylindrical, plexiglass testing chamber with raised, rod flooring for 30 minutes. During habituation in the chamber, animals had access to water. Animals were habituated for five days prior to the start of the experiment.

Following recovery from surgery and habituation, animals were sodium-depleted as described in chapter 2. Two groups were sodium-depleted, while a non-deplete control group received isotonic saline injections. Twenty-two hours following injections, animals were weighed. All animals' cannulae were then opened, stomachs rinsed, and cannulae closed. Animals were returned to their cages and food was removed. Two hours after rinsing, animals were given a sodium appetite test. This two hour delay was intended to allow any stress associated with stomach rinsing to dissipate before testing

commenced. The cannulae of deplete animals in the sham-drinking (Sham) group were reopened, while the cannulae of non-deplete (Control) and deplete real-drinking (Real) animals remained closed. At this time, animals were placed in the testing chambers and given access to 0.3M NaCl solution. It is known that sham-drinking provokes significantly greater ingestion, compared to real-drinking (Frankmann et al., 1996, Roitman et al., 1997). Roitman et al. (1997) reported that sham-drinking rats ingested 61.5 ml of 0.3M NaCl, as compared to the 18 ml ingested by real-drinking rats during a 120 minute test. Although sham drinkers absorb little or no ingested fluids, they do experience significantly greater taste stimulation as a result of prolonged drinking. Therefore, in order to control for the possible effects of NaCl taste stimulation and ingestive effort, the volume of solution given to the sham-drinkers was yoked to the intake of the real-drinkers. Each sham-drinker was paired with a real-drinker who was tested first. The sham-drinkers were given only as much NaCl as had been consumed by their paired real-drinker. Animals were tested for 90 minutes, with intakes measured at 30m, 60m and 90m. At the end of testing, animals were returned to their home cages, open cannulae were closed and distilled or tap water was returned to prevent any potential effects of thirst. Thirty minutes following the end of testing, animals were anesthetized with pentobarbital and transcardially perfused with phosphate buffered saline, followed by 4% paraformaldehyde.

Initial analysis revealed strong expression of Fos-IR in the sham drinking animals. Therefore, we sought to identify regional differences in expression of Fos-IR in the NAc shell in a subset of these animals. This was done by dividing the shell into three subregions: dorsomedial, intermediate, and ventrolateral, according to established parameters (Figure 3.2; Todtenkopf & Stellar, 2000; Zahm et al.,1998). All clearly labeled neurons were quantified throughout the entirety of the traced NAc shell. The percentage of Fos-IR cells per subregion was calculated as the total number of labeled cells in that subregion divided by the total number of cells in the shell.

Study 3c

To test the hypothesis that Fos-IR is increased in the NAc in response to elevated hormones combined with NaCl taste, a need-free sodium appetite was induced using a combination of aldosterone and angiotensin II, and animals were allowed to consume NaCl solution.

Animals were randomly assigned to injection groups. Food and water were removed from the cage, and animals were given a subcutaneous injection of a combination of aldosterone and angiotensin II (0.1 mg/kg Aldo, 0.2 mg/kg ANGII) or isotonic saline (1 ml/kg). These doses were the same as those used in chapter 2. Following injections, animals were placed in a clean cage identical to their home cage and returned to the same location on the cage rack. Immediately following injections, animals were given access to 0.3M NaCl solution or dH_2O for 120 minutes and intakes were recorded. Two hours after the beginning of appetite testing, animals were anesthetized with pentobarbital and transcardially perfused with phosphate buffered saline, followed by 4% paraformaldehyde.

Study 3d

The results of study 3b raise the possibility that there is something inherently motivating about the sham-drinking state. One notable feature of this state is the open cannula and the effects it may have on the gastric environment. To test the hypothesis that cannula state (open versus closed) may have an effect on reward motivation, animals with gastric cannulae were sodium-depleted and tested for breakpoint in an operant conditioning paradigm.

Prior to the start of this experiment, rats were implanted with gastric cannulae as described in study 3b. Following one week of recovery, animals were habituated to cannulae rinsing for 5 days. On the 6^{th} day of daily cannulae rinsing, operant conditioning began, according to the methodology of Clark & Bernstein (2006). Animals were then restricted to one hour of water access per day, in addition to the water received during conditioning. During the first two days of conditioning, animals received 30 minutes of magazine training in sound-attenuated operant chambers (Coulbourn

Instruments, Allentown, PA). Animals were then trained to lever-press for a 0.01ml water reward, starting with a fixed-ratio schedule that rewarded every lever press (FR1). This FR1 schedule was used for 30 minutes/day, for 3 days, followed by 3 days of FR3 schedule training for 30 minutes/day. The reinforcement schedule was then switched to a progressive ratio schedule, in which the number of responses required for each reward during a trial increased by 3 presses (PR3). In the PR3 schedule, rats were given 3 minutes to make the required number of lever presses for each trial. When 3 minutes elapsed without the requisite responses being made, the session was terminated, and the number of lever presses made for the last rewarded trial was recorded as the breakpoint. After 3 days of lever pressing for water on the PR3 schedule of reinforcement, the reward was switched to 0.9% (isotonic) NaCl, to preview the availability of NaCl in the operant chamber. Rats were trained to respond for 0.9% NaCl on the PR3 schedule for 10 days.

Following conditioning, animals were returned to *ad libidum* water access for 2 days, after which all animals were sodium-depleted as described in chapter 2. Twenty-two hours following furosemide injections, animals were weighed. All animals' cannulae were then opened, stomachs rinsed, and cannulae closed. Animals were returned to their cages and food was removed. Two hours after rinsing, animals were placed in the operant chamber. Half of the rats' cannulae were opened at this time, while the other half remained closed. The rats were allowed to lever-press for 0.3M NaCl solution on a PR3 schedule. When a reward was not earned within 3 minutes, the session was terminated and the breakpoint was recorded as a measure of motivation. Because each reward was 0.01ml of 0.03M NaCl, it is highly unlikely that the real-drinking animals experienced significant repletion of their sodium appetite. Likewise, it is unlikely that the sham-drinking animals were able to consume enough to experience drainage of the solution through their cannulae. The purpose of this study was to look at the differences in motivation experienced by animals with open versus closed cannulae, and the tiny amount of solution ingested by the animals prevented these extraneous variables from affecting the outcome.

Immunohistochemistry

In studies 3a-c, immunohistochemical labeling was used to visualize Fos expression. Fos-IR cells were labeled according to the methodology described in chapter 2.

Stereological Quantification

Fos-IR cells were quantified using the stereological techniques described in chapter 2.

Statistical analysis

The SPSS statistical program was used to analyze behavioral and Fos-IR data for all groups in these studies. Groups in studies 3a-c were compared using ANOVA to determine significant differences in Fos-IR and behavioral responses between groups. Planned comparisons were performed to determine the level of significance for between-group differences. A t-test was used to compare breakpoints between groups in study 3d.

Results

Study 3a

In order to examine activation of the NAc by the expression of sodium appetite in a consummatory model, Fos-IR was examined in the NAc of sodium-deplete or control animals, two hours after IO infusions of 0.5M NaCl or dH_2O (Deplete-NaCl, n=6; Deplete-dH_2O, n=5; Control-NaCl, n=6; Control-dH_2O, n=6).

As shown in figure 3.3, the results of this study indicate that expression of sodium appetite in an IO model does not increase Fos-IR in the NAc. This study also supported the previous findings that sodium depletion alone does not affect Fos expression in the NAc. Statistical analysis showed no significant differences in Fos-IR in the NAc shell ($F_{3,19} = 0.631, p = .60$) and core ($F_{3,19} = 1.07, p = .39$) between groups.

Study 3b

In order to examine activation of the NAc by the expression of sodium appetite in an appetitive model, Fos-IR was examined in the NAc following NaCl ingestion by deplete animals. In order to independently examine the effects of NaCl taste stimulation and sodium repletion, these effects were compared in sodium-deplete animals allowed to

real-drink (Real, n = 15) and sham-drink (Sham, n = 10) NaCl solution, and in non-deplete control animals (Control, n = 5) who also had real-drinking access to NaCl. Cumulative intake in depleted animals averaged 12.36 ml, which was significantly more than the 1.4 ml average intake by the control animals ($t_{27} = 3.49, p < 0.01$). Intake was measured at 30m, 60m and 90m. There were no significant differences in intake volumes between the Real and Sham groups at any of the three time points.

Sham-drinking during expression of a sodium appetite resulted in dramatic activation of the NAc. ANOVA analysis of Fos-IR in the NAc revealed significant differences between groups in both the shell ($F_{(2,27)} = 26.76, p < 0.001$) and core ($F_{(2,27)} = 19.26, p < 0.001$) subregions. As shown in figure 3.4, there were significant increases in Fos-IR in both the shell and core of rats in the Sham group relative to both the Real (shell: $t_{27} = 6.77, p < 0.001$, core: $t_{27} = 5.82, p < 0.001$) and Control (shell: $t_{27} = 5.52, p < 0.001$, core: $t_{27} = 4.53, p < 0.001$) groups. In contrast to the Sham group, Fos-IR in the Real group did not significantly differ from that in the control group in either shell or core (shell: $t_{27} = 0.50, p = 0.62$, core: $t_{27} = 0.21, p = 0.84$).

Fos expression was clearly not uniform in its distribution throughout the NAc shell. As shown in table 3.1, regional analysis revealed that the majority of Fos-IR nuclei were concentrated in the dorsomedial subregion of the shell.

Study 3c

To test the hypothesis that elevated hormone levels, combined with NaCl taste, are responsible for the Fos response seen in the NAc of sham-drinking animals, Fos-IR was examined following need-free sodium appetite expression by animals injected with a combination of aldosterone and angiotensin II (Hormone-NaCl, n = 5). This was compared to Fos-IR in animals treated with aldosterone and angiotensin II and given access to dH_2O (Hormone-dH_2O, n = 5), treated with aldosterone and angiotensin II alone (Hormone only, n = 5), and control animals injected with isotonic saline and given access to both NaCl and dH_2O (Control, n = 4).

Statistical analysis indicated that hormone administration significantly increased intake of 0.3M NaCl compared to intake in the control group ($t_7 = 3.72, p < 0.01$; Figure 3.5). Water intake was not affected by hormone administration ($t_7 = 0.03, p = 0.98$)

As shown in figure 3.6, there was no effect of hormone administration, with or without subsequent NaCl or dH_2O ingestion, on Fos-IR in the NAc. Statistical analyses show that there were no between-group differences in either the shell ($F_{3, 15} = 0.18, p = 0.91$) or the core ($F_{3, 15} = 0.56, p = 0.65$). These findings suggest that elevated hormone levels associated with persistent sodium depletion are not responsible for the Fos-IR seen in sham-drinking animals.

To ascertain the efficacy of the hormone doses and assay used, Fos-IR was also examined in the SFO and SON, which are known to be activated by these hormones. Significant differences were revealed in both regions (SFO: $F_{4,19} = 7.87, p < 0.001$; SON: $F_{4,17} = 7.60, p < 0.001$). These results confirmed that, relative to control animals, administration of aldosterone and angiotensin II significantly increased Fos-IR in the SFO ($t_{19} = 3.84, p < 0.001$) and SON ($t_{17} = 2.16, p < 0.05$; Figure 3.7). Fos-IR was further increased in the SON by sodium appetite expression (NaCl vs. hormones only: $t_{17} = 2.27, p < 0.05$; NaCl vs. dH_2O: $t_{17} = 2.93, p < 0.01$).

Study 3d

To rule out the possibility that the open-cannula state of sham drinking is associated with inherently greater motivation, the breakpoint on a progressive reinforcement schedule was used to measure motivation for NaCl. Breakpoint in sham- (n = 8) and real-drinking (n = 8) animals was compared. Statistical analysis revealed no difference between groups ($t_{14} = 0.54, p = .60$; Figure 3.8), indicating that an open cannula is not sufficient to increase motivation.

Discussion

The present studies demonstrate a strong increase in Fos-IR in both the core and shell of the NAc when sodium-deplete rats sham-drink NaCl. In contrast, neither real-drinking during expression of a sodium appetite, nor sodium depletion itself, was associated with significant elevations in Fos-IR in this region. The sham-drinking

preparation used in study 3b has been documented to provoke exaggerated, vigorous and prolonged NaCl drinking in sodium-deplete animals (Roitman et al., 1997; Frankmann et al., 1996). However, in the present study, NaCl intake by sham- and real-drinkers was yoked, so strong NAc activation in the sham condition cannot be attributed to increased NaCl taste stimulation or intake. Additionally, intake was measured throughout the appetite test, and no differences were found in the rate of consumption between the Real and Sham groups. The difference between sham- and real-drinkers, then, was not in the amount consumed, pattern of ingestion, or extent of NaCl stimulation experienced, but in the lack of sodium absorption and consequent persistence of the deplete state in the Sham, but not Real, group. While it has been shown that sham drinking does not completely prevent any absorption of ingested nutrients, it is reasonable to assume that the sham-drinking animals in study 3b absorbed minimal sodium and remained deplete. Certainly the avid and extended ingestion reported by Roitman et al. (1997) and Frankmann et al. (1996) support this assumption.

The present results also fail to support a role for elevated levels of the hormones aldosterone and angiotensin II in activation of the NAc. Although these hormones have been associated with sodium depletion and their rapid return to baseline levels is a marker of sodium repletion following NaCl ingestion (Tordoff et al., 1991), there was no effect of administration of aldosterone and angiotensin II on Fos-IR in the NAc. Further, while hormone administration did significantly increase NaCl ingestion and Fos-IR in the SFO and SON, the combination of hormone and NaCl taste had no measurable effect on NAc Fos-IR.

Persistent sodium depletion and NaCl taste stimulation would each appear to be necessary for NAc activation, but neither stimulus alone is sufficient. The synergism evident here is striking because of the absence of any evidence of an activating effect of either sodium depletion or NaCl drinking individually. Research does not support persistent motivation for sodium when animals express salt appetite in a real-drinking model. Levels of renin activity and plasma aldosterone have been shown to return to replete control concentrations within 60 minutes of ingesting NaCl, and deplete rats

reliably consume over twice the amount of lost sodium within the first 15 minutes of a salt appetite test (Tordoff et al., 1991). Further, real-drinking stops long before sham-drinking subsides (Roitman et al., 1997; Frankmann et al., 1996). These hormone and behavioral measures indicate that real-drinking rapidly satiates salt appetite, and eliminates the reward motivation experienced by a deplete rat. This evidence supports the hypothesis that a difference in satiety plays a role in the activation seen in the NAc. However, since no difference was found in Fos-IR in the NAc of Deplete-dH_2O and Control-dH_2O animals in study 3a, the present results suggest that NAc activation in this model requires the combined effect of stimulation by the taste of NaCl and persistent motivation for sodium.

