



Unilateral hypothalamic lesion results in compensatory sprouting of contralateral magnocellular neurosecretory axons in the rat neural lobe  
by John Andrew Watt

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in  
Biological Sciences  
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**Abstract:**

Sprouting in the magnocellular neurosecretory system was investigated using a unilateral hypothalamic knife-cut of the hypothalamo-neurohypophysial tract to partially denervate the rat neural lobe (NL). Densitometric, stereometric and ultrastructural methods were utilized to demonstrate the compensatory response to denervation in this system. Quantitative immunocytochemical analysis of vasopressin levels in the NL demonstrated a transient reduction by 10 days post-surgery with a subsequent trend toward recovery reaching 88% of normal 3 months following surgery. Measures of neural lobe cross-sectional area demonstrated a similar trend with an initial reduction in size followed by a recovery to normal cross-sectional area over an identical time course. Morphometric measurement of contralateral supraoptic (SON) and paraventricular (PVN) magnocellular neurons known to project to the neural lobe revealed a persistent hypertrophy of both soma and cell nuclei in both areas throughout the post-surgical period examined. Ultrastructural investigations revealed a reduction in axon number in the NL following surgery which paralleled the decrease in vasopressin immunoreactivity and cross-sectional area. This reduction was followed by a partial recovery of axon number closely correlated with the increase in normal vasopressin immunoreactivity and size of the neural lobe. These results suggest that contralateral magnocellular vasopressinergic efferents will undergo compensatory sprouting as a result of partial denervation of the neural lobe.

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IN THE RAT NEURAL LOBE

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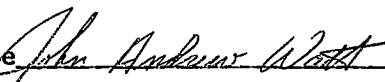
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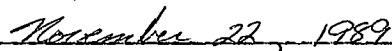
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Date

A handwritten date "November 22, 1989" written in cursive script over a horizontal line.

This thesis is dedicated to the late George Michael Carson whose unbounded friendship and undaunted spirit will forever provide a source of strength to those who knew and loved him.

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## ABSTRACT

Sprouting in the magnocellular neurosecretory system was investigated using a unilateral hypothalamic knife-cut of the hypothalamo-neurohypophysial tract to partially denervate the rat neural lobe (NL). Densitometric, stereometric and ultrastructural methods were utilized to demonstrate the compensatory response to denervation in this system. Quantitative immunocytochemical analysis of vasopressin levels in the NL demonstrated a transient reduction by 10 days post-surgery with a subsequent trend toward recovery reaching 88% of normal 3 months following surgery. Measures of neural lobe cross-sectional area demonstrated a similar trend with an initial reduction in size followed by a recovery to normal cross-sectional area over an identical time course. Morphometric measurement of contralateral supraoptic (SON) and paraventricular (PVN) magnocellular neurons known to project to the neural lobe revealed a persistent hypertrophy of both soma and cell nuclei in both areas throughout the post-surgical period examined. Ultrastructural investigations revealed a reduction in axon number in the NL following surgery which paralleled the decrease in vasopressin immunoreactivity and cross-sectional area. This reduction was followed by a partial recovery of axon number closely correlated with the increase in normal vasopressin immunoreactivity and size of the neural lobe. These results suggest that contralateral magnocellular vasopressinergic efferents will undergo compensatory sprouting as a result of partial denervation of the neural lobe.

1  
INTRODUCTION

Statement of Purpose

The purpose of this study was to determine if compensatory collateral sprouting of intact magnocellular vasopressinergic axons will occur within the neurohypophysis of the rat following unilateral transection of the hypothalamo-neurohypophysial tract.

A brief survey of the history of research into neural regeneration within the mammalian central nervous system (CNS) will be presented. This will be followed by a comparison of the different forms of injury-induced central neural reorganization, and a discussion of the current evidence for functional recovery following injury and the possible correlates observed within the CNS. Emphasis will then be placed on the occurrence and types of axonal sprouting observed within the magnocellular and parvocellular neurosecretory systems with an anatomical description of this system provided for the reader's orientation.

Historical Perspectives

Understanding neuronal response to injury and the regenerative capacity of the central nervous system (CNS) has been a basic goal of neurobiological research since the pioneering work of the late nineteenth and early twentieth centuries. The existence of regenerative nerve fibers was first reported by Eichhorst and Naunyn in 1874 (Clemente, 1964). These investigators observed very thin nerve fibers penetrating the scar tissue barrier resulting from intradural spinal

crush in rabbits and dogs. Twenty years later Stroebe described nerve fibers penetrating the scar tissue barrier formed following complete spinal transection in dogs (Clemente and Windle, 1954). These findings were extended by Bielchowsky who, in 1906, reported finding newly grown nerve fibers in the peripheral regions of tumors in brain and spinal cord (Cotman et al., 1981). Bielchowsky recognized the existence of terminal boutons within the growth plexus and concluded that a true regeneration of central axons had taken place. It was during this same period that Ramon Y. Cajal examined the effects of spinal transection in dogs and cats. He too described what appeared to be newly formed neuronal fibers and growth cone profiles in the scar tissue barrier which bridged the site of transection. Cajal (1928) reported a diminishment in the fiber density over a period of 4-5 weeks, leading him to conclude that the regenerative process was aborted. These early studies led to the formulation of what was until recently the widely accepted doctrine of abortive regeneration in the (CNS); it stated that an initial regenerative state is entered by the injured axon but will shortly thereafter be terminated. In Cajal's own words, "Once the development was ended the founts of growth and regeneration of the axons and dendrites dried up irrevocably. In adult centers the nerve paths are something fixed, ended, immutable. Everything may die, nothing may be regenerated." (Cajal, 1928; pg. 286).

Many investigators began to examine the factors underlying the apparent termination of the regenerative process. Cajal (1928) postulated that the CNS was lacking in a Schwann cell-like supportive element necessary for the sustenance and guidance of growing fibers.

Cajal went on to suggest that peripheral nerve implants into sites of CNS necrosis might stimulate an ingrowth of CNS fibers owing to the "neurohumoral" factors released by the Schwann cells (Clemente, 1964). Subsequent research lent support to this hypothesis, but again the regenerative process was terminated as the implanted tissue was resorbed in the weeks following surgery (Clemente, 1964). Others suggested that vascular damage resulting from CNS lesions may induce an "isolation dystrophy" leading to cell death in the ischemic region hence limiting opportunities for neuronal outgrowth (Clemente, 1964). Impenetrable scar formation was thought to be a factor by others (Windle et al., 1952; Clemente, 1955; Kao, 1977; Brown, 1947). Clark (1943) suggested the density of the mature CNS was incompatible with regrowth. Similarly, Arteta (1956) believed central neural regeneration was limited by the lack of an adequately arranged matrix for neurite guidance rather than by an intrinsic inability of axons to regenerate.

These suggestions were all based upon numerous studies demonstrating a severe limitation in the CNS capability for regrowth. However, these investigators were limited by the anatomical methods then available, principally silver impregnation techniques. With the advent of modern anatomical techniques and in vitro systems for monitoring neurite outgrowth, it has become apparent that the neurons of the CNS display a tremendous propensity for regrowth and terminal reorganization following injury. As a result, renewed interest has arisen in the capacity for repair within the central nervous system, and numerous reports are now available describing this phenomenon in a variety of CNS systems.

Plasticity in the Mammalian CNS

These recent studies show that the mammalian central nervous system is capable of modifying synaptic contacts in response to injury, hormonal perturbations or environmental stimuli; this capacity is generally referred to as plasticity (Cotman et al., 1984). The principal evidence for plasticity comes from investigations of injury-induced responses (Cotman et al., 1984). Injury-induced responses commonly occur in two forms, regeneration and sprouting, which differ in several ways. First, regeneration is a term generally used to describe the regrowth of a single fiber from the proximal end of the severed axon and is most frequently observed following injury to peripheral nerves; sprouting refers to the outgrowth of multiple collateral fibers from the proximal end of the severed axon or from neighboring intact axons. Second, the distance over which regrowth may occur is generally more extensive in regenerating axons than in sprouting axons (Cotman et al., 1984). Sprouts may arise from collaterals of intact fibers sharing the partially denervated terminal field (compensatory collateral sprouting), or from sustaining collaterals of injured axons proximal to the site of injury (regenerative sprouting; Kiernan, 1971). Injury-induced sprouting, often termed reactive synaptogenesis, may occur in several different forms, e.g., sprouts arising from nodes of Ranvier (nodal sprouting), from preexisting axon collaterals (collateral sprouting), or from the terminal bouton of intact axons innervating a partially denervated terminal field (terminal sprouting; Cotman et al., 1984). Although sprouting in any form can be extensive, it is generally more limited in the distance over which it may occur (Cotman et al., 1984).

and tends to occur more rapidly (Murray, 1986) than regeneration.

#### Neuronal Reorganization and Recovery of Function

If regeneration and sprouting represent forms of anatomical recovery from injury, the question arises whether or not such changes coincide with some degree of functional recovery. Although numerous studies have described some form of axonal reorganization following axotomy (eg., regeneration, sprouting of intact collaterals or sprouting of injured axons), relatively few have demonstrated a correlation between axonal growth and recovery of function (Finger and Almlı, 1985). In fact, the associations made between neural regrowth and functional recovery have been so sporadic that several apposing hypotheses have arisen regarding the underlying purpose of the reorganizational process. Some authors maintain that the reorganizational process is strictly governed by the need of the system to achieve a level of function close to that maintained prior to the injury. Others suggest that the neural changes associated with injury are related to developmental processes and may result from an alteration in the balance between growth stimulating and growth suppressing factors (Finger, 1985). Later authors argue that any recovery of function associated with the reorganizational process is strictly coincidental. Support for the developmental hypothesis comes from studies of age-related factors and sprouting (Kawamoto and Kawashima, 1985a, 1985b, 1987). These studies have demonstrated that the initiation of the sprouting response is most rapid in the developing brain, decreases in the mature brain and is slower still in the aged brain (Cotman et al., 1984; Finger and Almlı,

1985).

If indeed injury induced reorganization is related to a developmental process, it follows that this reorganization would be most likely to occur or to occur more rapidly during periods of rapid brain growth (Gall et al., 1981; Tsukahara et al., 1981; Cotman and Lynch, 1984; Tsukahara, 1985). This does not, however, indicate that neural reorganization cannot take place in adult tissue. As mentioned above, there is ample evidence suggesting that mature systems do retain this ability but generally to a more limited extent (Cotman et al., 1984; Kawamoto et al., 1985; Needles et al., 1986). Indeed, neural reorganization following injury can lead to behavioral dysfunction rather than recovery. For example, reflex spasticity is observed secondary to spinal injuries (McCouch, 1958) and abnormal feeding behavior is observed in hamsters following contralateral compensatory sprouting in the superior colliculus (Schneider, 1976). However, there are conditions under which sprouting in a previously denervated zone may contribute to recovery of function. Loecshe and Stewart (1975) demonstrated that unilateral damage to the entorhinal cortex resulted in initial impairment of performance of a trained task in rats; subsequently, task performance improved. Histologic examination demonstrated that sprouting had occurred within the contralateral entorhinal cortex and that the time course of sprouting paralleled that of behavioral recovery.

Wuttke et al. (1977) reported functional recovery paralleling axonal regrowth in the anterior hypothalamus. Serotonergic input to the hypothalamus was interrupted using 5,7-dihydroxytryptamine (DHT)

chemical axotomy. Initially there was a marked reduction in plasma luteinizing hormone (LH) levels; however, two months following surgery the LH levels returned to normal values. Axonal regeneration was evidenced by an increase in  $^3\text{H}$ -5HT uptake and release in the anterior hypothalamus which paralleled the recovery of LH levels.

Functional recovery following unilateral chemical axotomy of the descending serotonergic bulbospinal afferents was described by Nygen et al. (1974). Using the hindlimb extensor reflex as a measure of recovery, these investigators demonstrated that the initial 5-HT hypersensitivity observed following chemical axotomy disappeared within three months following surgery. This recovery was shown to closely parallel the time course of sprouting.

The prolific regrowth observed following damage to the axons of the magnocellular neurosecretory system has been repeatedly correlated with functional recovery (Raisman, 1977; Kawamoto, 1985; Kawamoto and Kawashima, 1987). Hypophysectomy, the removal of the pituitary gland and distal infundibulum, is followed by the reestablishment of neurosecretory contacts with the capillaries of the mantle plexus in the zona externa of the median eminence (Raisman, 1977) and within the pseudo-neural lobe formed proximal to the stalk section (Kawamoto and Kawashima, 1987). Coincident with the development of these neurosecretory contacts, the polyuria and polydipsia associated with hypophysectomy disappear. The functional recovery achieved is such that the animal not only maintains homeostatic hydration levels but is able to resist moderate dehydration stress (Moll and DeWeid, 1962). Polenov (1974) found that salt loading hypophysectomized rats with a 1% NaCl

drinking solution results in a substantial decrease in the amount of neurosecretory materials present within the pseudo-neural lobe. These animals demonstrate a direct correlation between alleviation of diabetes insipidus and release of secretory products from the reorganized neurosecretory endings. The functional competence achieved by the reorganized stalk was such that animals were able to withstand a twenty day salt loading regimen without deleterious effect.

#### Sprouting of Non-Neurosecretory Systems

Murray and Thompson (1956) partially denervated the superior cervical ganglion unilaterally in the cat by surgically transecting the rami communicantes from their respective thoracic spinal segments. Following surgery up to 90% of the preganglionic fibers within the sympathetic ganglion degenerate. Electrophysiological stimulation of the surviving fibers produced a very weak contraction of the nictitating membrane. However, 4-8 weeks following the denervating lesion, stimulation of the surviving fibers resulted in a contraction of the nictitating membrane equal to that produced by stimulation of the intact contralateral side. Histological examination indicated that sprouts arose from surviving preganglionic fibers within 5 days after denervation. Functionally mature synapses were reestablished with previously denervated post-synaptic sympathetic ganglion cells by 4 weeks after denervation.

It was not until the work of Raisman (1969) that direct ultrastructural evidence of sprouting was reported. Raisman examined the changes which occurred within the medial septal nuclei following

discrete electrolytic lesions of either the fimbria or medial forebrain bundle (MFB) in the rat. The medial septal nuclei are characterized by the intermingling of hippocampal and hypothalamic projection fibers in a shared terminal field. The anatomical separation of the fimbria and MFB allows for a discrete localized lesion restricted to either pathway. In order to facilitate recognition of the changes that could take place following partial denervation, Raisman first categorized the types of synaptic profiles observed in the intact septum. It was found that the fimbrial-hippocampal afferents selectively terminate on dendrites while the MFB-hypothalamic afferents display both axodendritic and axosomatic terminals. Synapses were also categorized according to the number of post-synaptic profiles encountered in a given plane of section, termed the multiple synapse index (MSI).

In the first of two separate experiments, Raisman produced localized electrolytic lesions in the fimbria thereby targeting the hippocampal projection fibers. Post-lesion ultrastructural analysis revealed an increase in the number of double and multiple synapse profiles from an MSI of 2.0 in normal rats to an MSI of 5.0 in lesioned rats (Raisman, 1969). These data were interpreted as evidence of reinnervation of previously denervated sites by terminals of normal, intact fibers in close proximity to the denervated postsynaptic sites.

In the second experiment, the preoptic portion of the MFB sustained a long term lesion resulting in the deafferentation of the hypothalamic projection fibers within the medial septal nuclei. Two days prior to sacrifice, these animals received fimbrial lesions. Large numbers of axosomatic terminals were subsequently revealed by orthograde

degeneration. Since a lesion of the fimbria does not normally give rise to large numbers of axosomatic profiles, these data were interpreted as indicating that intact hippocampal fibers had reinnervated previously denervated somatic post-synaptic sites.

