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The sensitization of sodium appetite: Plasticity in neural networks governing body fluid homeostasis and motivated behavior

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THE SENSITIZATION OF SODIUM APPETITE: PLASTICITY IN NEURAL NETWORKS GOVERNING BODY FLUID HOMEOSTASIS AND MOTIVATED BEHAVIOR

by

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CERTIFICATE OF APPROVAL

PH.D. THESIS

This is to certify that the Ph.D. thesis of

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Abstract

When most omnivores and herbivores become sodium depleted they engage in the motivated behavior of sodium appetite (AKA salt appetite), or the seeking out and ingestion of salty substances. Sodium appetite is associated with psychological processes that serve to enhance the incentive and rewarding value of salty substances. These processes attract animals to salty substances and reinforce the ingestion of salty substances. The experience of sodium depletion also produces long-lasting changes in behavior; one of the most apparent changes being a seemingly life-long increase in hypertonic salt intake which indicates sodium appetite is sensitized. Two neural circuits have been implicated in the sensitization of sodium appetite: 1) a forebrain neural circuit that regulates body fluid homeostasis, and 2) the mesolimbic dopamine system which mediates motivated behaviors. This dissertation has three aims that serve the overall purpose of providing a better understanding of the neurobiological mechanisms that mediate sodium appetite and the sensitization of sodium appetite. The first aim is to develop a model of sodium depletion that is amenable to pharmacological manipulation in order to determine whether the blockade of N-methyl-d-aspartate receptors, which are critical for neural plasticity, will prevent the sensitization of sodium appetite. The second aim is to determine whether sensitization is associated with relatively long-term molecular changes in forebrain areas that regulate body fluid homeostasis. The third aim is to identify how forebrain areas involved in body fluid homeostasis connect to and influence activity in the mesolimbic dopamine system. Taken together, these experiments provide support for a neural circuit that exhibits plasticity after sodium depletion that is comprised of brain structures that sense and process body fluid balance, integrate homeostatic state with motivation and reward systems, and motivation and reward systems themselves.

Public abstract

People in Westernized societies tend to eat too much salt (also known as sodium). This "salt gluttony" has been implicated in many diseases including cardiovascular disease and autoimmune disease. When many mammals become sodium depleted (through excess vomiting, diarrhea, or hemorrhage) they enter a state of sodium deficiency that causes them to seek out and ingest foods and drinks that contain salt. Interestingly, when animals are repeatedly depleted of sodium they progressively increase their salt intake and essentially become salt gluttons. This dissertation investigates how sodium depletion changes the brain in order to elicit salt gluttony. The goal of this work is to provide a foundation for potential behavioral or pharmacological treatments that may one day help prevent excess salt intake.

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Chapter 1: Introduction and Background[1]

1.1 Thirst and sodium appetite

Animals maintain body fluid homeostasis by regulating the distribution and concentration of water and sodium. Animals face different challenges to body water and sodium content depending on their environmental niche [135]. For example, the distribution and types of ions present in the intracellular and extracellular space of aquatic animals is dependent on whether they live in fresh or salt water environments. Aquatic animals are characterized as either osmoconformers that regulate extracellular fluids to match the osmolality of their environment or osmoregulators that regulate osmolality within a strict range (~300mOsm; [100]). By the nature of their environment, terrestrial animals are osmoregulators that continually lose body water and sodium through normal physiological processes and environmental demands. In order to replenish lost water and sodium, terrestrial animals must engage in behaviors related to thirst and sodium appetite, or the seeking and ingestion of water and salty substances. Terrestrial animals exhibit drinking (i.e., thirst) intermittently because water is frequently lost from the body due to respiration, transpiration, and the formation of urine. Sodium is lost along with water through normal physiological processes including urination, defecation, salivation, and sweating. However, because the body has reserves of sodium [128], sodium must be taxed to a fairly great extent before salt appetite is evoked. Thus, sodium appetite results from less-common circumstances that deplete the body of the ion including pregnancy, excess vomiting, sweating, hemorrhage, diarrhea, and long-term maintenance on a sodium deficient diet [18, 38, 110].

[1] Portions of Chapter 1 are derived from reviews published by the author (see Refs. 73 and 74).

Sodium-bereft diets provided the selection pressure that shaped neural mechanisms which promote sodium appetite in herbivores and omnivores [38, 56, 87]. Carnivores ingest sufficient amounts of sodium from the viscera and muscle tissue of their prey [38, 148], and when tested under sodium deficient conditions, carnivores either fail to exhibit sodium appetite or exhibit a meager sodium appetite. In contrast, herbivores subsist off of plants that tend to be very low in sodium. As such, herbivores exhibit a robust sodium appetite under sodium-deplete conditions [37, 148]. Perhaps one of the most profound examples of the herbivore's robust sodium appetite comes from elephants in Kenya that travel deep into Mount Elgon where they extract salt from cave walls [14]. Omnivores also exhibit sodium appetite under sodium-replete conditions [2, 136, 161]. Early humans may have been particularly prone to sodium depletion as they primarily lived in environments that were bereft of sodium and a majority of their diet was composed of plant matter that was low in sodium [38]. These environmental conditions provided the selection pressure for a set of physiological mechanisms that would help maintain and replenish sodium stores in humans.

Most of our knowledge of the neurobiological mechanisms that mediate salt intake has come from studies examining rats. In fact, Curt Richter was the first to demonstrate sodium appetite in the laboratory in an experiment that used rats as subjects [136]. In rodent models of sodium appetite, the ingestion of hypertonic saline solutions (1.5-9.0% w/v) is the dependent variable that is used to assess sodium appetite. These hypertonic saline solutions are aversive to rats that are in sodium balance and, as such, rats will only seek out and ingest significant quantities of these hypertonic saline solutions when they become sodium deficient [5]. Several experimental protocols have been employed to evoke sodium appetite (for review see Ref. [75]). At the present time, administration of the diuretic and natriuretic drug furosemide is one of the

most commonly used sodium depletion protocols. In this protocol rats receive injections of furosemide and normal rat chow is replaced with sodium deficient diet. 20-24 hours after furosemide treatment rats are allowed access to hypertonic saline and sodium appetite is assessed. Injections of furosemide produce a very robust sodium appetite (10-15 mls of 1.8% hypertonic saline intake in 3 hours). With the furosemide model of depletion sodium appetite develops approximately 20 hours after furosemide treatment. However, furosemide can also be combined with a low dose of a blood pressure lowering drug, such as captopril (5 mg/kg), a method of sodium depletion referred to as furo/cap (see Table 1 for abbreviations), to produce a rapid onset sodium appetite (~ 1 hour; [54]). When co-administered, these drugs cause a state of hypovolemia coupled with a slight drop in blood pressure [54, 165] that elicits rapid thirst and salt appetite responses. This protocol produces a reliable but modest sodium appetite (3-7 mls of 1.8% hypertonic saline intake in 3 hours).

1.2 Body fluid homeostasis systems

Sodium appetite is initiated through the coordinated activity of physiological mechanisms in the central nervous system and periphery [81, 85]. Sodium depletion results from a state of extracellular dehydration (water and sodium loss) that is associated with reduced total fluid volume. The decrease in total fluid volume has drastic effects on cardiovascular function and, as a result, physiological responses are engaged to protect and restore fluid volume. Blood volume is monitored by baroreceptors located in organs that regulate cardiovascular function including the kidneys, heart, and vasculature [16]. Baroreceptors detect the decrease in blood volume and pressure caused by extracellular dehydration and trigger hormonal and sympathetic responses to promote water and sodium retention, redistribution of blood to ensure organ perfusion, and the behavioral responses of thirst and sodium appetite [129]. These physiological and behavioral

responses are mediated by two renin-angiotensin-aldosterone-systems (RAASs) which include a peripheral hormonal system and a central neurotransmitter/neuromodulator system [43, 45, 60, 176].

The RAASs are essentially signaling cascades located in the periphery and central nervous system that serve to protect body fluid homeostasis. During a state of extracellular dehydration the peripheral RAAS signaling cascade (endocrine) is engaged which begins with the synthesis of renin by the kidneys and its subsequent release into circulation. Renin circulates in the plasma where it cleaves constitutively present angiotensinogen into angiotensin I. Angiotensin I is biologically inert and serves as an intermediate peptide that is converted by angiotensin converting enzyme (primarily located in the lungs and vasculature) into the octapeptide angiotensin II (Ang II). In many ways Ang II is the centerpiece of restoring body fluid homeostasis - Ang II coordinates many of the physiological and behavioral responses necessary to defend and restore body fluids [52, 133]. Physiologically, Ang II induces vasoconstriction and triggers the release of two hormones critical for regulating body fluids – vasopressin and aldosterone [21, 22, 88]. Vasopressin is a peptide hormone synthesized in the magnocellular neurons of the hypothalamus and secreted from the anterior pituitary into the circulation to promote vasoconstriction and water retention by the kidneys [171]. Aldosterone is a steroid hormone released from the adrenal cortex that promotes renal sodium retention and the motivated state of sodium appetite. Ang II also acts at the adrenals to stimulate aldosterone synthesis and release. With respect to behavior, Ang II induces thirst and synergizes with aldosterone to elicit sodium appetite [43, 58]. In addition to the peripheral RAAS, there is also a central RAAS. The synthetic enzymes and precursors necessary for Ang II and aldosterone synthesis are present in the central nervous system [52, 61, 64]. Ang II is capable of acting as a

neurotransmitter and aldosterone can act as a neuromodulator where they function as synaptic and paracrine signals to increase sympathetic tone, raise blood pressure, and promote thirst and salt appetite.

The neural circuitry that regulates fluid balance involves the coordinated action of hindbrain and forebrain nuclei (see Figure 1 for an overview of the neural circuitry mediating sodium appetite; [85]). In the hindbrain, the nucleus of the solitary tract (NST) and adjacent area postrema play a critical role in monitoring body fluid homeostasis [85, 114]. These areas are capable of sensing and processing levels of circulating Ang II and aldosterone, extracellular fluid osmolality, and changes in blood pressure. The NST projects to the lateral parabrachial nucleus (LPBN), and together these structures inhibit sodium appetite tonically [29]. It is likely that a confluence of internal signals including a decrease in blood pressure and an increase in both circulating and central Ang II and aldosterone alter activity in the NST and AP to result in a disinhibition of sodium appetite.

In the forebrain, an ensemble of brain nuclei located along a region of the brain known as the lamina terminalis (LT) contribute to fluid balance regulation [82, 111]. The nuclei located along the LT include the subfornical organ (SFO), median preoptic area (MnPO), and organum vasculosum of the lamina terminalis (OVLT). The SFO and OVLT deserve significant attention because they lack a blood-brain barrier. This grants these structures the capability of monitoring the osmotic status of circulating plasma in addition to concentrations of circulating Ang II and aldosterone [83]. Changes in peripheral osmolality and an elevation in circulating levels of Ang II and aldosterone trigger SFO and OVLT activation to ultimately cause thirst and sodium appetite [111]. The MnPO appears to act as an integrative relay that receives information from the SFO and OVLT [100].

1.3 Motivation and reward systems

Motivation is a psychological construct that was developed as a heuristic to explain why animals engage in non-reflexive, goal-directed behaviors [10, 11]. Within the behavioral sciences, motivation has often been evoked to explain why animals avoid punishers and search for and procure rewards [4]. Rewards can also be conceived of as incentives, a term originally coined by Rietta Simmons [153] which she described as "the end which serves to arouse, to direct, and to bring to a conclusion some persistent activity." In other words, incentives are goal objects that animals will seek out in order to engage in a consummatory behavior (e.g., the act of copulation, engaging in various maternal behaviors, drinking, or eating). In the case of ingestive behaviors, the seeking and consumption of goal-objects can be divided into appetitive and consummatory phases of motivation [31]. The appetitive phase is marked by a state of arousal and flexible (i.e., non-reflexive) seeking of goal objects. For example, a hungry rat may become aroused and begin searching for food, most likely relying on past experience and sensory information to locate food in the environment. Once an animal comes into contact with a goal object it enters a consummatory phase that is defined by the consummation of the appetitive state [22]. For example, the ingestion of food by a hungry rat is the consummatory phase of hunger.

Initially the concept of motivation was a heuristic intended to aid in the understanding of behavior and, like most heuristics, it had limitations. However, the greatest advances in understanding motivation have come from physiological psychologists who have applied a reductionist approach to the study of motivation [4, 44]. This effort has yielded physiological underpinnings of motivation including neural circuits, molecular mechanisms, and endogenous ligands that modulate motivated behaviors. These discoveries have advanced the study of motivation from a heuristic to defined physiological and neural systems that can be

experimentally manipulated. Although many brain regions influence the motivated state of an animal, the dopaminergic projections from the ventral tegmental area (VTA) to the nucleus accumbens (NAc; AKA the mesolimbic or A10 dopamine system) appears to play a key role in all appetitive motivated behaviors [77, 89, 147].

Although structures along the LT and hindbrain detect when the body is in a state of a sodium deficit (see Section 1.2), these sensory areas must work in tandem with neural circuitry that promotes motivated behaviors in order to elicit thirst and salt appetite. In other words, sodium depleted animals need to become active and start navigating the environment to find the nearest source of salt. The mesolimbic dopamine system essentially energizes behavior (i.e., promotes a state of arousal), directs behavior towards a goal object in the environment, and aids in reinforcement when the goal-object is obtained [10, 11]. Increased neural activity in the NAc and dopamine release in the NAc is correlated with the presence of a sodium appetite [99, 124, 140].

Sodium depletion evokes processes in the central nervous system that induce the motivated state of sodium appetite. Sodium appetite is associated with central nervous system mechanisms that alter the appraisal of salty substances. One such process is referred to as the *hedonic shift* that occurs during sodium depletion [5]. The hedonic shift is characterized by a change in the hedonic appraisal and incentive value of salty substances. In other words, during sodium depletion animals begin to evaluate salty substances as more palatable (i.e., the hedonic quality of sodium becomes increasingly positive; [5, 137]) and animals begin to seek out and ingest salty substances (i.e., the incentive value of salty substances is increased [27, 137]). Perhaps the most striking demonstration of the hedonic shift associated with sodium appetite has come from the work of Kent Berridge and colleagues. They have been assessing sodium appetite

by measuring the intake of Dead Sea concentrations of hypertonic saline (9% NaCl; [137]). When sodium-replete rats taste this very high salt concentration they exhibit a pattern of species-specific orofacial and forelimb behaviors that are indicative of aversion/disgust. Furthermore, when placed in a cage with a lever that can be pressed to produce intra-oral delivery of Dead Sea hypertonic saline, rats will learn to associate the lever with the aversive flavor of hypertonic saline and as such they will avoid and bury it [137]. However, when rats are made sodium deficient they perform approach behaviors towards the lever, lick the lever, and press it to obtain sodium. Once they taste the concentrated saline rats will engage in a pattern of species-specific behavioral responses consistent with those that indicate that the taste of salt is rewarding [65]. The same set of responses is also emitted when rats taste sweet sucrose solutions. Therefore, sodium depletion is capable of shifting the hedonic value of salt from a negative (aversive) to a positive (rewarding) stimulus [5, 7]. Furthermore, it appears that the hedonic shift for salt occurs before sodium depleted rats have even had the opportunity to voluntarily ingest salt (i.e., the hedonic shift occurs independently of learning; [137]). The first time rats become sodium depleted they will avidly lever press to obtain Dead Sea hypertonic saline despite the fact that all of their prior experience with this solution had been negative.

The hedonic shift that occurs during sodium depletion alters how the nervous system, including structures within the mesolimbic dopamine system, processes the taste of salt [28, 79, 101]. In the periphery, gustatory nerves exhibit reduced responses to the taste of salt during sodium deficiency [28]. Neurons within the NST, which receives afferent information from the gustatory nerves, also exhibit altered firing patterns to the taste of salt during deficiency [79]. Similar to the changes in firing observed in the gustatory nerves, the salt-responsive neurons in the NST exhibit reduced firing during deficiency. Interestingly, neurons in the NST that respond

to sweet tastes such as sucrose become activated in response to the taste of salt in the depleted animal [79]. Areas in the forebrain that code the motivational and rewarding value of stimuli exhibit a different profile of responses to the taste of salt during sodium deficiency [101, 141, 142]. Neurons in the NAc appear to be involved in evaluating the motivational valence of stimuli [141, 142]. In other words, the NAc plays a role in determining whether stimuli in the environment should be approached or avoided [50]. An inverse relationship exists between neuron firing rate in the NAc and the rewarding properties of taste stimuli [141]. Specifically, neurons in the NAc exhibit an increase in activity in response to aversive tastes such as quinine. In contrast, palatable tastes like sweet sucrose solutions reduce the firing rate in the NAc. The inhibition of NAc neurons that occurs in response to palatable tastes appears to be caused by the release of the neurotransmitter dopamine from neurons originating in the VTA into the NAc [142]. With respect to sodium appetite, NAc neurons exhibit either an increase or decrease in firing rate in response to the taste of hypertonic saline solutions depending on the homeostatic state [101]. Rats that are in sodium balance show increased firing of NAc neurons to the taste of hypertonic saline solutions. However, sodium deficient rats exhibit a reduced firing rate [101]. In other words, in sodium-replete rats the NAc processes salty tastes similar to how it processes the taste of bitter quinine solutions [101, 141]. Sodium deficiency changes how the NAc processes salty tastes such that firing patterns are consistent with the response to sweet sucrose solutions [101, 141]. This indicates that the dynamic evaluation of the rewarding or aversive quality of salty tastes is associated with a change in the activity of NAc neurons. Interestingly, sodium depletion also alters how neurons respond to salty tastes in the posterior ventral pallidum (VP), a brain region that has been implicated in generating reward [154]. Neurons in the VP are normally unresponsive to the taste of hypertonic saline solutions [166]. However, during sodium

deficiency neurons in the VP exhibit increased firing in response to the hypertonic saline solutions. These experiments suggest that the experience of sodium depletion can produce significant changes in how brain areas that code reward and the motivational valence of environmental stimuli process salty tastes. It is likely that the coding of salty tastes in the NAc and VP is influenced by activity in structures along the LT and hindbrain that are involved in sensing deficits in body fluid homeostasis. The changes in how the NST, NAc, and VP process salty tastes may be necessary for the hedonic shift that occurs during sodium depletion.

1.4 The sensitization of sodium appetite and neural plasticity

The average sodium intake in most Westernized societies is far in excess of that which is required for normal physiological function [17, 34]. Excess salt intake, also known as salt gluttony [148], can have detrimental effects on cardiovascular health, especially in salt-sensitive individuals [17, 33-35]. Therefore, it is likely that reducing salt intake would have beneficial effects on public health. For example, one projection predicts that a nation-wide reduction of salt intake in the United States could save 92,000 lives and reduce health-care costs by $10 to $24 billion per year [9]. A recent study concluded that in 2010, 1.65 million cardiovascular disease-related deaths worldwide were attributable to high salt intake [120]. Several ideas have been proposed to explain excess salt intake (for review see Ref. [98]). These ideas range from stress-induced salt intake [39, 71] to learned preferences for salty foods [177]. One potential determinant of salt gluttony that has been gaining increased experimental scrutiny is the sensitization of sodium appetite, or the seemingly life-long increase in salt intake that occurs after sodium depletion [75, 144].

The sensitization of sodium appetite was originally discovered by John Falk in 1965 [48]. Falk had sodium depleted rats on two separate occasions and found that rats exhibited enhanced

salt intake on the second depletion. Since this seminal finding, sodium appetite sensitization has been replicated by numerous laboratories that have used protocols to elicit sodium appetite through either sodium depletion [36, 48, 145] or pharmacological means [19]. Perhaps the most translationally relevant aspect of the sensitization of sodium appetite is that it also produces a seemingly life-long increase in daily need-free salt intake [46, 144, 145]. Sodium appetite sensitization appears to be a form of non-associative learning that is expressed through enhanced sodium intake [49, 59]. Sodium intake plateaus after 1 to 3 depletions, depending on the sodium depletion protocol employed [48, 72, 144, 145]. Furthermore, sodium appetite sensitization also potentiates the drive to obtain sodium that is exhibited during sodium deficiency [27].

