1996

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Yong-San Huang
Iowa State University

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An immunohistological and electron microscopic investigation of neurosecretory neuronal regeneration following hypophysial stalk transection in animals subjected to chronic intermittent salt loading

by

Yong-San Huang

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Veterinary Anatomy

Major Professor: H. Dieter Dellmann

Iowa State University

Ames, Iowa

1996

Graduate College

Iowa State University

This is to certify that the Doctoral dissertation of

Yong-San Huang

has met the dissertation requirements of Iowa State University

Signature was redacted for privacy.

Major Professor

Signature was redacted for privacy.

For the Major Program

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For the Graduate College

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GENERAL INTRODUCTION

1. Introduction

The peripheral nervous system (PNS) and the central nervous system (CNS) do not respond in like manner to axotomy. In the PNS neurons usually survive axotomy, regenerate an axon and establish functional contact with their target area [122,197]. In the CNS neurons through retrograde degeneration almost always die after axotomy [122,197,262]. This is because the axon either does not regenerate or attempts at regeneration are unsuccessful or abortive [122,197]. Evidence is accumulating that the failure of adult mammalian CNS neurons to regenerate a new axon after injury is not due to an intrinsic incapability to regenerate; it is due rather to a combination of factors. These factors include: the microenvironment of the CNS (which forms a glial scar that impedes the growth of regenerating axons [117,201-203] and produces myelin-associated neurite growth inhibitors [219,220]); massive cell loss or atrophy of neurons (caused either by the axotomy or the development of secondary postlesional effects [22,47,56,164,195]); an unfavorable non supportive glial microenvironment; the absence of or presence of insufficient quantities of growth factors [243]; the failure of CNS neurons to upregulate the expression of growth- or regeneration-associated genes (such as those encoding for tubulin, actin and growth-associated protein (GAP-43) [130,152,222,244]; and last, inappropriate synapse formation [122]. There are, however, a few select areas in the CNS where spontaneous postaxotomy regeneration occurs: these are the olfactory system and the hypothalamo-neurohypophysial system [47,121-123,237].

The first report on the remarkable capacity of hypothalamic magnocellular neurosecretory neurons to regenerate was published in 1954; in this publication, Stutinsky [237] described the development of a miniature neural lobe following hypophysectomy. Subsequent papers confirmed and expanded his findings. They also reported considerable functional recovery from postaxotomy polydipsia [56,102,135,164,196]. However, the capacity for spontaneous regeneration in the hypothalamo-neurohypophysial system (HNS) is limited: Only when axons are transected in the median eminence or hypophysial stalk does regeneration occur. When axons are lesioned within the hypothalamus, the neurosecretory neurons behave like most CNS neurons and do not regenerate [48,122]. Strategies have been devised to explain why intrahypothalamic regeneration does not occur.

It has been shown that neural lobe or sciatic nerve transplanted into contact with the proximal stumps of transected neurosecretory neurons support vigorous regeneration, whereas transplants of optic nerve and cryotreated neural lobe or sciatic nerve induce only moderate or no regeneration [48,49,52,53]. Whenever the ventral surface of the hypothalamus is lesioned together with the hypothalamo-neurohypophysial tract (HNT), the leptomeninges prove to be a suitable microenvironment for robust regeneration [51]. Fine structural analyses of the tissues into which neurosecretory axons regenerate, have provided unequivocal evidence that regenerating axons invariably are associated with glial cells, i.e., pituicytes in the neural lobe, Schwann cells in the sciatic nerve, and neurolemmocyte-like cells in the leptomeninges. Neurosecretory axons do not regenerate into cryotreated transplants that were devoid of viable glial cells [48]. These studies culminated in this

conclusion: specific glial cells in the hypothalamo-neurohypophysial system are a prerequisite for successful neurosecretory axon regeneration.

Neurosecretory axons terminate in the neural lobe at fenestrated capillaries. In other words they terminate in an area of the CNS characterized by the absence of the blood-brain barrier (BBB) [122]. Spontaneous regeneration of axotomized neurosecretory neurons is observed only in areas in which the BBB is likewise absent, i.e., in the median eminence and hypophysial stalk. Spontaneous regeneration of axotomized neurosecretory neurons does not occur in the hypothalamus, where a BBB is present. The contention, that regeneration of neurosecretory axons requires absence of the BBB was tested in a series of experiments that led to this conclusion: Only tissues that lack a BBB, support neurosecretory axon regeneration [53,54,184]. And, of course, axons regenerate only in association with glial cells.

Obviously, successful regeneration in the HNS occurs only into a microenvironment that has supportive glial cells and in which the lack of a BBB provides serum-derived factors to the proximal axon stump -- and via retrograde axoplasmic transport -- to the injured neuronal cell body.

Interruption of the HNT causes the following major morphological and physiological changes [19,22,47,56,57,92,120,164,258,259]: Axotomized neurons, especially in the SON and to a lesser degree in the PVN, undergo massive retrograde degeneration, and only between approximately 14 % and 33 % survive [47,56,102,164,195]. Failure of secretion of vasopressin (AVP) into the general circulation prompts loss of antidiuretic function, causing diabetes insipidus [42,47,56,57,102,135,164]. The severity of this loss of the antidiuretic

function subsides when axons regenerate, but even after long survival periods and the establishment of new perivascular terminals, water consumption still remains above normal level [135,164]. The surviving neurons are incapable of secreting sufficient amounts of AVP and, therefore, they can not compensate for the massive postaxotomy cell loss. Consequently, the lesioned animals only partially recover from diabetes insipidus. Improved or complete restoration of antidiuretic function could conceivably be achieved through strategies aimed at rescuing more lesioned neurons.

The administration of vasopressin is a strategy often used in human hypophysectomized patients in order to alleviate postoperative diabetes insipidus [56,57]. In hypophysectomized rats, pharmacological doses of vasopressin have been shown to inhibit AVP synthesis and to cause a significant and selective decrease of the number of surviving vasopressinergic neurons with concomitant loss of antidiuretic function [92,93]. Even though AVP administration has an immediate and positive effect on posthypophysectomy diabetes insipidus, by reducing water consumption, in the long run, it has a deleterious rather than a beneficial effect.

We hypothesize that, if administration of AVP suppresses or decreases AVP synthesis by magnocellular neurosecretory neurons, and leads to postaxotomy cell death, then osmotic stimuli that increase and stimulate AVP synthesis, would have the opposite effect and would enhance postaxotomy neurosecretory neuronal survival and stimulate axonal sprouting. The observation that chronic consumption of 2 % NaCl induces non-injured neurosecretory neurons to sprout in the median eminence and to grow into the third ventricle lent further support to this hypothesis [50]. As the oral administration of a 2 % NaCl solution proved to

be fatal to stalk-transected rats, a chronic intermittent salt-loading regimen (CISL) was adopted, and in the first paper it was reported that CISL caused a significant increased number of neurosecretory neurons in the SON to survive axotomy, and caused complete functional recovery from polydipsia [102].

Based on these observations, we further hypothesized "that CISL, through stimulation of microtubular protein synthesis and/or their assembly into microtubules, accelerates neurosecretory neuronal regeneration and establishment of perivascular contacts. Glia-derived and blood-borne (serum-derived) factors could be transported retrogradely to the neuronal perikarya earlier after transection and in greater quantities, because of the presence of more microtubules, in animals subjected to CISL than in euhydrated animals. Consequently more neurons are rescued" [102].

Several observations support this hypothesis. Dehydration through oral administration of 2 % NaCl or water deprivation increases metabolic activity [77], AVP biosynthesis [26,34,64,168,180] and synthesis of proteins [68,140-142,175]. The proteins include cytoskeletal structural proteins such as microtubules and microfilaments [140,175]. Microtubules are necessary not only for axoplasmic transport of neurosecretory granulated vesicles (NGVs) [34], but also for the growth of axons [181]. Recently, βIII-tubulin, one of the isotypes of β-tubulin (which in turn is one of the two microtubular tubulins) has been proposed as being essential to regrowing axons [170,171]. Upregulation of βIII-tubulin was observed in neurons of the transected sciatic nerve in which regeneration occurred [112,170,171], and upregulation of βIII-tubulin persisted until the regenerating axons reached

their target areas [112]. Lesioning of CNS neurons, which do not regenerate, did not result in tubulin upregulation [130,152,153].

Recent papers have reported that several immediate early genes (IEGs), such as c-jun or c-fos, which are inducible by external stimuli, encode proteins that act as transcription factors to regulate the expression of target genes [39,162,224]. Jun forms dimers with itself or other IEG products -- such as Fos, Jun-B or Jun-D -- to form the transcriptionally active complex, AP-1 [134,176]. In these dimerized forms, Jun can bind to DNA and can act as transcription regulator to affect the expression of target genes [162,224]. Axotomized neurons in the PNS express c-jun [45,65,86,88,90,91,108,109,137]. That expression is downregulated when regrowing peripheral axons have reached their target areas [45,90,137]. But if regeneration is prevented, expression of c-jun persists in the injured neurons [65,86,137]. Such correlation between regeneration, or attempts at regeneration, and c-jun expression is apparently not a consistent feature in the CNS, and neuronal responses are highly variable [89,110,111,136].

Neuronal injury, such as axon transection, typically does not induce expression of c-fos [90,137]. However, in a few select areas of the CNS neurons respond to a variety of stimuli with the expression of c-fos [95]. Fos, the product of the c-fos gene, promotes the formation of heterodimers with products of the jun family [40,176]. Like Jun, through this dimerization, Fos acts as a factor to regulate the transcription of target genes [162,224]. Fos increases the DNA binding activity of Jun [80,176], and Fos/Jun heterodimers exert a stimulating effect on their target gene expression [36,218].

Nothing is known about postaxotomy c-jun expression in the HNS. However, given the regenerative capacity of this system, it seemed logical to assume that c-jun is similarly expressed in the PNS. Moreover, since either dehydration through water deprivation or substitution of drinking water with 2 % NaCl has been shown to cause expression of c-fos in magnocellular neurosecretory neurons [95,139,269], we hypothesize that CISL likewise induces c-fos expression. Fos, then, could conceivably enhance the binding of Jun to target genes, promote their transcription and stimulate the survival and regeneration of the axotomized neurosecretory neurons.

The observed coexpression of c-jun and of genes encoding for GAP-43 and tubulin isoforms in extrinsic axotomized neurons [65,86,111,244] is the basis of the following hypothesis: the expression of genes encoding for tubulin and GAP-43, whose products are considered essential for axon regeneration and/or collateral sprouting, is dependent on the preceding expression of c-jun.

In the second report, we first describe the patterns of expression of Jun, Fos and βIII-tubulin in the neurosecretory neurons of stalk-transected and/or CISL-treated animals; then we describe their relationship to axon regeneration and recovery from polydipsia. We present data supporting the hypothesis that CISL, through the induction of Fos, causes earlier expression of βIII-tubulin, faster postaxotomy collateral sprouting, and thus faster establishment of perivascular terminals. It is from these new perivascular terminals that serum-derived factors are presumed to be carried through retrograde axoplasmic transport to the neuronal perikarya earlier and in greater quantities than would be the case in euhydrated animals. As a result a greater number of AVP-synthesizing neurons would be rescued.

2. Dissertation organization

The body of the dissertation consists of two manuscripts. The first one is published in Brain Research [102]. The second one will be submitted to the same journal. The papers are preceded by a general introduction and followed by general conclusions. The literature cited in the general introduction and general conclusions is presented after the general conclusions.

3. Literature review

3.1. Hypothalamo-neurohypophysial system (HNS) in euhydrated animals

The hypothalamo-neurohypophysial system is composed of the magnocellular neurons of the hypothalamic paraventricular and supraoptic nuclei, their axons that from the hypothalamo-neurohypophysial tract (HNT), and their terminals in the neural lobe [82,230].

3.1.1. Morphology

3.1.1.1. Supraoptic nuclei (SON)

In the rat, the SON is extremely well defined. It straddles the anterior part of the optic tract just behind the optic chiasm and is divided by the optic tract into the principal part and the retrochiasmatic part [9,206]. The principal part is situated rostrally while the retrochiasmatic part is situated posterio-medially to the optic tract and lateral to the midline [9,187]. The magnocellular neurons of the SON are spherical or ovoid in shape, with diameters of 20-35 μm [234]. The extensive Nissl substance is usually located at the periphery of the cell body. Magnocellular neurons synthesize and process the vasopressin and oxytocin precursors into vasopressin and oxytocin and their respective neurophysins.

These are transported into the neural lobe via axons in the internal zone of the median eminence [63,270]. Hence, using immunohistochemical methods, vasopressin- and oxytocin-containing neurons and their axons can be easily and clearly identified [206,252]. In the principal part of the SON, the oxytocin neurons tend to be situated rostrally and dorsally while the vasopressin neurons tend to be situated caudally and ventrally [9,100,118,206,252].

Ultrastructurally, the euchromatic nucleus is pale, large and usually in the center of the cell, but sometimes it is eccentrically placed [116,198]. The nucleolus is very prominent. In animals at rest fewer than 5 % of the neurons in the SON contain more than one nucleolus 84]. The nuclear envelope has indentations of variable depth [116,198]. The matrix of the cytoplasm is relatively electronlucent. Mitochondria are ovoid in shape, numerous, and fairly evenly distributed in the cytoplasm. Abundant rough endoplasmic reticulum (rER) is composed of parallel stacks of long, flattened cisternae with numerous ribosomes attached, and they are usually localized at the periphery of the neurons [116]. In addition, many free ribosomes are present in the cytoplasm. Each neurosecretory neuron has several Golgi configurations, generally in perinuclear array [34]. Each Golgi stack is composed of 3 to 5 closely apposed cisterns and additional GERL (Golgi associated endoplasmic reticulum that gives rise to lysosomes) elements aligned at a slight distance from the last or innermost Golgi cistern [25,115]. Although it is usual to describe the convex, cis or condensing face of each Golgi stack as contrasting to the concave, trans or forming face, this description of secretory polarity within the Golgi system may not be true in the magnocellular neurosecretory neurons. Broadwell and Oliver [25] suggested that normally GERL produces both neurosecretory granulated vesicles (NGVs) and lysosomes, while during osmotic stress both

Golgi and GERL may produce NGVs. However, Picard et al. [188] proposed that a Golgi-GERL system is functional at all times with Golgi proper producing NGVs and GERL producing lysosomes. Mature membrane-bounded NGVs characterized by a dense granular core are dispersed throughout the cytoplasm. These granules are around 160-200 nm in size [116]. Also, there are immature NGVs in the vicinity of the Golgi apparatus as well as in their periphery.

The total number of cells in the SON ranges from 4000 to 7000 [41,240]. Rhodes et al. [206] stated that in immunohistochemically prepared materials the average number of vasopressin and oxytocin neurons in the SON was 3236 and 1443 respectively.

Rasmussen [194] concluded that most neurons in the SON project to the neural lobe because of the massive loss of neurons in this nucleus after hypophysectomy. Also, using neural tracing methods, Sherlock et al. [225], Wiegand and Price [261] and Armstrong et al. [10] demonstrated that virtually every neuron in the SON sends an axon to the neural lobe. The fact that some of the tracer injected into the ME was retrogradely taken up by the neurons in the SON led Wiegand and Price [261] to infer that a few neurons in the SON send collateral branches to the median eminence (ME). However, the fact that ^{3}H-amino acid injected into the SON is not anterogradely transported to the external zone of the ME indicates that the SON does not send collateral branches to the external zone of the ME [5]. Nevertheless, electrophysiological studies suggest that a few cells in SON may send axon collaterals to the ME [192] and lateral hypothalamus [138,149].

3.1.1.2. Paraventricular nuclei (PVN)

The PVN is situated in the dorsomedial hypothalamus adjacent to the third ventricle [240]. The terminology of the subnuclei of the paraventricular nucleus is not standard and consequently is used differently used by many authors. Swanson and Kuypers [240] using Nissl method subdivided the paraventricular nucleus into three magnocellular and five parvocellular subnuclei. The subnuclei for the magnocellular are anterior, medial and posterior part. The subnuclei for parvocellular subnuclei are periventricular, anterior, medial, dorsal and lateral part. The posterior magnocellular subnucleus is the largest and classically called PVN --this corresponds to the middle part used by Hou-Yu et al. [100] and the medial and lateral magnocellular PVN defined by Rhodes et al. [206]. The Rhodes terminology will be used for magnocellular PVN in this thesis. The anterior and medial part magnocellular subnuclei correspond to the anterior commissural nucleus (ACN) described by Rhodes et al., [206], Hou-Yu et al., [100] and Peterson [187]. Most neurons in the ACN are oxytocin-containing [100], and project to the neural lobe [5]. The medial part of the magnocellular PVN consists primarily of oxytocinergic neurons, while the lateral PVN is formed by a core of vasopressinergic neurons with a rim of oxytocinergic neurons [100,206]. In the magnocellular PVN the characteristics of the neural perikarya, at both light- and electron-microscopic levels, are the same as in the SON [116].

The cell number in the magnocellular PVN of rats ranges between 1300 and 2000 in Nissl stained materials [240]. Rhodes et al. [206] reported that on the average the PVN had 1174 oxytocinergic cells and 976 vasopressinergic cells.

Less severe retrograde cell loss in the PVN than SON after hypophysectomy led Rasmussen [194] to infer that axons from the magnocellular PVN end at varying levels within the hypothalamus and ME, and only a few axons actually reach the neural lobe. After fluorescent tracers or horseradish peroxidase were injected into the neural lobe, labeled cells could be found in the magnocellular lateral subdivision of the PVN as well as in the magnocellular accessory nuclei, and only a few scattered labeled cells could be found in the parvocellular nuclei [225,240,261]. However, Armstrong [10] stated that both medial and lateral magnocellular PVN send fibers to the hypophysis. Neurosecretory axons not only are present in the internal zone of the ME but also project to the external zone where they terminate on the primary portal plexus [230]. Most axons in the external zone of the ME are vasopressinergic, and only very few are oxytocinergic [253]. Lesion studies demonstrated that the cellular origin of these axons resides in the PVN [8,253]. Applying horseradish peroxidase to the ME , Wiegand and Price [261] and Swanson et al. [241] found that cells projecting to the external zone are different in both size and distribution from those that innervate the neural lobe, and that these cells are predominately in the parvocellular nuclei, such as the periventricular and medial parts of the PVN. The periventricular part of parvocellular PVN is composed of small vertically oriented fusiform cells that surround the ventricle and lie medial to the rest of PVN. The medial parvocellular PVN consist of densely packed small cells medial to the medial part of the magnocellular PVN [240].

3.1.1.3. Hypothalamo-neurohypophysial tract (HNT)

The HNT consists of axons derived from the PVN and the SON. Fibers leave the SON dorsally, follow the dorsal surface of the optic chiasm, and run caudomedially to the ME [37,82,242]. Those from the PVN leave the nucleus laterally, turn around the fornix and after a short caudal course join the supraoptic axons at the posteriolateral edge of the optic chiasm. They then pass through the extreme ventromedial area of hypothalamus, the internal zone of the ME and infundibular stalk, and then branch into the neural lobe [82,83]. The average length of the axons from the SON to the neural lobe is about 5 mm in the adult rat [82,83]. Axons from the PVN are located at the periphery of the stalk and usually terminate at the peripheral area of the neural lobe. By contrast, those from the SON are usually situated at the central area of the stalk and diffusely terminate in the neural lobe [5].

3.1.1.3.1. Structure of the median eminence

In rats, the ME is divisible into internal and external zones. The internal zone can further be subdivided into ependymal, subependymal, and fibrous layer, and the external zone can further be subdivided into the reticular and palisade layer [128,210,221]. The portal venules, and the pars tuberalis are superficial to the palisade layer [127,128,166,221].

The *ependymal layer* is a single layer of ependymal cells (tanycytes) with different shapes of microvilli and these project from the apical surface of the cells into the third ventricle. Judging by the shapes of the microvilli, Kobayashi et al. [128] stated that these microvilli have the function of secretion or absorption. The long basal tanycyte processes extend throughout the ME and contact the outer basal lamina of the pericapillary spaces

[128,221]. The *subependymal layer* lies just beneath the ependymal layer and is composed of one or two layers of subependymal cells whose morphology is similar to that of ependymal cells except for the lack of microvilli [128]. The *fibrous layer* is a narrow layer containing pituicytes and axons of the HNT which course horizontally through the ME to the neural lobe. Most axons measure from 2-8 microns in diameter and contain many neurosecretory granulated vesicles which range in size from 160 to 200 nm. These are about the same size as those in axon terminals of the neural lobe [166,221]. The processes of pituicytes and ependymal cells interweave with the axons here and extend to the palisade layer of the ME [128].

In the external zone the *reticular layer* is superficial to the fibrous layer. Axons of different types and processes of pituicytes and ependymal cells are interwoven in this layer and are the preterminal segments of the tubero-hypophysial tract [196]. The *palisade layer* is beneath the reticular layer. Here the pituicyte and ependymal cellular processes diverge into small processes and end feet, and mix with the axons. Most axons and glial processes run perpendicularly through this layer to the contact zone where axon terminals and the end feet of the pituicytes or ependymal cells appose the outer basal lamina of the perivascular space of the primary portal capillaries [128,196].

Immunohistochemical studies show that vasopressinergic and oxytocinergic axons with many axonal dilatations are mainly in the fiber layer of the internal zone [230,253]. Neurosecretory axons are not only present in the internal zone of the ME but also project to the external zone where they terminate on the primary capillary plexus [186,274]. However, most neurosecretory axons in the external zone are vasopressinergic; only very few are

oxytocinergic [253]. As mentioned before, these axons originate from the parvocellular nuclei in the PVN. The parvocellular vasopressinergic NGVs (about 100 nm) in the axonal terminals are smaller than that of magnocellular NGVs (about 175 nm) [263]. Thus, the distinction between the parvocellular axons in the external zone and the magnocellular axons in the internal zone is easily made at the EM level because of the difference in the diameters of NGVs [263]. Also, axons in the external zone containing 100 nm NGVs are never stained for oxytocin. In the NGVs of the parvocellular axons, vasopressin is colocalized with corticotropin releasing factor (CRF), and both are released synergistically to cause release of corticotropin from the adenohypophysis [251].

The median eminence is vascularized by a dense capillary network which is made up of two vascular systems: the surface network and the deep network. The surface network covers the entire ventral surface of the ME, and the network itself is covered by the pars tuberalis. The surface capillary network has a dense irregular mesh-like structure in which the arterioles, which supply the network, and the venules, which drain the network to the adenohypophysis, are found. Some capillaries penetrate into the palisade layer of the ME and constitute the short capillary loops. Some arterioles of the surface network give rise to long capillary loops[59,166]. The capillaries in the ME are of the fenestrated type and are permeable to blood-borne factors and/or proteins. They are characteristic of the capillaries in the circumventricular organs, i.e., areas of the CNS that lack a blood-brain barrier and that include the ME, hypophysial stalk and neural lobe.

3.1.1.3.2. The structure of the infundibular stalk

The hypophysial stalk is a solid structure that lacks ependymal cells. Apart from that, it has the same structural organization as the external and internal zones of the ME. The HNT passes longitudinally through the center of the stalk toward the neural lobe. Only a few vasopressinergic axons from parvocellular nuclei are present in the external zone of the stalk. The ventral surface capillary network in the ME also extends to the surface of the hypophysial stalk. Capillary loops are fewer or absent [165]. The capillaries in the stalk are of the fenestrated type and lack a blood-brain barrier (BBB).