Reinnervation of previously denervated post-synaptic sites by neighboring, intact axons has also been demonstrated in the hippocampus. The entorhinal cortex gives rise to a large pathway projecting to the granule cells of the ipsilateral dentate gyrus, the perforant pathway (Hjorth-Simonsen, 1972). This pathway is known to terminate in the distal 73% of the molecular layer of the dentate gyrus, whereas the remaining 27% of the molecular layer is comprised of the terminal field of the hippocampal commissural and associational systems (Gall and Lynch, 1981). Following unilateral lesions of the entorhinal cortex a prolific sprouting response will occur arising from the axons of the associational-commissural terminal field. When performed in neonate rats e.g., 1-14 days of age, this expansion will result in the reinnervation of the entire outer molecular layer, a depth of approximately 300um, with reinnervation complete by 48hrs post-lesion (Gall and Lynch, 1981). In adult rats, under identical conditions, the commissural fibers will expand their field to occupy approximately 37% of the outer molecular layer, a value close to that of their normal terminal contribution to the inner layer. However, there is a delay of approximately four days in the onset of the sprouting response in the adult while no delay is apparent in the neonate. The more extensive sprouting observed in neonates is characterized by the formation of two terminal zones. The inner (50um) field is characteristic of both the

neonatal and adult pattern while the expansive outer zone of reinnervation is characterized by the outgrowth of long, thick collateral fibers from which the outer terminals arise. The number of these collaterals diminishes as the age of the animal, at time of surgery, increases (Gall and Lynch, 1981).

More recent autoradiographic evidence also indicates the existence of a sparse crossed projection which innervates the rostral contralateral dentate gyrus, the temporo-dentate projection (Steward and Lynch, 1976). This projection, like the ipsilateral homologous perforant pathway, typically terminates within the outer molecular layer of the dentate gyrus (Steward and Lynch, 1976). In response to unilateral ablation of the entorhinal cortex this crossed input will undergo a prolific sprouting response, increasing its terminal contribution to the contralateral dentate gyrus by 500% (Steward and Lynch, 1976). Autoradiographic and electrophysiological evidence demonstrate that the reorganized terminals are intact and retain the functional specificity of the normal dentate gyrus (Steward and Lynch, 1976).

Different types of central neurons differ in their capacity to regenerate following injury. Those with lightly myelinated or unmyelinated axons generally display a higher propensity for regrowth than heavily myelinated systems (Cotman et al., 1981). Monoaminergic neurons will regenerate over considerable distances in the brain and spinal cord following axotomy with reestablishment of some of their original connections (Bjorklund et al., 1979). The extent of this regeneration and, more generally, of regeneration throughout the CNS,

depends largely on the degree of cellular damage sustained. Damage restricted to the distal axonal fields will generally result in a more prolific response than when the damage is in close proximity to the cell body (Raisman, 1977).

Sprouting has been reported in a number of central catecholaminergic systems including rat mesencephalon (Katzman et al., 1970), cat spinal cord (Bjorklund et al., 1971), and rat lateral geniculate body (Stenevi et al., 1972). Following surgical transection or mechanical crush of central catecholaminergic axons, there is a rapid accumulation of neurotransmitter proximal to the site of lesion. This rapid buildup becomes demonstrable histochemically within hours of the lesion. These fibers generally display a rough-varicose morphology for the first few days following axotomy. Over time, however, bundles or plexuses of fine varicose fibers begin to appear surrounding or penetrating the site of injury (Katzman et al., 1971; Bjorklund and Stenevi, 1979; Dellman et al., 1987).

The most extensive regeneration seen in the monoaminergic systems occurs in the descending serotonergic (5-HT) bulbospinal pathways following 5,6-dihydroxytryptamine (DHT) treatment. In this system sprouting from the proximal stumps has been observed as early as 4-5 days following chemical axotomy (Bjorklund and Stenevi, 1979). Following almost complete destruction of the 5-HT spinal afferents, normal to near normal reinnervation patterns are reestablished in the cervical spinal segments after 2 months. By 8 months a partial recovery is seen in the more distal thoracic and lumbar segments.

This considerable propensity for regrowth was further demonstrated

by Wuttke et al. (1977), following unilateral intraventricular injection of 5,7-DHT. This systemic chemical axotomy was followed by an almost complete reorganization of the serotonergic neurons throughout the central nervous system. A similar phenomenon has been observed in the descending noradrenergic bulbospinal afferents following intracisternal injection of 6-hydroxydopamine (6-OHDA)(Nygren et al., 1976). This treatment destroyed approximately 90% of the descending noradrenergic afferents. As was seen in the 5-HT system, a gradual return of immunofluorescent fibers occurred first as an essentially complete and normal reinnervation of the cervical cord segments, followed by the partial reinnervation of the thoracic and lumbar segments by 6-7 months post-surgery (Nygren et al., 1976).

The red nucleus, which plays a key role in motor control, has also served as a model system for investigation of sprouting in the central nervous system. The red nucleus receives afferents from two sources, the cerebrum via the cerebral peduncles and the cerebellum from axons arising from the interpositus nuclei. The terminal fields of these sets of afferents are topographically segregated. The cerebral afferents terminate exclusively on the distal dendritic field while the afferents from the nucleus interpositus are limited to axosomatic synapses. Tsukahara (1985) and associates have provided both electrophysiological and ultrastructural evidence for sprouting in this system in both kittens and adult cats under a variety of experimental conditions. These investigators found that electrolytic interruption of the cerebral afferents in kittens resulted in sprouting from three sources; the contralateral cerebral peduncles, the ipsilateral interpositus nucleus

and the contralateral interpositus nucleus. Similar lesions of the interpositus input in kittens resulted in sprouting from the contralateral cerebral input. Effects of identically placed lesions in the adult cat differed from those in kittens in that sprouting occurred in the contralateral cerebral peduncle following interruption of the interpositus input but not after interruption of the cerebral input. However, the topographical specificity of these two input sources was preserved in both kittens and adult cats. In summary, kittens demonstrated a higher propensity for sprouting than did adults. In addition, the topographical arrangement of the segregated afferents was preserved indicating that distinct postsynaptic guidance cues may play a role in the reorganizational process.

Cellular events accompanying the sprouting process have also been observed. For example, cellular hypertrophy following injury has been well documented. Hypertrophy of both somata and nuclei in supraoptic (SON) neurons paralleled the formation of a pseudo-neural lobe in the proximal infundibular stalk following hypophysectomy (Moll and DeWeid, 1962; Raisman, 1973). This response is an example of regenerative sprouting e.g., sprouts arising from the severed axons at the proximal side of the stalk lesion. Moll and DeWeid (1962) attributed this enlargement to a heightened level of activity in these cells. Pearson et al. (1987a) described an increase in cell size in the contralateral pars compacta and ipsilateral pars reticulata of the substantia nigra, and the contralateral globus pallidus following unilateral ablation of the left striatum and overlying cortex. These investigators also demonstrated hypertrophy of cholinergic cells of the rat basal forebrain

following damage to the contralateral cortex. This enlargement was apparent by 7 days post-surgery and persisted for at least 300 days post-surgery (Pearson et al., 1984). The degree of hypertrophy observed was related to the age of the animal (Pearson et al., 1985) and the degree of damage sustained (Pearson et al., 1987). They went on to conclude that the hypertrophy represented, "a cell soma event accompanying a distant axonal sprouting" (Pearson et al., 1987).

Cotman et al. (1977) have reported that increase in cell size is one of the earliest manifestations of the sprouting response. In several cases a correlation has been demonstrated between cellular hypertrophy and sprouting of intact efferents of the enlarged cells contralateral to the site of injury (Goldschmidt and Steward, 1980; Hendrickson, 1982; Headon, 1985; Pearson, 1987), in a manner similar to that observed in this investigation. Thus, cellular hypertrophy in response to direct axonal injury or in response to partial denervation of a terminal field shared with other lesioned neurons is a well described cellular event.

#### The Magnocellular Neurosecretory System

The magnocellular neurosecretory system (MNS) is composed of the large oxytocinergic and vasopressinergic cells of the paraventricular, supraoptic and accessory nuclei, which project to the hypothalamo-neurohypophysial tract (Silverman, 1983; Ju et al., 1986).

The supraoptic nucleus (SON) is comprised of a compact mass of magnocellular neurons lying just anterolateral to the optic chiasm and optic tract termed the principle division (Ju et al., 1986), and a loose aggregation of magnocellular neurons positioned along the dorsolateral

aspect of the optic tract and ventral surface of the brain at the level of the arcuate nucleus termed the retrochiasmatic division (Rhodes et al., 1981). Within the principle division of the SON there exists a partial segregation of vasopressinergic (VP) and oxytocinergic (OT) neurons. The VP neurons are located predominately in the posteroventral region while the OT neurons are found predominately within the anterodorsal region (Vandesande and Dierickx, 1977; Rhodes et al., 1981; Swanson and Sawchenko, 1983).

The paraventricular nucleus (PVN), once thought to be a uniform aggregation of magnocellular neurons (Christ, 1969), is now known to be composed of eight topographically distinct magnocellular and parvocellular subdivisions (Armstrong et al., 1980; Swanson and Sawchenko, 1983; Silverman, 1983). These subdivisions are comprised of three magnocellular and five parvocellular cell groups. The three magnocellular subgroups are described as the anterior, the medial and the larger posterior magnocellular subdivisions. The five parvocellular subdivisions are comprised of the anterior, medial, posterior, lateral and periventricular cell groups (Swanson and Sawchenko, 1983). A topographically distinct arrangement of VP and OT neurons also exists in the PVN. The anterior and medial magnocellular subdivisions are comprised almost exclusively of OT neurons. In the posterior magnocellular subdivision the VP neurons are located predominately within the posterodorsolateral region and the OT producing neurons are found predominately in the anteroventromedial region (Swanson and Sawchenko, 1983).

In addition to vasopressin and oxytocin, at least 30 other

neuropeptides have been immunocytochemically localized within the MNS including: somatostatin, dynorphin, enkephalin, gastrin, and luteinizing hormone releasing hormone (LHRH), to name but a few. However, their functions remain largely unclear (Silverman, 1983; Swanson and Sawchenko, 1983; Ju et al., 1986).

Retrograde transport studies utilizing horseradish peroxidase (HRP) injections into the neural lobe (NL) have further delineated the cell groups contributing terminals to the NL (Armstrong et al., 1980; Kelly and Swanson, 1980; Ju et al., 1986). Almost all of the cells of the SON are labeled following these injections. SON axons are seen rising in a fan-like array from the dorsal aspect of the principle division of the SON before coursing ventromedially toward the internal lamina of the median eminence (ME) and infundibular stalk. Axons arising from the more lateral regions of this division first run anterolaterally while the medially placed cells send axons anteromedially. Both groups then stream caudally over the optic tract to join the fibers arising from the dorsal SON. Those axons originating from the retrochiasmatic division of the SON were seen to emerge from the dorsal aspect and stream medially, dorsolaterally and anterodorsally. However, only the anterodorsal axons were traceable for any distance and were seen to join their principle division counterparts along the dorsal surface of the optic tract (Ju et al., 1986).

Within the PVN the cell groups most heavily labeled following retrograde transport of HRP from the NL are predominately concentrated within the medial and posterior magnocellular subnuclei (Silverman, 1983) corresponding to the PVM and PVL of Armstrong et al. (1980).

However, scattered large and small neurons within several parvocellular subnuclei are also labeled, with the periventricular division being the most substantial contributor (Swanson and Kuypers, 1980; Ju et al., 1986). The axons arising from the magnocellular divisions of the PVN project ventroposterolaterally from the PVN to course around the dorsal and ventral aspects of the fornix then sweep posteroventromedially to occupy the lateral horns of the zona interna of the median eminence on their way to the NL (Ju et al., 1986; Swanson and Sawchenko, 1983).

The magnocellular projections arising from the PVN and SON remain topographically segregated throughout their course through the hypothalamo-neurohypophysial tract. The SON efferents occupy the central two thirds of the zona interna, decussate within the median eminence, then continue through the infundibular stalk to form a widespread terminal distribution throughout the entire NL, with a higher concentration in the central core. The PVN fibers, on the other hand, maintain an ipsilateral distribution throughout their course through the lateral zona interna and infundibular stalk, and terminate primarily in the periphery of the NL (Silverman, 1983; Alonso and Assenmacher, 1981). The functional correlates, if any, of this anatomical separation remain unknown (Silverman, 1983). Silverman et al. (1980) have also reported reciprocal connections, predominately oxytocinergic, between the two PVN and between each ipsilateral PVN and SON. The regions receiving afferents from the contralateral PVN appear to be the PVL and PVM subnuclei (Silverman, 1983).

Efferents arising from the PVN contribute to the functionally and morphologically distinct parvocellular and extrahypothalamic

neurosecretory systems as well as the magnocellular system. Parvocellular neurosecretory efferents are labeled by HRP or true blue injections localized to the zona externa of the ME. This preparation results in cellular labeling restricted to the periventricular, medial and anterior parvocellular subdivisions (Swanson and Sawchenko, 1983). Immunocytochemical analysis suggests that the majority of axons innervating the zona externa are vasopressinergic (Silverman, 1983; Dierickx et al., 1976), although oxytocinergic fibers have also been observed (Vandesande and Dierickx, 1977). Immunocytochemical evidence further indicates that the terminals in the external ME are not collaterals of the axons projecting to the NL via the zona interna (Antunes et al., 1977), suggesting that the PVN is the sole source of fibers to the zona externa (Alonso and Assenmacher, 1981). The axons of the parvocellular neurosecretory system terminate on the primary portal plexus and are thought to be part of a neurovascular link between the neurosecretory system and the adenohipophysis. The release of neurosecretory products may exert some degree of control over adenohipophysial hormonal secretion (Swanson and Sawchenko, 1983; Antunes et al., 1977). Further indications of functional differences between these systems have been reported. Severe dehydration will result in a depletion of neurosecretory granules (NSG) from the NL but has no effect on the NSG populations in the external ME. Likewise, adrenalectomy will cause an increase in the size of the NSG population in the external ME but has no effect on NSG populations in the NL (Silverman, 1983).

In addition to the hypothalamic projections described above,

extrahypothalamic projections arising from the PVN have been shown to terminate within various nuclei of the forebrain, midbrain and spinal cord (Sofroniew et al., 1980; Rhodes et al., 1981) as well as within preganglionic cell groups of the sympathetic and parasympathetic autonomic nervous system (Swanson and Sawchenko, 1983; Silverman, 1983). The parvocellular neurosecretory cells projecting to the NL (Swanson and Kuypers, 1980) or ME (Swanson et al., 1980) are distinct from those innervating the spinal cord (Swanson and Sawchenko, 1983).

In summary, the magnocellular, parvocellular and extrahypothalamic efferents of the PVN form anatomically and functionally distinct neurosecretory pathways within the central nervous system.

#### Regeneration in the Magnocellular Neurosecretory System

Actual regeneration of MNS axons has been reported in the ferret (Adams et al., 1969). In this study the infundibular stalk was severed but the cut ends were left juxtaposed, and a Gomori stain for neurosecretory material was used to follow the course of regeneration from two weeks to twelve months. By two weeks the proximal portion of the lower infundibular stalk displayed clear indications of regenerative fibers having penetrated the scar tissue barrier. From one to three months following surgery the regenerating axons had grown as far as the caudal end of the infundibular stalk. At five months the regenerating fibers had invaded the neural lobe and in two animals sacrificed at one year post-surgery the entire neural lobe had been reinnervated. Several animals also developed a neural lobe-like structure just distal to the site of lesion. This growth is similar to the pseudo-neural lobe-like

structure formed in the transected infundibular stalk following hypophysectomy as described above.