Although previous studies have documented a distinct difference in the intake of sham- and real-drinking animals (Roitman et al., 1997; Frankmann et al., 1996), there are no data to support an a priori difference in motivation between these two groups. The present results indicate that a sham drinking rat will not work harder than a real drinking rat for a reward. The NaCl solution reward for each successful set of lever presses was 0.01 ml; an amount small enough to limit the likelihood of repletion in real drinkers or actual drainage experienced by sham drinkers. The results of this study showed that there is no inherent difference in behavioral motivation between sham and real drinkers as measured by breakpoint for NaCl, which suggests that the Fos-IR seen in the NAc of sham-drinking animals is not due the open-cannula, or its possible effects on the gastric environment. Both real and sham drinkers start out with similar levels of motivation, but real drinkers are rapidly satiated, leading to a marked decrease in motivation. The prolonged behavioral motivation manifested as vigorous sham drinking can be attributed to ongoing sodium depletion and the effort to satisfy the appetite. The abundant Fos-IR in the NAc of sham drinking animals appears to be due to the very specific combination of NaCl taste and persistent depletion.

However, we cannot exclude other differences between sham and real drinking animals as an explanation for these findings. For example, it is likely that the sham-drinking animals in study 3b experienced significant frustration when they ran out of the

limited NaCl solution, which was yoked to the intake of a paired real-drinker. This frustration could have contributed to activation of the NAc. Two potential studies would be of interest to explore the effects of frustration. One study might compare the Fos response in the NAc following limited and unlimited sham-drinking. And the other study could explore the effects of sham-drinking NaCl when a need-free appetite has been provoked with hormone administration. These two studies might further elucidate the role of frustration and how it relates to sodium appetite and the activation of the mesolimbic circuit. Stress is another factor which may have influenced the present results. Several stressors have been shown to activate the neurons of the NAc, including food restriction, footshock, restraint and amphetamine administration (Copeland et al., 2005; Carr & Kutchikhidze, 2000; Kalivas & Duffy, 1995). However, sodium depletion is not associated with increases in corticosterone, a concomitant of stress (Roitman et al., 1999). Further, sodium depletion alone also failed to increase Fos-IR in the NAc in the present studies. This suggests that the stress of sodium depletion is not sufficient to activate the NAc. However, it remains possible that the stress associated with the frustration of sham drinking might be a contributing factor. These and many other potential factors remain that might explain the differential Fos-IR in the NAc of sham and real drinkers in the present studies.

It is important to note that the absence of significant elevations in Fos-IR in the NAc cannot be interpreted to mean that no neural activation of this region occurred after sodium depletion or drinking NaCl. Fos-IR is not considered an extremely sensitive index of neuronal activation. Furthermore, since brains were analyzed at only a single time point, it remains possible that elevations in Fos expression would have been seen at some other time point. Interestingly, strong elevations in Fos expression have been demonstrated in regions such as OVLT and SFO both two and 24 hours following sodium depletion with furosemide (Roland & Morian, 1999; Thunhorst et al., 1998; Rowland et al., 1996), suggesting that in those regions, at least, elevated Fos is not transient. What is evident here is that both the immunohistochemical method and the temporal parameters used in the present study were clearly capable of demonstrating dramatic elevations of

Fos expression in NAc in response to sham drinking of NaCl, which provides a striking contrast to the absence of effects under other conditions.

A notable feature of the sham-drinking paradigm is that the persistent appetitive behaviors and prolonged ingestion seen in sham-drinking animals are similar to the behavior of animals responding for drugs or electrical stimulation of the medial forebrain bundle. There is no evidence of satiation in either paradigm. Further, the concentration of Fos expression in the dorsomedial NAc shell of sham-drinking animals is similar to patterns of neuronal activation seen following drug administration. This subregion has been implicated in the induction of behavioral sensitization and dopaminergic response to repeated cocaine administration (Todtenkopf et al., 2002; Todtenkopf & Stellar, 2000). Another feature of the sham-drinking paradigm is that the experience of extensive gustatory stimulation by NaCl, without repletion, could be viewed as a violation of the animals' expectancy of reward. Although these rats had no prior experience with sodium appetite which could have generated such expectancies, sodium appetite in rats has been well documented to be a strong, innate response and thus such an expectancy may well be innate. Both of these features provide a link between sham-drinking NaCl and the hypothesized functions of the NAc, whose dopamine has been hypothesized to mediate the response to persistent motivation concurrent with violation of reward expectancy (Schultz, 2002).

These findings suggest that sham drinking may provide an intriguing approach to the study of motivational circuitry because it strongly engages the NAc, perhaps in response to the failure of an anticipated reward outcome. Neuronal activation by the violation of reward expectancy has been shown in other experimental models. Schultz et al. have shown repeatedly that DA neurons fire in response to reward prediction errors, that is, the omission of an expected reward or the presentation of an unexpected reward (Waelti et al., 2001; Mirenowicz & Schultz, 1994; Schultz et al., 1993). And increased Fos-IR has been found in the NAc shell in response to an environment that has been paired with a palatable meal (Park & Carr, 1998). It is unclear from that study whether the activation was in response to reward anticipation or the failure of the cue environment

to be paired with the expected reward. As these examples demonstrate, the mesolimbic DA system, and the NAc in particular, are activated by unfulfilled reward expectancies. The results of the present studies also support a role for NAc neurons in the response to the omission of expected reward. Although motivation by sodium depletion was not sufficient to increase Fos-IR, significant expression was provoked in the region by the failure of a rewarding taste (NaCl) to produce the expected outcome (sodium repletion).

While it remains to be determined what mechanisms underlie the activation of the NAc in this model, the present results support the hypothesis that the NAc is active in the response to sham drinking, making this a unique model of motivation. Research utilizing this model may further elucidate the role of the NAc in responding to reward stimuli which evade negative feedback, and thus contribute to our understanding of the neural mechanism underlying appetitive behaviors that become pathological, such as drug tolerance and sensitization and obesity-related behaviors.

Table 3.1. Mean percentage of Fos-IR cells in subregions of the NAc shell of sham-drinkers.

Subregion	**Percentage of NAc shell Fos-IR cells**
Dorsal Medial NAc Shell	67.3%
Intermediate NAc Shell	18.4%
Ventrolateral NAc Shell	14.3%

Figure 3.1. Illustration of a sham-drinking rat. When the cannula is open, ingested fluids drain out of the stomach, minimizing absorption of nutrients.

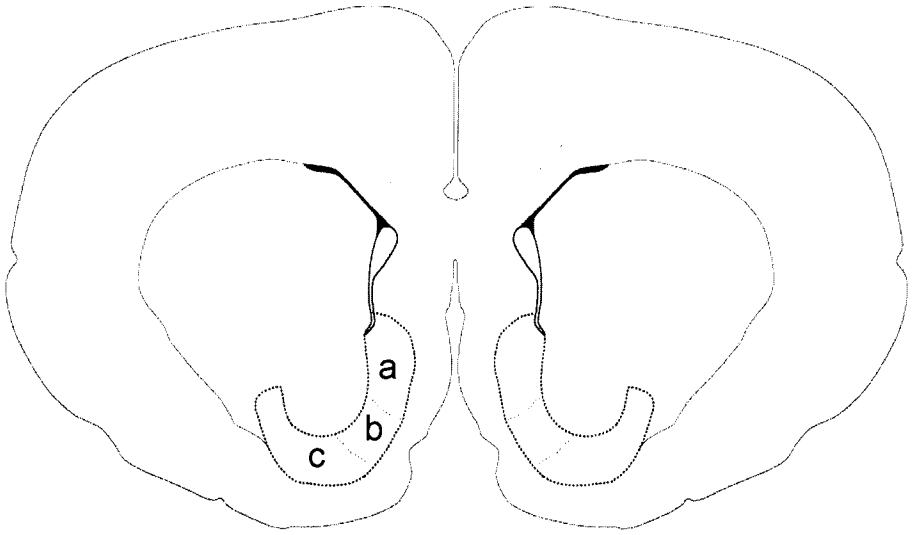
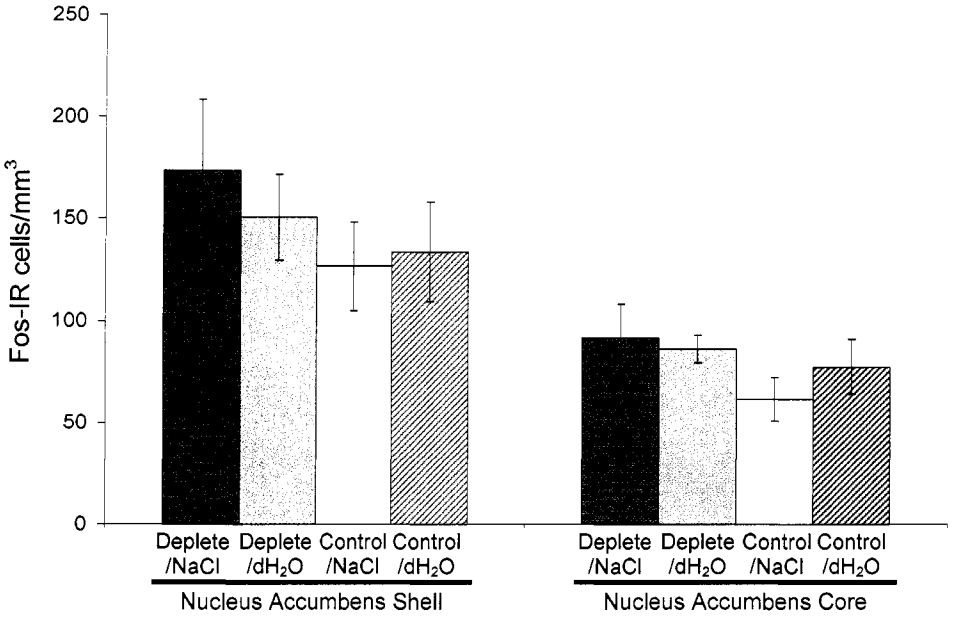


Figure 3.2. Representative diagram of NAc shell subregions: a) dorsomedial shell, b) intermediate shell, c) ventrolateral shell.

A.



B.

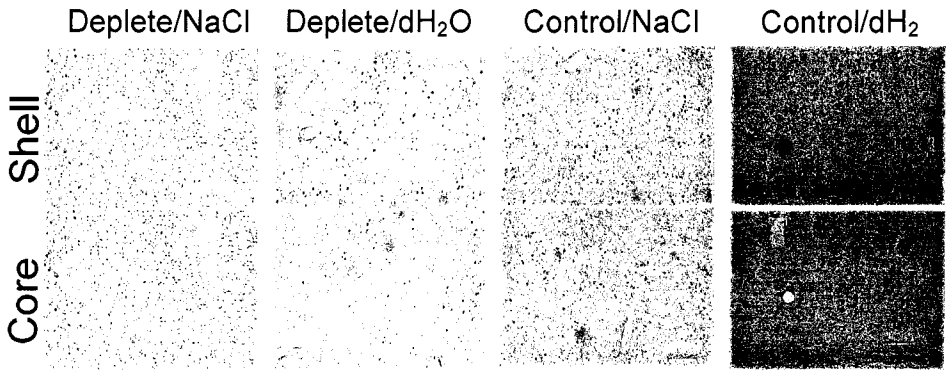
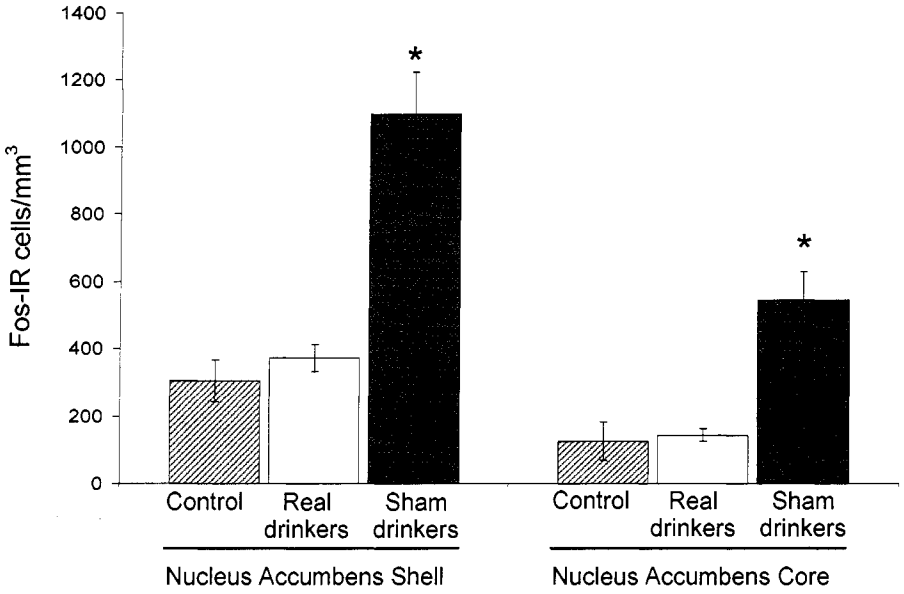


Figure 3.3. A) Average Fos-IR cells per mm^3 in the NAc shell and core of animals in study 3a. There were no effects of either sodium depletion or expression of sodium appetite on Fos-IR in either the shell or core subregions.
B) Representative micrographs of Fos-IR in the NAc shell and core of animals in the Deplete-NaCl, Deplete-dH_2O, Control-NaCl and Control-dH_2O groups of study 3a.

A.



B.

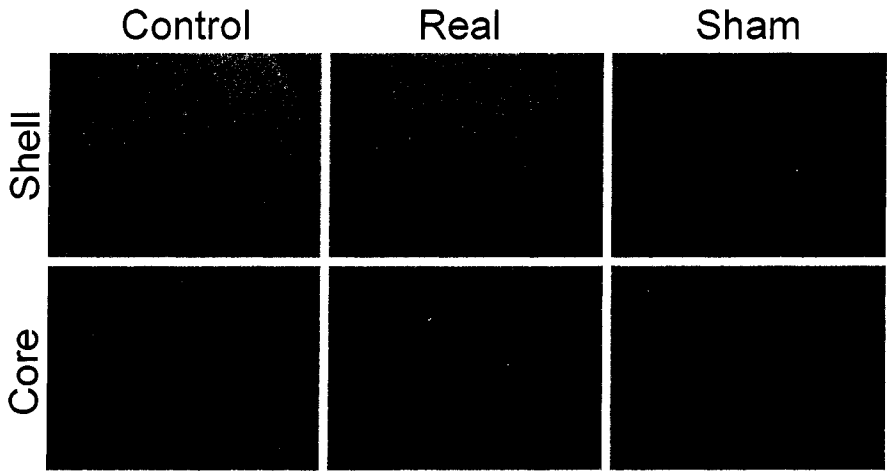


Figure 3.4. A) Average Fos-IR cells per mm^3 in the NAc shell and core of animals in study 3b. There was a significant between-group difference in both the shell and core subregions. In both regions, the sham-drinking group had significantly more Fos-IR relative to both the control and real-drinking groups. $*p < 0.001$.
B) Representative micrographs of Fos-IR in the NAc shell and core of animals from Control, Real and Sham groups of study 3b.