3.1.1.4. The structure of the neural lobe

The neural lobe contains axons (mainly magnocellular neurosecretory axons), axon terminals, blood vessels, pituicytes and microglia. As the neurosecretory axons enter the neural lobe, they branch and form axonal enlargements or dilatations. The dilatations which do not appose the pericapillary basal lamina are classified as axon swellings. Those which abut the basal lamina are called axon endings or terminals. Axon swellings and terminals contain typical axonal organelles including lysosomes, mitochondria, NGVs and microvesicles. NGVs about 175 nm in diameter contain either vasopressin or oxytocin. AVP and OT in the NGVs are released by exocytosis into the capillary lumen via the pericapillary space [82]. Spherical microvesicles similar to synaptic vesicles are found mainly in the axon ending and very often form clusters [168]. It has been suggested that microvesicles, along with the mitochondria, play a significant role in sequestering and buffering the calcium that enters the neurosecretory axon endings during exocytosis [168].

Intermingled with the axon swellings and terminals are the pituicytes -- the predominant non-neural cells in the neural lobe. The morphological characteristics of these cells are their ovoid, pale nuclei with a rim of condensed chromatin and a well-developed Golgi complex with granular vesicles that indicate a specific secretory capacity. Cisterns of smooth endoplasmic reticulum are dispersed throughout the perikaryon but rough endoplasmic reticulum is always poorly developed. Many lipoprotein granules ranging from 0.5 to 2 μm in diameter (or sometimes grapelike clusters) are distributed throughout the cytoplasm [265]. This specific feature makes it possible to differentiate them from astrocytes, although they share some of the characteristics of astrocytes such as their neuroectodermal origin and positive reaction for GFAP [265]. They extend their processes to surround and engulf neurosecretory axonal processes, and together with neurosecretory axon terminals use the endfeet of their processes to make direct contact with the basal lamina of the pericapillary space [265]. The percentage of the pericapillary contact zone on the basal lamina occupied by the pituicyte endfeet is between 26 % [31] and 48 % [247]. The remainder is covered by neurosecretory axon terminals. But stimuli, such as water deprivation, saline drinking, parturition and lactation, can induce the pituicytes to release the engulfed axons and permit the axon terminals to contact more of the basal lamina [82,248,265].

3.1.2. Physiology of the HNS

AVP and OT are synthesized in the magnocellular neurons of the SON and PVN, transported through the HNT and then released from the axon terminals of the neural lobe

into the general circulation [63]. AVP increases the reabsorption of water from the distal tubules and the collecting ducts of the kidney in order to maintain the water balance [7,150], and OT causes the contraction of the uterine smooth muscle during parturition. It also causes contraction of the myoepithelial cells in the mammary gland so that milk is ejected in response to suckling stimuli [150]. Lack or insufficient AVP in the plasma, due to massive vasopressinergic cell loss in the SON and PVN after surgical, traumatic or electrolytic interruption of the HNT, results in loss of the water reabsorption function of the kidney [47,56,91,135,164]. Hence, lesioned animals produce large quantities of hypotonic urine and are polydipsic. However, electrolytic lesion only of the PVN does not increase the water consumption of the lesioned animals [73,94,273].

3.2. Hypothalamo-neurohypophysial system in dehydrated animals

3.2.1. SON and PVN

Osmotic challenge causes a variety of changes in the neuroscretory cells of the SON and PVN. Regardless of whether the osmotic stimulus is dehydration through water deprivation or oral administration of a 2 % NaCl solution, the morphologic responses are remarkably similar if not identical, and they reflect the increased biosynthetic activities of the neurosecretory cells. They also involve both AVP- and OT-synthesizing cells.

Neurosecretory cells in the SON react to water deprivation by increasing in size: Size increase has been reported at 5 % as early as 1.5 days after the beginning of dehydration [204] and this increase reaches 180 % after 12 days of water deprivation [168]. Oral 2 %

NaCl administration has somewhat less drastic though similar effects and culminates in 140 % larger cells after 25 days of dehydration [168].

As cell size increase through dehydration by water deprivation and 2 % NaCl ingestion, multiple nucleoli appear in many SON cells [11,84]. A morphometric analysis of the SON in acutely dehydrated animals and animals subjected to chronic intermittent salt loading for 6 days did not reveal a significant increase in the number of nucleoli; nucleolar volume did, however, increase significantly [133], suggesting elevated ribosome biosynthesis. The nucleolar response is amazingly rapid: it occurs as early as 2 hours after the beginning of water deprivation [84].

It is not surprising then, that there is an increase in the percentage of cells in which expansion and dilation of the rough endoplasmic reticulum occurs, as was first described by Zambrano and De Robertis [271] [60,115,132,169,198,250]. This is one of the morphologic signs that reflects the state of activity and the increased protein synthesis of the cell [169,271]. Expansion and dilation is likewise observed early during dehydration [249]. It is apparently in only a relatively small number of SON cells that dilation of rER is observed, the maximum reported percentage being 25 % after 3 days of oral 2 % NaCl [169]. The observed repeated decreases and increases in the number of cells with dilated rER, despite continued dehydration [132,169,250,271], seem to contradict the notion that the dilated rER indicates increased cellular activity; one has to keep in mind, however, that at the onset of dehydration not all cells are in the same state of activity and, therefore, probably do not react immediately nor synchronously to the osmotic stimulus.

Concomitant with the dehydration-induced changes in the rER modifications occur in the Golgi complex: it increases in size through proliferation of its cisternae and becomes fragmented [60,189-191,198,204]. This obviously reflects the increased biosynthesis of vasopressin to be expected in response to dehydration.

The number of the NGVs in the perikaryon of the neurosecretory cells tends to be decreased after dehydration [60,115,191,198,271]. Morphometric studies, however, have not substantiated these findings [169,204,250] and have led to the conclusion that dehydration does not cause any significant change in the number of NGVs. Not surprisingly, because of a more rapid turnover of NGVs, there appears to be a significant increase in the number of immature NGVs [169].

Autoradiographic studies reported increases in the rate at which labeled amino acids are incorporated into proteins of the neurosecretory neurons in the SON and PVN, confirming the finding that in osmotically challenged rats, protein synthesis is increased [68,140-142,175]. While it is more than likely that this increased protein synthesis represents increased biosynthesis of the precursor molecules of AVP, and to a lesser degree of OT, it is also possible that it additionally reflects increased biosynthesis of other proteins [142], such as membrane proteins and those of the cytoskeleton. Interestingly, hypertonic saline ingestion causes a weaker response than dehydration through water deprivation [142], but that response appears to be more selective in the SON than in the PVN [68,175]. Apparently, water deprivation is a much stronger stimulus than hypertonic saline ingestion, insofar as it causes neurosecretory neurons to increase the rate of glucose utilization (reflecting increased metabolic activity), while hypertonic saline ingestion does not [77].

With the development of probes for the detection of the AVP messenger RNA, it has become possible to examine specifically the AVP gene expression in both magnocellular neurons in euhydrated and dehydrated animals [270]. Chronic ingestion of saline has been shown to increase AVP mRNA content in the SON and PVN to between 2 and 7 times above basal level [12,28,105,144,148,256,270], with the response being greater in the SON than in the PVN [155]. Water deprivation, on the other hand, has just the opposite effect [155]. Individual cellular content of AVP mRNA and the number of cells expressing AVP mRNA rise rapidly in response to water deprivation, peak at 48 hours and then decline at 72 hours [155]. This observation correlates well with the changes in rER reported above and the changes in AVP content of the magnocellular nuclei early during dehydration [272]. Prolonged salt-loading (7 to 14 days of hypertonic saline ingestion) has been shown to increase gene expression for AVP [157,226-228] OT [157,255] and for several peptides colocalized in vasopressin and oxytocin neurons in both magnocellular nuclei [156]. During rehydration there is an immediate and gradual decrease in AVP and OT mRNA and these return to normal levels only when AVP replenishment of the neural lobe is completed [12].

In animals subjected to salt loading there is a substantial decrease in immunoreactive AVP and OT in the SON and PVN for 1 to 4 weeks as compared to the opposite for untreated animals [50,157,268]. This is a reflection of an imbalance between secretion and synthesis, the secretion being higher than the synthesis.

If one correlates the changes in gene expression with those in immunoreactive AVP and OT levels after salt-loading, it becomes obvious that elevated transcription leads to heightened translation, increased transport and increased secretion [157,226]. Biosynthesis

of hormones is augmented about fivefold [26,64,164,180]. Posttranslational processing is likewise three times more rapid [34]. These changes are consistent with the heightened demand for hormone to counteract the effects of dehydration; the changes are equivalent to a fivefold increase in the amount of newly synthesized neurosecretory material being transported [34,64].

3.2.2. Median eminence (ME)

In experimental animals -- as compared with control animals -- after 3 days of water deprivation no change is observed in the AVP content of the ME, but following 7 days of water deprivation, a significant decline in vasopressin content is detected [177]. After 7 days and 30 days of dehydration through 2 % NaCl loading, the immunostaining intensity of AVP and OT in the internal zone of the ME is decreased (the opposite is found for the control animals), but the decline is more pronounced in OT-positive than in AVP-positive axons [50]. Choy and Walkins [37] stated that immunostaining intensity of AVP and OT in the internal zone of the ME in salt-loaded animals decreases drastically after salt-loading for 1 day, then returns to the control level by 14 days, and decreases to a very low level at 21 days.

Interestingly, but not surprisingly, salt-loading for 7 days does not affect the immunoreactivity for either AVP or OT in the external zone of the ME [50,160], supporting the notion that the AVP axons in the external zone are not involved in the maintenance of osmotic equilibrium and ion balance [21]. Long term drinking of 2 % NaCl induces AVP- and OT-containing fibers to sprout in the ME and grow into the third ventricle [50]. This is an important observation with serious implications for the use of oral NaCl to stimulate

sprouting of neurosecretory fibers. Likewise, with the increased biosynthesis of AVP in the perikarya, there is an increase in the number of NGVs transported to the neural lobe [34,64]. This is accompanied by an increase of axonal diameters and microtubular content in the fibers of the neurohypophysial tract [75].

3.2.3. Hypophysial stalk

No information is currently available on dehydration-induced changes in the immunostaining intensity of AVP and OT in the hypophysial stalk, but given the structural similarities between ME and hypophysial stalk, changes in this area may be assumed to be the same as in the ME.

3.2.4. Neural lobe

Dehydration either through water deprivation or salt-loading causes secretion of AVP and OT from the neural lobe and increases plasma concentration of AVP and OT [82,226]. Therefore, the amount of AVP and OT [177,226], and immunostaining intensity for AVP and OT [37,50,160] in the neural lobe are decreased. The decrease in the immunostaining intensity for AVP and OT is most pronounced at 7 days, and the AVP decline is stronger than the OT decline [50]. Ultrastructurally, depletion of NGVs from axonal dilatations during dehydration first occurs in the nerve endings and only later in the swellings. Similarly, at the onset of rehydration newly synthesized NGVs move first to the endings [179].

3.2.5. Paradigm of chronic intermittent salt-loading (CISL)

In this paradigm, experimental animals are dehydrated with 2 % NaCl for 18 hours per day as drinking water, and then allowed to rehydrate on normal tap water for the remaining 6 hours. Unlike continuos salt-loading and water deprivation, CISL is a moderate osmotic stimulus; this is because 6 days of CISL does not significantly affect the body weight and hormone content in the hypothalamo-pituitary-adrenal axis [226]. After 6 days of CISL, the amounts of mRNA encoding for AVP and OT in the SON and PVN are increased, and about 70 % of AVP and 60 % of OT in the neural lobe are depleted [226]. With the exception of one publication by Lafarga et al.[133] who reported increased nucleolar size after 6 days of CISL, there are no reports in the literature on the morphologic effects of CISL on the HNS.

3.3. Hypothalamo-neurohypophysial system (HNS) in axotomized rats

3.3.1. Morphological changes of SON and PVN after axotomy of the HNT

Retrograde degeneration occurs in the magnocellular cells in the SON and PVN after axotomy of the HNT [47,124,125,173,174,195]. During the so-called obstructive phase, beginning 1 or 2 days after hypophysectomy and lasting for 10 days [195], accumulated neurosecretory material spreads back from the proximal transection site to the cell bodies [195]. The staining intensities of the vasopressinergic and oxytocinergic cells in the rats hypophysectomized for 10 days are higher than those in intact animals [118]. After 5 days, no clear-cut changes in the number of AVP- and OT-immunoreactive cells in the SON and PVN were observed. However, from the fifth to fourteenth day of survival, a progressive

decrease in the number of cells and in the intensity of the immunostaining was observed , especially in vasopressinergic cells [118,259]. After 36 days, there was no major difference in the number of the surviving oxytocinergic cells between the hypophysectomized and normal rats, whereas vasopressinergic cells showed a clear reduction in number, both in the SON and PVN [259].

Ultrastructurally, from 1 to 6 days after hypophysectomy, the cells become shrunk. The nuclei and cytoplasm become progressively more dense. The cytoplasm contains autophagic vacuoles and dense bodies [173,174]. Between 6 and 9 postoperative days, the degenerating cells become even more shrunk and spherical, and contain more dense lamellar bodies. In fact, the original organelles in the cell bodies become unrecognizable [47]. The nucleolus loses its normal appearance and becomes an amorphorus mass of fine granular material. The cytoplasm completely loses ribosome and electron density. Between 6 and 9 operative days, the glial cells gradually increase their phagocytotic activities to eliminate the degenerated cells [47]. There is agreement that the degree of degeneration and the rapidity with which it develops are largely dependent upon the distance between the site of lesion and perikarya --the shorter the remaining axons, the faster degeneration occurs and the greater the number of cells involved [42]. One plausible explanation for that phenomenon is that axotomy close to the cell body may interrupt and deprive the neuron of axon collateral branches which are capable of transporting target-derived factors to the perikaryon to support the survival of the cell.

Degeneration of the cells in the SON and PVN is not the inevitable result after interruption of the HNT. Some cells undergo the so-called reaction change and eventually

survive from the insult of the axotomy and become responsible for the functional recovery from the postsurgical DI [42,135,195].

Ultrastructurally, the reaction changes in axotomized neurons which are not degenerating are first recognized one day after axotomy, appearing as a few NGVs and dense bodies in the cell bodies [124,125]. After three days, they become more conspicuous and are characterized by an extensive rER which is either fragmented or in a peripheral location [123,124,173,174, 195]. These cells also contain many ribosomes and NGVs which are most abundantly accumulated in the periphery and are intermingled with dense bodies and multivesicular bodies [173,174]. Between 9 to 12 postoperative days, the surviving cells are enlarged and contain more NGVs, dense bodies and multivesicular bodies [47]. From 13 days onward, the accumulated granulated vesicles in the perikarya are decreasing. At 18 days, the perikarya contain only a few NGVs and well-developed Golgi apparatus which indicate their highly secretory activity [47]. Between 2 to 8 months, the surviving neurons gradually become hypertrophic in order to compensate for the loss of many magnocellular neurosecretory neurons. These enlarged neurons have extensive rER and Golgi apparatus [195]. The NGVs are no longer accumulated in the periphery and dense bodies are smaller and less numerous [173,174].

3.3.2. Proportion of the magnocellular cell loss after axotomy

Disconnection of the HNT through hypophysectomy, neural lobectomy or stalk transection causes retrograde degeneration and loss of magnocellular cells in the SON [22,56,92,135,164,183,259]. The percentage of cell loss is between 75 % and 90 % [47]

depending on the method used: After hypophysectomy, Rasmussen [194] reported a cell loss of 80 %; Raisman [195], O'Connor [183], and Moll and DeWied [164] reported a cell loss of 75 %. Fourteen percent of the cells were left after stalk transection and 24 %, after neural lobectomy [183]. With immunohistochemical methods, it becomes possible to assess the survival of various cell types. It is interesting, that after intracranial stalk compression, only 35 % of the vasopressinergic cells but 69 % of the oxytocinergic cells survive [56]. There is general agreement that the further the lesion is from the perikarya the greater the number of cells survive [42,47,183].

Retrograde degeneration and cellular death occurring in the PVN after axotomy is comparable to that occurring in the SON after axotomy. However, the percentage of cell loss in the PVN is usually less than that occurring in the SON. This is because there is a longer distance between the perikaryon and transection site, and there are more collateral branches projecting to other areas [42,47] such as to the spinal cord, the brain stem or the forebrain. The proportion of cells surviving after hypophysectomy is between 25 % and 50 % [47]. The percentage of cells surviving after neural lobectomy is 31 % according to O'Connor [183] and 33 % according to Moll and De Wied [164]. Using immunohistochemical staining methods, Dohanics et al. [56] found that 27 % of the vasopressinergic cells and 65 % of the oxytocinergic cells survived in the PVN after stalk compression.

3.3.3. Morphological changes in the median eminence (ME) after transection of the HNT

Within the first 5 days after interruption of the HNT, neurosecretory material cannot be released from the damaged neurons. As a result, it piles up in the axons proximal to the

lesion site in the fiber layer of the ME, and the staining intensity of neurosecretory axons is higher than it normally is [19,42,196]. In an immunohistochemical study, Villar et al. [258] reported that 5 days after hypophysectomy the staining intensity of vasopressin in the fiber layer of the ME is higher than in normal animals, but that of oxytocin is decreased. Beyond 5 days, the staining intensity and number of neurosecretory axons in the fiber layer decreases because the neurosecretory material accumulated in the axotomized axons disappears [19,118,119,196].

Two days after hypophysectomy, a few regenerating AVP- and OT-positive axons outgrow toward the external zone of the ME [193,258]. Five days after hypophysectomy, their number in the external zone increases [258], and by six days, a few regenerating neurosecretory axons reach the pars tuberalis [47]. By 10 days, both the staining intensity and number of AVP- and OT-positive axons increase, but regenerating oxytocinergic axons are less numerous than that the vasopressinergic axons [118,119]. After still longer survival times, the number and staining intensity of vasopressinergic and oxytocinergic axons in the external zone further increase. From two months onward, both kinds of axons form two distinct groups, one in the fiber layer of the internal zone, one in the external zone [258]. In a recent report, Alonso et al. [3] confirmed that distribution pattern of axons but emphasized that 35 days after stalk transection the majority of the regenerated axons in the external zone of the ME were oxytocinergic, interestingly vasopressinergic axons were scarce.

The first observation by Stutinsky that neurosecretory axons regenerate into the ependymal layer [236], into the third ventricle [236] and into the pars tuberalis [237] has

been confirmed by others [42,47,164,193]. Axons also regenerate into the meninges ventral to the pars tuberalis [2,51,193].

Most publications on the structure of the miniature neural lobe that forms after hypophysectomy mention the rich vascularity of this lobe, but a detailed description of the vascular reorganization of the lesion site awaited a publication by Moll [165]. This was followed by accounts of the fine structural characteristics of the vascular reorganization [19,47,193,196]. During the first week, the blood vessels in the ME dilate, endothelial cells proliferate [19,193], and the superficial capillary loops elongate [165]. Between 6 and 9 days after hypophysectomy, massive capillary invasion occurs at the lesion site [47,173,174], then capillaries from the vascular collar penetrate into the hypophysial stalk [165] and form several rows of capillaries [196]. The high vascularity obviously attracts the regenerating axons which grow toward the newly proliferated capillaries; they then become surrounded by a dense network of vasopressinergic and oxytocinergic axons [196,258].

Ultrastructurally, during the first week after hypophysectomy, morphological changes in the ME involve the appearance in the fiber layer of axons which are massively dilated and engorged with NGVs, with autophagic vacuoles, with lamellated dense bodies and with slender mitochondria [196]. Occasionally, in the external zone a few large axons appear; these are the regenerating axons from the fiber layer and contain various lamellated dense bodies and NGVs. The diameters of these regenerating axons are larger than those normally present in the external zone [196]. The newly formed regenerating axons are ensheathed by hypertrophic pituicyte or tanycyte processes, and grow toward the proliferating blood vessels. They are released from these sheaths when they make direct contact with the basal lamina of

the pericapillary space. By two weeks, some of the axonal terminals with NGVs and microvesicles make neurohemal contacts on the pericapillary space. From two weeks on, the blood vessels continue to proliferate, as do the regenerating axons, and there is an increase in the number of neurohemal contacts on the pericapillary space [196]. Regenerating neurosecretory axons search for and grow toward the capillaries and make contact with them [47,196,258]. It is suggested that the chances for the axotomized axons to survive and subsequently to form a new neural lobe depend on their reaching the fenestrated vessels to imbibe the necessary blood-derived growth factor from the capillary [258]. This hypothesis was first proposed by Dellmann [47] and tested by Ouassat [184]. They reported that the degree of vascular permeability to horseradish peroxidase in various grafts (such as neural lobe, sciatic nerve or optic nerve) transplanted into contact with the intrahypothalamically lesioned HNT area, correlates with the number of regenerating neurosecretory axons. In neural lobe grafts, where fenestrated capillaries are present and the BBB is permanently absent, blood-derived factors are available, and the regeneration of neurosecretory axons is extensive. This is not so for other grafts, such as sciatic and optic nerve grafts. In optic nerve grafts, where the BBB was restored after 10 days, only relatively few neurosecretory axons regenerate [184].

3.3.4. Morphological changes in the hypophysial stalk proximal to the lesion site after axotomy of the HNT

Degenerative events not only do occur in the distal transected axons, but retrograde degenerative changes also occur in axons proximal to the lesion site. One day after hypophysectomy or stalk transection, Murakami et al. [173] reported no changes in the

proximal area, but Dellmann [47] observed more dense lamellar bodies and axoplasmic reticulum. Five days later, the axons are either shrunk or swollen and contain aggregates of microtubules. Dense lamellar bodies and swollen, aggregated NGVs are increasing in number. After ten days, the degenerative changes are more noticeable: swollen axons contain polymorphic vesicles, numerous dense lamellar bodies and autophagic vacuoles. By twenty days, most degenerative axons are eliminated through phagocytic pituicytes [47].

Although retrograde degeneration is obviously substantial and affects many of the transected axons, the HNS possesses outstanding regenerative capability. Since Stutinsky [237] described the generation of a new neural lobe at the proximal end of the transected neurosecretory axons, numerous studies have been performed to study the sequential changes after axotomy of the HNT [22,47,56 57,92,93,164,165,193,195,196,258,259]. Within one day after hypophysectomy, proximal transected axons are clearly loaded with neurosecretory material and become more engorged between 2 to 5 days [19,47]. At the lesion site the blood vessels are congested immediately after interruption of the HNT [42,47,196]. By 5 days, a proliferation of endothelial cells occurs initiating reorganization of the vascularity. After two weeks, the morphological features are very different: The entire area has become extremely vascular and the amount of neurosecretory material has decreased. The proximal end of the transected stalk subsequently becomes rounded off [19], enlarges and gradually transforms into neural lobe-like tissue. By 30 days, the size of the reorganized neural lobe is about 1/4 of the size of a normal neural lobe [164]. From this point on, the neurosecretory material gradually accumulates in the regenerated axons near the blood vessels. By 2 months, there is

a considerable reaccumulation of neurosecretory material and the reorganized end of the stalk has the histological appearance of a normal neural lobe [19].

At the fine structural level, one day after transection axons undergoing regeneration contain variable numbers of NGVs and extensive axoplasmic reticulum [47]. Between 2 and 9 days after transection the regenerating neurosecretory axons are characterized by dilatations. These contain many NGVs and axoplasmic reticulum that become progressively more extensive and develop many dilatations with a somewhat electron dense content [47,124]. After 9 days, the regenerating axons begin to contact pericapillary spaces and the new axonal terminals typically contain NGVs, microvesicles and mitochondria, but axoplasmic reticulum is no longer present [173,174,124]. At the same time, regenerating axon terminals in the ME are also reaching pericapillary spaces. As time passes, more new axon terminals morphologically identical to those seen in the neural lobe, contact pericapillary spaces [47].

3.3.5. Morphological changes in the neural lobe after stalk transection

After interruption of the HNT, the axons distal to the transection site inevitably degenerate. The most conspicuous change after interruption is the appearance of retraction balls which represent enlarged neurosecretory axons filled with numerous NGVs [42,47]. They extend about 0.2 mm from the transection site, then disappear about 10 days after the interruption [47].