Regeneration of severed MNS axons was also reported in rats following replacement of the neural lobe immediately after its removal, by suction, from the sella turcica. However, successful reinnervation of the neural lobe was only observed in two cases where the proximal infundibular stalk had not degenerated (Kiernan, 1971).

Within the central nervous system proper, the penetration of intrahypothalamic allografts of sciatic nerve, optic nerve, hypodermal connective tissue or neural lobe by regenerating neurosecretory fibers following stereotaxic placement of the grafts between the SON and PVN has been repeatedly demonstrated (Dellman et al., 1985a, 1985b, 1986a, 1987). The invading neurosecretory axons form plexuses in the periphery of the grafts. Ultrastructural analysis of these plexuses indicate that the terminals formed at the basement membrane surrounding the vessels invading the grafts are functional (Dellman et al., 1987). Although the presence of graft material is required for growth of axons within the hypothalamus proper, it is not required for growth of neurosecretory axons into the ventral leptomeninges of the rat (Dellman et al., 1988). Following unilateral knife cuts of the ventral hypothalamic surface, neurosecretory axons grew into all layers of the leptomeninges for distances up to 3.3mm from the lesion site by 40 days post-surgery. In addition, these axons showed morphologic signs of neurosecretory activity (Dellman et al., 1988). Accumulations of neurosecretory granules within the perivascular space surrounding small blood vessels were also observed near the lesion site following a hypothalamic

knifecut in the monkey (*Macaca mullata*). These accumulations were found in both SON and PVN efferent pathways (Antunes et al., 1977).

#### Sprouting in the Magnocellular and Parvocellular Neurosecretory Systems

The magnocellular neurosecretory system has been the subject of extensive investigations of axonal sprouting in a variety of experimental preparations. Interruption of the hypothalamo-neurohypophysial tract either by hypophysectomy, neurolobectomy, infundibular stalk transection or hypothalamic lesion will result in morphological reorganization of the neurosecretory fibers proximal to the site of lesion in a variety of species. This effect was first described by Stutinsky in the rat (1951, 1952). He later confirmed his findings in the eel (1957). These initial reports were followed by further studies in the rat (Billenstein and Leveque, 1955; Moll, 1957; Moll and De Weid, 1962; Dellman and Owsley, 1969; Raisman, 1973; Polenov et al., 1974; Polenov et al., 1981; Yulis and Rodriguez, 1982; Kawamoto and Kawashima, 1986, 1987), mouse (Kawamoto, 1985; Kawamoto and Kawashima, 1985), goat (Beck, et al., 1969), ferret (Adams, Daniel and Prichard, 1969), rabbit (Gaupp and Spatz, 1955), frog (Rodriguez and Dellman, 1970), Rhesus monkey (Antunes et al., 1978), and human (Beck and Daniel, 1959).

Following hypophysectomy in rats, two reorganizational events have been observed. First, the magnocellular neurosecretory axons which are normally confined to the internal zone of the median eminence grow ventrally into the external zone. Here they establish functionally competent contacts with fenestrated capillaries. These "synaptoid

contacts" differ morphologically from axoaxonic, axodendritic or axosomatic synapses. The contact occurs between the terminal ending of the axon and the outer of two basement membranes enclosing the perivascular space. When in an inactive state, these terminals are separated from the basement membrane by the active inclusion of a pituicyte foot process. Upon metabolic demand the pituicyte withdraws and contact is reestablished. Hence these synaptoid contacts are dynamic and display an inherent functional plasticity in both the SON nuclei and neurohypophysis (Theodosis and Poulain, 1987).

Ultrastructural examination found these neurosecretory contacts to be identical to those of the intact neural lobe (Raisman, 1977). These investigators found that the neurosecretory axons reorganized into a miniature neural lobe-like structure at the proximal end of the transected infundibulum (Billenstein and Leveque, 1955; Moll and DeWeid, 1962; Kiernan, 1971; Raisman, 1973; Polenov et al., 1974; Kawamoto and Kawashima, 1987). Formation of new neurohemal contacts in both the ME and proximal infundibular stalk is accompanied by a general hypertrophy of the capillary mantle plexus (Raisman, 1973). A similar process has been described in ferrets (Adams et al., 1969) and mice (Kawamoto and Kawashima, 1985).

Silverman and Zimmerman (1982) have demonstrated a sprouting response within the parvocellular neurosecretory system following unilateral electrolytic lesion of the paraventricular nucleus in adrenalectomized rats. As described above, the parvocellular neurosecretory axons projecting from each PVN normally remain ipsilateral within their terminal field in the external zone of the

median eminence (Alonso and Assenmacher, 1981; Silverman and Kimmerman, 1982; Vandesande et al., 1977; Antunes et al., 1977). Approximately 21 days following unilateral destruction of the PVN, however, both HRP injections into the intact contralateral PVN and immunocytochemical localization of vasopressin-neurophysin within the median eminence revealed a bilateral innervation of the external zone. It was concluded that these previously undetected fibers were sprouts from the undamaged terminals of the contralateral PVN efferents.

#### Justification, Hypothesis and Experimental Design

As indicated in the discussion above, the magnocellular neurosecretory system has displayed a tremendous propensity for regrowth following a variety of experimental perturbations. However, under all experimental conditions reported to date, the regrowth has been restricted to a sprouting or regenerative response arising from the transected fibers. Only within the parvocellular neurosecretory system has the occurrence of compensatory collateral sprouting arising from intact contralateral neurosecretory fibers been demonstrated following injury (Silverman and Zimmerman, 1982). Hence, there exists a gap in our understanding of the response of intact magnocellular neurosecretory efferents to injury or partial denervation.

The anatomy of the magnocellular neurosecretory system provides an excellent environment in which to investigate the possible occurrence of compensatory collateral sprouting. Magnocellular neurosecretory efferents originating from the contralateral SON and PVN project to the neurohypophysis, at some distance from the site of origin, to terminate

in a shared terminal field. Unilateral transection of the magnocellular efferents via hypothalamic knifecut would result in partial denervation of the common terminal field. Any compensatory sprouting response subsequently observed within the neurohypophysis could then be attributed to the surviving contralateral magnocellular efferents. Hence, the following hypothesis was proposed: Following unilateral hypothalamic knife-cut the surviving, intact, contralateral magnocellular efferents within the neurohypophysis will undergo compensatory collateral sprouting in an effort to reinnervate the partially denervated terminal field.

In order to test the strength of the hypothesis a series of four experiments were done. In the first experiment, the cross-sectional area of the contralateral SON and magnocellular PVN cell bodies and their respective nuclei were measured as an indicator of the cellular response to the injury. As noted above, the occurrence of cellular hypertrophy has been correlated with the sprouting response in other CNS systems following injury. In the second experiment, measures of neural lobe cross-sectional area were compiled in a manner similar to that used in the first experiment. It seemed likely that an extensive loss of efferents following surgery would result in a measurable reduction in the cross-sectional area of the neural lobe. Any subsequent trend toward recovery of neural lobe cross-sectional area could indicate an increase in the population of axons within the terminal field thus providing additional, indirect evidence for a sprouting event.

In experiment 3, a quantitative immunohistochemical technique was employed. It was reasoned that an injury-induced loss and subsequent

trend toward recovery of vasopressin staining density within the neural lobe would reflect the loss and subsequent recovery of vasopressinergic efferents, thus providing a more direct indication of a sprouting response. However, the quantitative immunohistochemical evidence, although providing strong support, would not conclusively demonstrate a sprouting response over other possible explanations for the shift in staining density. Therefore, in experiment 4, an ultrastructural survey of the axonal population within the neural lobe was undertaken in order to directly measure changes in axon number. In addition, the relative area of neural lobe occupied by axons, glia and extracellular space was determined in order to elucidate the degree of axonal recovery and other possible manifestations of the response of the magnocellular neurosecretory system to partial denervation.

## METHODS

Experimental Design

The experiments described below were segregated into two major experimental divisions, light level and ultrastructural. The surgical procedures and subject age at time of surgery (35 days post-parturition), were identical throughout this investigation. Table 1 lists the experimental groups prepared, and the age and number of animals within each group at the time of sacrifice.

The post-surgical (PS) survival periods selected for light level analysis, e.g., PS 10-218 days, were used to determine the time table of events following surgery at each level of analysis described below. Littermate sham controls were prepared for each experimental group using identical surgical procedures except the lesion extended only - 5mm below bregma to insure that the ipsilateral PVN and SON sustained no damage.

Animals

Young male Holtzman rats were used. A higher propensity for sprouting exists in neonates than adult or aged animals (Cotman and Lynch, 1976; Tsukahara, 1981), and this capacity decreases with age (Cotman, 1984; Finger and Almlı, 1985; Kawamoto and Kawashima, 1987). Therefore, we chose to use young rats to avoid age-related factors that might inhibit the sprouting response. The precise age was arbitrarily chosen at 35 days post-parturition. Animals were individually housed under 12L:12D light cycle with ad lib access to lab chow and water.

Table 1. Summary of animals used for light and ultrastructural analysis.

Exp. Group	Light level analysis			ultrastructural analysis	
	# exp. animals	# controls sham	intact	# exp. animals	# cont. intact
PS 10 Day	5	3	3	3	2
PS 20 Day	5	3	3	-	-
PS 30 Day	6	3	3	-	-
PS 32 Day	-	-	-	3	2
PS 90 Day	5	3	3	3	2
PS 218 Day	2	-	-	-	-

PS=post surgery, each subsequent number denotes the post-surgical survival period in days.

#### Surgery

Prior to surgery each animal received Na-Pentobarbital, 50mg/kg intraperitoneal (IP) to induce a surgical plane of anesthesia. Animals received methoxyflurane to prolong or deepen the plane of anesthesia as necessary. Each animal received a preanesthetic injection of atropine sulfate, 0.5mg subcutaneous (SC), to reduce mucous secretions.

Following anesthesia, rats' heads were shaved, washed with 100% ethanol (ETOH) and positioned in the stereotaxic apparatus with the incisor bar in the horizontal position. Incisions were then made in a caudal to rostral direction and the dorsal surface of the cranium exposed, cleared of fascia and dried. The tip of the stereotaxic knife blade (see appendix for materials and design), was positioned at bregma and the coordinates recorded. The lesion coordinates, AP -2mm to +5mm, ML - 0.5mm, (Paxinos and Watson, 1986) were then marked on the surface of the cranium with bregma serving as stereotaxic zero. A burr hole was then made through the skull using a Dremel moto flex drill and #3 carbide

burr, and care was taken to insure that the hole had clean, smooth edges to reduce the possibility of scalp irritation after surgery. The dura mater was incised using a sterile, small gauge hypodermic needle. Any excessive bleeding was interrupted by applying pressure with a Gelfoam pad directly to the wound site. Once the incision was completed and cleared of debris, the stereotaxic knife blade was then repositioned at -2mm AP, and lowered to the ventral surface of the cranial cavity. When the knife blade contacted the skull surface, the blade flexed slightly. The blade was then retracted 1mm and the knife slowly brought forward to the rostral extent of the lesion. The blade was then retracted and any blood and/or debris was cleared from the site. The wound was closed by first filling the burr hole with Gelfoam. The surface of the cranium was then gently wiped clean and nitrofurazone topical antibiotic applied liberally prior to suturing of the scalp. The animal was then removed from the stereotaxic apparatus and replaced in his home cage for recovery.

#### Fixation and Embedment

At the time of sacrifice animals were anesthetized by ether inhalation prior to intraventricular perfusion with Zamboni fixative (125mls 37% formaldehyde, 150mls saturated picric acid, and 725mls 0.1M  $\text{NaPO}_4$  buffer, pH 7.4) for 30 min. or until 1000 mls of perfusant had run through the specimen. Animals were then decapitated, their brains and neural lobes removed intact, blocked, and post-fixed in perfusant for 24 hours. Following post-fixation, the brains and attached neural lobes were dehydrated through a series of increasing concentrations of

ETOH to 100% ETOH, cleared in 100% xylene, then paraffin infiltrated slowly under increasing vacuum for twenty-four hours at 58 degrees centigrade. Specimens were then embedded using standard tissue embedding molds and rings (see appendix for procedures).

#### Paraffin Microtomy

Microtomy was performed on a Leitz Wetzlab paraffin microtome. Ten micron serial sections were collected from each specimen block, float-mounted singly on gelatin coated slides and dried overnight on a slide warmer prior to use.

#### Immunohistochemistry

NL vasopressin staining density was measured in each of the seven experimental groups listed above as an indication of the initial loss and subsequent recovery of vasopressinergic efferents, via sprouting, following partial denervation of the NL. Sham controls were included to control for any hemorrhage-induced alteration in NL vasopressin staining levels.

Fifteen paraffin sections of neural lobe were selected from each of six subjects for every group. Sections were selected from similar depths through the neural lobe across animals of all groups in order to ensure that the levels of neural lobe analyzed were roughly equivalent. Sections were then cleared of all paraffin traces with xylene and rehydrated through a decreasing series of ETOH concentrations leading to distilled water. All sections were then incubated in rabbit anti-arginine vasopressin antisera (1:5000)(gift of Dr. Gaj Nilaver) in 0.1M phosphate buffered normal saline (PBS) pH 7.4 at 4°C overnight. Tissue

sections were then rinsed in PBS and then exposed in succession to the secondary antibody, biotinylated goat anti-mouse IgG (1:100)(Cappel) for 60 min. at room temperature; two ten min. PBS rinses; avidin-biotin complex (Vector laboratories) 30-60 min.; two ten min. PBS rinses; and 3,3'-diaminobenzidine (DAB)(Sigma) in 0.003% H<sub>2</sub>O<sub>2</sub> for 45 min. at room temperature. Each slide was then dehydrated through an ETOH series, cleared in xylene and coverslipped.

The large number of sections to be stained required that batches, each composed of 30-slides, be prepared in series. In order to ensure variations in staining density across batches not be misinterpreted as alterations in relative levels of immunoreactive vasopressin between groups, each batch was composed of sections chosen across experimental groups.

#### Image Analysis and Morphometric Measurements

Quantification of vasopressin immunoreactivity was performed on an MCID image analysis system (Imaging Research Inc.). Microscope illumination was stabilized with a SOLA CVS transformer to prevent fluctuations in illumination from biasing densitometric measurements. Caution was taken when leaving and reaccessing the program that illumination intensities and aperture settings were precisely the same across all measurements. Calibrations were tested prior to each sampling session against a known sample. Images were projected from an Olympus BH-2 light microscope using a 2X objective through a Sierra Scientific CCD high resolution camera for relay to the MCID program and displayed on an Electrohome monitor for image editing and analysis. The

neural lobe perimeter was then traced using a PC mouse and the system automatically determined the area in  $\mu\text{m}^2$  and the mean relative staining density within the trace. An estimate of nonspecific background staining was obtained by measuring the mean density of the intermediate lobe (IL) of each sample analyzed. All sample data were recorded in a file and later downloaded to a spreadsheet program (Lotus 1-2-3) for graphic illustration and statistical analysis. Once all data had been acquired, individual mean background (IL) staining densities were subtracted from the corresponding individual mean NL staining densities. This corrected value represented the vasopressin-specific staining density observed in each sample. The group mean specific-density value and standard error were then computed for each experimental group.

The cross-sectional area of the somata and their nuclei in the SON and PVN were measured contralateral to the site of injury. The purpose of these measures was to demonstrate the cellular response to lesion and to attempt to correlate this response with the degeneration and sprouting of the partially denervated NL. Measures of nuclei cross-sectional area were to provide further evidence of enhanced cellular activity and verification of the somal measures (see discussion). Similar measures were made of cross-sectional area of NL, for the purpose of illustrating the initial reduction and subsequent recovery of cross-sectional area and correlating this response with the loss and recovery of axonal number.

