MONTANA STATE UNIVERSITY

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
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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 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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Christopher Wade Moffett

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This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

Jan 23, 1996
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Chairperson, Graduate Committee

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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

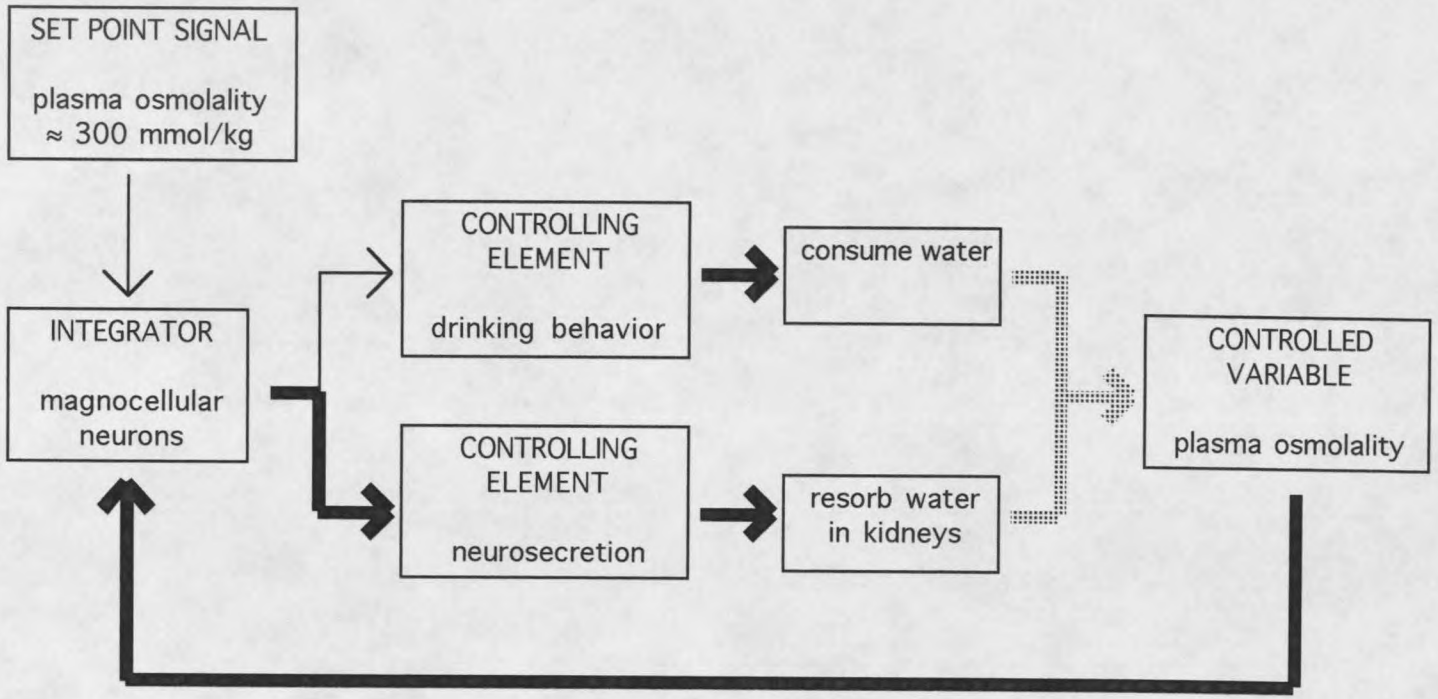


Figure 1. Schematic of a control system which regulates plasma osmolality. Solid lines indicate a positive influence and dotted lines indicate a negative influence. The line thickness indicates the relative strength of the relationship between the two elements.

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

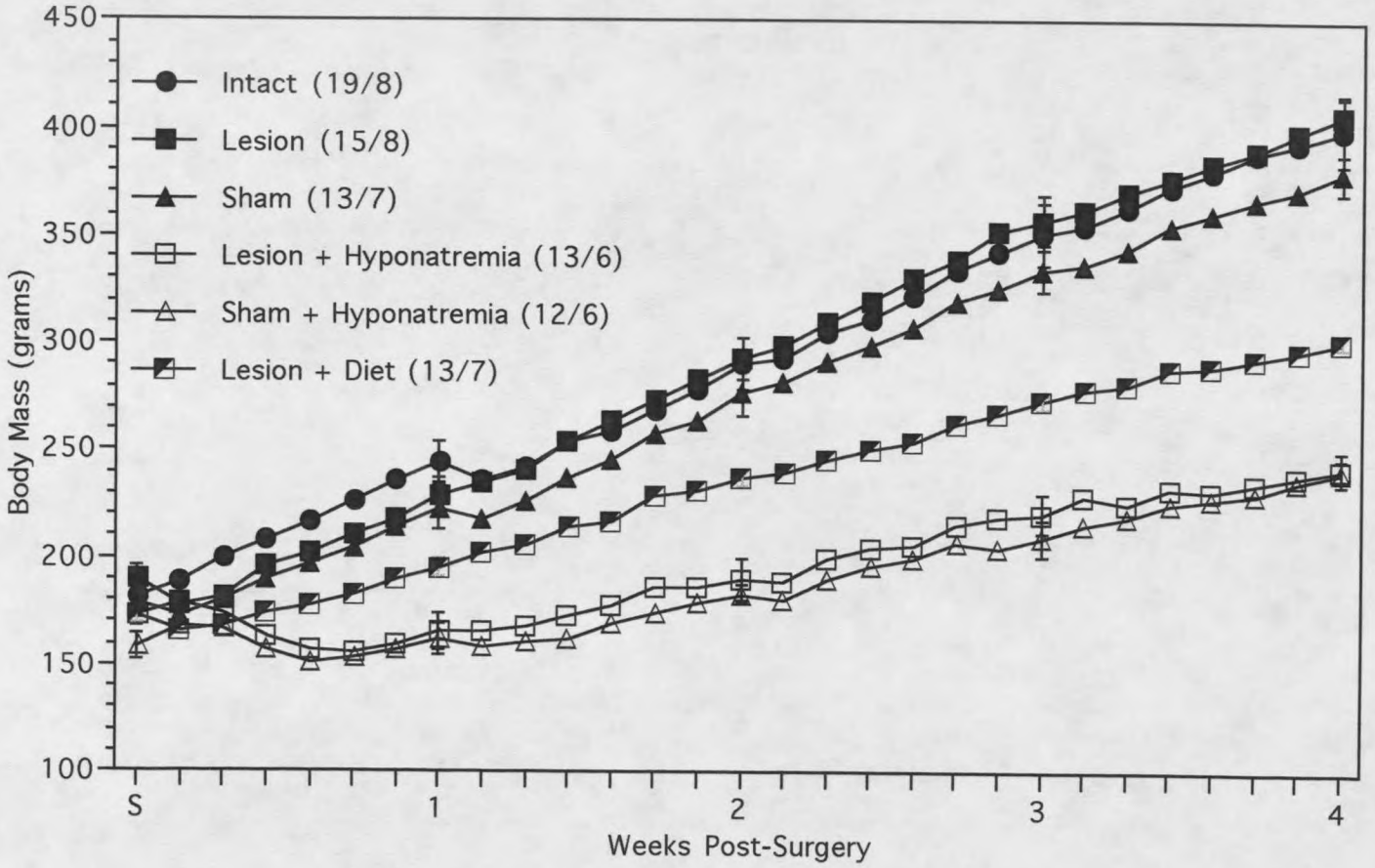


Figure 2. The average body mass of the rats in each experimental group from the time of surgery (S) through four weeks post-surgery. The numbers in parentheses indicate n at times less than or equal to one week/greater than one week. The error bars, calculated at weekly intervals, represent the standard error of the mean.

mask any possible effects produced by unilateral destruction of the hypothalamo-neurohypophysial tract.

Drinking Behavior

The volume of liquid diet drunk by each rat was measured daily, and the average volume consumed by animals in each experimental group was calculated. Animals in the lesioned + diet group consistently drank 35 to 40 ml of diet each day. In contrast, animals in the hyponatremic groups (lesioned and sham) drank very little diet during the first week following surgery and then progressively drank more throughout the second week. After the second post-surgical week, the hyponatremic groups consumed nearly the same amount of diet as the lesioned + diet group. No statistical comparisons were made, but the average amount of diet consumed by animals within each group is plotted across time in Figure 3.

Plasma Analyses

Plasma Osmolality

Blood samples were drawn at weekly intervals from all rats, and the average plasma osmolality was determined for each experimental group. The average plasma osmolalities for the intact, lesioned, sham, and lesioned + diet groups were always between 295 and 303 mmol/kg, while the hyponatremic (lesioned and sham) groups were depressed to between 222 and 242 mmol/kg. Furthermore, the average plasma osmolality of most experimental groups was

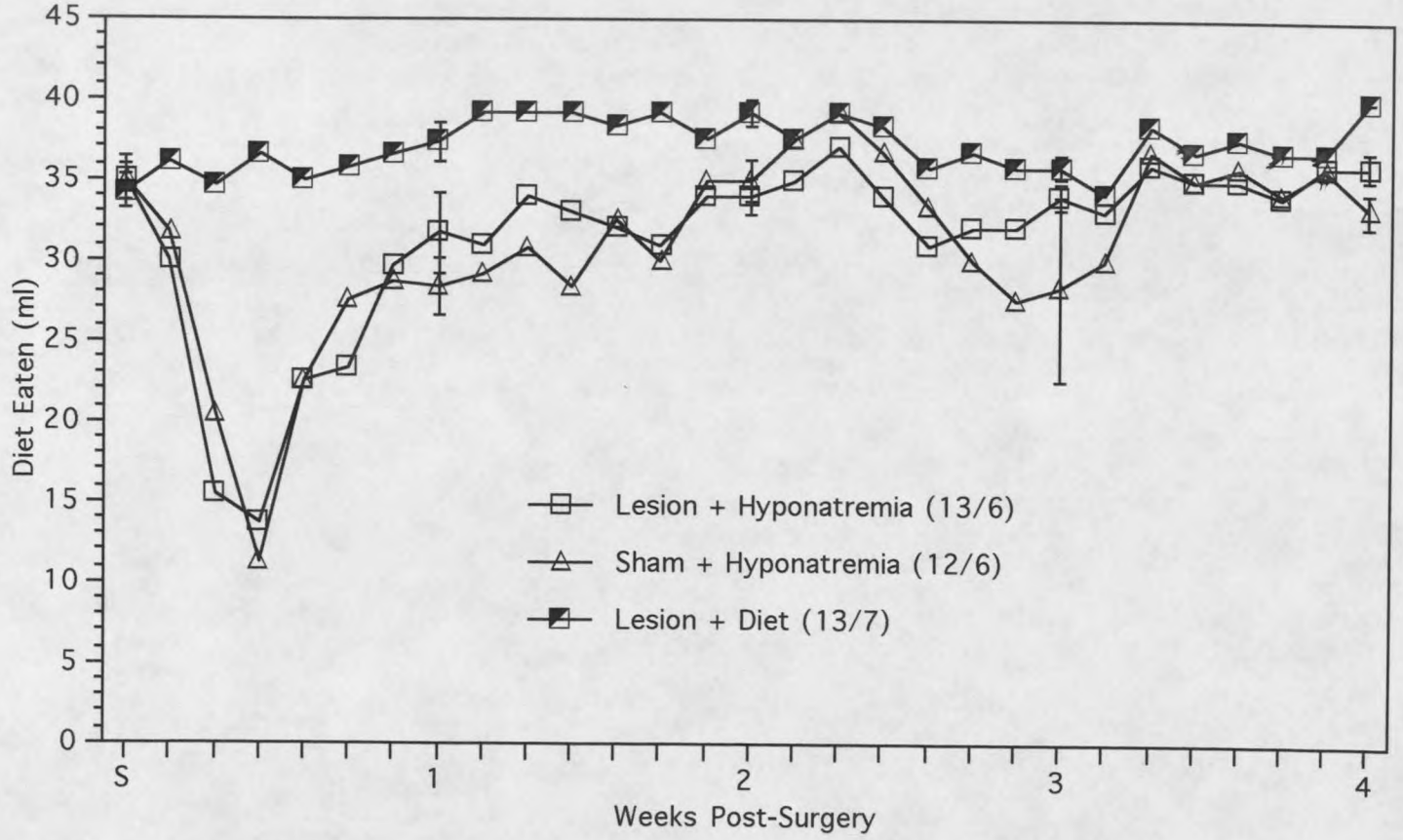


Figure 3. The average volume of diet eaten by the rats in each experimental group given liquid diet from the time of surgery (S) through four weeks post-surgery. The numbers in parentheses indicate n at times less than or equal to one week/greater than one week. The error bars, calculated at weekly intervals, represent the standard error of the mean.

slightly higher at four weeks post-surgery than at the earlier time points. Two-way ANOVA indicated significant changes with respect to experimental group ($df = 5$; $F = 617.15$; $p < 0.0001$) and time ($df = 3$; $F = 6.53$; $p = 0.0004$). LSD comparisons ($p < 0.05$) of plasma osmolalities revealed that the hyponatremic (lesioned and sham) groups were significantly lower than the other groups, and that the fourth post-surgical week was significantly higher than the other time points--see Appendices A and B. The average plasma osmolalities for each animal group are plotted across time in Figure 4.

Plasma Potassium Concentration

The concentration of plasma potassium was determined from the trunk blood of at least four rats within each experimental group at times corresponding to post-surgical weeks one and four. Regardless of the experimental group or time point examined, the concentration of plasma potassium was approximately five to six mM. Thus, plasma potassium concentration was not significantly altered by any of the experimental manipulations, nor did it change across time (data not shown).

Plasma Sodium Concentration

Plasma sodium concentrations were determined from the trunk blood of at least four rats from each experimental group at times corresponding to post-surgical weeks one and four. While the average concentration of plasma sodium was between 136 and 140 mM in the intact, lesioned, sham, and lesioned + diet groups, it was depressed to between 100 and 107 mM in the

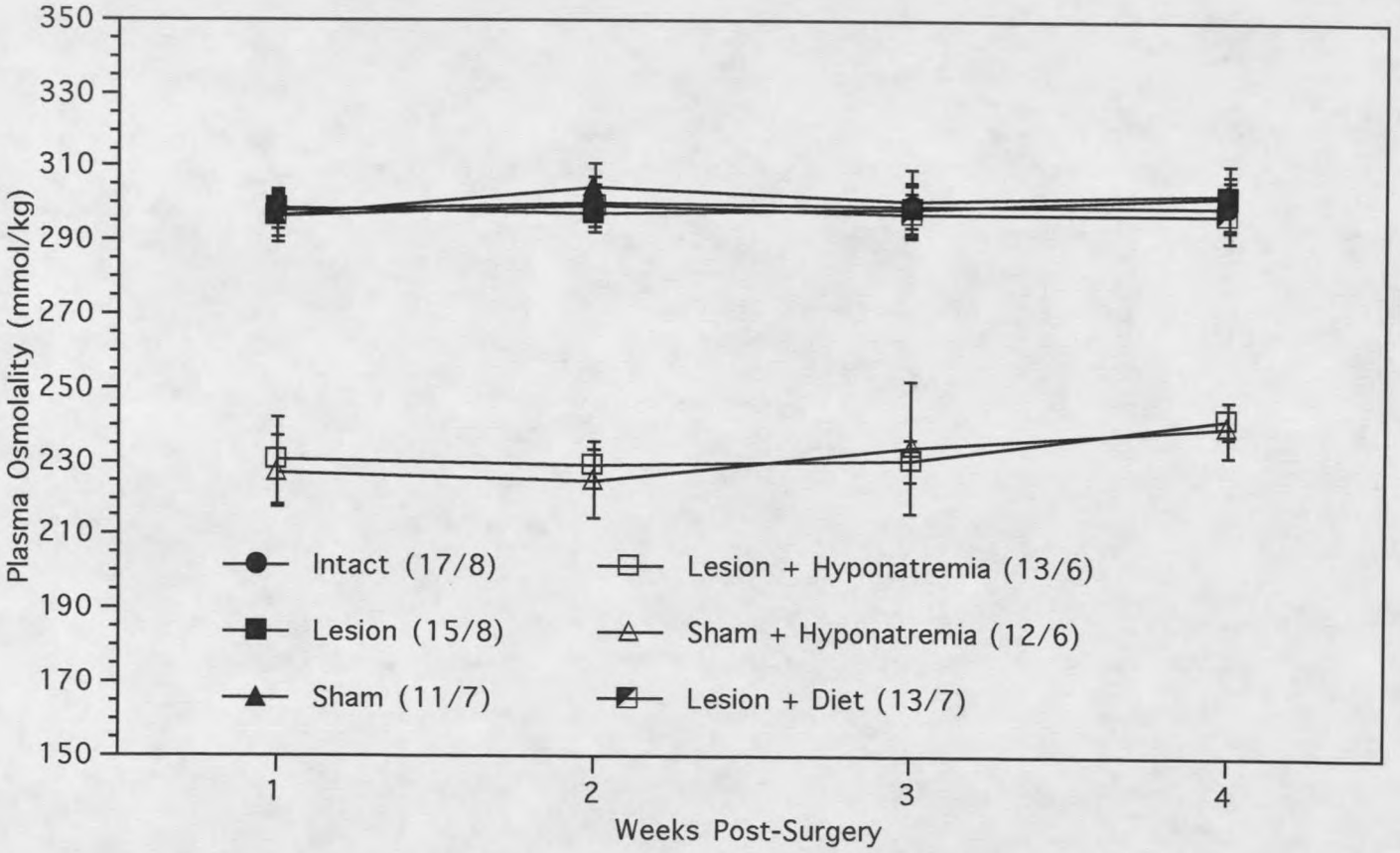


Figure 4. The average plasma osmolality of the rats in each experimental group from one through four weeks post-surgery. Two-way ANOVA indicates statistically significant changes take place with respect to group ($df = 5$; $F = 617.15$; $p < 0.0001$) and time ($df = 3$; $F = 6.35$; $p = 0.0004$). LSD comparisons ($p < 0.05$) show that the plasma osmolalities of the hyponatremic groups are below those of all the other groups, and that the overall values at four weeks post-surgery are higher than those at the earlier times. The numbers in parentheses indicate n at times less than or equal to one week/greater than one week. The error bars represent the standard deviation.

hyponatremic (lesioned and sham) animals. Furthermore, plasma sodium concentrations were slightly higher from animals sampled at post-surgical week four than those sampled at week one. Two-way ANOVA indicated significant changes with respect to experimental group ($df = 5$; $F = 661.07$; $p < 0.0001$) and time ($df = 1$; $F = 16.10$; $p < 0.0001$). LSD comparisons ($p < 0.05$) of plasma sodium concentrations revealed that the hyponatremic (lesioned and sham) groups were significantly lower than the other groups, and that the overall values were significantly higher at four weeks post-surgery than at one week post-surgery--see Appendices D and E. The average plasma sodium concentrations for each experimental group are graphed in Figure 5.