Ultrastructurally, the degenerating neurosecretory axons are characterized by densely packed, enlarged and clustered NGVs. Rupture of the limiting membrane of NGVs, loss of

their content and eventual fusion are commonly observed. An increased number of dense lamellar bodies as well as vacuolation and cyst formation are also observed [47]. All degenerated axons are phagocytosed by the microglia and pituicytes between 10-14 days after the interruption [47]. The remaining neural lobe is very small, devoid of axons and consequently devoid of immunostaining for AVP and OT, and consists only of pituicytes and capillaries.

3.3.6. Possible explanations for spontaneous regeneration after axotomy of the HNT

The capacity for spontaneous regeneration in the hypothalamo-neurohypophysial system is limited. Only when axons are transected in the median eminence or hypophysial stalk does regeneration occur. When axons are lesioned proximal to these areas (i.e., within the hypothalamus) the neurosecretory neurons behave like most CNS neurons and do not regenerate. Experimental works from this laboratory using a variety of transplants to promote regeneration of intrahypothalamically transected neurosecretory axons have provided unequivocal evidence that the regenerating axons invariably are associated with glial cells, and that they fail to regenerate when viable glial cells are lacking (48,49,52-54]. These studies, together with observations from this and other laboratories of successful regeneration into the ME, hypophysial stalk and HNT [47], culminated in the conclusion, that the presence of specific glial cells, such as pituicytes in the ME and hypophysial stalk and Schwann cells in the meninges, is an indispensable prerequisite for successful neurosecretory axon regeneration. Whether glial cells support axon growth by direct contact between the outgrowing axons and surface molecules on the glial plasma membrane, such as adhesion

molecules or whether glial cells support axon growth by diffusible neurotrophic factors, or both, remains to be investigated.

However, one additional element requires attention: Neurosecretory axons terminate in the neural lobe at fenestrated capillaries; in other words, they are in an area of the CNS characterized by the absence of the blood-brain barrier (BBB). Spontaneous regeneration of axotomized neurosecretory neurons is observed only in areas in which the BBB is likewise absent --i.e., in the median eminence and hypophysial stalk; but spontaneous regeneration does not occur in the hypothalamus, where a BBB is present [121,122]. The contention, that regeneration of neurosecretory axons requires absence of the BBB was tested by Ouassat [184]. He reported that the degree of vascular permeability to horseradish peroxidase in various intrahypothalamic grafts is correlated with the magnitude of neurosecretory axon regeneration. In neural lobe grafts, where the BBB is permanently absent, blood-derived factors are available, and the regeneration of neurosecretory axons is extensive as compared to the regeneration in optic nerve grafts. In optic nerve grafts, where the BBB was restored after 10 days, only relatively few neurosecretory axons regenerated. Since neural lobe grafts possess pituicytes to support regeneration, it is not clear whether specific glial cells are more important than fenestrated capillaries as a prerequisite for neurosecretory regeneration.

Obviously, successful regeneration in the HNS occurs only in a microenvironment that has supportive glial cells and in which the lack of a BBB provides serum-derived factors to the proximal axon stump and via retrograde axoplasmic transport to the injured neuronal cell body.

3.3.7. Physiology after axotomy of the HNT

The patterns of water consumption after hypophysectomy, neural lobectomy and stalk transection differ one from the other. Only the pattern of water consumption after stalk transection will be discussed here. Symptoms of diabetes insipidus (DI), comprising polydipsia and polyuria follow after severance of the HNT. This is because the antidiuretic hormone is no longer released into the general circulation from the disconnected neural lobe. These symptoms may occur within a few hours and last for 1 or 2 days [56,57,135]. Following this stage -- called the acute phase of diabetes insipidus -- there is an interphase of 2 or 3 days during which there is normal water intake and urine output. The interphase is due to the release of the antidiuretic hormone from the degenerating axonal terminals in the neural lobe [47,135]. This conclusion is supported by the observation that normal water intake does not occur in the neural lobetomized rat, that the amount of the excreted antidiuretic hormone in the urine at two days after stalk transection is higher than it is after one day, and that implantation of the neural lobe under the kidney capsule of the stalk transected rats extends the duration of the interphase [135]. This interphase is then followed by permanent DI.

Daily water intake is around 125 ml/day in the rat after stalk compression, and about 160 ml/day after stalk transection [56,57,135]. Recovery of water intake has not occurred three weeks after stalk compression [57]. Two months after stalk transection, water intake of some animals regresses, but it is still two times higher than normal, while water intake of some animals does not decrease at all [135]. Even though the reorganized neural lobe has the same histological appearance as the normal neural lobe, water intake is still higher than in

normal animals [164]. This is actually not surprising, since posttransectional cell loss in the hyphothalamic nuclei is massive; the surviving cells can either not at all or only partially compensate and secrete enough AVP to maintain the water balance.

3.4. Regeneration in the nervous system

In general, axons in the mammalian CNS are not capable of regeneration after injury. By contrast, damaged axons in the mammalian PNS or in the CNS of lower vertebrates can regenerate and restore their function. For a successful regeneration, the damaged neurons need first of all to survive the insult; then the lesioned axons have to sprout, elongate and reinnervate their target area. The regeneration process needs not only certain perikaryal reactions but also a suitable microenvironment at the lesion area.

3.4.1. Regeneration in the PNS after axotomy

After injury, the axons distal to the lesion site invariably degenerate by a process known as Wallerian degeneration. Wallerian degeneration is manifested by rapid and total disintegration of the axons and by concomitant dissolution of associated myelin sheaths. Both the axons and myelin sheaths are removed by macrophages and Schwann cells [208]. Concurrently, the Schwann cells proliferate and form columns that are surrounded by a basal lamina, known as endoneurial tubes or bands of Büngner. These are thought to guide the regenerating axons from the proximal segment to their targets [6]. Schwann cells are considered to be the key element of successful regeneration [78]: Schwann cells synthesize neurotrophic molecules such as the nerve growth factor (NGF) [14], the brain-derived

neurotrophic factor (BDNF) [1] and the ciliary neurotrophic factor (CNTF) [205]. Schwann cells also express integrins and cell adhesion molecules [43,214], and produce extracellular matrix (ECM) molecules such as laminin and collagens [27]. All these components affect neuron survival and axon elongation. However, Ide et al [106] pointed out that vital Schwann cells are not necessary in order for grafts of sciatic nerve to support the regeneration, since the basal laminae in the cryotreated grafts in which the cells had been killed can serve as guidance for the regenerating axons. The fact that regenerating axons are attached to the inner surface of the basal laminae along their entire length implies that basal laminae serve as contact guidance for the regenerating axons.

3.4.2. Regeneration in the CNS after axotomy

Injured neurons in the CNS, except olfactory sensory neurons and magnocellular hypothalamo-neurosecretory neurons do not undergo spontaneous functional regeneration,. Several hypotheses have been formulated to explain the failure of regeneration in the CNS.

3.4.2.1. Glial scar (scar formation)

The destruction of tissue by mechanical injury leads to the formation of scars in the CNS: These are to protect and maintain the homeostasis of the CNS [203]. The most important cell type in the CNS scar is the astrocytes, but there are also oligodendrocytes, microglial cells, and macrophages in the scar tissue [201,202]. The dense and complex nature of scars, and the fact that regenerative sprouts rarely grow through scar tissue has led to the postulate that the glial scar has a barrier function in regeneration [197,262]. Processes of

hypertrophic astrocytes forming multilayer interfaces between the intact CNS and the lesion area are thought to impede the regenerating axons [117].

The most compelling evidence in favor of the glial scar hypothesis was obtained when regeneration of injured dorsal root axons was studied. Regenerating axons of centrally transected dorsal roots are prevented from growing into the spinal cord and stop at the dorsal root entry zone. Here astrocytes form a multilayer structure similar to that of glial scars [231]. Ingrowth of the regenerating axons into the gray matter of the spinal cord occurs, however, when dorsal roots are directly implanted into the gray mater of the spinal cord [229] or when the dorsal root entry zone is bridged with nitrocellulose filters that are coated with growth-permissive immature astrocytes [126]. Nevertheless, it has also been argued that glial scars do not appear to be the main factor causing the failure of regeneration in the CNS, as robust axonal outgrowth is not seen when glial reactivity is absent [70,72,79]. Furthermore, it has been shown that glial scar in the hypothalamus does not impede the regeneration of the neurosecretory axons [52].

3.4.2.2. Oligodendrocyte and myelin associated neurite growth inhibitors

Schwab and Thoenen [220] postulated that inhibitory activity is present in adult CNS tissue that can not be overcome by stimulatory effects of neurotrophic factors since in coculture of dissociated perinatal rat sensory, sympathetic or retinal neurons with explants of adult sciatic nerve or optic nerve in the presence of NGF or BDNF, many axons grew into the sciatic nerve but not into the optic nerve. There is a strong growth inhibiting effect for neurons if membranes from oligodendrocyte-enriched cultures and CNS myelin membranes

are used as a substrate [32,254]. Schwab and colleagues then isolated two active proteins, and called these neurite growth inhibitors NI-35 and NI-250. These molecules are found in all CNS white matter , including the optic nerve, but are absent from the sciatic nerve. In the presence of neutralizing monoclonal antibodies (IN-1) against NI-35 and NI-250 neurites can grow over oligodendrocytes in culture and into optic nerve explants [13,32]. In vivo, the application of IN-1 antibodies to an injured corticospinal tract results in long-distance growth of regenerating axons of this tract where regeneration normally is limited [215-217]. It demonstrates the intrinsic capability of corticospinal axons to regenerate when certain inhibitory mechanisms are eliminated [219]. Also, application of IN-1 antibodies leads to an accelerated regeneration of the transected cholinergic-septohippocampal tract and to regrowth over several millimeters of intracranially transected optic nerve in which regeneration usually does not occur [30,260]. Although myelin-associated neurite growth inhibitors NI-35 and NI-250 seem to represent a major non-permissive component for successful regeneration in the CNS, recent evidences show that some other inhibitor molecules may also inhibit the regeneration; for example, myelin-associated glycoprotein (MAG) [152,172] and collapsin which can inhibit neurite outgrowth or induce collapse of the growth cone [147].

3.4.2.3. Presence of blood-brain barrier (BBB)

The finding that regenerating axons grow into skin transplants in the brain led Heinicke and Kiernan [85] to propose that plasma-derived proteins that have escaped from the capillaries of the grafts and enter the extracellular fluid around the growth cones of these axons might be responsible for the regeneration. In the adult intact CNS, blood-derived

proteins are unavailable due to the presence of a BBB to serum proteins [24,101]. But they escape from the breached blood vessels into the injured CNS, are endocytosed by the injured axons and transported to the neuronal perikarya. This leads to the initiation of axonal growth [121]. Thus, regeneration occurs when blood vessels are breached. But, once the BBB is reestablished the regenerating axons are deprived of blood-derived proteins, and regeneration is aborted [122].

3.4.2.4. Ineffective cell body reaction by CNS after axotomy

In contrast to most hypotheses, which attribute the failure of CNS regeneration to the environmental conditions at the growing proximal tip of the damaged axon, Barron et al. [15,16] suggested that intrinsic CNS axons fail to regenerate because of the perikaryal reactions to the injury. Basically, the soma of extrinsic neurons generally mount an anabolic response to axon injury. This includes an increased neuronal content of RNA and protein, coupled with heightened rates at which radiolabelled precursor are incorporated into these compounds [16]. These metabolic changes form the basis for an increased production of specific proteins needed for axon sprouting and elongation [154]. By contrast, intrinsic neurons respond to the axon injury by cytoplasmic, nuclear and nucleolar atrophy, accompanied by a decrease in the soma content of RNA and protein [16]. The reaction in intrinsic neurons is regressive and innately incapable to initiating or sustaining reconnection of the damaged neurites [17]. Thus a regressive cell reaction is an unlikely base for successful reconstitution of a CNS axon.

Grafstein's investigations do, in fact, support this point of view [74]. In goldfish retinal ganglion cells which have an unusual regenerative potential, axotomy-induced increases in the number of polyribosomes and proliferation of rER are considered to be of prime importance in promoting axon regeneration, since they lead to a massive increase in tubulin synthesis.

3.5. Selective changes of protein after axotomy

3.5.1. Expression of GAP-43 after axotomy

GAP-43 is a membrane-associated phosphoprotein abundantly present in the developing growth cone but the level decreases after developmental synaptogenesis [18,178,232]. It can be reexpressed in axotomized neurons after axotomy of an adult peripheral or extrinsic nerve such as the hypoglossal and facial nerve [97,233,244]. Interestingly, in intrinsic neurons the expression of GAP-43 after axotomy depends on the distance between lesion and neuronal cell body. Axotomy of corticospinal tract, optic nerve or rubrospinal tract at long distances from their cell bodies fails to induce expression of GAP-43 in their respective neurons [114,199,200,233,244]. However, axotomy near their cell bodies, e.g., intraorbital transection of the optic nerve or transection of the rubrospinal tract at the cervical level, induces GAP-43 expression in their neurons [58,244]. Transplantation of a peripheral nerve at the lesion area of the proximally transected intrinsic nerve induced GAP-43 expression in the cell bodies and regeneration occurs but in distally transected intrinsic nerve with the same grafts, GAP-43 is not induced and regeneration does not occur [58,207,209,222,244]. The correlation between the expression of GAP-43 and the

regenerative propensity of axotomized neurons indicates that GAP-43 expression represents a prerequisite for axonal regeneration. The recent discovery that increased expression of GAP-43 in the cell body is not necessarily required for motoneuronal collateral sprouting [20] may provide an explanation for the surprising observation that GAP-43 cannot be found in extensively regenerating vasopressinergic and oxytocinergic axons in the median eminence [4].

3.5.2. Expression of cytoskeletal proteins after axotomy

There are three phases in the regrowth of transected axons: The first phase is the sprouting phase. During this phase fine caliber sprouts emerge from the parent stump of the axon and grow distally. Actin is necessary during this phase for growth cone motility. The next is the elongation phase. Microtubules are essential to this stage. When regenerating axons reach their targets, they enter the maturation phase. The maturation phase involves radial growth of the new axons, which requires the presence of neurofilaments [181].

3.5.2.1. Changes of actin and microtubules after axotomy

Microtubules are assembled from α- and β-tubulin, the products of two small multigene families. In vertebrates, 6 functional genes for α-tubulin and 7 for β-tubulin have been identified [38,238]. But only 5 α-tubulin isotypes and 5 β-tubulin are expressed in the mammalian brain [238]. All of β-tubulin isotypes are highly homologous, differing from each other primarily in the 15 amino acids at the carboxy-terminal region of the protein [38,143,238,239]. Among the 5 isotypic β-tubulins in the mammalian nervous system, only

β_{III}-tubulin is neuron specific in avian species and almost neuron-specific in the rat [29,238,239]. β_{III}-tubulin is also the only phosphorylated tubulin [146]. Since different tubulin genes are expressed differentially during brain development, it is suggested that certain tubulin gene products are suited better for the requirements of growing neurons, while others are required for mature neurons [23,143,159]. In mammals, six different α-tubulin genes are expressed in neural and nonneural tissue during development [257]. Of these, two distinct α-tubulin m-RNAs, termed Tα1 and Tα26, are known to be expressed in the developing and mature brain of the rat [159].

Changes in the total amount of actin and tubulin in injured neurons differ between extrinsic and intrinsic neurons. In axotomized extrinsic neurons, the total amount of actin and tubulin increases for several weeks until regenerating axons reach their targets [244,245], whereas in axotomized rubrospinal (intrinsic) neurons the total amount of actin and tubulin increases one week after axotomy, then decrease at 2 and 3 weeks [244]. In these intrinsic neurons, the initiation of cell body reaction including upregulation of actin and tubulin expression, is similar to that of extrinsic neurons. But the inability of the intrinsic neurons to sustain the increased actin and tubulin expression may cause them to terminate the regeneration. It is thought that upregulation of tubulin and increased delivery of microtubules to the regrowing axons are essential for successful regeneration after injury [244].

Microtubules are composed of α- and β-tubulin, and there are many isotypes in each kind of tubulin. It is not known at this point whether all kinds of isotypes of tubulin genes are upregulated. Apparently, some specific isotypes of tubulin genes are selectively induced after injury. For example, α_1-tubulin gene [158,244], and β_{II}- and β_{III}-tubulin genes are

preferentially upregulated after transection or crush [66,97,98,112,170,171], while βI- andβIV-tubulin genes are only slightly or not at all affected after the nerve is injured [98,182,267]. The real meaning of this selectively upregulated gene expression is not known. However, observations that various tubulin genes are differentially regulated during brain development support the hypothesis that some tubulin isotypes such as αI- and βII-tubulin are preferentially suited for the requirements of growing neurons [23,143,159].

Selective upregulation of βIII- [170,171] or β-tubulin [267] in axotomized neurons is thought to be an effective neuronal cell body response for successful regeneration. Indeed, sciatic nerve transection, which is followed by regeneration, evokes upregulation of specific βIII- or β-tubulin genes for at least 4 weeks, while corticospinal tract transection or crush of the central root of a dorsal root ganglion, which do not regenerate, fail to induce upregulation of βIII- or β-tubulin [130,267].

Upregulation of αI-tubulin occurs as early as 4 hours after transection or crush of the facial or sciatic nerve, reaches a peak between 3 and 7 days in the facial nerve and between 7 and 15 days in the sciatic nerve. Then after the regenerating axons reach their target area, upregulation slowly declines to control levels [158]. The duration and magnitude of the upregulation of specific tubulin genes after crush of the sciatic nerve --a condition in which regeneration occurs faster-- is shorter and lower than the regeneration after transection of the nerve [112,158]. The cellular mechanisms involved in the induction of specific tubulin gene expression are not known but they could involve either retrograde transport of signals from the site of injury, depletion of retrograde trophic factors derived from target tissue, or other as

yet undefined signals. However, administration of NGF cannot prevent the β-tubulin gene expression in axotomized neurons [266].

Induction of βII-tubulin gene expression is not observed in retinal ganglion cells (RGCs) after intracranial transection of the optic nerve [96]. On the other hand, Mckerracher et al. [152] stated that intracranial transection of optic nerve causes an early (1 day after) increase in β-tubulin mRNAs in the axotomized RGCs. This is followed by a later decrease for three weeks, and suggests that the transient increase in tubulin mRNAs reflects an early regenerative response, the persistence of which depends on the further interaction of the growth cone with the microenvironment. Indeed, when a peripheral nerve is transplanted into the intraorbital transection site to provide a suitable environment for regeneration and to sustain the survival of the axotomized RGCs, long lasting expression of β-tubulin was observed in those neurons whose axons regenerated into the peripheral nerve [153]. This indicates that even in intrinsic neurons the upregulation of a specific tubulin gene marks effective regeneration.

Expression of αI- and βII-tubulin was observed both in the controlateral sprouting and surviving neurosecretory neurons in the SON and PVN, especially 10 days, but also 30 days, after unilateral intrahypothalamic lesion of the HNT [185]. Since both long-term dehydration and CISL for 2 weeks induces axons to sprout into the third ventricle [50,102], and ingestion of 2 % NaCl solution for one week causes increases in axonal diameters and microtubular content in the axons of neurohypophysial tract [75], it is reasonable to assume that induction of the tubulin gene may occur in the dehydrated neurosecretory neurons as it does in growing neurons during development and in regenerating neurons. CISL enhances survival and

regeneration capability of axotomized neurosecretory neurons [102]. Conceivably, CISL induces more cells to express tubulin and earlier than after axotomy only, thereby accelerating the growth of transected axons and the establishment of pericapillary contacts in the ME and proximal lesion site. Retrograde transport of target-derived factors or serum-derived factors to the perikarya would also occur earlier after transection and assure the survival of more neurons. As in the sciatic nerve-crush condition, in which regeneration and disappearance of upregulation of tubulin are faster than in nerve-transection, the accelerated establishment of perivascular contacts in the stalk-transected and CISL-treated animals may lead to the disappearance of upregulation of tubulin faster than in the stalk-transected and euhydrated animals. In the second part of this research project the above working hypothesis is tested, and the pattern of induction of βIII-tubulin in axotomized neurosecretory neurons is correlated with their regeneration activities.

3.5.2.2. Changes of neurofilaments after axotomy

Following injury of either extrinsic or intrinsic nerves, their respective neuronal cell bodies invariably respond with a rapid suppression of neurofilament synthesis [96,98,99,112,152,245,267]. This decrease in the neurofilament gene expression is a response only to axonal injury and is unrelated to the neuron's capability for either survival or effective axon regeneration [96]. Decrease of the neurofilament synthesis in axotomized intrinsic neurons is greater than in extrinsic neurons. Also, the amount of neurofilament returns to control levels when the regenerating axons reach their targets [76]. This suggests

that neurofilament protein synthesis may be regulated by retrogradely transported materials from the axon terminals.

3.6. Immediate early genes

Immediate early genes (IEGs) originally were defined as a class of genes that are rapidly and transiently expressed in cells following stimulation by growth factors [39]. Many IEGs encode nuclear proteins which function as transcription factors and are regarded as the third messengers in an intracellular signal transduction cascade between cell surface receptor (first messenger), cytoplasmic second messenger system and nucleus to regulate the cell function for the response to stimuli.

Activation of receptors located in the cytoplasmic membrane by extracellular stimuli is followed by the induction of different cytoplasmic secondary messengers; these subsequently lead to the activation or inhibition of IEG. After translation of the IEG proteins in the cytoplasm, these proteins enter the nucleus and form homodimers or heterodimers to bind with the DNA at the promoter of their respective target gene [162]. Using immunohistochemical staining methods Fos or Jun proteins can be localized in the nucleus.

Among the many IEGs, the fos and jun family genes are best understood. The fos family comprises c-fos and fos-related antigen (FRA) including fra-1, fra-2 and fos-B; the jun family comprises c-jun, jun-B and jun-D. These two IEG families belong to the basic zipper superfamily whose protein products possess a leucine zipper and a basic zipper DNA binding domain. The leucine zipper domain has an α-helical structure consisting of four or five leucines in orderly arrangement at 7-residue intervals and is aligned along one face of the

helix. The side chains of the leucine, extending from the Jun helical domain, interact with those of either the Fos or Jun helix, thereby promoting dimerization [134]. Adjacent to the leucine zipper domain there is a highly basic domain that required for DNA binding [67,131]. Each member of the jun family can form both homodimers with itself, and heterodimers with other members of the jun and fos family, while each member of the fos family can not form homodimers with itself [40,176]. All the different combinations of Jun-Jun or Jun-Fos form a dimeric complex called activator protein-1 complex (AP-1 complex), and bind to the AP-1 binding site of DNA. But they possess different DNA binding specificity, affinities and transcriptional activities. Fos-Jun dimers bind to the AP-1 binding site with 50 times greater efficiency than do Jun-Jun dimers [80,176]. Also, Fos-Jun dimers activate the transcription of genes containing on AP-1 binding site [36,218].

3.6.1. Expression of IEGs might reflect the regenerative propensity of central nervous system

3.6.1.1. c-jun expression after transection of peripheral and cranial nerves

After axotomy in the mammalian PNS, some of the injured neurons die while some survive and regenerate their axons to their target tissue and restore their function. But in the CNS, transected axons initially sprout after transection, but then regeneration is aborted, and massive neuronal death occurs. Furthermore, in the central nervous system, neurons of different ages (young vs old) or in different areas (HNS or olfactory system vs other areas) are conspicuously different in their endogenous potential to survive and to elongate their axons [61]. Many neurobiological events, ranging from cell death to successful regeneration, occur after axotomy. The initiation and maintenance of these events are genetically

controlled. Some IEGs, such as c-jun and c-fos, have been shown to encode transcription factors which have been thought to control specific target genes --genes whose products would in turn have a specific effector function in the neuronal response to noxious stimulation [162,224]. Therefore, investigation of gene expression after axotomy is an important way to explain the mechanisms operating in cell death and axon regeneration.