To make the measurements the microscope image of the cells in the contralateral SON was projected onto a Summagraphics digitizing pad via an Olympus drawing tube. A pen was used to trace the perimeter of each

cell and its nucleus and the information relayed through an interface to a DEC 11/2 computer equipped with a stereometric program (developed by Dr. Steven Young, UCSD). Fifty individual cell somata and nuclei were measured per animal (n=4 animals per group). The criterion for cell selection was that a distinct cell boundary, a clearly defined nuclear membrane and a distinct nucleolus all be present. The total number of sections sampled was determined by the number required to acquire 50 individual samples meeting the sampling criteria. Mean cross-sectional areas of cell bodies and nuclei were then calculated for each animal and each experimental group.

#### Ultrastructural Analysis

Ultrastructural confirmation of the sprouting response was obtained by determining the axon number in a series of 15 electron micrographs collected at random through the NL of subject animals and intact littermate controls. The post-surgical groups were selected to determine the ultrastructural correlates of the loss in vasopressinergic staining density apparent at 10 days post-surgery and of the subsequent partial recovery observed at 32 and 90 days post-surgery. Additional measurements were made of the relative area occupied by axons, glia, and extracellular space (including capillary space), in each micrograph. The purpose of these measures was threefold: 1) to investigate the possible hypertrophy of the glial elements in response to denervation, 2) to correlate the change in axon number with corresponding changes in the relative area of neuropil occupied by neural elements, and 3) to determine the change in volume of space within the neuropil and

correlate these changes with the axonal and glial response to denervation.

Animals were sacrificed under ether anesthesia and perfused with 2% glutaraldehyde + 4% paraformaldehyde in 0.2M NaPO<sub>4</sub> (pH 7.4) at 5, 10, 32 and 90 days post-surgery. Each NL was removed intact and post-fixed by immersion at 4°C overnight, followed by secondary fixation in 4% OsO<sub>4</sub> for 4 hours at room temperature. Individual NL were then dehydrated through increasing concentrations of methanol, infiltrated with propylene oxide and embedded in Spurr's resin (8 hrs at 70°C). Flat Beem capsule molds were used with the rostral pole of the NL positioned at the tip of the capsule. All Beem capsules were trimmed so that the area from which the samples were taken was always the central region of the NL, e.g., that region predominately occupied by SON fibers (Alonso and Assenmacher, 1981).

Silver to gold ultrathin sections were cut on a Reichert ultramicrotome using glass knives, mounted on standard 300 mesh copper grids, and counterstained in uranyl acetate and Reynold's lead citrate using standard procedures. Sections were examined using a Zeiss EM 10/CR transmission electron microscope at a magnification of 5K (40kv with a .003mm objective aperture). Fifteen micrographs were randomly collected at successive levels throughout the NL of each animal (n=3 animals per group). All micrographs were photographically enlarged 2.7X for a final magnification of 13.5K.

### Quantification of Sprouting

In order to determine the change in number and density of axons in the neural lobe following surgery, every axon profile in each of the 15 micrographs collected from a given animal was counted. Morphological criteria for distinguishing axon profiles from other structures within the neuropil (pituicyte and perivascular cell processes) were the presence of neurosecretory vesicles and neurofilaments. In order to exclude the possibility of experimental bias, micrographs from all experimental and control groups (n=235) were randomized and analyzed blind. Once the counting process was complete, the axon number per micrograph was compiled for each animal, and the mean axon number and standard error calculated for each group.

The total area occupied by axons, glia (pituicyte and perivascular cell bodies and processes), and extracellular space including capillary space was next determined. Measurements were performed using a Zeiss Interactive Digital Analysis System (ZIDAS). Each measured element was traced using a cross hair cursor interfaced with a digitizing pad upon which the micrograph was positioned. The net sum of all measurements of a given cellular element was then recorded for each micrograph and compiled for each animal and each experimental group. Axon density was determined by dividing the total number of axons in each group by the total area examined, 11070  $\mu\text{m}^2$ .

### Statistical Analysis

One way analysis of variance was performed using the AV1W program in the MSUSTAT statistical package (developed by Dr. R.D. Lund),

followed by testing the significance of differences in group means using the MSUSTAT program COMPARE and the Newman-Keuls test.

## RESULTS

Post-Mortem Observations

The efficacy of the hypothalamic lesion was determined by examining brain sections stained with cresyl violet which were collected from the most rostral to the most caudal aspect of the knife cut in each animal. Only those animals that sustained a complete unilateral transection of the hypothalamo-neurohypophysial tract were used in any phase of this investigation. A large cavitation extended from the dorsal surface of the brain to varying depths of the hypothalamus, expanding the lesion tract and the lateral ventricle through which the knife blade passed in all experimental animals and, to a limited extent, sham controls (fig. 1). The cavitation sometimes, but not always, resulted in the lateral displacement of the contralateral PVN. However, the ipsilateral and contralateral SON were never directly affected by the cavitation. In those sites where the medial and lateral walls of the lesion site were juxtaposed, extensive gliosis was observed.

Cell death was apparent as early as 10 days post-surgery, and was usually complete by 30 days post-surgery. Cell death in the axotomized PVN was never complete except in those cases where lateral displacement by cavitation contributed to the loss (fig. 1). Partial to complete loss of cell populations in the axotomized SON was observed, with gliosis very apparent within the damaged nuclei. A similar gliotic response was observed in the SON following hypophysectomy in the rat (Raiman, 1977).

Although no attempt was made to quantify the effect, an increase in the occurrence of multiple nucleoli in both SON and magnocellular PVN nuclei was seen in many, but not all, lesioned animals.

#### Quantitative Immunohistochemistry of the Neural Lobe

Changes in the density of vasopressin immunoreactivity and in the cross-sectional area of the NL following surgery were monitored as an indirect measure of alterations in the density of vasopressinergic axons. It was reasoned that any decrease in VP staining density occurring subsequent to the surgery would reflect the loss of VP neurosecretory efferents. Any subsequent increase in VP staining densities would then provide preliminary indication of a compensatory sprouting response. The occurrence of a similar trend in NL size would aid in discriminating between a true shift in axon population and an increase in vasopressin content of surviving axons, termed a loading response.

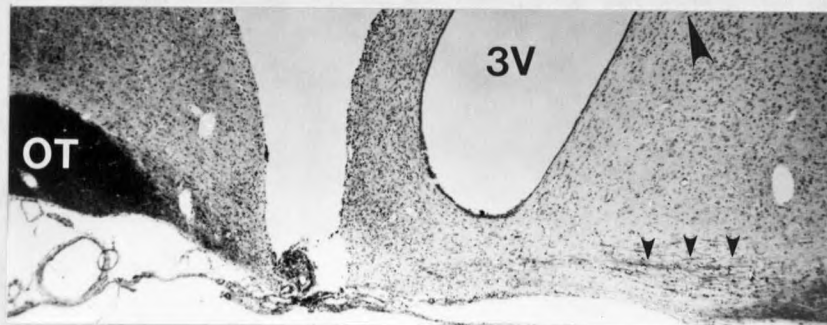


Figure 1. Light micrograph of cavitation surrounding the lesion site. The large arrows indicate the PVN nuclei. The smaller arrows indicate fibers of the hypothalamo-neurohypophysial tract projecting toward the ME. (OT), optic tract, (3V), third ventricular.

Quantitative densitometry demonstrated a significant change in vasopressin staining density ( $P < .02$ ,  $F = 3.02$ ) and NL cross-sectional area ( $P < .02$ ,  $F = 3.47$ ) throughout the post-surgical survival period. Newman-Keuls analysis indicated a 42% ( $P < .05$ ) reduction in VP immunoreactivity 10 days following surgery when compared to controls (fig. 2). Neural lobe cross-sectional area was reduced an almost identical 41.3% ( $P < .05$ ) below control values at this time (fig. 3).

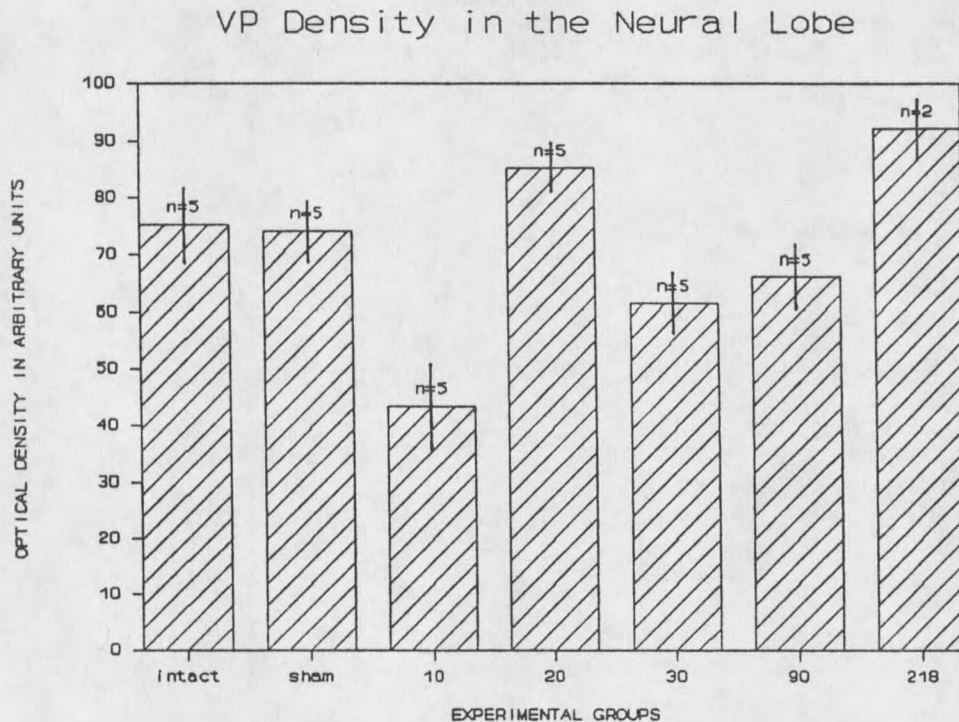


Figure 2. Changes in VP staining density in the neural lobe as a result of partial denervation. Bars from left to right represent control, sham, 10 day, 20 day, 30 day, 90 day, and 218 day post-surgical group. Vertical bars represent standard error of the mean (SEM).

At twenty days post-surgery a significant increase in VP staining density occurred ( $p < .05$ ). However, while the cross-sectional area of the NL did undergo a partial recovery, it was still 20.4% below the control value, although not significantly different from either control or 10 day post-surgical values. Hence, there was no significant difference between any time points in fig. 3 except at 10 days post-surgery.

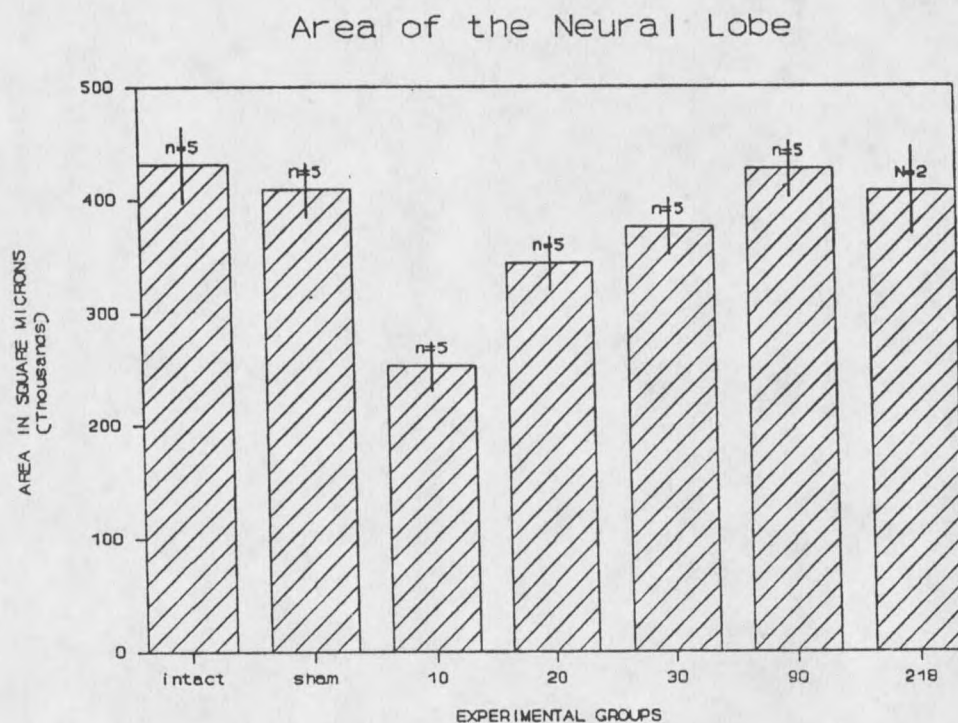


Figure 3. Changes in cross-sectional area of the neural lobe as a result of partial denervation. Bars from left to right represent control, sham, 10 day, 20 day, 30 day, 90 day, and 218 day post-surgical groups. Vertical bars represent (SEM).

This apparent imbalance between vasopressin immunoreactivity and size of the NL may reflect a loading response. One may speculate that by 20 days the synthetic activity of the surviving VP neurons entered a heightened metabolic state. If, however, the reestablishment of functionally competent neurosecretory contacts had not yet occurred, the result could be a loading of VP neurosecretory granules in the terminals. At thirty days post-surgery the VP staining level was again below normal (18.4% below the control value), but not significantly different from either the intact or 10 day post-surgical group. However, the size of the NL demonstrated a continued recovery to within 13% of normal. If the speculations regarding the staining density at 20 days post-surgery are correct, these data could indicate the reestablishment of functionally competent neurosecretory contacts by 30 days post-surgery with the subsequent release of backloaded vasopressin. This trend toward recovery of normal VP staining levels continued in the 90 day post-surgical group with density reaching 88% of normal, while NL size recovered completely. Neither value was significantly different from their respective control. However, at 218 days post-surgery the staining density had continued to increase to levels 18% above control values while the size of the NL remained constant. These data support the hypothesis of compensatory sprouting in the NL in response to partial denervation. The correlation between the quantitative densitometry data and the change in size of the NL suggest the changes observed in VP staining density are due to a loss and recovery of VP efferents as opposed to a loading response.

Stereometric Analysis of SON and PVN Soma and  
Cell Nuclear Cross-Sectional Area

Stereometric analysis of cross-sectional areas of soma and cell nuclei in the contralateral SON and PVN were performed as a method of determining cellular reaction to denervation.

It has been well established that experimentally induced hyperosmolality via salt loading (Paterson and Leblond, 1977) and increased levels of metabolic activity (Bandaranayake, 1976) such as hyperstimulation of the antidiuretic and oxytocinergic systems by dehydration and lactation (Watson, 1965), will result in cellular hypertrophy. Furthermore, cellular hypertrophy following partial denervation of a shared terminal field has been correlated with sprouting of axons arising contralateral to the site of injury (Goldschmidt and Steward, 1980). Hence, cellular hypertrophy can be used as a means of determining heightened cellular activity in response to a variety of metabolic perturbations.