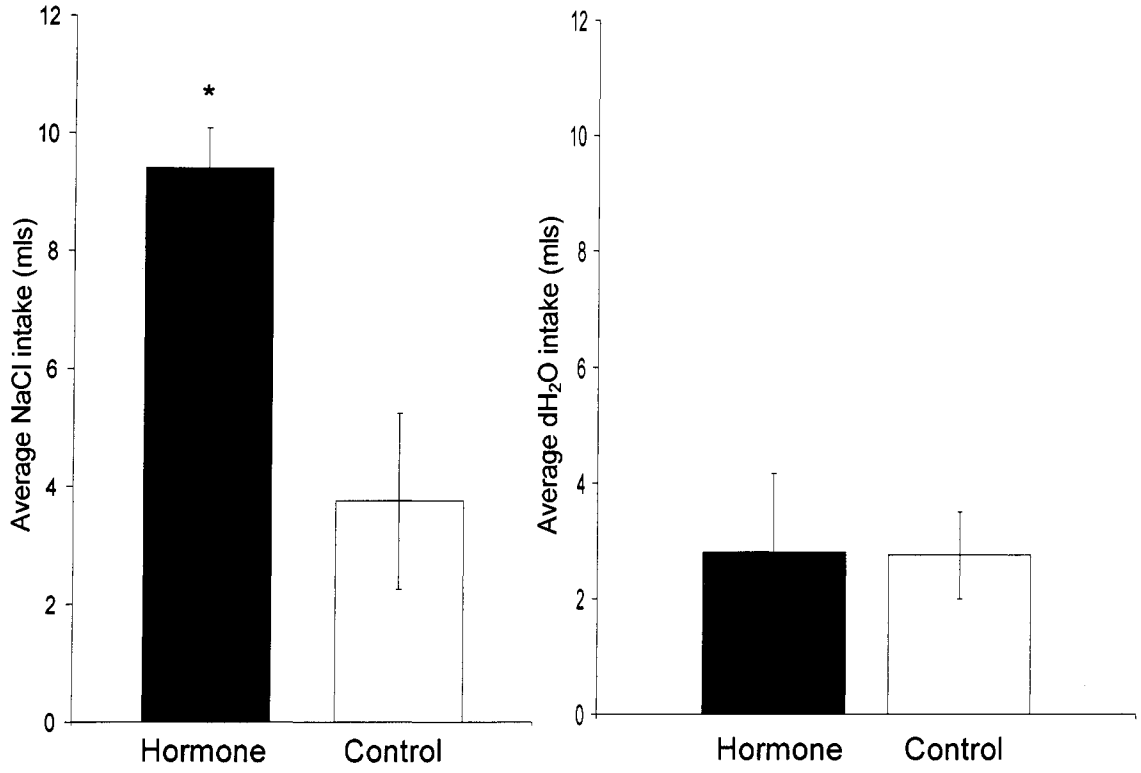
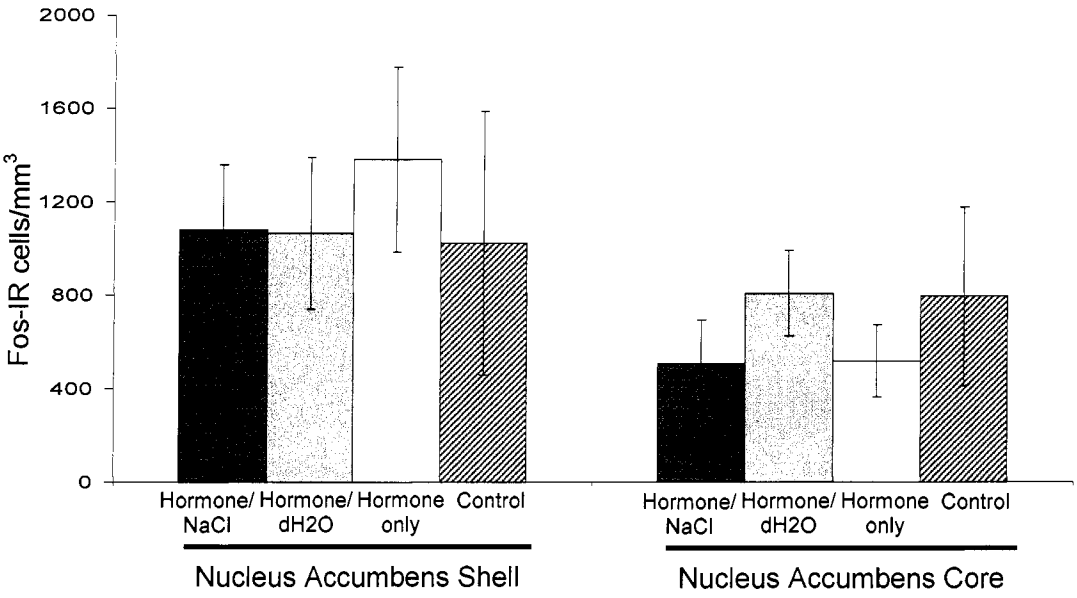


Figure 3.5. Average intake of 0.3M NaCl and dH_2O following exogenous administration of aldosterone and angiotensin II. Hormone administration significantly increased NaCl ingestion, but did not affect water intake. $*p < 0.01$

A.



B.

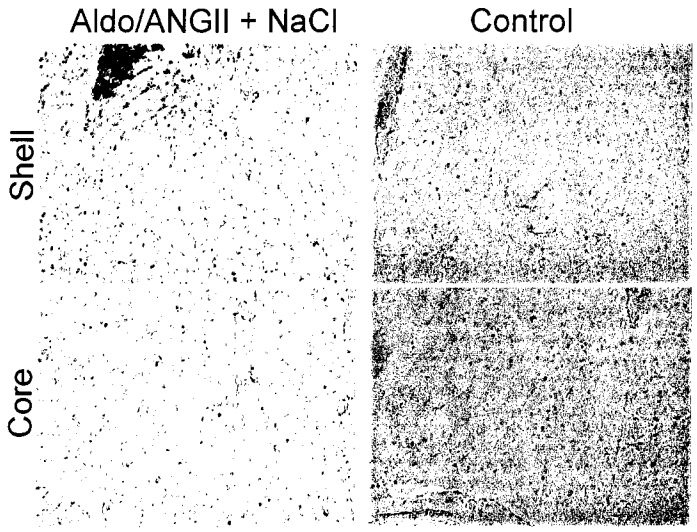
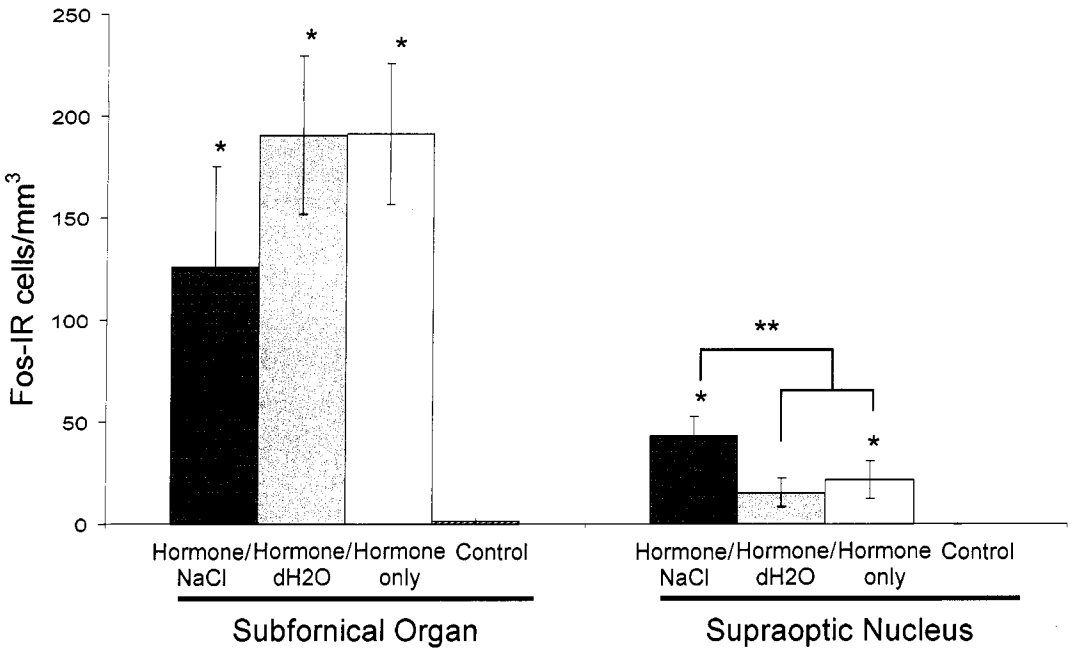


Figure 3.6. A) Average Fos-IR cells per mm^3 in the NAc shell and core of animals in study 3c. Exogenous administration of aldosterone and angiotensin II, with or without expression of sodium appetite, had no effect on Fos-IR in either the shell or core subregion.
B) Representative micrographs of Fos-IR in the NAc shell and core of animals in the Hormone-NaCl and Control groups of study 3c.

A.



B.

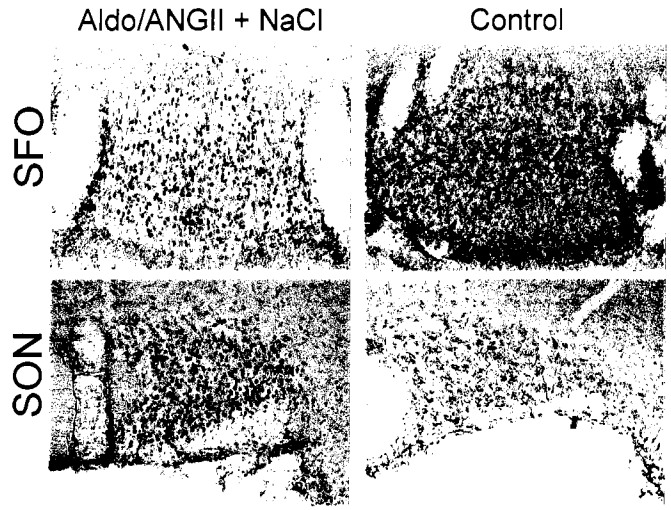


Figure 3.7. A) Average Fos-IR cells per mm^3 in the subfornical organ (SFO) and supraoptic nucleus (SON) of animals in study 3c. There was a significant effect of exogenous aldosterone and angiotensin II administration on Fos-IR in both regions. In the SON this effect was further increased in animals who consumed NaCl following hormone administration. $*p < 0.05$ relative to control, $**p < 0.05$ relative to hormone + NaCl ingestion.
B) Representative micrographs of Fos-IR in the NAc shell and core of animals in the Hormone-NaCl and Control groups of study 3c.

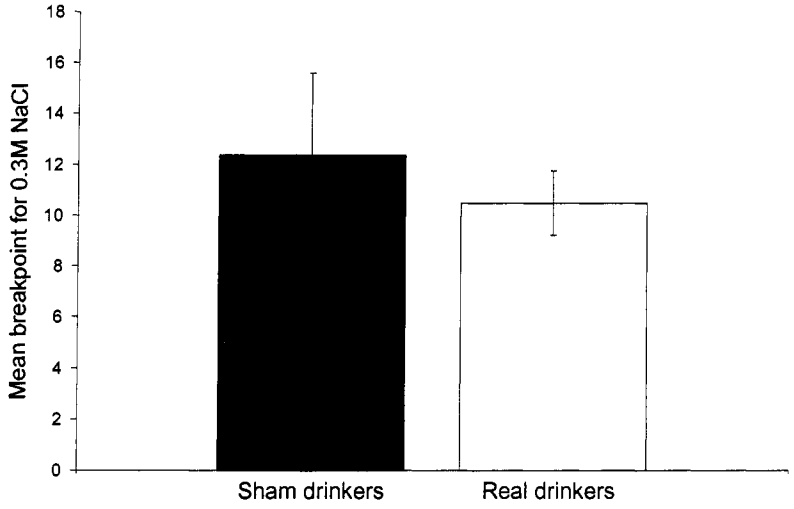


Figure 3.8. Average breakpoint for NaCl in sham- and real-drinking animals. There was no significant difference in motivation for NaCl between real and sham drinking animals.

CHAPTER 4
DOPAMINE AND OPIATE RECEPTOR INVOLVEMENT IN BEHAVIORAL MOTIVATION FOR SODIUM IN DEPLETE ANIMALS

Introduction

Behavioral response for reward activates both the dopamine (DA) and opiate receptor systems. Increased DA release in the mesolimbic system is correlated with natural rewards such as sexual experience (Pfaus et al., 1995; Wenkstern et al., 1993) and palatable tastes (Bassareo et al., 2002). Blockade of the DA system attenuates the response for these rewards (Wise, 1985). Specifically, DA D1 receptors are critical for reward-seeking behaviors such as responding for food, lateral hypothalamic self-stimulation and drug administration (Hunt & McGregor, 2002; Beninger & Miller, 1998). DA D2 receptor blockade has also been found to attenuate reward-seeking and response to drug rewards (Chausmer & Katz, 2001; Roitman et al., 1997; Ranaldi & Beninger, 1993). Thus, it appears that activation of DA receptors is necessary for the behavioral response for rewards. However, other findings have suggested a more nuanced dependence on DA receptor activation. Roitman et al. (1997) demonstrated that sham drinking, but not real drinking, depends on DA D2 receptor activation. That study showed that a highly specific DA D2 antagonist (raclopride) significantly decreases expression of sodium appetite while sham drinking, although real drinking of NaCl solution by sodium-deplete rats is unaffected. The role of DA D1 receptors in real and sham drinking remained to be explored.

To examine the effects of blockade of DA D1 receptors on expression of sodium appetite, study 4a compared sodium intake in real-drinking animals following administration of the D1 antagonist, SCH-23390, or saline. The results of this study indicated that DA D1 receptor antagonism does not attenuate expression of salt appetite, which suggests that D1 receptors are not necessary for expression of sodium appetite. However, as a role for the DA D2 receptor pathway was only unmasked by a sham drinking protocol, a similar condition-dependent role may exist for the DA D1. Study 4b combined a sham drinking protocol with a pharmacological DA D1 antagonist to investigate a role for D1 receptors in the expression of salt appetite. The D1 antagonist,

SCH-23390, and dosage used, have been shown by others to attenuate reward-seeking behaviors (Hunt & McGregor, 2002; Beninger & Miller, 1998).

In addition to data on DA receptor involvement in reward responses, research has also demonstrated that opiate receptors are critical to the incentive properties of natural rewards. Opiate receptor agonists enhance taste palatability (Doyle et al., 1993) and the opiate receptor antagonists naloxone and naltrexone reduce preference for highly palatable substances (Apfelbaum & Mandenoff, 1981), amphetamine-induced locomotor activity (Schad et al., 1995; Hooks et al., 1992; Andrews & Holzman, 1987), responding for an operant reinforcer (Schaefer & Michael, 1990; Franklin & Robertson, 1982; Harris & Snell, 1980; Holtzman, 1974) and development of conditioned place preference (Trujillo et al., 1991). While these findings indicate that opiate receptors are involved in the response to many types of rewards, a role for opiate receptors in the expression of sodium appetite has not been fully elucidated. It is likely that blocking opiate receptors will result in attenuation of sodium appetite expression, similar to the results seen in other reward models. To test this hypothesis, study 4c examined the effects of the general opiate receptor antagonist, naltrexone, on expression of sodium appetite in real-drinking animals.

