Metabolic investigations of the hypothalamo-neurohypophysical system during axonal sprouting : efects of hyponatremia
by Christopher Wade Moffett

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biological Sciences
Montana State University

Abstract:
Initial studies of compensatory collateral axonal sprouting in the magnocellular neurosecretory system in the Department of Biological Sciences at Montana State University-Bozeman have suggested that central peptidergic neurons become hyperactive during the growth of new axons (J. A. Watt and C. M. Paden , 1991, Experimental Neurology, volume 111, pages 9-24). Accordingly, elucidating the possible correlation between neuronal activity and axonal sprouting was at the heart of this thesis. In order to more fully understand this potential relationship, two hypotheses were tested: First, could the activity of magnocellular neurosecretory neurons be accurately monitored during compensatory collateral axonal sprouting? Second, could the activity of the magnocellular neurosecretory neurons be reduced throughout the period in which axonal sprouting is known to occur? To address the first hypothesis, rats underwent unilateral hypothalamic knife lesions in order to induce axonal sprouting by intact magnocellular neurosecretory neurons; then plasma osmolality, plasma sodium concentration, cytochrome oxidase histochemistry of the supraoptic nuclei and neurohypophysis, and in situ hybridization for oxytocin and vasopressin messenger ribonucleic acid pools in the supraoptic nuclei were used as measures of neuronal activity. To address the second hypothesis, a chronic hyponatremia protocol was used to suppress the activity of the magnocellular neurosecretory system in Iesioned and control rats. All of the measures examined indicated some degree of neuronal hyperactivity during axonal sprouting. Furthermore, these same measures indicated that the increase in activity normally associated with axonal sprouting was reduced or eliminated by the hyponatremia protocol. Thus, it is concluded that the activity of intact magnocellular neurosecretory neurons is elevated during the process of compensatory collateral axonal sprouting. Also, this increase in activity can be attenuated significantly through the use of the chronic hyponatremia protocol. Thus, increased neuronal activity may be an essential component of axonal growth in the central nervous system, and further investigation of axonal sprouting by magnocellular neurosecretory neurons in hyponatremic animals should elucidate this relationship.

METABOLIC INVESTIGATIONS OF THE HYPOTHALAMO-NEUROHYPOPHYSIAL SYSTEM DURING AXONAL SPROUTING: EFFECTS OF HYPONATREMIA

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Jan 23, 1996
Date

Chairperson, Graduate Committee

Approved for the Major Department

Jan 23/96
Date

Head, Major Department

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Date January 23, 1996

There is a Tide in the Affairs of Men
Which taken at the Flood, leads on to Fortune;
Omitted, all the Voyage of their Life
Is bound in Shallows and in Miseries.
On such a full Sea are we now a-float,
And we must take the Current when it serves,
Or lose our Ventures.

Excerpt from William Shakespeare (1680) JULIUS CÆSAR, Actus Quartus, pages 52-53. Reproduced by Cornmarket Press Limited (1969) London.

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ABSTRACT

Initial studies of compensatory collateral axonal sprouting in the magnocellular neurosecretory system in the Department of Biological Sciences at Montana State University--Bozeman have suggested that central peptidergic neurons become hyperactive during the growth of new axons (J. A. Watt and C. M. Paden , 1991, Experimental Neurology, volume 111, pages 9-24). Accordingly, elucidating the possible correlation between neuronal activity and axonal sprouting was at the heart of this thesis. In order to more fully understand this potential relationship, two hypotheses were tested: First, could the activity of magnocellular neurosecretory neurons be accurately monitored during compensatory collateral axonal sprouting? Second, could the activity of the magnocellular neurosecretory neurons be reduced throughout the period in which axonal sprouting is known to occur? To address the first hypothesis, rats underwent unilateral hypothalamic knife lesions in order to induce axonal sprouting by intact magnocellular neurosecretory neurons; then plasma osmolality, plasma sodium concentration, cytochrome oxidase histochemistry of the supraoptic nuclei and neurohypophysis, and *in situ* hybridization for oxytocin and vasopressin messenger ribonucleic acid pools in the supraoptic nuclei were used as measures of neuronal activity. To address the second hypothesis, a chronic hyponatremia protocol was used to suppress the activity of the magnocellular neurosecretory system in lesioned and control rats. All of the measures examined indicated some degree of neuronal hyperactivity during axonal sprouting. Furthermore, these same measures indicated that the increase in activity normally associated with axonal sprouting was reduced or eliminated by the hyponatremia protocol. Thus, it is concluded that the activity of intact magnocellular neurosecretory neurons is elevated during the process of compensatory collateral axonal sprouting. Also, this increase in activity can be attenuated significantly through the use of the chronic hyponatremia protocol. Thus, increased neuronal activity may be an essential component of axonal growth in the central nervous system, and further investigation of axonal sprouting by magnocellular neurosecretory neurons in hyponatremic animals should elucidate this relationship.

CHAPTER 1

INTRODUCTION

Statement of Purpose

These studies were done with two goals in mind: First, to further investigate the increase in neuronal activity associated with collateral axonal sprouting suggested by previous studies in Dr. Paden's laboratory (Watt, 1989; Watt and Paden, 1991; Watt, 1993), and second, to produce rats in which the activity of the magnocellular neurosecretory system was diminished or eliminated throughout the period that axonal sprouting had been described to occur. These goals were designed to build a supporting framework for future experiments that will test the hypothesis that decreased neuronal activity inhibits axonal sprouting within the magnocellular neurosecretory system.

To address the first goal, several measures of the activity of the magnocellular neurosecretory system were made from rats in both control (intact and sham) and sprouting (lesioned) groups. These measures were plasma osmolality, plasma electrolyte (potassium and sodium) concentrations, cytochrome oxidase histochemistry, and *in situ* hybridization of oxytocin and vasopressin ribonucleic acids. To address the second goal, chronic hyponatremia, a protocol developed to study the minimum stimuli necessary for vasopressin secretion (Verbalis, 1984; Verbalis and Drutarosky, 1988), was used to inhibit the activity of the magnocellular neurosecretory system following

unilateral lesions. The same measures of activity listed above were also used to investigate the metabolic effects of hyponatremia in sham and lesioned animals.

Magnocellular Neurosecretory System

The magnocellular neurosecretory system is comprised chiefly of the neurons in the hypothalamic paraventricular and supraoptic nuclei and their axon terminals within the neurohypophysis, or neural lobe of the pituitary gland. While both nuclei contribute axons to the neurohypophysis in roughly equal numbers, the supraoptic nuclei represent a more homogeneous population of neurons (Armstrong, et al., 1980; Swanson and Kuypers, 1980) and have been shown to be more responsive during osmotic challenges (Bandaranayake, 1974; Brimble, et al., 1978; Sherman, et al., 1986; Dellmann, et al., 1988; Carter and Murphy, 1989) and collateral axonal sprouting than the paraventricular nuclei (Watt, 1989). For this reason, only the supraoptic nuclei and the neurohypophysis were examined in the following experiments and will be described in detail.