Enhanced sodium intake has been found to exist in humans who experienced sodium depletion perinatally [32, 96]. Specifically, the children of mothers who were repeatedly sodium depleted during pregnancy exhibit greater salt intake in adulthood [32]. Similarly, infants who experienced repeated bouts of salt loss due to vomiting or diarrhea show an increased salt intake during adolescence [96]. Currently there is no evidence that supports the idea that sodium depletion during adulthood produces life-long increases in need-free salt intake [97]. However, this may simply be due to the fact that virtually every adult human has experienced sodium deficiency during their lifetime and, as such, an adequate control group with no history of sodium depletion may not exist.

The enhanced sodium ingestion associated with sodium appetite sensitization is not caused by an increase in circulating levels of angiotensin II or aldosterone, as neither of these hormones is progressively elevated in the periphery with each successive depletion [145]. Therefore, one hypothesis is that sodium depletion alters central nervous system circuitry beyond the body-to-brain signaling roles of circulating angiotensin II and aldosterone [75]. In other

words, it is likely that sodium depletion induces neural plasticity in the central nervous system to drive elevated salt consumption. Neural plasticity is essentially the process by which neurons change in structure or function in response to experience [63, 178]. Neural plasticity can be observed as long-term changes in neuron morphology, receptor or mRNA expression, and neurotransmitter synthesis and release, amongst other changes. Many forms of plasticity require the activation of glutamatergic N-methyl-D-aspartate receptors (NMDA-Rs; [69, 86, 167]). The NMDA-R is a unique gated ionotropic receptor that only enters an open state when glutamate is bound to the receptor and the neuron upon which the receptor is present is sufficiently depolarized. Therefore, these properties of the NMDA-R provide a physiological mechanism that allows for Hebbian learning [70], where the co-activation of neurons increases the strength of the connection between those neurons (put simply - neurons that fire together, wire together; [30]).

Evidence suggests that sodium appetite sensitization is associated with neural plasticity within at least two neural circuits: the LT and the mesolimbic dopamine system [124, 139]. Rats with a history of sodium depletion exhibit elevated sodium depletion-induced c-*fos* expression in both the SFO and NAc [124]. Additionally, a history of sodium depletions enhances dendritic arborization and length in the NAc [139]. Currently, relatively little work has been conducted to determine whether neural plasticity in these areas has any functional significance, although lesions of the SFO appear to prevent sensitization [143]. With respect to the changes in neuron morphology observed in the NAc, it is worth considering the possibility that plasticity occurring in the VTA drives plasticity in the NAc [107].

1.5 The hypothalamus as an integrator of homeostatic state with motivation and reward systems

Over the last half-century, much of what we know about the neural mechanisms that underlie both body fluid homeostasis and motivation and reward has advanced significantly. However, the mechanism by which forebrain body fluid homeostasis systems (e.g., structures along the LT) interact with and modulate activity within the mesolimbic dopamine system is unclear. In order for a robust sodium appetite to occur, LT structures must work in tandem with the mesolimbic dopamine system [72, 102, 103, 140]. Furthermore, it appears that the experience of sodium depletion induces neural plasticity in both the LT and the mesolimbic dopamine system[124, 139], indicating that there may be important interactions between these two systems in promoting the sensitization of sodium appetite. There do not appear to be direct major projections from the LT to the VTA, although sparse projections from the MnPO have been reported [62, 134]. One possibility is that an intermediate structure or set of structures aids in integrating information related to body fluid status with motivation and reward systems. One region of the brain that may serve this purpose is the hypothalamus [73].

The hypothalamus appears to be capable of integrating information from systems that sense and process deficits in homeostasis (e.g., a shortage of calories or sodium) in order to elicit activation of downstream systems that promote adaptive autonomic and behavioral responses [159]. The hypothalamus is of particular interest because it contains a large number of neurons that project to the VTA [62, 134]. These neurons are distributed in an arc that extends across three nuclei in the hypothalamus - the dorsomedial hypothalamus (DMH), perifornical area (PeF), and the dorsal region of the lateral hypothalamic area (LHAd). It has been shown that the SFO, one area that plays an important part in regulating body fluid homeostasis, sends

projections to regions of the hypothalamus that tend to contain VTA-projecting neurons [158]. It is likely that VTA-projecting neurons in the hypothalamus receive inputs from nuclei that sense deficits in both body fluid and energy homeostasis [73, 158]. These neurons may serve the overall purpose of integrating information related to homeostatic status with motivation and reward systems in order to promote the seeking and ingestion of rewards that are necessary to restore homeostasis including food, water, and salt [73].

The neuropeptide orexin (Ox, also known as hypocretin) is expressed in neurons that are distributed in the caudal half of the hypothalamus. Similar to the medial-lateral distribution of VTA-projecting neurons in the hypothalamus, neurons that synthesize Ox extend in an arc from the DMH to the LHAd (Figure 2). Ox appears to be one of the few known centralized peptide neurotransmitter systems as Ox neurons from a relatively circumscribed region of the hypothalamus send distal projections to diverse areas of the brain [132]. Consistent with this idea, Ox neurons have been implicated in the mediation of numerous physiological and psychological phenomena including the stress response [93], pressor response [51], arousal [157], and motivated behaviors [12, 68, 162]. With respect to motivated behaviors, administration of Ox is capable of eliciting robust food (hence the name orexin; [146]) and water intake [92]. Ox neurons have also been implicated in promoting copulation [41, 121]. Importantly, a subset of Ox neurons project to the VTA (Figure 2; [47]), where Ox is capable of depolarizing both dopaminergic and non-dopaminergic neurons [91]. Ox release in the VTA also appears to be capable of eliciting plasticity in VTA neurons [13]. Preliminary data from retrograde tracing studies in our laboratory indicated that regions of the hypothalamus that tend to contain Ox neurons receive input from structures that regulate energy (the arcuate nucleus of the hypothalamus) and body fluid (the LT) homeostasis (Figure 3; [66]). It is possible that Ox

neurons aid in coupling information related to body fluid status from the LT with the mesolimbic dopamine system in order to promote both sodium appetite and thirst.

1.6 Specific Aims

The experiments presented in the following chapters have 3 major aims. Chapter 2 aimed to develop an experimental paradigm of sodium appetite sensitization that would allow for expedient application of pharmacological manipulations in order to test whether NMDA-Rs are critical for sodium appetite sensitization. The objective of Chapter 3 was to identify whether sensitization of sodium appetite is associated with long-lasting molecular changes in the LT and whether these changes are dependent on NMDA-R activation. Chapter 4 aimed to determine whether neurons in the hypothalamus that synthesize the neuropeptide Ox operate to couple body fluid status with motivation and reward systems. Together, the experiments presented in Chapters 2 and 3 indicate that the sensitization of sodium appetite is dependent on NMDA-R activation and is associated with relatively long-lasting molecular alterations in the LT. An additional experiment, presented in Chapter 5, found that activation of NMDA-Rs in the VTA are also critical for sodium appetite sensitization. The experiments from Chapter 4 support the idea that second-order orexinergic neurons in the hypothalamus aid in integrating body fluid status with motivation and reward systems. Finally, in Chapter 5 it is proposed that a LT-Hyp-VTA-NAc circuit is involved in the sensitization of sodium appetite.

1.7 Figures

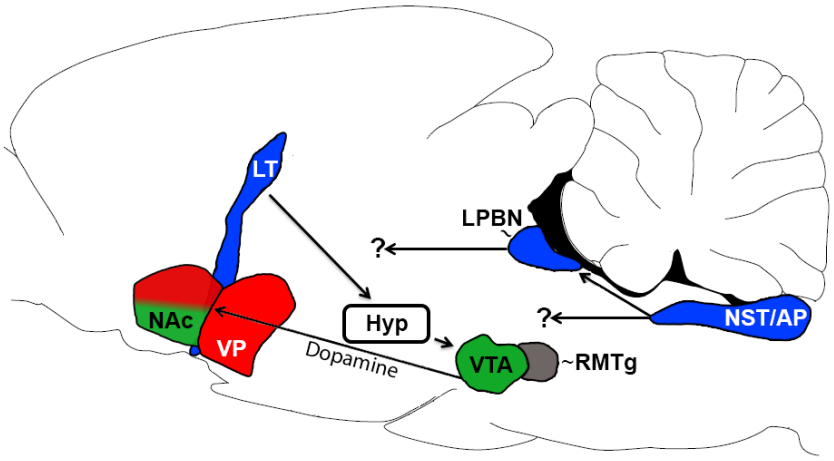

Figure 1 - The neural circuitry that mediates the various aspects of the biopsychology of salt appetite and sodium depletion. Regions in blue represent areas that play a role in detecting and communicating deficiency in mineralo-fluid balance or a decrease in blood pressure. Areas in green are likely to be involved in promoting goal directed behavior towards salt (i.e., a desire for salty substances). Areas in red appear to code the rewarding effect of ingesting salty foods. The NAc is highlighted in both green and red because it contains sub-regions that promote goal-directed behavior and reward [89, 130]. Finally, it is proposed that the rostromedial tegmental nucleus (RMTg), outlined in grey, may mediate aversive effects of sodium deficiency [80, 118]. The NTS and LPBN are likely to project to and influence activity of forebrain and midbrain nuclei that mediate motivated behavior and hedonics [152]. The lamina terminalis appears to project to neurons in the hypothalamus which may serve to integrate body fluid status with motivation and reward systems such as the VTA [73]. The VTA, in turn, sends dopaminergic projections to the NAc that mediate salt craving [6]. It is possible that opioid release in the dorsomedial accumbens is involved in the rewarding aspects of salt ingestion [123, 130]. Abbreviations and a brief description of brain areas are presented in Table 2.

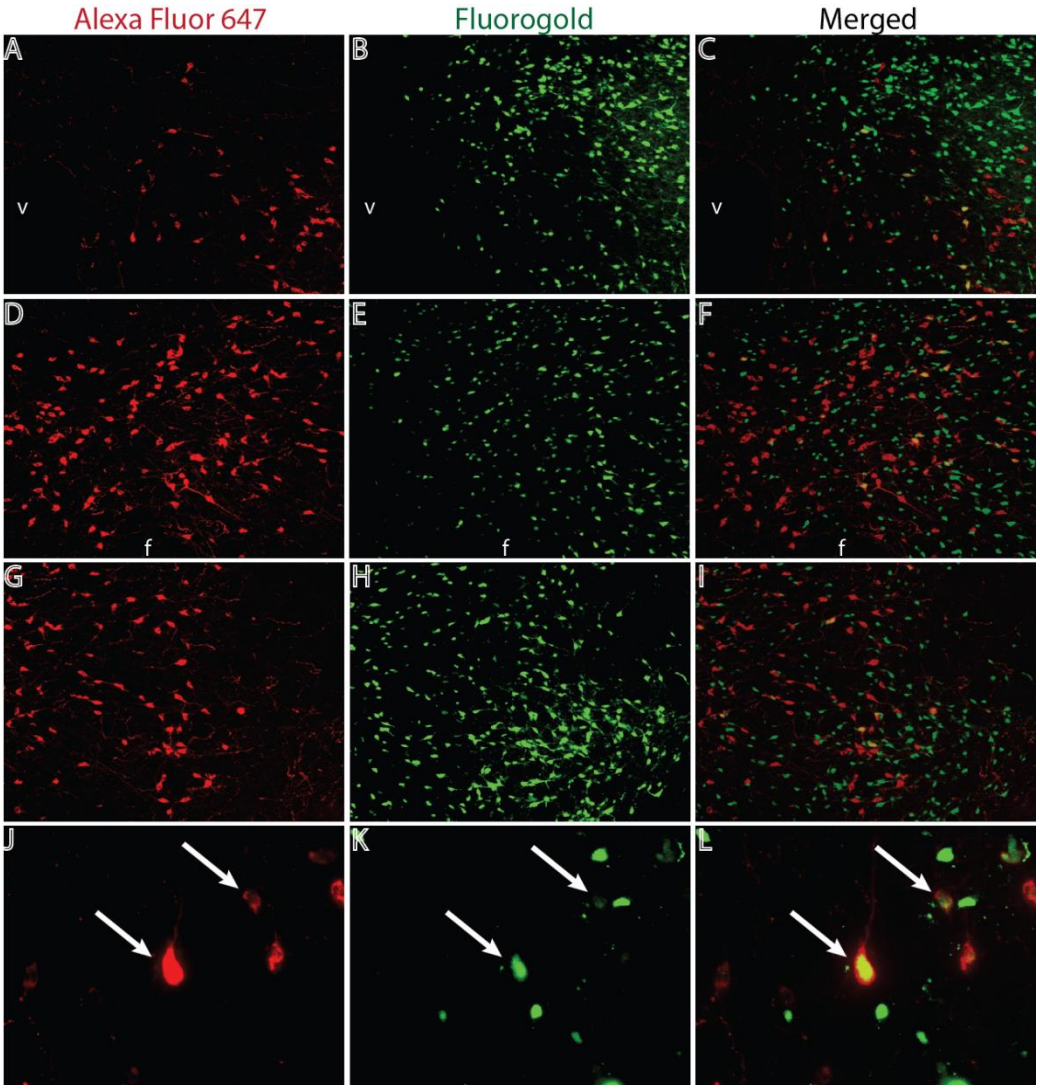

Figure 2 - Co-labeling between Ox and VTA projection neurons in the hypothalamus. The retrograde tracer Fluoro-Gold (Fluorochrome, Denver CO) was microinjected (2% in 250 nl) into the VTA, brains were collected and sliced at 40µm, and then tissue was stained for Ox A immunoreactivity via overnight incubation with an anti-Ox A antibody (1:8000, Phoenix Pharmaceuticals, Burlingame CA) and visualized through incubation with Alexa Fluor 647 (1:200, Jackson ImmunoResearch, West Grove PA) for 1 hour. Images A-I were taken at 10x magnification and images J-L were taken at 40x. In the DMH (A-C), significant Ox neuron

Figure 2 – continued: labeling was observed (A) in addition to retrogradely labeled neurons from the VTA (B), and some Ox neurons projected to the VTA (C, yellow labeling). In the PeF (D-F) a subset of Ox neurons (D) and retrogradely labeled neurons from the VTA (E) co-localized (F). In the LHAd (G-I) a subset of Ox neurons (G) and retrogradely neurons from the VTA (H) colocalized (I). A 40x magnification of labeling observed in the DMH is presented in panels J-L. Double-labeled neurons are indicated by white arrows. v = ventricle, f = fornix.

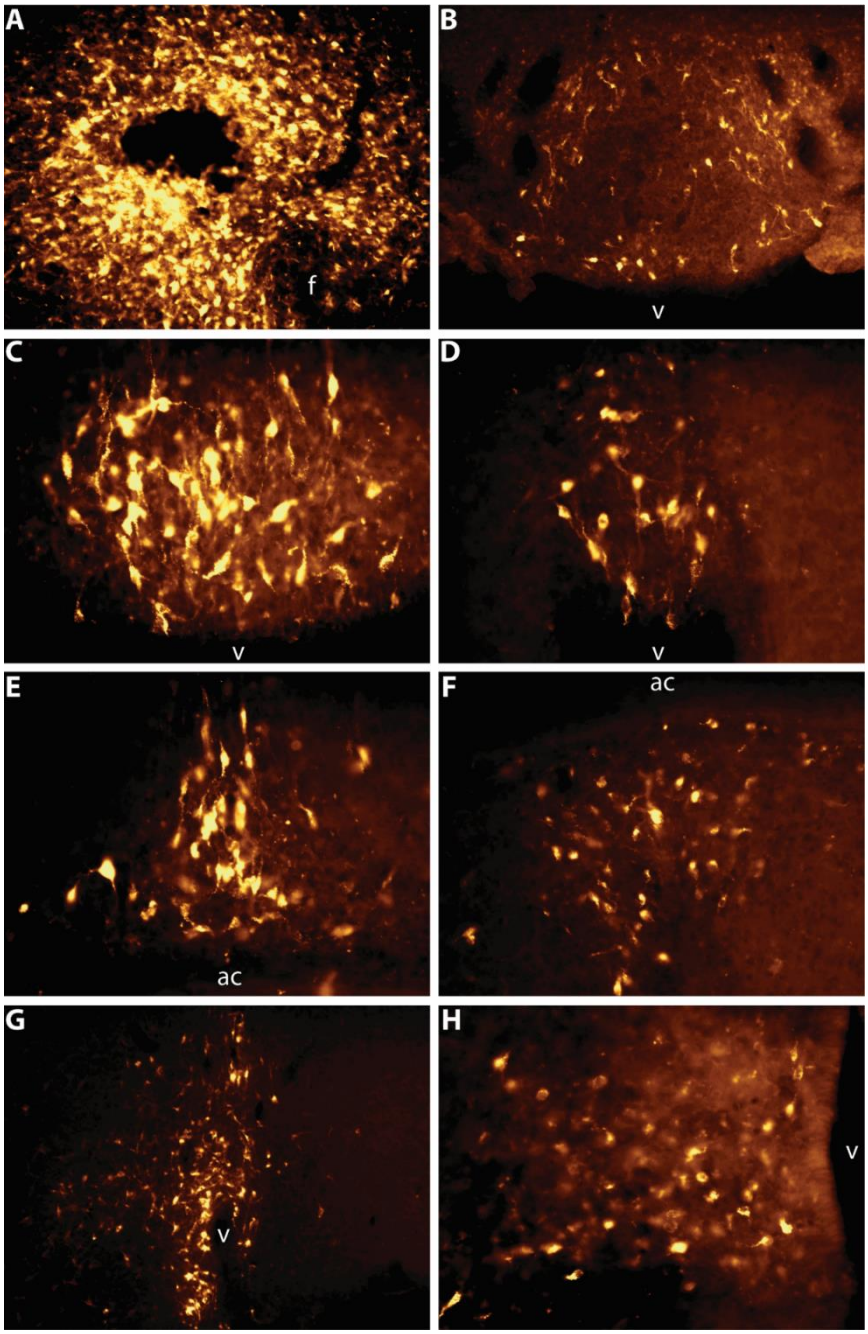

Figure 3 - Retrograde labeling from the LHAd and PeF to the LT and arcuate nucleus of the hypothalamus. 2% Fluoro-Gold in physiological saline was iontophoresed into the PeF and LHAd (A). Retrograde labeling was observed across the LT and 18 in the ARH. Specifically, significant retrograde-labeling was seen in the annulus of the SFO (B), 19 anterior SFO (C), stalk

Figure 3 – continued: of the SFO (D), dorsal and ventral MnPO (E, F), OVLT (G), and ARH 20

(H). Images were taken at different magnifications to compensate for the size of the brain area.

21 Panels A, B, and G were taken at 10x and C, D, E, F, and H were taken at 20x. f = fornix, v =

22 ventricle, ac = anterior commissure.

1.8 Tables

Abbreviation	Meaning
Ang II	Angiotensin II
Arc	Activity regulated cytoskeletal-associated protein
AP	Area postrema
AT1-R	Angiotensin II type 1 receptor
BDA	Biotinylated dextran amine (10,000 molecular weight)
Cap	Captopril
COIN	Co-injection of Fluorogold and biotynilated dextran amine
DARPP-32	Dopamine- and cAMP-regulated phosophoprotein 32 kDa
DMH	Dorsomedial Hypothalamus
FG	Fluorogold
Furo	Furosemide
Furo/cap	Furosemide and captopril
Hyp	Hypothalamus
ICV	Intracerebroventricular
LHAd	Dorsal region of the lateral hypothalamus
LPBN	Lateral parabrachial nucleus
LT	Lamina terminalis
MnPO	Median preoptic area
MR	Mineralocorticoid receptor
NAc	Nucleus accumbens
NMDA	N-Methyl-D-Aspartate
NST	Nucleus of the solitary tract
OVLT	Organum vasculosum of the lamina terminalis
Ox	Orexin
Ox-A	Orexin A
Ox-B	Orexin B
PeF	Perifornical Area
RAAS	Renin-angiotensin-aldosterone-system
RMTg	Rostromedial tegmental nucleus
SFO	Subfornical organ
SGK	Serum- and glucocorticoid-induced kinase
VP	Ventral pallidum
VTA	Ventral tegmental area

Table 1 – List of abbreviations.