Materials and Methods

Subjects

Adult, male Long-Evans rats (Charles-Rivers Laboratories) were individually housed in stainless steel wire cages on a 12-hour light-dark schedule. Rodent chow (Teklad, Madison, WI) and water were available *ad libidum* except as noted. All animals were accustomed to handling prior to beginning experimental procedures.

Study 4a

To investigate a role for DA D1 receptors in the expression of sodium appetite in real-drinking animals, NaCl ingestion was measured in sodium-deplete rats following administration of a DA D1 receptor antagonist, SCH-23390, or saline. Three different dosages of SCH-23390 were used.

All animals were sodium-depleted with furosemide, as described in chapter 2. Twenty three and a half hours following depletion, food was removed and the animals were weighed and randomly assigned to receive either the DA D1 antagonist SCH-23390 (0.05 mg/kg, i.p., n=6; 0.1 mg/kg, n = 5; or 0.5 mg/kg, n = 5) or saline (Control, n=8). Thirty minutes after injections, water was removed from the home cage and animals were given access to 0.3M NaCl for 120 minutes. After testing, NaCl intake was recorded, and regular food and water were returned to the home cage.

Study 4b

To test the hypothesis that DA D1 receptors play a role in expression of sodium appetite in sham-drinking animals, NaCl ingestion was measured in sham-drinking, sodium-deplete animals following administration of the DA D1 receptor antagonist SCH-23390, or saline.

Prior to the start of this experiment, all animals were implanted with a gastric cannula according to the procedure described in chapter 3. Following one week of recovery, animals were accustomed to having their cannulae opened, stomachs rinsed and then cannulae closed. Two hours after rinsing, animals were placed in a cylindrical, plexiglass testing chamber with raised, rod flooring for 30 minutes. During habituation in the chamber, animals had access to water. Animals were habituated for five days prior to the start of the experiment.

Following surgery and habituation to the testing chambers, animals were sodium-depleted as described in chapter 3. Twenty-two hours following depletion, food was removed and cannulae were opened, stomachs rinsed and then cannulae closed. 90 minutes later, animals were randomly assigned to one of three groups. One group received injections of the DA D1 antagonist SCH-23390 (0.1 mg/kg, i.p.) and two groups received saline injections (Control). The dosage of antagonist used was based on the outcome of study 4a, which found no differences in the effects of 0.05, 0.1, and 0.5 mg/kg SCH-23390 on real drinking. Thirty minutes later, the cannulae of the antagonist group (Sham-SCH-23390, n = 5) and one no-drug control group (Sham-Control, n = 5) were reopened, while the cannulae of the other no-drug control group (Real-Control, n =

4) was left closed. Animals were put into the testing chamber and given access to 0.3M NaCl solution for 120 minutes. After testing, all cannulae were closed and animals returned to their home cages.

Study 4c

To examine the effects of a general opiate antagonist on expression of sodium appetite, NaCl intake was measured in real-drinking rats following administration of naltrexone. Three dosages of naltrexone were used in this study, to identify possible dose-dependent effects of this drug on expression of sodium appetite.

All rats were depleted according to the procedure described in chapter 2. Twenty three and a half hours following depletion injections, animals were randomly assigned to groups, and received injections of the general opiate receptor antagonist naltrexone (0.5 mg/kg, i.p., n=6; 1 mg/kg, n = 6; 2 mg/kg, n = 6) or saline (n=6). 30 minutes after injections, 0.3M NaCl was provided in the home cage for 120 minutes. After testing, intakes were measured and regular chow and water were returned.

Statistical analysis

The SPSS statistical program was used to analyze behavioral data for all groups in studies 4a-c. ANOVAs were used in all studies to determine significant differences in intakes between groups. Post-hoc comparisons were used to determine the level of significance for between-group differences.

Results

Study 4a

To test the hypothesis that DA D1 receptors are involved in the expression of sodium appetite, NaCl intake by sodium-deplete rats was measured following treatment with either SCH-23390 or saline.

Results of an ANOVA comparing NaCl intake indicate that DA D1 receptors are not necessary for expression of sodium appetite. There was no difference in average NaCl consumption between any of the treatment groups ($F_{3, 20} = 0.668$, $p = 0.58$; Figure 4.1). Not only were no differences seen between groups receiving the antagonist and control animals, there were also no significant differences between groups receiving

different doses of the antagonist. These results fail to support a role for DA D1 receptors in the expression of sodium appetite.

Study 4b

Roitman et al. (1997) reported no effect of DA D2 receptors on the expression of sodium appetite in real-drinking animals, but showed a dramatic effect of D2 antagonism in a sham-drinking model. Thus, it remained possible that a role for DA D1 receptors might be revealed in a sham-drinking model. To follow up on study 4a and further explore the effects of DA D1 receptors on expression of sodium appetite, study 4b compared the intake of sham-drinking rats following administration of SCH-23390 or saline.

Following an initial statistical analysis, 2 rats were removed from the Sham-Control group as statistical outliers. Reported results do not reflect their intake volumes.

The results of an ANOVA revealed a significant effect of DA D1 receptors in sham-drinking rats. Subjects treated with SCH-23390 consumed significantly less NaCl solution relative to animals in the Sham-Control group ($t_6 = 2.42, p < 0.05$; Figure 4.2) and the Real-Control group ($t_7 = 2.55, p < 0.05$).

Study 4c

To investigate a role for opiate receptors in the expression of sodium appetite, NaCl intake was compared in rats following treatment with the general opiate antagonist naltrexone, or saline injections.

ANOVA results showed a significant effect of opiate receptors on expression of salt appetite ($F_{3,20} = 7.11$, $p < 0.01$, Figure 4.3). Post hoc comparisons revealed that rats injected with all three doses of naltrexone consumed significantly less NaCl compared to rats injected with saline (0.5 mg/kg: $p < 0.01$; 1 mg/kg: $p < 0.05$; 2 mg/kg: $p < 0.01$). However, no dose-dependent differences were detected between the groups receiving naltrexone.

Discussion

The present studies were aimed at further defining the role played by both dopamine and opiate receptor systems in the motivation for a natural reward. Specific

DA D1 and general opiate receptor antagonists were used to measure the effects of these receptors on expression of sodium appetite. The results of studies 4a and 4b, taken together, indicate that DA D1 receptors are specifically involved in expression of sodium appetite by sham-drinking animals, with no discernable role in expression during real-drinking. The results of study 4c revealed attenuation of sodium appetite expression by the opiate antagonist naltrexone.

Research has demonstrated that blockade of DA D2 receptors dramatically attenuates sham drinking, while real drinking is unaffected (Roitman et al., 1997). The results of that research suggest that real drinking, which is characterized by sodium repletion and satiety, does not rely on the DA D2 pathway. However, sham drinking, which is characterized by little-to-no sodium repletion, a lack of negative feedback, and much greater intake, relies heavily on the D2 pathway. The present research indicates that the role of DA in expression of sodium appetite is not specific to D2 receptors. Studies 4a and 4b found that, as with D2 receptors, pharmacological blockade of D1 receptors had no effect on real drinking, but significantly attenuated NaCl intake by sham-drinking animals.

Numerous previous studies have suggested a global role for DA D1 and D2 receptors in the mediation of response for rewards (reviewed in Beninger & Miller, 1998; Beninger, 1993). These studies have indicated that administration of either D1 or D2 antagonists will suppress incentive learning and reward responding. However, newer research has challenged this categorical assumption. Although infusions of D1 or D2 antagonists into the NAc suppress locomotor and exploratory activity, total food intake by hungry rats is unaffected (Baldo et al., 2002). These findings, along with the results of Roitman et al. (1997) and present study 4b, suggest that DA D1 and D2 antagonists are similar in their effects on the response to rewards. Interestingly though, taken together with the results of study 4a, these findings also indicate that DA receptors are not uniformly involved in the response to all reward situations.

It is possible that the different effects of DA antagonists on real and sham drinking are associated with the difference in the magnitude of the necessary response for

reward in each model. Research has shown that DA requirements vary according to the response cost of a task. A low response requirement in a lever-pressing model was not affected by DA depletions, whereas depletions did significantly disrupt responding on a schedule requiring high behavioral response (Correa et al., 2002). Study 4a demonstrated that DA D1 receptors, like D2 receptors (Roitman et al., 1997) are not necessary for expression of sodium appetite in real-drinking animals. Numerous studies have defined NaCl as a reward for sodium-deplete animals. Deplete animals consume NaCl at concentrations that are aversive to the non-deplete animal (Denton, 1984; Richter, 1956) and lever-press for NaCl (Clark & Bernstein, 2006). Additionally, antagonist-free, sodium-deplete groups in the current study showed normal, elevated NaCl intake. Thus, the lack of effect of the DA D1 antagonist seen in this study is certainly not due to a lack of motivation for NaCl in the real-drinking animal. In this model, relatively low motor output is required of the animal before the sodium appetite is satiated. It is likely that the comparatively low behavioral requirement does not rely on DA receptor activation. Alternately, real drinking may rely on non-specific DA receptor activation, such that blocking either D1 or D2 receptors had no effect because activation of the other system is sufficient for responding. The effect of simultaneous blockade of both D1 and D2 receptors on expression of sodium appetite has not been identified, due in part to the confounding neuroleptic effect such drug administration has on overall behavioral responding.

Conversely, the vigorous and prolonged response of sham drinking animals requires a much greater degree of behavioral output. The extended response of sham-drinking animals, relative to real-drinking animals, likely relies on significantly greater receptor activation to sustain the behavior. The results of study 4b, considered with those of Roitman et al. (1997), establish the necessity of DA receptor activity in this model. Further, these studies suggest that both D1 and D2 receptors are involved in sham drinking, as blockade of either type results in attenuation. The findings of studies 4a and 4b raise the possibility that the DA receptor system is only necessary to maintain high appetitive response requirements, but is not critical for incentive motivation on tasks with

low response requirements, including normal responding for 0.3M NaCl solution. However, Roitman et al. (1997) demonstrated that real-drinking a low concentration of NaCl (0.1M), which resulted in over twice as much intake as real-drinking 0.3M NaCl, was unaffected by a D2 receptor antagonist. This suggests that, while both D1 and D2 receptor subtypes are necessary in certain reward models, such as sham-drinking, tasks with higher appetitive response requirements are not solely maintained by DA receptor activation. This further suggests that more than one neural system may play an independent role in the response to sodium appetite.

The results of study 4c are consistent with previous research showing suppressed responding for rewards following administration of naltrexone. Low-doses, similar to those used in the present study, decrease electrical self-stimulation of the NAc (Trujillo et al., 1998) and operant responding for a scheduled reward (Harris & Snell, 1980). Other general opiate antagonists have also been shown to attenuate consumption of highly palatable substances (Apfelbaum & Mandenoff, 1981), amphetamine-induced hyperlocomotion (Schad et al., 1995; Hooks et al., 1992; Andrews & Holzman, 1987), responding for an operant reinforcer (Schaefer & Michael, 1990; Franklin & Robertson, 1982; Holtzman, 1974) and development of conditioned place preference (Trujillo et al., 1991). These studies suggest that opiate antagonists specifically decrease reward value, resulting in decreased motivation to respond. The current study extends this research by demonstrating that NaCl intake by deplete animals is also significantly attenuated by opiate receptor blockade.

The opiate system likely plays a critical role in the enhanced palatability of NaCl observed in sodium-deplete rats (Berridge et al., 1984). This hypothesis is supported by the finding that the opioid peptide, enkephalin, is associated with expression of sodium appetite. In situ hybridization revealed increased enkephalin-mRNA in the NAc shell of sodium-deplete animals following appetite expression (Lucas et al., 2003). The same study also revealed an attenuating effect of the opioid antagonist naltrindole on normal expression of sodium appetite, while the dopamine D2 antagonist raclopride had no effect (Lucas et al., 2003). Endogenous opiates have been implicated in the hedonic valuation

of rewards. Compared to the dopamine system, which is involved in the behavioral acquisition of rewards, the opiate system appears to determine the palatability of those rewards. Specifically, both the μ- and κ-opiate receptor subtypes have been implicated in determining the incentive properties of rewards. Injections of μ-opiate receptor agonists into the NAc result in increased intake of a high-fat diet, saccharin and salt (Zhang & Kelley, 2002; 2000). Administration of exogenous κ-opiate receptor agonists also induce hyperphagia (Gosnell & Levine, 1996), while κ-opiate receptor antagonists reduce food intake by attenuating hedonic value (Leventhal et al., 1995; Carr et al., 1993). The present findings support this hypothesis, and suggest that blocking opiate receptors decreases the palatability of NaCl which is enhanced by sodium depletion. There is no literature to suggest that naltrexone has an effect on sodium balance, and it is likely that all animals were equally in need of sodium following 24 hours of depletion. Therefore, the decrease in intake seen here may be attributed to a decreased hedonic value of NaCl, which resulted in attenuated expression of sodium appetite.

Although it is possible that the decrease in NaCl intake seen in study 4c may be due to a systemic decrease in motor activity or impaired ability to respond, this possibility is unlikely, as other research has found no effects on motor functions following similar and much larger doses of naltrexone (Trujillo et al., 1998). Further, ingestion was observed in all naltrexone-treated animals, albeit at significantly lower volumes compared to control animals. This supports the notion that the rats were able to make an appetitive response for NaCl, but chose not to do so.

Given the attenuating effects of naltrexone in real-drinking animals, it is likely that opiate receptors play a critical role in the expression of sodium appetite. This study was not repeated in a sham-drinking model. Sham drinking has been shown to produce vigorous and prolonged ingestion (Roitman et al., 1997; Frankmann et al., 1996), and likely depends on a greater degree of motivation than real drinking. Further, a real-drinking rat receives positive reinforcement for drinking NaCl solution via two modalities: oral stimulation by NaCl taste (as signaled by the chorda timpani branch of the facial nerve) and post-ingestive feedback following sodium repletion. If naltrexone

sufficiently reduces the reinforcement coming from both of these sources and eliminates reward motivation in the real-drinker, one can reasonably predict that it would reduce motivation similarly, or to a greater extent, in an animal experiencing reinforcement from only one of these sources.