Cytochrome Oxidase Histochemistry

Supraoptic Nucleus

In general, brain sections were faintly stained by cytochrome oxidase histochemistry. While white matter areas were essentially devoid of reaction product, gray matter areas were stained a nearly homogeneous light gray: Striatal regions interspersed with bundles of white matter appeared to be slightly darker than the cortex, thalamus, and hypothalamus, all of which were stained equally. Indeed, it was difficult to discern the exact shape and size of the supraoptic nuclei without referring to an adjacent section that was stained with cresyl violet. Visual analysis of all the brains revealed only three cases in which the clarity and density of staining warranted special mention. First, infrequent short tubular segments were stained much more than the

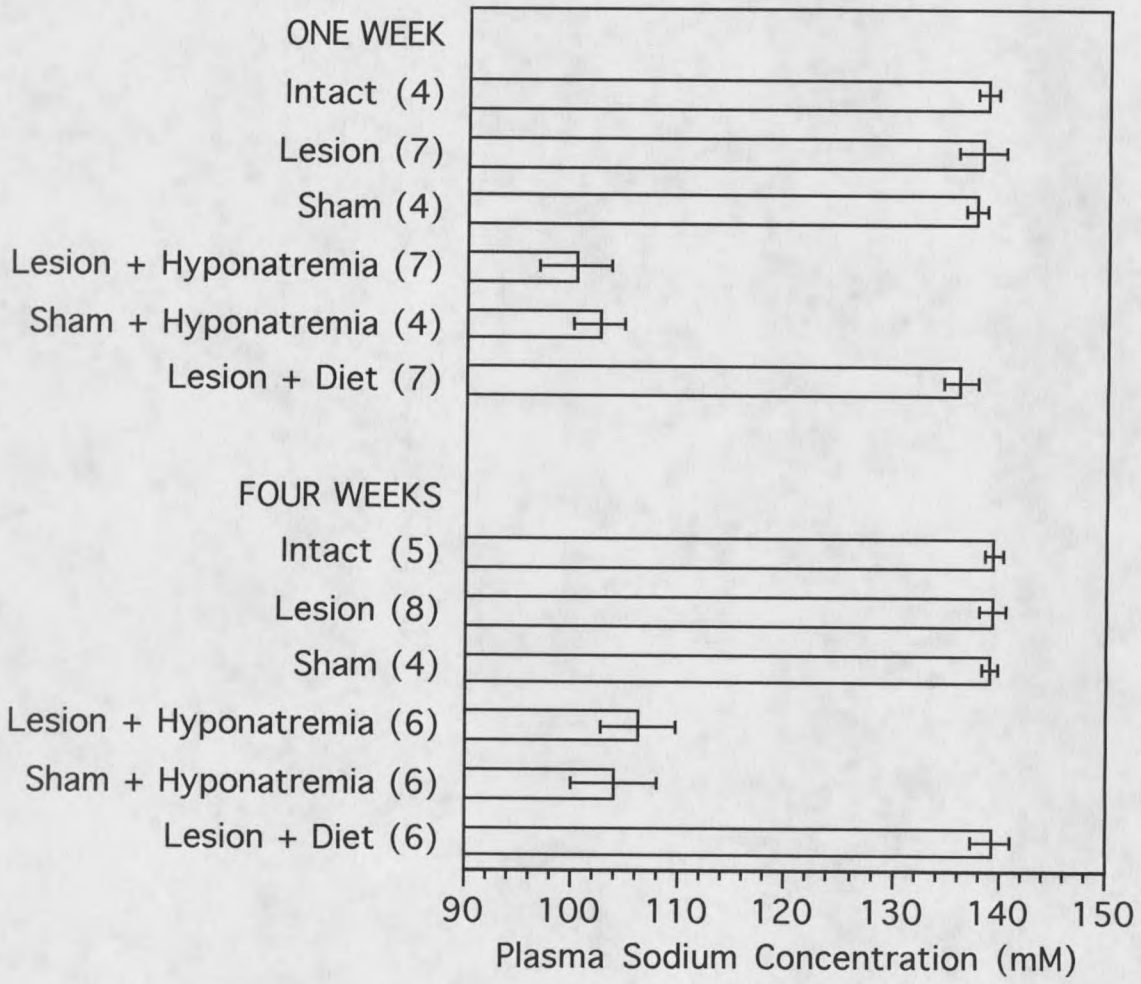


Figure 5. The average plasma sodium concentration of the rats in each experimental group at one and four weeks post-surgery. Two-way ANOVA indicates that statistically significant changes take place with respect to both group ($df = 5$; $F = 661.07$; $p < 0.0001$) and time ($df = 1$; $F = 16.10$; $p < 0.0001$). LSD comparisons ($p < 0.05$) show that the plasma sodium concentrations of the hyponatremic groups are below those of all the other groups, and there is a overall elevation in plasma sodium concentrations between one and four weeks post-surgery. The numbers in parentheses indicate n and the error bars represent the standard deviation.

surrounding neuropil so that they could easily be seen. These segments were not restricted to any particular brain region and were often seen to branch, and thus were assumed to be vascular. Second, brains from animals in all of the lesioned or sham groups that were killed one week following surgery often had a large number of extremely dark cellular profiles within and adjacent to the wound site. Due to the restricted location, uniform size and rounded morphology of these profiles, they were assumed to be phagocytic in nature (macrophages and/or activated microglia). Third, large neuronal profiles were occasionally seen in the axotomized supraoptic nucleus of animals in all of the lesioned groups. Although these neurons were only rarely seen, they were more common in animals killed one week rather than four weeks after surgery and thus were assumed to be magnocellular neurons that had survived axotomy. Most importantly, repeated observations of the supraoptic nuclei on the intact/sprouting side of all the animals indicated that there was very little, if any, change in staining density with respect to experimental group or time, as can be seen in Figures 6 and 7.

Neurohypophysis

Pituitary sections were stained to a greater degree than brain sections by cytochrome oxidase histochemistry. In general, the neurohypophysis (neural lobe) stained the darkest, the intermediate lobe stained the lightest, and the anterior lobe had an intermediate level of staining. As in the brain sections, the cellular morphology of the sections was indistinct. However, short tubular

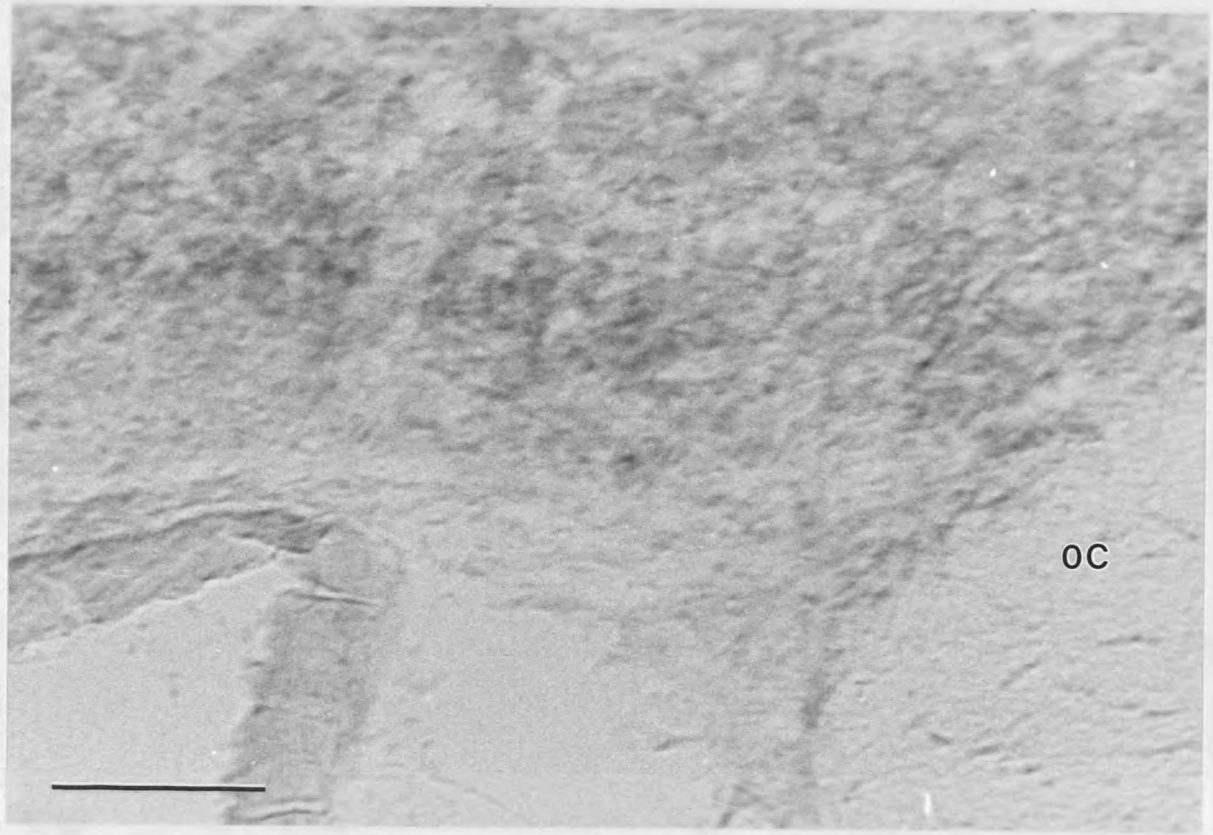


Figure 6. Photomicrograph of cytochrome oxidase histochemical staining in the sprouting supraoptic nucleus from a lesioned animal at four weeks post-surgery. In this figure, the supraoptic nucleus is located just above and to the left of the optic chiasm (OC), which stains very lightly. When compared to the sprouting supraoptic nucleus from a lesioned + hyponatremic animal (Figure 7), very little change in stain density may be seen. Scale bar equals 100 μm.

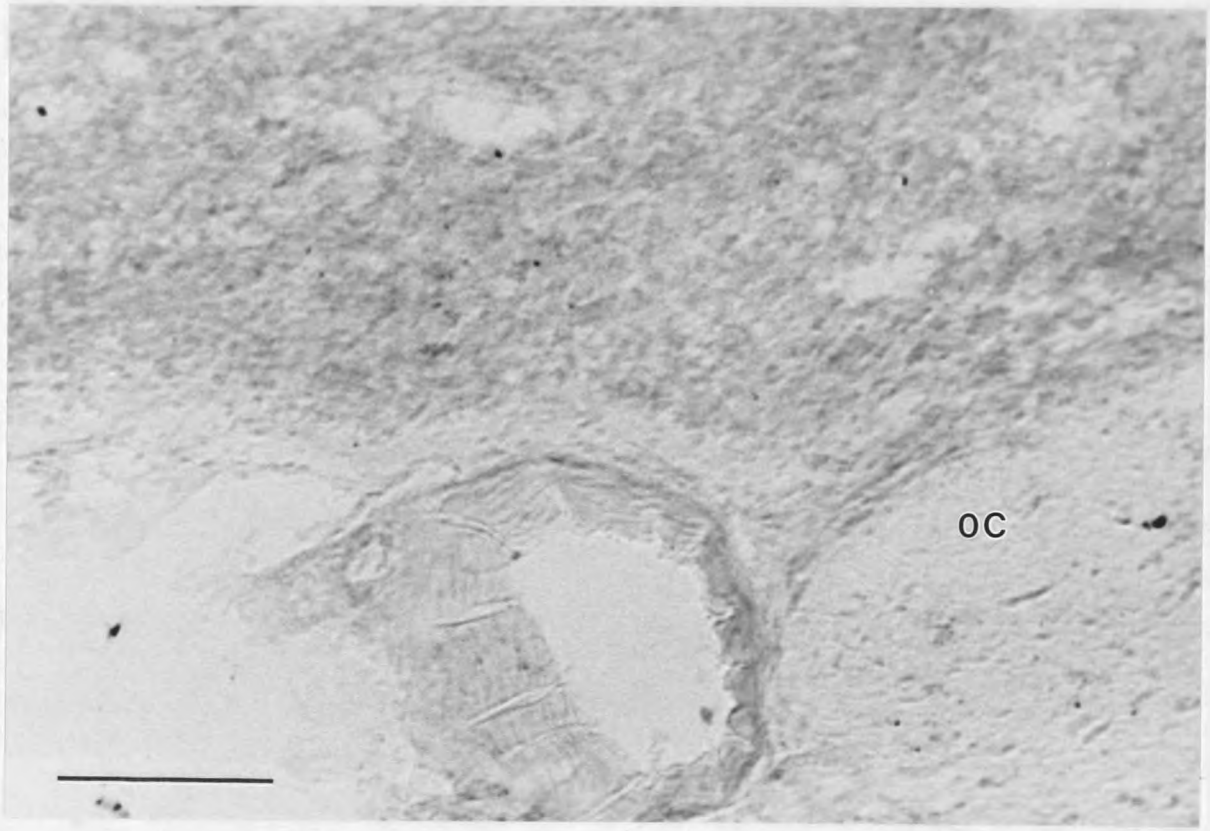


Figure 7. Photomicrograph of cytochrome oxidase histochemical staining in the sprouting supraoptic nucleus from a lesioned + hyponatremic animal at four weeks post-surgery. In this figure, the supraoptic nucleus is located just above and to the left of the optic chiasm (OC), which stains very lightly. When compared to the sprouting supraoptic nucleus from a lesioned animal (Figure 6), very little change in stain density may be seen. Scale bar equals 100 μm.

segments (presumably blood vessels) were occasionally seen (Figures 8 and 9).

The average cross-sectional area of the neurohypophysial sections changed with respect to both experimental group and time (Figure 10). Two-way ANOVA indicated significant changes with respect to experimental group ($df = 5$; $F = 35.62$; $p < 0.0001$) and time ($df = 1$; $F = 18.39$; $p < 0.0001$). LSD comparisons ($p < 0.05$) showed that sections of neurohypophyses from the intact and sham groups were the largest, those from lesioned and sham + hyponatremic groups were intermediate in size, and those from the lesioned + hyponatremic and lesioned + diet groups were consistently the smallest. In addition, LSD comparisons ($p < 0.05$) indicated that sections of neurohypophyses from groups examined at four weeks post-surgery were generally larger than those examined at one week post-surgery (see Appendices E and F). Furthermore, the average cross-sectional area of the neurohypophysial sections significantly increased following four days of salt-loading when compared to those from intact animals (Student's t; $p < 0.05$).

A variable number of intensely stained cells were readily apparent in the neurohypophyses of animals from each experimental group. These cells had a generally uniform morphology, being consistently about ten to twelve μm in diameter, rounded in shape and lacking any discernable processes (Figures 8 and 9). While the average number of intensely stained cells in neurohypophysial sections from hyponatremic (lesioned and sham) rats was extremely high at one week post-surgery, this value appeared to be

approximately equal in all the experimental groups by four weeks post surgery (Figure 11). However, when the alterations in sectional area were taken into account (Figure 12), statistically significant trends in the areal density of these intensely stained cells became apparent. Two-way ANOVA indicated significant changes with respect to experimental group ($df = 5$; $F = 10.73$; $p < 0.0001$), and LSD comparisons ($p < 0.05$) revealed that the areal density was not affected by surgery, but was increased by the chronic hyponatremia protocol (Appendices G and H). This increase was particularly apparent at one week post-surgery, but persisted to a much smaller degree at four weeks post-surgery. Although the changes in the areal density of these cells were quite dramatic, no changes in their morphology were apparent.

In the neurohypophyses from the majority of animals, two gradients were observed in the stain produced by cytochrome oxidase histochemistry. First, rostral sections tended to be darker than caudal sections from the same animal. Second, the ventral portion of the neurohypophysis (adjacent to the intermediate lobe) tended to be darker than the dorsal portion (near the free edge of the pituitary). The rostral-caudal gradient was very common: when the data were pooled across all animal groups and time points, rostral sections were determined to be darker than caudal sections 82 percent of the time. Further analysis of the pooled data demonstrated that the ventral-dorsal gradient was visually discernable in only 26 percent of the sections; however, it should be noted that the rest of the sections did not necessarily have an opposing gradient *per se*. Indeed, the majority of sections had no obvious

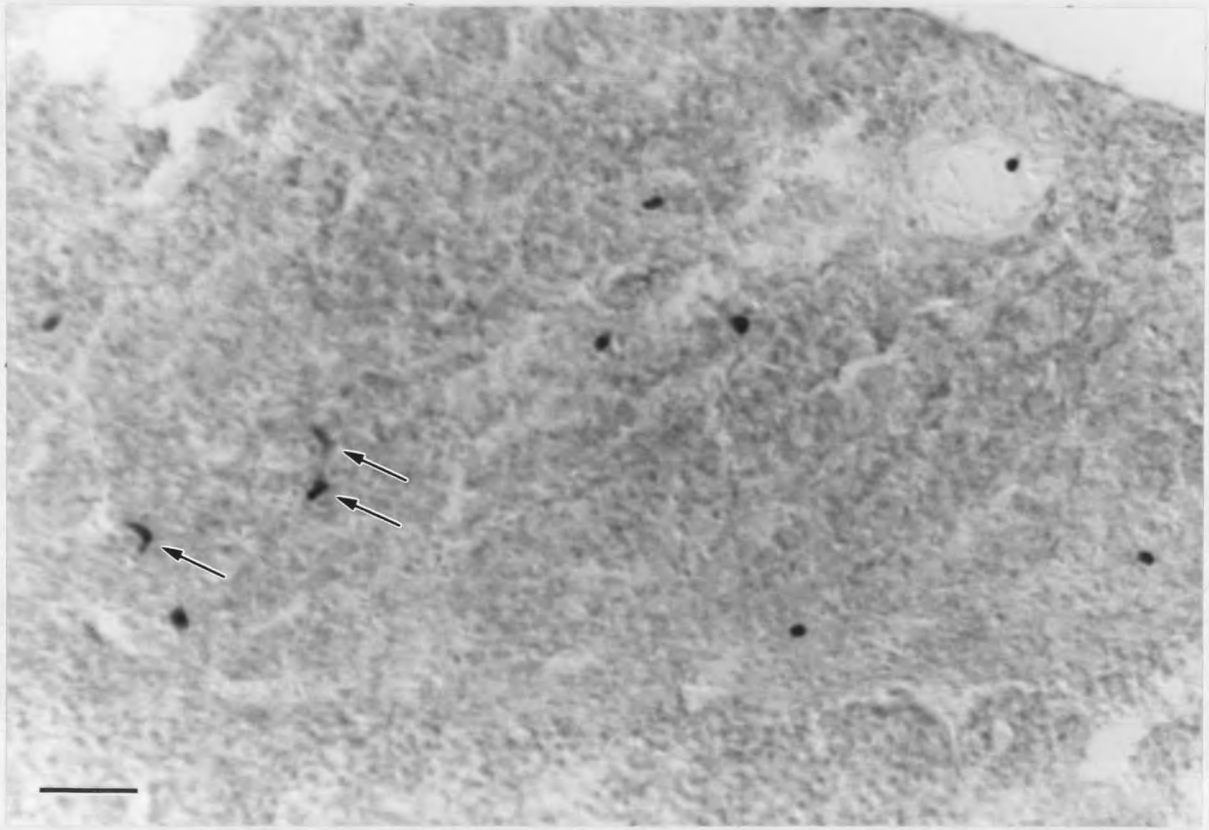


Figure 8. Photomicrograph of cytochrome oxidase histochemical staining in the pituitary from a lesioned + hyponatremic animal at one week post-surgery. In this figure, many intensely stained cells (black dot-like figures) and two tubular segments (arrows) may be seen within the neurohypophysis. These same elements may be seen at a higher magnification in Figure 9. Scale bar equals 50 μm.