Significant changes in cross-sectional area of both somata ( $P < .00001$ ,  $F = 15.19$ ) and nuclei ( $P < .00001$ ,  $F = 11.56$ ) occurred in the contralateral SON following surgery. Significant, but less pronounced changes also occurred in somata ( $P < .01$ ,  $F = 4.15$ ) and nuclei ( $P < .002$ ,  $F = 6.60$ ) of contralateral PVN magnocellular neurons following surgery (figs. 4-7). Soma and cell nuclei of the contralateral SON showed no significant change in size by 10 or 20 days post-surgery (fig. 5). By 30 days post-surgery a significant upward trend in cross-sectional area becomes demonstrable with SON cell bodies reaching an area 42.5% above normal ( $P < .01$ ). This trend is further reflected in nuclear size which

increased to 22% above normal at the same time point ( $P < .01$ ). The increases in both cell and nuclear area continued through the later time points with cell sizes reaching 82.4% of normal by 218 days post-surgery ( $P < .01$ ). Again, the nuclear response parallels the cellular response, reaching 83.6% above normal values by 218 days PS ( $P < .05$ ).

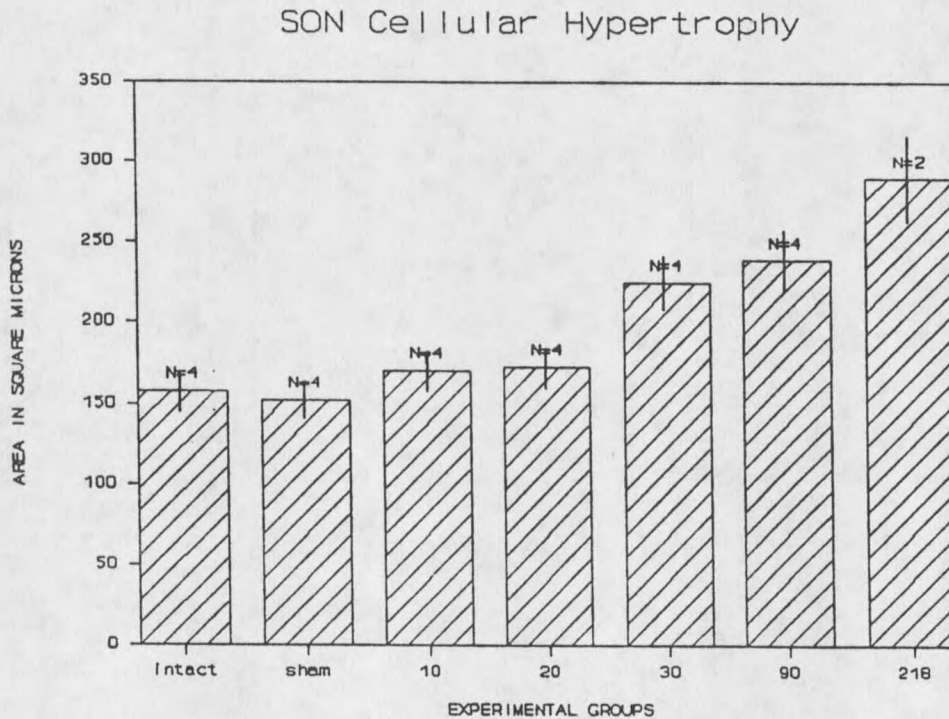


Figure 4. Changes in cross-sectional area of cell somas in the contralateral SON following hypothalamic lesion. Bars from left to right represent control, sham, 10 day, 20 day, 30 day, 90 day, and 218 day post-surgical groups. Vertical bars represent (SEM).

In contrast, the PVN neurons showed a delayed response, with no

significant change in size apparent until cell cross-sectional area increased to 32% above normal 90 days post-surgery ( $P < .01$ )(fig. 6). However, this increase was transient, with soma decreasing to normal cross-sectional area by 218 days post-surgery. PVN nuclear size enlarged to 32.6% above normal by 90 days post-surgery ( $P < .05$ )(fig. 7). By 218 days post-surgery nuclear cross-sectional area was not significantly different from controls.

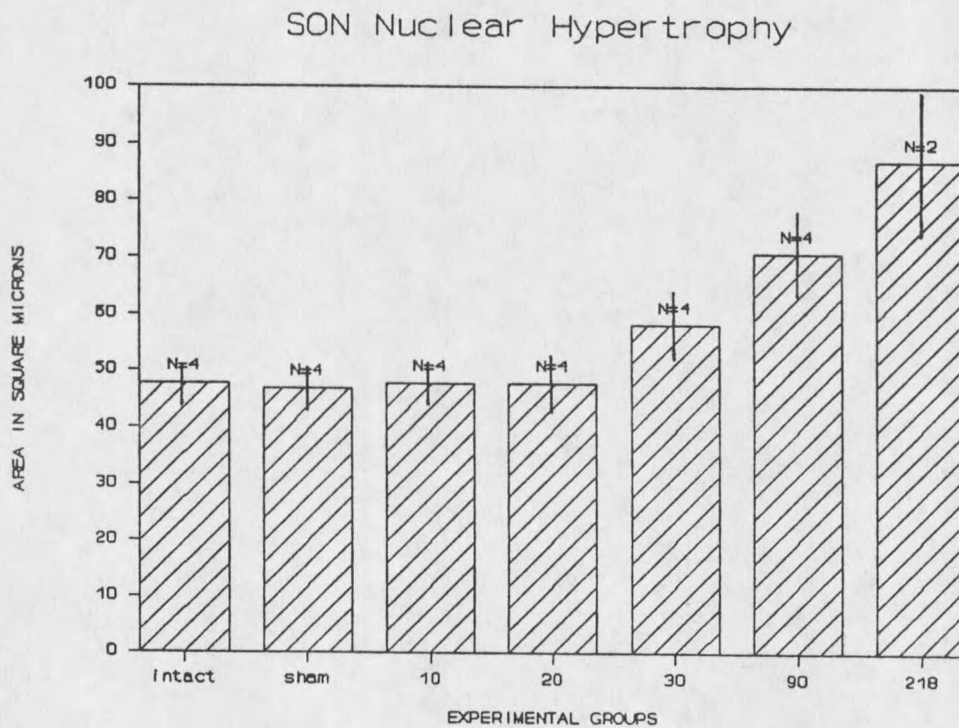


Figure 5. Changes in cross-sectional area of the cell nuclei in the contralateral SON following hypothalamic lesion. Bars from left to right represent control, sham, 10 day, 20 day, 30 day, 90 day, and 218 day post-surgical groups. Vertical bars represent (SEM).

Ultrastructural Examination of the NL

The ultrastructure of normal and partially denervated NL was examined in order to confirm the indications of sprouting observed at the light microscopic level, as well as to elucidate any changes in morphology that may contribute to understanding the neurohypophysial response to partial denervation.

## PVN Cellular Hypertrophy

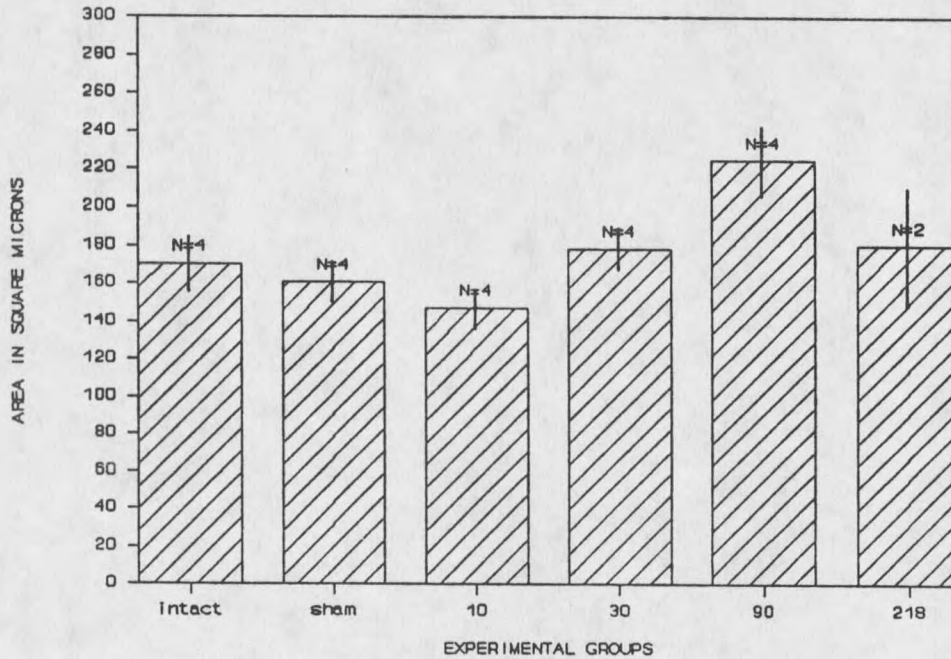


Figure 6. Changes in cross-sectional area of cell soma in the contralateral PVN following hypothalamic lesion. Bars from left to right represent control, sham, 10 day, 30 day, 90 day, and 218 day post-surgical groups. Vertical bars represent (SEM).

Several parameters of NL morphology were examined in each of 5 experimental groups (normal controls, and lesioned animals at 5, 10, 32 and 90 day post-surgical survival periods). Parameters examined were: 1) absolute number of axons, 2) the relative volume of NL occupied by axons; 3) axon density per unit area of NL;

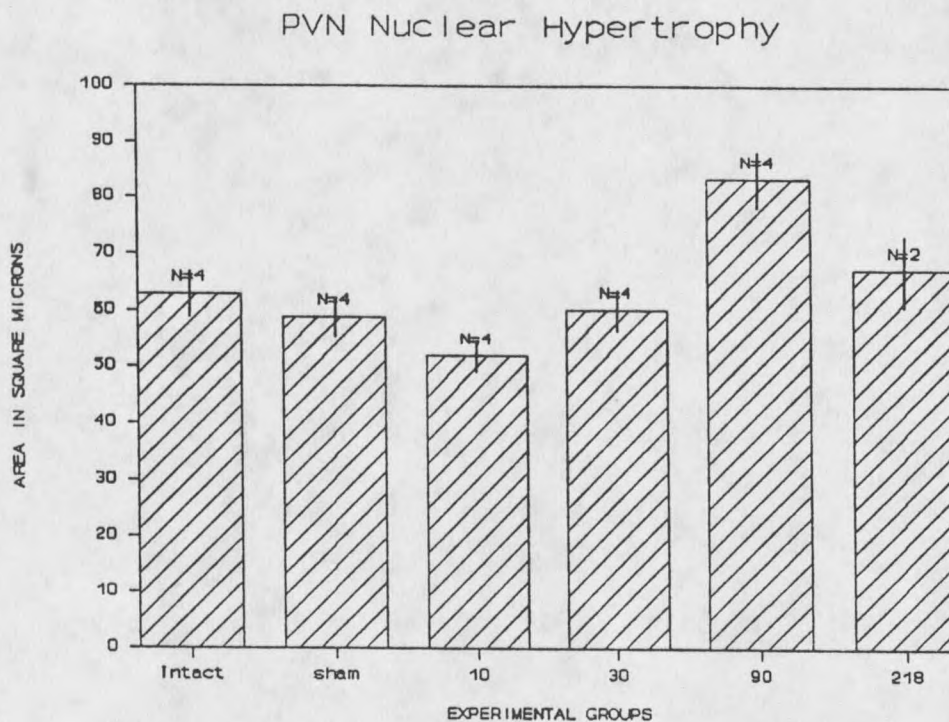


Figure 7. Changes in cross-sectional area of the cell nuclei in the contralateral PVN following hypothalamic lesion. Bars from left to right represent control, sham, 10 day, 30 day, 90 day, and 218 day post-surgical groups. Vertical bars represent (SEM).

4) the relative volume of NL occupied by support cells (eg. pituicyte and perivascular cells); and 5) volume of the NL occupied by

extracellular space, including capillary lumina (Tables 2,3).

#### Normal Rat Neural Lobe

Axons were counted in 15 micrographs from each of 3 normal rats. These micrographs contained a total of 3224 axons. Axons occupied 66.95% of the total area of NL examined (7405.0  $\mu\text{m}^2$  of 11070.0  $\mu\text{m}^2$  surveyed). Extracellular and capillary space occupied 7.5% of the total area of NL examined, with the remaining 25.5% occupied by pituicyte and perivascular cells. Axon density was calculated to be 29.12 axons/100.0  $\mu\text{m}^2$  observed (Tables 2,3).

Neural lobe morphology was very similar to that reported by others (Kurosumi et al., 1961; Monroe, 1967; Zambrano, and de Robertis, 1968). Axons were of varying caliber ranging from very small cylinders with numerous neurofilaments and few or no neurosecretory vesicles to large vesicle filled axonal swellings with numerous mitochondria and both electron dense and electron lucent neurosecretory vesicles but no apparent neurofilaments. Axonal endings in the proximity of perivascular space often contained small synaptic vesicles in addition to the larger neurosecretory vesicles. Synaptic vesicles were also observed within axonal swellings at some distance from the site of termination. Pituicyte ramifications were extensive and typically contained substantial numbers of lipid droplets, rough endoplasmic reticulum (RER), mitochondria, glycogen particles and polysomal clusters (fig. 8). Axons were typically in close apposition to, and sometimes completely enclosed within, pituicytes. Perivascular cells were limited in number and displayed an electron dense cytoplasm with

extensive cisternae of RER, highly condensed chromatin (particularly surrounding the inner membrane of the nuclear envelope), lipid droplets, mitochondria, and polysomal clusters scattered throughout a more limited amount of cytoplasm than seen in pituicytes. Perivascular cell ramifications were typically of smaller caliber and less extensive than pituicyte processes and were often in direct apposition to axons.

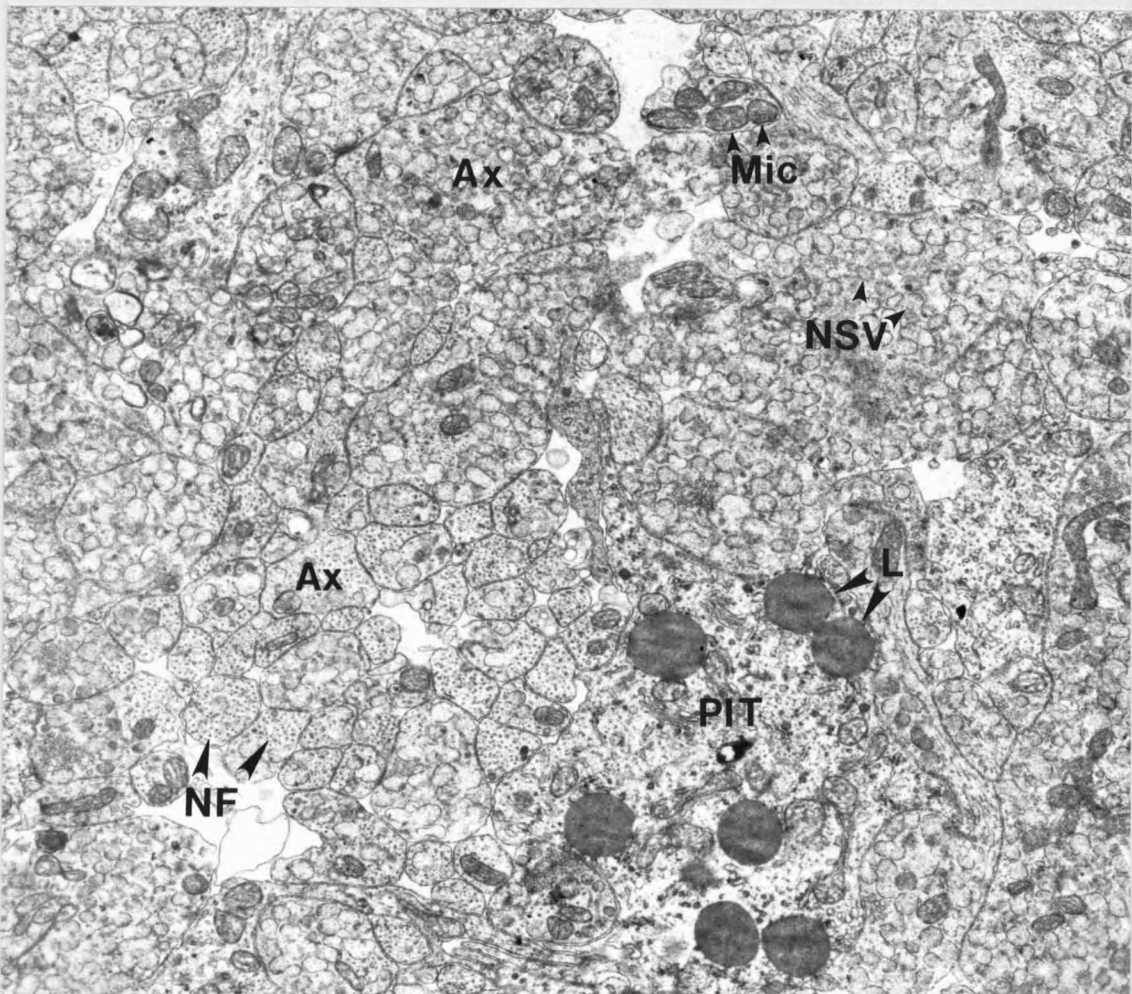


Figure 8. Electron micrograph of intact neural lobe. (Ax) axon, (NF) neural filaments, (PIT) pituicyte process, (Mic) mitochondria, (NSV) neurosecretory vesicles, (L) lipid vesicles.