Abbreviation	Description
Hyp	Hypothalamus – Structures along the LT project to the hypothalamus and the hypothalamus contains dense projections to the VTA. It is likely that the hypothalamus aids in integrating signaling from the LT related to body fluid status with the mesolimbic dopamine system.
LPBN	Lateral parabrachial nucleus – An area in the hindbrain that appears to chronically inhibit sodium appetite. Disinhibition of this region results in a robust salt appetite.
LT	Lamina terminalis – A region of the forebrain that contains nuclei that detect disturbances in fluid balance and initiate thirst and salt appetite.
NAc	Nucleus accumbens – A region that has been implicated in motivated behaviors. Dopaminergic projections from the VTA promote craving for rewards in the environment. Opioid release in the dorsomedial accumbens appears to promote reward associated with reward consumption.
NST/AP	Nucleus of the solitary tract/Area Postrema – A region in the hindbrain that receives afferents from baroreceptors and detects peripheral signals related to fluid balance.
RMTg	Rostromedial tegmental nucleus – A region of the midbrain that appears to code aversion. This structure inhibits dopamine neurons in the VTA.
VP	Ventral pallidum – A region of the forebrain that is involved in coding reward.
VTA	Ventral tegmental area – An area of the midbrain that contains the dopamine neurons that project to the nucleus accumbens.

Table 2 - Table associated with Figure 1.

Chapter 2: Dissociation of thirst and sodium appetite in the furo/cap model of extracellular dehydration and a role for N-methyl-D-aspartate receptors in the sensitization of sodium appetite.[2]

2.1 Introduction

Rats with a history of sodium depletions exhibit enhanced sodium intake [48, 49, 144, 145]. When extracellular dehydration is induced repeatedly with the diuretic and natriuretic drug, furosemide, sodium intake gradually increases until it plateaus after 3 or 4 depletions [144, 145]. Although a majority of studies have provided evidence for increased sodium intake after sodium depletion, Falk had found evidence for sensitized water intake when water was offered in a single bottle test [48]. Therefore, the experience of extracellular dehydration is capable of sensitizing sodium appetite and, in some instances, thirst.

Sensitization of fluid (e.g., water or hypertonic saline) intake is an example of a change in behavior due to previous experience. Behavior is a product of the nervous system and, as such, any change in behavior is may be associated with neural plasticity – a change in neuron structure or function. Behavioral and physiological studies of the mechanisms of neural plasticity provide strong support for the idea that NMDA-R activation is necessary for the neural plasticity that mediates sensitization, habituation, and associative forms of learning and memory [108, 167, 172]. Based upon this, it is likely that neural plasticity mediates sensitization of sodium appetite. Therefore, it was hypothesized that NMDA-R blockade would prevent sensitization of fluid intake.

[2] Chapter 2 is adapted from a publication from the author (see Ref. 72).

Sodium appetite can be induced through depletion of extracellular fluids or directly stimulated through pharmacological means [75]. Generally these manipulations require a relatively long latency (i.e., on the order of hours to days) between the treatment and expression of sodium appetite. However, the co-administration of the diuretic furosemide (furo) and the angiotensin converting enzyme inhibitor captopril (cap) induces a rapid sodium appetite and thirst with a latency of approximately an hour, a model referred to as furo/cap [54, 109]. The rapid induction of sodium appetite that is observed in this model is the result of extracellular dehydration along with a small decrease in blood pressure [164].

The rapid induction of sodium appetite in the furo/cap model allows for the expedient application of pharmacological manipulations prior to the induction and expression of salt appetite. However, the downside to the furo/cap protocol is that it elicits thirst and sodium appetite concurrently. As such, accurate measurements of sodium and water intake can become problematic when both behaviors are measured at once.

In the present work we dissociated thirst from sodium appetite in the furo/cap model by altering the timing of water and sodium access and tested whether sensitization of thirst or sodium appetite would occur in the modified furo/cap paradigms. Additionally, we investigated whether systemic NMDA-R blockade with MK-801 would prevent sensitization of sodium appetite or thirst in two variations of the furo/cap model. The results of these experiments suggest that 1) altering the timing of water and sodium access during extracellular dehydration alters the dynamics of the sensitization of fluid intake, and 2) NMDA-R activation is necessary for the sensitization of fluid intake.

2.2 Materials and methods

Subjects

All experiments were conducted in accordance with the National Institute of Health *Guide for the Care and Use of Laboratory Animals* and were approved by The University of Iowa Animal Care and Use Committee. Male Sprague Dawley rats (Harlan Teklad) that weighed between 275-300 grams upon arrival were used. Rats were allowed one week to acclimate to the environment prior to experimentation. They were maintained on a 12/12 light/dark cycle and housed in opaque shoebox (40.5 x 28.5 x 17.5 cm), translucent square (28.5 x 28.5 x17.5 cm), or wire mesh suspended (24 x 17.2 x 17.0 cm) cages in a temperature and humidity controlled room. Unless noted otherwise, rats had ad libitum access to filtered tap water, 1.8% w/v NaCl solution, and NIH-31, irradiated, modified open-formula mouse/rat diet. Rats had at least 3 days of ad libitum 1.8% saline access before experimentation. In all experiments 1.8% saline intake was used to assess sodium appetite.

Dissociating thirst from sodium appetite in the furo/cap model

Initial experiments using furo/cap to induce thirst and sodium appetite in our laboratory found that when water and saline were offered concurrently, animals displayed sensitization of both thirst and saline intake. The furo/cap protocol was modified to determine whether sensitization of water or saline intake would occur if the timing of saline and water presentation was altered.

Water and saline were removed and rats received subcutaneous furosemide (10 mg/kg, Hospira Inc, Lake Forest IL) and captopril (5 mg/kg, Sigma-Aldrich) injections. In one group of rats, water access was allowed immediately after furo/cap injections (n=7). Water intake was

measured at 30 minutes and 60 minutes post-furo/cap treatment and then at 15-minute intervals until rats exhibited satiation of thirst, defined as the ingestion of <0.4 mls of water within a 15-minute interval. Upon satiation of thirst, rats were allowed access to hypertonic saline. Saline intakes were measured for 90 minutes at 15-minute intervals during the first hour and then at 90 minutes. This fluid presentation order will be referred to as the water-first/saline-second protocol. In addition, a 'water only' control group (n=7) received furo/cap with access to water alone over the same time period.

A separate group of rats (n=10) received furo/cap treatment but were not allowed access to water prior to saline presentation. In this experiment, saline was offered 90 minutes after furo/cap treatment to time-lock saline access with the water-first/saline-second protocol. Rats had access to saline alone for a duration of 90 minutes during which saline intake was recorded at 15, 30, and 90 minutes. Water was then provided and the intakes of water and saline were recorded for an additional 90 minutes at 15-minute intervals for the first 30 minutes and then at 90 minutes. This protocol is referred to as the saline-first/water-second protocol.

Sensitization of fluid intake

Rats that were tested to develop the water-first/saline-second protocol were given 3 additional furo/cap treatments for a total of 4 depletions and allowed to recover for 4 days between treatments. To control for observed changes in water intake over successive depletions, a separate group of rats was run in an identical protocol; however they were limited to 4 mls of water intake. An intake of 4 mls was chosen because that was the mean amount of water animals drank during the 4th depletion. To determine whether order of fluid presentation would affect

fluid intake sensitization, rats from the saline-first/water second protocol were also repeatedly treated with furo/cap for a total of 4 depletions, each separated by 4 days.

Effect of MK-801 on fluid intake sensitization

All experiments investigating the effect of NMDA-R antagonism on sodium appetite sensitization used MK-801 (0.15 mg/kg, IP) or vehicle (95% saline, 5% DMSO). 0.15 mg/kg MK-801 was chosen based upon studies investigating NMDA-Rs in sensitization which found that a dose of 0.1 mg/kg MK-801 was sufficient to block those types of learning [173]. A slightly higher dose was used in the present experiments in order to compensate for drug excretion induced by furosemide. Drug or vehicle was administered 20 minutes prior to furo/cap treatment. In the concurrent fluid presentation protocol rats (n=14 per group) were treated with furo/cap and were offered water and saline concurrently 60 minutes after furo/cap treatment. This time was chosen because approximately 60 minutes is required for rats to develop a sodium appetite after furo/cap treatment [164]. Intakes were measured over a 3-hour time period at 15-minute intervals for the first hour, then at 30-minute intervals for the second hour and a final recording was taken at 180 minutes. A total of 3 furo/cap treatments, each separated by 1 week, were administered. Urine was collected during the first hour and 20 minutes after MK-801 or vehicle injection. Urine volume, sodium concentration, and potassium concentration were determined with an ion-selective electrode electrolyte analyzer (Nova Biomedical, Waltham MA).

To provide further confirmation of the reliability of the effect of MK-801 on sensitization of sodium appetite, the saline-first/water-second protocol was employed. Given that rats rapidly displayed sensitization of sodium appetite in this protocol (see Figure 8A), rats (n=9 in the vehicle group and n=8 in the MK-801 pretreated group) were pretreated with vehicle or MK-801

(0.15 mg/kg) during the first furo/cap treatment only. Rats were allowed to recover for 4 days and then received a second furo/cap treatment. During the second furo/cap treatment all subjects were pretreated with vehicle. Therefore, rats were tested in a drug-free state on their second depletion. Based upon the results from the saline-first/water-second protocol where rats exhibited sensitization of sodium appetite during the second depletion with no changes in water intake (see Figure 8), only saline intake was measured during this experiment. Saline intake was measured for a total of 90 minutes at 15-minute intervals for the first 30 minutes and then at 30-minute intervals for the last hour.

Statistics

All results were analyzed using SigmaPlot 12.0 build 12.0.0.182 (Systat Software Inc, San Jose, CA) and α was set at 0.05. Water and saline intake in the studies investigating sensitization in modified furo/cap protocols were analyzed with one-way repeated measures ANOVAs. In studies examining the effect of MK-801 on sensitization, a two-way repeated measures ANOVA was utilized. When a significant main effect or interaction was observed from ANOVA analyses, Newman-Keul's post tests were applied to determine group and depletion differences.

2.3 Results

Dissociation of thirst from sodium appetite

Results as depicted in Figure 4, show that thirst satiated 90 minutes after furo/cap treatment. When saline was offered at the end of this period, saline intake occurred without additional water intake. Rats offered water without saline access displayed similar water intake. In the saline-first/water-second protocol rats rapidly drank saline in the first 15 minutes and then

drank only a small amount of saline over the next 75 minutes (Figure 5). After water was presented, rats drank minute amounts of saline; therefore it appears that water intake was also isolated from saline intake in this protocol.

Sensitization of sodium intake

Regardless of the order of access to water or saline, sensitization of saline intake occurs with repeated furo/cap treatments. Specifically, in the water-first/saline-second protocol a main effect of depletion number on saline intake was found, $F(3,18)=6.47$; $p<0.01$. Post-hoc tests revealed rats drank significantly more saline on the third and fourth depletion. Therefore, it appeared that a progressive increase in saline intake occurred over repeated furo/cap treatments in this protocol (Figure 6A). Water intake did not significantly change, $F(3,18)=2.09$; $p=0.14$ (Figure 6B), nor did total fluid intake, $F(3,18)=2.07$; $p=0.15$ (Figure 6C). However, saline intake gradually increased to comprise a greater percentage of total fluid intake, $F(3,18)=6.17$; $p<0.01$ (Figure 6D). Enhanced saline intake that occurred in this protocol is not due to a decrease in water intake over successive depletions as rats with limited water access still displayed increased saline intake, $F(3,18)=7.03$, $p<0.01$ (Figure 7).

A different profile of sensitization occurred in the saline-first/water-second protocol. Analyses identified a main effect of depletion number on sodium intake, $F(3,27)=4.56$; $p<0.05$, and post-hoc tests revealed that rats drank more saline on the second, third, and fourth depletions (Figure 8A). Water intake did not significantly differ over depletions, $F(3,27)=2.15$; $p=0.19$ (Figure 8B), however total fluid intake did show a progressive increase such that total fluid intake was greater on the third and fourth depletions compared to the first, $F(3,27)=4.61$; $p<0.05$

(Figure 8C). The proportion of water and sodium intake did not change over repeated furo/cap treatments, $F(3,27)=0.98$; $p=0.42$ (Figure 8D).

Effect of MK-801 on the sensitization of sodium appetite and thirst

Initial analyses examining combined water and saline intake revealed that there was a significant main effect of depletion, $F(2,52)=4.294$; $p<0.05$, and a significant interaction, $F(2,52)=4.26$; $p<0.05$. Post-hoc tests showed that vehicle pretreated rats exhibited a sensitized total fluid intake such that combined water and saline intake was significantly greater on depletions 2 and 3 (Figure 9). This effect was blocked by MK-801 pretreatment such that vehicle pretreated rats exhibited greater overall fluid intake on the third depletion compared to MK-801 pretreated rats. MK-801 pretreated rats failed to show a significant elevation of fluid intake during the second and third depletion. No significant differences were observed between MK-801 and vehicle pretreated rats in total fluid intake during the first or second furo/cap treatment. Upon closer inspection, rats could be dissociated into two groups – those that exhibited sensitized water or sodium intake (data not shown, but see Ref. [72]). Therefore, in the concurrent fluid access protocol sensitization of thirst or sodium appetite could not be clearly demonstrated. No significant differences in urine output were observed (Table 3).

Effect of MK-801 on the sensitization of sodium appetite per se

Given that rats developed rapid sodium appetite sensitization in the saline-first/water-second protocol, this protocol was chosen to further examine whether MK-801 would prevent sodium appetite sensitization. Rats were pretreated with MK-801 during the first depletion only, and all rats received a vehicle injection prior to the second depletion. Analyses identified a significant effect of drug pretreatment $F(1,15)=7.963$; $p<0.05$ and a significant interaction

$F(1,15)=7.634$, $p<0.05$**.** Post-hoc tests revealed that vehicle pretreated rats exhibited greater saline intake during the second depletion and MK-801 pretreated rats failed to show an increase in saline intake (Figure 10). Furthermore, MK-801 pretreated rats drank significantly less saline on the second depletion compared to vehicle pretreated rats. Therefore, a single pretreatment with MK-801 was sufficient to block the sensitization of sodium appetite. Importantly, no significant differences were observed during the first furo/cap treatment which suggests MK-801 pretreatment did not have non-specific effects to disrupt water or sodium intake, but specifically prevented the development of sensitization of sodium appetite.

2.4 Discussion

The present findings show that the dynamics of water and sodium intake as stimulated by furo/cap treatment are affected by the order in which the fluids are presented. Nonetheless, when the timing of water and saline presentation is controlled by the experimenter, the sensitization of sodium appetite occurs reliably and it is independent of whether water or saline access is offered first. Furthermore, NMDA-Rs, which have been heavily implicated in the induction of neural plasticity, are critical for sensitization of thirst and sodium appetite.

The order of fluid presentation alters the amount of water and sodium intake seen in the furo/cap model. When water is offered prior to sodium a progressive increase in sodium intake occurs. Interestingly, total fluid intake does not change, but a greater amount of sodium is ingested after each depletion. This suggests that rats exhibit a preference shift from water to sodium after repeated depletions. This preference shift could be an example of incentive relativity [57], or a change in reward preference produced by prior experience with rewards. Specifically, it is possible that the experience of ingesting hypertonic saline during extracellular dehydration causes rats to learn that hypertonic saline is more rewarding than ingesting water. It

is also possible that the ingestion of water prior to hypertonic saline leads to a hypo-osmotic state. Rats may learn to shift their preference from water to hypertonic saline in order to reduce hypo-osmosity. It is interesting to note that one common model used to study salt appetite is extracellular dehydration induced by administration of the diuretic furosemide alone [145]. In some ways this model reflects the water-first/saline-second protocol as water is provided after furosemide treatment but sodium access is delayed for 20-24 hours. Sodium appetite increases in a progressive fashion (i.e. sensitizes) in the furosemide-only model of extracellular dehydration, similar to the water-first/sodium-second protocol employed here.

When sodium is offered prior to water, rats exhibit a rapid and robust sensitization of sodium appetite that is apparent upon the second depletion. In addition, total fluid intake increases on the third and fourth depletion in this protocol. The increase in total fluid intake observed in this protocol may be due to the ingestion of relatively large amounts of sodium on the third and fourth depletions which may induce cellular dehydration. This state of cellular dehydration could elicit an osmotic thirst that ultimately yields elevated total fluid intake.

Unfortunately, the present experiments cannot address the circumstances that result in sensitization of thirst. Sensitization of thirst has been reported when water access alone was provided on a second extracellular dehydration [48]. Others have found that rats repeatedly treated with furo/cap exhibit sensitized thirst and sodium appetite when water and saline were offered concurrently [131]. In addition, repeated administration of intracerebroventricular angiotensin II results in enhanced angiotensin II-induced water intake when water is offered in a single bottle test [116]. Sensitization of thirst did occur in a subset of animals in the concurrent fluid access paradigm [72]. Despite this, our findings in general, in addition to prior research on sensitization of fluid intake [48, 49, 144, 145] suggest that sensitization of sodium appetite is

more reliable, especially when the timing of water and saline access is controlled by the experimenter.

With respect to the physiological mechanisms of sensitization of fluid intake, blockade of NMDA-Rs with systemic MK-801 was sufficient to prevent sensitization of sodium appetite, and when water and sodium were offered together, sensitization of fluid intake in general. These findings implicate glutamatergic NMDA-Rs in experience-dependent changes in fluid intake. Critically, MK-801 was capable of blocking sodium appetite sensitization in the sodium-first/water-second protocol when it was administered during the first depletion only paradigm. This provides strong evidence that NMDA-R blockade prevents the sensitization of sodium appetite without affecting the expression of sodium appetite. An alternate explanation of the present findings is that MK-801 treatment induced nausea or taste aversion. Admittedly, the dose of MK-801 employed in these experiments is higher than the commonly used 0.1 mg/kg dose. A higher dose (0.15 mg/kg) was chosen to compensate for enhanced urine excretion induced by furosemide. However, others have found that a dose of 0.3 mg/kg MK-801 is required to produce a conditioned taste aversion to saccharin [78]. Therefore, the 0.15 mg/kg dose employed here probably did not produce a conditioned taste aversion. In addition, our results cannot be explained by differences in urine excretion as we failed to find any significant effect of MK-801 or depletion number on diuretic/natriuretic-induced urine output.

2.5 Figures

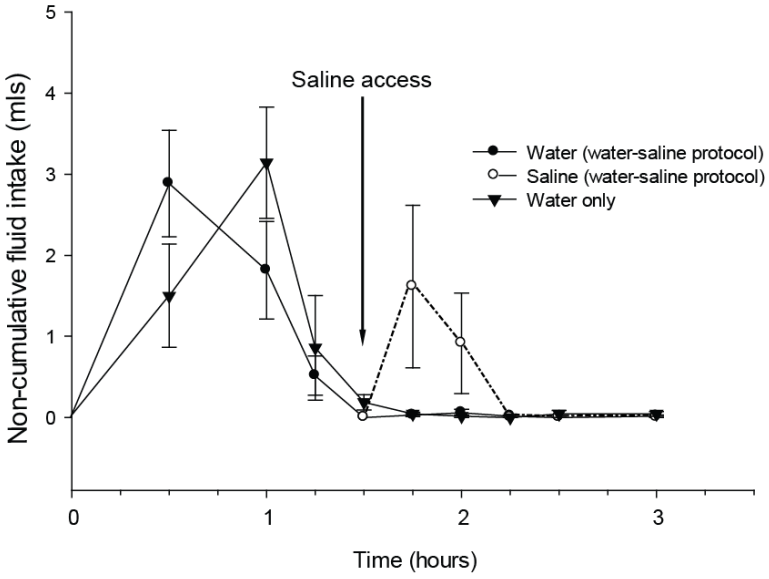

Figure 4 - Dissociation of water and 1.8% saline intake in the water-first/saline-second furo/cap protocol. Non-cumulative intakes are presented on the y-axis and time is on the x-axis. Rats were offered water immediately after furo/cap treatment and allowed to drink until satiation which occurs at approximately 90 minutes. Saline access was then offered in addition to water (arrow). During this time rats drank hypertonic saline and essentially no water. All data are expressed as mean ± SEM.