Supraoptic Nuclei

The anatomy of the rat supraoptic nuclei is very simple. The nuclei are located bilaterally just dorsal to the lateral edges of the optic chiasm and can be readily identified with a Nissl stain (Pellegrino, et al., 1979). The nuclei are comprised of a few thousand neurons all of which are morphologically similar

(Raisman, 1973b; Swaab, et al., 1975a; Rhodes, et al., 1981). The magnocellular neurons are ovoid with diameters of 20-35 μm and contain a large nucleolated nucleus, well-developed Golgi apparatus, and abundant rough endoplasmic reticulum (Rechardt, 1969; Morris and Dyball, 1974; Dyball, et al., 1979; Sofroniew and Glasmann, 1981; Dyball and Kemplay, 1982). Supraoptic neurons usually have less than four dendrites that are thick and rarely branch before terminating within the nucleus (Dyball, et al., 1979; Dyball and Kemplay, 1982); frequently these processes contact local blood vessels (Sofroniew and Glasmann, 1981) or project ventrally to the glia limitans (Armstrong, et al., 1982). The unmyelinated axons of the neurons exit the supraoptic nucleus dorsally and then turn toward the midline before running caudally through the internal zone of the median eminence and eventually terminate within the neurohypophysis (Armstrong, et al., 1982). When the dye is placed in the neurohypophysis, retrograde tracers stain nearly all of the supraoptic neurons, indicating that this is the neurons' primary, if not their only, target (Sherlock, et al., 1975; Ju, et al., 1986).

The magnocellular neurons within the supraoptic nuclei are often divided into oxytocinergic and vasopressinergic populations. Although the two types exist in roughly equal numbers (Vandesande and Dierickx, 1975; Sokol, et al., 1976), oxytocinergic neurons predominate in the rostral and dorsal regions while vasopressinergic neurons are more numerous in the caudal and ventral regions (Swaab, et al., 1975a; Swaab, et al., 1975b; Choy and Watkins, 1977; Rhodes, et al., 1981). In addition, a plethora of other neuropeptides and

neuroactive substances have been localized within the oxytocinergic and/or vasopressinergic neurons of the supraoptic nuclei; this topic has been the subject of excellent review papers (Brownstein and Mezey, 1986; Meister, 1993).

Neurohypophysis

The neurohypophysis represents a highly simplified peptidergic terminal field. The thin axons of magnocellular neurons branch profusely throughout the neurohypophysis (Nordmann, 1977) before ending as neurosecretory terminals on the numerous small capillaries present in this organ (Brown, 1925). Ultrastructural analyses show that the axon terminals synapse on the basal laminae of the perivascular spaces, presumably in order to release the contents of their neurosecretory granules into the blood (Palay, 1955; Monroe, 1967; Tian, et al., 1991). Autoradiographic studies have shown that the axons from each supraoptic nucleus spread throughout the entire neurohypophysis but are particularly dense in the center of the organ (Alonso and Assenmacher, 1981). Immunohistochemistry reveals a rostral-caudal gradient of oxytocinergic-to-vasopressinergic fibers with approximately equal proportions innervating the middle regions of the neurohypophysis (Vandesande and Dierickx, 1975).

The two forms of neuroglia within the neurohypophysis, pituicytes and microglia, appear to be actively intertwined with the magnocellular neurosecretory endings. Pituicytes, a form of astrocyte (Salm, et al., 1982), insinuate themselves between the axon terminals and the capillary endothelia

(Palay, 1957). Microglia appear to be constantly engulfing axonal endings, suggesting that the degradation and formation of terminals may be a normal phenomenon within the neurohypophysis (Olivieri-Sangiacomo, 1972; Pow, et al., 1989).

Physiology

The primary role of the magnocellular neurosecretory system is to regulate fluid homeostasis. If one considers this to be a control system like those proposed by Kupfermann (1991), the controlled variable is plasma osmolality and the set point is between 287 (Duncan, et al., 1989) and 321 mmol/kg (Zingg, et al., 1971) for adult male rats. The controlling elements are both behavioral and humoral: drinking provides the body with more fluid and lowers plasma osmolality. Secretion of hormones from the neurohypophysial axon terminals increases the recovery of water from the filtrate within the distal tubules and collecting ducts of the kidneys and thus also lowers plasma osmolality (Fernandez and Cox, 1984; Morgan, 1984). Although this function is most often attributed to vasopressin, it has been shown that oxytocin and vasopressin act synergistically to regulate renal function (Balment, et al., 1986). A diagram of this negative-feedback system is shown in Figure 1.

Electrophysiological and radioimmunoassay experiments have provided data that support the hypothesis that the activity of the magnocellular neurosecretory system is finely tuned to regulate plasma osmolality, as was first proposed nearly 50 years ago (Verney, 1947). The firing rate of both

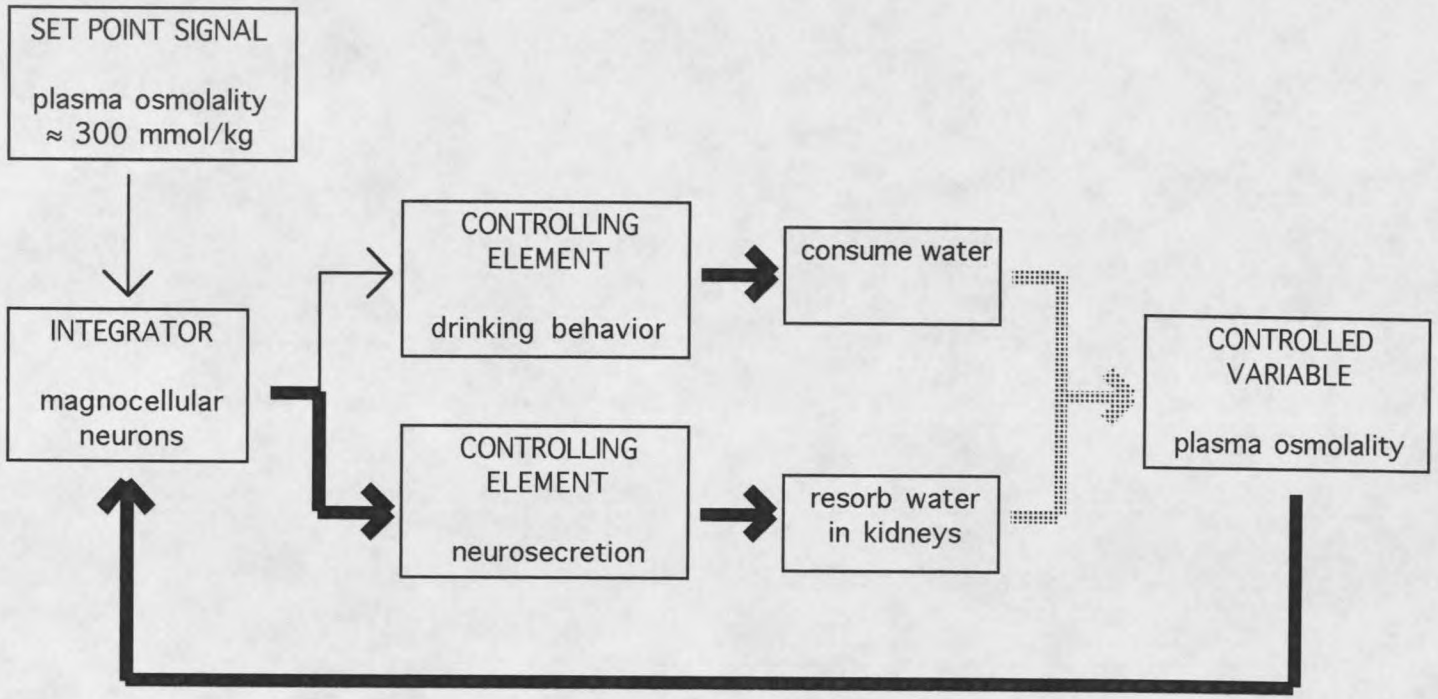

Figure 1. Schematic of a control system which regulates plasma osmolality. Solid lines indicate a positive influence and dotted lines indicate a negative influence. The line thickness indicates the relative strength of the relationship between the two elements.

oxytocinergic and vasopressinergic supraoptic neurons is positively correlated to plasma osmolality *in vivo* (Brimble and Dyball, 1977; Brimble, et al., 1978; Poulain and Wakerley, 1982). *In vitro* analyses using hypothalamic explants have shown that the secretion of vasopressin is responsive to changes in the osmolality of the culture medium as small as one percent throughout the physiological (280-305 mmol/kg) range (Sladek and Knigge, 1977). Furthermore, the plasma concentrations of oxytocin (Balment, et al., 1980) and vasopressin (Dunn, et al., 1973) are directly proportional to plasma osmolality. Thus, activity of the magnocellular neurosecretory system serves to decrease plasma osmolality and maintain fluid homeostasis.