Overall, the results of the present studies indicate that opiate and dopamine receptors have critical, overlapping roles in the expression of sodium appetite. When considered together, these findings suggest that in real-drinking rats, ingestion may be entirely driven by the sodium depletion-induced increase in palatability of NaCl. The opiate receptor-dependent hedonic valuation of NaCl may be sufficient to maintain ingestion to the point of satiety, at which point feedback signals decrease palatability, which then terminates ingestion. Indeed, an opiate receptor-dependent model of real drinking during expression of sodium appetite is supported by the findings that DA receptors are not involved in real drinking. However, sham drinking, which requires a much greater degree of behavioral effort, may require both the opiate receptor-dependent increase in NaCl palatability and DA receptor activation to sustain ingestive behavior. As discussed in chapter 3, the combination of NaCl taste and lack of satiety feedback inherent to sham drinking may result in the increased activity of DA neurons that has been associated with the violation of expectations (Schultz, 2002; Waelti et al., 2001; Mirenowicz & Schultz, 1994; Schultz et al., 1993). This suggests that while increased palatability may induce NaCl ingestion in sham-drinking animals, it is the increased DA activity resulting from the violated expectation of repletion that maintains the prolonged ingestion.

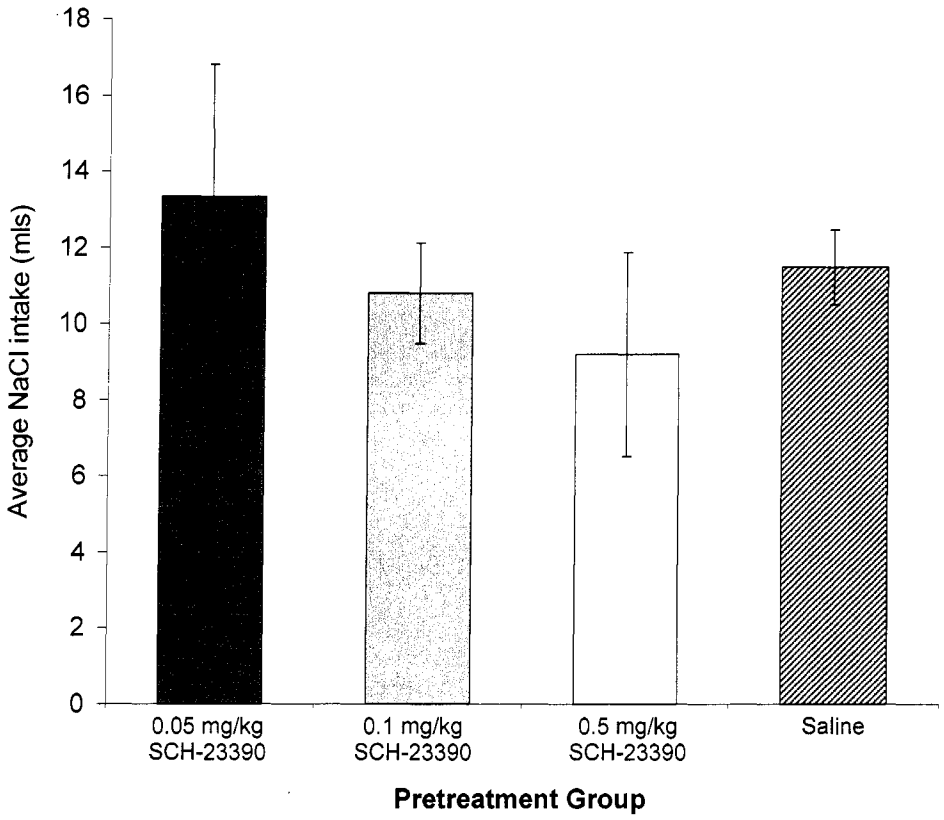


Figure 4.1. Mean 0.3M NaCl intake by sodium-deplete, real-drinking animals following SCH-23390 or saline pretreatment. There were no significant effects of this DA D1 antagonist, at any dose, on real drinking during expression of sodium appetite.

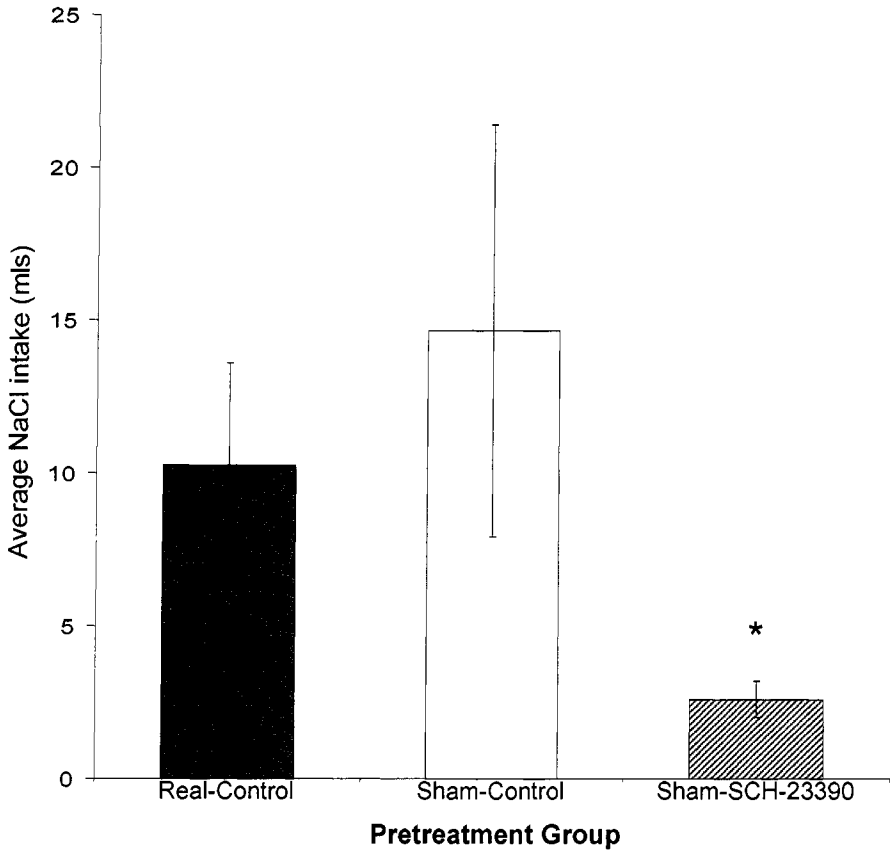


Figure 4.2. Mean 0.3M NaCl intake by sodium-deplete, real- and sham-drinking animals following SCH-23390 or saline pretreatment. There was a significant attenuation of sham drinking by the DA D1 antagonist. $*p < 0.05$ compared to the real-control and sham-control groups.

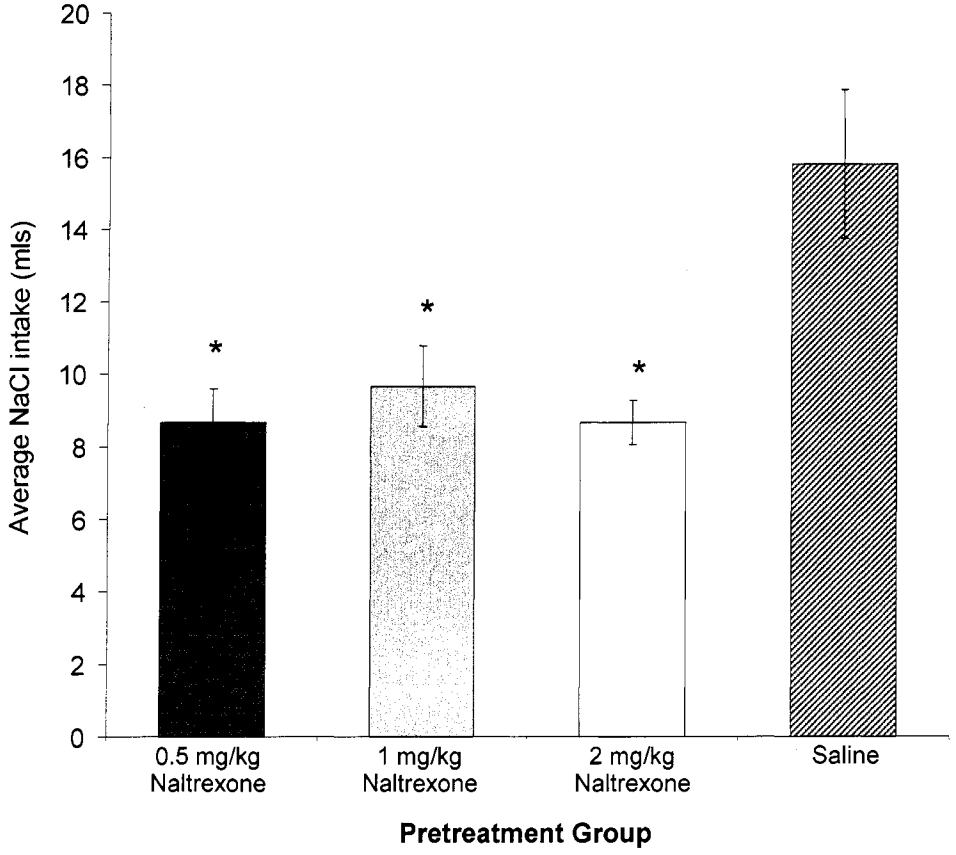


Figure 4.3. Mean 0.3M NaCl intake by sodium-deplete, real-drinking rats following naltrexone or saline pretreatment. Naltrexone significantly reduced intake at all doses. *$p < 0.05$ compared to the saline group.

CHAPTER 5
EFFECTS OF SODIUM DEPLETION ON MOTIVATION FOR SUCROSE

Introduction

Previous research, as well as findings reported in chapters 3 and 4, clearly demonstrate that sodium depletion results in highly motivated behavior directed at NaCl consumption. Sodium depletion elicits a strong sodium appetite and enhances the reward value of an otherwise avoided concentration of NaCl (Berridge et al., 1984). And deplete rats reliably consume more than twice the amount of lost sodium within the first 15 minutes of a salt appetite test (Tordoff et al., 1991). This motivated responding for NaCl is further magnified in sham-drinking animals, who consume approximately 3-5 times more 0.3M NaCl than real-drinking animals (Roitman et al., 1997; Frankmann et al., 1996). Expression of the motivated sodium appetite has been shown to rely on the opiate receptor system, as well as the dopamine receptor system and activation of the NAc in a sham drinking model (chapters 3 and 4). Although this is strong evidence that sodium depletion increases motivation, it remains unclear whether it is specific for sodium, or if depletion results in a general enhancement of reward motivation.

A substantial body of research has focused on the mechanisms underlying sodium appetite, and how depletion causes long-term changes in reward motivation. Results of these studies have shown that sodium appetite elevates levels of plasma aldosterone and angiotensin II (Rowland & Morian, 1999; Speilman & Davis, 1974), and increases enkephalin-mRNA in the striatum (Lucas et al., 2003), while decreasing dopamine transporter activity in the NAc (Roitman et al., 1999). Depletion changes the palatability of, and oromotor responses to, NaCl (Berridge et al., 1984). And multiple sodium depletions result in sensitized sodium appetite (Sakai et al., 1987), increased dendritic branching of neurons in the NAc shell, and cross-sensitization to amphetamine (Roitman et al., 2002). However, very little research has investigated the effect of sodium depletion on general reward motivation. A study by Conover et al. (1994) did examine the effects of sodium depletion on the value of lateral hypothalamic stimulation, and found that depletion does not increase the value of stimulation, but does increase the

ability of NaCl to compete with stimulation as a reward. Another study compared intakes of NaCl, water, sucrose and flavored solutions and found that NaCl was preferred over other solutions, including sucrose, by sodium-deplete animals (Nozaki et al., 2002). These results suggest a specificity of motivation for NaCl in deplete animals. To expand upon this research, and examine motivation for another natural reinforcer, the present studies compared the intakes of NaCl and sucrose by sodium-deplete animals. Sucrose was chosen for comparison to NaCl because its reward properties are well established, and it is commonly used as a reinforcer in operant-learning studies. Sucrose is both highly palatable and nutritive, and its rewarding properties require no prior depletion or deprivation.

In order to examine the effects of sodium depletion on preference for NaCl compared to another reward, study 5a measured ingestion by sodium-deplete animals given a choice of NaCl and sucrose. To characterize concentration-dependent reward motivation, intakes of multiple NaCl and sucrose concentrations were measured over a series of ingestion tests. A sham-drinking preparation was used in this study to prevent sodium repletion and caloric satiation. The effects of sodium repletion on reward preference were examined in study 5b, which compared preference for NaCl or sucrose in real-drinking animals. In this study, rats were given NaCl and sucrose at concentrations that had been found to be preferred in study 5a.

Studies 5a and 5b suggested that sodium depletion specifically raises the motivation for NaCl. However, the evident lack of enhanced motivation for another reward may be a function of having a choice which included the physiologically required mineral. It remains unknown if, in the absence of the appropriate reward (NaCl), the preference for an inappropriate reward (sucrose) will be increased by sodium depletion. In order to test the hypothesis that sodium depletion increases general motivation, sucrose intake by sodium-deplete rats was examined in study 5c. The results showed no effect of depletion on sucrose intake, indicating that sodium depletion does not increase general motivation. These findings contribute to previous findings on the specificity of sodium

appetite by showing that sodium appetite does not affect the response to another highly palatable, natural reward.

Materials and Methods

Subjects

Adult, male Long-Evans rats (Charles-Rivers Laboratories) were housed individually in stainless steel wire cages on a 12-hour light-dark schedule. Rodent chow (Teklad, Madison, WI) and water were available *ad libidum* except as noted. All animals were accustomed to handling prior to beginning experimental procedures.

Study 5a

In order to explore the specificity of motivation for NaCl in sodium-deplete animals, intake of various concentrations of NaCl and sucrose were compared in sham-drinking animals.

Prior to the start of this experiment, gastric cannulae were implanted as described in chapter 3. Following one week of recovery, animals (n = 15) were accustomed to having their cannulae opened, rinsed, and closed. Animals were also given access to NaCl and sucrose solutions during the habituation period, to minimize the effects of neophobia during testing. Animals were habituated for five days prior to the start of the experiment.

During the first week of the experiment, baseline NaCl and sucrose preference was measured in non-deplete rats. On the test day, animals had their cannulae rinsed and re-closed, and food was removed from the home cage. Two hours later, cannulae were reopened, and animals were given one of three concentrations of NaCl in counterbalanced order, along with a single concentration of sucrose. (All concentration pairs for all tests in study 5a are presented in Table 5.1.) Each NaCl/sucrose choice was given for 10m, followed by a 5 minute inter-trial interval with no solution before the next NaCl/sucrose choice was presented. Intakes were recorded at the end of each 10 minute trial. Solution side and order of presentation was alternated between trials. Following testing, all cannulae were closed, and food and water were returned to the cage.

Following baseline testing, animals were sodium-depleted as described in chapter 2. Twenty-two hours following injections, animals were weighed. All animals' cannulae were then opened, rinsed, and closed. Animals were returned to their cages and food was removed. Two hours after rinsing, all cannulae were reopened, and animals were presented with counterbalanced pairs of NaCl and sucrose, as described above. Intakes were recorded after each 10 minute intake trial. Following all three trials that constituted one test, cannulae were closed, and food and water were returned to the cage. This testing procedure was repeated 3 additional times, with a one-week interval between each depletion and test.