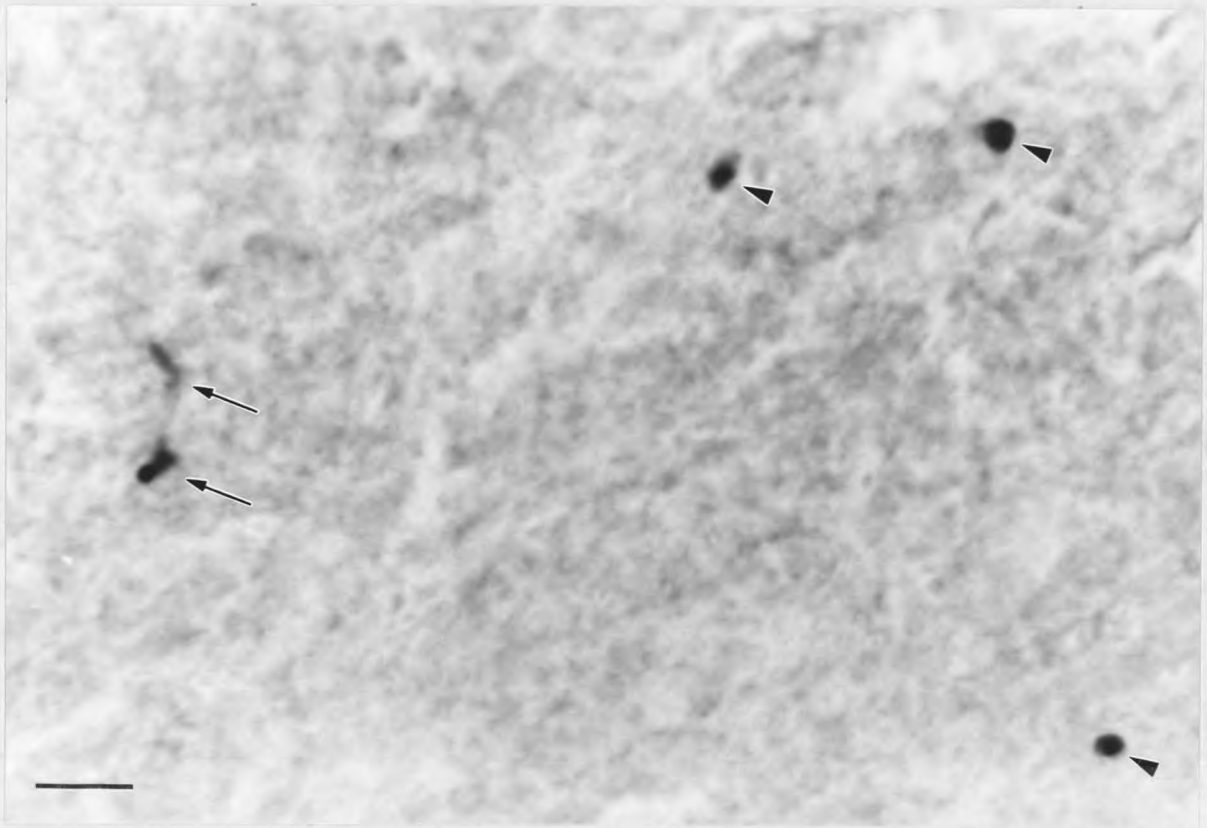


Figure 9. Photomicrograph of cytochrome oxidase histochemical staining in the neurohypophysis from a lesioned + hyponatremic animal at one week post-surgery. In this figure, three intensely stained cells (presumably macrophages and/or activated microglia; arrowheads) and a branching tubular segment (presumably a small blood vessel; arrows) are readily visible. These same elements may be seen at a lower magnification in Figure 8. Scale bar equals 25 μm.

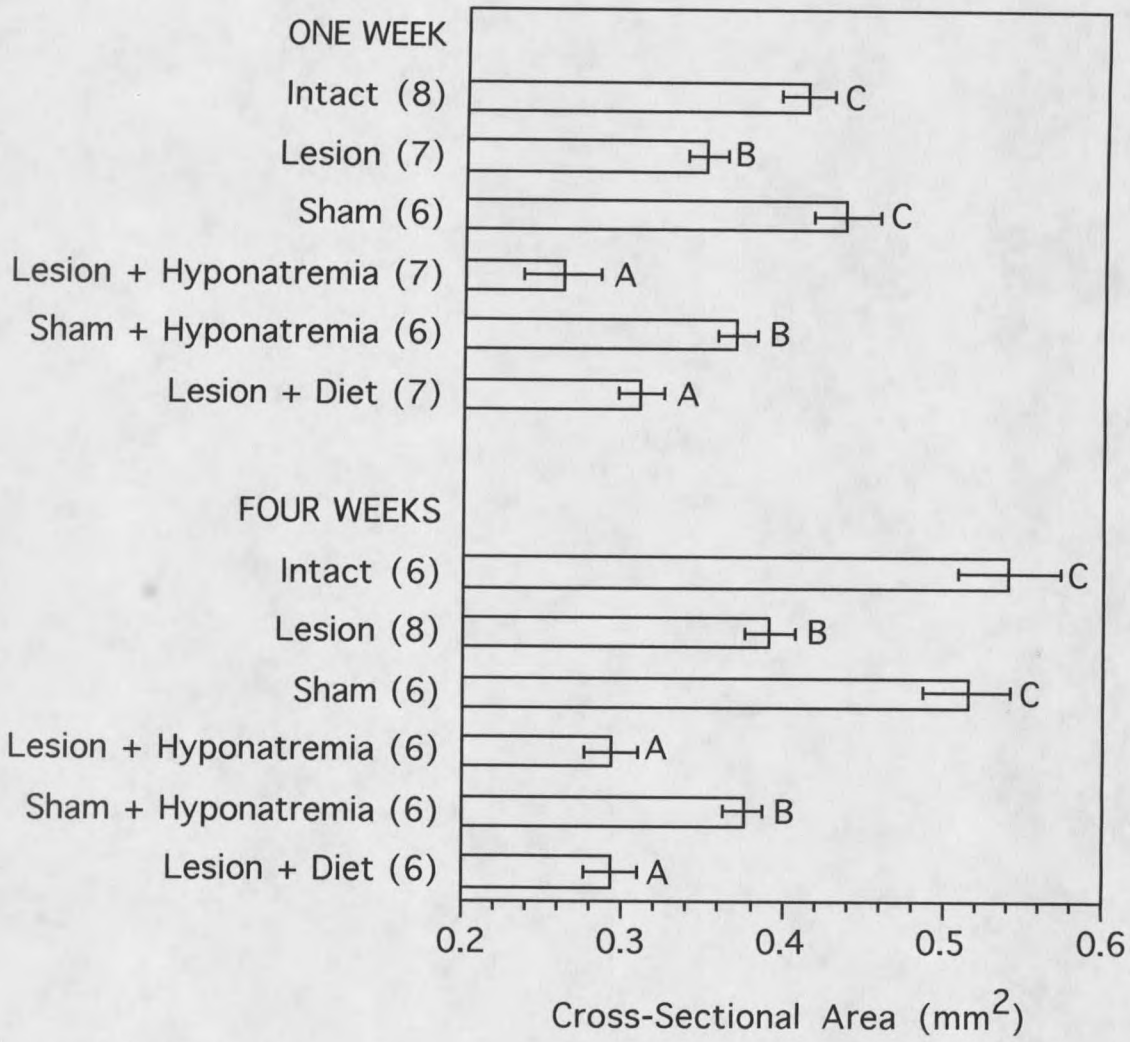


Figure 10. The average cross-sectional area of the neurohypophyses from rats in each experimental group at one and four weeks post-surgery. Two-way ANOVA indicates statistically significant changes take place with respect to both group ($df = 5$; $F = 35.62$; $p < 0.0001$) and time ($df = 1$; $F = 18.39$; $p < 0.0001$). LSD comparisons ($p < 0.05$) show that the neurohypophyses from intact and sham groups are the largest, those from lesioned and sham + hyponatremic groups are intermediate in size, and those from lesioned + hyponatremic and lesioned + diet groups are consistently the smallest (A, B and C represent statistically different sets). Furthermore, pooling the data across groups shows that neurohypophyses examined at four weeks post-surgery are generally larger than those examined after one week. The numbers in parentheses indicate n and the error bars represent the standard error of the mean.

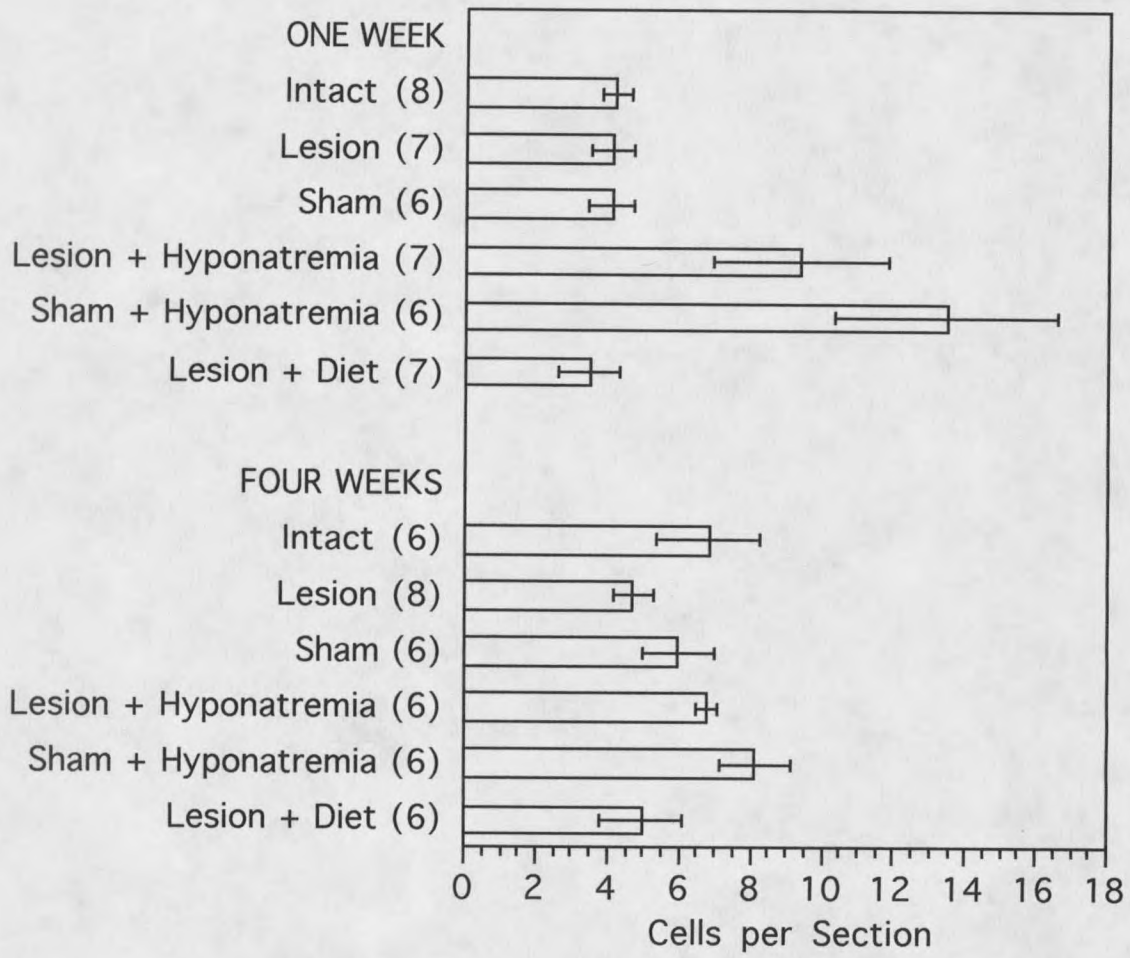


Figure 11. The average number of intensely stained cells in neurohypophysial sections from rats in each experimental group at one and four weeks post-surgery. All tissues were treated identically with cytochrome oxidase histochemistry. Sections from hyponatremic animals appear to have more intensely stained cells than sections from the other groups at one week post-surgery, but not at four weeks post-surgery. The numbers in parentheses indicate n and the error bars represent the standard error of the mean.

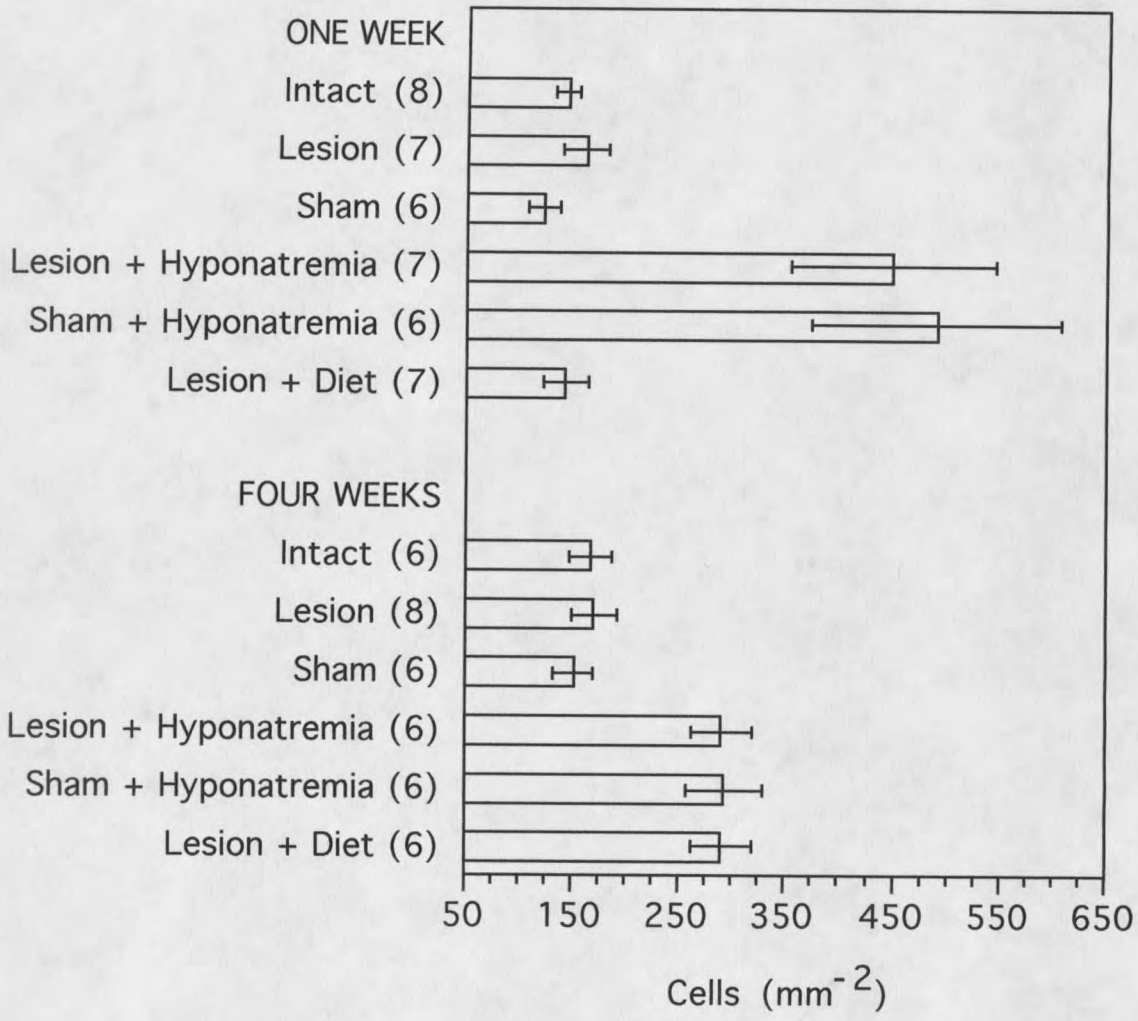


Figure 12. The average density of intensely stained cells within the neurohypophyses from rats in each experimental group at one and four weeks post-surgery. Two-way ANOVA indicates that statistically significant changes take place with respect to group ($df = 5$; $F = 10.63$; $p < 0.0001$) but not time. LSD comparisons ($p < 0.05$) show that the areal densities of the intensely stained cells in the hyponatremic groups are greater than those of all the other groups. The numbers in parentheses indicate n and the error bars represent the standard error of the mean.

gradient, and neurohypophysial sections in which the dorsal regions were darker than the ventral regions were extremely rare. When the data were analyzed by group or time point, no significant patterns were seen (data not shown). Examples of the ventral-dorsal gradient may be seen in Figures 13 and 14.