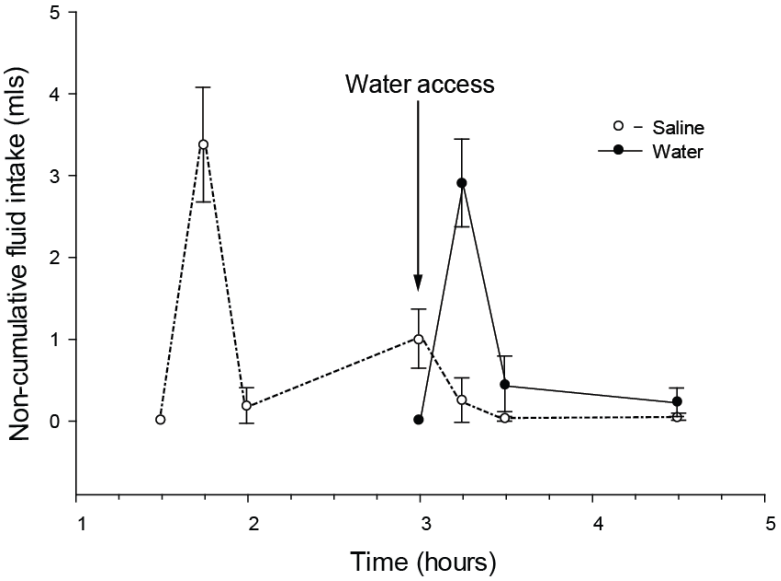

Figure 5 - Dissociation of water and saline intake in the saline-first/water-second protocol. Non-cumulative intakes are presented on the y-axis and time is on the x-axis. Rats were offered saline 90 minutes after furo/cap treatment. Water was provided after 90 minutes of saline access (arrow). While water was available rats drank water and miniscule amounts of saline. All data are expressed as mean ± SEM.

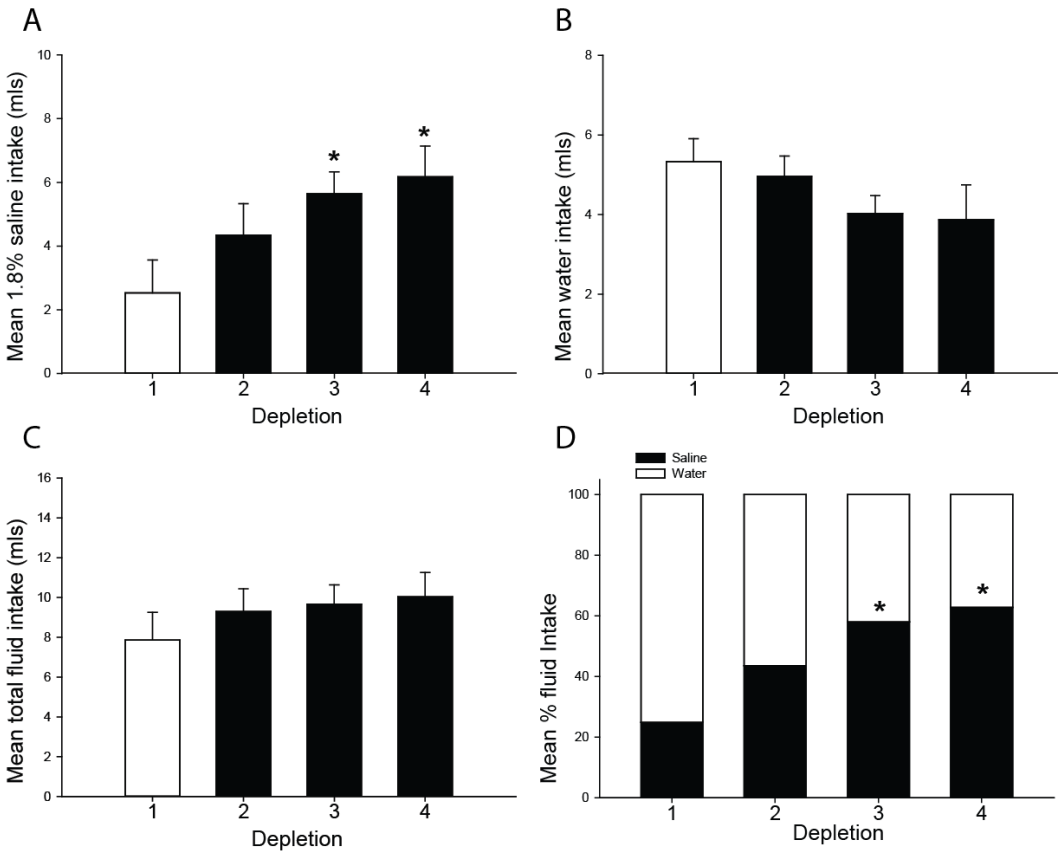

Figure 6 - Sensitization of saline intake in the furo/cap water-first/saline-second protocol. Repeated furo/cap treatments induce a progressive elevation in saline intake (A), but no change in water intake (B) or total fluid intake (C). Over repeated depletions saline intake comprises a greater percentage of fluid intake (D), suggesting a shift in preference from water to saline intake in the water-first/sodium-second protocol (*=$p<0.01$ vs. depletion 1). All data are expressed as mean, and where error bars appear, ± SEM. Please note differences in ordinate across graphs.

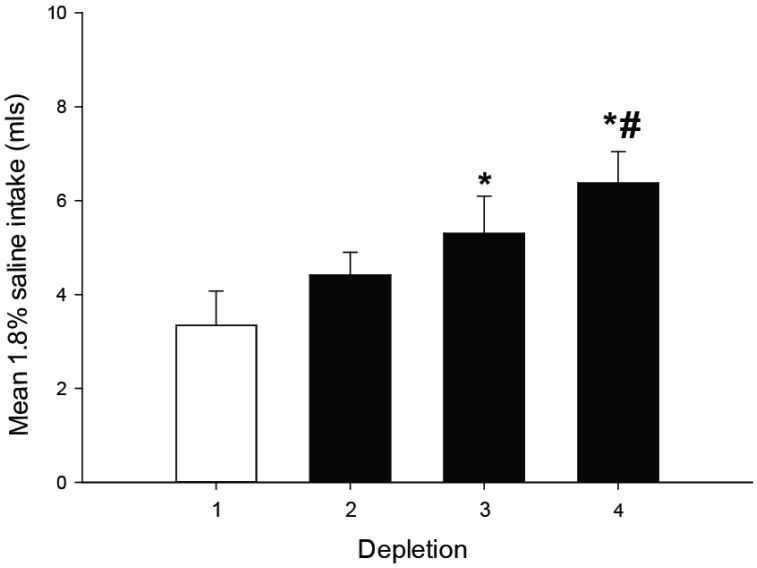

Figure 7 - Sensitization of saline intake in the water-first/saline-second protocol is not dependent on a decrease in water intake. Total 1.8% saline intake is displayed on the y-axis. When offered limited fluid access (4mls) during each test, rats displayed a progressive elevation of saline intake (*=$p<0.05$ vs. depletion 1, #=$p<0.01$ vs. depletion 1), similar to rats with unlimited water access. All data are expressed as mean ± SEM.

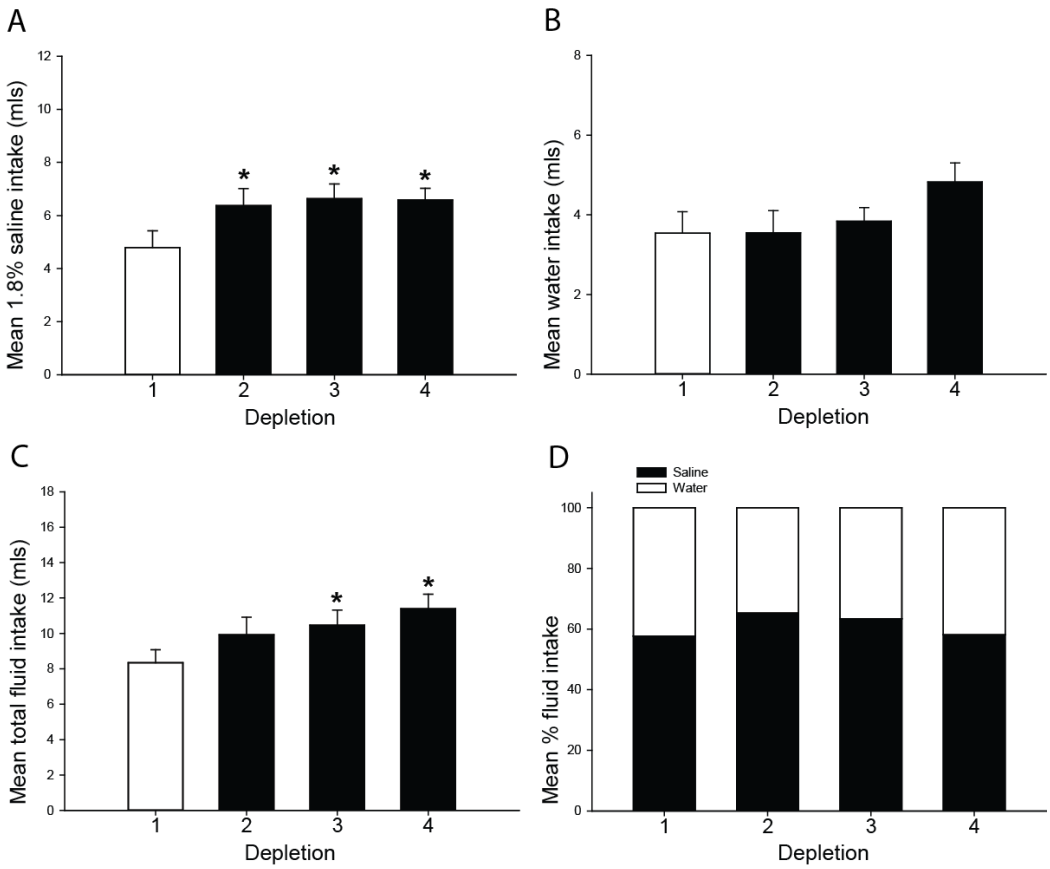

Figure 8 – Sensitization of saline intake and total fluid intake in the furo/cap sodium-first/water-second protocol. 1.8% saline intake rapidly sensitizes upon repeated furo/cap treatments (A). Water intake does not significantly change over depletions (B). However, rats exhibit a progressive sensitization of total fluid intake (C). No significant differences in the composition of water or sodium during each depletion were observed (D). *=$p<0.05$ vs. depletion 1. All data are expressed as mean, and where error bars appear, ± SEM. Please note differences in ordinate across graphs.

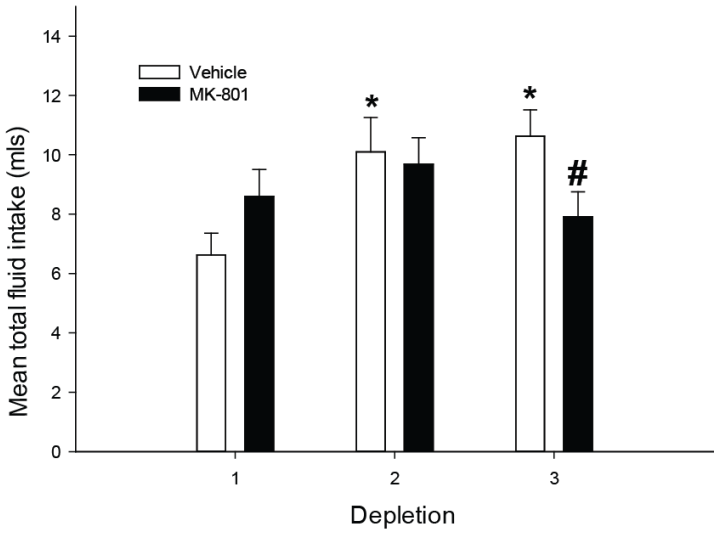

Figure 9 – Effect of MK-801 pretreatment on sensitization of fluid intake when water and sodium were offered concurrently in the furo/cap model. Vehicle pretreated rats exhibited sensitized fluid intake by the second depletion (*=$p<0.005$ vs vehicle, depletion 1) while MK-801 pretreated rats failed to show any increase in total fluid intake (#=$p<0.05$ vs vehicle, depletion 3). No significant differences were observed between vehicle and MK-801 pretreated rats during the first or second depletions. No significant differences were observed in total fluid intake during the first depletion. All data are expressed as mean ± SEM.

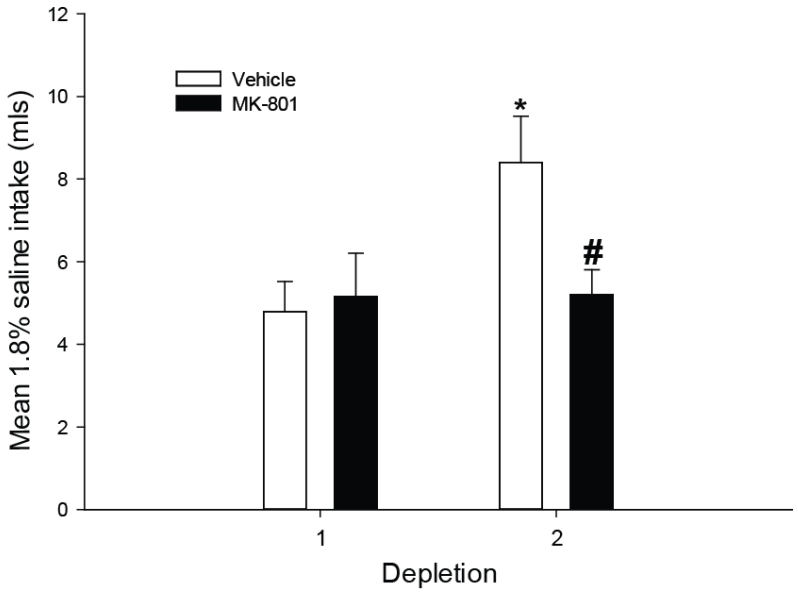

Figure 10 - Effect of MK-801 pretreatment on sensitization of saline intake in the saline-first/water-second protocol. Vehicle pretreated rats exhibit enhanced saline intake by the second depletion (*=p<0.01 vs. vehicle, depletion 1) while MK-801 pretreated rats fail to display increased intake (#=p<0.05 vs. vehicle, depletion 2). All data are expressed as mean ± SEM.

2.6 Tables

	Depletion	1	2	3
Urine	Vehicle	8.9	9.5	9.5
Volume (mls)	MK-801	10.3	10.4	10.4
Potassium	Vehicle	.31	.35	.35
Content (mmol)	MK-801	.31	.3	.34
Sodium	Vehicle	.95	1.1	1
Content (mmol)	MK-801	.96	1	1

Table 3 - Mean urine volume, potassium content, and sodium content in vehicle or MK-801 pretreated rats across depletions. No significant differences were observed.

Chapter 3: The sensitization of sodium appetite: Evidence for sustained molecular changes in the lamina terminalis[3]

3.1 Introduction

When omnivores and herbivores become sodium deficient they exhibit sodium appetite, which entails seeking and ingesting salty substances. Sodium appetite is commonly assessed by measuring intakes of hypertonic saline solutions (1.5-9.0% w/v NaCl) in rats. The expression of sodium appetite is mediated through the actions of the peptide angiotensin II and the steroid aldosterone. Angiotensin II and aldosterone act as hormones synthesized in the periphery (i.e., the circulating RAAS) and also as neurotransmitters/neuromodulators generated within the central nervous system (i.e., the central RAAS; [43, 52, 64]). Forebrain nuclei including the SFO, organum vasculosum of the lamina terminalis, and median preoptic nucleus are located along the LT and are critical for maintaining body fluid homeostasis; collectively they monitor and process information related to circulating angiotensin II, aldosterone, and plasma osmolality [82, 111]. Structures of the LT also house components of the central RAAS including the synthetic enzymes and precursors of angiotensin II and aldosterone as well as receptors for both the steroid and peptide [84]. When rats are repeatedly depleted of sodium they display elevated sodium intake [49]. Rats exhibit progressive [19, 144, 145] or immediate [49, 72] increases in sodium intake after one to three episodes of sodium deficiency depending on the depletion

[3] Chapter 3 is adapted from a publication from the author (see Ref. 76).

protocol employed. This increase in salt intake operationally defines sodium appetite sensitization.

Previous studies have demonstrated that the development of sodium appetite sensitization is dependent on angiotensin II, aldosterone, and N-methyl-D-aspartate receptor (NMDA-R) signaling in the central nervous system. Antagonism of angiotensin II type 1 receptors (AT_1-R; [131]) or blockade of angiotensin II synthesis concurrent with central mineralocorticoid receptor (MR) antagonism [144] prevents the sensitization of sodium appetite. Administration of central angiotensin II along with systemic aldosterone induces sensitization [144]. Blockade of NMDA-Rs prior to sodium depletion prevents sensitization of sodium appetite (see Chapter 2; [72]). It is likely that sodium depletion induces neural plasticity in the central nervous system through coordinated actions of angiotensin II, aldosterone, and glutamate, which are responsible for the elevation of sodium intake. Such plasticity has been related to long-lasting changes in gene transcription and mRNA translation, neuronal structure, receptor expression, among many other alterations in neural or glial molecular biology and function.

Many sodium depletion protocols require a long latency between the induction of sodium loss and the actual expression of sodium appetite (generally between 8 hours to several days; [75]). Co-administration of the diuretic furosemide along with the anti-hypertensive drug captopril (furo/cap) causes an extracellular dehydration concomitant with a slight drop in blood pressure [164]. These effects result in a rapid onset of thirst and sodium appetite with a latency of approximately 1 hour. Rats repeatedly treated with furo/cap reliably exhibit sensitization of sodium appetite when the order of water and sodium access is controlled by the experimenter [72]. Specifically, when water is offered for 90 minutes before sodium access (the water-

first/sodium-second iteration of the furo/cap protocol) rats exhibit a progressive increase in sodium intake over successive furo/cap treatments.

One goal of Chapter 3 was to extend the findings from Chapter 2 on the effect of NMDA-R blockade on the sensitization of sodium appetite in the water-first/sodium-second iteration of the furo/cap protocol. A second aim was to examine changes in the expression of molecular components indicative of sustained alterations in activity of the central RAAS and an additional putative marker of neural plasticity in the LT. In an initial experiment we tested whether NMDA-R antagonism would attenuate the sensitization of sodium appetite and whether repeated sodium depletions would result in an NMDA-R-dependent increase in mRNA expression for components of the RAAS and a MR-related molecular marker of plasticity, serum-and glucocorticoid-induced kinase (SGK), in the LT. In a second set of experiments it was hypothesized that sodium depletion would induce a pattern of *fos*-B expression in areas along the LT that would be consistent with the characteristics of the Δ*fos*-B isoform, which is associated with neural plasticity [126]. Consistent with our hypotheses, repeated sodium depletions induced increased expression of molecular markers related to both the brain RAAS and neural plasticity that correlated with sodium intake. Such changes lasted for several days after sodium depletion, and NMDA-R antagonism prevented them. These findings lend further support to the hypothesis that neural plasticity within the LT contributes to the sensitization of sodium appetite.

3.2 Materials and methods

Subjects

All experiments were conducted in accordance with the National Institute of Health *Guide for the Care and Use of Laboratory Animals* (36) and were approved by The University of

Iowa Animal Care and Use Committee. Male Sprague Dawley rats (Harlan Teklad, Indianapolis IN) weighing between 275-300 grams upon arrival were used as subjects. Rats were maintained on a 12/12 light/dark cycle and housed in translucent (28.5 x 28.5 x17.5 cm) or wire mesh suspended (24 x 17.2 x 17.0 cm) cages in a temperature and humidity controlled room. Unless noted otherwise, the animals had ad libitum access to filtered tap water, 1.8% w/v hypertonic saline (1.8 % NaCl), and NIH-31 irradiated modified open formula mouse/rat diet. Rats had at least 3 days of ad libitum 1.8% NaCl access prior to experimentation. The studies used 1.8% NaCl intake to assess sodium appetite and the consumption of deionized water to assess thirst.