Neural Lobe at 5 Days Post-Surgery

These animals displayed a marked reduction in axon number dropping from the control value of 3224 axons to 1938 axons (38.9% decrease) (fig. 9). Total area occupied by axons dropped a corresponding 36.6% below control values. Axon density dropped to 17.51/100  $\mu\text{m}^2$ . In contrast, the area occupied by pituicyte and perivascular cells increased 64% over control values. A similar increase of 57.5% in extracellular space was observed. Although fewer axon profiles were encountered, surprisingly few degenerating axons were observed at this time point.

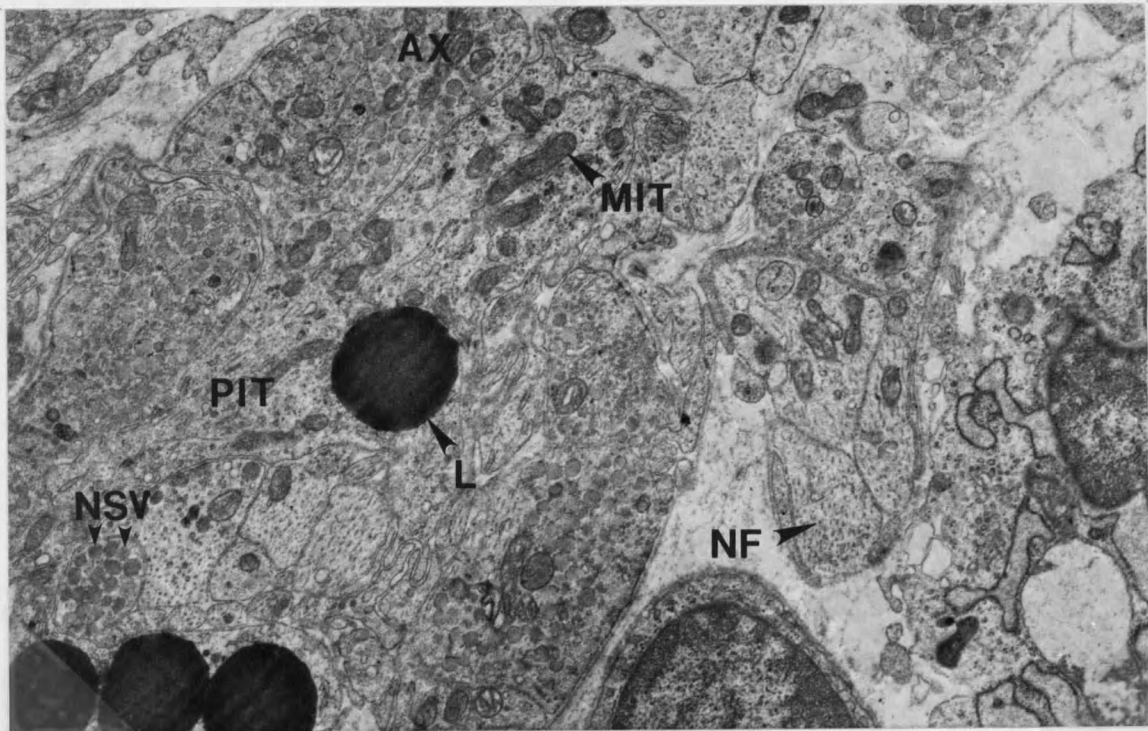


Figure 9. Electron micrograph of neural lobe at 5 days post-surgery. (Ax) axon, (NF) neural filaments, (PIT) pituicyte process, (Mic) mitochondria, (NSV) neurosecretory vesicles, (L) lipid vesicles.

The association between pituicyte processes and axons appeared normal, with no occurrence of phagocytic engulfment of degenerating axons by pituicytes observed. There was a marked increase in the number of perivascular cells encountered. These cells had very dilated RER cisternae filled with a granular amorphous substance (fig. 9), a heightened occurrence of granular inclusions indicative of phagocytic activity, and more extensive ramifications than previously observed.

Observations of axonal degeneration following infundibular stalk transection have revealed that a pituicytic engulfment of severed axons occurs by 5 days post-surgery and that complete disposal of the axons has occurred by 10 days post-surgery (Dellman et al, 1974). The present observations following surgery indicate the initial breakup of severed axons by 5 days post-surgery (fig. 10).

Elimination of severed axons is essentially complete by 5 days post-surgery as evidenced by the increased volumes of extracellular space, now cleared of degenerating axons, and the infrequent occurrence of degenerating axon profiles. Extensive extracellular space was also observed in the regenerating stump of the infundibulum following hypophysectomy (Kiernan, 1971). It is notable that those examples of phagocytosis encountered involved perivascular cells exclusively (fig. 11). Although pituicyte profiles contained higher numbers of lipid droplets and slightly dilated Golgi apparatus, there were no examples of phagocytosis observed.

These data are in agreement with similar observations of Zambrano and De Robertis (1968) following destruction of the PVN efferents to the NL. These investigators reported phagocytic activity to be limited to

the perivascular cell population of the NL, whose activity reached a peak about 6 days following the lesion.

Table 2. Results of EM Stereometric Analysis: Group Data.

Total area of neural lobe surveyed per group, 11,070  $\mu\text{m}^2$

Exp. Group	Axons	Area occupied by axons	Area occupied by glia	Extracellular space
intact	3224	7411.5 $\mu\text{m}^2$	2826.8 $\mu\text{m}^2$	831.7 $\mu\text{m}^2$
5 day	1938	4701.9 $\mu\text{m}^2$	4411.8 $\mu\text{m}^2$	1956.3 $\mu\text{m}^2$
10 day	1866	4523.0 $\mu\text{m}^2$	3938.4 $\mu\text{m}^2$	2608.6 $\mu\text{m}^2$
32 day	2334	5674.8 $\mu\text{m}^2$	3018.2 $\mu\text{m}^2$	2377.0 $\mu\text{m}^2$
90 day	2538	7039.4 $\mu\text{m}^2$	2200.4 $\mu\text{m}^2$	1830.1 $\mu\text{m}^2$

Table 3. Results of EM Stereometric Analysis: Individual Data.

Total area of neural lobe surveyed per animal, 3690.0  $\mu\text{m}^2$

Exp. Animal	# axons	Area occupied by axons	Area occupied by glia	extracellular space	axons/100 $\mu\text{m}^2$
intact #1*	817	2539.3	933.5	217.2	22.14
intact #2*	934	2379.06	940.45	177.53	25.31
intact #3*	1473	2257.41	952.83	437.01	39.91
5 day #2	480	1740.17	1251.99	652.54	13.01
5 day #3	653	1570.29	1240.67	879.04	17.7
5 day #4	775	1428.29	1919.14	424.77	20.32
10 day #3	593	1417.7	1288.73	1007.37	16.07
10 day #4	610	1721.17	1284.05	684.78	16.53
10 day #5	663	1407.91	1365.6	916.5	17.97
32 day #1	598	1527.89	1193	969.11	16.2
32 day #2	975	2062.35	789.78	837.87	26.42
32 day #3	761	2084.56	1035.41	570.03	20.62
90 day #2	960	2624.76	982	339.74	26.01
90 day #3	789	2263.09	626.33	800.58	21.38
90 day #4	789	2397.11	591.32	701.57	21.38

\* all intact animals were 125 days of age at time of sacrifice.  
all areas are in square microns ( $\mu\text{m}^2$ ).

Neural Lobe at 10 Days Post-Surgery

A total of 1866 axon profiles were counted, demonstrating a continued decline to levels 42.1% below control values with a corresponding decrease in area occupied by axons to 39% below control values. Axonal density dropped to  $16.85/100 \text{ um}^2$ . This continued decline in axon number occurred concomitantly with a further increase in extracellular space, which reached a peak value of 68.1% above normal (fig. 12). However, a partial reduction in area occupied by glial cells was observed, with pituicyte processes occupying 35.57% of the total area surveyed in this group. Nevertheless, total area occupied by glial cells remained above normal levels. Morphological observations were consistent with a decreased level of perivascular cell activity, as indicated by fewer granular inclusions and pycnotic nuclei, and more constricted cisternae of RER.

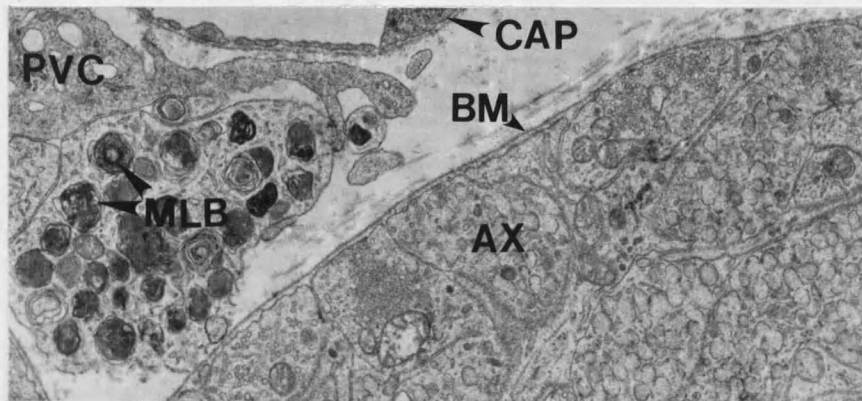


Figure 10. Electron micrograph of degenerating axon within perivascular space of the neural lobe. Multilaminar bodies (MLB), perivascular cell process (PVC), capillary endothelium (CAP), basement membrane (BM), axon terminals (Ax).

Neural Lobe at 32 and 90 Days Post-Surgery

A partial recovery of axon number was observed in this group with a total of 2334 axons counted, representing 72.4% of control values and an increase of 20% over the 10 day post-surgical group (fig. 13). Axon density increased to 21.08/100  $\mu\text{m}^2$  with the area occupied by axons increasing to 51.3%. There was further reduction in the area of neuropil occupied by glial cells with values returning to near normal levels of 27.26%. The volume of extracellular space decreased but remained well above normal at 21.5% of the measured area. A continued increase in axon number was apparent in the 90 day group with 2538 axon profiles counted, representing a recovery to 78.7% of the normal axon population. Axon density increased to 22.9 axons/100  $\mu\text{m}^2$  while the total area occupied by axons increased to 95.0% of normal. The area occupied by pituicyte and perivascular cells continued a downward trend to 22% below normal. The volume of extracellular space also continued to decline (to 16.53% of the area measured) but still remained greater than normal (fig.14).

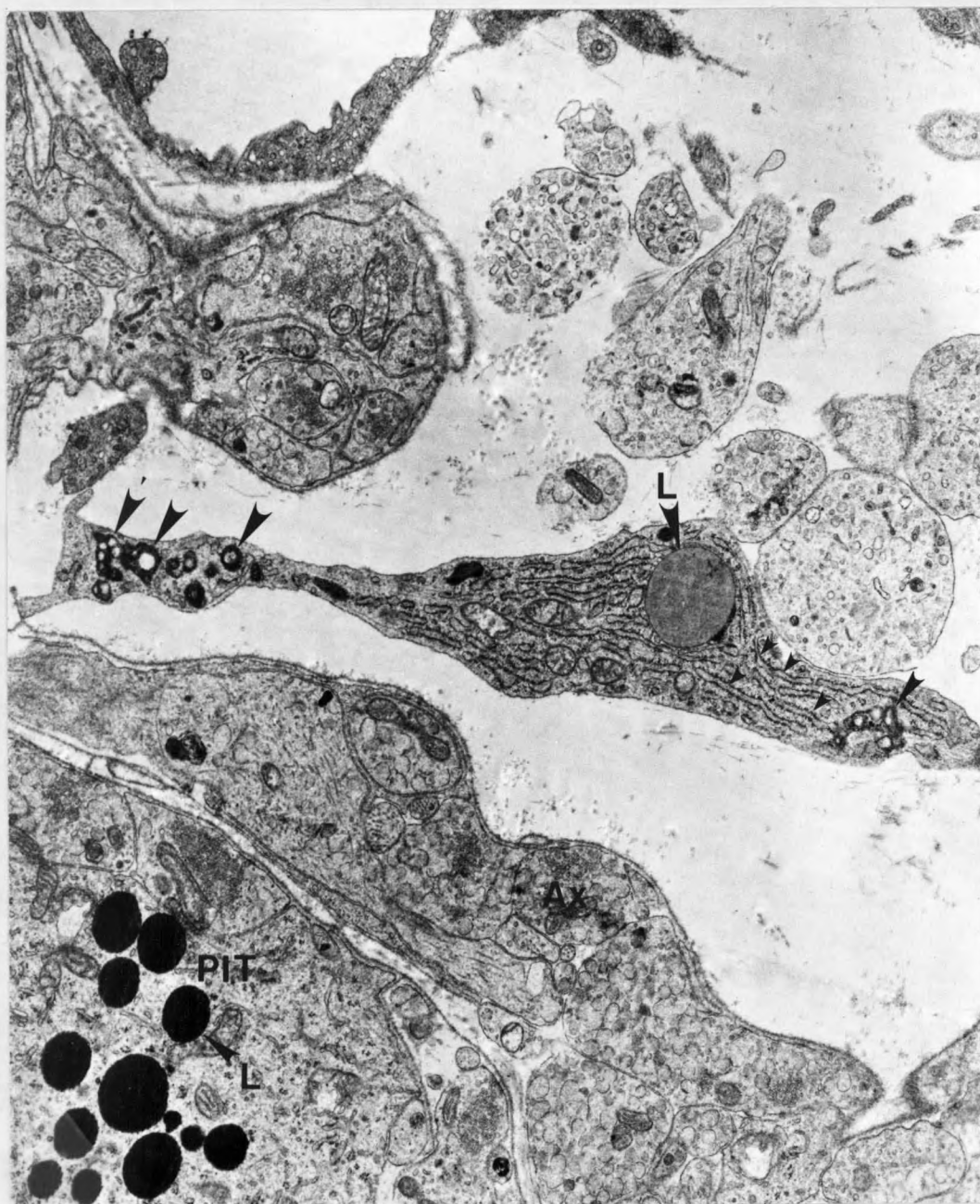


Figure 11. Electron Micrograph of Perivascular cell process with phagocytic debris (large arrow). Note the extensive RER (small arrows). (Pit) pituitary process, (L) lipid vesicles, (Ax) neurosecretory axon terminals.