The intensity of cytochrome oxidase histochemical staining (expressed as the proportional area darker than or equal to 78 gray levels) was shown to increase with enhanced activity of the magnocellular neurosecretory system. For example, four days of salt-loading increased this value from 0.32 to 0.72, which was statistically significant (Student's t; $p = 0.0136$). More importantly, by four weeks post-surgery, the intensity of staining had increased in the lesioned group and decreased in the lesioned + hyponatremic group. One way ANOVA ($df = 5$; $F = 3.04$; $p = 0.0234$) followed by LSD comparisons ($p < 0.05$) indicated that the contrast between the lesioned and lesioned + hyponatremic groups was statistically significant--see Appendices I and J. Photomicrographs showing the range of staining within the neurohypophyses of the intact, lesioned, and lesioned + hyponatremic groups at four weeks post-surgery may be seen in Figures 13, 14, and 15. Furthermore, the intensity of cytochrome oxidase histochemical staining in the neurohypophyses from all the groups at both time points is graphed in Figure 16.



Figure 13. Photomicrograph of cytochrome oxidase histochemical staining in the pituitary from an intact animal at 63 days of age (equivalent to four weeks post-surgery). In this figure, a ventral > dorsal gradient of stain intensity may be seen within the neurohypophysis (N). Furthermore, this neurohypophysis is stained to a lesser degree than that from a lesioned animal (Figure 14) and to a greater degree than that from a lesioned + hyponatremic animal (Figure 15). Scale bar equals 100 μm.



Figure 14. Photomicrograph of cytochrome oxidase histochemical staining in the pituitary from a lesioned animal at four weeks post-surgery. In this figure, a ventral > dorsal gradient of stain intensity may be seen within the neurohypophysis (N). Furthermore, this neurohypophysis is stained to a greater degree than those from both an intact animal (Figure 13) and a lesioned + hyponatremic animal (Figure 15). Scale bar equals 100 μm.



Figure 15. Photomicrograph of cytochrome oxidase histochemical staining in the pituitary from a lesioned + hyponatremic animal at four weeks post-surgery. This neurohypophysis (N) is stained to a lesser degree than those from both an intact animal (Figure 13) and a lesioned animal (Figure 14). Scale bar equals 100 μm.

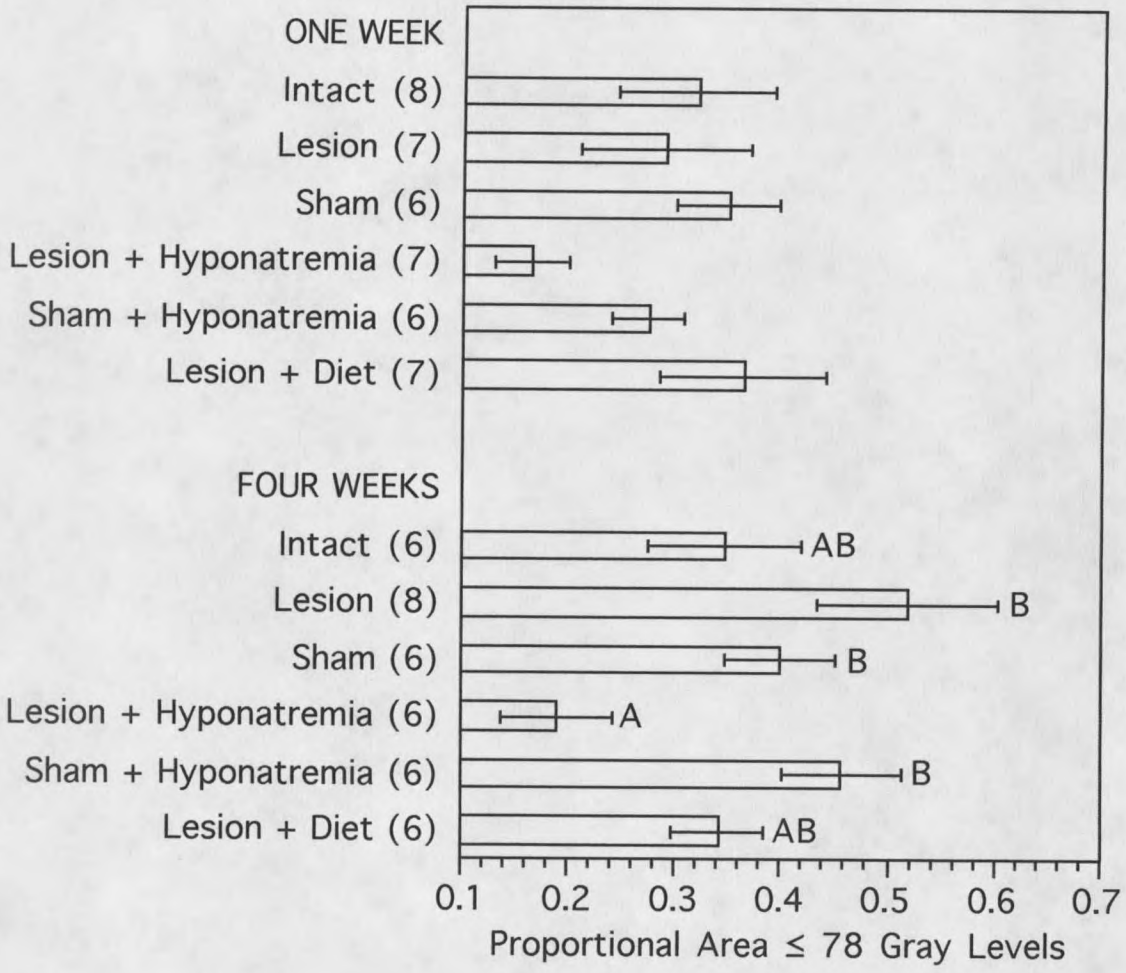


Figure 16. The intensity of cytochrome oxidase histochemical staining within the neurohypophyses from rats in each experimental group at one and four weeks post-surgery. One-way ANOVA indicates that statistically significant changes take place ($df = 5$; $F = 35.62$; $p < 0.0001$) at four weeks post-surgery. LSD comparisons ($p < 0.05$) show that neurohypophyses of the lesioned + hyponatremic group are significantly lighter than those of the lesioned group (A and B represent statistically different sets). Stain intensity is measured as the proportional area darker than or equal to 78 gray levels, numbers in parentheses indicate n, and error bars represent the standard error of the mean.

In Situ Hybridization

Oxytocin

In situ hybridization was used to determine the relative levels of oxytocin mRNA within the supraoptic nuclei of animals in all the experimental groups. When the autoradiographs were quantified as an integrated gray level (a measure that multiplies area and density), it was apparent that the oxytocin mRNA pools became elevated within the sprouting supraoptic nuclei following lesion surgery. At one week post-surgery, this increase was apparent and it became statistically significant by four weeks post-surgery (Student's t; $p = 0.0208$). Furthermore, mRNA pools in the lesioned + hyponatremic group were not only below those of the intact controls, but also significantly under those of the lesioned group at both one (Student's t; $p = 0.0287$) and four (Student's t; $p < 0.0001$) weeks post-surgery, suggesting that the hyponatremia protocol was an effective suppressor of the magnocellular neurosecretory system throughout the sprouting response. Data tables and statistical analyses are in Appendices K and L, and the integrated gray level of *in situ* hybridization for hypothalamic oxytocin mRNA at one and four weeks post-surgery is graphed in Figures 17 and 18.

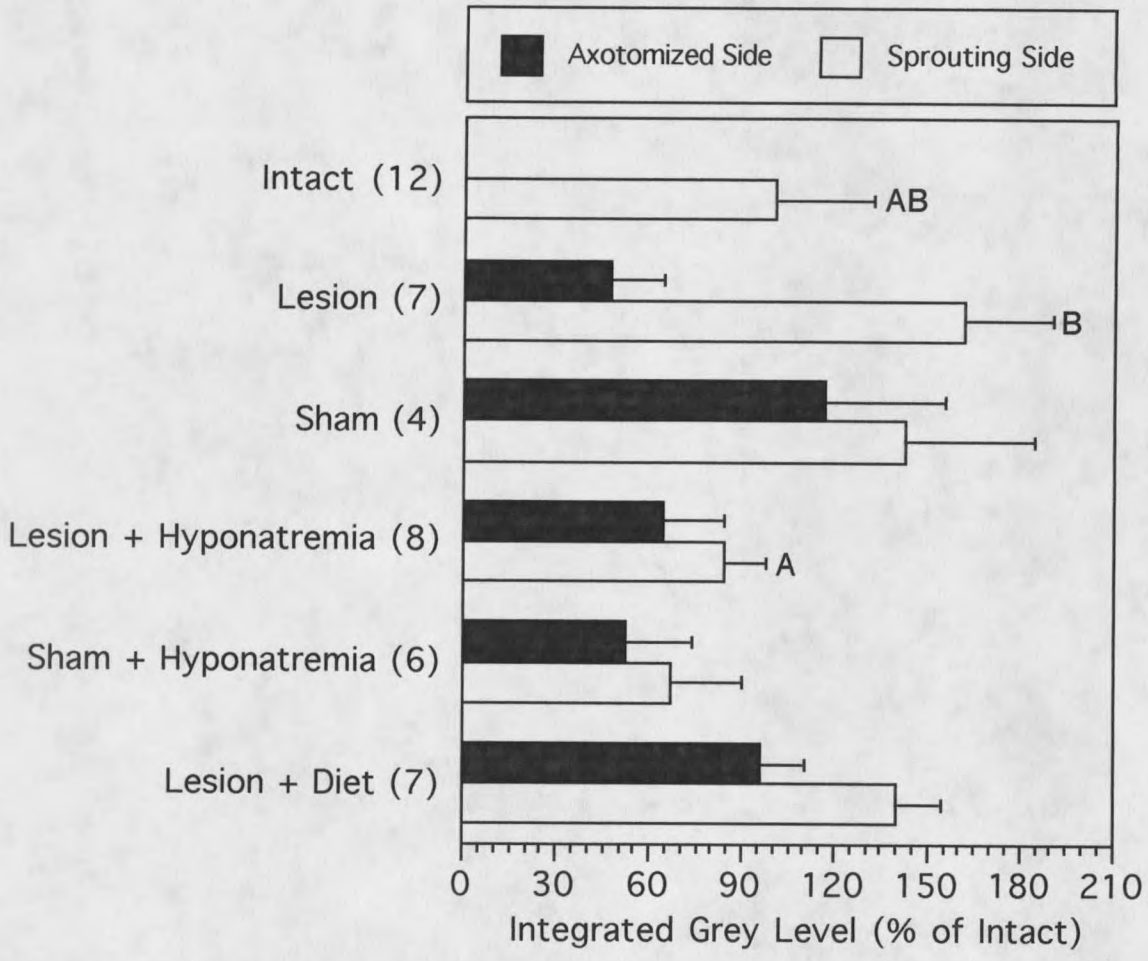


Figure 17. The relative size of the oxytocin mRNA pools within the supraoptic nuclei from rats in each experimental group at one week post-surgery. The magnitude of the *in situ* hybridization signal for oxytocin mRNA is determined through computerized image analysis and expressed as an integrated gray level. Student's t tests between the sprouting sides of the intact, lesioned, and lesioned + hyponatremic groups indicate that the signal from the lesioned group is significantly ($p < 0.05$) greater than that from the lesioned + hyponatremic group (A and B represent statistically different sets). The numbers in parentheses indicate n and the error bars represent the standard error of the mean.

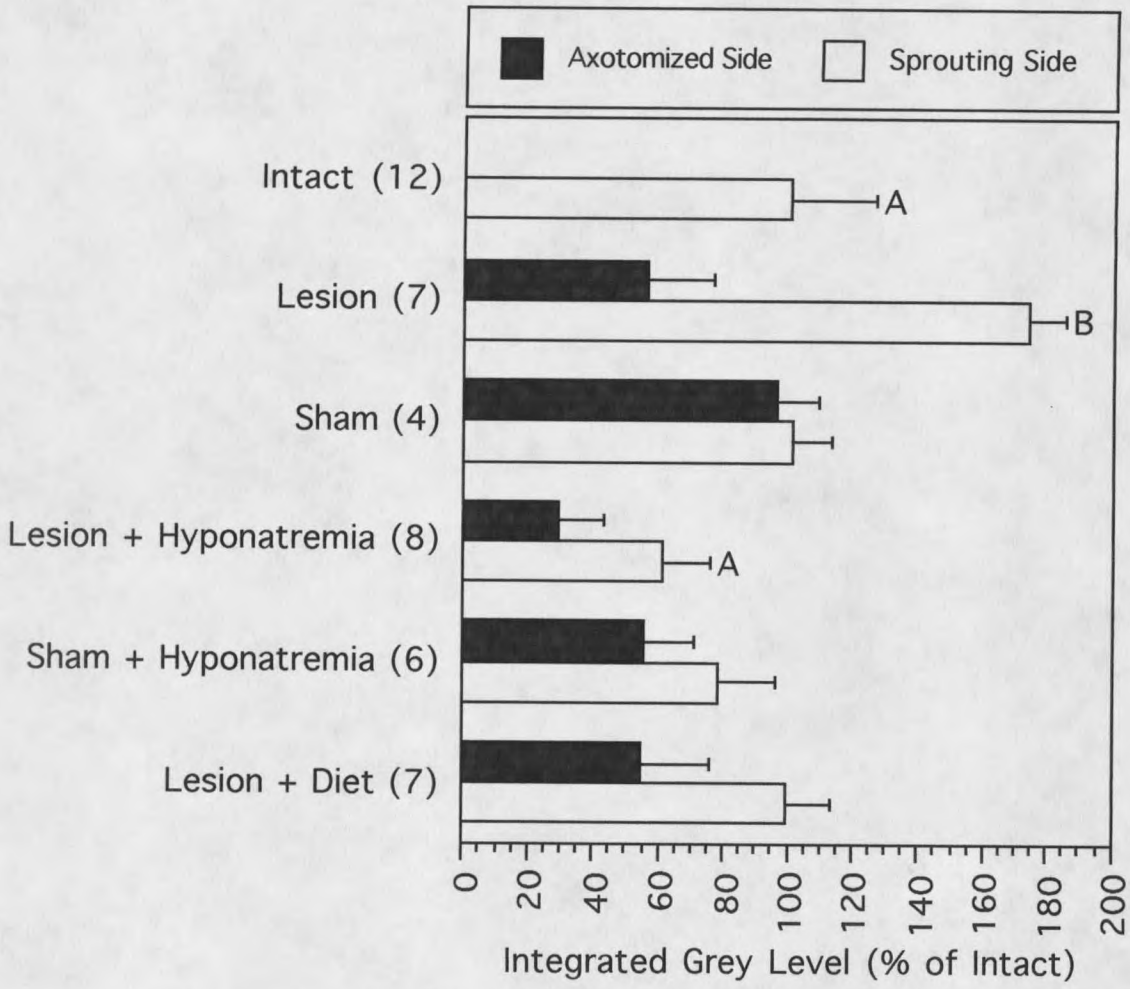


Figure 18. The relative size of the oxytocin mRNA pools within the supraoptic nuclei from rats in each experimental group at four weeks post-surgery. The magnitude of the *in situ* hybridization signal for oxytocin mRNA is determined through computerized image analysis and expressed as an integrated gray level. Student's t tests between the sprouting sides of the intact, lesioned, and lesioned + hyponatremic groups indicate that the signal from the lesioned group is significantly ($p < 0.05$) greater than those from both the intact and the lesioned + hyponatremic groups (A and B represent statistically different sets). The numbers in parentheses indicate n and the error bars represent the standard error of the mean.

Vasopressin

In situ hybridization for vasopressin mRNA pools within the hypothalamus provided a clear picture of the functional state of the magnocellular neurosecretory system during compensatory collateral sprouting and hyponatremia. In the sprouting supraoptic nucleus, vasopressin mRNA pools rose significantly (Student's t; $p = 0.0003$) in the lesioned group by one week post-surgery and then, by four weeks post-surgery, declined to levels only slightly above those of intact controls. In contrast, vasopressin mRNA pools in the lesioned + hyponatremic groups were depressed below those of intact controls and were significantly lower than the lesioned groups at both one (Student's t; $p < 0.0001$) and four (Student's t; $p = 0.0008$) weeks post-surgery--data tables and statistical analyses are in Appendices M and N. Consistent with previous reports of neuronal degeneration following axotomy (Raisman, 1973b; Herman, et al., 1986; Herman, et al., 1987; Van Tol, et al., 1989; Paden, et al., 1995), the axotomized supraoptic nuclei of animals in all the lesioned groups showed only minor amounts of vasopressin mRNA. Digitized images and graphs of the *in situ* hybridization of vasopressin mRNA are shown in Figures 19-22. Before leaving the *in situ* hybridization studies, it should be emphasized that while animals were prepared and brain tissues were sectioned by C. W. Moffett 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.