Extracellular dehydration protocol

Extracellular dehydration was induced through the furo/cap model which uses subcutaneous injection of furosemide (10mg/kg, Hospirca Inc. Lake Forest, IL) along with the anti-hypertensive drug captopril (5 mg/kg, Sigma Aldrich). The water-first/sodium-second iteration of the furo/cap protocol was used in all experiments - water access was provided immediately after furo/cap treatment and 90 minutes later 1.8% NaCl access was added.

Effect of MK-801 on sodium appetite sensitization and mRNA expression in the LT

In Chapter 2 it was found that administration of the NMDA-R antagonist MK-801 prevented sodium appetite sensitization in the furo/cap model when sodium access was offered prior to water (see also Ref. [72]). One purpose of our studies was to extend the generalizability of this finding by using a water-first/sodium-second protocol. Rats (n=12 in the vehicle group and 13 in the MK-801 group) were pretreated with vehicle (95% saline, 5% DMSO) or MK-801 (0.15 mg/kg) 20 minutes prior to receiving furo/cap treatment. Immediately after furo/cap treatment, they were allowed access to water for 90-minutes and intakes were recorded. At the

end of the 90 minute water access period, sodium access was allowed and fluid intakes were recorded for an additional 90 minutes. Rats were treated 3 times with this protocol with each treatment separated by 4 days.

Separate groups of rats were used to investigate whether repeated sodium depletions induce changes in mRNA expression in the LT. Three experimental groups (n=4) were employed: a vehicle pretreated and sham depleted group, a vehicle pretreated and furo/cap depleted group, and an MK-801 (0.15 mg/kg) pretreated and furo/cap depleted group. Each group received vehicle or MK-801 20 minutes prior to furo/cap or sham depletion. Rats received a total of 3 furo/cap or sham treatments each separated by 4 days. 5 days after the treatments rats were sacrificed by decapitation, the LT was dissected by hand, and mRNA expression for AT_1-R and angiotensin type 2 receptors (AT_2-R), angiotensin converting enzyme (ACE) 1 and 2, MR, and SGK was measured using real-time reverse transcription polymerase chain reaction (RT-PCR).

RT-PCR was performed as described previously (56, 57). Briefly, mRNA was isolated using TRIzol (Invitrogen). mRNA transcription was accomplished using random hexamers under the manufacturer's instructions (Applied Biosystems). cDNA amplification was performed by a C1000 thermocylcer system (Bio-Rad). mRNA levels were normalized to GAPDH levels and calculated using the ΔΔCt method. Each assay was run in triplicate to ensure reliable results. Results are expressed as relative fold change compared to sham-depleted rats.

Effect of repeated sodium depletions on sensitization of sodium appetite and fos-B/Δfos-B expression in the LT

To examine the effect of furo/cap on the sensitization of sodium appetite 3 groups of rats were used (n=11). One group of rats received 4 sham depletions, a second received 3 sham depletions prior to a single furo/cap depletion, and a third received 4 furo/cap depletions. Rats were treated with a depletion or sham depletion every 4 days and fluid access was offered in accordance with the water-first/sodium-second experimental protocol. Water and 1.8% NaCl intakes were recorded on the 4th depletion or sham depletion. Immediately following furo/cap treatment water was offered for 90 minutes and then sodium access was allowed. During the 4th depletion intakes of water and sodium were recorded for 180 minutes at 90-minute intervals.

A subset of rats (n=4) were sacrificed 4 days after the last depletion or sham depletion and perfused transcardially with phosphate buffered saline followed by 4% paraformaldehyde (PFA). To control for variability in staining efficiency, all cycles of tissue collection and staining were performed in cohorts such that an equal number of sham, single, and repeatedly depleted animals were run in parallel. Brains were post-fixed for 4-6 hours in 4% PFA and then transferred to vials containing 20% sucrose dissolved in phosphate buffered saline. Brains were left in sucrose overnight at 4° C, and the next morning 3 series of non-consecutive 40µm slices were sectioned with a cryostat and stored in cryoprotectant at -20° C. Tissue was removed from storage and washed 3 times in phosphate buffered saline, blocked in normal rabbit serum for 1 hour and then incubated with a goat-raised anti-*fos*-B antibody (1:2000-1:4000; sc-48-G, Santa Cruz, Dallas TX) for 48 hours at 4°C. This antibody detects both *fos*-B and the truncated isoform of *fos*-B known as Δ*fos*-B. Tissue was then washed 3 times and incubated with rabbit-raised biotinylated anti-goat anti-body for 1 hour (1:200, Santa Cruz, Dallas TX), washed 3 times and

incubated with avidin-biotin complex (Vector Labs Elite Kit, Burlingame CA) for 1 hour. Tissue was washed 3 times and exposed to DAB and hydrogen peroxide for approximately 4 minutes to visualize sites of protein expression. Sections were mounted and cover-slipped. A digital image of the SFO was taken at approximately equivalent rostral-caudal coordinates and cell counts were performed manually by an experimenter blind to the treatment conditions using ImageJ (NIH, version 1.46r).

Statistics

All results were analyzed using SigmaPlot 12.0 build 12.0.0.182 (Systat Software Inc). Statistical significance was set at $p<0.05$. In studies examining the effect of MK-801 on sensitization, the difference in sodium intake between the first and third depletion was used as the dependent variable and these data were analyzed with Student's *t*-test. Depletion, *fos*-B, and RT-PCR data were analyzed using a one-way ANOVA, and when a significant main effect was observed, Newman-Keul's post-tests were employed to probe group differences.

3.3 Results

Effect of MK-801 on sodium appetite sensitization and mRNA expression in the LT

MK-801 pretreatment significantly attenuated sensitization of sodium appetite such that the increase in total salt intake between the first and third depletions was reduced in the MK-801 pretreated group $t(23)=2.336$; $p<.05$ (Figure 11). No differences in salt intake during the first depletion were observed between vehicle and MK-801 pretreated rats $t(23)=0.265$; $p=.79$. No effects of MK-801 on water intake were observed (data not shown). Analyses revealed a main effect of treatment on mRNA expression for AT_1-R $F(2,9)=4.938$; $p<.05$, MR $F(2,9)=8.372$; $p<.01$, and SGK $F(2,9)=5.056$; $p<.05$. No treatment effects were observed in mRNA expression

for AT_2-R $F(2,9)=1.179$; p=.35, ACE1 $F(2,9)=2.037$; p=.18, and ACE2 $F(2,9)=2.458$; p=.14. mRNA data are displayed in Figure 12. Compared to sham-treated animals, repeated furo/cap treatments elevated message for AT_1-R MR and SGK. MK-801 treatment prevented these changes such that message for AT_1–R, MR, and SGK did not increase over sham treated values.

Effect of repeated sodium depletions on sensitization of sodium appetite and fos-B/Δfos-B expression in the LT

Analyses revealed a main effect of depletion number, $F(2,30)=23.819$; $p<0.001$, where rats with a history of sodium depletions exhibited greater salt intake than rats with a single depletion and both groups of depleted rats exhibited greater salt intake than sham depleted rats (Figure 13A). A main effect of depletion number on water intake was also found $F(2,30)=7.576$; $p<0.005$. Sodium depleted rats exhibited greater water intake compared to sham treated rats (Figure 13B), but no significant differences were observed between rats that received a single sodium depletion or repeated sodium depletions. A main effect of depletion number on total fluid intake was observed $F(2,30)=14.683$; p<.001. Rats that received extracellular dehydration exhibited greater total fluid intake, but there were no differences between rats that received a single or repeated depletions (Figure 13C). Finally, a greater proportion of total fluid intake consisted of sodium intake in rats with a history of sodium depletions compared to rats receiving a single depletion $t(20)=3.354$; $p<0.005$ (Figure 13D).

A survey of brains for immunohistochemical evidence of protein indicated that the SFO was the only region in the brain that exhibited consistent differences in *fos*-B/Δ*fos*-B expression across treatments. Therefore all *fos*-B/Δ*fos*-B analyses were performed on the SFO. These analyses revealed a main effect of depletion number on *fos*-B/Δ*fos*-B expression $F(2,9)=12.4$;

p<.005. Post-hoc tests identified that sham-depleted rats exhibited very low levels of *fos*-B/Δ*fos*-B staining, rats with a single depletion exhibited significantly increased *fos*-B/Δ*fos*-B expression than seen in sham-depleted rats, and animals with a history of 4 depletions exhibited greater levels of *fos*-B/Δ*fos*-B than sham- and single-depleted rats (Figures 14 and 15).

3.4 Discussion

The primary findings from the present experiments are that 1) MK-801 pretreatment attenuated the sensitization of sodium appetite in the water-first/sodium-second protocol, 2) sodium appetite sensitization is associated with maintained elevation of mRNA expression of key components of the brain RAAS in the LT region, and 3) salt preference during sodium depletion is associated with a sustained elevation of *fos*-B/Δ*fos*-B in the SFO. A list of the molecular markers investigated in the present study and their significance is presented in Table 4.

The behavioral results from these experiments are consistent with prior reports of enhanced salt appetite as a function of episodic sodium depletions [72, 144, 145]. It was found that when using the water-first/sodium-second protocol, rats with a history of sodium depletions drank more 1.8% NaCl than rats with a single depletion and they drank a greater proportion of sodium relative to water. Importantly, pretreatment with 0.15 mg/kg MK-801 attenuated sensitization of sodium appetite in this protocol but did not influence salt intake on the first depletion. The observed attenuation in sodium appetite is consistent with the idea that sensitization of sodium appetite is dependent on signaling involving NMDA-Rs, which are critical for the initiation of many forms of neural plasticity [30].

Rats with a history of furo/cap treatments displayed elevated message for components of the central RAAS. Specifically, AT_1-R and MR mRNA were upregulated 5 days after the last sodium depletion. Importantly, this effect was blocked by MK-801 pretreatment. To our knowledge, this is the first evidence that sodium depletions can have long-lasting NMDA-R-dependent effects on the central RAAS. Others have found that treatments that affect angiotensin II or MR levels can have long-lasting effects on the central expression of angiotensin II receptors [24, 90, 149]. In the present study, no significant differences were observed in components of the RAAS that have been found to serve counter-regulatory functions such as ACE2 or AT_2-R expression [20, 23].

SGK mRNA was increased after sodium depletion and NMDA-R antagonism prevented this increase. SGK is a MR-dependent kinase [125, 151] that has been linked to neural plasticity [94, 95, 168]. Rats transfected with a dominant negative isoform of SGK in the hippocampus display impaired expression of hippocampal long-term potentiation, fear conditioning, novel object recognition, and spatial recognition [94, 95, 168]. It is possible that SGK expression is increased after sodium depletion due to aldosterone action at the MR and that SGK may play an important role in shaping neural plasticity within components of the LT to sensitize sodium intake.

Expression of *fos*-B/Δ*fos*-B staining in the SFO was directly associated with salt preference. Rats with a history of sodium depletions exhibited a greater preference for sodium relative to water in addition to total sodium intake. Rats with a history of sodium depletion also exhibited greater *fos*-B/Δ*fos*-B expression relative to rats that were sodium depleted for the first time. *fos*-B is an immediate early gene that is expressed during neuronal activity [126, 127]. Δ*fos*-B is an isoform of *fos*-B. Δ*fos*-B lacks the catalytic domain present on *fos*-B which results

in it remaining elevated for weeks after induction [126, 127]. As such, Δ*fos*-B expression follows a characteristic pattern. Baseline expression of Δ*fos*-B is nearly absent in brain nuclei. However, in response to treatments associated with the induction of neural plasticity (e.g., repeated stress and administration of addictive drugs) Δ*fos*-B is expressed and remains elevated for weeks and it accumulates over repeated treatments. The pattern of *fos*-B/Δ*fos*-B staining observed in the present study follows the characteristics of the Δ*fos*-B isoform. A near complete absence of staining was observed in sham-depleted rats, whereas rats experiencing a single depletion exhibited a moderate amount of staining, and a greater amount of staining was observed in rats depleted repeatedly. Therefore, it is likely that sodium depletion induced increased expression of Δ*fos*-B in the SFO and that Δ*fos*-B accumulated over the course of repeated depletions.

It is possible that the expression of *fos*-B/Δ*fos*-B observed after sodium depletion was due to the ingestion of sodium (i.e., expressed due to associative learning). This may be unlikely as previous studies have suggested that the sensitization of sodium appetite is a non-associative phenomenon [49]. Rats will exhibit increased salt intake even when salt is replenished through means independent of ingestion. It is also unlikely that the expression of *fos*-B/Δ*fos*-B was due to a possible change in circulating angiotensin II or aldosterone caused by sodium depletion as repeated sodium depletions do not appear to change circulating levels of either hormone [145].

The present experiments have limitations that need to be acknowledged. The *fos*-B/Δ*fos*-B and mRNA data are correlative in nature and the functional significance of these changes remains to be tested. mRNA was collected from the entirety of the LT and we cannot pinpoint an exact structure or set of structures where these molecular changes may be occurring. Functional studies employing viral vectors could be used to elevate or eliminate AT_1–R or MR receptors in key brain areas in the LT in order to provide insight into the importance of receptor expression

and the sensitization of sodium appetite. Additionally, there are dominant negative forms of SGK and Δ*fos*-B that allow for the possibility of testing the functional importance of both of these molecules in sodium appetite sensitization.

Together, the present findings indicate that a history of sodium depletions elevates sodium intake in addition to inducing a long-lasting elevation of molecular markers related to neural plasticity (SGK and a pattern of staining that appears to be Δ*fos*-B) and those related to the central RAAS (AT_1-R and MR). Sensitization of sodium appetite and enhanced expression of AT_1-R, MR, and SGK mRNA were dependent on intact NMDA receptor signaling. Based upon this and previous findings [144, 145], it is possible that the coordinated actions of glutamate, angiotensin II, and aldosterone produce long-lasting changes in neural structures containing components of the RAAS. The amount of *fos*-B/Δ*fos*-B staining in the SFO was associated with sodium depletion history and may contribute to sodium appetite sensitization. Additional evidence implicates the SFO in sensitization of sodium appetite as the immediate early gene c-*fos* is elevated in the SFO in sodium-deficient rats with a history of depletion relative to rats with no prior history of sodium depletion [124]. Some of the molecular changes observed in the current findings have been shown to promote glutamatergic AMPA receptor insertion, which is critically involved in producing long-term potentiation [106]. Specifically, SGK induces surface expression of GluA1-containing AMPA receptors [156] and Δ*fos*-B has been linked to neural plasticity with respect to drugs of abuse where it aids in the insertion of GluA2-containing AMPA receptors into the neuronal membrane [126, 127]. These findings add to a growing body of evidence that supports the occurrence of neural plasticity in body fluid homeostasis neurocircuitry [72, 124, 174, 175], and they provide insight into important molecular changes that are likely to drive sensitization of sodium appetite.

3.5 Figures

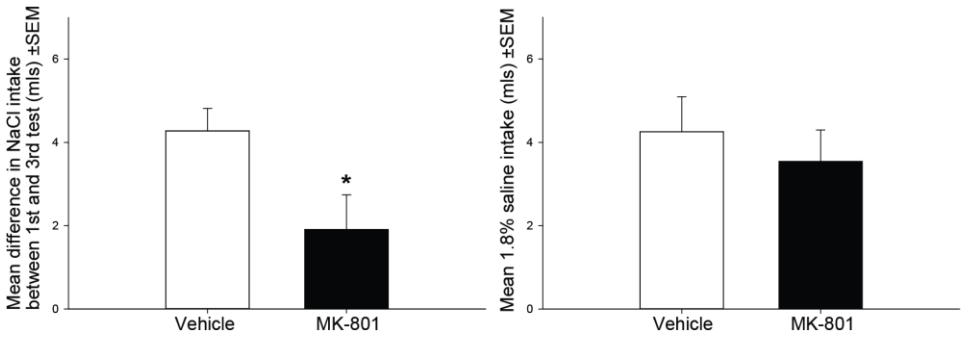

Figure 11 – Effect of MK-801 pretreatment on sodium appetite sensitization in the water-first protocol/sodium-second protocol. Rats pretreated with MK-801 display an attenuated sensitization of sodium appetite (left; *=p<.05), however MK-801 has no effect on sodium intake during the first furo/cap treatment (right).

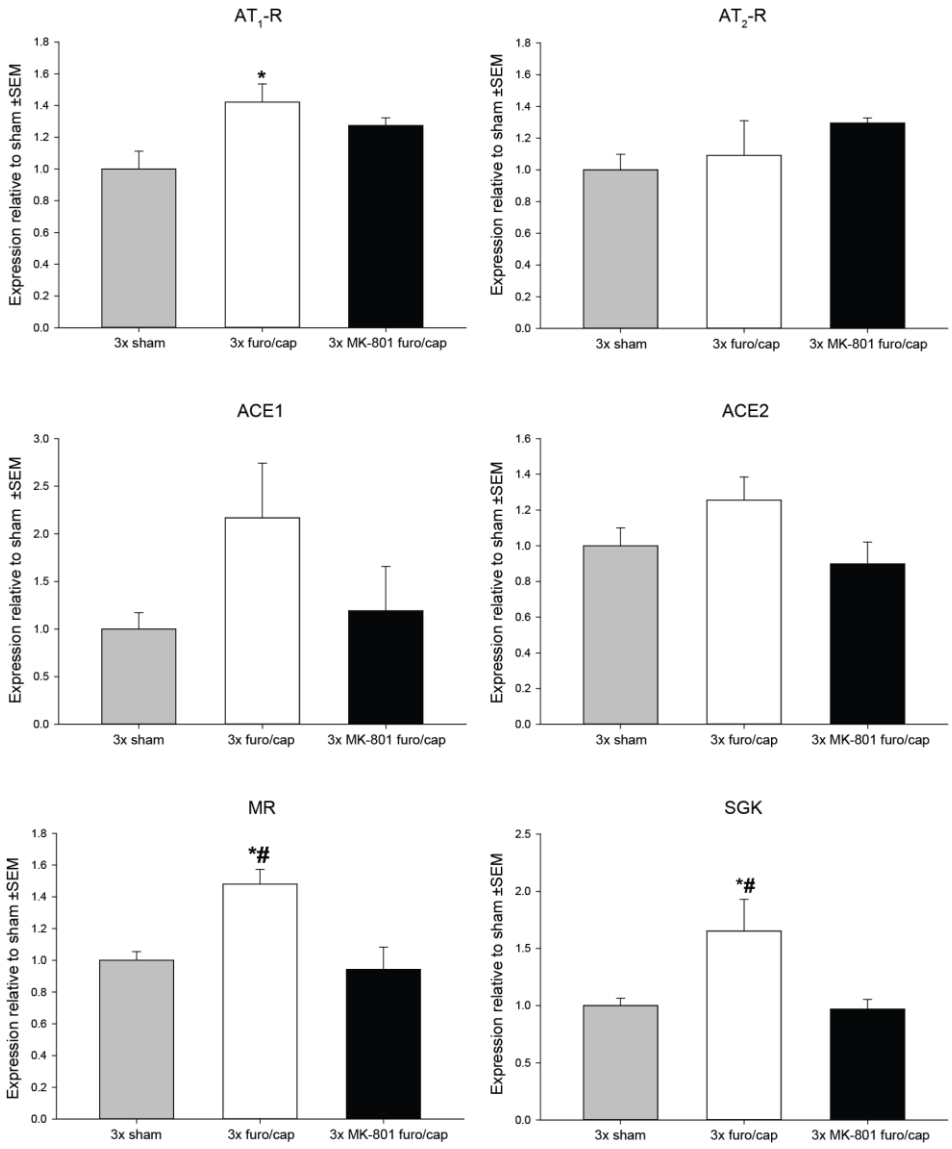

Figure 12 - Effect of repeated furo/cap treatments and MK-801 pretreatment on mRNA expression in the LT. Compared to sham-depleted rats, rats with a history of furo/cap treatments expressed greater levels of mRNA for the AT_1-R, MR, and SGK (*=p<.05 vs. 3x sham-treated rats). Rats pretreated with MK-801 failed to show an elevation of message for AT_1-R, and had significantly less message for MR and SGK compared to rats with a history of furo/cap treatments (#=p<.05 vs. 3x furo/cap). No significant differences were observed in AT_2 receptor, ACE1, or ACE2 mRNA expression. All data are expressed as arbitrary units.