Altering the Activity of Magnocellular Neurons

A major advantage of studying the magnocellular neurosecretory system is that its activity may easily be changed experimentally. Two manipulations depend on the sensitivity of the system to plasma osmolality--hyponatremia and salt-loading significantly decrease and increase neuronal activity, respectively. In addition, unilateral injury to the magnocellular neurosecretory system appears to cause the undamaged neurons to be hyperactive during collateral axonal sprouting. A major goal of this thesis is to determine if hyponatremia can be combined with unilateral lesions to inhibit the activity of neurons that are in the process of sprouting axons.

Salt-Loading

Providing dilute (usually two percent) NaCl as the only source of fluid, known as salt-loading, is a simple and effective procedure that increases plasma osmolality (Jones and Pickering, 1969; Sherman, et al., 1986) and thus the activity of the magnocellular neurosecretory system. Electrophysiological studies have shown that this procedure increases the firing rate of magnocellular neurons (Dyball and Pountney, 1973). Morphological studies, often done at the ultrastructural level, indicate that the supraoptic neurons increase in size (Enestrom and Hamberger, 1968; Morris and Dyball, 1974; Armstrong, et al., 1977; Dyball and Garten, 1988). Within these cells the nuclei and nucleoli increase in size (Bandaranayake, 1974) and the endoplasmic reticulum becomes swollen (Morris and Dyball, 1974). In addition, astrocytic processes in the supraoptic nucleus retract from between neurons and between dendrites, presumably permitting the formation of electrotonic synapses (Tweedle and Hatton, 1984; Perlmutter, et al., 1985).

Salt-loading causes many changes within the neurohypophysis as well as the supraoptic nucleus. The entire neurohypophysis becomes larger (Friesen and Astwood, 1967; Dellmann, et al., 1988), possibly due to proliferation of endothelial cells and pituicytes (Paterson and LeBlond, 1977). Similar to the glial retraction that takes place in the supraoptic nucleus, pituicytes reduce their ensheathment of axon terminals; this is believed to promote diffusion of the hormones into the blood (Tweedle and Hatton, 1987).

The salt-loading procedure clearly indicates the relationship between neuronal activity, neuropeptide gene regulation, and neurosecretion in the magnocellular neurosecretory system. It has been repeatedly shown that salt-loading increases the size of the oxytocin and vasopressin mRNA pools within the supraoptic neurons (Sherman, et al., 1986; Lightman and Young, 1987; Suemaru, et al., 1990). Accordingly, neurosecretion also increases such that plasma concentrations of oxytocin and vasopressin are elevated by salt-loading (Van Tol, et al., 1987). Elegant studies using intron-specific and exon-specific probes for vasopressin RNA and nuclear run-on assays indicate that the regulation of vasopressin gene transcription is extremely responsive to changes in neuronal activity: transcription of the vasopressin gene increases by over 250 percent only 30 minutes after an injection (presumably intraperitoneal) of two molar sodium chloride (Herman, et al., 1991). Thus, all measures indicate that salt-loading increases the activity of the magnocellular neurosecretory system and this procedure could serve as a positive control for the detection of metabolic changes within this system.

Hyponatremia

Chronic hyponatremia is a protocol in which plasma osmolality is depressed through the use of desmopressin, a potent vasopressin receptor (V_2) agonist, and liquid diet (Verbalis and Drutarosky, 1988). Desmopressin promotes water resorption by binding to the receptors in the distal tubules and collecting ducts of the kidneys. Given desmopressin alone, the rat will reduce

its drinking volume in order to match the decreased water loss and maintain normal plasma osmolality. However, when liquid diet represents the only source of calories, the rat must drink in order to meet its nutritional needs. Thus, the rats take in more water than they are able to excrete and plasma osmolality falls below the normal set point.

Chronic hyponatremia significantly inhibits the activity of the magnocellular neurosecretory system. Pronounced decreases in oxytocin and vasopressin mRNA content and synthesis are seen within the magnocellular nuclei of hyponatremic animals (Robinson, et al., 1990). Furthermore, increases in plasma oxytocin and vasopressin cannot be elicited until plasma sodium reaches normal levels (Verbalis, et al., 1986). Although the basal concentrations of oxytocin and vasopressin appear to be unaffected, these very low concentrations (approximately two pg/ml for oxytocin) are approaching the 0.5 pg limit of detection (Ogasa, et al., 1991; Iványi, et al., 1995) and thus make the detection of decreases in the concentration of these hormones extremely difficult.

It is currently believed that hyponatremia causes an active inhibition of the magnocellular neurosecretory system (Verbalis, 1993). It has been demonstrated that the rapid and significant rise in plasma hormone concentrations associated with cholecystokinin injections, hemorrhage, and hypovolemia is attenuated or absent in hyponatremic rats (Verbalis and Dohanics, 1991). Contemporary reports indicate that the inhibitory afferents to the supraoptic nuclei use gamma-amino butyric acid (GABA) and/or

endogenous opioid peptides as their neurotransmitters. GABA has been shown to decrease both the firing rate of supraoptic neurons (Randle, et al., 1986) and the secretion of vasopressin (Iovino, et al., 1983). GABAergic afferents to the supraoptic nuclei have been shown to exist (Theodosis, et al., 1986b) and it has been suggested that they arise from the ventral nucleus medianus (Renaud, et al., 1991)--a region implicated in the osmoregulatory control of thirst (Johnson, 1985). Opiates have been shown to elevate the osmotic threshold for vasopressin secretion (Kamoi and Robertson, 1985) while naloxone, an opiate antagonist, has been shown to potentiate the secretion of oxytocin to a variety of stimuli (Summy-Long, et al., 1986). Furthermore, naloxone can partially reverse the inhibitory effects of hyponatremia (Dohanics and Verbalis, 1992). Thus, although hyponatremia decreases the activity of the magnocellular neurosecretory system, it may increase the activity of inhibitory afferents synapsing within the supraoptic nucleus.

At this time, the morphology of the magnocellular neurosecretory system of hyponatremic animals has not been described. However, the space within the endoplasmic reticulum of supraoptic neurons is decreased following forced hydration (Zambrano and de Robertis, 1966). Furthermore, a lesion that partially deafferents the magnocellular neurosecretory system and causes adipsia has been shown to cause an increase in the glial coverage of neurosecretory terminals (Carithers, et al., 1981). Thus, one may expect that any metabolic and morphological changes that occur during chronic hyponatremia would be the opposite of those described for salt-loaded animals.