7 Days

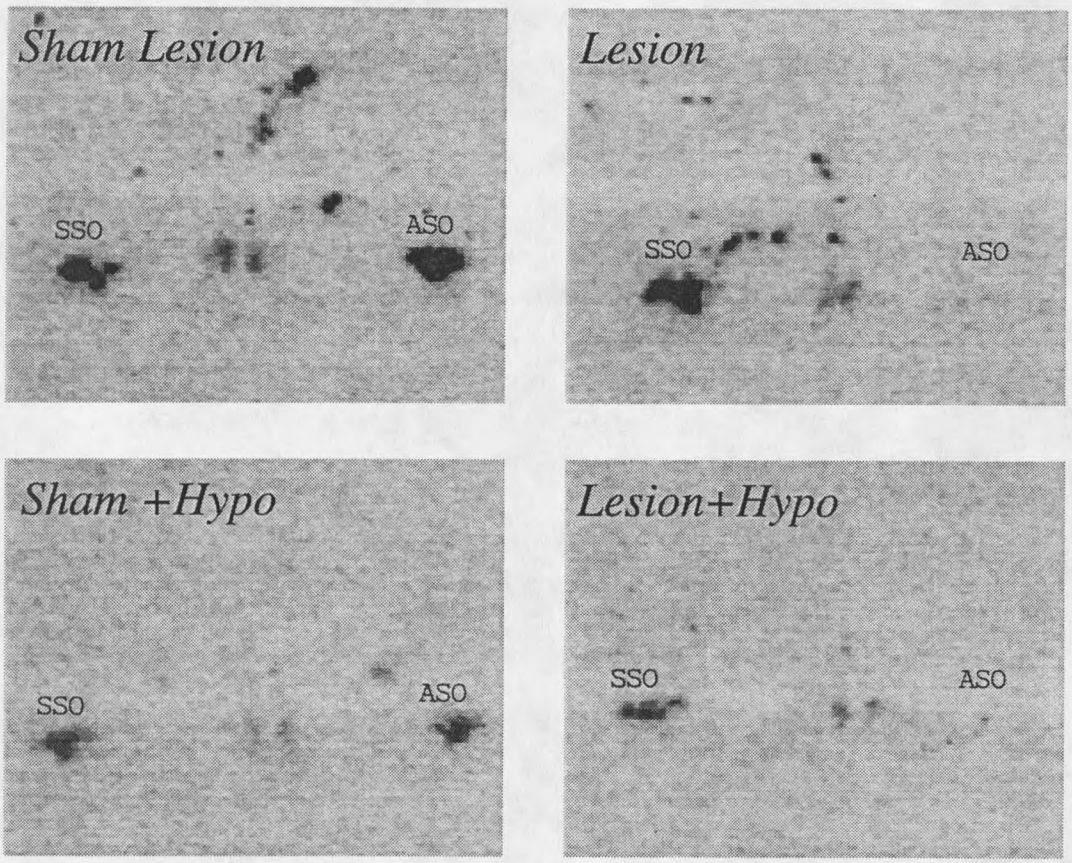


Figure 19. Digitized images (generously provided by Dr. J. P. Herman) of *in situ* hybridization autoradiographs from sections exposed to probes for vasopressin mRNA. The hypothalami shown here are from animals in the sham, lesion, sham + hypo(natremia), and lesion + hypo(natremia) groups at one week post-surgery. Axotomized supraoptic nuclei (ASO) from both types of lesioned animals show little, if any, signal. More importantly, hyponatremia can be seen to reduce the vasopressin mRNA pools within the sprouting supraoptic nuclei (SSO).

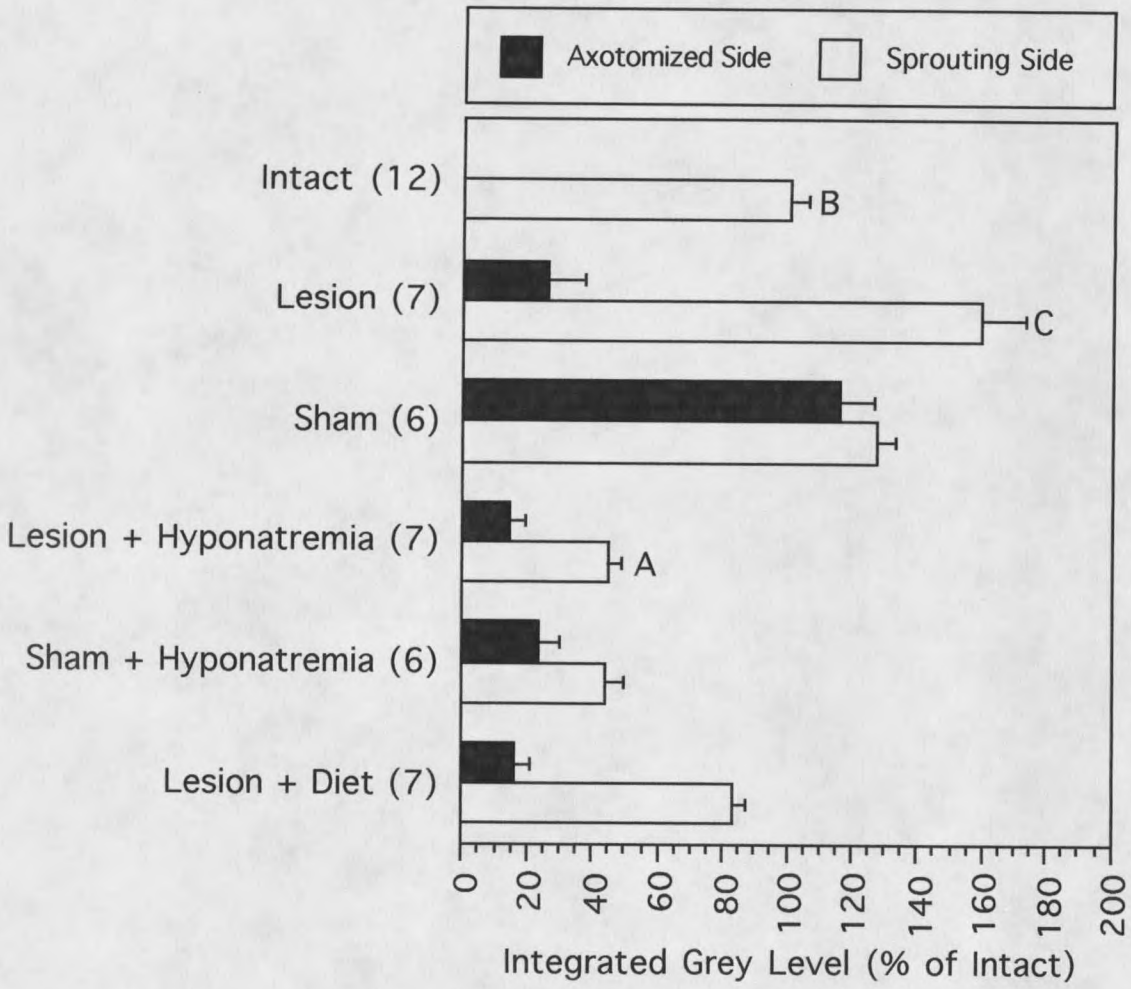


Figure 20. The relative size of the vasopressin mRNA pools within the supraoptic nuclei from rats in each experimental group at one week post-surgery. The magnitude of the *in situ* hybridization signal for vasopressin mRNA is determined through computerized image analysis and expressed as an integrated gray level. Student's t tests between the sprouting sides of the intact, lesioned, and lesioned + hyponatremic groups indicate that statistically significant differences ($p < 0.05$) exist in the signals from each of these groups: lesioned > intact > lesioned + hyponatremic (A, B and C represent statistically different sets). The numbers in parentheses indicate n and the error bars represent the standard error of the mean.

28 Days

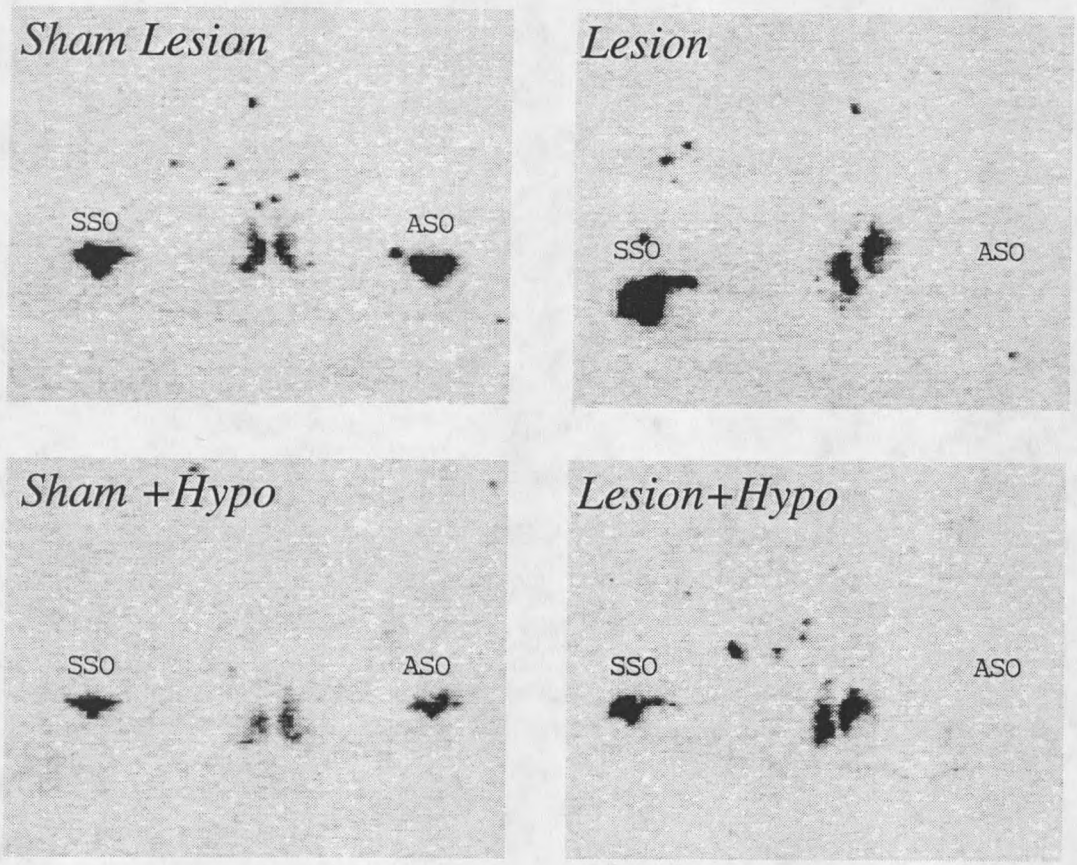


Figure 21. Digitized images (generously provided by Dr. J.P. Herman) of *in situ* hybridization autoradiographs from sections exposed to probes for vasopressin mRNA. The hypothalami shown here are from animals in the sham, lesion, sham + hypo(natremia), and lesion + hypo(natremia) groups at four weeks post-surgery. Axotomized supraoptic nuclei (ASO) from both types of lesioned animals show little, if any, signal. More importantly, hyponatremia can be seen to reduce the vasopressin mRNA pools within the sprouting supraoptic nuclei (SSO).

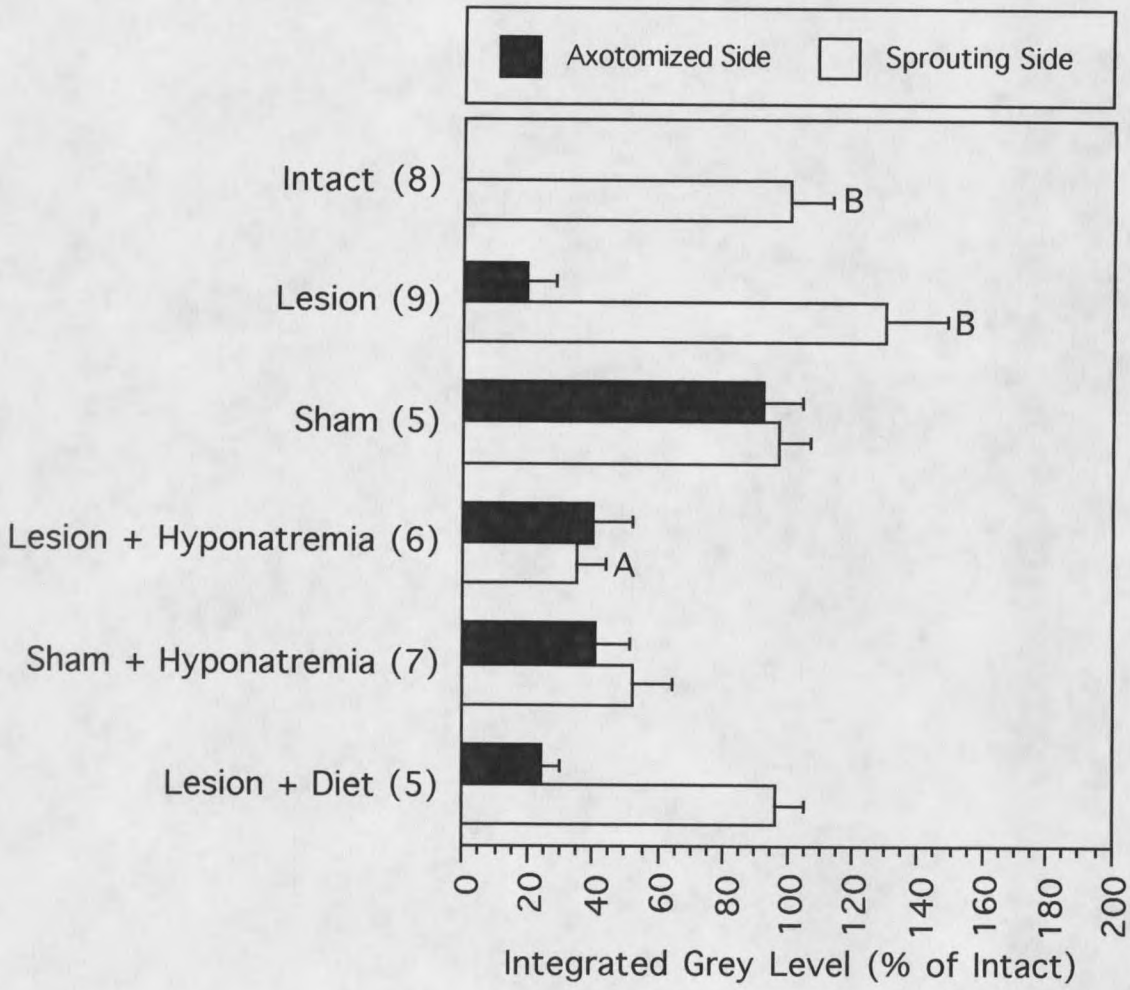


Figure 22. The relative size of the vasopressin mRNA pools within the supraoptic nuclei from rats in each experimental group at four weeks post-surgery. The magnitude of the *in situ* hybridization signal for vasopressin mRNA is determined through computerized image analysis and expressed as an integrated gray level. Student's t tests between the sprouting sides of the intact, lesioned, and lesioned + hyponatremic groups indicate that the signal from the lesioned + hyponatremic group is significantly ($p < 0.05$) less than those from both the intact and the lesioned groups (A and B represent statistically different sets). The numbers in parentheses indicate n and the error bars represent the standard error of the mean.

CHAPTER 4

DISCUSSION

The results of these experiments clearly indicate that the goals of this thesis, as they were defined in the Introduction, were achieved. Three independent measures demonstrated that the activity of intact magnocellular neurons increased during compensatory collateral axonal sprouting following unilateral destruction of the hypothalamo-neurohypophysial tract. *In situ* hybridization showed that the mRNA pools of both oxytocin and vasopressin were elevated within the supraoptic nucleus on the sprouting side. Furthermore, the intensity of cytochrome oxidase histochemical staining increased within the neurohypophysis, suggesting that the sprouting neurosecretory terminals had a heightened firing rate during the sprouting response. Also, each of these measures was significantly reduced in rats that underwent chronic hyponatremia in addition to lesion surgery, signifying that the activity of the magnocellular neurosecretory system was diminished throughout the period in which axonal sprouting has been described to occur. Further discussion of the results of all the experiments, including speculation as to possible mechanisms that produced the outcomes observed, follows below.

Body Mass

Since the average mass of the rats in the lesioned group was equal to that of intact animals (Figure 2), one must infer that the surgical procedure used to produce sprouting within the neurohypophysis did not immediately alter the set point for body weight. This observation is consistent with previous studies in which bilateral, rather than unilateral, hypothalamic lesions were necessary in order to change body mass (reviewed in Kupfermann, 1991). Conversely, chronic hyponatremia appeared to reduce the amount of weight the rats could gain each day: this may have been the result of several factors. First, the rats in this study were not only relatively immature at the time hyponatremia was established (weighing only about 175 grams), but also had just undergone extensive trauma. Second, desmopressin was administered to the rats at a rate of two ng/hour throughout the experimental period. A previous study of the chronic hyponatremia protocol showed that rats gained weight at a normal pace when they received desmopressin at a rate of one ng/hour, but lost weight when the rate was five ng/hour (Verbalis and Drutarosky, 1988). Thus, the desmopressin administered to the animals in these experiments may have been near a critical level at which weight gains are prevented. Third, the liquid diet may have provided the rats with less protein than the dry chow. Comparison of the certificates of analysis indicated that the liquid diet was less than 17 percent protein while the dry chow was 23 percent protein. This could explain why the lesioned + diet rats, which did not receive desmopressin, had

body masses consistently above those of the hyponatremic groups but not as great as those of the intact, lesioned or sham groups.

Before leaving the subject of body mass, two points must be emphasized. First, all of the rats given the liquid diet had access to enough calories to gain weight. Indeed, 30 ml of the liquid diet (less than the average amount consumed by lesioned + diet rats) provided 57 kcal (Verbalis and Drutarosky, 1988), while 15 grams of the dry chow (the maximum average amount eaten) provided 45.6 kcal of metabolizable energy (PMI Feeds data sheets). Second, all of the animals, regardless of their experimental group, increased in body mass over the experimental period. In fact, the lesioned + diet rats in these experiments gained weight at a faster rate than intact animals in another study given the same liquid diet for two weeks (Robinson, et al., 1990). Thus, it may be assumed that the rats described in these studies were supplied with enough nutrients to grow and maintain good health.

Drinking Behavior

The volume of diet consumed by animals on the liquid diet in these experiments (Figure 3) was very similar to data from the original studies of chronic plasma hypoosmolality. For example, the sharp depression in volume drunk by the lesioned + hyponatremic and sham + hyponatremic groups seen three days after the initial osmotic pump implantation was identical to that observed in hypoosmolar rats that had not undergone brain surgery: this sharp

depression is hypothesized to be the result of endogenously-generated water produced by tissue catabolism (Verbalis and Drutarosky, 1988). Perhaps the less pronounced recession in drinking behavior that occurred between two and three weeks post-surgery was an analogous response to the implantation of the second osmotic pump. Interestingly, the drinking behavior of the lesioned + diet group remained steady. This is in contrast to previous studies that indicated a sharp reduction in the volume drunk by lesioned rats, particularly in the first few days after surgery (Watt and Paden, 1991; Watt, 1993). Maybe, in the case of the lesioned + diet group, the need for daily water balance was superseded by caloric requirements. In support of this, the 35 to 40 ml of diet consumed each day by the lesioned + diet group was consistent with the drinking behavior of intact rats given osmotic pumps filled with saline (Verbalis and Drutarosky, 1988). Furthermore, when one considers that the liquid diet was only 57 percent water by weight , the lesioned + diet animals were drinking less than 23 ml of water per day: a volume less than that consumed by the lesioned rats in the original studies (Watt and Paden, 1991; Watt, 1993). Thus, either caloric or fluid needs could explain the drinking behavior of the lesioned + diet rats.