Study 5b

To examine the effect of sodium repletion on preference for NaCl and sucrose in sodium-deplete animals, the intakes of real-drinking rats were measured during a 120 minute test.

Prior to the start of the experiment, animals were given access to sucrose and NaCl solutions for 5 days, to prevent the effects of neophobia on testing. Following this habituation, all animals (n = 14) were sodium-depleted as previously described. Twenty-four hours after depletion injections, animals were weighed, and food and water were removed from the cage. Rats were then given NaCl (1.8%) and sucrose (10%) solutions for 120 minutes. These concentrations were used because they were preferred in study 5a. Intakes were measured at 15, 30, 45, 60, 90 and 120 minutes. Following testing, food and water were returned to the cage.

Study 5c

To test the hypothesis that sodium depletion results in a general increase in motivation, intake of sucrose by deplete rats was measured.

Prior to the start of the experiment, all animals (n = 13) were exposed to sucrose and NaCl solutions for 5 days to eliminate neophobia. Following this habituation, animals were randomly assigned to either sodium-deplete or non-deplete control groups. Animals in the deplete group were sodium-depleted as described in chapter 2, and animals in the control group received an equivalent injection of isotonic saline.

Twenty-four hours following injections, animals were weighed, and food and water were removed from the cage. All animals were given one bottle of 10% sucrose for 60 minutes. Intakes were recorded. In order to ascertain that deplete animals were normally motivated for NaCl, all animals were subsequently given one bottle of 0.3M (1.8%) NaCl for 60 minutes, and intakes were recorded. The order of these tests was not randomized, in order to maintain a sodium-deplete state during the sucrose intake test.

Statistical analysis

The SPSS statistical program was used to analyze intake data for all groups in these studies. Data from tests 1 and 2 in study 5a were combined to compare several NaCl concentrations to 10% sucrose. Data from tests 3 and 4 were analyzed individually. Intake of NaCl and sucrose in studies 5a and 5b were compared using dependent t-tests to determine significant differences. Independent t-tests were used in study 5c to compare the intakes of the deplete and non-deplete groups.

Results

Study 5a

Intakes of various concentrations of NaCl and sucrose were compared in sodium-deplete, sham-drinking animals to examine the effects of depletion on reward preference.

As shown in figure 5.1, a baseline intake test in non-deplete animals indicated that rats consistently preferred sucrose to NaCl at all concentrations given. Statistical analysis showed that 10% sucrose is significantly preferred over 1% ($t_{14} = 5.71, p < 0.0001$), 2% ($t_{14} = 5.93, p < 0.0001$), and 3% ($t_{14} = 3.27, p < 0.01$), NaCl solutions.

The first intake tests, which compared NaCl to 10% sucrose in sodium-deplete animals, revealed that sodium depletion increases the preference for NaCl to levels similar to that for sucrose. At lower NaCl concentrations, there was no significant difference between NaCl and sucrose intake, indicating a marked increase in NaCl preference (0.5% NaCl: $t_4 = 0.74, p = 0.50$; 1% NaCl: $t_{19} = 1.10, p = 0.28$; 1.5% NaCl: $t_4 = 0.67, p = 0.54$; Figure 5.2). As concentration increased, preference for NaCl decreased relative to sucrose (2% NaCl: $t_{14} = 9.97, p < 0.0001$; 3% NaCl: $t_{14} = 9.38, p < 0.0001$).

The intake tests comparing NaCl to lower concentrations of sucrose confirmed that sodium depletion increases NaCl preference in a concentration-dependent pattern. In a comparison of NaCl and 5% sucrose intake, statistical tests showed that sucrose was only significantly preferred relative to 0.5% NaCl ($t_{14} = 5.88, p < 0.0001$; Figure 5.3). However, both concentrations of hypertonic NaCl were consumed in volumes similar to 5% sucrose intake (1% NaCl: $t_{14} = 0.14, p = 0.89$; 1.5% NaCl: $t_{14} = 0.98, p = 0.34$). These findings were replicated in a comparison of NaCl to 2.5% sucrose. As shown in figure 5.4, sucrose was preferred relative to 0.5% NaCl ($t_{13} = 3.21, p < 0.01$), but there was no difference between 2.5% sucrose and 1% ($t_{13} = 0.54, p = .60$) or 1.5% NaCl($t_{13} = 0.85, p = .41$).

The percentages of total intake for each NaCl and sucrose concentration pair are illustrated in figure 5.5. Although NaCl intake did not significantly exceed sucrose intake, the increase in NaCl palatability is striking. Further, it appears that the increase in motivation was specific to NaCl, as sucrose intake did not increase parallel to NaCl intake.

Study 5b

As study 5a compared intakes in sham-drinking animals, the effects of sodium repletion on motivation for NaCl and sucrose over the course of an appetite test remained to be explored. To do so, study 5b examined the effects of sodium repletion on NaCl and sucrose preference in a 120 minute appetite test, in sodium-deplete, real-drinking rats.

The result of this study confirmed the increase in palatability following sodium depletion. A dependent t-test indicated that there was no significant difference between cumulative intake of 1.8% NaCl and 10% sucrose ($t_{13} = 0.5, p = 0.63$). However, closer analysis showed that preference for NaCl decreased over time. Comparison of intakes at individual time points revealed a significant difference between NaCl and sucrose intake at 90 minutes ($t_{13} = 2.46, p < 0.05$) and 120 minutes ($t_{13} = 1.87, p < 0.05$; Figure 5.6).

Study 5c

The results of studies 5a and 5b suggested that the motivation for sucrose is not increased by sodium depletion. In order to test this hypothesis, intake of 10% sucrose was compared in deplete and non-deplete, real-drinking rats.

This comparison indicated that sodium depletion does not affect motivation for a highly palatable, non-essential reward. Statistical analysis confirmed that there was no difference in sucrose intake between sodium-deplete and non-deplete rats ($t_{11} = 0.28, p = 0.78$; Figure 5.7).

A follow-up test ascertained that sodium appetite was normally expressed in these rats. Sodium-deplete rats consumed significantly more 1.8% NaCl compared to non-deplete animals ($t_{11} = 3.85, p < 0.01$). These findings suggest a high degree of specificity in the motivation provoked by sodium depletion.

Discussion

The present studies indicate a strong, highly specific motivation for NaCl in sodium-deplete animals. In contrast, sodium depletion was not found to affect motivation for sucrose. Sucrose, a palatable natural reward, has been shown to be highly reinforcing and preferred (Levine et al., 2003; Smith & Sclafani, 2002; Pfaffmann, 1982). The baseline data of study 5a supported this, as rats showed a consistent preference for 10% sucrose relative to all NaCl concentrations given. Conversely, NaCl solutions are normally avoided by sodium replete rats (Richter, 1936). Given the a priori disparity between taste preference for sucrose and NaCl, it seemed possible that the availability of sucrose would override the motivation for NaCl in sodium-deplete animals. Alternately, the established shift in palatability of NaCl following depletion (Berridge, et al., 1984) could alter the preference ordering of NaCl and sucrose. In light of these possibilities, the present results are intriguing. Studies 5a and 5b demonstrate that sodium depletion elevates NaCl intake with little effect on sucrose intake. Only at high NaCl concentrations did sucrose intake override the motivation for NaCl. These findings suggest that while NaCl preference is increased by sodium depletion, the motivation for sucrose is unaffected. This was underscored by study 5c, which directly compared the

intake of sucrose by sodium-deplete and non-deplete rats. This study provided clear evidence that the sucrose intake is not affected by sodium depletion.

In addition to confirming that motivation for NaCl and sucrose are similar following sodium depletion, study 5b also demonstrated that the increase in motivation for NaCl is dependent upon the deplete state, and recedes in real drinking animals at a time point previously associated with other hallmarks of sodium repletion. Research has shown that sodium repletion occurs rapidly, following ingestion of NaCl. Deplete rats typically consume 2-3 times the amount of lost sodium within the first 15 minutes of a sodium appetite test. And levels of renin and plasma aldosterone are restored to normal concentrations within 60 minutes of ingesting NaCl (Tordoff et al., 1991). The results of study 5b correspond to this timeline. Seventy-five percent of the total NaCl intake was consumed during the first 15 minutes of the test. And although sucrose and NaCl intakes were similar at 15, 30, and 45 minutes, NaCl intake reached a plateau by 60 minutes, while sucrose intake began to increase. By 90 minutes, the difference was significant. These results add compelling evidence that sodium appetite is not only specific, but it adjusts rapidly to physiological sodium balance.

Although a great deal of previous research has focused on natural reward models, such as food, water and sex, little work has been done to evaluate sodium depletion and its effects on reward motivation. The results of the present studies support the small amount of research that has previously shown that sodium depletion does not affect the overall salience of rewards other than NaCl. Conover et al. (1994) found that sodium depletion has no effect on the reward value of lateral hypothalamic (LH) stimulation, but does increase the reward value of NaCl compared to stimulation in a forced-choice test. And Nozaki et al. (2002) found a preference for NaCl in sodium-deplete rats given a choice of water, NaCl and sucrose. Similarly, the present results indicate that while sodium depletion has no effect on the reward value of sucrose, it does increase the intake of NaCl to comparable levels, suggesting that depletion allows NaCl to compete with sucrose as a reward. Taken together, these studies suggest a highly specific pathway underlying the motivation for NaCl in the deplete animal. Although it is likely that

appetitive responses for rewards follow a final common pathway, it is apparent here that sodium depletion provokes activity in a unique pathway that is dedicated to the behavioral acquisition of NaCl. The lack of effect on response for sucrose or LH stimulation (Conover et al., 1994) suggests that this neural pathway remains independent of the neural regions associated with other rewards until a point further downstream.

Table 5.1. Concentrations of NaCl and sucrose compared in study 5a. Each weekly test compared the intake of three NaCl concentrations to intake of one sucrose concentration.

Test	NaCl concentrations	Sucrose concentration
Baseline (non-deplete)	1%, 2%, 3%	10%
Test 1	1%, 2%, 3%	10%
Test 2	0.5%, 1%, 1.5%	10%
Test 3	0.5%, 1%, 1.5%	5%
Test 4	0.5%, 1%, 1.5%	2.5%

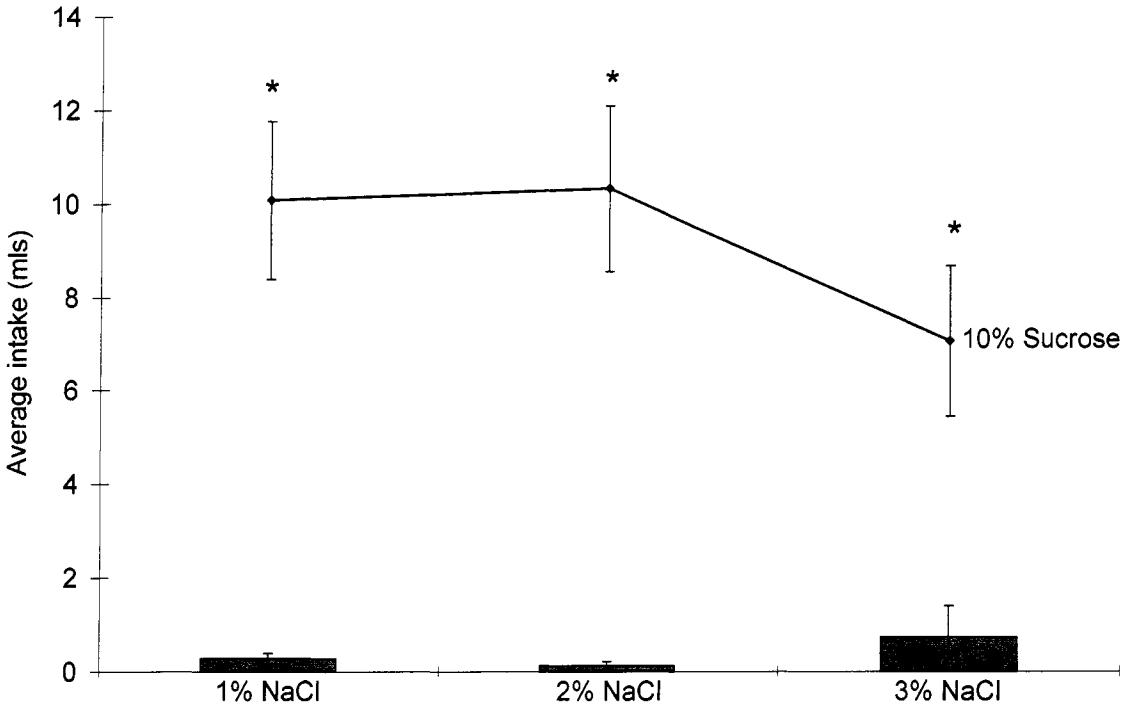


Figure 5.1. Baseline intake of sucrose and NaCl in non-deplete, sham-drinking rats. 10% sucrose is significantly preferred over 1%, 2% and 3% NaCl. *$p < 0.01$ versus paired NaCl.

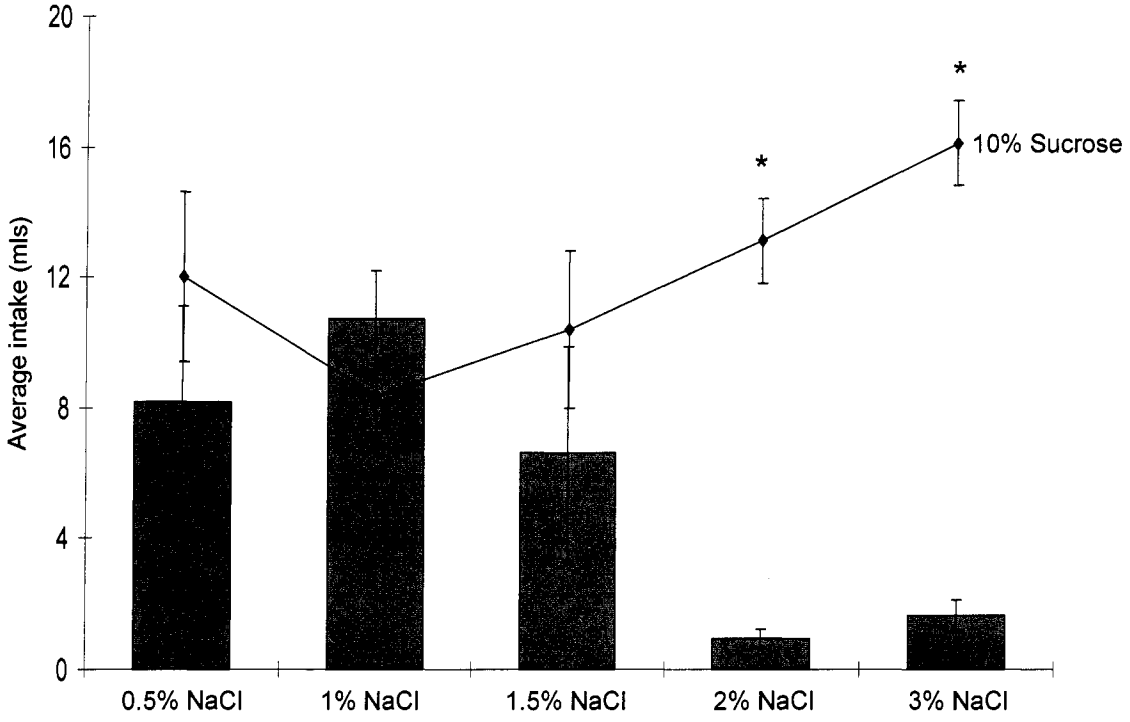


Figure 5.2. Intake of NaCl and 10% sucrose in sodium-deplete, sham-drinking rats. Sodium depletion increases the intake of lower concentrations of NaCl to that of sucrose. *$p < 0.0001$ versus paired NaCl.