Axonal Sprouting

Although the capability of magnocellular neurons to regenerate new neurohemal contacts following axotomy was first described in the 1950s (Billenstien and Leveque, 1955; Moll, 1957), the ability of uninjured magnocellular neurons to undergo compensatory axonal sprouting has only recently been discovered (Watt, 1989; Watt and Paden, 1991) and is currently the primary research topic within Dr. Paden's laboratory. This sprouting response is robust; the total number of axons within the neurohypophysis reach 76 percent of normal 32 days after a unilateral hypothalamic knife cut that separates all of the magnocellular neurons on the ipsilateral side from the neural lobe. During the sprouting response, drinking and urine volumes decrease and urine osmolality increases, suggesting an increase in the plasma concentration of vasopressin. Furthermore, a significant amount of cellular and nuclear hypertrophy occurs within the sprouting supraoptic nuclei by 30 days post-surgery. Thus, substantial evidence suggests that magnocellular neurons are hyperactive during compensatory collateral sprouting.

Indicators of Magnocellular Neuronal Activity

Since neuronal activity is at the heart of this thesis, it was absolutely essential that the functional state of the magnocellular neurosecretory system be measured accurately. To this end, several different variables were selected as indicators of neuronal activity. In general, analyses of plasma osmolality and electrolyte (potassium and sodium) concentrations indicate the ability of the

entire system to regulate fluid homeostasis. Cytochrome oxidase histochemistry acts as a measure of cellular metabolism within the cell bodies of the supraoptic nucleus and the axon terminals of the neurohypophysis. *In situ* hybridization with riboprobes for oxytocin and vasopressin exons and introns gives insights as to the gene expression within the magnocellular neurons. Thus, measurement of all these variables indicates the activity of the magnocellular neurosecretory system at the systemic, cellular, and molecular levels; these measures may be used not only to monitor neuronal activity during axonal sprouting, but also to prove that chronic hyponatremia decreases neuronal activity following unilateral hypothalamic lesions.

Plasma Analyses

In normal animals, plasma osmolality is the principle regulator of neuronal activity within the magnocellular neurosecretory system and it is kept at a set point between 287 (Duncan, et al., 1989) and 321 mmol/kg (Zingg, et al., 1971). Since the primary function of the neurosecretory system is to maintain plasma osmolality at the set point, both the firing rate of (Brimble and Dyball, 1977; Mason, 1980), and secretion by (Dunn, et al., 1973; Balment, et al., 1980) magnocellular neurons is positively correlated with plasma osmolality. This correlation is the basis for the effects of both salt-loading and chronic hyponatremia on the magnocellular neurosecretory system: three days of salt-loading increases plasma osmolality to approximately 345 mmol/kg, while hyponatremia decreases it to between 225 and 242 mmol/kg (Verbalis

and Drutarosky, 1988; Iványi, et al., 1995). Thus, analysis of plasma osmolality may be used not only to check the effectiveness of chronic hyponatremia, but also to find the set point of the osmotic control system in lesioned animals during the sprouting response. However, it is important to emphasize that, since plasma osmolality may be affected by changes in drinking behavior and/or neurosecretion, measurement of this variable does not necessarily indicate the activity of the magnocellular neurosecretory neurons *per se*.

Plasma potassium concentration, unlike plasma osmolality, is not under the jurisdiction of the magnocellular neurosecretory system. The concentration of this electrolyte is regulated by secretion of mineralocorticoids (chiefly aldosterone) from the adrenal cortex (Fernandez and Cox, 1984; Morgan, 1984). Accordingly, the hyponatremia protocol does not alter plasma potassium levels (Verbalis, 1984). Thus, it may be assumed that measurement of this electrolyte serves as a specificity control for the action of lesion and hyponatremia paradigms on the magnocellular neurosecretory system.

Very elegant electrophysiological studies have shown that magnocellular neurons respond to changes in plasma osmolality rather than sodium concentration (Brimble and Dyball, 1977; Brimble, et al., 1978). However, since the concentration of plasma sodium is normally about 140 mM (Verbalis and Drutarosky, 1988; Verbalis, et al., 1989; Iványi, et al., 1995), it accounts for approximately half of the osmolality of plasma and one expects the two measures to be covariant. Indeed, plasma sodium concentration is depressed in hyponatremic (Verbalis and Drutarosky, 1988; Iványi, et al., 1995) and

elevated in salt-loaded animals (Jones and Pickering, 1969) in the same manner as plasma osmolality. Thus, the concentration of plasma sodium may be used as a duplicate measure to check the effectiveness of the hyponatremia protocol and the set point of the osmotic control system in lesioned animals during axonal sprouting.

Cytochrome Oxidase Histochemistry

Cytochrome oxidase histochemistry produces a staining pattern that indicates the recent respiratory metabolism of neurons and their processes (Wong-Riley, 1989; Gonzalez-Lima and Jones, 1994). Correspondingly, ultrastructural studies have shown that the reaction product is essentially limited to mitochondria (Seligman, et al., 1968; Wong-Riley, 1976; Kageyama and Wong-Riley, 1982). Further studies have indicated that the density of the reaction product is indicative of the amount of cytochrome oxidase present within the tissue rather than a modulation of the activity of a constant amount of enzyme (Hevner and Wong-Riley, 1989; Hevner and Wong-Riley, 1990; Chandrasekaran, et al., 1992; Hevner and Wong-Riley, 1993). Although the longevity of cytochrome oxidase within the central nervous system is unknown, the half-life of this enzyme within the liver has been shown to be approximately one week (Ip, et al., 1974). In accordance with this, alterations in cytochrome oxidase histochemical staining within the central nervous system take place over days or weeks, as can be seen in the examples below.

All of the evidence to date suggests that the pumping of ions consumes the majority of neuronal energy (Roland, 1993) and thus the levels of cytochrome oxidase within a cell are proportional to its firing rate (Erecinska and Silver, 1989; Hevner, et al., 1992). In support of this, within the gastric mucosa, the secretory chief cells stain to a lesser degree than the ion-pumping parietal cells (Wong-Riley, et al., 1987). Moreover, during development of Purkinje neurons, somatic staining is most intense on postnatal days three through seven, while excitatory climbing fiber input predominates, but then declines by postnatal day 15 as inhibitory basket cell terminals establish connections (Mjaatvedt and Wong-Riley, 1988). Thus, the stain produced by cytochrome oxidase histochemistry is an indirect measure of neuronal activity due to firing rate.

Cytochrome oxidase histochemistry has been used successfully as a marker of neuronal activity within many areas of the central nervous system. In the auditory system, unilateral deafness for 18 weeks produces a decrease in the reactivity of the ipsilateral cochlear nuclei, contralateral nuclei of the lateral lemniscus, and the monaural portion of the contralateral inferior colliculus (Wong-Riley, et al., 1978). These reductions in staining can be reversed by four weeks of continuous electrical stimulation of the cochlear nerve (Wong-Riley, et al., 1981). Similarly, two weeks following hemilabyrinthectomy a decrease in staining of the vestibular ganglion on the ipsilateral side can be detected (Kevetter and Perachio, 1994). In the visual system, removal of an eye or suturing one eye closed shortly after birth changes the pattern of reactivity

within layer IV of cortical area 17 from a continuous band to alternating columns of light and dark staining suggestive of ocular dominance columns and, more importantly, causes a decrease in the staining of the appropriate layers of the lateral geniculate nuclei once the animals are several months old (Wong-Riley, 1978; Wong-Riley, 1979). Furthermore, unilateral intraocular injections of tetrodotoxin for three days produce the same changes throughout the visual system (Wong-Riley and Carroll, 1984), but the effects of tetrodotoxin are not permanent since normal staining levels return six weeks after cessation of treatment (Wong-Riley and Riley, 1983). In the somatosensory system, a decrease in the reactivity of the corresponding cortical barrels is produced by ten weeks after the removal of whiskers (Wong-Riley and Welt, 1980). In the hippocampus, the dendrites and somata of CA3 pyramidal neurons stain the darkest; it is interesting to note that these neurons receive the majority of the excitatory afferents to this region (Kageyama and Wong-Riley, 1982).