Plasma Analyses

Plasma Osmolality

Weekly measurement of plasma osmolality indicated that the set point of the magnocellular neurosecretory system was not affected by stereotaxic

surgery (Figure 4). Indeed, throughout the experimental period, the plasma osmolalities of the lesioned, sham, and lesioned + diet groups were essentially identical to those from the intact controls. Interestingly, the inability of the lesion surgery to affect plasma osmolality emphatically suggests that magnocellular neurosecretory neurons increased their activity during the process of compensatory collateral axonal sprouting. Operating under the assumption that the activity of the magnocellular neurons was regulated by a control system that stabilized plasma osmolality (Figure 1), any prolonged decrease in drinking behavior would necessitate an increase in neurosecretion by these neurons in order to maintain water balance. Since the lesion surgery has been shown to cause such a decrease in drinking behavior (Watt and Paden, 1991; Watt, 1993) and plasma osmolality was held at a constant value, then the intact magnocellular neurons must have been hypersecretory throughout the sprouting process.

The chronic hyponatremia protocol was extremely effective in lowering plasma osmolality throughout the experimental period in rats with either lesion or sham surgery. Again, if one assumes the control system that regulates the magnocellular neurosecretory system was intact, then the magnocellular neurons must have been less active throughout the period in which axonal sprouting has been shown to take place in normonatremic animals (Watt, 1989; Watt and Paden, 1991).

A slight, but statistically significant increase was seen in the plasma osmolalities of the pooled groups between one and four weeks post-surgery

(Appendix B). Although this rise was particularly apparent in the hyponatremic groups, the values from these rats continued to be substantially below those of all the other groups; therefore, the hyponatremia protocol was effective throughout the experimental period. Since the intact controls showed a slight elevation in plasma osmolality, it is likely that developmental changes take place in the regulation of the magnocellular neurosecretory system during adolescence; while no such changes have yet been reported, the function of this system has been shown to become compromised in aged rats (Watkins and Choy, 1980; Fliers and Swaab, 1983; Goudsmit, et al., 1988). Furthermore, because identical increases were seen in both the lesioned and sham groups, it is unlikely that the slight increase observed was the result of axonal sprouting. The larger increase in the plasma osmolalities of the hyponatremic groups than the other groups may have been the result of "renal escape", an elusively defined phenomenon that was discussed in previous reports of chronic hyponatremia (Verbalis, 1984; Verbalis and Drutarosky, 1988). Perhaps this phenomenon involves downregulation of vasopressin receptors in the kidneys and a concomitant decrease in the ability of desmopressin to promote water resorption.

Plasma Potassium Concentration

In these studies, plasma potassium concentration was affected by neither stereotaxic surgery nor the hyponatremia protocol (data not shown). This lack of effect was predictable, since the magnocellular neurosecretory system is not

normally associated with the regulation of plasma potassium concentrations (Fernandez and Cox, 1984; Morgan, 1984). Accordingly, the concentration of plasma potassium was shown to remain relatively constant in a previous study of chronic hyponatremia (Verbalis, 1984). Thus, measurement of this electrolyte served as a control to indicate only logical plasma variables, such as osmolality and sodium concentration, were affected by the chronic hyponatremia protocol.

Plasma Sodium Concentration

Plasma sodium concentration responded to stereotaxic surgery and the hyponatremia protocol in exactly the same manner as plasma osmolality: while unilateral destruction of the hypothalamo-neurohypophysial tract did not have any effect, substantial decreases were produced by the hyponatremia protocol (Figure 5). Since the normal concentration of sodium accounts for nearly half of the osmotic pressure in plasma, this pattern is not surprising. Indeed, numerous attempts to alter either plasma osmolality or sodium have suggested that the two variables are covariant (Jones and Pickering, 1969; Verbalis, et al., 1986; Verbalis and Drutarosky, 1988; Kadekaro, et al., 1990; Verbalis and Dohanics, 1991; Iványi, et al., 1995).

The dramatic reduction in plasma sodium concentrations of the lesioned + hyponatremic rats, when compared to animals in the lesioned group, implies that the hyponatremia protocol was effective in chronically reducing the activity of the magnocellular neurosecretory system throughout the sprouting response. Nearly identical plasma sodium concentrations have been shown to

significantly decrease the quantity of oxytocin and vasopressin mRNA within the supraoptic nuclei (Robinson, et al., 1990), the synthesis of these neuropeptides, (Robinson, et al., 1990), and their secretion into the blood (Verbalis, et al., 1986; Verbalis, et al., 1989; Verbalis and Dohanics, 1991; Iványi, et al., 1995). Thus, when one considers that the magnocellular neurosecretory neurons undergoing axonal sprouting were likely to be hyperactive, it seems certain that the activity of these cells was attenuated in the animals of the lesioned + hyponatremic group.

As with plasma osmolality, a general rise in plasma sodium concentration was seen between one and four weeks post-surgery (Appendix D). This is likely to be the result of a combination of developmental factors and renal escape, but not axonal sprouting, as described above.

Cytochrome Oxidase Histochemistry

Supraoptic Nucleus

Although it would have been interesting to observe alterations in the cytochrome oxidase staining of the sprouting supraoptic nucleus, no such changes were seen (Figures 6 and 7). However, because an abundance of literature indicates that only the most profound changes in hypothalamic neuronal activity may be detected by cytochrome oxidase histochemistry, this observation should be considered neither surprising nor unusual.

The low density of mitochondria within magnocellular neurosecretory neurons, a value that appears to be immutable, is partly responsible for the insensitivity of the cytochrome oxidase stain within the supraoptic nuclei. Indeed, mitochondria have been shown to make up less than ten percent of the cytoplasmic volume within supraoptic neurons (Eneström and Hamberger, 1968)--a value that was not altered by either one week of dehydration (Rechardt, 1969) or salt-loading (Eneström and Hamberger, 1968). Considering that the intensity of cytochrome oxidase histochemical staining has been shown to correspond with the amount of enzyme present (Hevner and Wong-Riley, 1989; Hevner and Wong-Riley, 1990; Chandrasekaran, et al., 1992; Hevner and Wong-Riley, 1993) and that the reaction product is essentially limited to mitochondria (Seligman, et al., 1968; Wong-Riley, 1976; Kageyama and Wong-Riley, 1982), one would expect that only the most profound increases in stain density could be detected within the supraoptic nuclei. Indeed, the only report that has yet indicated a qualitative increase in cytochrome oxidase histochemical staining within this region used Brattleboro rats, whose magnocellular neurosecretory systems are constantly stimulated because they are genetically unable to produce vasopressin (Krukoff, et al., 1983).

In addition, it is likely that inhibitory afferents form a mitochondrial pool with a staining pattern converse to that of the magnocellular neurons in the supraoptic nucleus. Indeed, it has been hypothesized that the activity of the neurons within the magnocellular neurosecretory system is controlled

predominantly by inhibitory GABAergic and opiate afferents (Bourque and Renaud, 1991; Verbalis, 1993). GABAergic terminals have been localized within the supraoptic nuclei (Theodosis, et al., 1986b) and exposure to GABA decreases both the firing rate of these neurons (Randle, et al., 1986) and their secretion of vasopressin (Iovino, et al., 1983). Furthermore, opiates have been shown to elevate the osmotic threshold for the secretion of vasopressin (Kamoi and Robertson, 1985) while the secretion of oxytocin is potentiated by naloxone, an opiate antagonist (Summy-Long, et al., 1986). Additionally, recent evidence suggests neurons that stain heavily for nitric oxide synthase, such as the supraoptic neurons (Sagar and Ferriero, 1987; Bredt, et al., 1990; Bredt, et al., 1991; Dawson, et al., 1991; Arévalo, et al., 1992; Pow, 1992; Vincent and Kimura, 1992), but lightly for cytochrome oxidase, are driven primarily by inhibitory afferents (Zhang, et al., 1995). Thus, when thick sections of hypothalamus are quantified under low magnification, as was done in these studies, any potential increases in neuronal staining are likely to be nullified by decreases in the staining of the surrounding neuropil.

An overall shortage of reaction product throughout the hypothalamus made it difficult to detect decreases in the staining of that region. The light staining pattern observed was consistent with previous reports (Arvy, 1962; Hevner, et al., 1995). In fact, the sprouting supraoptic nuclei in this study frequently registered an average density of more than 100 gray levels--a score only 30 levels darker than a blank slide. Since the hyponatremia protocol depresses plasma osmolality near the limit of viability, it would be extremely

difficult to further reduce the activity of the magnocellular neurosecretory system. Thus, it may be considered impossible to produce significant decreases in the cytochrome oxidase staining of the supraoptic nucleus.

Several lines of evidence suggest that the large, heavily-stained neuronal profiles that were occasionally seen in the axotomized supraoptic nuclei were surviving magnocellular neurons. Their morphological characteristics (location, shape, and size) were all consistent with those of neurosecretory neurons. Furthermore, a small number of neurons have been reported to survive within the axotomized supraoptic nucleus following unilateral destruction of the hypothalamo-neurohypophysial tract (Watt, 1989; Paden, et al., 1995). In these exceptional neurons, the mRNA pools for growth-associated tubulins were shown to be elevated to levels similar to, or above, those of cells undergoing axonal sprouting (Paden, et al., 1995). This suggests that the axotomized neurons may be extending axons towards new targets, such as the ependymal lining of the third ventricle (Wu, et al., 1989) or hypothalamic blood vessels (Dellmann, et al., 1987; J. A. Watt, personal communication). Moreover, investigations of cytochrome oxidase staining within axotomized neurons of the trochlear nucleus have indicated that surviving cells undergo a permanent increase in oxidative metabolism (Iannuzzelli, et al., 1994). Thus, it seems reasonable to conclude that the large cells in the axotomized supraoptic nucleus that stain intensely were surviving neurosecretory neurons, and that they had undergone a dramatic increase in activity.

Neurohypophysis

Analysis of the average cross-sectional area of the neurohypophysial sections indicated that the relative size of this organ was affected by at least three factors (Figure 10). First, because the size of the sections from the intact and sham groups increased between one and four weeks post-surgery, it is likely that the neurohypophysis continued to grow throughout the experimental period. This finding is not surprising considering that developmental studies have shown changes in the structure of the neurohypophysis in rats between one and two months of age (Galabov and Schiebler, 1978; Dellmann, 1981). Second, the consistently smaller size of sections from the hyponatremic (lesioned and sham) animals when compared to those of their normonatremic counterparts implies that the size of the neurohypophysis is correlated to its functional state. This is supported by reports that indicate salt-loading caused a dramatic increase in the size of the neurohypophysis (Friesen and Astwood, 1967; Dellmann, et al., 1988). Third, the diminutive size of sections from the lesioned and lesioned + hyponatremic groups as contrasted to those from the corresponding sham controls suggests that the neurohypophysis shrinks following partial denervation. These data conform to previous descriptions of the effect of unilateral destruction of the hypothalamo-neurohypophysial tract on the size of the neurohypophysis (Watt, 1989; Watt and Paden, 1991). Furthermore, it is possible that these factors can act in an additive manner: speculating on the data from the lesioned + diet group, a modest decrease in neurosecretory activity (caused by ingestion of the liquid diet) combined with

shrinkage (caused by the lesion surgery) may have produced the relatively small cross-sectional area of these neurohypophysial sections. While the relationship between neurohypophysial size and experimental group is certainly intriguing, it also appears to be a contributing factor in the areal density of phagocytic cells within the organ, as discussed below.

The presence of cells within the neurohypophysis that stain extremely intensely with cytochrome oxidase histochemistry, and the robust increase in the density of these cells seen within the tissues of hyponatremic rats, represent novel findings (Figures 8, 9, and 11). At least three lines of evidence indicate that the intensely stained cells are phagocytes such as macrophages or activated microglia. First, the fairly uniform size (about 10 μm in diameter) and rounded shape of the cells is consistent with descriptions of activated microglia and macrophages (Perry, et al., 1985; Perry, et al., 1987; Perry and Gordon, 1988; Moffett and Paden, 1993). Second, macrophages are capable of respiratory oxidative bursts that produce superoxide anions and hydrogen peroxide (Bernard, et al., 1976; Sies and de Groot, 1992). These oxidants are capable of not only assisting in the breakdown of phagocytized materials, but also precipitating soluble diaminobenzidine--thus simulating the reaction product of cytochrome oxidase (Graham and Karnovsky, 1966; Seligman, et al., 1968). Assuming that activated microglia are also capable of respiratory oxidative bursts, the presence of oxidant-producing enzymes could explain the dramatic degree of staining observed. Finally, neurohypophysial microglia are believed to be especially active as phagocytes, even in normal animals, and

have been hypothesized to be involved in the continuous remodeling of neurosecretory terminals (Olivieri-Sangiacomo, 1972; Pow, et al., 1989). If this is true, then chronic hyponatremia may cause extensive remodeling and/or elimination of axon terminals in the neurohypophysis that requires a substantially increased density of phagocytic cells. Before leaving the subject of the intensely stained cells, it should be emphasized that these elements represented only a minuscule fraction of the total neurohypophysial area. Thus, they did not contribute significantly to the gradients seen in, or the overall stain density of, the neurohypophysial sections.

The gradients observed in the quantity of cytochrome oxidase staining across the neurohypophysis are particularly interesting. The presence of more reaction product in rostral sections than caudal sections is consistent with the distribution of oxytocin fibers within the neurohypophysis (Vandesande and Dierickx, 1975). In addition, the staining gradient in which ventral portions of the neurohypophysis are darker than dorsal portions is distinctly opposite to figures that show vasopressin immunoreactivity in the pituitary of a salt-loaded rat (Dellmann, et al., 1988). Thus, it is likely that cytochrome oxidase histochemistry stains oxytocinergic axons more than vasopressinergic ones. Unfortunately, it is impossible to use this information to make a definitive connection between oxidative metabolism and firing rate since, under normal conditions, oxytocin neurons fire at a relatively constant rate while vasopressin neurons fire phasically (Poulain and Wakerley, 1982). However, several reports suggest that oxytocin neurons are more plastic than vasopressin

neurons (Chapman, et al., 1986; Theodosis, et al., 1986a; Montagnese, et al., 1988; Dohanics, et al., 1992; Watt and Paden, 1993). Thus, an implied relationship exists between the degree to which cytochrome oxidase histochemistry stains the axons of a neuron and that cell's potential plasticity--this relationship is consistent with the hypothesis that neuronal activity is associated with axonal sprouting.

Most importantly, analysis of cytochrome oxidase staining within the neurohypophysis suggests that the metabolism of magnocellular neurosecretory terminals was increased during collateral axonal sprouting, and that this increase was eliminated by chronically decreasing plasma osmolality (Figures 12-15). Unfortunately, cytochrome oxidase histochemistry does not differentiate between the mitochondria contained in axon terminals and those in neuroglia. Consequently, if neuroglial mitochondria were contributing significantly to the overall deposition of reaction product, then the changes in the overall stain intensity of the neurohypophysis may represent modifications in glial activation. In support of this, microglia and pituicytes have been shown to respond to changes in osmotic stimulation (Paterson and LeBlond, 1977; Dellmann, et al., 1979; Theodosis, 1979; Tweedle and Hatton, 1980; Hatton, et al., 1984; Kawamoto and Kawashima, 1984; Tweedle and Hatton, 1987; Beagley and Hatton, 1992) and to lesion-induced degeneration of neurosecretory axons (Moffett and Paden, 1993; Watt and Paden, 1993). Conversely, with the exception of the easily identifiable intensely stained cells, cellular contours or profiles were not seen following cytochrome oxidase

staining of the neurohypophysis. This lack of morphological detail is consistent with the pattern one would expect from the staining of numerous small axons branching throughout the neurohypophysis (Nordmann, 1977). Furthermore, analysis of cytochrome oxidase staining within the cerebral cortex (Chandrasekaran, et al., 1992) and spinal cord (Wong-Riley and Kageyama, 1986) have indicated that synaptic elements, such as axon terminals and dendrites, were more reactive than neuroglia. Thus, considering that the question of axonal versus neuroglial staining will remain a conundrum without elaborate investigations of neurohypophysial glia at the electron microscopic level, and that these experiments were designed to address neuronal activity, all further discussion will include the assumption that the reaction product produced by cytochrome oxidase histochemistry is indicative of neurosecretory terminal activity.

At one week post-surgery, the cytochrome oxidase staining in the neurohypophyses from the lesioned group was essentially the same as that from the intact group, while tissues from the lesioned + hyponatremic group were much lighter (Figure 16). Since it is known that the neurohypophyses from the lesioned rats contained only about 62 percent of the axon terminals present in the intact organ at this time (Watt, 1989; Watt and Paden, 1991), the equivalent staining of the two groups suggests that the terminals remaining in lesioned neurohypophyses were hyperactive. This implied elevation in neurosecretion is consistent with the chronically decreased drinking behavior of the lesioned animals seen immediately following surgery (Watt and Paden,

1991; Watt, 1993). Applying similar reasoning to the data from the lesioned + hyponatremic group, the very low level of cytochrome oxidase staining was presumably due to not only a loss of about 38 percent of the axonal population, but also a decrease in the activity of the remaining axons.

At four weeks post-surgery, the cytochrome oxidase staining within the neurohypophyses from the lesioned group was greater than those from the intact group, while tissues from the lesioned + hyponatremic group were significantly lighter. Even if the axonal population within the neurohypophyses from intact and lesioned animals was identical, this would suggest that the axons in the latter group were more active. However, since the neurohypophyses in the lesioned group contained only about 76 percent of the normal axon number at this time (Watt, 1989; Watt and Paden, 1991), it is almost certain that these axons were hyperactive. These data correspond very well to other measures of the activity of the magnocellular neurosecretory system, including decreased drinking behavior as well as cellular and nuclear hypertrophy in the sprouting supraoptic nucleus (Watt and Paden, 1991; Watt, 1993). Although the low amount of reaction product deposited in the neurohypophyses of the lesioned + hyponatremic group certainly indicates that these neurosecretory axons were less active than normal, it could also imply that sprouting did not proceed to the same degree as in the lesioned group. This very interesting implication has been shown to be true for oxytocinergic parvocellular neurosecretory neurons, since chronic hyponatremia reduced their ability to sprout axons into the median eminence (Dohanics, et al., 1994).