Transection of the sciatic nerve results in persistent expression of c-jun --but not c-fos [86,88,90,137]-- in the axotomized sensory neurons in dorsal root ganglia (DRG) and motoneurons in the ventral horn of the spinal cord [46,71,90,108,109,137]. A higher basal immunohistochemical staining intensity of Jun was observed in the DRG 10 hours after the transection, and in motoneurons 15 to 20 hours after the transection [90,137]. Maximal staining intensity was found for both DRG and motoneurons 2 or 4 days after transection [90,137]. Increased staining intensity was maintained if regeneration was prevented. Otherwise it returned to basal levels when axons successfully regenerated into their target areas, 40 to 60 days after transection. After sciatic nerve transection, the staining intensity in the sensory neurons and motoneurons remained higher than basal level up to 150 days [45,88,90].

After injury of the vagus nerve, c-jun is also induced in the sensory neurons in the nodose ganglion and dorsal motor nucleus. One day after injury the Jun immunohistochemical staining intensity is higher than basal level, and reaches its maximum at two days. It still remains higher than basal level 100 days after vagotomy, whereas it returns to basal level 100 days after vagal nerve crush because of the faster regeneration in

that situation [91]. Transection of the facial nerve or cervical sympathetic trunk also induces the expression of c-jun in the neurons of the superior cervical ganglion [81,91].

Following sciatic nerve transection, the peripheral territory vacated by sensory sciatic nerve fibers is innervated by undamaged axons from the adjacent saphenous nerve. One to three weeks after sciatic nerve transection, a substantial number of Jun-positive cells is present not only in the sciatic nerve-related DRGs but also within L3 DRG. This ganglion does not contribute sensory axons to the sciatic nerve, but does do so to the saphenous nerve [109]. It thus appears that c-Jun expression not only does occur in response to axon damage, but also may be associated with axonal growth [109].

It has been hypothesized that the induction of c-Jun expression in axotomized neurons may result from the relative lack of growth factors or inhibitory factors that normally reach the perikaryon through retrograde transport from the target organ. In support of this hypothesis, it has been observed that block of axonal transport through application of colchicine or vinblastine into the sciatic nerve or vagal nerve causes c-jun expression in primary afferent neurons and motoneurons [91,137]. In further support of this hypothesis, the onset of c-jun expression is dependent on the distance between the transection site and the axotomized neuronal cell bodies; the shorter the distance, the faster the onset [90,103,104]. In proximal axotomy, after which most of the axon is severed from the cell body, only relatively small amounts of the neurotrophic factor remain in the proximal axon, and once their supply by retrograde transport to the perikaryon is exhausted, c-jun is expressed.

The injury-induced increase in c-jun expression in DRG neurons is reduced by a continuous injection of nerve growth factor (NGF) into the ganglion; on the other hand,

continuous injection of NGF antibody causes c-jun expression in the normal DRG neurons. These findings indicate that a deprivation of target tissue-derived NGF, as after axotomy, leads to the induction of c-jun expression [71]. However, adding NGF or BDNF (brain-derived neurotrophic factor) into a DRG cell culture does not inhibit c-jun expression. This suggests that c-jun expression in the DRG cells cannot simply be attributed to the deficiency of NGF or BDNF [44].

3.6.1.2. c-Jun expression after axotomy of intrinsic central neurons

Transection or crush of the optic nerve induces c-jun expression in the retinal ganglion cells (RGCs) [87,103,104,211,212,222]. In a normal retina, basal expression of c-jun is weak in most RGCs. One day after proximal intraorbital transection of the optic nerve (3 mm away from the optic disc), RGCs start to increase expression of c-jun. At two days, nearly all RGCs express c-jun. After five to eight days the expression reaches a maximum. Thereafter it abruptly decreases as a substantial number of RGCs die. After two weeks, only a few RGCs express c-jun. After three weeks no more c-jun positive cells can be found [87]. Following distal transection of the optic nerve (9 mm away from the optic disc), c-jun expression is weaker. It begins later and declines faster than c-jun expression after proximal lesion [103,104]. Transection of the medial forebrain bundle (MFB) induces c-jun expression in the neurons in the substantia nigra compacta (SNC) and ventral tegmental area (VTR) [89,136]. The increase of c-jun expression was first observed 24 to 36 hours after transection, reached maximum after 2 to 3 days, declined after 10 days and disappeared after

30 days [89,136]. Cessation of expression of c-jun in the RGCs, SNC and VTR is thought to cause failure of regeneration in the CNS [89,110].

Two or three weeks after proximal axotomy and after peripheral nerve tissue was grafted in contact with the proximal stump of the transected optic nerve, more Jun-positive cells are found in the retinal ganglion cell layer [103,222] than in the retinal ganglion cell layer of animals that did not receive a graft. After proximal transection of the rubrospinal tract at cervical level 3 (C3) --but not distal transection at thoracic level 10 (T10)-- Jun immunoreactivity is first seen at 12 hours after axotomy and it reaches maximum at 8 days. Additionally, if a peripheral nerve is grafted near the transection site after proximal transection --but not after distal transection-- regeneration occurs accompanied by upregulation of the regeneration-associated proteins, such as growth-associated protein (GAP-43) and tubulin [103,111,222,243,244]. The proximal transection causes c-jun expression, along with the upregulation of the regeneration-related genes, and regeneration occurs; on the other hand when conditions such as distal optic nerve transection, low thoracic transection of the rubrospinal tract, transection of collaterals of hippocampal and cortical neurons, and transection of dorsal root fibers fail to induce c-jun expression [104,109,111,136], regeneration does not take place. These results seem to imply that even in the CNS c-jun expression might be associated with the expression of some effector genes such as GAP-43, tubulin, and cytoskeletal proteins necessary for the axonal regeneration [111,232,244].

Nothing is known about c-jun expression in the HNS after HNT transection. Given the regenerative capabilities of magnocellular neurosecretory neurons and their postaxotomy

functional recovery, will c-jun expression in the neurosecretory neurons follow the same pattern as it does in the PNS, i.e., will c-jun expression persists until the transected axons reach their target areas? If so, how may c-jun expression be related to functional recovery after axotomy? Another question is: how is c-jun expression related to regeneration? Is there a different pattern of c-jun expression between stalk-transected animals subjected to chronic intermittent salt-loading and stalk-transected euhydrated animals?

3.6.2. c-fos expression in the magnocellular neurons of the SON and PVN

c-fos is only transiently induced after osmotic stimuli and is a sensitive marker for acute neuronal stimulation [33,69,95,213,223]. Maximal c-fos expression is induced in the magnocellular neurons of the SON and PVN at 1 to 2 hours after intraperitoneal injection of hypertonic saline, but it is only barely present at 4 to 8 hours [223]. c-fos expression is slight in activated oxytocinergic magnocellular neurons of lactating animals [62]. c-fos is not found in oxytocinergic neurons of rats under a continuous stress situation [107]. However, Miyata et al. [161] demonstrated that c-fos is markedly induced in the magnocellular neurons of the SON and PVN in rats loaded with 2 % NaCl for 10 days, or deprived of water for 5 days. Thus, c-fos expression can be maintained by a long-lasting osmotic stimulus [161].

Fos is not consistently expressed after axotomy [90,137]. Because Fos increases the DNA-binding activity of Jun [80,176], and Fos/Jun heterodimers exert a stimulatory effect on the expression of their target genes [36], it seems logical to assume that Fos increases the efficiency of Jun in promoting the transcription of target genes related to neuronal

regeneration --the genes that would enhance survival and regeneration of axotomized neurons.

Chronic dehydration induces c-fos expression in neurosecretory neurons, but it is not known whether CISL has the same capability. If CISL does in fact induce c-fos expression in neurosecretory neurons, one possible mechanism by which CISL could act on neurosecretory neurons is that Fos in combination with Jun promotes the expression of an as yet undetermined target gene whose transcriptional product enhances neurosecretory neuronal survival and axon regeneration.

In the second paper, c-fos expression is monitored in stalk-transected and CISL animals and correlated with the expression of c-jun in axotomized neurosecretory neurons, with survival of neurosecretory neurons, with regeneration of their axons in the median eminence and stalk, and with the recovery from diabetes insipidus.

CHAPTER ONE. CHRONIC INTERMITTENT SALT LOADING ENHANCES FUNCTIONAL RECOVERY FROM POLYDIPSIA AND SURVIVAL OF VASOPRESSINERGIC CELLS IN THE HYPOTHALAMIC SUPRAOPTIC NUCLEUS FOLLOWING TRANSECTION OF THE HYPOPHYSIAL STALK

A paper published in Brain Research

Yong-San Huang and H. Dieter Dellmann

Abstract

Hypophysial stalk-transected (ST) and sham operated animals were subjected to a chronic intermittent salt loading regimen (CISL) for 14 days beginning 1 day post surgery (dps). Animals were sacrificed at 15 and 36 dps. Three days after the termination of CISL, water consumption in *ST + CISL* animals decreased to the same level as that of sham-operated animals, while that of *ST + water* animals was maintained at a significantly higher level. The number of the surviving vasopressinergic neurons in the supraoptic nuclei of the *ST + CISL* group was significantly higher than that of *ST + water* group. CISL induced vasopressinergic axonal sprouting into the external zone of the median eminence, and formation of subependymal perivascular plexus. While CISL also enhanced regeneration of oxytocinergic axons into the external zone, it did, however, not have any effect on the number of oxytocinergic neurons surviving axotomy.

1. Introduction

An exception to the general rule that after transection of axons in the central nervous system spontaneous regeneration does not occur is the hypothalamo-neurohypophysial system (HNS) [14,16,25,32]. This system comprises two main groups of magnocellular neurons, the supraoptic nuclei (SON) and paraventricular nuclei (PVN), whose axons form the hypothalamo-neurohypophysial tract, which courses through the median eminence and hypophysial stalk to terminate at pericapillary spaces in the neural lobe [15,20].

Interruption of the hypothalamo-neurohypophysial tract through hypophysectomy [8,25,38,40,47,50], neural lobectomy [32] , or stalk transection [1,8,24] causes several major morphologic and functional changes. Axons distal to the transection site degenerate and are eliminated through phagocytosis by activated microglia and pituicytes [9,28,52]. Axons proximal to the transection site sprout into the external zone of the median eminence [24,25,32,38], the proximal stump of the transected stalk [8,32], third ventricular lumen [40,50], and leptomeninges [12], or reinnervade the neural lobe and grow into the adenohypophysis if the hypophysis is left in situ [8]. Regenerating axons preferentially grow toward areas where proliferation of vessels occurs subsequent to tract transection [38], such as median eminence and proximal stalk, or into the highly vascular environment of the leptomeninges and adenohypophysis [8,12]. Neurons, especially in the SON and to a lesser degree in the PVN [8], undergo retrograde degeneration, and only between approximately 14 % and 33 % of the neurosecretory neurons survive axotomy [4,9,37].

Hypothalamo-neurohypophysial tract transection invariably causes diabetes insipidus [9,16,21,26], but the degree and pattern of posttransectional water consumption varies within

wide limits and is mostly dependent on whether or not neural lobectomy or hypophysectomy is performed, and on the percentage of transected axons [8,32]. To what degree recovery of antidiuretic function occurs, is largely contingent on the level of transection of the hypothalamo-neurohypophysial tract and the degree of regeneration of the transected axons. As a general rule, the farther the transection occurred from the perikarya, the better the chances are for recovery, and for a more pronounced recovery [8,35], but even after long survival periods and establishment of new perivascular terminals, water consumption still remains above normal level [32].

The experiments of this report were designed to test the working hypothesis that stimulation of AVP synthesis through oral administration of 2 % NaCl would increase the rate of postaxotomy neurosecretory neuronal survival and stimulate axonal sprouting. Two observations were at the origin of this working hypothesis. In animals, whose drinking water was substituted with a 2 % NaCl solution, a stimulus of AVP synthesis [5,30,41,42,45,51], neurosecretory axons were induced to sprout in the median eminence and to grow into the third ventricular lumen [11]. In contrast, the administration of pharmacological doses of vasopressin to hypophysectomized rats inhibited AVP synthesis and caused a significant and selective decrease of the number of surviving vasopressinergic neurons with concomitant loss of antidiuretic function [21,22]. In pilot experiments, stalk transected animals that had 2 % NaCl as drinking fluid, had prolonged postsurgical recovery periods, consumed little food, failed to groom and became lethargic to the point where they had to be sacrificed at around 2 days post surgery. We subsequently established that chronic intermittent salt-loading, which involves replacement of 2 % NaCl for drinking water for 18 h/day, and which has been

shown to cause increased AVP synthesis [41], had no noticeable negative effects in stalk-transected animals. Here we report, that 14 days of posttransectional intermittent salt-loading markedly enhanced the survival and functional recovery of axotomized vasopressinergic cells in the hypothalamic supraoptic nucleus.

2. Material and Methods

2.1. Animals

Male Holzman rats (250-300 g body weight) were individually housed, lights on from 06.00 to 18.00 h, with free access to tap water or 2 % saline (see below) and standard rat food throughout the investigation.

2.2. Surgical procedures and treatment

Animals were anesthetized with ketamine (60 mg/kg i.m.) and pentobarbital (20 mg/kg i.p.). The hypophysial stalk was transected stereotactically by lowering a 1.5 mm-wide wire loop knife in the midline vertical plane (3.8 mm caudal to bregma) from the surface of the brain to the basisphenoid; once in contact with the bone, the knife was moved bilaterally for 0.5 mm and then left in place for 30 seconds. In sham-operated control animals, the knife was lowered only 9 mm from the brain surface, causing a brain lesion identical to that in stalk-transected animals, but not transecting the stalk.

One day after the surgery, stalk-transected (ST) and sham-operated animals were randomly divided into 2 groups. In group 1, one half of the animals was subjected to chronic intermittent salt loading (CISL) for 14 days, i.e., animals had access to water from 10.00 h to

16.00 h and to a 2 % NaCl solution from 16.00 h to 10.00 h; the other half had free access to water. In group 2, one half of the animals was subjected to the same regimen as group 1, followed by 21 days of ad libitum access to water; the other half had free access to water. Animals were sacrificed 15 days after surgery in group 1 and 36 days after surgery in group 2. Based on these regimens, the following categories were established:

- 15 days: - *ST* + *CISL* (n=4) - *Sham* + *CISL* (n=4)
  - *ST* + *Water* (n=4) - *Sham* + *Water* (n=3)
- 36 days: - *ST* + *CISL* (n=5) - *Sham* + *CISL* (n=6)
  - *ST* + *Water* (n=6) - *Sham* + *Water* (n=3).

In addition, two animals that had not undergone any treatment, were used for control purposes. Water consumption of all animals was measured daily (at 10.00 h and 16.00 h) 3 days prior to surgery and during the postsurgical survival time.

2.3. Tissue preparation

Animals were deeply anesthetized with pentobarbital (50 mg/kg, i.p.) and perfused transaortically for 15 minutes with a phosphate-buffered (pH 7.4, 0.1 M) mixture of 4 % paraformaldehyde and 0.5 % glutaraldehyde. Following removal of the brain from the skull, the hypothalamus and hypophysis together with the surrounding meninges were isolated and postfixed in the same fixative for 14 hours at 4°C. The entire hypothalamus was cut with a vibratome into 50 µm serial frontal sections. The rostral and caudal ends of the pars principalis of the SON were identified using a 0.05 % methylene blue solution [14]. Starting with a randomly selected section, the lead section, one out of four sections was

immunostained for neurophysin-AVP (AVP). Beginning with the second section after the lead section, one out of four sections was immunostained for oxytocin (OT). Vibratome-cut 50 μm thick sections from the median eminence and neural lobe were immunostained for AVP and OT.

2.4. Immunohistochemical methods

Tissue sections were rinsed in PBS for 20 min, and treated for 20 min each with 1 % sodium borohydride in 0.1 M PBS to quench the aldehyde groups, and for 20 min. in 0.3 % hydrogen peroxide. Following 2 hours in a blocking solution containing 2 % bovine serum albumin, 2 % normal horse serum and 0.4 % Triton X-100 in Tris-phosphate buffer (pH 7.8), the sections were incubated with NP-AVP antibody (1:100) or OT antibody (1:200) overnight at room temperature. The monoclonal NP-AVP antibody (a gift from H. Gainer) does not cross-react with NP-OT or OT [49]. The monoclonal OT antibody (a gift from A.J. Silverman) does not cross react with NP-AVP or AVP [23]. After triple washing in TBS (5 min. each), the tissue sections were incubated in biotinylated rat serum preabsorbed horse antimouse IgG (1:100; Vector) for 2 hours, rinsed 15 min., reacted with avidin-biotin complex (ABC; Vector Elite Kit, 1:220 for 1 hour), and exposed to a substrate composed of 0.04 % 3,3-diaminobenzidine and 0.006 % hydrogen peroxide diluted in 0.1 M sodium acetate for 10 minutes. Tissue sections were then rinsed in 0.9 % sodium chloride, mounted on polylysine-coated slides, dehydrated in graded alcohols, cleaned in xylene and coverslipped with permount mounting medium. Control sections were run through the

immunohistochemical procedure after omitting either the primary or the secondary antibodies.

2.5. Cell counts

In all immunostained sections, the number of vasopressinergic and oxytocinergic cells was determined by counting all immunoreactive cells in both supraoptic nuclei at 400 X magnification. The sections were randomly mixed and coded with a code unknown to the person performing the count, and decoded after the count. The total number of neurosecretory cells for each experimental animal was calculated by the following formula: Sum of the number of immunostained cells divided by the number of the sections in which the cells were counted, multiplied by the total number of sections containing the SON.

2.6. Statistics

Daily water consumption data and the number of AVP and OT neurons were analyzed with three way ANOVA, followed by either two-way or one-way ANOVA, depending on which effects remained significant. Then the means of each group were compared by Tukey's multiple range test.

3. Results

3.1. Water consumption

3.1.1. in animals drinking water (Fig. 1)

In the *ST + water* group, during the first 24 hours after stalk transection, water consumption was drastically increased. On the second day post surgery (dps), water consumption returned to presurgery levels, and remained there for 24 hours. Thereafter, it gradually increased to reach its highest level at around the sixth day after stalk transection. Subsequently, it gradually declined, but remained at high levels throughout the experimental period.

In the *sham + water* group, water consumption remained at presurgical levels throughout the observation period.

3.1.2. in animals on CISL (Fig. 1)

In *ST + CISL* animals, daily fluid consumption, i.e., 2 % NaCl and water, followed a pattern similar to that observed in the *ST + water* group, with a less drastic and gradual decline of fluid consumption through 3 dps, and a subsequent increase to 7 dps and more or less leveling off at that level for the remainder of the CISL. Following cessation of CISL, water consumption declined drastically during the first five days and less rapidly thereafter.

In the *sham + CISL* group, fluid consumption increased at 2 dps and continued to rise steadily throughout the CISL period. After termination of CISL, water consumption declined to the level of that of sham-operated control animals.

3.1.3. Statistical analysis of water consumption

With three-way ANOVA, the water consumption data in group 2 were analyzed from 16 to 36 dps (i.e., one day after termination of CISL to the end of the experiment). In the analysis the factors were stalk transection (ST or sham), fluid consumption (salt or water) and date (days post surgery). It showed that all three factors, stalk transection ($F_{(1,19)} = 62.48$, $P<0.0001$), fluid consumption ($F_{(1,19)} = 14.94$, $P<0.0014$) and date ($F_{(20,19)} = 18.12$, $P<0.0001$) differed significantly, as did the two-way interactions between these factors; however, the three-factor interaction was not significant. We then analyzed the difference among four groups (*ST + CISL*, *ST + water*, *sham + CISL* and *sham + water*) at each day with one-way ANOVA, followed by Tukey's multiple range test at $\alpha = 0.05$. Tukey's test revealed that there was a significant difference among the groups at each day. Comparison of the means of each group with Tukey's test, showed that water consumption in the *ST + water* group was significantly higher than that in other groups on each day except at 16 dps, when there was no significant difference between *ST + water* and *ST + CISL* groups. Beyond 18 dps, i.e., 3 days after the termination of CISL, there was no significant difference among the *ST + CISL*, *sham + CISL*, and *sham + water* groups. Water consumption in the *ST + CISL* group at 36 dps was significantly lower than that at 16 dps.

3.2. Neurosecretory cells in the SON (Figs. 2 - 3)

3.2.1. Immunostaining

No differences were observed in the pattern and intensity of immunostaining for AVP and OT between intact and sham + water animals; our observations are in agreement with those reported by other investigators.

In the *sham + CISL* and *ST + CISL* groups, a decrease in the intensity of immunostaining for AVP and OT was observed, that ranged from subtle to drastic between individuals within groups and between groups. In the *ST + water* groups there was no appreciable change in the intensity of immunostaining for either peptide.

3.2.2. Morphometry

Three-way ANOVA of the number of surviving AVP and OT neurons in the SON at 15 dps and 36 dps showed that stalk transection ($F_{(1,34)} = 67.47$, $P<0.0001$) and CISL ($F_{(1,34)} = 25.3$, $P<0.0001$) significantly affected the number of AVP neurons. Since only two factors, ST and CISL, showed significant difference in AVP neurons, these two were further analyzed as independent factors by two-way ANOVA: both stalk transection ($F_{(1,34)} = 72.96$, $P<0.0001$) and CISL ($F_{(1,34)} = 26.91$, $P<0.0001$) significantly affected the cell number. Then these two factors were separated into 4 groups and one-way ANOVA was used, followed by Tukey's test, revealing that the number of AVP cells in the *ST + CISL* group was significantly higher ($P<0.05$) than that in the *ST + water* group (Fig. 4). While in the latter group only 35 % of the cells survived stalk transection, almost twice as many, i.e., 66 %, survived in the former. Although the cell number of both sham operated groups was significantly higher

than that of the *ST + water* group, there was no significant difference between the sham + water and *ST + CISL* groups.

Three-way ANOVA of the number of surviving OT neurons showed that only stalk transection ($F_{(1,34)} = 34.6$, $P<0.0001$) had a significant effect on the number of OT neurons, which was unaffected by CISL. Comparison of the total number of OT neurons, at 15 dps and 36 dps combined, in sham-operated and stalk-transected animals showed that stalk transection caused significant cell loss ($P<0.05$) (Fig. 5).

3.3. Hypothalamo-neurohypophysial tract

In *intact* and *sham + water* animals at 15 and 36 dps, no difference was observed in the immunohistochemical staining and morphology of vasopressinergic and oxytocinergic neurosecretory axons in the internal zone of the median eminence. Neurosecretory axons were stained intensely and had numerous small dilatations. In the external zone of the median eminence, AVP-positive, finely beaded and thin axons were present (Fig. 6A); occasionally, OT-positive axons were present, either singly or in bundles (Fig.7A).

In *sham + CISL* animals at 15 and 36 dps, AVP-positive axons in the internal zone had many large dilatations (Fig. 6B), and occasional subependymal single axons were observed. Conversely, an appreciable decrease in oxytocin immunoreactivity was observed (Fig. 7B). In the external zone, AVP-positive axons were more numerous than in the intact and sham + water groups. No changes were observed in OT-positive fibers.

In the *ST + water* animals at 15 and 36 dps, the morphology and intensity of immunostaining of both types of axons in the internal zone resembled that observed in intact

and sham + water animals, except for the presence of a few subependymal axons and axons projecting into the third ventricular lumen. In the external zone, an increased number of more intensely stained AVP-positive axons (as compared to both sham + water and sham + CISL animals) was present, especially around capillaries; these changes were more intense at 36 dps (Fig. 6C) than at 15 dps (not shown). OT-immunoreactive axons in the external zone were relatively numerous, often had a tortuous course and were particularly concentrated toward the pars tuberalis; again, these changes were slightly more intense at 36 dps (not shown) than at 15 dps (Fig. 7C).