Of particular relevance to this thesis, cytochrome oxidase histochemistry has been used to demonstrate changes in neuronal activity within the magnocellular neurosecretory system. The paraventricular and supraoptic nuclei of Brattleboro rats, which are constantly stimulated because the animals are genetically unable to produce vasopressin, stain more heavily than those of normal rats (Krukoff, et al., 1983). Furthermore, the staining within the hypothalamus of Brattleboro rats is reduced to normal levels when the animals are infused with exogenous vasopressin for one week (Krukoff and Calaresu, 1984). At present, only a single abstract exists that describes cytochrome

oxidase histochemical staining in the neurohypophysis, but no experimental manipulations were done (Kageyama and Wong-Riley, 1987). Consequently, analysis of cytochrome oxidase histochemical staining within the magnocellular neurosecretory system during hyponatremia and compensatory axonal sprouting will provide new insights regarding the activity of these neurons.

In Situ Hybridization

The use of radioactively labeled riboprobes to quantify specific mRNA pools *in vivo* was first described within the magnocellular neurosecretory system (Uhl, et al., 1985). Since that time, this method has been used to show increases in oxytocin and vasopressin mRNA within magnocellular neurons following salt-loading (Sherman, et al., 1986; Lightman and Young, 1987; Van Tol, et al., 1987; Suemaru, et al., 1990). Conversely, hyponatremia decreases the mRNA content for both these hormones within the paraventricular and supraoptic nuclei (Robinson, et al., 1990). Further investigations of this type have indicated that the regulation of transcription and translation within the magnocellular neurons is rapid and specific. For example, intron-specific probes show that transcription of the vasopressin gene is increased within 30 minutes after an intraperitoneal injection of hypertonic saline (Herman, et al., 1991). Furthermore, removal of the neurohypophysis causes a transient decrease followed by an increase in oxytocin mRNA, but a transient increase followed by a decrease in vasopressin mRNA (Villar, et al., 1990), proving that the two types of magnocellular neurons do not always act in parallel. Also,

intracerebroventricular injection of oligonucleotides antisense to vasopressin mRNA causes an increase in drinking behavior and decreases in both urine osmolality and vasopressin immunoreactivity within the supraoptic nuclei after only three hours, suggesting that the translation of the hormone is closely tied to its release (Skutella, et al., 1994). Thus, *in situ* hybridization for oxytocin and vasopressin RNA may be used to study the molecular regulation of these hormones in both types of magnocellular neurons during the process of compensatory axonal sprouting in hyponatremic rats and rats with normal plasma osmolalities.

Justification, Hypotheses, and Specific Objectives

The magnocellular neurons of the neurosecretory system exhibit unusually vigorous regenerative capabilities. These neurons have been repeatedly shown to regenerate axons severed by removal of the neurohypophysis (Billenstien and Leveque, 1955; Moll, 1957; Raisman, 1973a; Kawamoto and Kawashima, 1987). Recently, Dr. Paden's laboratory has demonstrated that the axon terminals of uninjured magnocellular neurons undergo robust collateral axonal sprouting in the neurohypophysis in response to unilateral destruction of the hypothalamo-neurohypophysial tract (Watt, 1989; Watt and Paden, 1991). The long-term objective of Dr. Paden's research is to determine what special characteristics of the magnocellular neurosecretory system provide the basis for this plasticity.

Although it is unlikely that any single mechanism can account for such a complex biological phenomenon as axonal sprouting, neuronal activity appears likely to play a role. *In vitro*, repetitive stimulation of neurons increases the number of filopodial extensions (Manivannan and Terakawa, 1994). *In vivo*, the sprouting of noradrenergic fibers from the superior cervical ganglion into the hippocampus following destruction of the medial septal nuclei is attenuated or eliminated when the preganglionic afferents are severed (Crutcher, et al., 1979). Similarly, preganglionic innervation is essential for the compensatory collateral sprouting of uninjured fibers from the superior cervical ganglion that project to the pineal gland (Dornay, et al., 1986). Within the hypothalamus, sprouting of axons into the external zone of the median eminence by parvocellular neurons of the paraventricular nuclei, which are involved in regulation of the pituitary-adrenal axis, is enhanced when negative feedback of neurosecretory activity is eliminated through adrenalectomy (Silverman and Zimmerman, 1982). Conversely, hyponatremia inhibits the sprouting of oxytocinergic fibers into this area following bilateral loss of the paraventricular nuclei (Dohanics, et al., 1994). During the sprouting response of magnocellular neurons, the cells enlarge, drinking and urine volume decrease, and urine osmolality increases, suggesting that the system has increased its activity (Watt and Paden, 1991; Watt, 1993). Thus, the link between the sprouting of new axon terminals and the activity of the sprouting neurons has been implied in many ways but remains to be tested in a direct manner.

Elucidating the relationship between axonal sprouting and neuronal activity not only is interesting in its own right but also may eventually be applicable to the neuropathology of epilepsy. Epileptic or seizure-induced increases in global brain activity have been correlated with sprouting of mossy fibers within the hippocampus (Ben-Ari and Represa, 1990). Furthermore, synchronous hyperactivity of mossy fibers within the brains of stargazer mice has been implicated in the formation of axonal sprouts within the hippocampi of these animals (Qiao and Noebels, 1993). This phenomenon appears to act as a positive-feedback loop in which excessive neuronal activity promotes the formation of axonal sprouts that, in turn, facilitate the organization of an epileptic focus (Mathern, et al., 1993). Analysis of tissue from epileptic humans supports the hypothesis that axonal sprouting is advanced by neuronal hyperactivity (Mathern, et al., 1995). Thus, there exists a need for a conclusive and direct analysis of the relationship between axonal sprouting and neuronal activity.

The advantage of using the magnocellular neurosecretory system as an experimental model is that its regulatory role in fluid homeostasis and its morphological simplicity make it relatively easy to manipulate neuronal activity and examine the results. Chronic hyponatremia markedly down-regulates magnocellular neuronal activity. Oxytocin and vasopressin mRNA are significantly reduced within the paraventricular and supraoptic nuclei of hyponatremic rats (Robinson, et al., 1990). Also, intraperitoneal injections of saline that normally produce rapid increases in neurosecretion are ineffective in eliciting a response from hyponatremic animals (Verbalis, et al., 1986).

Furthermore, the relatively homogeneous neural composition of the supraoptic nuclei and the neurohypophysis lends itself to measures of metabolic activity such as cytochrome oxidase histochemistry (Krukoff, et al., 1983; Krukoff and Calaresu, 1984). Thus, the magnocellular neurosecretory system is a natural choice as a model system for studies that seek to alter and describe neuronal activity.

The design of this thesis is based on two hypotheses: First, the activity of magnocellular neurons increases during the process of compensatory collateral axonal sprouting in the neurohypophysis. Second, this increase in activity can be eliminated by chronic hyponatremia. Proving these hypotheses through the analysis of several measures of neuronal activity is the goal of this thesis.

The specific objectives of this thesis are all based on the examination of normonatremic and hyponatremic rats with unilateral hypothalamic lesions in comparison to animals from the appropriate control groups. Analyses of plasma osmolality, potassium concentration and sodium concentration will be used to measure the systemic activity of the magnocellular neurosecretory system and determine if the set point has been altered. Histochemical evaluation of cytochrome oxidase within the neurohypophysis and supraoptic nuclei will indicate the metabolic activity of the magnocellular neurons at a cellular level. Measurement of oxytocin and vasopressin RNA levels within the supraoptic nuclei by *in situ* hybridization will elucidate changes in neuronal activity at the molecular level. Achieving these objectives will provide not only novel information regarding the process of compensatory axonal sprouting, but also a

base from which one may design experiments to determine if increased neuronal activity facilitates collateral sprouting whereas decreased activity inhibits it.