It is likely that changes in cytochrome oxidase staining of neurohypophysial axons are due to alterations in both mitochondrial density and reactivity. In support of this mechanism, an electron microscopic study showed that darkly stained neurons had a greater packing density of intensely stained mitochondria, while lightly stained cells had fewer mitochondria, most of which contained very little reaction product (Wong-Riley and Kageyama, 1986). In terms of mitochondrial density, changes at the axon terminals are likely to be the result of anterograde transport of mitochondria or mitochondrial components consistent with metabolic demands. Indeed, it has been shown that the rate at which nuclear-encoded mitochondrial precursor proteins were delivered to axon terminals was linked to neuronal activity (Liu and Wong-Riley, 1994). For magnocellular neurosecretory neurons, this mechanism is likely to be quite responsive, since the length of the hypothalamo-neurohypophysial tract is only about two mm, and mitochondria and/or mitochondrial components can be conveyed from the cell body to the axon terminals in only a few hours (Jones and Pickering, 1972; Gainer, et al., 1977; Brownstein, et al., 1980). In terms of mitochondrial reactivity, modifications are likely to represent changes in the quantity of functional cytochrome oxidase within the organelle. Since the cytochrome oxidase complex requires components encoded by both mitochondrial and nuclear genes in order to function (Capaldi, 1990), an imbalance in subunit production from these two genomes could create mitochondria that stain very lightly. In support of this, an elegant study of the mitochondrial-encoded and nuclear-encoded mRNA pools for several

cytochrome oxidase subunits indicated that acute regulatory changes in cytochrome oxidase activity were chiefly controlled by regulation of mitochondrial genes (Hevner and Wong-Riley, 1993). As a concluding illustration of the coupling between neuronal firing rate, ionic pumping and metabolism, levels of cytochrome oxidase and sodium, potassium-ATPase have been shown to be regulated in parallel (Hevner, et al., 1992).

In Situ Hybridization

In situ hybridization for oxytocin and vasopressin mRNA pools indicated that magnocellular neurosecretory neurons increased their activity during compensatory collateral axonal sprouting, and that this increase was eliminated through the use of the chronic hyponatremia protocol (Figures 17-22). Interestingly, however, oxytocinergic and vasopressinergic neurons did not respond identically, so the reaction of each neuronal type will be discussed individually.

Oxytocin

Oxytocinergic neurons in the axotomized supraoptic nucleus displayed fairly high pools of oxytocin mRNA compared to vasopressin mRNA at both one and four weeks post-surgery: this is probably attributable to the resiliency of the oxytocinergic neurons (J. G. Verbalis, personal communication). Although the results described in one abstract indicate that induction of chronic hyponatremia

for ten days prior to and six days after compression of the pituitary stalk (a procedure that effectively axotomizes the magnocellular neurosecretory axons and causes axonal regeneration), kills 86 percent of the oxytocinergic neurons in the supraoptic nuclei (Dohanics, et al., 1990), several other manuscripts suggest that these neurons are quite hardy. For instance, both immunofluorescence for oxytocin and *in situ* hybridization for oxytocin mRNA showed a decline of only about 25 percent in the supraoptic nucleus one week following neurohypophysectomy, and then a recovery to nearly normal levels by five weeks post-surgery (Villar, et al., 1990). More akin to the studies presented here, the oxytocin mRNA content of the supraoptic nuclei was reduced by only about 20 percent one week after a complete transection of the neurohypophysial stalk, and this decline was not exacerbated by continuous subcutaneous infusion of vasopressin (Van Tol, et al., 1989). Furthermore, eight weeks following hypophysectomy and vasopressin substitution, substantial numbers of oxytocinergic neurons have been shown to remain in the supraoptic nucleus (Herman, et al., 1987). Thus, the consistently high levels of oxytocin mRNA observed in the axotomized supraoptic nuclei should be considered neither surprising nor unusual.

More importantly, the relative size of the oxytocin mRNA pools within the supraoptic nucleus increased during the process of axonal sprouting and this increase was eliminated by chronically lowering plasma osmolality: both of these trends reach significant levels by four weeks post-surgery. Although there are no other studies that directly measured neurotransmitter mRNA pools in

intact peptidergic neurons that were sprouting new axons, several indirect comparisons are possible. First, pituitary stalk compression caused the secretion of oxytocin to become extremely elevated between one and four weeks post-surgery (Makara, et al., 1995). Second, *in situ* hybridization for two growth-associated tubulin isotypes, alpha 1 and beta II, in the supraoptic nucleus showed that these mRNA pools rose during collateral axonal sprouting (Paden, et al., 1995). Third, immunohistochemical and morphometric analyses demonstrated that, between ten and 30 days following unilateral destruction of the hypothalamo-neurohypophysial tract, the oxytocinergic somata in the intact supraoptic nucleus were significantly larger (Watt, 1993).

However, the relatively high levels (more than 50 percent of intact controls) of oxytocin mRNA seen in the hyponatremic animals at one and four weeks post-surgery are somewhat baffling. These values are considerably greater than the levels (20 to 30 percent of normal) previously seen in rats that were made hyponatremic for one to two weeks (Robinson, et al., 1990). Of course, this discrepancy could be the result of technical differences between the two laboratories such as the specificity of the riboprobes or the sensitivity of the computerized analysis. Perhaps, the results seen here indicate a situation in which oxytocinergic and vasopressinergic neurons do not respond to the experimental protocol in a parallel manner. In any case, the fact remains that oxytocin mRNA pools were elevated in intact magnocellular neurons sprouting new axon terminals and that this increase was annulled by the chronic hyponatremia protocol.

Vasopressin

In situ hybridization indicated that unilateral destruction of the hypothalamo-neurohypophysial tract drastically reduced the mRNA pools in axotomized neurons of the supraoptic nucleus (Figures 19-22). Indeed, the reduction in vasopressin mRNA observed at one week post-surgery was much more dramatic than that produced by neurohypophysectomy procedures (Van Tol, et al., 1989; Villar, et al., 1990). This may have been due to the proximity of the axotomy to the neuronal cell body, since the unilateral lesion was very close to the magnocellular somata and thus was likely to decimate the population of axotomized neurons more quickly and completely than transection of the pituitary stalk. In addition, it is likely that chronic plasma hypoosmolality profoundly decreased the ability of the axotomized vasopressinergic neurons to survive. This is supported by the observation that the majority of vasopressinergic neurons die as a result of axotomy followed by chronic infusion of vasopressin (Herman, et al., 1986; Herman, et al., 1987). Likewise, induction of chronic hyponatremia for ten days prior to and six days after pituitary stalk compression killed over 95 percent of the vasopressinergic neurons in the supraoptic nuclei (Dohanics, et al., 1990). Thus, the fact that vasopressin mRNA pools were above trace levels in the axotomized supraoptic nuclei is somewhat surprising--perhaps the lesion surgery was incomplete in a small number of animals and this error boosted the overall values for each of the lesioned groups (J. P. Herman, personal communication).

At four weeks post-surgery, a slight rebound occurred in the mRNA pools of the axotomized supraoptic neurons. This may be due to extreme activation of the few vasopressinergic neurons that had survived the lesion. In support of this, axotomized supraoptic neurons have been shown to stain intensely with cytochrome oxidase histochemistry (see above) and to contain very high levels of mRNA for alpha 1 and beta II tubulins (Paden, et al., 1995). Furthermore, four weeks following compression of the pituitary stalk, it has been indicated that regenerating vasopressinergic neurons secrete vasopressin at six times the normal rate (Makara, et al., 1995). Nevertheless, emphasis should be placed on the fact that the overall vasopressin mRNA pools in the axotomized supraoptic nucleus were permanently reduced, since these levels continued to be well below normal at four weeks post-surgery.

Consistent with the hypothesis that neurosecretory neurons are hyperactive during collateral axonal sprouting, vasopressin mRNA pools in the sprouting supraoptic nucleus of lesioned rats were significantly above normal at one week post-surgery and remained elevated at four weeks post-surgery. Furthermore, combination of the lesion protocol with chronic hyponatremia lowered the mRNA pools in the sprouting supraoptic nucleus significantly below those of the lesioned group throughout the experimental period. This strongly suggests that comparison of the axon densities in the neurohypophyses of the lesioned and lesioned + hyponatremic groups would be a direct test of the hypothesized relationship between neuronal activity and axonal sprouting.

Concluding Statements

The results of this thesis provide detailed evidence that the activity of undamaged magnocellular neurosecretory neurons increases during compensatory collateral axonal sprouting. They support earlier studies of fluid homeostasis and cellular and nuclear size that also indicated that these peptidergic neurons were hyperactive throughout the sprouting response (Watt, 1989; Watt and Paden, 1991; Watt, 1993). Also, previous studies have indicated elevations in neuronal activity associated with axonal sprouting, but they involved other neuronal populations: Loss of negative feedback due to adrenalectomy increased the axonal sprouting of parvocellular neurosecretory neurons into the median eminence (Silverman and Zimmerman, 1982). Epileptic or seizure activity has been shown to correspond with axonal sprouting in the hippocampus (Ben-Ari and Represa, 1990; Mathern, et al., 1993; Qiao and Noebels, 1993). Electrical stimulation of neurons *in vitro* promoted the extension of filopodial sprouts (Manivannan and Terakawa, 1994). In order to directly test the hypothesis that axonal sprouting is facilitated by increased neuronal activity, experiments are currently underway to examine the extent of axonal sprouting in the neurohypophysis of rats that have been salt-loaded following unilateral lesion of the hypothalamo-neurohypophysial tract (C. M. Paden and J. A. Watt, personal communications).

In addition, this thesis lays the foundation for further experiments that will test the hypothesis that axonal sprouting is suppressed by decreased neuronal

activity. Certainly, every measure examined here indicated that the increased activity normally associated with axonal sprouting by magnocellular neurons was curtailed by the chronic hyponatremia protocol. Other studies have reduced neuronal activity during the process of axonal sprouting, but none of them involved magnocellular neurosecretory neurons. Deafferentation of the superior cervical ganglion reduced sprouting by these neurons in the hippocampus (Crutcher, et al., 1979) and the pineal gland (Dornay, et al., 1986). Hyponatremia inhibited the sprouting of parvocellular oxytocinergic neurons in the median eminence (Dohanics, et al., 1994). Thus, further examination of the effects of decreased activity on axonal sprouting in the magnocellular neurosecretory system will provide truly novel results.

In conclusion, it should be emphasized that the experiments that comprise this thesis are part of a larger ongoing project. This project is worthy of investigation not only due to its own unique merits, but also because neuronal death is at the heart of many forms of neuronal disease and trauma. Thus, elucidation of the mechanism of compensatory collateral axonal sprouting "has potentially important implications for understanding the extent to which the brain can recover from injury" (Paden, 1995).

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APPENDICES

Appendix A: Plasma Osmolality Data (mmol/kg)

G1 = intact
G2 = lesion
G3 = sham
G4 = lesion + hyponatremia
G5 = sham + hyponatremia
G6 = lesion + diet
W = week post-surgery

###:	G	W	OSM
1:	1	1	306
2:	1	1	291
3:	1	1	302
4:	1	1	296
5:	1	1	294
6:	1	1	295
7:	1	1	307
8:	1	1	305
9:	1	1	302
10:	1	1	295
11:	1	1	291
12:	1	1	293
13:	1	1	293
14:	1	1	297
15:	1	1	301
16:	1	1	296
17:	1	1	303
18:	1	2	307
19:	1	2	302
20:	1	2	295
21:	1	2	294
22:	1	2	307
23:	1	2	300
24:	1	2	297
25:	1	2	300
26:	1	3	304
27:	1	3	304
28:	1	3	290
29:	1	3	291
30:	1	3	302
31:	1	3	301
32:	1	3	301
33:	1	3	304
34:	1	4	311
35:	1	4	307
36:	1	4	298
37:	1	4	294
38:	1	4	304
39:	1	4	295
40:	1	4	292
41:	1	4	297
42:	2	1	299
43:	2	1	295
44:	2	1	295
45:	2	1	304
46:	2	1	303
47:	2	1	303
48:	2	1	305
49:	2	1	297
50:	2	1	294
51:	2	1	295
52:	2	1	297
53:	2	1	294
54:	2	1	301
55:	2	1	300
56:	2	1	298
57:	2	2	295
58:	2	2	293
59:	2	2	292
60:	2	2	300
61:	2	2	300
62:	2	2	299
63:	2	2	300
64:	2	2	302
65:	2	3	294
66:	2	3	292
67:	2	3	289
68:	2	3	303
69:	2	3	302
70:	2	3	309
71:	2	3	301
72:	2	3	303
73:	2	4	308
74:	2	4	313
75:	2	4	317
76:	2	4	297
77:	2	4	295
78:	2	4	297
79:	2	4	296
80:	2	4	296
81:	3	1	304
82:	3	1	288
83:	3	1	290
84:	3	1	303
85:	3	1	307
86:	3	1	296
87:	3	1	286
88:	3	1	288
89:	3	1	302
90:	3	1	301
91:	3	1	298
92:	3	2	314
93:	3	2	304
94:	3	2	296
95:	3	2	299
96:	3	2	301
97:	3	2	311
98:	3	2	308
99:	3	3	301
100:	3	3	305
101:	3	3	290
102:	3	3	287
103:	3	3	302
104:	3	3	308
105:	3	3	312
106:	3	4	307
107:	3	4	305
108:	3	4	299
109:	3	4	294
110:	3	4	305
111:	3	4	309
112:	3	4	302
113:	4	1	236
114:	4	1	251
115:	4	1	212
116:	4	1	233
117:	4	1	231

Appendix A (continued)

###:	G	W	OSM
118:	4	1	227
119:	4	1	221
120:	4	1	224
121:	4	1	245
122:	4	1	235
123:	4	1	219
124:	4	1	219
125:	4	1	221
126:	4	2	232
127:	4	2	232
128:	4	2	222
129:	4	2	233
130:	4	2	227
131:	4	2	228
132:	4	3	241
133:	4	3	228
134:	4	3	224
135:	4	3	228
136:	4	3	229
137:	4	3	233
138:	4	4	244
139:	4	4	238
140:	4	4	248
141:	4	4	242
142:	4	4	245
143:	4	4	234
144:	5	1	236
145:	5	1	245
146:	5	1	223
147:	5	1	222

###:	G	W	OSM
148:	5	1	236
149:	5	1	232
150:	5	1	217
151:	5	1	213
152:	5	1	217
153:	5	1	221
154:	5	1	233
155:	5	1	230
156:	5	2	223
157:	5	2	215
158:	5	2	213
159:	5	2	233
160:	5	2	225
161:	5	2	241
162:	5	3	209
163:	5	3	217
164:	5	3	231
165:	5	3	250
166:	5	3	250
167:	5	3	247
168:	5	4	233
169:	5	4	244
170:	5	4	245
171:	5	4	241
172:	5	4	228
173:	5	4	246
174:	6	1	293
175:	6	1	286
176:	6	1	297
177:	6	1	308

###:	G	W	OSM
178:	6	1	304
179:	6	1	300
180:	6	1	299
181:	6	1	298
182:	6	1	301
183:	6	1	291
184:	6	1	291
185:	6	1	293
186:	6	1	296
187:	6	2	288
188:	6	2	295
189:	6	2	303
190:	6	2	299
191:	6	2	305
192:	6	2	309
193:	6	3	294
194:	6	3	297
195:	6	3	293
196:	6	3	290
197:	6	3	303
198:	6	3	306
199:	6	4	299
200:	6	4	291
201:	6	4	291
202:	6	4	292
203:	6	4	306
204:	6	4	307

Also, three rats were given two percent NaCl to drink between 38 and 42 days of age. At 42 days of age (equal to one week post-surgery) the plasma osmolalities of these animals were 399, 378, and 387 mmol/kg.

Appendix B: Statistical Analysis of Plasma Osmolalities (GLMODEL with type 3 SUM-SQRS)

Sample Sizes by Data Classification

	G1	G2	G3	G4	G5	G6	W sum
W1	17	15	11	13	12	13	81
W2	8	8	7	6	6	6	41
W3	8	8	7	6	6	6	41
W4	8	8	7	6	6	6	41
G sum	41	39	32	31	30	31	

Analysis of Variance

Model structure: G|W
For variable: OSM
Grand mean (204) = 278.8
R-squared = 0.9509

Source	df	SS	MS	F	p
Total	203	.21086E+6			
Model	23	.20050E+6	8717.3	151.43	.0000
Residual	180	10362	57.567		
G	5	.17764E+6	35527	617.15	.0000
W	3	1097.2	365.74	6.53	.0004
G*W	15	1203.2	80.215	1.39	.1544

Multiple Comparisons Based on LSD
(Student's t; letters represent statistically different sets)

	wt N	MEAN	
G1	36.9	299.4	B
G2	36.2	299.4	B
G3	30.8	301.3	B
G4	27.7	232.5	A
G5	27.4	231.4	A
G6	27.7	297.8	B
W1	79.4	274.3	A
W2	40.3	276.1	A
W3	40.3	276.9	A
W4	40.3	280.7	B

Appendix C: Plasma Sodium Concentration Data (mM)

G1 = intact
G2 = lesion
G3 = sham
G4 = lesion + hyponatremia
G5 = sham + hyponatremia
G6 = lesion + diet
W = week post-surgery

##:	G	W	NA
1:	1	1	138
2:	1	1	138
3:	1	1	140
4:	1	1	138
5:	1	4	140
6:	1	4	138
7:	1	4	139
8:	1	4	140
9:	1	4	140
10:	2	1	138
11:	2	1	142
12:	2	1	139
13:	2	1	136
14:	2	1	135
15:	2	1	138
16:	2	1	138
17:	2	4	139
18:	2	4	142
19:	2	4	139
20:	2	4	139
21:	2	4	138
22:	2	4	138
23:	2	4	139
24:	2	4	140
25:	3	1	138
26:	3	1	138
27:	3	1	138
28:	3	1	136
29:	3	4	139
30:	3	4	138
31:	3	4	140
32:	3	4	139
33:	4	1	102
34:	4	1	107
35:	4	1	98
36:	4	1	100
37:	4	1	99
38:	4	1	99
39:	4	1	97
40:	4	4	108
41:	4	4	107
42:	4	4	111
43:	4	4	104
44:	4	4	107
45:	4	4	101
46:	5	1	99
47:	5	1	103
48:	5	1	104
49:	5	1	104
50:	5	4	106
51:	5	4	105
52:	5	4	105
53:	5	4	105
54:	5	4	96
55:	5	4	107
56:	6	1	139
57:	6	1	137
58:	6	1	134
59:	6	1	135
60:	6	1	135
61:	6	1	136
62:	6	1	137
63:	6	4	138
64:	6	4	136
65:	6	4	140
66:	6	4	141
67:	6	4	141
68:	6	4	139

Also, three rats were given two percent NaCl to drink between 38 and 42 days of age. At 42 days of age (equal to one week post-surgery) the plasma sodium concentrations of these animals were 201, 187, and 195 mM.