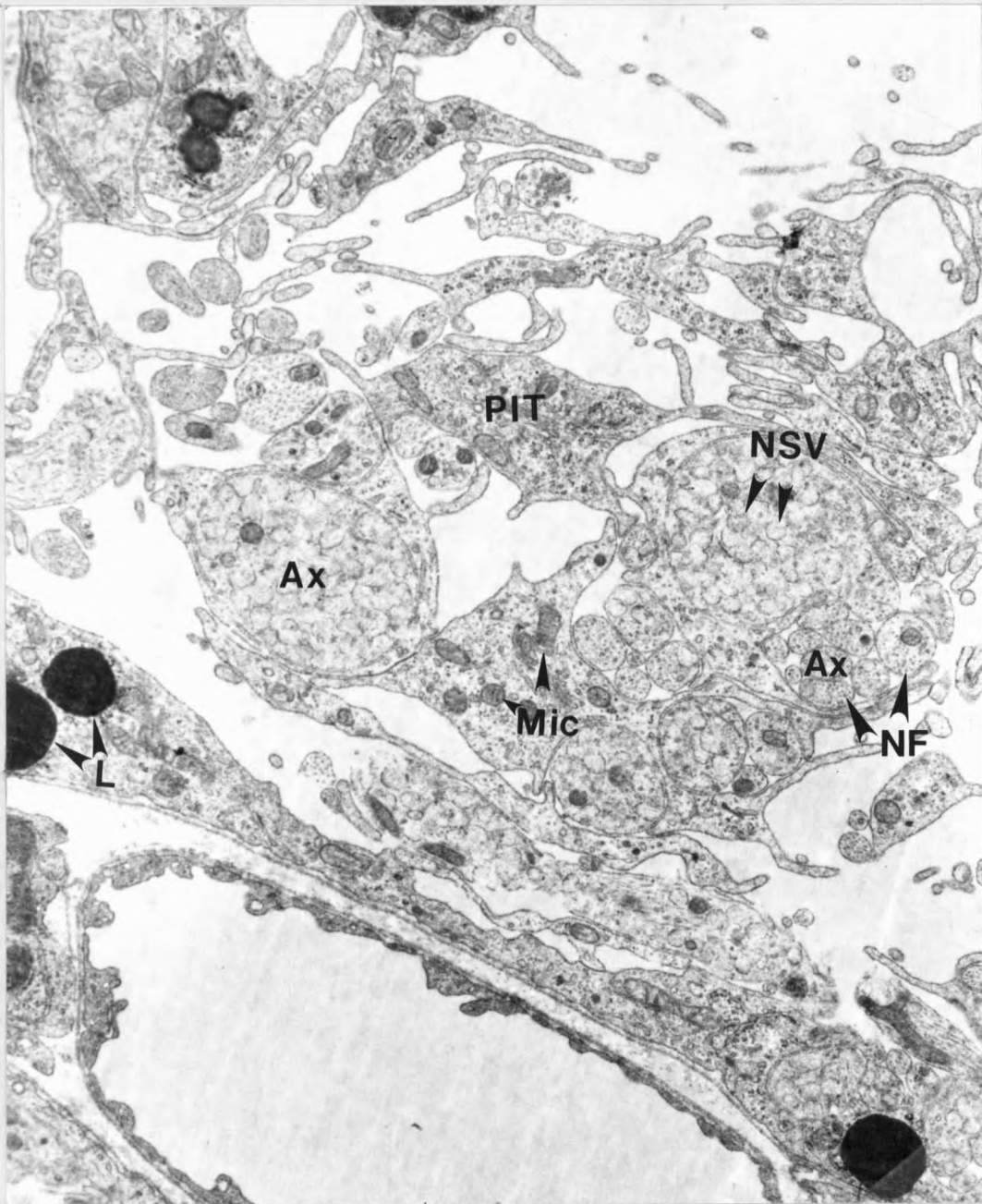


Figure 12. Electron micrograph of neural lobe at 10 days post-surgery. (Ax) axon, (NF) neural filaments, (PIT) pituicyte process, (Mic) mitochondria, (NSV) neurosecretory vesicles, (L) lipid vesicles.

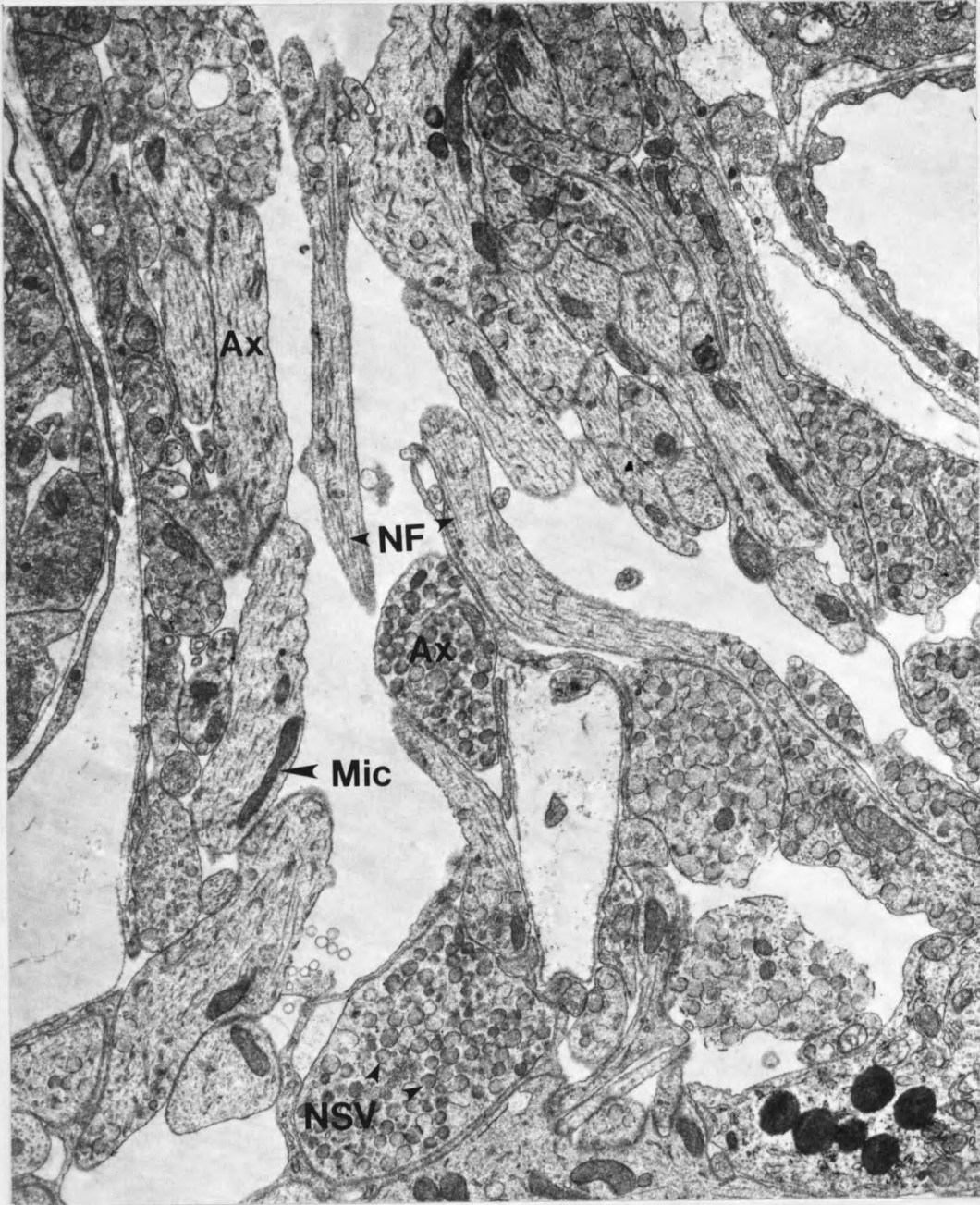


Figure 13. Electron micrograph of neural lobe at 32 days post-surgery. (Ax) axon, (NF) neural filaments, (Mic) mitochondria, (NSV) neurosecretory vesicles.

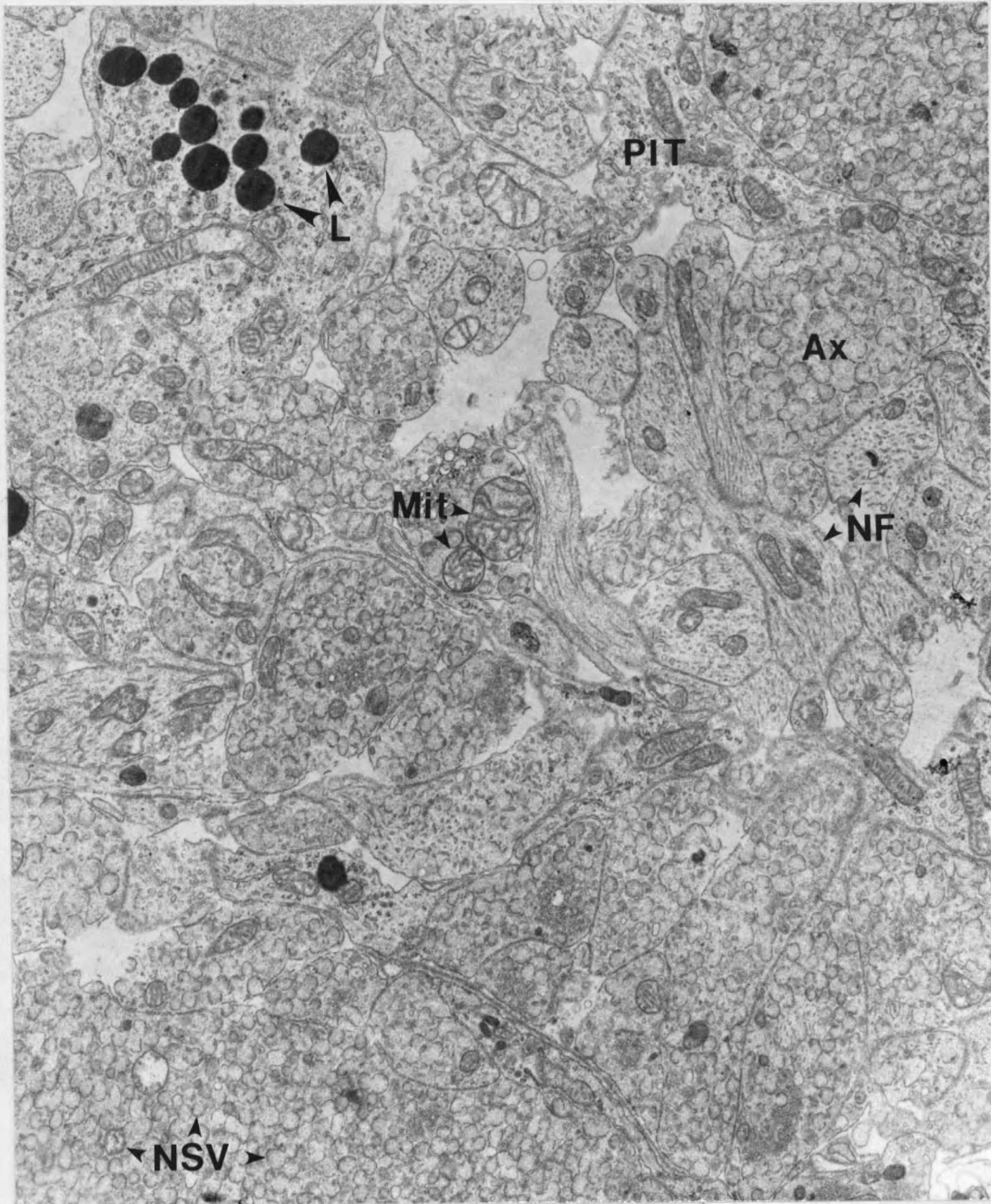


Figure 14. Electron micrograph of neural lobe at 90 days post-surgery. (Ax) axon, (NF) neural filaments, (PIT) pituicyte process, (Mit) mitochondria, (NSV) neurosecretory vesicles, (L) lipid vesicles.

## DISCUSSION

The presence of extensive cavitation, vascular hypertrophy and gliosis surrounding the hypothalamic knifecut (Antunes, 1978) are consistent with previous observations in the rhesus monkey (Kiernan, 1971), the ferret (Adams et al., 1971) and the rat (Raisman, 1973).

In this investigation a large variation in the degree of cell death was observed throughout the post-surgical survival period, with no observed differences in the extent of the lesion between those specimens having a high degree of cell death and those that did not. Raisman (1977) has suggested that the variability in SON cell death following hypophysectomy may result from differing degrees of trauma induced by the mechanical removal of the pituitary. A similar effect could be involved in this preparation but would prove difficult to demonstrate. It is generally accepted that the more proximal the site of axotomy is to the cell body, the more likely it is that cell death will result. This has been attributed to the existence of sustaining collateral fibers along the length of the injured axon (Adams et al., 1971). The existence of SON collateral fibers has been demonstrated immunohistochemically (Silverman, 1983) and electrophysiologically (Pittman et al., 1978) within the hypothalamus and ME, respectively. However, the close proximity of the knifecut to the SON in this investigation seems to preclude this explanation. One may speculate that recurrent collaterals may exist within the boundaries of the SON nuclei and provide the necessary sustaining input.

An increase in the number of nucleoli, similar to the increase observed in this investigation, has been observed in the rat in response to dehydration (Hatton et al., 1973, 1984) and may therefore result from the increased metabolic demands placed on the surviving magnocellular neurons.

The possibility of increased metabolic activity was investigated by measuring the cross-sectional area of magnocellular neurons in the contralateral SON and PVN nuclei. Results indicate a general hypertrophy of contralateral SON and PVN magnocellular neurons known to project to the neural lobe (Alonso et al., 1986)(figs. 4-6). However, the dynamics of this response differ between the two nuclei.

Observations of the PVN show the cellular enlargement to be less pronounced and to occur more slowly than that observed in the SON (compare figs. 4 and 6). This may be a reflection of both the relative commitment of axons and terminals each nuclei make to the NL and their respective peptide products. It has been reported that the PVN contributes approximately 20% of the axons and terminals to the NL (Olivecrona, 1957; Zambrano and De Robertis, 1968), most of which are oxytocinergic, while the SON contributes the remaining 80% of the magnocellular neurosecretory compliment. It seems possible that the smaller population with correspondingly fewer terminals, coupled with a different peptide product may result in less metabolic demand placed on these neurons thereby reducing the degree of cellular enlargement. This possibility is consistent with the return to normal cell cross-sectional area seen in these neurons by 218 days post-surgery.

However, the apparent increase in the cross-sectional area of the

somata may result from a change in the distribution of Nissl substance rather than actual enlargement of the cell (Goldschmidt and Steward, 1980). Observations of cresyl violet stained brain sections of neurons in the SON and PVN nuclei indicate a clear halo surrounding the nuclear envelope of many of the enlarged cell bodies which may reflect a redistribution of the Nissl material to the cell periphery. Measures of the cross-sectional area of the nuclei are not subject to this potential artifact, since the well defined nuclear envelope permits accurate measure of enlargement without the problem of Nissl redistribution. An increase in nuclear (Moll and De Wied, 1962) and nucleolar (Bandaranayake, 1976) cross-sectional area of SON and PVN neurons has been reported to occur in response to heightened metabolic demand. Paterson and Leblond (1977) demonstrated that salt-induced plasma hyperosmolality will result in the enlargement of cell nuclei and nucleoli in the SON. These responses are believed to be indicative of increased cellular activity, probably heightened protein synthesis.