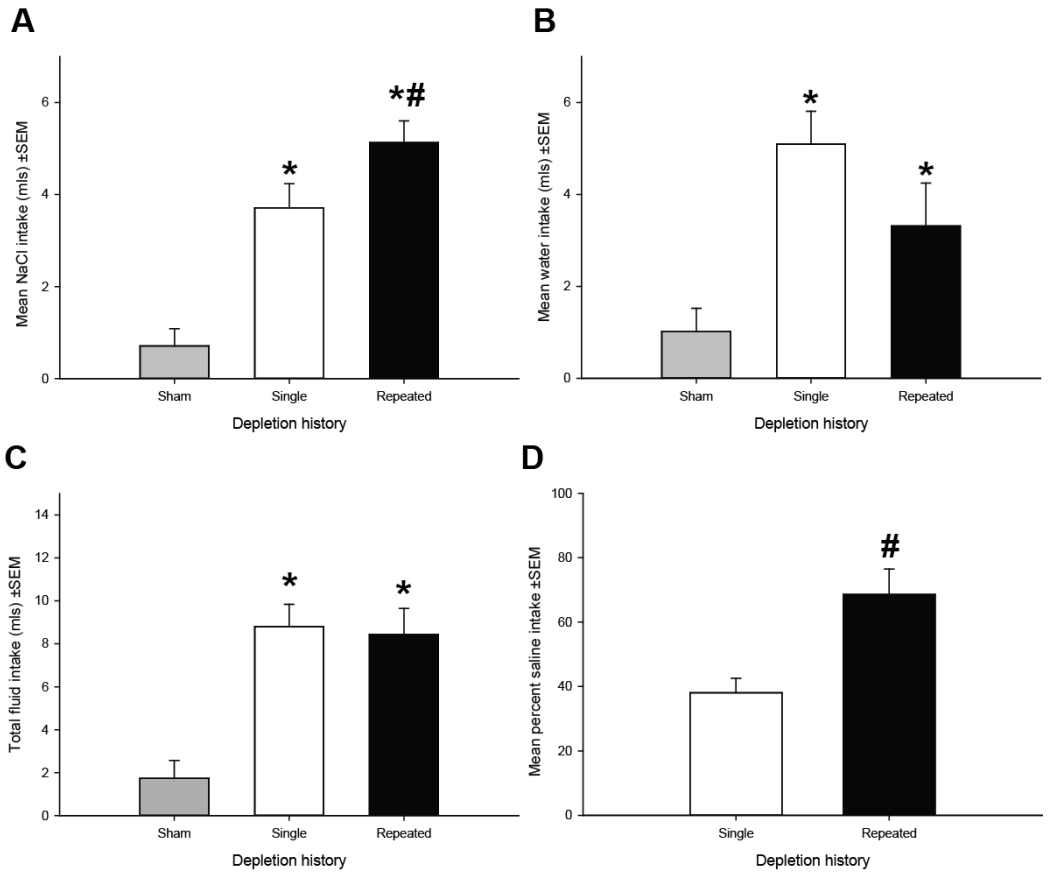

Figure 13 - Sensitization of sodium appetite. Rats with a history of sodium depletions display greater 1.8% NaCl intake than rats with a single depletion (A). Depleted rats drank more 1.8% NaCl and water than sham-treated rats (A and B). Combined water and sodium intake was elevated in single and repeatedly depleted rats compared to sham depleted rats (C). A greater proportion of total fluid intake consisted of sodium intake in repeatedly depleted rats compared to rats with a single depletion (D). *=p<.05 vs. sham, #=p<.05 vs. single depleted rats.

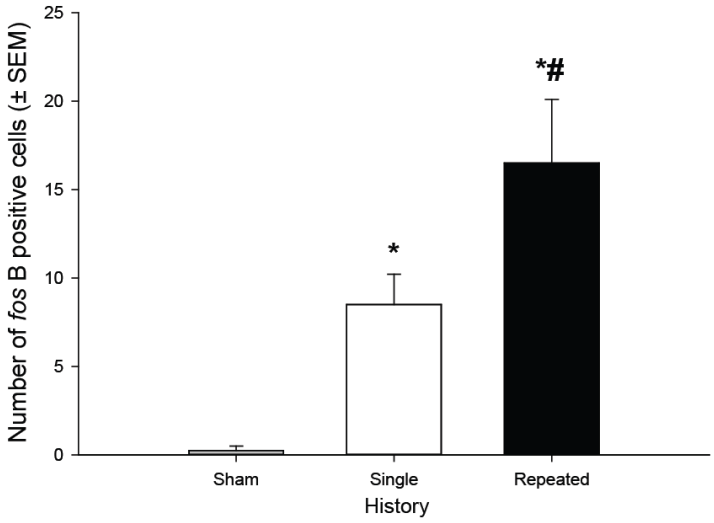

Figure 14 - Effect of sodium depletions on *fos*-B/Δ*fos*-B expression in the SFO. Sham-pretreated rats exhibited nearly absent *fos*-B/Δ*fos*-B staining, rats with a single sodium depletion exhibited moderate amounts of *fos*-B/Δ*fos*-B expression and rats with repeated sodium depletions exhibited robust expression of *fos*-B/Δ*fos*-B. *=p<.05 vs. sham-treated rats. #=p<.05 vs. single-depletion rats.

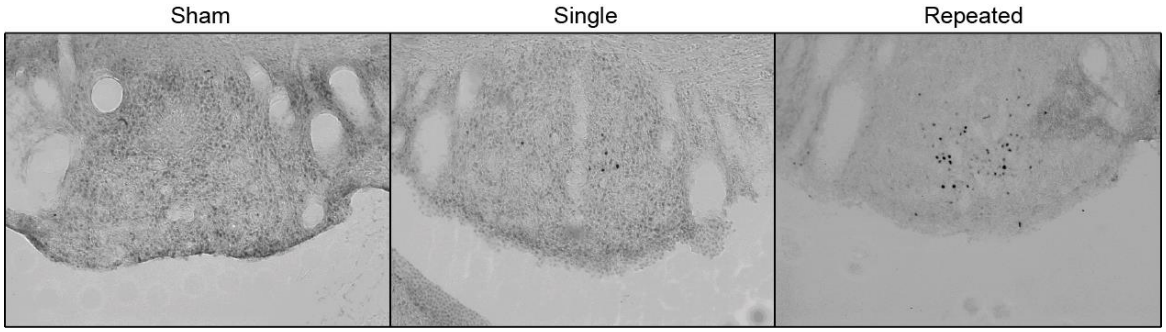

Figure 15 - Representative *fos*-B/Δ*fos*-B staining in the SFO. 10x images were taken of the posterior portion of the SFO approximately 1.4 mm caudal from bregma.

3.6 Tables

Abbreviation	Description
ACE1	Angiotensin converting enyzme type 1 – An enzyme that converts angiotensin I into angiotensin II.
ACE2	Angiotensin converting enzyme type 2 – A converting enzyme that appears to promote effects opposite of ACE1 by deactivating angiotensin II and converting angiotensin I into angiotensin 1-9.
AT_1-R	Angiotensin type 1 receptor – The primary receptor that mediates the actions of angiotensin II on water and sodium intake and vasoconstriction.
AT_2-R	Angiotensin type 2 receptor – Receptor for angiotensin II that may serve counter-regulatory functions.
MR	Mineralocorticoid receptor – Receptor for aldosterone that promotes sodium intake, vasoconstriction, and sodium retention.
SGK	Serum-and glucuocorticoid-induced kinase – Second messenger induced by MR activation that appears to be involved in some forms of neural plasticity.
fos-B	An immediate early gene that is upregulated after neurons become activated.
Δ*fos*-B	A truncated isoform of *fos*-B that lacks the catalytic moeity allowing it to remain elevated for weeks after induction. Δ*fos*-B appears to be involved in some forms of neural plasticity.

Table 4 – List of molecular markers examined in Chapter 3.

Chapter 4: The translation of sensed deficits in fluid balance to ingestive behavior: a role for orexin neurons in promoting the consummatory phase of thirst and sodium appetite

4.1 Introduction

When animals are depleted of water and sodium they manifest the motivated states of thirst and salt appetite, which are associated with behaviors involved in the seeking and ingestion of water and salt. Similar to nearly every motivated behavior, thirst and salt appetite can be divided into two phases: an appetitive phase that is marked by a seeking out of rewards in the environment and a consummatory phase that is defined by the termination of the appetitive state [31, 159]. For ingestive behaviors (e.g., eating and drinking) the consummatory phase is marked by the ingestion of a goal object. It is likely that interactions between neural circuitry involved in sensing deficits in fluid balance [85] and circuitry that promotes motivation and reward [73, 101] underlie the appetitive and consummatory phases of thirst and salt appetite.

A set of forebrain structures located along the rostral wall of the third ventricle, or the lamina terminalis (LT), are capable of sensing and processing information related to deficits in body fluid homeostasis [85, 113]. These structures include the subfornical organ (SFO), median preoptic area (MnPO), and organum vasculosum of the lamina terminalis (OVLT). Importantly, these LT structures are capable of modulating the activity of downstream brain areas in order to promote thirst and sodium appetite [158].

The mesolimbic dopamine system consists of dopaminergic cell bodies located in the ventral tegmental area (VTA) that project to the NAc and it is critical for the performance of seemingly every appetitive motivated behavior [89, 117]. Information related to body fluid status is likely to be transmitted from LT structures to motivation and reward systems in order to

promote thirst and salt appetite [74]. We sought to determine whether there is a neural pathway that allows for information processed in key LT structures to modulate mesolimbic activity. Specifically, it was hypothesized that hypothalamic neurons that synthesize the neuropeptide orexin (Ox) aid in coupling information related to deficits in fluid balance from structures in the LT with the mesolimbic dopamine system.

Ox is a neuropeptide that is distributed along the caudal half of the hypothalamus in an arc that extends laterally from the dorsomedial hypothalamus (DMH) to the dorsal region of the lateral hypothalamus (LHAd) [160]. Ox neurons have been partialed into 3 cell-clusters: one cell cluster lies in the DMH, a second in the perifornical area (PeF), and a third in the LHAd [67]. Ox exists in two forms – orexin A (Ox-A; also known as hypocretin-1) and orexin B (Ox-B; also known as hypocretin-2). Ox-A primarily binds to Ox type 1 receptors while both Ox-A and Ox-B bind to Ox type 2 receptors [42]. Ox neurons have been heavily implicated in promoting the motivation to obtain and ingest highly salient rewards [162] and, in the context of the present experiments, infusions of Ox into the cerebral ventricles produces a robust drinking response [92]. One mechanism by which Ox neurons may promote the consumption of salient rewards is through the action of Ox on the VTA [47, 91, 132, 180].

The present experiments investigated whether Ox neurons aid in integrating information from body fluid homeostasis systems with motivation and reward systems in order to promote thirst and sodium appetite. A secondary objective was to determine whether Ox promotes the appetitive or consummatory phases of thirst and sodium appetite. The first set of experiments employed anterograde and retrograde tracing to identify whether structures along the LT project to Ox-A neurons in PeF and whether the PeF projects to dopamine neurons in the VTA. In a second set of experiments c-*fos* and Ox-A double-labeling were used to determine whether

conditions related to extracellular dehydration, the seeking of water and sodium, or the ingestion of water and sodium would induce activation of Ox-A neurons. In a final experiment it was tested whether antagonism of Ox type 1 receptors within the VTA would attenuate extracellular dehydration-induced fluid intake. Together, the findings from these experiments support the presence of a LT-Ox-VTA neural pathway. However, Ox neurons appeared to be selectively recruited during the consummatory phase of thirst and sodium appetite rather than the appetitive phase.

4.2 Methods

Subjects

All experiments were conducted in accordance with the National Institute of Health *Guide for the Care and Use of Laboratory Animals* and were approved by The University of Iowa Animal Care and Use Committee. Male Sprague-Dawley rats (Harlan Teklad, Indianapolis IN) weighing between 275-300 grams upon arrival were used as subjects. Rats were maintained on a 12/12 light/dark cycle and housed in translucent (28.5 x 28.5 x17.5 cm) or suspended wire mesh (24 x 17.2 x 17.0 cm) cages in a temperature and humidity controlled room. Unless noted otherwise, the animals had *ad libitum* access to filtered tap water and NIH-31 irradiated modified open formula mouse/rat diet. Rats that received extracellular dehydration had at least 3 days of 1.8% W/V NaCl access prior to experimentation. 1.8% w/v hypertonic saline and deionized water were used to assess sodium appetite and thirst.

Extracellular dehydration

An acute model of extracellular dehydration was used that involves co-administration of the diuretic furosemide (furo; 10 mg/kg, SC; Hospirca Inc. Lake Forest, IL) and a low dose of

the antihypertensive drug captopril (cap; 5 mg/kg, SC, Sigma Aldrich). When these drugs are administered together they induce water and sodium depletion (i.e., extracellular dehydration) and slightly reduce blood pressure [164]. The combination of extracellular dehydration along with the slight decrease in blood pressure evokes a rapid thirst and sodium appetite (~1 hour after injections; [54, 55]).

Retrograde and anterograde tracing experiments

The first set of experiments aimed to identify whether a LT-Ox-VTA neural pathway is present in the rat brain by using anterograde and retrograde tracing in combination with immunohistochemistry for Ox-A and tyrosine hydroxylase. In the first anterograde tracing experiment, rats were anesthetized with Nembutal (60 mg/kg) and 10,000 molecular weight biotynilated dextran amine (BDA; 10% in physiological saline) was iontophoresed (+5 μA 7s on/off for 15 minutes) into the SFO (coordinates: -1.1 AP, -5.2 DV) or dorsal MnPO (coordinates: -.4 AP, -6.7 DV). In a second retrograde tracing experiment Fluorogold (FG; 2% in physiological saline) was iontophoresed ipsilaterally into the PeF (coordinates: -3.2 AP, +1.4 ML, -.8.8 DV). In a third experiment a co-injection (COIN) of 1.3% FG and 2.5% BDA was iontophoresed into the PeF (coordinates -3.0 AP, +1.2 AP, -8.8 DV) in order to determine whether LT neurons project to Ox neurons in the PeF and PeF neurons project to dopamine neurons in the VTA. 7-8 days after tracer injections rats were perfused and brains were collected.

c-fos expression during the appetitive and consummatory phases of thirst and salt appetite

The second set of experiments examined whether c-*fos* expression in Ox neurons is associated with the drive to obtain water and sodium (the appetitive phase of thirst and salt appetite) or the actual consumption of water and sodium (the consummatory phase of thirst and

salt appetite). In the first experiment, the effects of fluid ingestion on c-*fos* expression in Ox neurons was examined. Rats received vehicle or furo/cap treatment and 90 minutes later they were given access to water and 1.8% hypertonic saline (n=3 per group). Rats were allowed to drink for 90 minutes and then anesthetized and perfused. A separate second experiment examined the effects of extracellular dehydration without fluid access on c-*fos* expression in Ox neurons. Rats received vehicle or furo/cap treatment (n=4 per group) and 180 minutes after treatment they were anesthetized and perfused. In a third experiment, it was examined whether the act of seeking out and attempting to ingest fluids would induce c-*fos* expression in Ox neurons. Rats (n=3 per group) received either furo/cap or sham depletion. 90 minutes after furo/cap or sham depletion, rats were offered access to empty burettes (sham reward) and 90 minutes after burette access rats were anesthetized and perfused. It should be noted that in all three experiments rats were sacrificed 180 minutes after vehicle or furo/cap treatment.

Orexin type 1 receptor antagonism in the VTA

The final experiment aimed to determine whether Ox type 1 receptor antagonism in the VTA attenuates extracellular dehydration-induced water or sodium intake. Rats (n=6 in the vehicle group and n=5 in the SB-408124 group) were anesthetized with Nembutal (60 mg/kg) and implanted with 26 gauge bilateral guide cannula aimed at the VTA (coordinates: -5.2 AP, +1.8 ML, -6.2 DV). 1 week after surgery, a 32 gauge injector that extended 2mm beyond the guide cannula was inserted and vehicle (DMSO) or 300 ng/hemisphere of the Ox type 1 receptor antagonist SB-408124 (Sigma Aldrich, St. Louis Missouri) was microinjected in a volume of 300 nl per side over 1 minute. Ten minutes after VTA microinjection, rats received furo/cap treatment. In an effort to dissociate water drinking from hypertonic saline intake a water-first/saline-second presentation after furo/cap administration was used [72]: rats were

immediately provided water after furo/cap treatment and 90 minutes later they were given access to hypertonic saline as well. Water and sodium intakes were recorded at 0, 60, 90, 105, 120, and 180 minutes after fluid access. At the end of behavioral testing, rats were anesthetized and dye was microinjected to identify cannula placements. Rats with missed injections were removed from analysis. One rat in the vehicle pretreated group exhibited ablation of the VTA and was removed from analysis.

Perfusion and sectioning

Rats were heavily anesthetized with Nembutal (50 mg) and transcardially perfused with PBS followed by 4% paraformaldehyde. Brains were post-fixed in paraformaldehyde for 4-6 hours and then transferred to 20% sucrose in phosphate buffer and stored at 4ºC overnight. The next day slices were sectioned at 30 µm (tracing experiments) or 40 µm (c-*fos* and Ox labeling) with a cryostat set at -20ºC. A series of 2 non-consecutive sections were obtained for all tract tracing studies and a series of 3 non-consecutive sections were collected for c-*fos* and Ox double-labeling. Tissue was either immediately stained after sectioning or stored in cryoprotectant at 20ºC and stained at a later time.

Immunohistochemistry, tract tracing, and c-fos quantification

To ensure reliability of histological procedures, all tissue staining was performed in cohorts such that each batch of processed tissue contained an equal number of rats from each experimental and control group. Tissue was blocked with normal goat serum prior to all staining. c-*fos* was labeled by using a rabbit-raised anti-c-*fos* antibody (1:2000, Santa Cruz Biotechnology, Dallas Texas) and Ox-A was labeled with a rabbit-raised anti-orexin-A antibody (1:8000, Phoenix Peptides, Burlingame CA). This Ox-A antibody does not cross-react with other known

hypothalamic peptides and staining of cell-bodies was not observed outside of the hypothalamus. Tissue was incubated with the primary antibody for 20 hours at room temperature and then incubated with a biotinylated goat-raised anti-rabbit antibody (1:200, Vector Laboratories, Burlingame CA) for 1 hour. Tissue was then exposed to avidin-biotin complex for 1 hour (Vector Laboratories, Burlingame CA). To dissociate c-*fos* from Ox staining in the double-labeling experiment, tissue was stained for c-*fos* first. c-*fos* was visualized using a DAB peroxidase reaction in the presence of nickel and cobalt chloride, which yielded a dark nuclear stain. Afterwards tissue was incubated with an Ox-A antibody that was ultimately visualized with a DAB peroxidase reaction in the absence of nickel and cobalt chloride to produce a light brown cell body stain.

For retrograde and anterograde tracing studies a similar staining protocol was employed, however anti-rabbit Alexa Fluor 647 was used to visualize Ox neurons (1:1000, Jackson ImmunoResearch Laboratories Inc., West Grove PA) except in one case where DAB was used to visualize anterograde projections (in the presence of nickel and cobalt chloride) and Ox-A expression (in the absence of nickel and cobalt). In all other cases anterograde tracing was visualized through the use of an Alexa Fluor 555 Streptavidin conjugate (1:1000, Life Technologies, Grand Island NY). In the COIN experiment, dopamine neurons were visualized with a rabbit-raised anti-tyrosine hydroxylase antibody (1:1000, EMD Millipore Billerica MA) and Alexa Fluor 647. Fluorescence was captured using TRITC and Cy5 (far red) filters and images were pseudo-colored. No cross expression was observed between Alexa Fluor 555 and Alexa Fluor 647.

Digital images of Ox neurons, the VTA, and the LT were taken using a confocal microscope (Olympus IX81). To improve identification of c-*fos* positive Ox neurons a stack of

digital images were taken at a range of Z-coordinates. Ox and *fos* positive neurons were manually counted by an experimenter blind to the treatment conditions using ImageJ (version 1.48, National Institutes of Health). In the experiment where rats were depleted and allowed access to water and 1.8% saline, 3 images of the anterior portion of the VTA were captured and examined for c-*fos* immunoreactivity. The anterior VTA was chosen based upon the coordinates used in the SB-408124 experiment.