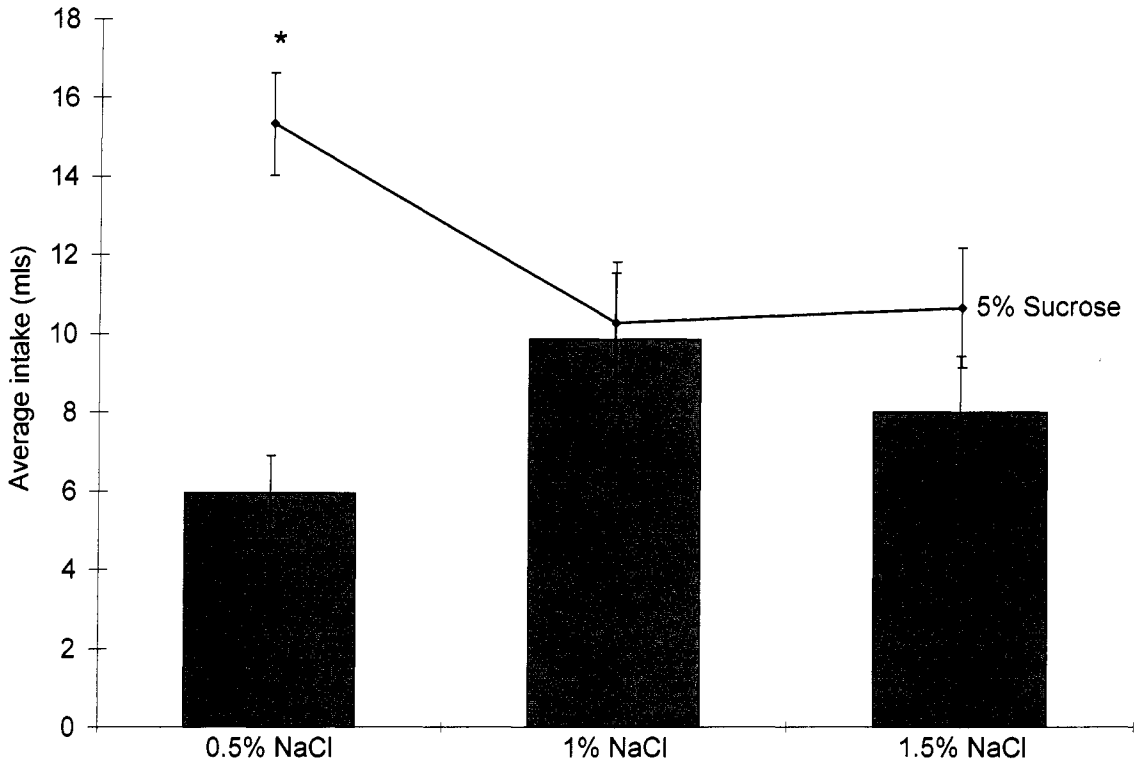


Figure 5.3. Intake of NaCl and 5% sucrose in sodium-deplete, sham-drinking rats. Intake of hypertonic NaCl was similar to that for sucrose. *$p < 0.0001$ versus paired NaCl.

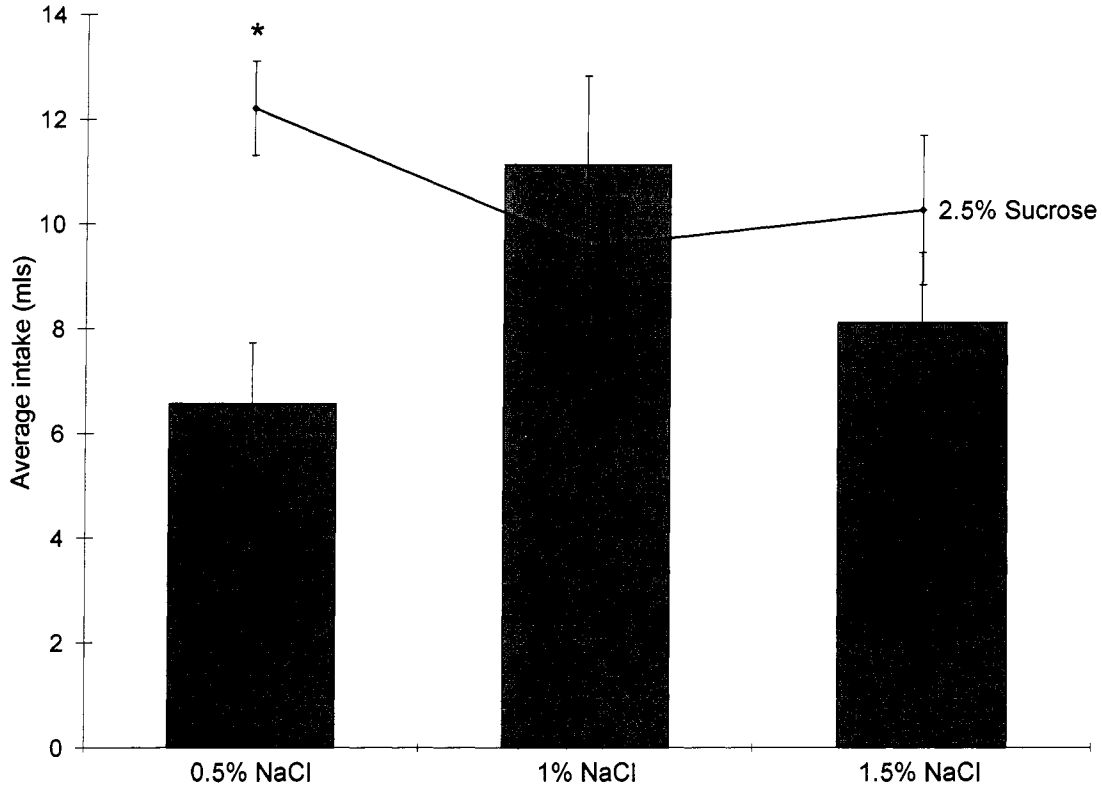


Figure 5.4. Intake of NaCl and 2.5% sucrose in sodium-deplete, sham-drinking rats. Sodium depletion increased intake of hypertonic NaCl to volumes similar to sucrose intake. $*p < 0.01$ versus paired NaCl.

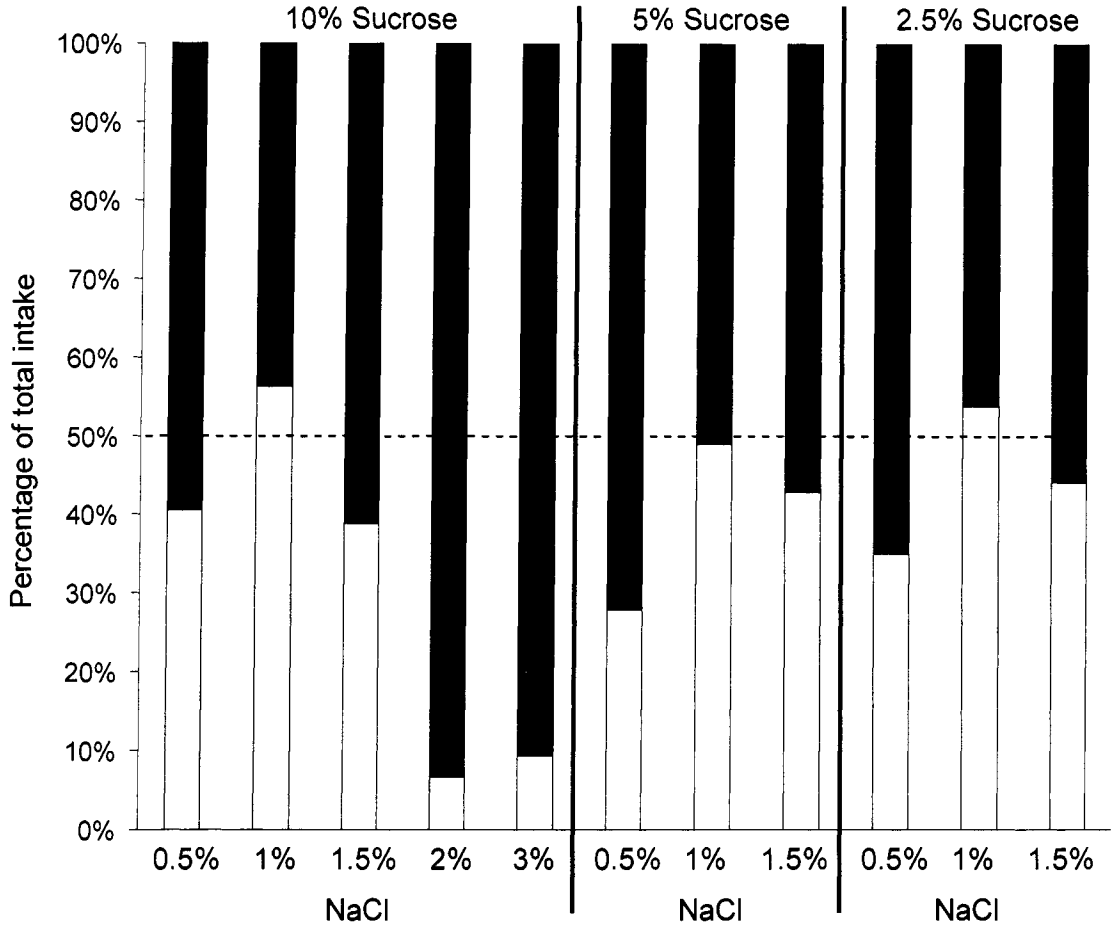


Figure 5.5. Percentage of total intake for each NaCl/sucrose comparison in study 5a. White bars represent NaCl intake, dark bars represent sucrose intake in each pairing.

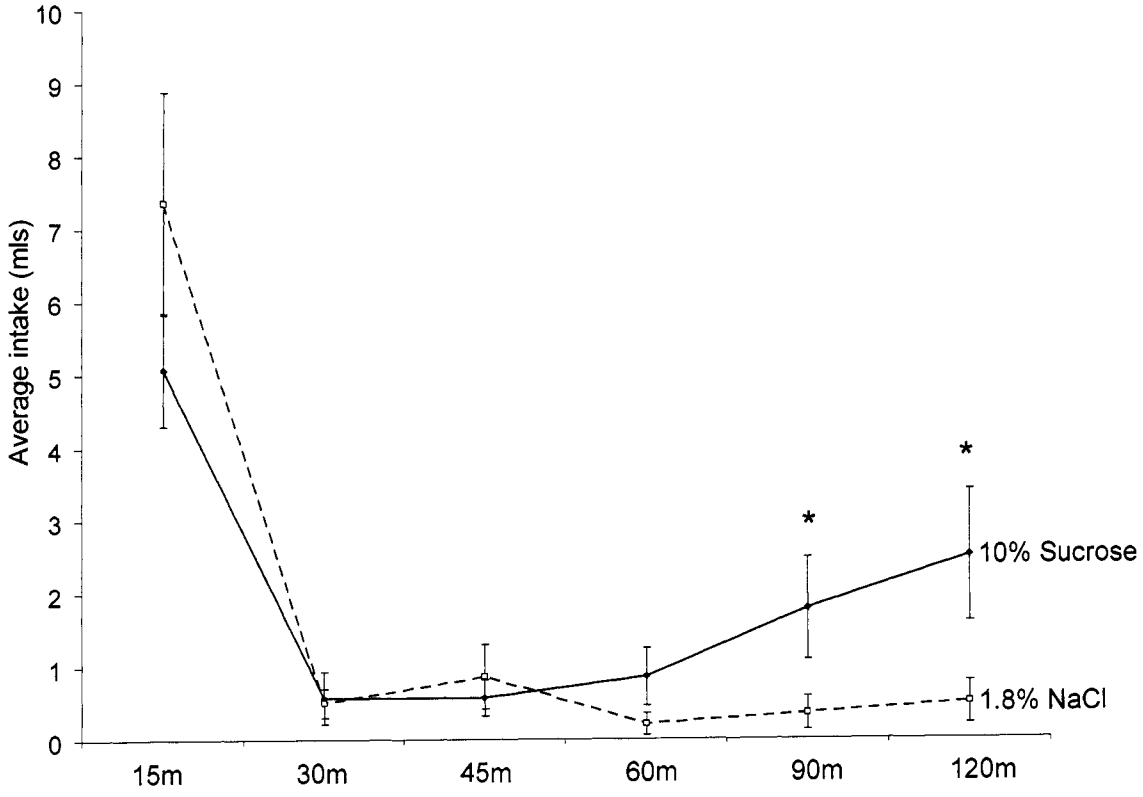


Figure 5.6. Intake of 1.8% NaCl and 10% sucrose in sodium-deplete, real-drinking rats. Intake of NaCl was similar to that for sucrose during the first 60 minutes of the test. Preference for sucrose increased significantly at a timepoint associated with sodium repletion. $*p < 0.05$ versus NaCl.