In the *ST + CISL* animals, both types of immunoreactive axons in the internal zone stained intensely, had many large dilatations, formed dense perivascular plexus, and some of them projected into the third ventricular lumen (Fig. 6D). There was no assessable difference between 15 and 36 dps. In the external zone, there were more AVP- and OT-positive axons than in any other group; the increase was more pronounced in AVP- than in OT-positive axons, and also at 36 dps than at 15 dps (Fig. 7D). OT-positive axons in the external zone were characterized by their large diameter, tortuous course, and often entwined or coiled terminals. Both types of axons were frequently densely packed at the boundary toward the pars tuberalis.

In the vicinity of the transection site, the median eminence became enlarged (Fig. 8). This enlargement was more pronounced, and AVP-positive axons were considerably more numerous and more intensely stained in the CISL than in the water groups.

The use of 50 μm thick sections precluded any assessment of changes in immunoreactivity in the neural lobe of the sham groups. In all *ST* animals, immunoreactive

axons were absent at 15 and 36 dps, indicative of complete stalk transection and absence of regeneration; the fine structural analysis of these neural lobes confirmed the light microscopic observations.

4. Discussion

4.1. Water consumption

Animals that are subjected to interruption of the hypothalamo-neurohypophysial tract characteristically develop posttransectional diabetes insipidus, i.e., they become polydipsic and excrete large amounts of dilute urine [8,9,21,26,32,36]. Water consumption after hypophysial stalk transection follows a distinctive triphasic pattern, confirming previous reports from this and other laboratories [8,16,26,36]. The high water consumption during the first day after surgery, in the ST + water group, coincides with polyuria and is due to the inability of the lesioned neurons to release vasopressin [26]. At 2 to 3 dps, a drastic decrease in water consumption occurs to the same level as that of animals in the sham + water group, and that decrease coincides with oliguria [26]. This temporary recovery is caused by the release of vasopressin from the degenerating vasopressinergic axon terminals in the neural lobe [9,16,26,36], since it is absent in hypophysectomized and neural lobectomized animals. After 3 dps, water consumption increases gradually to reach its highest level at around 6 dps. At this time, the axon terminals in the neural lobe are either no longer capable of releasing hormones or they release them in quantities that are not physiologically effective. The pattern of water consumption in the ST + CISL group is identical to that in the ST + water group, except that the decrease at 2 and 3 dps is not as drastic, because the animals are

consuming large quantities of hypertonic saline. For the same reason, the subsequent increase in fluid consumption, i.e., combined water and hypertonic saline, is much greater than in the ST + water group. This is also true in the sham + CISL group, in which the amount of consumed fluid is about two times higher than in the sham + water group, while the animals are subjected to salt-loading. In contrast, in the sham + water group, no change occurs in the postsurgery water consumption, which is maintained at presurgical levels and thus identical to that of intact animals.

After termination of the salt loading, between 16 and 36 dps, water consumption in the ST + water group remains elevated and is significantly higher than that in the ST + CISL and sham-operated groups. There is, however, a gradual decline in water intake, which at the end of the observation period reaches a level that is significantly lower than at the beginning. This confirms observations by other authors [16,26,32]; it is important to point out, however, that tract-transected animals do not recover completely from diabetes insipidus, and their water consumption remains significantly higher than that of control animals [32]. In the ST + CISL group, a radical change is observed after termination of CISL, as water consumption drastically declines; 3 days later, i.e., at 18 dps, it reaches a level that is no longer significantly different from that in the sham-operated groups, and that continues to decrease further throughout the observation period (Fig. 1). Obviously, the intermittent consumption of hypertonic saline caused vasopressin to be synthesized and released in quantities sufficient for complete recovery from polydipsia. In the sham + CISL group, water consumption rapidly decreases to the same level as that in the sham + water group, as early as 16 dps, i.e., 1 day after the termination of CISL.

4.2. Cell loss in the SON

After interruption of the hypophysial stalk through hypophysectomy, stalk transection, or neural lobectomy, many neurosecretory neurons in the SON undergo retrograde degeneration [4,8,9,21,25,32,35,37]. The percentage of total cell loss ranges from 67 % to 86 % [4,32,35,37] and is dependent on the level of transection [2,8,35], type of surgical procedure [32], and degree of regeneration of the transected axons [9]. In our experiments, 35 % of the AVP-positive cells survived in the ST + water group, while 66 % survived in the ST + CISL group, which most plausibly accounts for the spectacular recovery from polydipsia in that group. The question as to why more cells survive in the CISL than in the water group is addressed in the next section.

Oxytocinergic neurons have a much greater capacity for posttransectional survival than vasopressinergic ones [16,25]. More OT-positive cells than AVP-positive cells survive hypophysectomy, although their number is significantly reduced as compared to control animals [25]. Our experiments confirm these findings, showing that 70 % of the OT-positive cells survive stalk transection, but that their survival is not affected by CISL. Dehydration through either water deprivation or NaCl administration is known to cause changes in both vasopressinergic and oxytocinergic neurons. Oxytocinergic neurons are endowed with exceptional inherent plasticity [44], and their axons regenerate vigorously in response to various stimuli, such as neural lobectomy [32] or hypophysial stalk compression [16]. Reasons for the apparent unresponsiveness of oxytocinergic neurons to CISL remain to be established.

4.3. Changes in the median eminence

AVP and OT immunoreactivities in the median eminence of intact and sham-operated animals are differentially affected by osmostimulation through either CISL or 2 % NaCl. CISL has no effect on AVP immunoreactivity in the internal zone, but that of OT is clearly diminished. After 2 % NaCl, however, both immunoreactivities are less intense [3,11]. In the external zone, CISL causes an earlier (15 days) noticeable increase in AVP immunoreactivity than 2 % NaCl (30 days) [11]. CISL has no effect on OT immunoreactivity in the external zone, while 30 days of 2% NaCl cause a definite increase [11]. At this point, we have no explanation for the apparent lack of a reaction to CISL in AVP axons in the internal zone. CISL, however, apparently greatly accelerates AVP accumulation in the external zone. Whether this accumulation occurs in parvocellular ones that originate in the paraventricular nucleus [39] or in magnocellular ones that have sprouted into the external zone (see below) is not clear. Obviously CISL does induce limited sprouting of vasopressinergic axons into the subependymal neuropil of the median eminence, and it is conceivable that the highly vascular microenvironment of the external zone is more conducive to sprouting than the internal one. Future experiments will have to address that question.

Following hypophysial stalk transection or hypophysectomy, neurosecretory axons vigorously regenerate throughout the entire median eminence, and especially immediately proximal to the transection site, where they form a functional neural-lobe like structure that can respond to a dehydration stimulus [9,32,38,43]. Regenerating axons grow into the external zone of the median eminence where they form dense perivascular plexus, they also

sprout into the ependymal layer, the third ventricle and the proximal lesion area. Conspicuous differences in the number and intensity of the immunoreaction of the vasopressinergic axons were found between *ST + CISL* and *ST + water* group at both survival times, but especially at 36 dps. Not only are there considerably more and more intensely stained AVP-positive axons in the external zone of CISL groups, but the pattern observed at 36 dps (see Fig. 6D) closely resembles that reported by Villar et al. [47] 2 months after hypophysectomy. CISL apparently has accelerated posttransectional "maturation" of the AVP neurosecretory system. It is interesting to note that even though CISL fails to affect the number of oxytocin neurons surviving stalk transection, it has a similar posttransectional "maturation" effect on oxytocin-positive axons in the external zone.

The function of neurosecretory neurons is closely dependent on their ability to release their hormones into the circulation. When a neurosecretory neuron is deprived of its axon terminal at a capillary within the neural lobe, i.e., a location outside the blood brain-barrier, it no longer releases hormones in physiologically effective quantities. When transected neurosecretory axons regenerate, they preferentially grow into areas where fenestrated capillaries are present, and once they have established perivascular contacts, they secrete hormones, as evidenced by both functional and morphologic findings [6,9,12,13,21,31,32,36,38,46,48]. The microenvironment of the median eminence is ideally suited for neurosecretory axon regeneration in that it contains both fenestrated capillaries and glial cells. The latter have been shown to be a prerequisite for neurosecretory axon regeneration, since regeneration does not occur when they are absent [10]. In the median eminence, tanycytes apparently guide regenerating neurosecretory axons toward the

capillaries in the external zone, or possibly provide them with a factor or factors that promote their regeneration [7]. It is conceivable and even likely that these putative factors act synergistically with putative serum-derived factors/substances, and that the survival of axotomized neurosecretory neurons may be critically dependent on the availability of these factors. The faster a neurosecretory neuron establishes perivascular contact, the better its chances are for recovery. There may be a critical posttransectional time during which such terminals have to be established to prevent neuronal death. Hyperosmotic stimuli increase metabolic activity [19], vasopressin biosynthesis [17,29,41,51] and the synthesis of proteins [27], including those of cytoskeletal structures such as microtubules, microfilaments and neurofilaments [27,33]. These changes are paralleled by an increase in the rate of hormone transport [17]. Thus, an increased number of neurosecretory vesicles is transported intraaxonally to the neurohypophysis. In water-deprived rats, it has been shown, that increased axonal transport is accompanied by an increase of axonal diameters and the number of microtubules [18]. It seems reasonable to assume that identical changes occur in animals dehydrated through administration of NaCl. Microtubules are not only involved in axoplasmic transport, but they are also essential for elongation of axons [34]. We hypothesize that CISL, through stimulation of microtubular protein synthesis and/or their assembly into microtubules accelerates neurosecretory axonal regeneration and establishment of perivascular contacts. Glia-derived and blood-borne (serum-derived) factors could be transported retrogradely to the neuronal perikarya earlier after transection and in greater quantities, because of the presence of more microtubules, than in animals that were not

subjected to salt-loading, and consequently more neurons are rescued. Experiments are now in progress to test that hypothesis.

In conclusion, stimulation of synthetic activity of axotomized neurosecretory neurons with CISL increases the number of surviving vasopressinergic neurons, accompanied by complete and accelerated recovery from polydipsia. This is a simple and inexpensive method that could conceivably be used for treatment of posttraumatic or postsurgical diabetes insipidus in human patients.

Acknowledgements. We thank H. Gainer and A.J. Silverman for the donation of antibodies, and L. Wolins and M. Shelley for the statistical analysis of the data. The excellent technical assistance of R. DenAdel and C. Martens is gratefully acknowledged.

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Fig. 1. This graph depicts daily fluid consumption following sham operation or stalk transection (ST) at time 0, and subsequent chronic intermittent salt loading (CISL) beginning at 1 day post surgery (dps) for 14 days. Notice the fast decrease of water consumption to the same level as in sham-operated animals 3 days after termination of CISL (at 18 dps) in the ST + CISL group ($P>0.05$). The water consumption of rats in the ST + water group is significantly higher than in all other groups ($P<0.05$) from 17 dps to 36 dps. All values represent mean ± SEM.

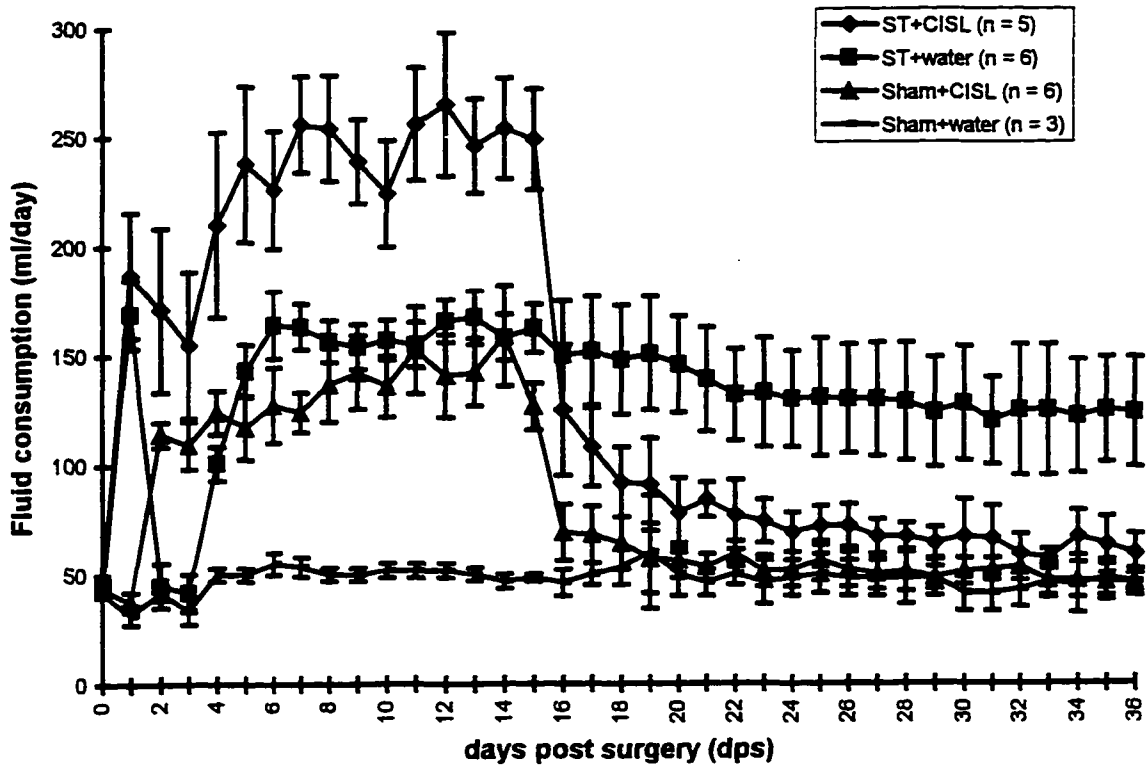

ST+CISL (n = 5)
ST+water (n = 6)
Sham+CISL (n = 6)
Sham+water (n = 3)
Fluid consumption (ml/day)
300
250
200
150
100
50
0
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
days post surgery (dps)

Fig. 2. OT immunoreactive cells in the supraoptic nucleus of (A) sham + water, 36 dps; (B) sham + CISL, 15 dps; (C) ST + water, 15 days; (D) ST + CISL, 15 dps. The number of cells is significantly lower in (C) and (D) as compared to (A) and (B), but there is no difference between (C) and (D). X 90.

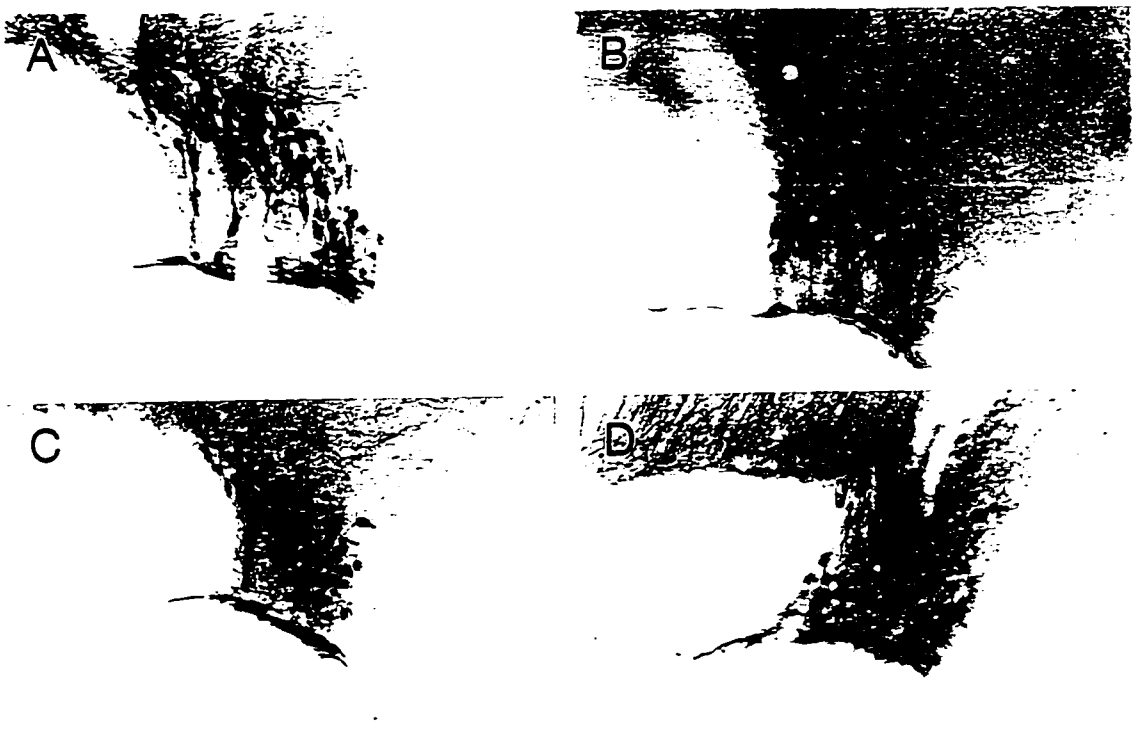

A
B
C
D

Fig. 3. AVP immunoreactive cells in the supraoptic nucleus of (A) sham + water, 36 dps; (B) sham + CISL, 15 dps; (C) ST + water, 15 dps; (D) ST + CISL, 15 dps. All sections are from approximately the same level. Notice especially the difference in the number of cells between (C) and (D). X 90.

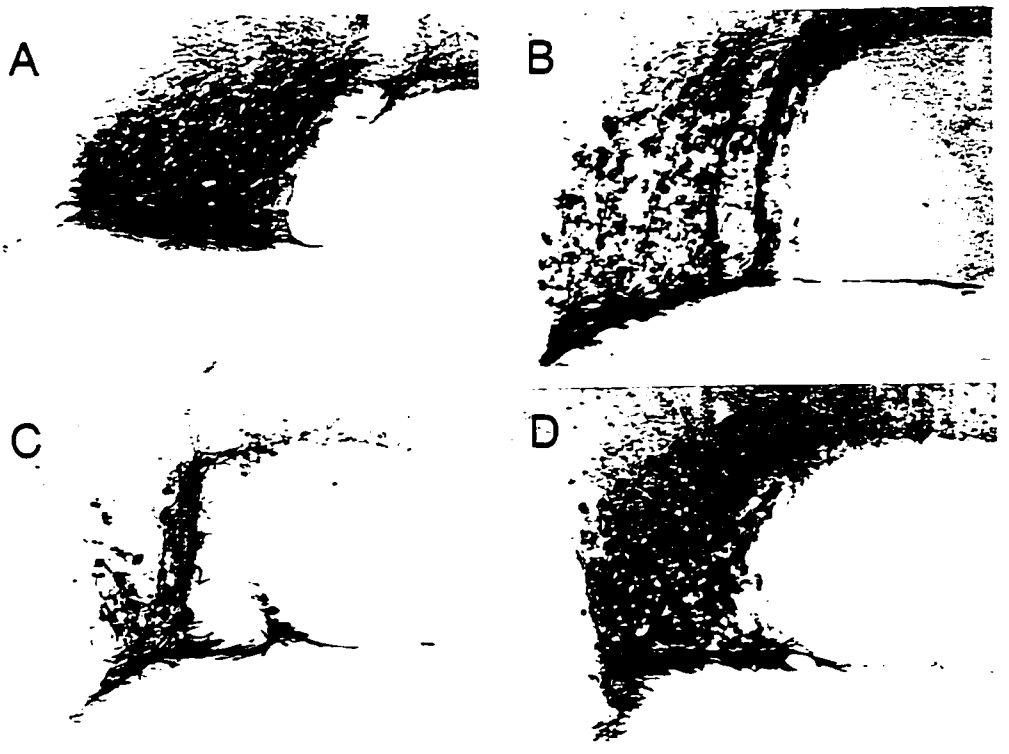

A
B
C
D

Fig. 4. Histogram depicting the number of surviving AVP cells (average of 15 and 36 dps combined) within the supraoptic nucleus (SON) in sham-operated and stalk-transected (ST) groups on water and CISL. The number in the ST + CISL group is significantly higher than in the ST + water group. Although the number of cells in both sham-operated groups is significantly higher than in the ST + water group, there is no significant difference between sham + water and ST + CISL groups. Values represent mean ± SEM. * $P<0.05$.

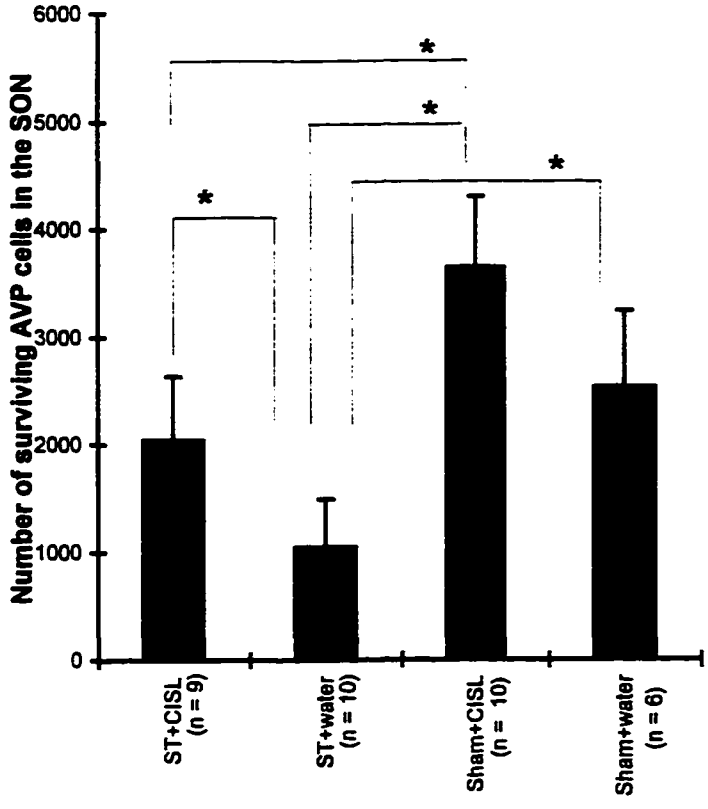

Number of surviving AVP cells in the SON
6000
5000
4000
3000
2000
1000
0
*
*
*
*
ST+CISL (n = 9)
ST+water (n = 10)
Sham+CISL (n = 10)
Sham+water (n = 6)

Fig. 5. Histogram depicting the number of surviving OT cells in the supraoptic nucleus. The number of OT cells in stalk-transected (ST) animals is significantly lower than that of sham-operated animals. Values represent mean ± SEM. * $P < 0.05$.

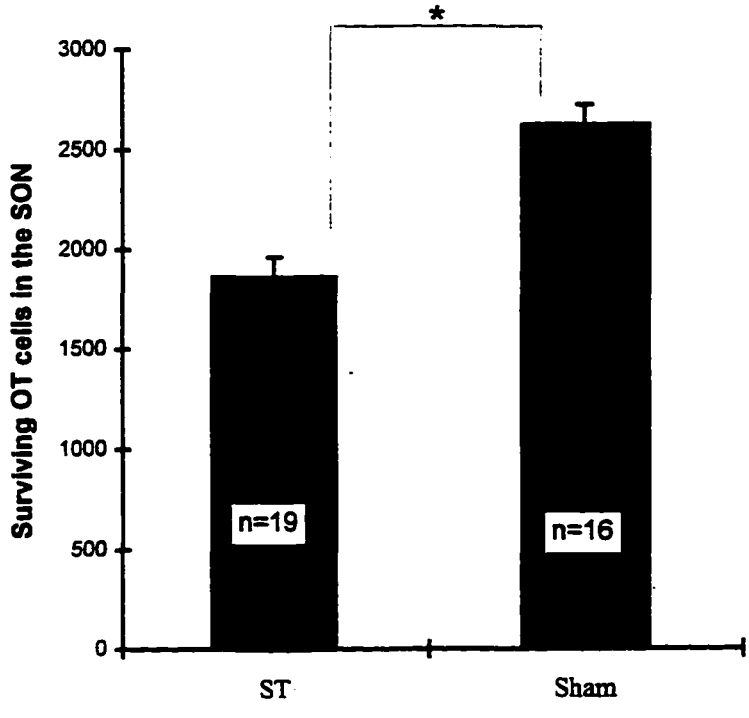

*
3000
2500
2000
1500
1000
500
0
Surviving OT cells in the SON
n=19
n=16
ST
Sham

Fig. 6. Sections of the median eminence, immunostained for AVP, from (A) sham + water, 15 dps; (B) sham + CISL, 15 dps; (C) ST + water, 36 dps; (D) ST + CISL, 36 dps. In the sham + CISL group (B), notice the increase in immunoreactivity in the external zone. In the ST + water group (C), the distribution of AVP is relatively uniform, while a distinct concentration in the internal zone and the periphery of the external zone is present in the ST + CISL group (D). X 90.