The morphometric data indicate a significant increase in nuclear cross-sectional area. The increase in cell and nuclear cross-sectional area is probably not due to secondary effects of the lesion (e.g., hemorrhage or trauma) as these are very transient reactions (Goldschmidt and Steward, 1980), whereas the hypertrophy noted here persists for 90 (PVN) to 218 (SON) days post-surgery. It is noteworthy that the hypertrophic response displayed by the SON and PVN cell nuclei essentially mirrors that of the respective cell bodies. This serves to validate the cellular measurements reported above. Although the hypertrophy demonstrated in the cell bodies may be due to the

redistribution of the Nissl substance to the cell periphery, it nonetheless appears to reflect the expansion of the cell boundaries.

The quantitative immunocytochemical measures made of NL vasopressinergic staining density provide indirect indications of lesion-induced loss and subsequent recovery of vasopressinergic efferents.

A marked reduction in vasopressin immunoreactivity in the NL occurred within 10 days after partial denervation. This initial decrease was followed by a gradual increase until the density of immunostaining exceeded normal levels by 218 days post-surgery (fig. 2). Data from sham controls indicated that hemorrhage and/or surgical trauma alone had no measurable effect on NL vasopressin staining densities. There are two possible mechanisms which could contribute to the recovery in VP staining density. First, the observed recovery could be due solely to a loading response of the system in the absence of a sprouting response. In order to maintain homeostatic plasma VP levels, the remaining intact neurosecretory neurons may enter a hyperactive state resulting in a buildup of neurosecretory peptides in axons. The second possibility is that compensatory sprouting occurred from neurosecretory efferents of the intact contralateral magnocellular neurons. The results are most consistent with a combination of these two events. The transient increase in staining density observed at 20 days post-surgery (fig. 2) which precedes the hypertrophic response of cell somata and nuclei discussed above, may reflect loading of VP axons prior to the reestablishment of functionally competent neurosecretory contacts. This is supported by the ultrastructural evidence indicating a continued loss

of axons at 10 days post-surgery with a subsequent partial recovery at 30 days post-surgery. The continued elevation of staining density through later time points may reflect a tendency of the system to regain the homeostatic levels maintained prior to the surgical insult. It is interesting that the VP levels remain well above normal levels at 218 days post-surgery in spite of the decrease in axons within the terminal field. This may reflect a tendency of the system to rebuild the VP reserve capacity to pre-surgical levels, but within a system now relying on a reduced cellular complement. Thus, each given axon and respective complement of terminals must provide storage for an increased number of neurosecretory granules. It would be interesting for future investigations to determine the actual number of neurosecretory granules per axon within the 218 day neural lobe in an effort to confirm these speculations.

Either the loading response or the sprouting response would likely be preceded by or coincide with an increase in the metabolic activity of the surviving magnocellular neurons for production of increased quantities of neurosecretory peptides and/or cytoplasmic and membrane components necessary to sustain axon outgrowth. Since the time course of cellular hypertrophy and recovery of vasopressin immunoreactivity coincide so sharply, they provide strong evidence for a compensatory response of the contralateral SON and PVN magnocellular neurons to the unilateral transection of the hypothalamo-neurohypophysial tract. Unfortunately, the ubiquitous distribution of vasopressinergic axons and terminals within the NL precludes using anatomical redistribution of immunocytochemically identified axons as a means of confirming the

presence of sprouts.

Therefore, an ultrastructural survey of the effects of partial denervation of the NL was performed. Both the number of axon profiles and the volume of the NL occupied by axons were substantially reduced at 5 and 10 days post-surgery (Table 2,3). Concurrent with this loss of axons was a significant decrease in the cross-sectional area of the NL ( $P < .05$ ). Murray (1986) reported a similar reduction in the area of the tectal lamina following partial denervation caused by intra-orbital optic nerve crush. This initial loss in area was followed by an increase of 17% over control values. She associated this increase with the reinnervation of the target area by regenerating axons.

The possibility exists that the change in NL cross-sectional area observed in this investigation may be due to shrinkage induced by the histological preparation. In addition, the methods used to prepare tissue for light and electron microscopic analysis utilized different fixation and embedding procedures which may have resulted in differences in the degree of tissue shrinkage. Even so, the degree of shrinkage and the extent of axon loss are quite comparable (Table 1; fig. 2). Since the contents of the NL are comprised predominately of neurosecretory axons, pituicytes, perivascular cells, perivascular space, and capillaries, the considerable increase in the volume of NL occupied by extracellular and capillary space and the glial hypertrophy observed following surgery suggests the reduction in size of the NL is a direct result of the loss of axons.

The results provide ultrastructural confirmation that partial denervation of the NL resulted from unilateral knife-cut of the

hypothalamo-neurohypophysial tract, further supporting the assumption that the reduction in VP immunoreactivity and contralateral cellular hypertrophy seen following surgery are the direct result of denervation.

The recovery of axon numbers and the corresponding increase in NL area occupied by axons coincided with a partial recovery of the cross-sectional area of the NL. However, by 30 days post-surgery the areas occupied by pituicyte processes had returned to control values with a corresponding decrease in volume of extracellular space. At 90 days, the latest post-surgical survival period examined, there was a continued recovery reaching 95% of normal area occupied. These data again correlate with a continued recovery in NL cross-sectional area, which reached 98.8% of normal. Again, a corresponding decrease in glial area was observed, declining to values below those seen in control animals. The volume of extracellular space also continued to decline but remained considerably greater than control values throughout the post-surgical period. It is notable that the area occupied by axons continued to increase to a level close to normal when in fact the actual number of axon profiles remained below control values at the latest time point examined. It can therefore be inferred that the size of the axons actually increased as the sprouting system matured (from a control diameter of  $2.3\mu\text{m}^2$  to  $2.77\mu\text{m}^2$  at 90 days post-surgery). The decline in area occupied by glia may stem from the gradual reduction in perivascular cells observed following the degenerative phase, possibly in combination with a reduced metabolic demand on the pituicyte population stemming from the reduced number of axons within the terminal field.

This ultrastructural analysis indicates that the recovery in NL cross-sectional area is a direct result of the partial recovery of axon numbers. All of the data obtained support the hypothesis that the partial recovery in axon numbers is a result of a compensatory sprouting response within the NL which arises from intact magnocellular efferents of the contralateral SON and PVN. The data indicate that vasopressinergic cells are involved in this response, but do not exclude the possible involvement of oxytocinergic neurons as well. This is the first evidence of sprouting by intact magnocellular neurons, and thus represents a novel model for the study of sprouting in central peptidergic systems. Only one other example of compensatory sprouting of intact, contralateral neurosecretory efferents has been reported (Silverman and Zimmerman, 1982). However, that system is both anatomically and functionally distinct from the system described herein, being restricted to the axons of the parvocellular PVN terminating in the zona externa of the median eminence (see introduction). All other reports of sprouting in neurosecretory systems involve regeneration of severed axons. The prolific sprouting seen in the infundibulum following hypophysectomy is due to the sprouting from the distal end of injured neurosecretory fibers (Kiernan, 1971). This is also true of the hypothalamic sprouting following knifecut (Antunes et al., 1978) allographic transplants of neural lobe (Dellman et al., 1987), and reinnervation of the NL following infundibular transection (Adams et al., 1969).

In addition to axons, the NL consists primarily of pituicytes, perivascular cells and perivascular connective tissue spaces and

capillary endothelium. It thus provides an ideal environment for the study of axonal/glial interactions during sprouting, trophic activity of the support matrix, and the reestablishment of functional neurosecretory contacts.

The mechanisms involved in the initiation and outgrowth of neuronal sprouts have gained increasing attention as our understanding of the extent of this phenomenon progresses. The precise role the pituicytes may play in this sprouting response remains in question. It has been suggested that they may provide a structural matrix for axonal growth (Barer and Lederis, 1966; Dellman et al., 1987). There is also evidence of a trophic role of pituicytes in axonal outgrowth. During development of the NL the axons and pituicytes in contact exchange small vesicles thought to contain a proteinaceous substance (Galabov and Schiebler, 1978). Galabov and Schiebler (1978) suggest that the pituicytes exude a substance necessary for axonal outgrowth. They also believe this stimulating function exists in adult animals as well as in the developing rat.

Others have suggested that the endothelial hypertrophy which proceeds axonal sprouting in this system may provide the stimulatory input for initiation of the sprouting response (Raisman, 1973). There is some indirect evidence for this hypothesis. Five days after stalk transection the capillaries of the mantle plexus react with a vigorous ingrowth of blood vessels into the area of the reorganizing proximal infundibular stalk (Billenstein and Leveque, 1955). However, these authors also reported the appearance of mitotic pituicyte cell profiles by 3 days post-surgery. Adams et al. (1971) reported the concomitant

formation of an ectopic neural lobe and functionally competent reinnervation of the denervated NL in the ferret following infundibular stalk transection. There was an invasion of capillaries in the region of the ectopic neural lobe, but no pituicyte response was reported. It seems unlikely that the ectopic sprouting would result from a functional requirement in animals within which the NL had been reinnervated, suggesting the response was induced by capillary endothelial activity.

However, there is evidence of pituicyte hypertrophy occurring concurrently with capillary hypertrophy. This was noted in the reorganizing proximal infundibulum following posterior lobectomy (Moll and DeWeid, 1962) or hypophysectomy in the frog (Dellman and Owsley, 1969). Studies of the development of the neural lobe indicate that the differentiation and development of the blood vessels, pituicytes, perivascular cells and neurosecretory axons occur in parallel (Galabov and Scheibler, 1978). Perhaps an interaction between these elements is required for axonal outgrowth and formation of functional neurosecretory contacts. Dellman et al. (1987b) demonstrated that interrupted neurosecretory axons in the hypothalamus tend to reinnervate allografts of neural lobe preferentially over those of peripheral nerve (Dellman et al., 1986), and optic nerve or connective tissue (Dellman et al., 1985). These authors further noted that the areas of highest reinnervation in the neural lobe allografts were those areas showing the highest capillary density. In those areas of allograft where capillaries were either absent or not patent, there were no neurosecretory fibers, although these areas were likely to contain pituicytes. The author deemed the pituicyte to play a decisive role in reinnervation but one

that is secondary to that of capillaries (Dellman et al., 1987a). Hence, a strong correlation exists between the ability and degree of reinnervation and the extent of revascularization. In other regions of the brain e.g., non-neurosecretory systems, there does not appear to be a capillary hypertrophy preceding or concurrent to the reinnervation process. In these systems it seems likely the factors involved in the initiation of sprouting are released by the post-synaptic site. However, in the neural lobe the "post-synaptic" sites lack the specificity of classic synapses, being comprised predominately of the external basement membrane of the perivascular space. It seems reasonable to predict the involvement of capillary endothelial activity in the initiation and possible guidance of the neurosecretory sprouts. It would be interesting, in future investigations of this system, to determine the extent of the capillary hypertrophy in the partially denervated neural lobe and its relation to the sprouting response.

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## APPENDIX

Experimental ProtocolsABC Immunohistochemical Procedures for Light Microscopy

## 1) Clearing and Rehydration:

- a) two changes 10 min. each in xylene.
- b) two changes 10 min. each in 100% ETOH.
- c) one 5 min. rinse in 95% ETOH.
- d) one 5 min. rinse in 70% ETOH.
- e) one 5 min. rinse in 50% ETOH.

## 2) Primary Antibody:

- a) add 10ul AVP (1:100) to 150ml's PBS, then add 10ml's .5% thyroglobulin (THY) and a small amount of rat liver powder (RLP), agitate lightly and let stand 30 min., then centrifuge to remove suspended liver powder.
- b) incubate sections in primary antibody overnight at 4°C (1:5000 in PBS).
- c) two changes 10 min. each in PBS.

## 3) Secondary Antibody:

- a) incubate sections in biotin-conjugated goat anti-rabbit (1:100 in PBS) for 60 min. at room temperature.
- b) two changes 10 min. each in PBS.

## 4) Avidin-Biotin Complex

- a) mix 10ul avidin reagent A in 1ml of .1M NaPO<sub>4</sub> for every milliliter needed, then add equivalent volume of biotin reagent

- B, let stand for 30 min. at room temp.
- b) incubate sections for 30 min. at room temp.
  - c) two changes 10 min. each in .1M NaPO<sub>4</sub>
- 5) Diaminobenzidine (DAB) color reaction:
- a) add one 10mg DAB tablet to 20ml's .1M NaPO<sub>4</sub>, sonicate until completely dissolved then filter. Immediately prior to use add .05ml of .003% H<sub>2</sub>O<sub>2</sub> to every 50ml DAB prepared.
  - b) incubate sections 30 min. in dark.
  - c) two changes 10 min. each in PBS
- 6) Dehydration and Clearing:
- a) one 5 min. rinse in 70% ETOH.
  - b) one 5 min. rinse in 95% ETOH.
  - c) two changes 10 min. each in 100% ETOH.
  - d) two changes 10 min. each in xylene.
  - e) coverslip with permount.

#### Tissue Preparation for Transmission Electron Microscopy

##### 1) Primary Fixation:

- a) perfuse all animals via the intraventricular route with 4% glutaraldehyde + 2% paraformaldehyde in .1M NaPO<sub>4</sub>.
- b) remove brain, block area of interest and post-fix. for 3 hours in primary fixative.
- c) three changes, 10 min. each under hood in cold Ringer's solution to remove all trace of primary fixative.

##### 2) Secondary Fixation:

a) four hours at room temp. under hood in 2%  $\text{OsO}_4$  in .1M potassium-sodium phosphate buffer (PSPB) pH 7.2-7.4.

b) three changes 5 min. each in cold PSPB under hood.

3) Dehydration:

a) seven changes 15 min each, in order, under hood; 50% ETOH, 50% ETOH, 70% ETOH, 95% ETOH, 100% ETOH, 100% ETOH, 100% ETOH, (tissue may be stored in 70% or 95% ETOH overnight if necessary).

b) two changes propylene oxide (PO) 15 min. each under hood.

4) Infiltration/Polymerization:

a) Place tissue in a PO/Spurr's resin (2:1) mixture on shaker for 30 min., under hood.

b) continue infiltration in PO/Spurrs (1:2) mixture on shaker for 60 min., under hood.

c) continue infiltration in 100% Spurr's resin in refrigerator overnight.

5) Polymerization:

a) transfer one tissue sample to each beam capsule chamber using a flat beam capsule mold. Orient tissue so that the rostral pole abuts the tip of the mold. Fill each mold with 100% Spurr's then place capsules in a  $70^\circ\text{C}$  oven for 8 hours.

Materials and Design of Stereotaxic Knife

1) Supplier:

a) All materials were purchased from;

Small Parts Inc., 6901 N.E. 3<sup>rd</sup> Ave.  
P.O. Box 381736, Miami, FL 33238-1736

## 2) Materials:

a) part#	qty	description	size & length
HTX-33	2	stainless steel hypodermic tubing	33 gauge, 6"
HTX-24	2	stainless steel hypodermic tubing	24 gauge, 6"
ZRA-5	1	aluminum rod	5/16" diameter, 12"

## 3) Construction:

- a) cut the aluminum rod into three 10 centimeter (cm) lengths, then at one end of each bore a .008" hole approximately .25" in depth.
- b) Cut both the HTX-33 and HTX-24 hypodermic tubing into 5cm lengths. Spread a small amount of epoxy bonding medium over approximately 1cm of one end of the HTX-33 tubing and insert this end into the bore hole of the aluminum rod, allow this to dry overnight.
- c) apply a small amount of epoxy to approximately 1cm of one end of the HTX-24 hypodermic tubing and insert into the free end of the HTX-33 tubing in a telescopic fashion and allow to dry.
- d) flatten the lower 2cm of the HTX-24 tubing into a blade-like shape by squeezing forcefully with large hemostats, then bend the lower 1cm in a 30° angle from vertical so that an angled blade is formed, this will reduce the flexure of the blade as it passes through the target area.

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