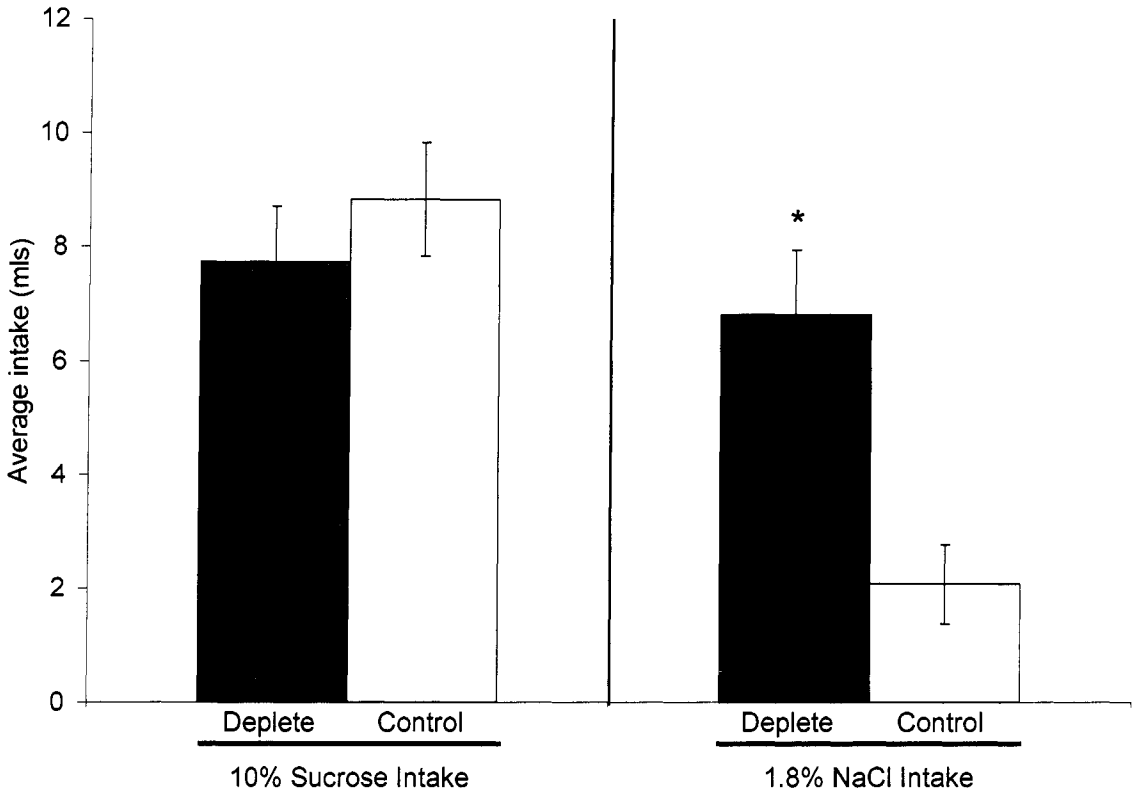


Figure 5.7. Intake of sucrose and NaCl by sodium-deplete and non-deplete control animals in separate tests. There was no effect of sodium depletion on 10% sucrose intake during the first test. A subsequent intake test confirmed a significant sodium appetite in the deplete animals. $*p < 0.01$ versus control.

CHAPTER 6
GENERAL DISCUSSION

The neural and hormonal correlates of sodium depletion have long been understood in terms of renal function, hormone involvement and NaCl intake. However, less work has focused on the motivational properties of sodium depletion and its effects on the neural reward system. The present studies extend what is known about the neural and behavioral effects of sodium depletion as a motivator. These studies have demonstrated that sodium depletion does not strongly activate the nucleus accumbens, nor does expression of sodium appetite depend on dopamine receptor activity. Further, sodium depletion produces a highly specific motivation for NaCl, without affecting motivation for another reward.

A major conclusion drawn from these studies is that sodium depletion and expression of sodium appetite do not strongly activate the nucleus accumbens. This is intriguing, as it differs significantly from the conclusions of research on numerous other models of motivation. Drugs of abuse, food, water, sucrose, sex, brain stimulation: response to all of these rewards results in robust NAc activation (Smith, 2004; Crombag et al., 2002; Hedou et al., 2002; Roop et al., 2002; Vanderschuren et al., 2002; Bradley & Meisel, 2001; Park & Carr, 1998; Chergui et al., 1996; Wenkstern et al., 1993; Hope et al., 1992; Graybiel et al., 1990). Many of these natural and artificial rewards specifically increase Fos-IR in the NAc (Lopez & Ettenberg, 2002; Vanderschuren et al., 2002; Bradley & Meisel, 2001; Park & Carr, 1998; Hope et al., 1992; Graybiel et al., 1990); activation that was conspicuously absent in the studies of chapters 2 and 3. Although no increases in NAc Fos-IR were found following expression of sodium appetite, this model does share similarities with other natural reward models in terms of behavioral motivation. Sodium appetite is highly context-dependent; animals will only increase NaCl intake while sodium-deplete. Further, the behavior produced is robust and directed at obtaining sodium. Sodium-deplete animals will work for NaCl solutions, and show increased preference for these solutions relative to other, nonsodium-containing solutions. Thus, it is surprising that no increased Fos-IR was associated with expression

of sodium appetite. However, it remains possible that activation of the NAc in the present model may be detectable with a more sensitive measure, and it is also possible that lesion studies may reveal a role for the NAc in expression of sodium appetite. Because the present studies did not incorporate such measures, it is not possible to entirely rule out the involvement of the NAc in the response to sodium depletion. Nonetheless, it is surprising that this model of motivation did not produce Fos-IR similar to that seen in other models of reward motivation.

These findings suggest that regions associated with fluid and sodium balance play a greater role in sodium appetite than does the mesolimbic circuitry. The subfornical organ and supraoptic nucleus have both been shown to be activated by sodium depletion (Thunhorst et al., 1998; Rowland et al., 1996). Further, increased levels of the hormones aldosterone and angiotensin II are associated with sodium appetite (Sakai, 1986; Speilman, 1974). Administration of these hormones in chapters 2 and 3 significantly activated the SFO and SON, but had no effect on the NAc. Although these studies did not examine Fos-IR in the SFO or SON following sodium depletion, other studies using the same method of depletion have demonstrated activation of these regions after similar procedures (Rowland & Morian, 1999; Thunhorst et al., 1998; Rowland et al., 1996). In the present experiments that tested its expression, animals showed normal, robust sodium appetite, confirming the presence of the motivation. Clearly, neural control of the behavior was present, though seemingly not in the NAc.

The present findings also indicate that activation of dopamine D1 receptors is not necessary for expression of sodium appetite. Previous research has indicated that DA D2 receptors are likewise, not critical for normal expression of sodium appetite (Roitman et al., 1997). This is surprising, as sodium depletion has been shown to decrease dopamine transporter activity in the NAc (Roitman et al., 1999), which would indicate that dopamine plays a role in the response to depletion. However, these studies looked only at inhibition of D1 and D2 receptors individually, without simultaneous inhibition. Thus, it remains possible that parallel pathways exist, and either D1 or D2 receptor activation alone is sufficient for the expression of sodium appetite. However, it is also possible that

DA receptor activation is simply not critical in this reward model. Other reward models have shown that dopamine is not necessary for the appetitive or consummatory response to reward (Ikemoto & Panksepp, 1999), and DA-deficient mice express normal preference for sucrose, including approaching and initiating licking the bottle (Cannon & Bseikri, 2004). Further, no increase in dopamine release is found during the consumption of a conditioned, predicted reward (Mirenowicz & Schultz, 1994).

As results presented in chapter 4 suggest, normal expression of sodium appetite relies on opiate receptors. General opiate receptor blockade resulted in significant attenuation of sodium appetite expression, without entirely eliminating ingestive behavior. These receptors are known to mediate the hedonic value of rewards, including NaCl (Kelley et al., 2002). And sodium depletion significantly increases the palatability of NaCl (Berridge et al., 1984). Thus, it is likely that normal expression of sodium appetite is motivated by an opiate receptor-mediated increase in the hedonic value of NaCl. Because sodium repletion requires a minimal amount of behavioral output, it is possible that this response could be entirely supported by the increased hedonic value of NaCl. Along with the lack of dependence on DA in the real-drinking model, these results contribute to the definition of the neurochemical systems supporting the response to sodium depletion.

Another major conclusion of these studies is that sham drinking during expression of sodium appetite involves the NAc, and relies on DA receptor activation. Despite the lack of strong NAc activation in a real-drinking model, Fos-IR was dramatically increased by sham-drinking. And unlike real drinking, sham drinking also relies on both DA D1 and D2 receptors. Results from chapter 4, along with the findings of Roitman et al. (1997), demonstrate that blockade of either DA receptor subtype will reduce the intake of sham drinkers to that of real drinkers. These findings support previous indications that sham drinking relies on the mesolimbic circuitry to a much greater extent than real drinking. However, this raises the question of whether this model is simply a magnification of effects that are present but undetected in the real drinking model, or

whether this is a unique model that does not bear direct comparison to the real drinking model.

There are numerous differences between the real and sham drinking models. Real drinking results in rapid sodium repletion, which eliminates the motivation to continue drinking; sham drinking does not. While real drinking provides feedback to the brain to signal satiety, sham drinking lacks any such feedback. Because of this, real drinkers only must make the motor responses of drinking for a limited time, whereas sham drinkers will persist at this behavior for a much greater duration. Along with persistent ingestive behaviors and depletion, sham drinkers also experience ongoing elevated hormone levels. All of these things separate the sham drinking model from the real drinking model of sodium appetite expression. At this point, it remains unclear whether the differences intrinsic to each model account for the differences found in neuronal activation and dopamine involvement, or whether these findings are attributable to a presently unidentified variable.

The attenuation of sham drinking by systemic DA D1 or D2 receptor antagonism raises the question of what neuronal populations mediate this behavioral effect. The dramatic increase in Fos-IR seen in sham-drinking animals indicates a role for the NAc, and it is likely that antagonists affect DA neurons that synapse in this region. This suggests that the neuronal population activated in response to sham drinking may be the same population involved in producing the attenuated intake associated with DA antagonist administration. The NAc receives dopaminergic input from the VTA (Kalivas et al., 1993) and dopamine receptors are found on GABA neurons in the NAc. These neurons are colocalized with either dynorphin or enkephalin, and project to the midbrain (Gerfen et al., 1991). This raises the possibility that the cells activated by sham drinking may be dynorphin- or enkephalin-expressing cells. Previous work has shown that sodium depletion, followed by access to 2% NaCl, significantly elevates enkephalin mRNA in the NAc (Lucas et al., 2003). The same study reported no change in dynorphin levels in deplete animals drinking salt compared to depleted animals drinking water or non-deplete control animals. However, in contrast to the present studies, Lucas et al. (2003)

examined mRNA expression only in real-drinking animals. While these findings suggest that the cells activated by expression of salt appetite in chapter 3 may be enkephalin-containing cells, the present studies did not find significant activation of NAc cells following normal expression of sodium appetite. It is possible that enkephalin-expressing neurons were not sufficiently activated by real drinking as they were in Lucas et al. (2003). However, cFos is not a highly sensitive marker of activation, and it is more likely that increased enkephalin mRNA was present, though undetected by our methods, in these animals. Notably, dramatic elevations in cFos-IR were detected in sham-drinking animals. This not only suggests that a higher magnitude of activation occurred in these animals, but that multiple populations of neurons may be activated by sham drinking that are not activated by real drinking. It is possible that the activation of enkephalin-expressing cells by real drinking is insufficient for detection by cFos, but that sham drinking activates both enkephalin- and dynorphin-containing cells, increasing both the number of cells and magnitude of activation. The involvement of an additional population of cells may explain the dramatic increase in Fos-IR following sham drinking. Enkephalin-containing neurons are known to primarily express DA D2 receptors and project to the ventral pallidum (VP), while dynorphin-containing neurons express DA D1 receptors and project to the substantia nigra (SN; Badiani et al., 1999). Thus, this hypothesis suggests that sham drinking may activate not only the NAc, but also the VP and SN. Given the reciprocal connections between these areas, this may be a critical loop in the circuit maintaining the high level of behavioral output characteristic of sham-drinking.

While normal expression of sodium appetite appears to be controlled by neural circuitry other than the mesolimbic dopamine system, the characteristic qualities of the sham drinking paradigm suggest that this model of natural reward is comparable to other highly motivating models of reward, including psychostimulant drug administration and electrical brain stimulation. The lack of negative feedback to signal satiety and terminate ingestive behavior makes sham drinking a unique model of natural reward. An initially sodium-deplete rat, normally drinking NaCl solution, experiences negative feedback in

the form of increased plasma sodium. But lacking this feedback, the sham-drinking animal will continue responding for reward for an extended period of time. This response pattern seen in sham-drinking animals is remarkably similar to that seen in animals responding for drug administration or brain stimulation. In these artificial reward models, no feedback system exists to terminate behavior. In light of this, the current findings also raise the possibility that models lacking a negative feedback signal not only produce more robust behavioral responses for reward, but also depend on the mesolimbic dopamine circuit to a much greater extent than rewards whose intake is moderated by satiety.

Recent analysis holds that dopamine does not mediate the reinforcing properties of a reward. Rather, dopamine is believed to signal the presence of novel stimuli, both appetitive and aversive, as well as the omission of predicted rewards. This signaling is critical to establishing the response-reward associations that lead to learned behaviors (Schultz, 2006; 2002; 2001). In light of this theory, the current results suggest that normal expression of sodium appetite does not involve a learning process. Sodium appetite is a biologically driven motivation, and its expression is innate. The animal does not need to learn to respond to depletion. In the presence of NaCl, it will respond with robust drinking on the very first exposure. Thus, it may be that there is no need for learning to occur, and no increased DA activity in this model. It is only in the sham-drinking rat that dopamine receptor activation is critical for behavioral response. This suggests that the persistence of sham drinking may rely on an error signal. It is possible that the activation seen in the NAc following sham drinking results from DA neurons signaling the omission of an expected reward; that is, lack of repletion following ingestion of NaCl. Although this was not directly measured in the present studies, if the activation seen in the NAc did result from an error signal, it is also possible that by blocking that signal with dopamine receptor antagonists, the impetus to continue drinking was eliminated. It is unlikely that the brain relies on an error signal to persist in drinking; however, an error signal may be a significant contributor to the prolonged sham drinking in a rat with functional DA receptors.

Alternately, it is also possible that the dopamine receptors are critical for maintaining behavioral responses. It is well established that DA is necessary for motor output in many behavioral models (Koob & Swerdlow, 1988; Gershanik et al., 1983). Perhaps blockade of DA receptors simply reduces the normally exuberant behavior of sham drinkers to output levels similar to those generated by real-drinking rats. Even real drinking NaCl at very low concentrations does not result in the same degree of behavioral output as sham-drinking at any NaCl concentration (Roitman et al., 1997). Real-drinking behavior may not rely on DA activity because it does not necessitate a behavioral response as strenuous as sham-drinking. This raises the possibility that sham-drinking depends on DA receptor activation because of the high degree of behavioral response required by this model. However, it is also possible that sham drinking relies on DA receptor activity for reasons independent of its behavioral response requirements.

The present studies have extended what is known about the effects of sodium depletion on motivation and neural reward circuitry. Sodium appetite is a distinct model of reward motivation, comparable to other models of natural reward on behavioral measures, though it does not appear to strongly depend on the NAc or DA system. However, the current findings also indicate that sham drinking during sodium appetite expression is a robust and unconventional model of natural reward. This model, though based on a natural drive to maintain homeostatic balance, mimics the behavioral and neural effects of artificial reward models. The results of these studies reveal a role for the NAc and DA receptors in mediating the response for NaCl in sham-drinking rats, suggesting that the mesolimbic system is critical for mediating behavioral responses that lack moderating negative feedback, despite playing little or no role in normal feedback situations.

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Publications

Journal Articles

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D.L. Adkins , A.C. Voorhies, and T.A. Jones. Behavioral and neuroplastic effects of Focal endothelin-1 induced sensorimotor cortex lesions. Neuroscience, 128 (2004).

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