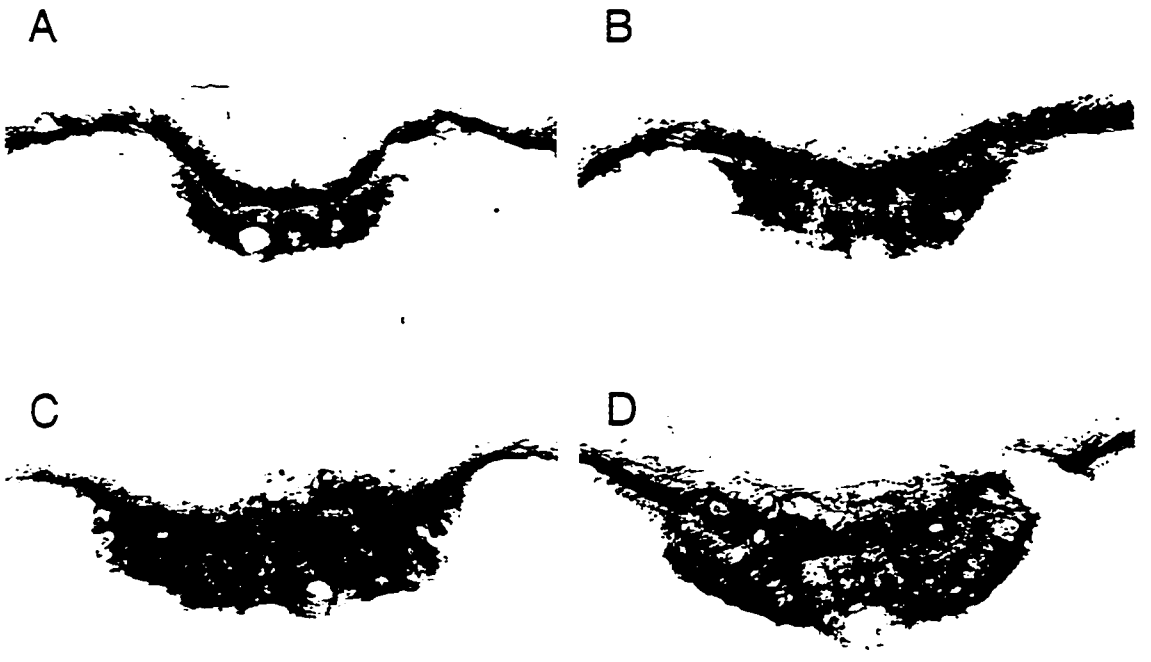

A
B
C
D

Fig. 7. Sections of the median eminence, immunostained for OT, from (A) an intact animal; (B) sham + CISL, 15 dps; (C) ST + water, 15 dps; (D) ST + CISL, 15 dps. CISL causes a decrease in immunoreactivity in the internal zone of the sham groups but has no effect in the external zone. The sprouting of OT axons into the external zone of ST animals (C) is greatly enhanced by CISL (D). X 90.

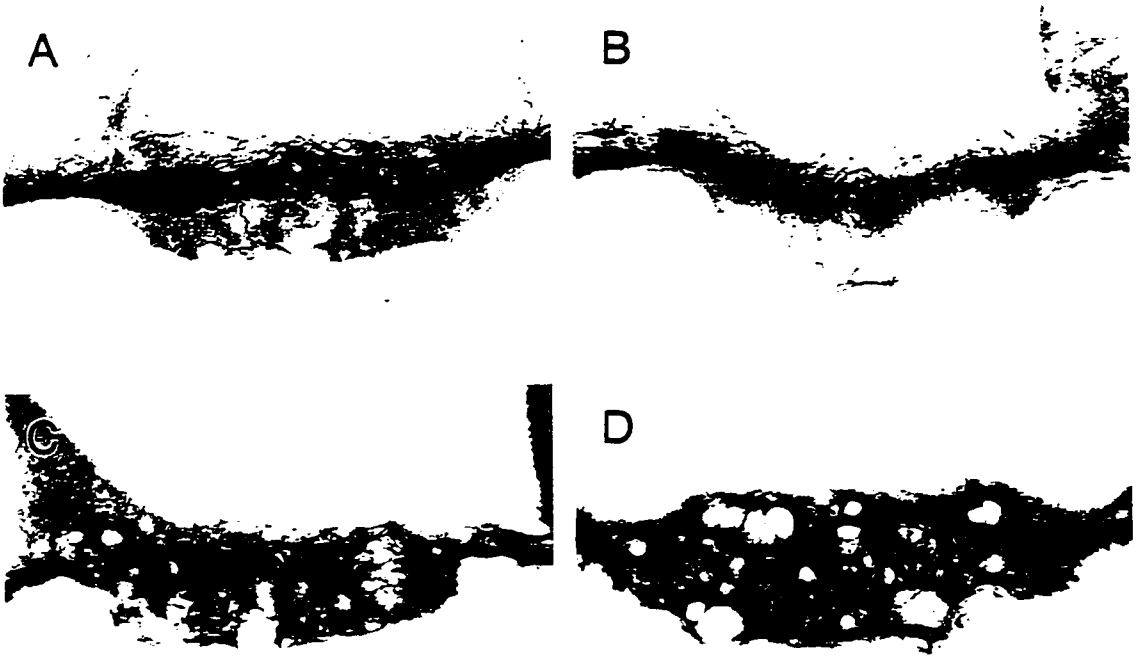
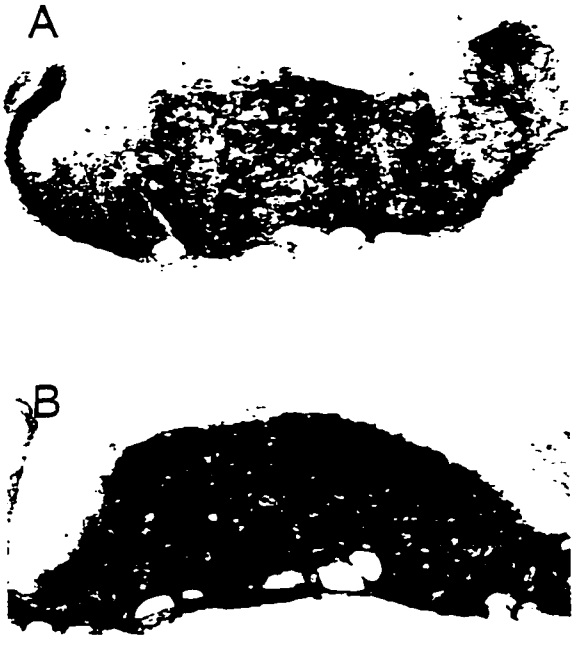

A
B
D

Fig. 8. Sections of the median eminence, immunostained for AVP from (A) ST + water and (B) ST + CISL groups at 36 dps. AVP-positive axons are considerably more numerous in the ST + CISL animals than in the ST + water animal. The median eminence is enlarged and bulges into the lumen of the third ventricle. These sections are located close to the lesion site, and regenerating axons have sprouted vigorously so that a clear distinction between internal and external zone is no longer possible. X 90.


A
B

CHAPTER TWO. AN IMMUNOHISTOLOGICAL AND ELECTRON MICROSCOPIC INVESTIGATION OF THE EFFECTS OF CHRONIC INTERMITTENT SALT LOADING ON THE SURVIVAL AND AXON REGENERATION OF NEUROSECRETORY NEURONS FOLLOWING HYPOPHYSIAL STALK TRANSECTION

A paper to be submitted to Brain Research

Yong-San Huang and H. Dieter Dellmann

Abstract

Stalk-transected (ST) and sham-operated animals either drank water ad lib or were subjected to CISL for various time periods and sacrificed at 2, 5, 10 and 15 dps, and at 18 dps, i.e., 3 days after cessation of chronic intermittent salt loading (CISL). We confirmed the previous observation that, at 18 dps, the water consumption in the ST + CISL group was not significantly different from that in the sham-operated group. From 10 dps on, the number of surviving vasopressinergic neurons in the SON in the ST + CISL group was significantly higher than that in the ST + water group. There was also a significant difference in the number of surviving oxytocinergic neurons between ST + CISL and ST + water group at 10 and 18 dps, but not at 15 dps, but the difference between these two groups was less pronounced than in vasopressinergic neurons.

CISL, but not stalk transection, induced expression of c-fos in both vasopressinergic and oxytocinergic neurons beginning at 2 dps and persisting throughout the period of salt loading. Stalk transection on the other hand, induced the expression of c-jun in two types of

neurosecretory neurons. The earlier cessation of Jun expression in the ST + CISL group than in the ST + water group is interpreted as an indication of establishment of functional neurovascular contacts by regenerating neurosecretory axons. Both CISL and stalk transection induced increased expression of βIII-tubulin in the neurosecretory neurons. However, stalk transection in combination with CISL cause an earlier (2 dps) increase in tubulin immunoreactivity in neurosecretory neurons in the ST + CISL than in the ST + water group. From 5 dps on, CISL induced a noticeably higher number of vasopressin- and βIII-tubulin-positive axons to sprout into the external zone of the ME and lesion area, where they made perivascular contacts with the proliferating capillaries. Only at 18 dps, was there a slightly higher number of oxytocinergic axons present in the ST + CISL than in the ST + water group.

In conclusion, the following explanation is proposed: ST induces c-jun expression, CISL induces additional expression of c-fos. Fos then enhances the binding of Jun to one of its presumptive target genes, gene for βIII-tubulin, and promotes its expression. βIII-tubulin is present earlier and in larger quantities in the ST + CISL group and causes faster postaxotomy collateral sprouting and establishment of perivascular contacts, target derived factors are transported retrogradely to the neuronal perikarya earlier and in a greater quantities, and thus induces the postaxotomy survival of a significantly greater number of vasopressinergic neurons capable to achieve complete recovery from post-transectional polydipsia.

1. Introduction

Damaged axons in the mammalian peripheral nervous system (PNS) and in the central nervous system (CNS) of lower vertebrate animals successfully regenerate. However, in the mammalian CNS axons are either incapable of regeneration after injury, or they initiate but then quickly abort regeneration [86]. There are a few exceptions to this generalization: notably the axons of the olfactory system and the hypothalamo-neurohypophysial system (HNS) are capable of limited regeneration [15,52,97]. In the HNS spontaneous regeneration is observed when the hypothalamo-neurohypophysial tract is transected in the median eminence or hypophysial stalk [1,3,5,15,25,50,85,101]. Not all axotomized neurons survive the injury [24,25,73,84], and those that do survive and regenerate an axon are capable of improving but are not capable of restoring the posttransectional polydipsia [58,73].

Recently, we reported that subjecting hypophysial stalk-transected animals to chronic intermittent salt loading (CISL), enhanced the survival of vasopressin- (AVP) positive cells in the supraoptic nucleus and brought about total recovery of antidiuretic function [39]. We are proposing that CISL stimulates the synthesis of microtubular proteins and/or their polymerization, and thus expedites axon elongation and/or sprouting, and the establishment of pericapillary contacts. Consequently, factors derived from the glial microenvironments and blood-borne factors from the fenestrated capillaries in the median eminence and hypophysial stalk are expected to reach neuronal perikarya earlier after transection and in greater quantities than would otherwise be the case, and assure the survival of more neurons.

Hypertonic saline stimuli have various effects on AVP-synthesizing cells. They increase metabolic activity [31], vasopressin biosynthesis [4,26,27,28,48,67,93-95,104] and

the synthesis of other proteins, including those of cytoskeletal structural proteins [63,64,77]; they also induce non-injured neurosecretory neurons to sprout in the median eminence and to grow into the third ventricle [15]. In addition, they induce axonal transport of an increased number of neurosecretory granulated vesicles (NGVs) within which AVP is carried from the perikaryon to the axon terminals [8,28,79]. This is accompanied by an increase in the number of microtubules and of axonal diameters [30].

Microtubules not only are involved in axoplasmic transport, but are also essential for the elongation of axons [80]. Indeed, upregulation of βIII-tubulin was observed in neurons of the transected sciatic nerve in which regeneration occurred [47,75,76], and that upregulation of βIII-tubulin persisted until the regenerating axons reached their target areas [47]. Lesioning of CNS neurons, which do not regenerate, did not result in tubulin upregulation [54]. Therefore, it is thought that upregulation of βIII-tubulin is essential for successful regeneration after injury [75,76].

Recent papers have reported that several immediate early genes (IEGs) such as c-jun or c-fos, which are inducible by external stimuli, encode proteins that act as transcription factors to regulate the expression of target genes [71,92]. Jun forms dimers with itself or other IEG products such as Fos, Jun-B or Jun-D to form the transcriptionally active complex AP-1 [55,57]. In these dimerized forms, Jun can bind to DNA; and it acts as transcription regulator to effect the expression of target genes [71,92]. Axotomized neurons in the PNS express c-jun [14,29,34,35,46,59]. That expression is downregulated when regrowing peripheral axons have reached their target areas [35,36,59], but if regeneration is prevented, expression of c-jun persists in the injured neurons [35,59]. Such correlation between

regeneration, or attempts at regeneration, and c-jun expression is apparently not a consistent feature in the CNS, and neuronal responses are subject to great variability [44,45,60].

Neuronal injury, such as axon transection, typically does not induce expression of c-fos [35,59]. Neurons in a few select areas of the CNS, however, do respond to various stimuli with the expression of c-fos [38]. Fos, the product of c-fos gene, forms heterodimers with products of the Jun family. Like Jun, through this dimerization, Fos acts as a factor to regulate the transcription of target genes [71,92]. Fos increases the DNA binding activity of Jun [32,78], and Fos/Jun heterodimers exert a stimulating effect on their target gene expression [10,90].

Nothing is known about postaxotomy c-jun expression in the HNS. Given the regenerative capacity of this system, it seemed logical to assume that c-jun is expressed in the HNS as it is in the PNS. Moreover, since dehydration through water deprivation or the substitution of drinking water with 2 % NaCl has been shown to cause expression of c-fos in magnocellular neurosecretory neurons [38,56,61,70,105], we hypothesize that CISL likewise induces c-fos expression. Fos then could conceivably enhance the binding of Jun to target genes, promote their transcription and stimulate the survival and regeneration of the axotomized neurosecretory neurons.

The observed coexpression of c-jun and of genes encoding for GAP-43 and tubulin isoforms in extrinsic axotomized neurons [45,99] led to our hypothesis that the expression of the latter genes, whose products are considered essential for axon regeneration and/or collateral sprouting, is dependent on the preceding expression of c-jun.

In this report, we present data supporting our working hypothesis. We show that ST induces expression of c-jun which in combination with CISL-induced c-fos-expression causes earlier expression of βIII-tubulin, faster postaxotomy collateral sprouting, and thus faster establishment of perivascular terminals, rescue of a greater number of AVP-synthesizing neurons, and complete recovery from polydipsia.

2. Material and Methods

2.1. Animals

Male Holzman rats (250-300 g body weight) were individually housed, lights on from 0600 to 1800 h, with free access to tap water or 2 % saline (see below) and standard rat food throughout the investigation.

2.2. Surgical procedures and treatment

Animals were anesthetized with ketamine (60 mg/kg i.m.) and pentobarbital (20 mg/kg i.p.). The hypophysial stalk was transected stereotactically by lowering a 1.5 mm-wide wire loop knife in the midline vertical plane (3.8 mm caudal to bregma) from the surface of the brain to the basisphenoid; once in contact with the bone, the knife was moved bilaterally for 0.5 mm and then left in place for 30 seconds. In sham-operated control animals, the knife was lowered only 9 mm from the brain surface, causing a brain lesion identical to that in stalk-transected animals, but not transecting the stalk.

One day after the surgery, stalk-transected (ST) and sham-operated animals were randomly divided into 5 groups. In every group , one half of the animals was randomly

selected and subjected to chronic intermittent salt loading (CISL) for different time periods, i.e., animals had access to water from 1000 h to 1600 h and to a 2 % NaCl solution from 1600 h to 1000 h; the other half had free access to water. In group 1, 2, 3, 4 and 5, animals were sacrificed at 2, 5, 10, 15 and 18 days post surgery (dps), respectively. Animals in group 1 to 4 were on CISL for 1, 4, 9 and 14 days, and sacrificed when they were still on CISL, while those in group 5 were on CISL for 14 days and sacrificed after rehydration for 3 days. Based on these regimens, the following categories were established:

2 days: *ST* + *CISL* (n=4); *Sham* + *CISL* (n=4)

ST + *Water* (n=5); *Sham* + *Water* (n=4).

5 days: *ST* + *CISL* (n=5); *Sham* + *CISL* (n=4)

ST + *Water* (n=5); *Sham* + *Water* (n=4).

10 days: *ST* + *CISL* (n=9); *Sham* + *CISL* (n=6).

ST + *Water* (n=7); *Sham* + *Water* (n=6).

15 days: *ST* + *CISL* (n=5); *Sham* + *CISL* (n=6).

ST + *Water* (n=6); *Sham* + *Water* (n=6).

18 days: *ST* + *CISL* (n=10); *Sham* + *CISL* (n=6).

ST + *Water* (n=10); *Sham* + *Water* (n=6).

Furthermore, at 2, 10 and 18 dps, two animals were sacrificed in each treatment group, and at 5 dps in the stalk-transected groups for electron microscopic studies. In addition, two intact animals were sacrificed after 14 days of CISL. Water consumption of all animals was measured daily (at 1000 h and 1600 h) 3 days prior to surgery and during the postsurgical survival time.

2.3. Tissue preparation

Animals were deeply anesthetized with pentobarbital (50 mg/kg, i.p.) and perfused transaortically for 15 minutes with phosphate-buffered (pH 7.4, 0.1 M) 4 % paraformaldehyde. Following removal of the brain from the skull, the hypothalamus and hypophysis together with the surrounding meninges were isolated and postfixed in the same fixative for 24 hours at 4°C. The entire hypothalamus was cut with a vibratome into 50 μm serial frontal sections. The rostral and caudal ends of the pars principalis of the SON were identified using a 0.05% methylene blue solution [14]. Starting with a randomly selected section, the lead section, one out of six sections was dual immunostained for neurophysin-AVP (AVP) and Jun. The following three sections were processed for dual immunohistochemical stain for the following antibody combinations: OT + Fos; AVP + Fos; OT + Jun. The fifth section was immunostained for βIII-tubulin antibody. The sixth section was used for control procedures.

Vibratome-cut 50 μm thick sections from the median eminence, lesioned hypophysial stalk and neural lobe were immunostained for AVP, OT and βIII-tubulin.

Animals for electron microscopic (EM) studies were perfused with a phosphate-buffered (pH 7.4, 0.1 M) mixture of 4 % paraformaldehyde and 0.5 % glutaldehyde and postfixed in the same solution for 14 hours at 4 °C. Hypothalamus, median eminence, lesion area and hypophysis were vibratome-cut into 50 μm sections.

2.4. Immunohistochemical methods

For dual immunohistochemical staining, section were first stained for either Jun or Fos. After a 20 min. rince in PBS, tissue sections were treated for 20 min. with 1 % sodium borohydride in 0.1 M PBS to quench the aldehyde groups, and for 20 min. in 0.3 % hydrogen peroxide. Following 2 hours in a blocking solution containing 2 % bovine serum albumin, either 2 % normal horse serum (for monoclonal antibody) or 2 % normal goat serum (for polyclonal antibody) and 0.4 % Triton X-100 in Tris-phosphate buffer (pH 7.8), the sections were incubated with either Jun (1:10,000, monoclonal, Santa Cruz) or Fos (1:50,000, polyclonal, Oncogen) antibody overnight at room temperature. After triple washing in TBS (5 min. each), the tissue sections were incubated in either biotinylated rat serum preabsorbed horse antimouse IgG (1:100; Vector) or biotinilated goat antirabbit IgG (1:500; Vector) for 2 hours, rinsed 15 min., reacted with avidin-biotin complex (ABC; Vector Elite Kit, 1:220 for 1 hour), and exposed to a substrate composed of 0.04 % 3,3-diaminobenzidine, 2.5 % nickel sulfate and 0.006 % hydrogen peroxide diluted in 0.1 M sodium acetate for 10 minutes. Tissue sections were then rinsed in 0.9 % sodium chloride for 15 min. Tissues were then processed following the same methods as above for the secondary staining for either AVP (1:800) or OT (1:1600). The monoclonal NP-AVP antibody (a gift from H. Gainer) does not cross-react with NP-OT or OT [103]. The monoclonal OT antibody (a gift from A.J. Silverman) does not cross react with NP-AVP or AVP [40]. We used the same protocol to stain with monoclonal βIII-tubulin antibody (1:160,000, a gift from A. Frankfurter) except that 8 % bovine serum albumin and 8 % normal horse serum was used for a blocking solution. The stained sections were mounted on gelatin-coated slides, dehydrated in graded

alcohols, cleaned in xylene and coverslipped with permount mounting medium. Control sections were run through the immunohistochemical procedure after omitting either the primary or the secondary antibodies.

Tissue sections from the same level were selected for EM studies. Some sections were immunostained for AVP or OT before processing for EM studies. Immunohistochemical staining procedures for EM studies followed the same protocol as that for light microscopic studies, but dilutions were 1:100 for AVP antibody and 1:200 for OT antibody.

2.5. Electron microscopic methods

Immunostained or conventional sections were postfixed for one hour in 1 % OsO_4 containing 0.75 % potassium ferricyanide, stained en bloc in 2 % uranyl acetate, dehydrated in graded alcohols, infiltrated in an epon-araldite mixture, and flat-embedded. Thin sections were stained with lead citrate and viewed in a Hitachi HU-12 A electron microscope.

2.6. Cell count

2.6.1. Sections stained with AVP, OT, Jun or Fos

In all immunostained sections, the number of AVP-positive cells or OT-positive cells, and the number of AVP-positive or OT-positive cells which were either Jun- or Fos-positive was counted at 400 X magnification. Before counting the cells, sections were mixed and coded with a code unknown to the person performing the counting, and decoded after the count. The total number of the cells stained with different antibodies in each experimental

animal was calculated by the following formula: Sum of the number of the immunostained cells divided by the number of the sections in which the cells were counted, and multiplied by the total number of sections containing the SON.

2.6.2. Sections stained with βIII-tubulin

We only did the cell counting in the stalk-transected animals sacrificed at 5 dps and 10 dps. The same protocol as above was followed to count the number of the βIII-tubulin-positive cells.

2.7. Statistics

Daily water consumption data and the number of AVP- and OT-positive cells were analyzed with three-way ANOVA, and the means of each group were compared by Tukey's multiple range test. Data of the percentage of the Jun- or Fos-positive cells in either AVP- or OT-positive cells were analyzed with two-way ANOVA, followed by comparing the means of each group with Tukey's multiple test.

3. Results

3.1. Water consumption (Fig. 1)

3.1.1. in animals drinking water

In the sham + water group, water consumption remained at presurgical levels throughout the observation period.