CHAPTER 2

METHODS

Animals and Experimental Groups

Male Holtzman rats, bred at the Montana State University Animal Resource Center, were used for all studies. All animals were caged individually within the same room with a cycle of 12 hours dark/12 hours light and were between 33 and 63 days of age during the experiments. Shortly after birth, each animal was assigned a code number and, in order to prevent bias, was not decoded until after completion of the analyses.

Since the investigations completed in this thesis required the combination of two different animal protocols, unilateral hypothalamic lesions and hyponatremia, several different experimental groups were necessary to form a complete and balanced study. The intact group consisted of rats given *ad libitum* access to dry chow (PMI Feeds #5001) and tap water in order to establish normal values. Rats in the lesioned group were fed in the same manner as intact animals, but underwent stereotaxic surgery (described below) to unilaterally destroy the hypothalamo-neurohypophysial tract at 35 days of age--this procedure was developed by Dr. Paden's laboratory and has been shown to cause robust collateral axonal sprouting within the neurohypophysis by the contralateral undamaged magnocellular neurons (Watt, 1989; Watt and Paden, 1991; Watt, 1993). The sham group consisted of rats that were identical

to the lesioned animals except that the surgery was designed to damage cortical structures while leaving the magnocellular neurosecretory system unscathed. Thus, the sham group served as a control for the possible effects of anesthesia, hemorrhage, and non-hypothalamic trauma. The lesioned + hyponatremic group was composed of rats in which the sprout-producing surgery was combined with a protocol designed to chronically depress plasma osmolality (detailed below). These lesioned + hyponatremic animals were used to investigate the possibility of reducing the activity of the magnocellular neurosecretory system throughout the process of axonal sprouting. The rats in the sham + hyponatremic group underwent both the sham surgery and the chronic hyponatremia protocol. Thus, they served as controls for the effects of hyponatremia in animals with intact magnocellular neurosecretory systems. Furthermore, since the hyponatremia protocol required that the dry chow be replaced with a liquid diet, a lesioned + diet group was necessary. These rats underwent unilateral hypothalamic surgery and were given access to the same diet as animals in the hyponatremic groups, but had normal plasma osmolalities. Thus, the lesioned + diet group served as a control for any nutritional variation between dry chow and liquid diet.

Rats within each of the six experimental groups were analyzed at both 42 and 63 days of age. These two time points corresponded to one and four weeks post-surgery: Previous studies of the neurohypophysis have shown that, at one week post-surgery, degeneration has caused the number of axon terminals in the neurohypophysis to be depressed to approximately 61 percent

of normal and, at four weeks, sprouting has produced a significant recovery of terminals (Watt, 1989; Watt and Paden, 1991). Thus, the combination of the six experimental groups and two time points was designed to fully examine, in a carefully controlled manner, the effects of decreased neuronal activity within the magnocellular neurosecretory system throughout both the degenerative and sprouting phases following unilateral hypothalamic insult.

As a final note, it should be known that each group/time point was made of rats obtained from at least two different litters. This was done to ensure that the effects observed were truly representative of the experimental manipulations rather than "litter effect" variables such as age of mother or litter size.

Stereotaxic Surgery

All brain surgeries were done when the rats were 35 days of age in a manner nearly identical to that which was developed within Dr. Paden's laboratory (Watt, 1989). Each animal was given a subcutaneous injection of atropine sulfate (0.05 mg/kg mass; Anthony Products) to reduce mucous secretion within the respiratory tract and then an intraperitoneal injection of sodium pentobarbital (45 mg/kg mass; Sigma) to induce anesthesia. Once each rat was unconscious, its head was shaved, washed with surgical scrub and 100% ethanol, and placed in the stereotaxic apparatus (David Kopf Instruments). A two cm incision was made along the dorsal surface of the cranium just to the right of the midline, the skin was retracted, and any loose

connective tissue was pushed aside. The tip of the stereotaxic wire knife was placed at bregma and then the lesion coordinates were calculated (AP -3 to +5 mm and ML -0.08 mm) so that marks could be made on the skull. A slot was made through the cranium with a #3 carbide burr (Roboz) driven by a flexible drill (Dremel) and the underlying dura was punctured and sectioned with a 0.4 mm x 12 mm (27 gauge; Monoject/Sherwood) needle. For animals in any of the lesioned groups, the wire knife was lowered at the caudal edge of the slot until it could be seen to flex slightly as it contacted the skull, partially retracted (approximately 0.5 mm), driven rostrally until it contacted the anterior edge of the slot, and then removed; for animals in either of the sham groups, the blade was lowered to DV -5 mm. During this time, any blood that might interfere with visualization of the wire knife was drawn away with a Kimwipe (Kimberly-Clark). A small piece of Gelfoam (Upjohn) was placed in the slot, the skin was closed with sutures (Davis & Geck #3-0 Dexon S), and nitrofurazone powder (Fermenta) was applied to the site to reduce the risk of infection. For those animals that required supplemental anesthesia after the sodium pentobarbital injection, vaporized methoxyflurane (Pitman-Moore "Metofane") was used. Please note that all surgical instruments were heat sterilized prior to use and that the surgeon wore gloves, hat, and mask throughout the procedure.

Hyponatremia

The chronic hyponatremia protocol developed by Verbalis' laboratory (Verbalis and Drutarosky, 1988) was used to establish plasma hypoosmolality

in lesioned and sham animals. Beginning at 33 days of age, the rats were given 45 ml of concentrated liquid diet instead of solid chow. This diet was replaced daily in a clean 50 ml feeding tube (Bio-Serv #9019) and contained 140 g dextrose (Sigma) and 496 g diet (Bio-Serv #F1268SP) per liter of water. Immediately following stereotaxic surgery at 35 days of age, a one cm incision was made in the skin of the rats near the scapulae, an osmotic pump (Alzet #2002) was implanted subcutaneously, and the incision was sutured closed. Each pump contained 4 ng/μl desmopressin acetate (a vasopressin analogue; Rhône-Poulenc Rorer)--this concentration was made by diluting the commercially available 0.01% nasal spray in 0.15 M NaCl (Sigma) that had been autoclaved. To ensure immediate delivery of the desmopressin after implantation, each pump had been loaded and soaked in sterile 0.15 M NaCl at 37° C for at least four hours before use. When the rats were 36 days of age, their water bottles were removed. Since the life of each osmotic pump was 14 days, rats in the four-week groups had their pumps replaced at 49 days of age. This was done in exactly the same manner as described above at the same time that blood was drawn to measure plasma osmolality (see below).

Measurement of Body Mass and Diet Eaten

Each rat was weighed daily using a one-armed balance (Ohaus "dial-o-gram"). Furthermore, the approximate volume of liquid diet eaten by the hyponatremic and lesioned + diet animals was monitored daily. Due to the high viscosity of the liquid diet and a lack of precision in the calibrations on the

feeding tubes, the volume of diet eaten was rounded to the nearest multiple of five ml.