Appendix D: Statistical Analysis of Plasma Sodium Concentrations (GLMODEL with type 3 SUM-SQRS)

Sample Sizes by Data Classification

	G1	G2	G3	G4	G5	G6	W sum
W1	4	7	4	7	4	7	33
W4	5	8	4	6	6	6	35
G sum	9	15	8	13	10	13	

Analysis of Variance

Model structure: G|W
For variable: NA
Grand mean (68) = 126.5
R-squared = 0.9838

Source	df	SS	MS	F	p
Total	67	19261			
Model	11	18949	1722.6	309.04	.0000
Residual	56	312.15	5.5741		
G	5	18424	3684.9	661.07	.0000
W	1	89.746	89.746	16.10	.0000
G*W	5	57.453	11.491	2.06	.0839

Multiple Comparisons Based on LSD
(Student's t; letters represent statistically different sets)

	wt N	MEAN	
G1	8.9	139.0	B
G2	14.9	138.6	B
G3	8.0	138.3	B
G4	12.9	103.3	A
G5	9.6	103.2	A
G6	12.9	137.7	B
W1	30.5	125.5	A
W4	33.5	127.9	B

Appendix E: Average Cross-Sectional Area Data (mm^2)

G1 = intact
G2 = lesion
G3 = sham
G4 = lesion + hyponatremia
G5 = sham + hyponatremia
G6 = lesion + diet
W = week post-surgery

##:	G	W	AREA
1:	1	1	.380
2:	1	1	.333
3:	1	1	.491
4:	1	1	.439
5:	1	1	.396
6:	1	1	.438
7:	1	1	.393
8:	1	1	.427
9:	1	4	.482
10:	1	4	.677
11:	1	4	.486
12:	1	4	.597
13:	1	4	.519
14:	1	4	.484
15:	2	1	.312
16:	2	1	.371
17:	2	1	.351
18:	2	1	.386
19:	2	1	.302
20:	2	1	.358
21:	2	1	.370
22:	2	4	.418
23:	2	4	.430
24:	2	4	.356
25:	2	4	.326
26:	2	4	.400
27:	2	4	.420
28:	2	4	.443
29:	2	4	.336
30:	3	1	.441
31:	3	1	.354
32:	3	1	.462
33:	3	1	.445
34:	3	1	.413
35:	3	1	.512
36:	3	4	.511
37:	3	4	.641
38:	3	4	.473
39:	3	4	.466
40:	3	4	.532
41:	3	4	.469
42:	4	1	.245
43:	4	1	.341
44:	4	1	.270
45:	4	1	.284
46:	4	1	.217
47:	4	1	.320
48:	4	1	.147
49:	4	4	.275
50:	4	4	.287
51:	4	4	.320
52:	4	4	.297
53:	4	4	.346
54:	4	4	.231
55:	5	1	.316
56:	5	1	.371
57:	5	1	.370
58:	5	1	.378
59:	5	1	.378
60:	5	1	.407
61:	5	4	.363
62:	5	4	.370
63:	5	4	.383
64:	5	4	.355
65:	5	4	.345
66:	5	4	.433
67:	6	1	.291
68:	6	1	.281
69:	6	1	.284
70:	6	1	.291
71:	6	1	.306
72:	6	1	.333
73:	6	1	.386
74:	6	4	.345
75:	6	4	.263
76:	6	4	.311
77:	6	4	.379
78:	6	4	.318
79:	6	4	.274

Please note that areas were originally collected as pixels and then were converted to mm^2 (348,100 pixels = 1 mm^2).

Also, three rats were given two percent NaCl to drink between 38 and 42 days of age. At 42 days of age (equal to one week post-surgery) the average neurohypophysial cross-sectional areas of these animals were 0.477, 0.590, and 0.431 mm^2.

Appendix F: Statistical Analysis of Cross-Sectional Areas (GLMODEL with type 3 SUM-SQRS)

Sample Sizes by Data Classification

	G1	G2	G3	G4	G5	G6	W sum
W1	8	7	6	7	6	7	41
W4	6	8	6	6	6	6	38
G sum	14	15	12	13	12	13	

Analysis of Variance

Model structure: G|W (two-way)
For variable: AREA
Grand mean (79) = .3791
R-squared = 0.7544

Source	df	SS	MS	F	p
Total	78	.67127			
Model	11	.50638	.46035E-1	18.71	.0000
Residual	67	.16489	.24610E-2		
G	5	.43834	.87668E-1	35.62	.0000
W	1	.45256E-1	.45256E-1	18.39	.0000
G*W	5	.37563E-1	.75126E-2	3.05	.0153

Multiple Comparisons Based on LSD
(Student's t; letters represent statistically different sets)

	wt N	MEAN	
G1	13.7	.4765	C
G2	14.9	.3706	B
G3	12.0	.4766	C
G4	12.9	.2766	A
G5	12.0	.3724	B
G6	12.9	.3126	A
W1	40.6	.3568	A
W4	37.6	.4050	B

Appendix G: Density of Intensely Stained Cells Data (mm^{-2})

G1 = intact
G2 = lesion
G3 = sham
G4 = lesion + hyponatremia
G5 = sham + hyponatremia
G6 = lesion + diet
W = week post-surgery

##:	G	W	ISC
1:	1	1	153
2:	1	1	120
3:	1	1	136
4:	1	1	205
5:	1	1	172
6:	1	1	114
7:	1	1	114
8:	1	1	143
9:	1	4	116
10:	1	4	217
11:	1	4	169
12:	1	4	196
13:	1	4	108
14:	1	4	201
15:	2	1	125
16:	2	1	210
17:	2	1	228
18:	2	1	88
19:	2	1	106
20:	2	1	164
21:	2	1	213
22:	2	4	65
23:	2	4	182
24:	2	4	239
25:	2	4	169
26:	2	4	266
27:	2	4	124
28:	2	4	174
29:	2	4	155
30:	3	1	129
31:	3	1	127
32:	3	1	169
33:	3	1	63
34:	3	1	143
35:	3	1	111
36:	3	4	235
37:	3	4	147
38:	3	4	175
39:	3	4	107
40:	3	4	122
41:	3	4	124
42:	4	1	176
43:	4	1	481
44:	4	1	478
45:	4	1	973
46:	4	1	321
47:	4	1	391
48:	4	1	326
49:	4	4	378
50:	4	4	258
51:	4	4	190
52:	4	4	245
53:	4	4	335
54:	4	4	333
55:	5	1	811
56:	5	1	558
57:	5	1	789
58:	5	1	143
59:	5	1	188
60:	5	1	457
61:	5	4	176
62:	5	4	281
63:	5	4	279
64:	5	4	293
65:	5	4	455
66:	5	4	275
67:	6	1	172
68:	6	1	125
69:	6	1	99
70:	6	1	89
71:	6	1	95
72:	6	1	183
73:	6	1	246
74:	6	4	388
75:	6	4	319
76:	6	4	103
77:	6	4	135
78:	6	4	88
79:	6	4	274

Also, three rats were given two percent NaCl to drink between 38 and 42 days of age. At 42 days of age (equal to one week post-surgery) the densities of intensely stained cells in the neurohypophyses of these animals were 111, 135, and 125 mm^{-2}.

Appendix H: Statistical Analysis of Intensely Stained Cell Densities (GLMODEL with type 3 SUM-SQRS)

Sample Sizes by Data Classification

	G1	G2	G3	G4	G5	G6	W sum
W1	8	7	6	7	6	7	41
W4	6	8	6	6	6	6	38
G sum	14	15	12	13	12	13	

Analysis of Variance

Model structure: G|W (two-way)
For variable: ISC
Grand mean (79) = 230.8
R-squared = 0.5110

Source	df	SS	MS	F	p
Total	78	.21317E+7			
Model	11	.10893E+7	99027	6.36	.0000
Residual	67	.10424E+7	15559		
G	5	.83466E+6	.16693E+6	10.73	.0000
W	1	26897	26897	1.73	.1931
G*W	5	.19885E+6	39771	2.56	.0354

Multiple Comparisons Based on LSD
(Student's t; letters represent statistically different sets)

	wt N	MEAN	
G1	13.7	156.2	A
G2	14.9	166.9	A
G3	12.0	137.7	A
G4	12.9	369.6	B
G5	12.0	392.1	B
G6	12.9	181.0	A

Appendix I: Proportional Area ≤ 78 Gray Levels Data

G1 = intact
G2 = lesion
G3 = sham
G4 = lesion + hyponatremia
G5 = sham + hyponatremia
G6 = lesion + diet

At one week post-surgery

##:	G	COX
1:	1	.199
2:	1	.165
3:	1	.165
4:	1	.262
5:	1	.293
6:	1	.181
7:	1	.720
8:	1	.559
9:	2	.031
10:	2	.277
11:	2	.505
12:	2	.223
13:	2	.071
14:	2	.612
15:	2	.301
16:	3	.350
17:	3	.398
18:	3	.271
19:	3	.363
20:	3	.525
21:	3	.179
22:	4	.043
23:	4	.336
24:	4	.166
25:	4	.205
26:	4	.086
27:	4	.147
28:	4	.162
29:	5	.317
30:	5	.127
31:	5	.253
32:	5	.337
33:	5	.352
34:	5	.250
35:	6	.624
36:	6	.242
37:	6	.447
38:	6	.152
39:	6	.226
40:	6	.651
41:	6	.196

At four weeks post-surgery

##:	G	COX
1:	1	.056
2:	1	.441
3:	1	.470
4:	1	.350
5:	1	.223
6:	1	.540
7:	2	.503
8:	2	.705
9:	2	.390
10:	2	.907
11:	2	.610
12:	2	.263
13:	2	.181
14:	2	.541
15:	3	.459
16:	3	.140
17:	3	.453
18:	3	.430
19:	3	.501
20:	3	.411
21:	4	.098
22:	4	.100
23:	4	.156
24:	4	.093
25:	4	.397
26:	4	.293
27:	5	.542
28:	5	.650
29:	5	.540
30:	5	.351
31:	5	.302
32:	5	.359
33:	6	.410
34:	6	.149
35:	6	.466
36:	6	.301
37:	6	.368
38:	6	.349

2% NaCl (see Appendix A)

1:	1	.696
2:	1	.841
3:	1	.608

Appendix J: Statistical Analysis of Proportional Area ≤ 78 Gray Levels (GLMODEL with type 3 SUM-SQRS)

Sample Sizes by Data Classification

	G1	G2	G3	G4	G5	G6	W sum
W1	8	7	6	7	6	7	41
W4	6	8	6	6	6	6	38

Analysis of Variance

Model structure: G (one-way)
For variable: COX (at one week post-surgery)
Grand mean (41) = .2907
R-squared = 0.1603

Source	df	SS	MS	F	p
Total	40	1.1839			
Model	5	.18978	.37955E-1	1.34	.2720
Residual	35	.99410	.28403E-1		

Analysis of Variance

Model structure: G (one-way)
For variable: COX (at four weeks post-surgery)
Grand mean (38) = .3827
R-squared = 0.3222

Source	df	SS	MS	F	p
Total	37	1.3163			
Model	5	.42417	.84833E-1	3.04	.0234
Residual	32	.89218	.27881E-1		

Multiple Comparisons Based on LSD
(Student's t; letters represent statistically different sets)

	wt N	MEAN	
G1	6.0	.3467	AB
G2	8.0	.5181	B
G3	6.0	.3990	B
G4	6.0	.1895	A
G5	6.0	.4573	B
G6	6.0	.3405	AB

Appendix K: Integrated Gray Level for Oxytocin mRNA Pools Data (% of Intact)

At one week post-surgery:

Group	Side	N	MEAN	STD	SEM
Intact	Axotomized	6	95.250	70.168	28.646
Intact	Sprouting	6	104.750	87.837	35.859
Lesion	Axotomized	7	47.321	44.930	16.982
Lesion	Sprouting	7	160.879	77.845	29.423
Sham	Axotomized	4	116.398	77.273	38.637
Sham	Sprouting	4	142.753	83.459	41.729
L + hNa	Axotomized	8	63.776	57.893	20.468
L + hNa	Sprouting	8	83.845	40.064	14.165
S + hNa	Axotomized	6	52.172	52.381	21.384
S + hNa	Sprouting	6	66.688	56.433	23.039
L + diet	Axotomized	7	95.600	40.510	15.311
L + diet	Sprouting	7	139.427	38.836	14.679
*Intact	*Pooled	*12	*100	*79.002	*32.252

At four weeks post-surgery:

GROUP	SIDE	N	MEAN	STD	SEM
Intact	Axotomized	4	100.980	55.267	27.633
Intact	Sprouting	4	99.020	50.595	25.297
Lesion	Axotomized	8	56.017	58.970	20.849
Lesion	Sprouting	8	174.446	30.535	10.796
Sham	Axotomized	7	101.130	32.642	12.338
Sham	Sprouting	7	96.207	34.618	13.084
L + hNa	Axotomized	7	29.023	37.047	14.003
L + hNa	Sprouting	7	60.709	39.507	14.932
S + hNa	Axotomized	7	55.056	42.500	16.064
S + hNa	Sprouting	7	78.133	47.360	17.900
L + diet	Axotomized	6	54.855	51.277	20.934
L + diet	Sprouting	6	99.187	34.580	14.117
*Intact	*Pooled	*8	*100	*52.931	*26.465

Please note that this information (except *) was generously provided by Dr. J.P. Herman. Raw data were not provided.

* Indicates values created by averaging the data from the axotomized and sprouting sides of the intact group.

Appendix L: Statistical Analysis of Oxytocin mRNA Pools (INSTAT)

At one week post-surgery:

Student's t Tests (unpaired, two-tailed)

GROUP	SIDE	N	MEAN	SEM	p
Intact	Pooled	12	100	32.252	
					0.2224
Lesion	Sprouting	7	160.88	29.423	
					0.0287
L + hNa	Sprouting	8	83.845	14.165	
					0.7014
Intact	Pooled	12	100	32.252	

At four weeks post-surgery:

Student's t Tests (unpaired, two-tailed)

GROUP	SIDE	N	MEAN	SEM	p
Intact	Pooled	8	100	26.465	
					0.0208
Lesion	Sprouting	8	174.45	10.796	
					< 0.0001
L + hNa	Sprouting	7	60.709	14.932	
					0.2362
Intact	Pooled	8	100	26.465	

Please note that each p value was generated by comparing the data on the line above and the line below the p value.

Appendix M: Integrated Gray Level for Vasopressin mRNA Pools Data (% of Intact)

At one week post-surgery:

Group	Side	N	MEAN	STD	SEM
Intact	Axotomized	6	86.848	12.867	5.253
Intact	Sprouting	6	109.160	15.372	6.276
Lesion	Axotomized	7	26.151	29.441	11.127
Lesion	Sprouting	7	159.084	37.200	14.060
Sham	Axotomized	6	115.823	25.488	10.405
Sham	Sprouting	6	127.572	14.568	5.948
L + hNa	Axotomized	7	14.046	14.184	5.361
L + hNa	Sprouting	7	44.671	11.527	4.357
S + hNa	Axotomized	6	23.773	14.294	5.836
S + hNa	Sprouting	6	44.097	14.344	5.856
L + diet	Axotomized	7	15.781	17.590	6.648
L + diet	Sprouting	7	82.944	19.733	7.458
*Intact	*Pooled	*12	**100	*14.120	*5.764

At four weeks post-surgery:

GROUP	SIDE	N	MEAN	STD	SEM
Intact	Axotomized	4	92.690	16.078	8.039
Intact	Sprouting	4	107.310	37.063	18.531
Lesion	Axotomized	9	19.273	27.137	9.046
Lesion	Sprouting	9	129.777	56.238	16.746
Sham	Axotomized	5	92.198	26.771	11.973
Sham	Sprouting	5	97.384	20.729	9.270
L + hNa	Axotomized	6	39.493	30.266	12.356
L + hNa	Sprouting	6	34.768	21.847	8.919
S + hNa	Axotomized	7	40.420	28.410	10.738
S + hNa	Sprouting	7	52.410	32.593	12.319
L + diet	Axotomized	5	24.122	13.907	6.219
L + diet	Sprouting	5	96.696	29.914	13.378
*Intact	*Pooled	*8	*100	*26.570	*13.285

Please note that this information (except *) was generously provided by Dr. J.P. Herman. Raw data were not provided.

* Indicates values created by averaging the data from the axotomized and sprouting sides of the intact group.

** At one week post-surgery, the average of the intact axotomized and intact sprouting data is 98.004, so this value was arbitrarily changed to 100.

Appendix N: Statistical Analysis of Vasopressin mRNA Pools (INSTAT)

At one week post-surgery:

Student's t Tests (unpaired, two-tailed)

GROUP	SIDE	N	MEAN	SEM	p
Intact	Pooled	12	100	5.764	
					0.0003
Lesion	Sprouting	7	159.08	14.060	
					< 0.0001
L + hNa	Sprouting	7	44.761	4.357	
					< 0.0001
Intact	Pooled	12	100	5.764	

At four weeks post-surgery:

Student's t Tests (unpaired, two-tailed)

GROUP	SIDE	N	MEAN	SEM	p
Intact	Pooled	8	100	13.285	
					0.1913
Lesion	Sprouting	9	129.78	16.746	
					0.0008
L + hNa	Sprouting	6	34.768	8.919	
					0.0026
Intact	Pooled	8	100	13.285	

Please note that each p value was generated by comparing the data on the line above and the line below the p value.

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