Water consumption in the ST + water group, followed the previously reported three-phase pattern [102]. One day after stalk transection, it drastically increased. It then rapidly returned to the presurgical level on the second day, and remained there for one day. Thereafter, it gradually increased to reach its highest level at around the seventh day after stalk transection, and remained at high levels throughout the experimental period.

3.1.2. in animals on CISL

In the sham + CISL group, fluid consumption increased at 2 dps and continued to rise steadily throughout the CISL period. One day after termination of CISL, water consumption declined to the level of that of sham-operated euhydrated animals. Two out of six animals in this group, which were sacrificed at 10 dps, did drink considerably less than the other animals. Nevertheless, the pattern of water consumption of these animals was similar to those of others.

In ST + CISL animals, daily fluid consumption, i.e., 2 % NaCl and water, followed a pattern similar to that observed in the ST + water group. Because CISL began at the second day, the rapid decrease of water consumption observed in the ST + water did not occur in this group, and the drastic increase on the first day was followed by only a small decrease at 2 and 3 dps. Water consumption then gradually increased to 8 dps and then remained more or less at that level during the remainder of CISL. After termination of CISL, the water consumption declined rapidly during the first day (16 dps), followed by a small decrease at 17 dps, and a pronounced decrease at 18 dps.

3.1.3. Statistical analysis of water consumption

With three-way ANOVA, the water consumption data in group 5, in which animals were sacrificed at 18 dps, were analyzed from 16 to 18 dps (i.e., one day after termination of CISL to the end of the experiment). In the analysis the factors were stalk transection (ST or sham), fluid consumption (salt or water) and time (days post surgery). It showed that all three factors, stalk transection ($F_{(1,28)} = 44.27$, $P<0.0001$), fluid consumption ($F_{(1,28)} = 4.71$, $P<0.05$) and time ($F_{(2, 56)} = 12.23$, $P<0.0001$) differed significantly, as did the two-way and three-way interactions between these factors. We then analyzed the difference among four groups (ST + CISL, ST + water, sham + CISL and sham + water) at each day with one-way ANOVA, followed by Tukey's multiple range test at $\alpha = 0.05$. Tukey's test revealed that there were some significant differences among the groups at each day. Comparison of the means of each group with Tukey's test showed that water consumption in either ST + water group or ST + CISL group was significantly higher than that in either sham + water or sham + CISL groups at both 16 dps and 17 dps. At 18 dps, the water consumption in the ST + CISL group decreased to a level where it was no longer significantly different from that in the sham + CISL and sham + water groups. Thus, the water consumption in the ST + CISL group had recovered to control level but that in the ST + water group was still significantly higher than in any other group.

3.2. Neurosecretory cells in the SON

3.2.1. Light microscopic observations

3.2.1.1. Vasopressinergic cells

3.2.1.1.1. Immunostaining

In the sham + water animals, the pattern and intensity of the immunostaining for AVP were as previous published [102] and as reported by other investigators for euhydrated animals, and no differences were observed among the sham + water animals at different time periods.

In the sham + CISL animals, a gradual and progressively greater decrease in the intensity of immunostaining for AVP was observed as dehydration through CISL progressed, that ranged from subtle to drastic between individuals at the same time period, and between different time periods. At 18 dps, three days after cessation of CISL, the immunostaining intensity returned to the level observed in the sham + water group.

In the ST + water animals, at 2 dps, some cells stained more intensely than the cells in the sham + water group. At 5 dps and at subsequent observation periods, most cells were strongly immunoreactive (Fig. 2A,C).

In the ST + CISL animals, at 2 dps and 5 dps (Fig. 2B), the intensity of immunostaining increased similar to that in the ST + water animals. However, at 10 and 15 dps, immunoreactive intensity progressively declined as in the sham + CISL animals. Upon rehydration, at 18 dps, the intensity of immunostaining was at the same level as that in the sham + water group (Fig. 2D).

3.2.1.1.2. Morphometry (Fig. 3)

Three-way ANOVA of the number of the surviving vasopressinergic cells in the SON at different time periods showed that all three factors, stalk transection ($F_{(1,97)} = 480$, $P<0.001$), CISL ($F_{(1,97)} = 96$, $P<0.001$ and time ($F_{(4,97)} = 25$, $P<0.001$), significantly affected the number of the surviving AVP cells, as well as did all interactions. The differences among four groups (ST + CISL, ST + water, sham + CISL and sham + water) at each time period were then analyzed with one-way ANOVA, followed by Tukey's multiple range test at $\alpha = 0.05$. Tukey's test revealed that there was no significant difference among the four groups at 2 dps. By 5 dps, there was no significant difference between ST + water and ST + CISL group. Also, there was no significant difference between sham + water and both stalk transected groups, but the number of the AVP-positive cells in the sham + CISL was significantly higher than that in both stalk transected groups. However, from 10 dps on, the number of the surviving AVP cells in the ST + CISL group was significantly higher than that in the ST + water group. Also, the number of AVP cells in the sham animals was significantly higher than that of stalk-transected animals from 10 dps on. From 5 dps on, the number of vasopressinergic neurons increased gradually in the sham + CISL group and was significantly higher than in any other group at 15 and 18 dps. Comparison of the number between days in both ST + CISL and ST + water group showed that in both groups cell loss was particularly pronounced between 5 dps and 10 dps, especially in the ST + water group.

3.2.1.2. Oxytocinergic cells

3.2.1.2.1. Immunostaining

The pattern and intensity of the immunostaining of oxytocin in the sham + water group were as previously published [102] and reported in the literature for control animals with ad lib access to water. CISL did not have any noticeable effect on OT immunoreaction in the SON, and throughout the observation periods both the pattern and staining intensity remained virtually identical to that observed in the sham + water group. In the stalk-transected animals, at 2 dps, many cells increased in staining intensity (Fig. 4A), but there was no difference between ST + water and ST + CISL group. At 5 dps, the staining intensity in the stalk-transected animals had returned to the level of sham + water group, and no changes were noticed thereafter (Fig. 4B,C,D). There was no perceptible difference between the ST + water and ST + CISL group.

3.2.1.2.2. Morphometry (Fig. 5)

Three-way ANOVA of the number of the surviving oxytocinergic cells in the SON at different time periods revealed that all three factors, including stalk transection ($F_{(1,97)}=142$, $P<0.001$), CISL ($F_{(1,97)}=19$, $P<0.001$), time ($F_{(4,97)}=6$, $P<0.01$), significantly affected the number of the surviving oxytocinergic cells, as well as did the interactions. There was no significant difference between the means among the four groups (sham + water, sham + CISL, ST + water and ST + CISL group) at 2 dps. By 5 dps, the number of the surviving oxytocinergic cells in both sham groups was significantly higher than that in the ST + water group, but there was no significant difference between sham groups and ST + CISL group

and between ST + water and ST + CISL group. However, there was a significant difference between any two groups from 10 dps on, and the number in the sham animals was higher than that in the stalk-transected animals, and that in the CISL groups was greater than in the water groups. There was, however, no significant difference between sham + water and ST + CISL groups at 10 dps, and ST + water and ST + CISL groups at 15 dps.

3.2.1.3. Immunostaining for βIII-tubulin

Table 1 shows that no differences were observed in the pattern and intensity of immunostaining for βIII-tubulin in the SON among the sham + water groups at different time periods. Most neurosecretory cells in the SON were faintly and uniformly stained with no preference of location (Fig. 6A). This was considered as the basal level. Some axons in the SON showed a stronger reaction for tubulin.

At 2 dps, the intensity of immunostaining was low in the sham + CISL and ST + water groups (Fig. 6E), and the same as in the sham + water groups. In the ST + CISL group, however, in a few cells and many axons there was an increase in the intensity of immunoreaction (Fig. 6F).

At 5 dps, in the sham + CISL group, a few cells were moderately stained (Fig. 6B). In both the ST + water and ST + CISL groups, more cells showed increased immunoreactivity. The number of βIII-tubulin positive cells in the ST + CISL group was significantly higher than in the ST + water group (Fig. 7A,B and Fig. 8).

At 10 dps, the immunoreactivity in the sham + CISL group was the same as at 5 dps. In both the ST + CISL and ST + water groups, however, there was a decrease in the intensity

of the immunostaining (Fig. 7C,D). That was particularly pronounced in the ST + CISL group; most βIII-tubulin-positive cells in that group were only moderately immunoreactive. The number of positive cells, whose immunoreactivity was above the basal level of that of the sham + water group, was significantly higher than that in the ST + water group (Fig. 8).

At 15 dps, the immunoreactivity in the sham + CISL group had reached a peak (Fig. 6C). Most cells showed intense immunoreactive staining. In the ST + water group the immunoreactivity had returned to control level (Fig. 7E). The intense immunoreactivity observed in the ST + CISL group was the same as in the sham + CISL group (Fig. 7F). At 18 dps, the immunoreactivity in all groups returned back to the basal level of the sham + water group (Fig. 6D and 7G,H).

3.2.1.4. Jun expression

3.2.1.4.1. Jun expression in vasopressinergic cells (Fig. 2 and 9)

During the entire experimental period in the sham groups, the vasopressinergic cells lacked Jun immunoreactivity. At 2 dps, in both stalk-transected groups, about 40 % of AVP-cells were Jun positive. By 5 dps, the percentage of the Jun-positive cells in the ST + water group increased to 74 %, while that in the ST + CISL group decreased to 35 %. At 10 dps, the percentage of Jun-positive cells decreased vastly in both groups. At 15 dps and 18 dps, the percentage of Jun-positive cells in the SON was less than 3 % in both groups.

Two-way ANOVA showed that both CISL ($F_{(1,57)} = 148$, $P<0.001$) and time ($F_{(4,57)} = 27$, $P<0.001$) affected the percentage of Jun-positive vasopressinergic cells, as did the interactions. Comparison of the means of the percentage between ST + water and ST + CISL

groups at different time periods showed that only at 5 dps the percentage of Jun-positive cell in the ST + water group was significantly higher than in the ST + CISL group. At other periods, there was no significant difference in the percentage of Jun-positive vasopressinergic cells between these groups.

3.2.1.4.2. Jun expression in oxytocinergic cells (Fig. 4 and 10)

As in the vasopressinergic cells, no Jun immunoreactivity was observed in the oxytocinergic cells in both sham groups through the entire experimental period. In the stalk-transected animals, the percentage of Jun-positive oxytocinergic cells was lower than that in AVP-positive cells. In both stalk-transected groups, it was highest at 2 dps, then gradually decreased, especially at 10 dps, and by 15 dps it was less than 1 % in both groups.

Two-way ANOVA revealed that CISL significantly ($F_{(1,57)} = 8.7$, $P<0.01$) decreased the percentage of Jun-positive oxytocinergic cells. Also, the decrease of the percentage was significantly ($F_{(4,57)} = 16.5$, $P<0.001$) affected by time. But there was no interaction between these two factors.

3.2.1.5. Fos expression

3.2.1.5.1. Fos expression in vasopressinergic cells (Fig. 11)

Fos was not expressed in the vasopressinergic cells of the SON in the sham + water and ST + water groups. Animals on CISL, in either the sham + CISL or ST + CISL group, showed various degrees of Fos immunoreactivity in the vasopressinergic cells at different time periods.

At 2 dps, in both groups about 12 % of the vasopressinergic cells expressed Fos. By 5 dps, in both groups, there was a pronounced increase in Fos immunoreactivity in the vasopressinergic cells that continued moderately through 15 dps. At 18 dps, three days after rehydration, almost no vasopressinergic cells expressed Fos.

Two-way ANOVA of the percentage of Fos-positive vasopressinergic cells in the sham + CISL and ST + CISL groups at different time periods revealed that only time ($F_{(4,49)}$ = 12.6, $P<0.01$) affected the percentage of Fos-positive cells, as well as the interaction, but not the factor of stalk transection ($F_{(4,49)} = 0.59$, $P>0.05$). Comparison of the percentage between sham + CISL and ST + CISL groups at different time periods with Tukey's test revealed that there was no significant difference between the two groups. Also, comparison of the percentage of vasopressinergic cells expressing Fos at different time periods within groups with Tukey's test showed that there was no significant difference between 5, 10 and 15 dps in both ST + CISL and sham + CISL groups, but the percentages of these three periods in both groups were significantly higher than those at 2 dps, except that in the sham + CISL group there was no significant difference between 2 and 10 dps.

3.2.1.5.2. Fos expression in oxytocinergic cells (Fig. 12)

Oxytocinergic cells expressing Fos were not observed in the water groups, while animals on CISL had different degrees of Fos expression at different time periods. Generally, in a given animal, the degree of Fos expression in oxytocinergic cells parallels that in vasopressinergic cells.

Two-way ANOVA of the percentage of Fos-positive oxytocinergic cells in the sham + CISL and ST + CISL groups at different time periods revealed that only days post surgery ($F_{(4,47)} = 12.8$, $P<0.001$) affected the percentage, but not stalk transection ($F_{(1,47)} = 4.6$, $P>0.05$). Only at 5 dps, was the percentage of Fos-positive cells significantly higher in the ST + CISL group than in the sham + CISL groups. At other time periods, there was no significant difference between groups. The percentage of the oxytocinergic cells expressing Fos in the ST + CISL group at 5 dps was significantly higher than at 2 dps and 10 dps; at 15 dps it was significantly higher than at 2 dps. Other than that no significant difference could be found between any two of them. In the sham + CISL group there was no significant difference between any two time periods.

3.2.2. Electron microscopic observations

Magnocellular neurons in the sham + water groups (Fig. 13A) had short and elongated cisternae of peripherally located rough ER interspersed with ribosomes and polyribosomes. The cisternae had narrow lumens, and were either empty or had a moderately electron-dense content. The Golgi complex, consisting of varying numbers of stacks of cisternae, was in perinuclear position. Golgi-associated vesicles were numerous. These vesicles were either empty or were immature neurosecretory granulated vesicles (NGVs), i.e., they contained extremely electron-dense material. Immature NGVs also occurred throughout the cytoplasm, as did mature NGVs, identified by both their larger size and lesser electron density of their granules. Mature NGVs not only occurred singly but also in clusters, especially in the cell periphery and at the origin of dendrites. Large electron-dense vesicles,

considered to be lysosomes, were frequent. Nuclei contained little heterochromatin and usually one nucleolus. In the intact + CISL group (Fig.13B), neurosecretory cells with enlarged cisternae of rER, containing material of varying electron density, ranging from very dense in elongated cisternae to light in short ones, were numerous. The rER was mostly located peripherally in the cell, and the perinuclear area was occupied by multilocular Golgi complexes, together with numerous immature NGVs and lysosomes. Mature NGVs were virtually absent. Nuclei had usually one or several deep indentations and contained one or two large nucleoli.

At 2 dps, in the sham + CISL and the ST + water groups, neurosecretory cells had the same fine structural characteristics as those in the sham + water group. In the ST + water group, some cells contained, however, randomly distributed clusters of NGVs. Neurosecretory cells in the ST + CISL group either had a similar fine structure than those in the ST + water group, or they typically had short and predominantly ovoid cisternae of peripherally located rER and occasional subplasmalemmal clusters of mature NGVs, surrounding a wider perinuclear region containing an extensive Golgi complex, associated with many NGVs and lysosomes.

At 10 dps, in the sham + CISL group, in virtually all cells the rER was considerably more extensive as compared to the sham + water group (Fig. 13C). In many cells, the cisternae had become more elongated and contained moderately electron-dense material; cisternal constrictions were frequent and short cisternae appeared to segregate from the long ones. In other cells, the rER consisted of short ovoid and dilated, empty-appearing cisternae. The Golgi complex was more extensive than in the cells of the sham + water group, often

occurred in several superimposed stacks, and was usually associated with immature NGVs. Mature NGVs were essentially absent, but when present they occurred only singly. No obvious changes were observed in the lysosomes. Nuclei were euchromatic, nucleoli were larger and often two nucleoli were present in one nucleus. In the ST + water group (Fig. 14A), neurosecretory cells were characterized by an extensive perinuclear Golgi complex associated with many immature NGVs, peripherally located vast rER, and large euchromatic nuclei with shallow indentations. Cells with greatly fragmented rER but otherwise identical fine structure were not unusual. In the ST + CISL group (Fig. 14B), most cells were characterized by extensive, peripherally located rER consisting mainly of short and dilated cisternae filled with moderately electron-dense material. Nuclei usually had several deep indentations, and were surrounded by an area containing an extensive Golgi complex, immature NGVs, and lysosomes.

At 18 dps, in the sham + CISL group (Fig. 13D), the rER was less extensive than at 10 dps, and cisternae were still rather elongated in most cells. In some cells, however, many short non-dilated cisternae were present, often containing moderately electron-dense material. The extent of the Golgi complex appeared to be reduced, with no apparent change in immature NGVs. Mature NGVs were virtually absent. The number of lysosomes was increased. No changes were observed in nuclei and nucleoli. In the ST + water group (Fig. 14C), neurosecretory cell fine structure differed from that in the sham + water group primarily by a wider perinuclear region containing the Golgi complex, immature and especially mature NGVs, and lysosomes. The cell periphery contained the bulk of usually elongated and often stacked cisternae of rER and typically a few single NGVs, and only

exceptionally small clusters of NGVs. In the ST + CISL group (Fig. 14D), cells with peripherally located, elongated and usually non-dilated cisternae of rER containing moderately electron-dense material predominated, whereas only occasional cells contained short, ovoid non-dilated cisternae. Many mature NGVs, mostly single but also in clusters, coexisted with the rER. The wide perinuclear region was occupied by an extensive Golgi complex, many predominantly immature NGVs, and lysosomes.

3.3. Median eminence (ME)

3.3.1. Vasopressin (AVP) immunoreactivity

In the sham + water animals (Fig. 15A) vasopressinergic axons with numerous small dilatations were found in the fiber layer of the internal zone. In the external zone, many finely beaded and thin AVP-positive axons were present.

In the sham + CISL animals, at 2 dps, there were no changes in the immunostaining in either zone of the ME. From 5 dps on, the immunostaining intensity in the fiber layer decreased. But at 18 dps, it returned to the level of the sham + water group. Occasional single subependymal axons were observed after 10 dps. Through 10 dps, no change in the number of AVP-positive axons was observed in the external zone; however, at 15 dps (Fig. 15B) and to a lesser degree at 18 dps, there were more AVP-immunoreactive axons present in the external zone of the ME .

At 2 dps, in both stalk transected groups some intensely stained thick and beaded AVP-positive axons extended into the external zone and into the subependymal layer of the internal zone. A few of these AVP-positive axons reached the short capillary loops.

At 5 dps, in both stalk-transected groups more AVP-positive thick axons with axonal dilatations were present in the subependymal layer and in the external zone: they were more numerous and more intensely stained in the ST + CISL group than in the ST + water group (Fig. 15C,D). Many thick AVP-positive axons had reached the ventral capillaries.

At 10 dps, there were more AVP-positive axons than at 5 dps in both stalk-transected groups. AVP-positive axons surrounding proliferated capillaries especially in the external zone were more numerous in the ST + CISL group than in the ST + water group. At 15 dps and 18 dps there was a further increase in the number of AVP-positive axons in the external zone in both groups. That increase was greater in the ST + CISL groups than in the ST + water group (Fig. 15E,F).

3.3.2. Oxytocin (OT) immunoreactivity

In the sham + water and sham + CISL animals most oxytocin immunoreactive axons were located in the fiber layer of the internal zone. Occasionally, OT-positive axons were present in the external zone, either singly or in bundles (Fig. 16A). Between 5 dps and 15 dps, there was a progressive subtle decrease in intensity of the immunostaining for oxytocin in the internal zone in the sham + CISL group. At 18 dps, three days after the termination of CISL, the intensity returned to the level of the sham + water group.

At 2 dps, in the stalk-transected animals, a few tortuous, thick and intensely-stained oxytocin-positive axons were projecting from the fiber layer of the internal zone to the external zone. While the number of oxytocin-positive axons in the external zone in the ST +

CISL group was the same as in the ST + water group, the staining intensity in the ST + CISL group was less than in the ST + water one.

At 5 dps, more regenerating axons were present in the external zone of all stalk transected animals. Some beaded oxytocin-positive axons reached the short capillary loops. Differences in the number and staining intensity of these axons between the two groups were subtle.

At 10 dps and 15 dps, there was an increase in the number and staining intensity of oxytocin-positive axons in the external zone in all stalk-transected animals. No appreciable difference could be observed between ST + CISL and ST + water groups. At 18 dps, still more oxytocin-positive axons had grown into the external zone in both stalk-transected groups. While there was certainly no difference in the staining intensity between ST + CISL and ST + water groups, the number of axons in the ST + CISL group was higher than in the ST + water group (Fig. 16B,C).

3.3.3. βIII-tubulin immnoreactivity

In the sham + water group, many moderately reactive βIII-tubulin-positive axons were observed in the fiber layer of the internal zone, but not in the external zone (Fig. 17A). In the sham + CISL groups, no remarkable changes in the intensity of the immunostaining of βIII-tubulin in the fiber layer were observed at 2, 5 and 10 dps, but a few βIII-tubulin-positive axons projected into the subependymal layer at 5 and 10 dps (Fig. 17B). At 15 dps, there was a noticeable increase in the intensity of the immunostaining in the fiber layer and in the number of positive axons in the subependymal layer. A few tubulin-positive axons also

projected to the external zone (Fig. 17C). At 18 dps, however, the staining intensity and the number of the positive axons returned to control levels (Fig. 17D).

At 2 dps, in the stalk-transected animals, with the exception of a few single positive fibers in the external zone, no marked changes could be found in the ME. At 5 dps, in both groups, the number of tubulin-positive axons in the external zone had increased, but the increase was more substantial in the ST + CISL group and a particularly intense reaction was present at the ventral surface of the ME. Furthermore, many axons extended into the subependymal layer (Fig. 18A,B). From 10 dps to 18 dps, there was a further progressive increase in the number of tubulin-positive axons in the external zone and subependymal layer in both groups. This increase was most remarkable in the ST + CISL group, especially around short capillary loops (Fig. 18C,D,E,F,G,H). The staining intensity of the axons in the ST + CISL group gradually increased to peak at 15 dps and was moderately decreased at 18 dps (Fig. 18F,H). In the ST + water group tubulin immunoreactivity decreased to the basal level of the sham +water group at 15 dps and remained there at 18 dps.

3.3.4. Electron microscopic observations

Fine structural characteristics of the median eminence in intact animals and in the sham + water group are identical to those described in the literature [53,85,87,91]; therefore, only a summarizing description, primarily aimed at providing a basis for comparison with the experimental groups, will be given.

In the internal zone of the median eminence, ependymal cells and tanycytes provided the lining of the floor of the third ventricle. Their processes, together with those of

subependymal pituicytes, delineated compartments through which course the axons of the magnocellular hypothalamic nuclei. Most of these axons were small caliber axons, readily identified as magnocellular neurosecretory axons by the presence of typical NGVs (diameters above 160 nm); other axon profiles were devoid of NGVs; still other profiles were considerably larger (commonly referred to as Herring bodies), and were either filled with varying amounts of NGVs or did not contain any NGVs. In sections stained immunohistochemically for AVP, a positive reaction was observed not only in most axon profiles containing NGVs but also in many axon profiles and Herring bodies devoid of NGVs.

Deep in the external zone was an ill defined layer (reticular layer) comprising preterminal axon profiles originating from hypothalamic nuclei other than the magnocellular ones, containing dense core vesicles of considerably smaller diameter (average 100 nm). Superficial to this layer was the palisade layer, which is adjacent to the perivascular spaces of the primary capillaries of the hypophysial portal system or the cells of the pars tuberalis, and which is composed of axon terminals interspersed with processes of pituicytes and tanycytes Fig. 19A). Dense core vesicles within these axon terminals were exclusively of the smaller variety. Their number and electron-density varied within wide limits. Axon terminals also contained electron-lucent microvesicles (50 - 70 nm diameter), either alone or in combination with dense core vesicles. Immunohistochemical staining for arginine-vasopressin revealed numerous positive axons and axon terminals, confirming the light microscopic findings. It is important to note, that none of the positive axons contained large NGVs typical of

magnocellular neurosecretory neurons, but only small dense core vesicles, characteristic of parvocellular neurons.