Plasma Collection and Analyses

Plasma was collected from the rats in two ways. At time points other than the time of death, each animal was anesthetized with methoxyflurane and blood (less than one ml) was drawn from the tail vein with a 0.7 mm x 40 mm (22 gauge; Becton Dickinson) needle and a 5 cc syringe (Becton Dickinson). The blood was then transferred to a 600 μl microtainer containing lithium heparin and a plasma separator (Becton Dickinson #5969). The microtainer was then centrifuged at 13,000 rpm (approximately 13,000 x G) in a microcentrifuge (HBI) for two minutes to isolate plasma. These samples were stored at four degrees centigrade and were always analyzed less than 48 hours from the time of collection. At the time of death, the animals were anesthetized with methoxyflurane and decapitated. Trunk blood (approximately four ml) was collected with a polystyrene funnel (VWR) and a Vacutainer containing lithium heparin and a plasma separator (Becton Dickinson #6698). The Vacutainer was kept in a bath of ice water until it was centrifuged at 1,200 x G for ten minutes at 4° C in a bench centrifuge (MSE "Mistral 3000i") to isolate plasma. The plasma was then transferred with a Pasteur pipet (Fisher) to two 1.7 ml polypropylene microcentrifuge tubes (VWR): one sample was stored at 4° C for less than 48 hours prior to analysis while the other sample was immediately frozen (-80° C) for long-term storage.

The osmolality of each plasma sample was determined with a vapor-pressure osmometer (Wescor #5500) that had been standardized using standards ranging from 100 to 1000 mmol/kg. Due to a small amount of variability in the precision of the instrument (approximately 15 mmol/kg), each experimental sample was measured in triplicate and the mean of these measurements was used as the data point.

Electrolyte (potassium and sodium) concentrations were determined with a Dimension-AR (Dupont) plasma analyzer. This instrument was operated by Rhonda Craver at the Montana Department of Livestock Diagnostic Laboratory.

Tissue Preparation

At either one or four weeks post-surgery (42 or 63 days of age), each rat was briefly anesthetized with methoxyflurane and decapitated with a guillotine. Immediately after the trunk blood was collected, the brain was removed from the skull, blocked with a single-edged razor blade to remove structures anterior to the optic chiasm and posterior to the mammillary bodies, placed on a small square of aluminum foil that was resting on a block of dry ice, and then covered with a small amount of powdered dry ice. As the brain was freezing, the pituitary was also carefully removed from the skull, placed on a pre-cooled square of aluminum foil, and covered with dry ice. Following approximately one minute of freezing, the brain and pituitary were wrapped in the aluminum foil squares and stored in a plastic container at -80° C; in order to lessen the possibility of tissue dehydration, a small amount of water had previously been

frozen within each plastic container.

Brains were cryosectioned at -25° C with a Reichert-Jung Frigocut N equipped with a recently honed blade. A series of sections was taken repeatedly throughout the region of the supraoptic nuclei: 20 x 10 μm, 1 x 40 μm, and 1 x 20 μm. The 10 μm sections were used for *in situ* hybridization, the 40 μm sections for cytochrome oxidase histochemistry, and the 20 μm sections for cresyl violet staining. All sections were thaw-mounted onto precoated glass slides (Fisher "Superfrost/Plus") and then immediately refrozen in the walk-in freezer at -20° C. The majority, if not the entirety, of the supraoptic nuclei was always contained within four series (1,040 μm) of sections.

Pituitaries were also cryosectioned at -25° C. Serial 40 μm sections were thaw-mounted onto precoated glass slides and then immediately refrozen in the cryostat at -25° C. Alternate sections were used for cytochrome oxidase histochemistry and cresyl violet staining. The majority, if not the entirety, of each neurohypophysis was always contained within 30 sections (a total distance of 1,200 μm).

Cytochrome Oxidase Histochemistry

Brain and pituitary sections were histochemically stained for cytochrome oxidase activity using a method slightly modified from previous reports (Seligman, et al., 1968; Wong-Riley, 1976; Adams, 1981). Slides were air-dried in the draft created by a fume hood for 15 minutes at room temperature in order to promote tissue adherence and to warm the sections. Sections were then

immersed in a freshly-made reaction mixture containing 200 mg 3,3'-diaminobenzidine (Sigma; previously stored as a frozen solution, see Pelliniemi, et al., 1980), 50 grams sucrose (Sigma), five mg cobalt chloride (Baker), five mg nickelous ammonium sulfate (Baker) and 100 mg cytochrome c (Sigma #C-3006) per liter of Trizma buffer (0.05 M, pH 7.4 at 37° C) for 30 minutes at 37° C. The reaction was quenched by rinsing the sections in Trizma buffer (0.05 M, pH 7.4; two x one minute) at room temperature and then the sections were fixed by immersion in a solution of four percent Formalin (Sigma) in Trizma buffer (0.05 M, pH 7.4) for ten minutes at 4° C. The sections were then washed in Trizma buffer (0.05 M, pH 7.4; three x five minutes), dehydrated with a series of ethanols (70% ethanol, five minutes; 95% ethanol, ten minutes; 100% ethanol, two x ten minutes) and cleared with xylenes (two x ten minutes) before being coverslipped with Permount (Fisher). In order to minimize any effect of inter-run variability on final group means, each staining run contained all of the sections from animals in at least two different experimental groups.

Cresyl Violet Staining

Brain and pituitary sections were stained with cresyl violet using a procedure obtained from Matt Hirschfeld (personal communication). Sections were immersed for fifteen minutes in a fixative of four percent Formalin in Trizma buffer (0.05 M, pH 7.4) at 4° C and then washed in Trizma buffer (0.05 M, pH 7.4; three x five minutes) at room temperature. Sections were stained for five minutes in a solution of one gram cresyl violet acetate (Aldrich), 250 ml 100%

ethanol, one gram sodium acetate (Sigma), and one ml glacial acetic acid (EM Science) per liter of water. The sections were rinsed with water (two x one minute), dehydrated with a series of ethanols (50% ethanol, two x three minutes; 70% ethanol, five minutes; 95% ethanol, ten minutes; 100% ethanol, two x ten minutes) and cleared with xylenes (two x ten minutes) before being coverslipped with Permount.

Sections stained with cresyl violet served several purposes. Those from the brains of rats in the lesioned groups were used to verify that the wire knife had passed between the supraoptic nuclei and the third ventricle throughout the anterior hypothalamus. In addition, sections from the brains of all animals were used as aids in the determination of the location of the supraoptic nuclei in adjacent sections stained with cytochrome oxidase histochemistry. Also, pituitary sections were used in a general fashion to examine the integrity and location of the neurohypophyses; this was particularly helpful when examining pituitaries from hyponatremic animals.

Computerized Image Analysis

The degree to which cytochrome oxidase histochemistry stained neurohypophysial sections was quantified through the use of computerized image analysis. Images were captured with a high-resolution CCD camera (Sierra Scientific) and a MCID/M4 microcomputer imaging device (Imaging Research). The configuration of the microscope (Nikon "Optiphot-2") included filters (GIF, ND4 and ND32), a nearly closed diaphragm (second mark from the

right), a condenser (Nikon "Achr 0.15") set to 0.1, and a 4x objective (Nikon "Plan 4/0.13"). In order to properly focus the image for the camera, the stage was elevated approximately two mm above the normal setting. Also, to prevent fluctuations in illumination, the microscope was powered through a constant voltage transformer (Sola Electric).

Preliminary tests showed that the following process of data collection was the most sensitive in detecting subtle changes in stain density. Prior to analysis of any sections, a shading error was established to compensate for slight differences in the illumination across the field of view. In order to compensate for possible differences in coverslip/slide transmittance, a blank portion of each slide was analyzed and minor adjustments to the light control were made until the density was between 130.00 and 130.99 gray levels. Thus, the blank portion of each slide was in the middle of the range between absolute black (zero gray levels) and absolute white (256 gray levels).