Statistics

Student's t-tests were used to analyze data from all c-*fos* and intake experiments. Spearman's rho was used to analyze the relationship between water and sodium intake in SB-408124 pretreated rats.

4.3 Results

Retrograde and anterograde tracing studies

BDA infusions into the SFO (n=2) or dorsal MnPO (n=2) revealed labeling of anterograde projections with what appeared to be varicosities and axon terminals in apposition with Ox neurons (Figure 16). Projections that were in proximity of Ox neurons were observed in all Ox cell-clusters; however it appeared that a majority of projections were found in the PeF. Iontophoretic injections of Fluorogold and a COIN of FG and BDA into the PeF Ox cell-cluster (n=2 for FG alone, n=1 for the COIN) found that retrograde labeling occurred across all LT structures (Figure 17). Missed injections that were dorsal to Ox neurons either failed to retrogradely label structures along the LT or only found a paucity of retrograde labeling (data not shown). The COIN injection also revealed varicosities and axon terminals in apposition the VTA

dopamine neurons (Figure 17 G-H). No retrograde labeling in VTA dopamine neurons was observed after COIN.

c-fos expression during the appetitive and consummatory phases of thirst and salt appetite

Rats that received furo/cap and were allowed to ingest fluids exhibited a significant elevation of c-*fos* expression in Ox neurons (Figures 18 and 19). Specifically, a greater number of c-*fos* expressing Ox neurons were observed in the DMH $t(4)=5.164$; $p<.01$, PeF $t(4)=4.014$; $p<.05$, and LHAd $t(4)=3.56$; $p<.05$. No significant differences were observed in the total number of Ox neurons quantified in each cell-cluster (data not shown, $p>.30$). Additionally, depleted rats that were allowed access to fluids exhibited significantly greater c-*fos* expression in the anterior region of the VTA $t(4)=3.899$; $p<.05$ (Figure 18D). In contrast, rats that received depletion alone or that were depleted and received empty burettes exhibited nearly a complete lack of c-*fos* labeled Ox neurons (Figure 20). Since these conditions produced almost a complete absence of c-*fos* positive Ox neurons, double-labeling was not quantified in these experiments. The lack of *fos* expression was not due to a failure in the staining protocol as *fos* was observed in the central and medial nucleus of the amygdala within sections that contained Ox neurons and in the paraventricular nucleus of the hypothalamus in depleted rats (Figure 20). These areas have been associated with either water or sodium depletion [85].

Orexin type 1 receptor antagonism in the VTA

Analyses revealed that Ox antagonism within the VTA attenuated combined water and sodium intake $t(9)=2.78$; $p<.05$, but a reduction specific to either water or saline intake was not observed (Figure 21). A correlation analysis revealed that there was an inverse relationship between water and salt intake in SB-408124 treated rats $r(5)=-.87$, $p<.05$ (Figure 21D). Analysis

of vehicle pretreated rats did not yield a significant correlation between water and sodium intake (p=.18, data not shown).

4.4 Discussion

The present experiments found that 1) A LT-Ox-VTA neural pathway appears to be present in the brain, 2) Ox neurons in the hypothalamus exhibit increased c-*fos* expression during the ingestion of water and sodium but not from extracellular dehydration alone or the seeking out and attempted ingestion of water and sodium, and 3) Ox antagonism in the VTA attenuates fluid intake. Together, these findings indicate that a LT-Ox-VTA neural pathway operates to enhance the fluid intake exhibited during the consumption of water and sodium.

Retrograde and anterograde tracing studies found that LT structures project to Ox neurons, which in turn project to VTA dopamine neurons. BDA injections revealed that structures along the LT send projections that are in apposition with Ox neurons. Some of these projections consisted of varicosities while others appeared to be axon terminals. Neither the SFO nor the dorsal MnPO selectively projected to a single Ox cell cluster, although a majority of the projections appeared to terminate within the PeF. It should also be noted that there were significant projections to regions of the hypothalamus that did not contain Ox neurons. Retrograde labeling studies suggest that Ox neurons located in the PeF receive input from structures along the entirety of the LT. These findings are consistent with previous reports showing that the SFO innervates the hypothalamus and structures along the LT project to the PeF [66, 158]. Importantly, the pattern of retrograde labeling that was observed within the LT is comparable to the pattern of c-*fos* expression exhibited during intracellular dehydration and sodium depletion [112, 170]. The anterograde and retrograde tracing produced by the COIN of FG and BDA provides strong evidence that LT neurons project to the PeF which may relay

information to VTA dopamine neurons. Together, these findings suggest that the LT is capable of indirectly modulating VTA dopamine neurons through second-order Ox neurons.

When depleted rats were allowed to ingest water and sodium a significant elevation in the number of c-*fos* positive Ox neurons was observed in the hypothalamus across all Ox neuron cell clusters. In contrast, rats that received extracellular dehydration without fluid access or rats that received sham reward (an empty burette) failed to exhibit c-*fos* positive Ox neurons. Therefore, it appears that the *fos* expression observed in the present experiments was related specifically to the ingestion of fluids rather than either extracellular dehydration (i.e., a disruption in homeostasis) or the seeking out of fluids in the environment (i.e., the appetitive phase of thirst and sodium appetite). This finding is consistent with a previous report which found Ox neurons exhibit increased firing rate during the consumption of palatable food (chicken) reward [115]. Others have shown that Ox plays an important role in the consummatory phase of alcohol self-administration, but not the appetitive phase [3]. Most importantly, the present findings contribute to the idea that Ox neuron activation promotes the consummatory phase of motivated behaviors [3, 31, 40].

Ox is capable of promoting the performance of various motivated behaviors [162]. A mechanism by which Ox may operate to support motivated behaviors is through its release in the VTA [180], which appears to facilitate activity of the mesolimbic dopamine system [47, 91]. Ox neurons have been shown to project to the VTA [47, 132] and Ox can depolarize both dopaminergic and non-dopaminergic neurons in this structure [91]. Here it was found that administration of the Ox type 1 receptor antagonist SB-408124 into the VTA attenuated extracellular dehydration-induced fluid intake. SB-408124 pretreatment did not appear to selectively reduce water or salt intake; however an inverse relationship between water and salt

intake in SB-408124 pretreated rats was found. This finding is consistent with the idea that Ox plays a general role in promoting appetitive motivated behaviors [12, 73, 162]. The antagonist data buttresses the tracing data from the COIN experiment showing that the PeF Ox cell cluster projects to VTA dopamine neurons.

There are some limitations to the present findings that need to be acknowledged. One is that the Ox antagonism and c-fos experiments examined thirst and sodium appetite concurrently. Although we used a model of extracellular dehydration that has been found to dissociate thirst and salt appetite [72], this model may not have been ideal given that Ox appears to play a role in both thirst and sodium appetite. Additionally, the c-*fos* expression observed in the present experiments most likely reflects a combination of the effects of water and sodium intake. It is worth noting, however, that recent data from our laboratory indicate that the ingestion of sodium alone during sodium depletion can activate Ox neurons. Finally, the injections of BDA into the SFO and MnPO were limited to a small region of each of these structures. Because of the restricted diffusion of BDA a completely accurate representation of the projections from the entirety of the SFO and of the MnPO cannot be presented.

4.4 Perspectives and significance

It has been proposed that the hypothalamus functions to integrate internal homeostatic state with adaptive physiological and behavioral responses [155, 158, 159] and Ox neurons appear to contribute to this integrative capacity of the hypothalamus [8, 73, 179]. The present findings provide additional support for this concept by showing that structures along the LT, which function to regulate fluid balance, project to Ox neurons and Ox receptor blockade in the VTA attenuates fluid intake. A surprising finding was that Ox neurons selectively expressed *fos* when rats were allowed to consume water and sodium. Based upon this, it is likely that Ox

neurons function to enhance ongoing ingestion of highly salient rewards through the release of Ox in the VTA. Within this context, it is interesting to note that prior reports have stated that Ox appears to prolong the motivation to eat [163] and delay satiety [138]. Therefore, Ox release in the VTA may play a critical role in enhancing ongoing consummatory behaviors, including ingestion of water, sodium, and food intake. Under conditions of deprivation (e.g., food, water, or sodium deficiency) it is important that animals consume relatively large amounts of ingesta in order to restore homeostasis. Ox neurons may contribute to the considerable intakes observed under conditions of deficiency. It is also likely that highly salient rewards such as palatable foods or addictive drugs can activate Ox neurons to enhance intake independent of inputs from homeostatic systems (i.e., hedonic-driven feeding).

Inputs from homeostatic systems most likely affect Ox neuron function [8, 73]. An internal state of water and sodium deficit is capable of altering the saliency and rewarding value of both water and sodium to essentially transform these substances into highly salient rewards that are consumed in relatively large quantities [44, 74]. It is possible that the LT projections to Ox neurons serve the purpose of increasing the excitability of Ox neurons such that they are readily activated in response to the ingestion of water and sodium. Ox release in the VTA, in turn, serves to facilitate thirst and salt appetite. This would ultimately result in a greater intake of water and sodium to effectively restore fluid balance.

4.5 Figures

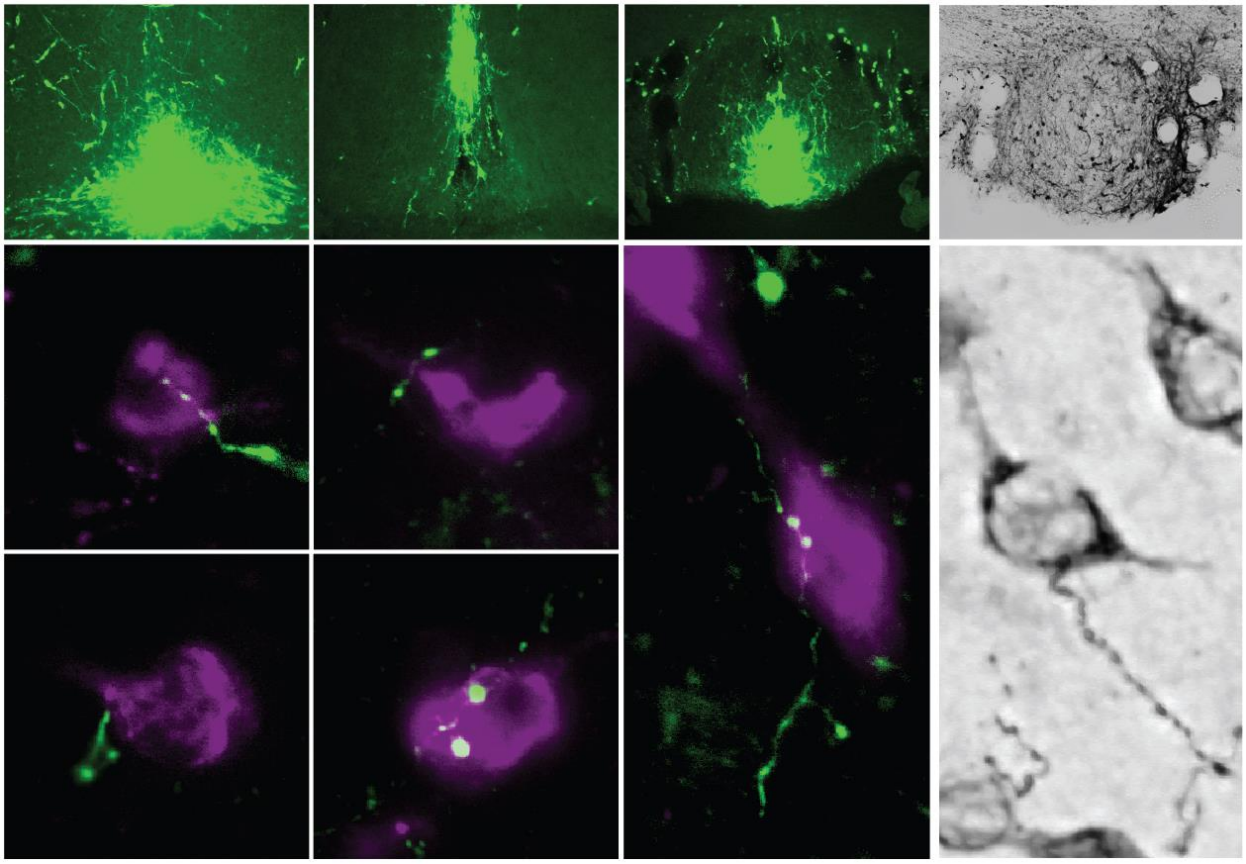

Figure 16 - Projections from the SFO and dorsal MnPO to Ox neuron cell bodies in the hypothalamus. Top row – 10x magnification of the focal point of injection sites in the dorsal MnPO and SFO (one set of tissue was visualized with DAB rather than immunofluorescence). All of the injection sites yielded anterograde labeling that consisted of varicosities or axon terminals in apposition with Ox cell bodies. Bottom – examples of projections in apposition with Ox neurons taken at 40x magnification. Green represents BDA labeling and magenta represents Ox labeling.

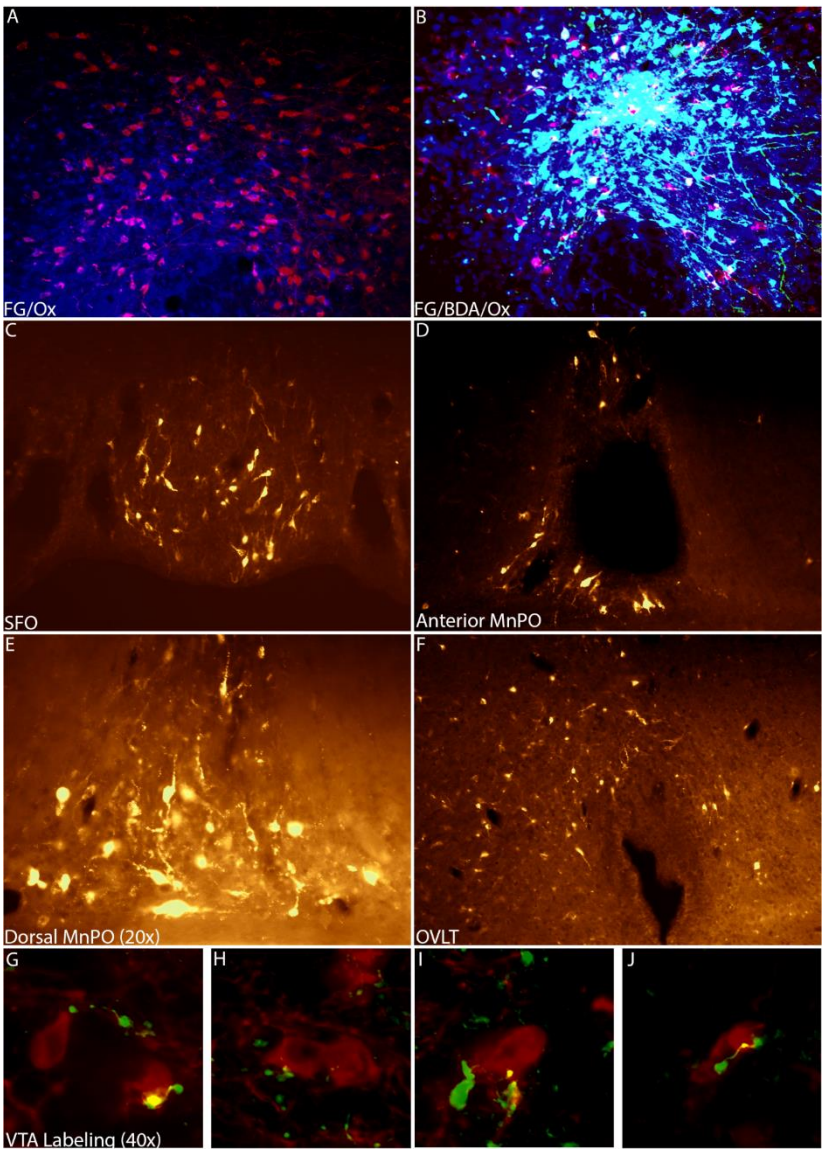

Figure 17 – Results from Fluorogold and COIN infusions in the PeF. Injection sites of 2% FG (blue) into the PeF Ox neurons (red) and a COIN of 1.33% FG (blue) and 2.5% BDA (green) in PeF Ox neurons (red) are presented in panels A and B, respectively. Retrograde labeling was observed along the entirety of the LT (panels C-F). Axon terminals and varicosities (green) were observed in apposition with tyrosine hydroxylase positive neurons in the VTA (red; panels G-J). All images were taken at 10x unless noted otherwise.

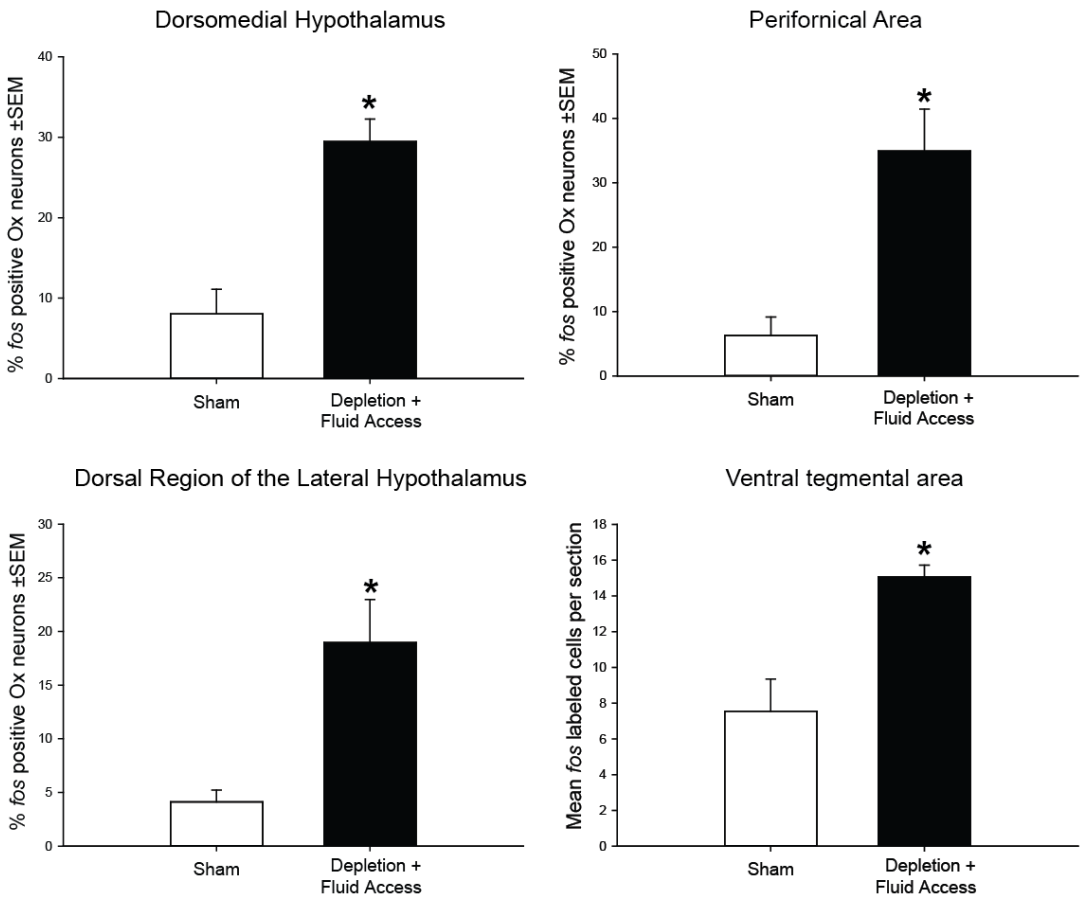

Figure 18 - Ingestion of water and salt in depleted rats activates hypothalamic Ox neurons and the VTA. The percentage of Ox neurons that expressed c-*fos* was greater in rats that were depleted and allowed to drink water and sodium across each Ox cluster (*=p<.05). The VTA also exhibited a greater amount of c-*fos* expression in this condition.