For each animal, the neurohypophysis was analyzed in at least nine pituitary sections and five measures were taken: the average density for the entire target (gray level), the area of the target (pixel), the number of small intensely stained cells within the target, the absence or presence of a stain gradient within the neurohypophysis, and the density histogram of the target (pixels at each gray level). These measures were then used to calculate several data points, described in detail below.

The average cross-sectional area of each neurohypophysial section was calculated for each animal in order to give a rough approximation of the size of

the entire organ. Shrinkage of the neurohypophysis following lesion surgery, followed by a gradual recovery in size had previously been described (Watt, 1989; Watt and Paden, 1991) and could possibly affect the optical density of the neurohypophysis following cytochrome oxidase histochemistry.

The areal density of round intensely stained cells within the neurohypophysis of each animal was calculated. This was done because not only had preliminary observations suggested that there was considerable variation between animals, but also glial activation was known to occur within the neurohypophysis following injury to the central nervous system (Moffett and Paden, 1993).

Preliminary observations indicated that cytochrome oxidase histochemical staining produced rostral > caudal and ventral > dorsal gradients within the neurohypophysis of each animal. The first gradient was examined by comparing the density of the two most rostral and the two most caudal sections from each animal. A gradient was said to be present when the average of the rostral sections was darker than the average of the caudal sections. The second gradient was examined by visually assessing the absence or presence of a ventral > dorsal gradient in every section. For each animal, the proportion of sections with a ventral > dorsal gradient was then calculated. These gradients could then be compared to an earlier description of the arrangement of oxytocinergic and vasopressinergic axon terminals within the neurohypophysis (Vandesande and Dierickx, 1975).

The proportional area of each neurohypophysis that was darker than or equal to 78 gray levels (less than or equal to 78 gray levels) was used as the measure of cytochrome oxidase histochemical staining. This was done by creating a pooled histogram (pixels at each gray level) from the pituitary sections of each animal. The cutoff at 78 gray levels was chosen because it was found to be the most sensitive in detecting subtle changes in the density of the neurohypophyses between the different experimental groups. Proportional, rather than absolute areas were chosen because the size of the neurohypophysis changes following the lesion surgery and subsequent axonal sprouting (Watt, 1989; Watt and Paden, 1991). Thus, the proportional area less than or equal to 78 gray levels measure should give an indication of the metabolic activity within the neurohypophysis of each animal.

In Situ Hybridization

While animals were prepared and brain tissues were sectioned at Montana State University--Bozeman, the hybridization of riboprobes to brain sections, development of autoradiographic films, and collection of data from these films was carried out in the laboratory of Dr. J. P. Herman at the University of Kentucky. Accordingly, this section of the thesis has been transcribed from personal communications with and previous manuscripts (Herman, et al., 1991) from that laboratory and should not be interpreted as the author's firsthand knowledge.

^{35}S-labeled RNA probes complementary to oxytocin exon C or vasopressin exon C were produced in the following manner. First, cDNA constructs were subcloned into plasmid systems with RNA polymerase promoter regions. Second, restriction enzymes were used to linearize the plasmids and labeled probes were created by reacting the plasmids with ^{35}S-labelled nucleotides and RNA polymerase. The probes were then separated from the free nucleotides in the reaction mixture with a Sephadex G50-50 column. In previous manuscripts, both the oxytocin probe (Sherman, et al., 1988) and the vasopressin probe (Herman, et al., 1991) were fully characterized.

Two to four brain sections per animal were prepared for hybridization through an elaborate series of active steps and passive washes. The active steps included fixation with paraformaldehyde, permeabilization with proteinase K, acetylation with acetic anhydride, and dehydration with graded ethanols. The passive washes that occurred between all of the active steps were done with sodium citrate buffer. All solutions were made with water that had been sterile filtered and autoclaved prior to use.

Once the brain sections had been prepared, the appropriate probe was diluted in a standard hybridization buffer (containing formamide, dextran sulfate, sodium citrate, and yeast tRNA) so that the activity was 1,000,000 to 2,000,000 dpm per 30 μl. Each slide was then treated with a 30 μl portion of the hybridization buffer, coverslipped, and placed in a hydrated chamber for approximately 24 hours. After this time, the coverslips were removed, any

unbound probe was digested with RNAse A, and the slides were washed before applying the slides to radiographic film. Controls consisted of either pretreatment of the slides with RNAse A or replacement of the antisense probe with sense probe within the hybridization buffer.

The NIH Image program was used to quantify the film autoradiographs made from each slide. In the analysis, the supraoptic nuclei were targeted and the integrated gray level (the average gray level multiplied by the number of pixels in the target) was calculated. This measure was chosen because it more accurately gauges mRNA pools in neurons of various volumes and thus would take into account the cellular hypertrophy known to occur during collateral axonal sprouting (Watt, 1989; Watt and Paden, 1991; Watt, 1993; Paden, et al., 1995). Prior to graphical representation and statistical analysis, the data were transformed into a percentage of the average intact value.

Statistical Analyses

Two computer programs were used to make statistical comparisons. MSUSTAT version 5.25 (developed by Dr. R.E. Lund at Montana State University) was used to run one-way and two-way analyses of variance (ANOVA) on several data sets. Data sets were entered using the ENDATA program with experimental group (G) and post-surgical week (W) as indexing variables, and then the GLMODEL program was used to make multiple comparisons. The GLMODEL program was selected because it is an ANOVA model capable of analyzing blocks of unequal size. In both one-way and two-

way analyses, the sum-of-squares type was set at 3, and in two-way analyses, the factorial structure was set at GIW. When contrasts across G and/or W produced statistically significant F values, least significant difference (LSD) multiple comparisons (based on the Student's t test; $p < 0.05$) were made. It should be noted that the LSD tests collapsed the data such that the comparisons were made between groups pooled across time or time points pooled across groups. Appendices A through J contain raw data tables and statistical analyses produced by MSUSTAT.

INSTAT version 2.01 (Graphpad Software; generously provided by Dr. D. E. Phillips) was used to compare selected sets of *in situ* hybridization data. This program was selected because it is capable of running unpaired, two-tailed Student's t tests on the data where only the sample size, mean, and standard error of the mean are known. Data from the following experimental groups were compared: intact versus lesioned, lesioned versus lesioned + hyponatremic, and intact versus lesioned + hyponatremic. Appendices K through N contain raw data tables and statistical analyses produced by INSTAT.

CHAPTER 3

RESULTS

Body Mass

Each rat was weighed daily throughout the experimental period, and the average body mass of animals within each animal group was calculated. While the average body mass of each group was essentially the same (approximately 175 grams) at 35 days of age, there were three patterns of weight gain during the next four weeks. Experimental groups given solid rat chow (intact, lesioned, and sham) gained weight in a steady manner and averaged at least 380 grams by the fourth week. In comparison, the lesioned + diet group gained weight at a slightly reduced rate and averaged a little over 300 grams by the fourth post-surgical week. The hyponatremic groups (lesioned and sham) gained weight, but at a much slower pace than the groups fed solid chow. The body mass of animals in the hyponatremic groups averaged 240 grams four weeks after surgery. No statistical comparisons were done, but the average body mass of the animal groups is plotted across time in Figure 2.

The body mass data are the first indication that the chronic hyponatremia protocol may have more profound effects than the lesion surgery. Indeed, as will be seen repeatedly throughout the results, the values from the lesioned + hyponatremic and sham + hyponatremic groups were nearly identical. Thus, one should consider the possibility that the effects produced by hyponatremia