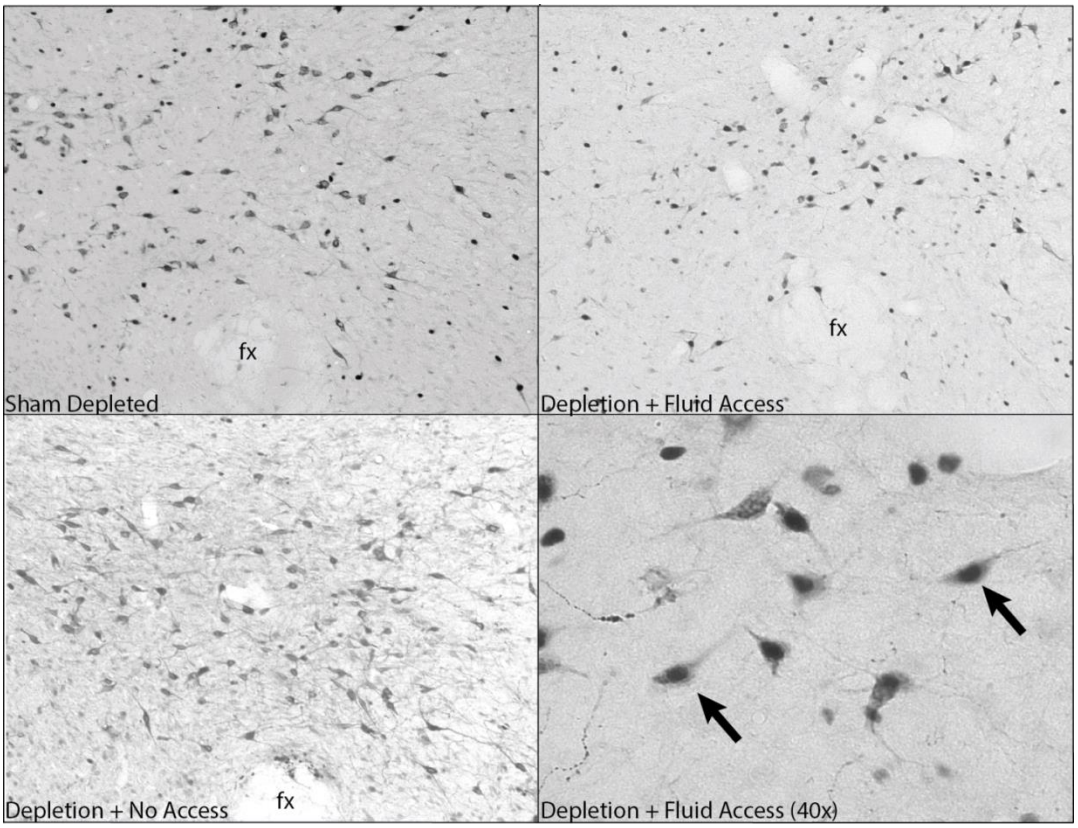

Figure 19 - Ox neuron staining in the PeF in rats that were sham depleted, depleted with fluid access, or depleted with no fluid access. Arrows indicate examples of double-labeled neurons in 40x magnification. fx = fornix.

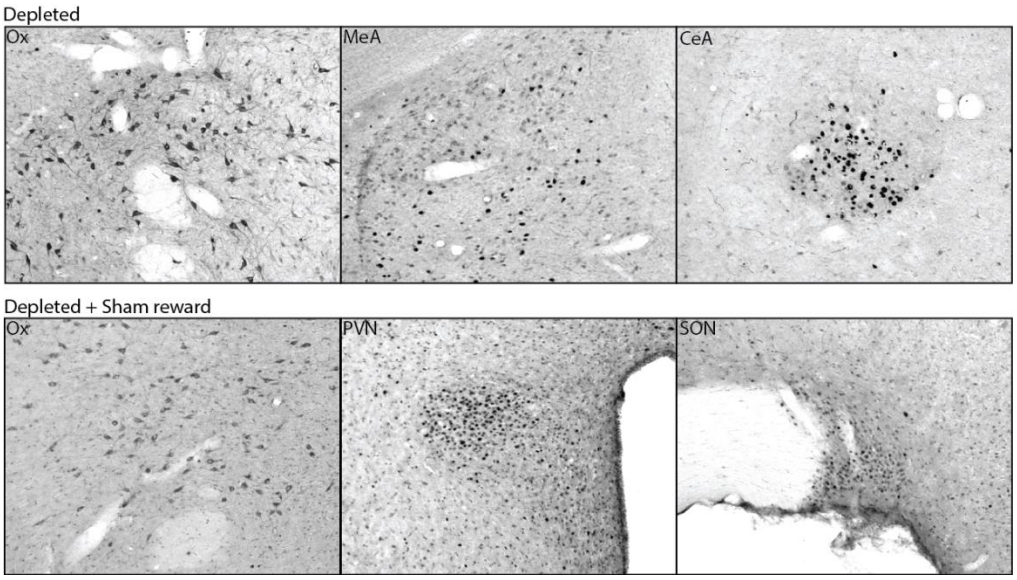

Figure 20 - Lack of *fos* positive Ox neurons in rats depleted of water and sodium and depleted with access to sham reward. Top – rats that received depletion alone failed to exhibit c-*fos* expression in Ox neurons, however *fos* was observed in the medial amygdala (MeA) and central amygdala (CeA) within sections that contained Ox neurons indicating that the lack of *fos* expression in Ox neurons was not due to a failure in the staining protocol. Bottom – rats that received depletion and were offered access to empty burettes failed to exhibit c-*fos* positive Ox neurons. c-*fos* labeling was observed in the paraventricular nucleus (PVN) and supraoptic nucleus (SON) indicating that rats were depleted.

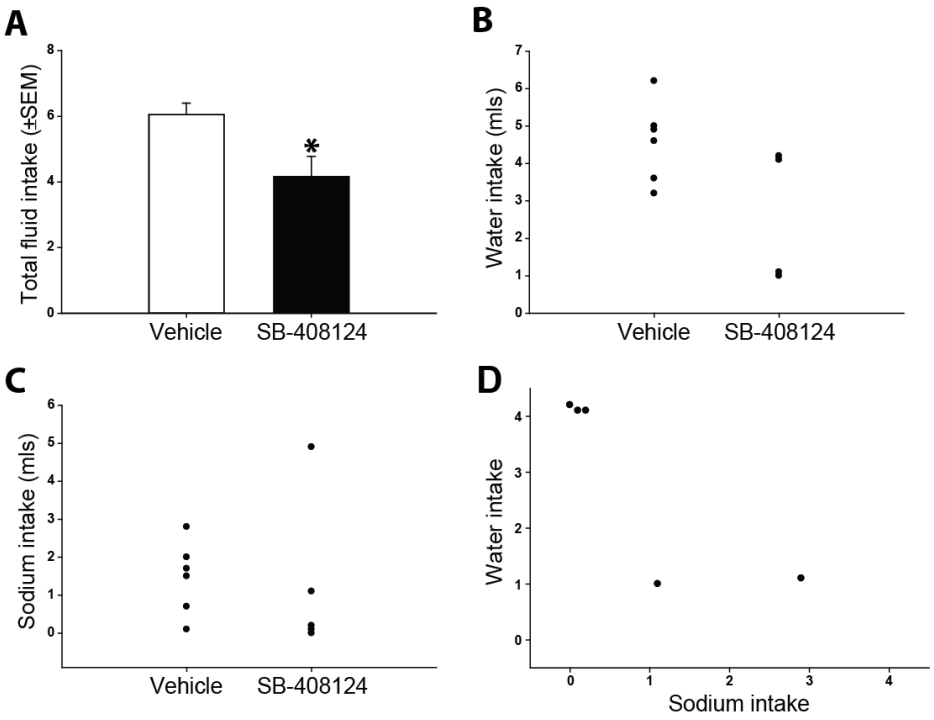

Figure 21 - Antagonism of Ox type 1 receptors within the VTA attenuates fluid intake in depleted rats. Rats pretreated with SB-408124 exhibited reduced total fluid intake (A; *=p<.05). Scatterplots of fluid intakes are presented in (B) and (C). An inverse relationship was found between water and sodium intake in the SB-408124 pretreated group group (D).

Chapter 5: Summary and Future Directions[4]

5.1 Summary

The overall goal of the present dissertation was to increase the understanding of the neurobiological basis of salt appetite and the sensitization of salt appetite through the use of pharmacological, molecular, and neuroanatomical methods. Systemic NMDA-R antagonism prior to sodium depletion prevents enhanced sodium intake [72], suggesting that NMDA-R-dependent neural plasticity is involved in the sensitization of sodium appetite. Sodium depletion also produces relatively long-lasting NMDA-R-dependent increases in mRNA expression for the mineralocorticoid receptor, angiotensin type 1 receptor, and SGK within the LT [76]. This finding supports the idea that sodium deficiency evokes neural plasticity in the LT through the upregulation of components of the central RAAS in this region. Additionally, other data from our laboratory found that NMDA-R antagonism directed at the VTA prevents the development of sodium appetite sensitization (Figure 22), which indicates that NMDA-R-dependent neural plasticity within the VTA may be necessary for sensitization to occur. Together, these experiments bolster previous reports that supported a role of neural plasticity in structures along the LT as well as the mesolimbic dopamine system [124, 139]. Additionally, the experiments outlined in Chapter 4 support the presence of a LT-Ox-VTA neural pathway that provides a mechanism for the LT to influence activity in the mesolimbic dopamine system.

So far, evidence suggests that two systems in the brain undergo plasticity in response to sodium depletion: the LT which serves as a forebrain system that regulates body fluid homeostasis and the mesolimbic dopamine system which mediates appetitive motivated

[4] A portion of this chapter is derived from a publication by the author (see Ref. 74).

behaviors (see Figure 23 for a summary of the available evidence for brain areas that may exhibit neural plasticity after sodium depletion). With respect to body fluid homeostasis systems, the SFO exhibits increased activity during sodium deficiency in sensitized rats [124]. Additionally, SGK, angiotensin type 1 receptor, and mineralocorticoid receptor mRNAs are elevated in the LT after repeated sodium depletions (Chapter 3, [76]). Whether hindbrain areas that sense and process body fluid status are important for sensitization remain to be tested. Sodium depletion also induces neural plasticity in the mesolimbic dopamine system [124, 139]. Repeated episodes of sodium depletion increase the dendritic length and arborization of NAc neurons [139]. Additionally, sensitized rats exhibit increased activity of NAc neurons during sodium deficiency [124]. Finally, NMDA-R antagonism within the VTA prevents the induction of sodium appetite sensitization (Figure 22). It is possible that plasticity occurring in the VTA is passed on to the nucleus accumbens [107].

Ox neurons are positioned to act as an intermediary between the LT and VTA dopamine neurons. Recent evidence implicates a role for Ox neurons in the sensitization of sodium appetite. For example, Ox is capable of inducing neural plasticity in VTA neurons [13] a finding that raised the hypothesis that Ox antagonism in the VTA would prevent sodium appetite sensitization. However, preliminary experiments in our laboratory have failed to conclusively demonstrate a role for Ox action in the VTA in sodium appetite sensitization. Another possibility is that Ox neurons themselves exhibit plasticity after depletion [99]. Activity-regulated cytoskeletal-associated protein (Arc) and dopamine and cAMP-regulated phosphoprotein 32kDa (DARPP-32) are intracellular messengers that have been implicated in plasticity [25, 150, 169]. Both of these intracellular messengers are upregulated after sodium depletion in PeF Ox neurons

[99]. As Ox action on the VTA appears to enhance fluid intake, plasticity that results in increased Ox neuron activity may contribute to the larger fluid intakes observed in sensitized rats.

A proposed neural circuit that mediates the sensitization of sodium appetite is presented in Figure 23. In short, neural plasticity may occur along a neural circuit that consists of areas that sense body fluid homeostasis in the forebrain (the LT), areas that integrate homeostatic state with adaptive autonomic and behavioral responses (the hypothalamus), and structures that promote goal-directed behaviors (the mesolimbic dopamine system). One major caveat to this circuit is that many of the experiments that have implicated neural plasticity along this circuit are correlational in nature and it is not clear if many of the changes are functionally significant.

5.2 Looking ahead: future directions

There are many experimental questions that need to be addressed in order to better understand the sensitization of sodium appetite. Perhaps one of the most important questions is whether hindbrain areas that are critical for regulating fluid balance exhibit neural plasticity after sodium depletion. To this author's knowledge, this question has not been explored yet. It will also be important to determine whether any of the molecular and neuroanatomical changes that occur after sodium depletion are functionally significant. For example, SGK is one intracellular messenger that may hold promise in abolishing sodium appetite sensitization. The function of SGK may be fairly specific to the learning and memory associated with body fluid homeostasis as this messenger is primarily elevated by MR activation [151]. Additionally, SGK appears to play an important role in the maintenance of neural plasticity rather than its induction [104].

Another area of research that is likely to be fruitful is the cross-sensitization between sodium depletion and other rewards. The observation that sodium depletion induces neural

plasticity in the mesolimbic dopamine system has raised the hypothesis that depletion may cross-sensitize other motivated behaviors [139]. For example, rats with a history of sodium depletions exhibit elevated drug-induced locomotor behavior [26, 122]. Recently we have found that a prior experience of repeated sodium depletions also elevates self-administration of some cocaine doses in rats. Our colleagues have also shown that sodium depletion cross-sensitizes need-free sucrose intake, however this effect appears to be associative in nature (personal communication with Dr. Laurival De Luca Jr). Future work examining cross-sensitization may yield important findings on how life experiences can alter motivation and reward neural circuitry to ultimately affect motivation and reward preferences.

5.3 Perspectives on the sensitization of sodium appetite

It is important to realize that the sensitization of sodium appetite can be conceived of as an adaptive form of simple, non-associative learning that aids animals in maintaining sodium balance when faced with environmental conditions and challenges that threaten body sodium content. When body sodium content is repeatedly depleted it is adaptive for an animal to seek out and ingest greater amounts of salt to help restore sodium balance and to protect against future sodium loss [53]. For example, humans working in hot environments can lose significant quantities of sodium through sweat [1, 15, 119] and it would be adaptive for these individuals to develop a propensity to ingest salty substances to protect and restore sodium balance. However, a negative consequence of sodium appetite sensitization is that it could promote pathological salt intake that may contribute to the development of many disorders [105] including cardiovascular disease.

One approach to explaining the sensitization of sodium appetite is that the neural processes that are engaged to promote salt seeking and ingestion during sodium appetite leave

behind a memory trace that permanently elevates animals' salt intake. In other words, sodium depletion induces a change in activity across neural circuits, including the LT and the mesolimbic dopamine system, which drives sodium appetite [85, 99, 140]. However, the change in activity that occurs along the LT and mesolimbic dopamine system may not return to baseline levels after sodium depletion. It is possible that, through mechanisms involving NMDA-R-dependent neural plasticity [72], the memory of salty tastes becoming increasingly desired [27] as a consequence of sodium deficiency remains in the central nervous system after sodium balance has been restored. This memory may ultimately alter behavior to cause enhanced seeking and ingestion of salty foods. If this is true, then many of the neural processes that are responsible for sodium appetite (e.g., changes in neurotransmitter release, mRNA expression, or receptor expression) should still be exhibited long after body sodium has been restored. The experiments from Chapter 3 support this idea as repeated episodes of sodium deficiency produce a long-lasting elevation in mRNA expression across the LT for AT_1, MR, and SGK.

5.4 Figures

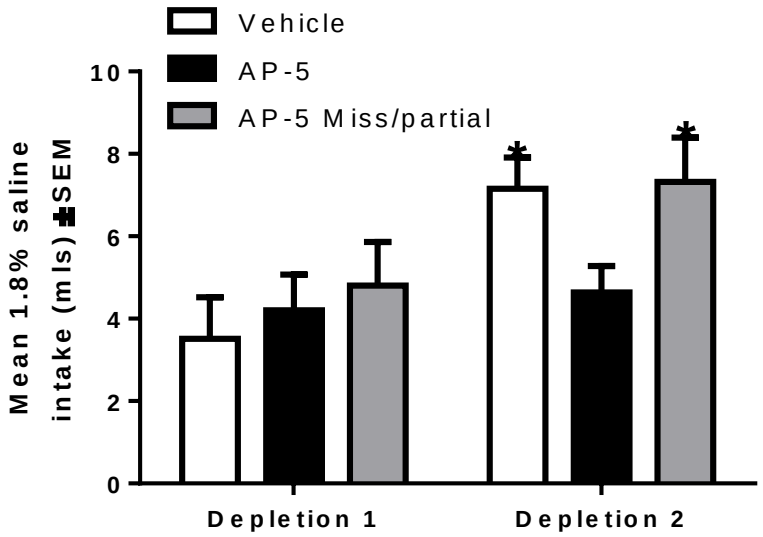

Figure 22 - Effect of AP-5 microinjection in the VTA on the sensitization of sodium appetite. Rats received 1µg AP-5 in .5µl aCSF or aCSF alone into the VTA prior to sodium depletion induced by furo/cap. Water and sodium intakes were recorded for 3 hours after depletion. 4 days later all rats received vehicle microinjection into the VTA and were then sodium depleted for a second time. Top – Mean hypertonic saline intakes during depletions 1 and 2. Rats that received vehicle in the VTA or microinjections of AP-5 that missed/partially spread into the VTA exhibited enhanced sodium intake on the second depletion while AP-5 pretreated rats failed to elevate sodium intake on the second depletion (*=p<.05 vs. sodium intake observed in the respective group on depletion 1).

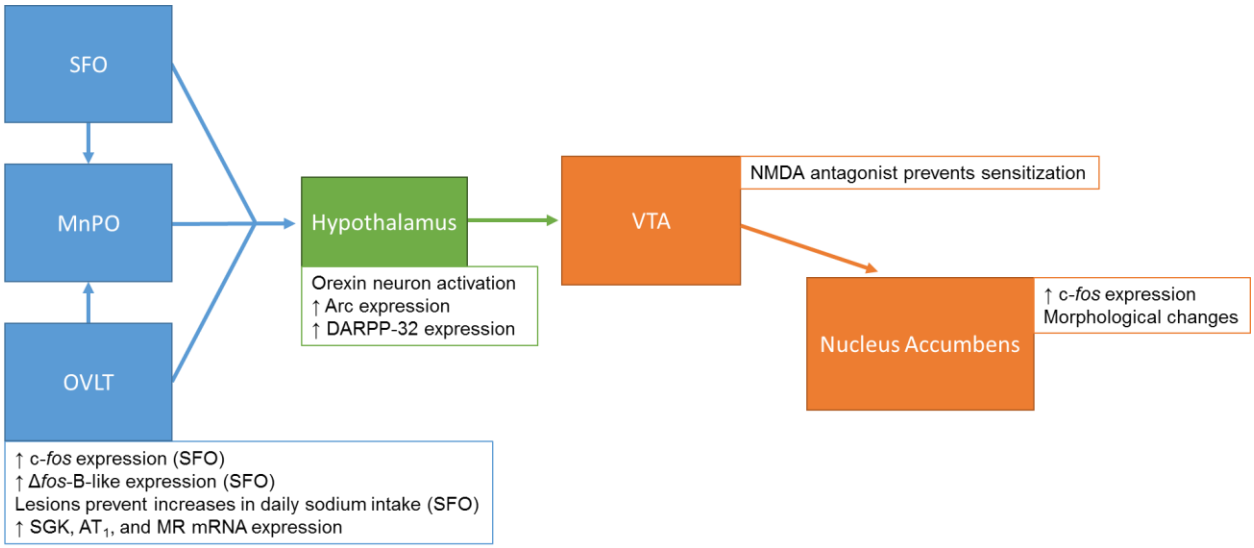

Figure 23 - Schematic summary of the proposed neural circuitry mediating the sensitization of sodium appetite. Areas along the LT regulate body fluid homeostasis (blue) and project to Ox-synthesizing neurons in the hypothalamus (green). The hypothalamus acts to integrate signaling related to body fluid balance with motivation and reward systems (orange). Boxes alongside each structure summarize the evidence indicating that they are involved in the sensitization of sodium appetite.

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