In the intact + CISL group, there was a conspicuous increase in the number and size of Herring bodies in the internal zone, as compared to intact and sham + water animals; most Herring bodies were devoid or almost devoid of NGVs. Neurosecretory axon profiles usually did not contain NGVs but were filled with microtubules. The external zone had the same structural characteristics as that of sham + water animals.

At 2 dps, in the sham + CISL group, no changes in the fine structure were observed in either the internal or the external zone. The median eminence in the ST + CISL and ST + water groups had identical fine structural characteristics. The light microscopic observation of subependymal neurosecretory axons was confirmed. In the deep layer of the external zone, occasional OT-positive and many AVP-positive magnocellular axons were coursing toward the palisade layer, parallel to and in close apposition with tanycyte processes (Fig. 19C). In the palisade layer only parvocellular vasopressinergic axons were abutting pericapillary spaces (Fig. 19B).

At 5 dps, conspicuous changes were observed in the palisade layer of both stalk-transected groups, but they were far more extensive in the ST + CISL than in the ST + water groups. Growth cones had not only advanced to the basal lamina of pericapillary connective tissue spaces, but had in many instances invaded these spaces (Fig. 20A). Axon terminals containing magnocellular NGVs and microvesicles were numerous at or in close proximity of these capillaries (Fig. 20B).

At 10 dps, in the sham + CISL group, large Herring bodies containing varying numbers of NGVs or no NGVs were present in the internal zone together with neurosecretory axon profiles, many of which were packed with microtubules. No changes were noticed in the external zone. In the ST + water group, many magnocellular vasopressinergic axons coursed through the palisade layer to terminate at pericapillary spaces, having displaced parvocellular axon terminals. Additionally, vasopressinergic magnocellular axons were present in the pericapillary connective tissue spaces adjacent to the pars tuberalis. They were almost always accompanied by pituicyte processes. In the ST + CISL group, the morphology of the internal zone resembled that of the internal zone in sham + CISL animals. In the external zone, more magnocellular neurosecretory axon terminals were present at pericapillary spaces than in the ST + water group.

At 18 dps, in the sham + CISL group, the fine structural morphology of the internal zone was identical to that observed at 10 dps. Those of the superficial external zone did not differ from those of the sham + water group, but deeper in the external zone, preterminal magnocellular axon profiles were present. In the ST + water group, the palisade layer had undergone substantial changes; parvocellular axon terminals were virtually absent, and the peripheries of perivascular spaces were almost exclusively occupied by magnocellular neurosecretory axon terminals. Their morphology ranged from those containing massive numbers of NGVs and clusters of microvesicles to those containing only a few NGVs but many microvesicles. In the ST + CISL group, the morphologic changes were indentical to those in the ST + water group, but quantitative differences were conspicuous and all

perivascular spaces were surrounded exclusively by magnocellular neurosecretory terminals (Fig. 20C,D).

3.4. Lesion area

3.4.1. Vasopressin immunoreactivity

Vasopressinergic axons in the hypophysial stalk of the sham + CISL animals and sham + water animals had identical morphology: axons with only a few dilatations coursed within the HNT through the center of the hypophysial stalk (Fig. 21A); a few finely beaded and thin axons originated from the tract and projected to the external zone ventrally in the stalk.

At 2 dps, a few thick AVP-positive axons were present at the transection site in all stalk-transected animals.

At 5 dps, there was an increase in thick vasopressinergic axons near the lesion area in both stalk-transected groups, but more axons were present in the ST + CISL group than in the ST + water group (Fig. 21B). At 10 dps and even more at 15 dps, the lesion area was enlarged. Many regenerated and often enlarged axons surrounded the capillaries that had distincly proliferated near and at the lesion site. Again, the number of vasopressin-positive axons was higher in the ST + CISL group than in the ST + water group (Fig. 21C,D). At 18 dps, the lesion area was filled with regenerated vasopressinergic axons with many dilatations, and closely resembled the neural lobe of sham + water animals. Fewer regenerating axons were present in the ST + water group than in the ST + CISL group (Fig. 21E,F).

3.4.2. Oxytocin immunoreactivity

In both sham + water and sham + CISL animals, the oxytocin immunoreactive axons were primarily located in the center of the hypophysial stalk, only an occasional axon extended to the periphery of the stalk (Fig. 22A).

At 2 dps, there was no difference in the number and staining intensity of oxytocinergic axons between ST + CISL and ST + water groups, and in all stalk-transected animals only a few oxytocinergic axons extended to the periphery of the stalk.

At 5 dps, more oxytocin-positive axons had grown out from the transection site in both stalk-transected groups, but there was no perceptible difference between the two groups (Fig. 22B). By 10 dps, oxytocinergic axons had grown further and surrounded the markedly proliferated capillaries, but at this time, there were distinctly more axons present in the ST + CISL group than in the ST + water group (Fig. 22C,D). Oxytocinergic axons continued to regenerate throughout the observation period and were always more numerous in the ST + CISL group than in the ST + water group (Fig. 22E,F).

3.4.3. βIII-tubulin immunoreactivity

βIII-tubulin-positive axons were observed in the center of the HNT in the hypophysial stalk in the sham + water groups (Fig. 23A). In the sham + CISL group, changes in the immunoreactivity at different postsurgical periods were similar to those observed in the ME.

At 2 dps, a few tubulin-positive axons had extended peripherally from the lesion site in the stalk-transected animals, but no differences in the staining intensity and number of positive axons were observed between ST + water and ST + CISL groups (Fig. 23B). At 5

dps, there was a noticeable increase in the number of tubulin-positive axons in the stalk-transected animals. In the ST + CISL animals, axons surrounding the blood vessels were more intensely stained and more numerous than in the ST + water group (Fig. 24A,B). At 10 dps, a further increase in number and staining intensity of the axons was especially noticeable in the ST + CISL group. Many heavily stained tubulin-positive axons radiated from the center toward the periphery of the stalk and surrounded the proliferated capillaries (Fig. 24C,D). At 15 dps and 18 dps, further axonal growth was found in both groups; more axons were present in the ST + CISL group than in the ST + water group (Fig. 24E,F,G,H). The staining intensity of the axons in the ST + CISL group reached a peak at 15 dps (Fig. 24F).

3.4.4. Electron microscopic observations

In intact animals and in the sham + water group, the fine structural characteristics of the lesion site differed from those in the median eminence only in a few respects. Ependymal cells were absent; the HNT occupied the center of the hypophysial stalk. In the sham + CISL group, the changes observed at the lesion site were identical to those in the median eminence.

At 2 dps, in the ST + water group, most axons of the HNT were filled with densely packed NGVs (Fig. 25A). At the site of transection, the original structure of the stalk was vastly disturbed; large spaces were present between axons and pituicytes, and the tissue was infiltrated with erythrocytes. Proximal to the transection site, there were numerous growth cones, characterized by abundant microtubules and profiles of axoplasmic reticulum. Many neurosecretory axons were filled with NGVs. Some axons contained enlarged and clustered NGVs, and were obviously degenerating. In many instances, neurosecretory axons were

present in the vicinity of perivascular spaces peripherally in the stalk. Occasionally, perivascular spaces were contacted by many magnocellular neurosecretory axons, more often only a few axons had established contact with the perivascular basal lamina (Fig. 25B). Such contacts contained the microvesicles typical of axon terminals. In the ST + CISL group, in addition to a strikingly high number of large growth cones, there were many enlarged neurosecretory axons and axon dilatations, either empty of filled with NGVs, extensive axoplasmic reticulum, or mitochondria and dense lamellar bodies at the lesion site (Fig. 25C). It was not unusual to find growth cones in pericapillary spaces (Fig. 25D). Other fine structural characteristics were identical to those observed in the ST + water group.

At 10 dps, in the ST + water group, there were extensive areas with many dilated axons that contained either only a few or no NGVs, together with microtubules and some mitochondria, or that were filled with elaborate axoplasmic reticulum or densely packed NGVs. Many axon terminals were present around the proliferated capillaries, but they also had invaded pericapillary connective tissue spaces, either alone or together with pituicyte filopodia. In the ST + CISL group, only quantitative differences, as compared to the ST + water group, were observed at the fine structural level.

At 18 dps, in the ST + water group, capillaries were more numerous and areas resembling neural lobe in situ were more extensive. In the ST + CISL group, fine structural characteristics were the same as in the ST + water group (Fig. 26).

4. Discussion

The hypothalamo-neurohypophysial system is ideally suited for spontaneous regeneration after transection of the axons of neurosecretory neurons in the hypophysial stalk. A glial scar does not develop; even though pituicytes contain GFAP filaments typical of astrocytes, they do not react to the transection with responses typical for astrocytes in the remainder of the CNS. On the contrary, the glial microenvironment is supportive of neurosecretory axon regeneration. Secondary effects, such as the formation of a postlesion cavity, do not occur in the hypophysial stalk. Furthermore, new posttransectional axon terminals, i.e., the proximal stumps of the transected neurosecretory axons, are located in a microenvironment that closely resembles that of the neural lobe: pituicytes are present and fenestrated capillaries are located nearby at the surface of the hypophysial stalk.

Despite the very favorable conditions for regeneration only between 14 % and 33% of the cells survive axotomy [7,15,24,73,81,84]. The remaining cells, even though they compensate for the cell loss by increasing their synthesis and secretion of AVP, are incapable of completely restoring antidiuretic function and normal fluid intake [24,39,73].

The results of the present experiments have confirmed and expanded earlier observations from this laboratory [39]. Evidence has been presented that provides a possible explanation for the mechanism through which CISL induces the survival and functional recovery of axotomized vasopressinergic neurons and the regeneration of their axons, and permits a number of important conclusions to be drawn.

4.1. Water consumption

Water consumption after stalk transection follows a triphasic pattern [24,25,39,58]. The acute phase, which is characterized by the symptoms of diabetes insipidus (DI), i.e., polydipsia and polyuria, occurs 1 day after transection and is due to vasopressin not being released from the axon terminals in the disconnected neural lobe [15,58]. In the following interphase, at 2 to 3 dps, water consumption decreases to normal levels. Since the interphase is absent in hypophysectomized or neural lobectomized animals, the source of vasopressin is considered to be the degenerating axon terminals in the neural lobe and not the recovered neurosecretory neurons [15,58]. Subsequently, in the third phase, water consumption gradually increases to the highest level at around 6 dps [24,25,39]. Even though degeneration of the neurosecretory axons in the neural lobe is not completed at this time, the neurosecretory axons apparently no longer release vasopressin or they release it in physiologically ineffective quantities. At 18 dps, water consumption is still at a level which is statistically not significantly lower than at 6 dps, even though at that time a new neural lobe structure has formed at the transection site. Apparently, neuronal recovery at this point is insufficient to decrease water intake. The restitution process does, however, continue and eventually water consumption declines to a significantly lower level [39]. But recovery is never complete and water intake never decreases to control levels [25,39,58].

Water consumption in stalk-transected animals subjected to CISL is initially similar to that in the ST + water group. One day after stalk transection, water consumption vastly increases, but the decrease in the interphase is not as drastic as that in the ST + water group because animals are drinking hypertonic saline. Ingestion of hypertonic saline, even in the

sham-operated group, causes animals to drink ever increasing quantities of fluid. Therefore, it is not surprising that fluid consumption in the ST + CISL group, even though it follows a pattern similar to that observed in the ST + water group (Fig.1), is considerably greater than that in the ST + water group, and remains at a high level until the termination of CISL. Within 24 h after cessation of CISL, water consumption abruptly declines and then further decreases at 18 dps to a level that is not significantly different from that in the sham-operated animals. This recovery is permanent [39], and therefore not due to a short-term continued high rate of synthesis and release of AVP induced by the NaCl consumption.

4.2. AVP and OT immunoreactivity and neurosecretory cell loss in the SON

Many neurosecretory cells undergo retrograde degeneration following the interruption of the hypophysial stalk through hypophysectomy, stalk transection, stalk compression or neural lobectomy [5,7,15,39,49,51,58,73,84,102]. Degenerating cells rarely occur during the first 5 days after axotomy [102]; most cells die between 5 and 14 dps [49,51,102]. In our experiments we confirmed these findings by showing that at 2 and 5 dps there is no significant difference in the number of neurosecretory cells between sham-operated and stalk-transected animals. At 10 dps, however, the number of vasopressinergic neurons in the ST + water group is drastically decreased to about 35 % of control values and then further declines only insignificantly to about 26 % at 18 dps. At this point, apparently, no further degeneration occurs since this figure is in agreement with that reported for 36 dps [39]. Between 5 and 10 days, cell loss in the ST + CISL animals is far less spectacular than that in the ST + water group; it reaches 67 % of control values at 10 dps, and further decreases

insignificantly to 56 % of control values at 18 dps. Cell loss is apparently likewise completed at 18 dps, as shown by comparing the figures of our current experiments with those reported for 36 dps [39]. In contrast, the number of surviving oxytocinergic neurons less drastically declines to 62 % in the ST + water group and to 78 % in the ST + CISL group. These observations are consistent with those of previous studies reporting that oxytocinergic neurons are more resistant to axonal damage, and regenerate more vigorously than vasopressinergic neurons [24,25,39,102]. A possible explanation of these phenomena is offered in the last section of this discussion.

Dehydration through either water deprivation or oral hypertonic saline consumption causes an increase in the biosynthesis of prohormones [8,26,48,104], a 3-fold increase in their posttranslational processing [8], and a 5-fold increase in axonal transport of hormones [8,28,79]. CISL is apparently a milder stimulus than continuous salt-loading. CISL does cause a decrease in immunoreactivity for vasopressin, but cells never become altogether depleted as they do after two weeks of hypertonic saline ingestion [68]. Interestingly, the number of vasopressinergic cells progressively increases during CISL and even after 3 days of rehydration the number is significantly higher than at the onset of CISL. We speculate, in the absence of any morphometric data in the literature, that in the supraoptic nucleus of euhydrated animals there are vasopressinergic cells that are at rest and are not immunoreactive. These cells are stimulated by CISL, and as dehydration progresses an increasing number of cells becomes immunoreactive. Whether this hypothesis is correct will have to be established in future experiments. The immunoreactivity of oxytocinergic cells is unaffected by CISL, which contrasts with the almost complete depletion of immunoreactive

oxytocin observed after 14 days of hypertonic saline ingestion [68]. There is also a progressive increase in the number of oxytocinergic cells through 18 dps, but the difference is statistically not significant.

The observation that more oxytocinergic cells survive in the ST + CISL than in the ST + water group at 10 and 18 dps is in contrast with our previous findings and probably for the same reasons responsible for the survival of vasopressinergic cells (see last section of this discussion). We do not have an explanation for the rise in the number of oxytocinergic cells in the ST + water group at 15 dps.

4.3. c-fos expression in the SON

c-fos is not expressed in neurosecretory neurons of euhydrated animals, and hypophysial stalk transection does not induce its expression. This is consistent with studies reporting the absence of expression of c-fos in axotomized PNS and CNS neurons [35,59]. CISL induces expression of c-fos in both vasopressinergic and oxytocinergic neurons as does dehydration through either water deprivation or oral administration of hypertonic saline [38,56,61,70,105].

In both types of neurosecretory cells, as salt loading progresses, the percentage of Fos-immunoreactive cells increases significantly in sham-operated and stalk-transected animals, and drops to zero after 3 days of rehydration. Stalk transection has no effect on the expression of Fos in vasopressinergic cells, but does induce a significantly higher percentage of oxytocinergic cells to express Fos at 5 dps (Fig. 12). The reason for this difference is not evident.

The functional significance of the expression of c-fos is unknown. Since there is no AP-1 binding site at the promotor area of the AVP and OT genes [61,72], c-fos does not stimulate the synthesis of either hormone. The similarity in the expression of c-fos and tubulin immunoreactivity prompts the hypothesis that Fos regulates the transcription of tubulin, and thus promotes the regeneration of the stalk transected neurosecretory neurons. Since Fos-Jun heterodimers bind to the AP-1 binding site with 50 times greater efficiency than do Jun-Jun dimers [32,78], the principal action of Fos could be to enhance Jun activity.

4.4. c-jun expression in the SON

Following transection of axons in various tracts of the CNS, the cells of origin express c-jun to varying degrees and over varying periods of time [34,44,45,60,88,89]. There is no apparently consistent pattern: c-jun may decline rather rapidly, preceding death of the axotomized cells, as for example in retinal ganglion cells after optic nerve transection, or its expression may be sometimes prolonged over extended periods of time, as in various nuclei whose axons were transected in the median forebrain bundle or mammillo-thalamic tract [34,44,45,60,88,89]. It is unclear what is responsible for the observed differences. It is notable, however, that there appears to be a relationship between the expression of c-jun and regeneration since the expression is prolonged when retinal ganglion cells regenerate through a peripheral nerve graft [42,88,89].

c-jun expression in neurosecretory neurons after stalk transection is similar to that in the PNS where there is an apparent correlation between the expression of c-jun and regeneration. Following sciatic nerve transection, upregulation of c-jun in the dorsal root

ganglia reaches a maximum within 2 days and decreases to basal levels when the regenerating axons have reached their targets [14,29,34,35,59].

In vasopressinergic cells, c-jun expression follows a distinctly different pattern in ST + CISL and ST + water groups. At 2 dps, c-jun expression is virtually identical in both stalk-transected groups. In the ST + water group, the number of Jun-positive cells peaks at 5 dps and then declines steeply at 10 dps and drops to zero at 18 dps. In the ST + CISL group, however, highest expression of c-jun is at 2 dps, and the decline already begins at 5 dps, and continues in a similar pattern to that in the ST + water group (Fig. 9). At 5 dps, more regenerating vasopressinergic axons had contacted capillaries in the external zone of the ME and lesion area in the ST + CISL group than in the ST + water group. Axons thus had reached their target, and consequently Jun returned to basal levels in their neurons of origin and therefore fewer neurons were Jun-positive.

The pattern of c-jun expression in the oxytocinergic neurons is different from that in the vasopressinergic neurons. It is highest at 2 dps and drops down from then on in both groups. Also, at all time periods, the percentage of Jun-positive oxytocinergic cells is lower than that of vasopressinergic neurons. Among the oxytocinergic neurons of the SON there are neurons that project axon collaterals to the lateral hypothalamus [2,62,66]. Conceivably, these neurons are less susceptible to the transection of their axons in the hypophysial stalk, and therefore do not express c-jun. Oxytocinergic neurons also have greater inherent plasticity than vasopressinergic neurons [33] and that may be the reason for the difference in Jun expression. We do not have any explanation for the significantly fewer cells that express c-jun at 2 dps in the ST + CISL group than in the ST + water group.

c-jun expression after axon transection corresponds to upregulation of regeneration-associated genes, such as those encoding for GAP-43 or tubulin [29,45,96,98,99]. Therefore, c-jun expression might be a prerequisite for initiation of synthesis of GAP-43, tubulin [45,96,99] or cytoskeletal proteins [96,99], associated with axonal sprouting and growth.

4.5. βIII-tubulin expression

In euhydrated sham-operated animals most neurosecretory neurons in the SON are faintly immunoreactive for βIII-tubulin. CISL does induce a few cells to become moderately immunoreactive at 5 and 10 dps and a moderate number of cells to become intensely immunoreactive at 15 dps; but three days after rehydration, the immunoreactivity is back to basic levels. Surprisingly, this increase is not matched in the median eminence, where an increase in tubulin immunoreactivity occurs only at 15 dps. In the SON, stalk-transection induces a reaction similar to that of CISL as early as 5 dps, but the gradual decline to basic levels occurs already at 15 dps. Similarly, in the median eminence tubulin-positive axons are increasingly more numerous between 5 and 18 dps, but their staining intensity is back to basic levels at 15 dps. The combination of stalk transection and CISL is obviously the strongest catalyst for tubulin expression, since it causes a pronounced increase in immunoreactivity in many cells at 5 dps and 10 dps, with a moderate number of cells still being intensely stained at 15 dps, and a return to basic levels at 18 dps. Changes in the median eminence in the ST + CISL group are almost exactly like those in the ST + water group, except that the increase in the number of axons and in their staining intensity is more pronounced and longer lasting.

CISL alone does not appear to strongly stimulate tubulin biosynthesis as judged from the results of immunohistochemical staining for βIII-tubulin in the median eminence. However, at the electron microscopic level it was obvious that at 10 dps neurosecretory axons in the internal zone of the median eminence contained more microtubules in sham + CISL animals than in sham + water animals. The increase was apparently not important enough to be expressed light microscopically. Conceivably and most likely, this was also the case at earlier times of CISL.

CISL does provide, however, an additional stimulus for tubulin biosynthesis in stalk-transected animals, thereby supplying the damaged neurosecretory neurons with a cytoskeletal protein required for axon elongation [80]. Because of the presence of βIII-tubulin immunoreactivity in the SON in the ST + CISL group (2 dps) earlier than in the ST + water group (5 dps), we predict that tubulin increases in neurosecretory axons in the median eminence shortly after 2 dps and earlier than in the ST + water group. βIII-tubulin, polymerized to form microtubules, would enable the damaged axons to grow and to sprout axon collaterals toward the capillaries and to establish earlier neurovascular contacts. At 10 dps, i.e., at a time when Jun immunoreactivity is virtually no longer present in the SON, βIII-tubulin immunoreactivity is still above basal level in a number of neurosecretory cells; this seems to indicate that regeneration is not yet completed and that axons continue to grow. That tentative conclusion is in fact supported by the fine structural analysis that shows quantitative changes in neurovascular contacts between 10 dps and 18 dps.

4.6. Fine structure of the SON

As could be expected from the light microscopic observations, the sham operation does not have any effect on the fine structural characteristics of magnocellular supraoptic neurons. In intact animals, the effects of 14 days of CISL are similar to those of dehydration through water deprivation or oral 2% NaCl [74]: there is an overall increase in the extent of the organelles involved in protein synthesis; particularly affected are the rough endoplasmic reticulum whose cisternae are characteristically dilated, and the Golgi complex. Whether the subjectively perceived decrease in the number of perikaryal NGVs is real or only a reflection of the increased cell size subsequent to osmotic stress remains to be determined. Three days after cessation of CISL, most cells have recovered from the osmotic stimulus and their fine structural morphology has returned to pre-CISL condition; some cells do, however, still have morphologic signs of increased secretory activity.

While at 2 dps only some cells react to the inflicted injury by an accumulation of NGVs in the ST + water group --this is the beginning of the "obstructive phase" [84]-- an appreciable number of cells in the ST + CISL group react to the combined challenge of transection and CISL with morphologic signs similar to those observed at later stages of CISL. At 2 dps, CISL alone does not affect the fine structure of supraoptic neurons in sham-operated animals. Axon transection, however, apparently renders some neurons more susceptible and reactive to salt-loading while other neurons, that were probably at a different stage of secretory activity at the time of transection, do not react. This early stimulus is probably not all that is required for eventual survival of the cell and regeneration of its axon, but it may be a key factor.

At 18 dps, the neurosecretory cells in both stalk-transected groups have virtually recovered from the inflicted injury, but their fine structural characteristics do reflect the need for increased amounts of AVP to compensate for the cell loss.

4.7. Changes in the ME and lesion area

Our experimental results indicate that changes in the expression of vasopressin, oxytocin and tubulin immunoreactivities range from moderate in the sham + CISL groups to spectacular in the stalk-transected animals. Twenty four hours of CISL was apparently not enough of a stimulus to produce any change in vasopressin and oxytocin immunoreactivities in the internal zone of the ME, which contrasts with the spectacular decrease reported in animals on 2 % NaCl [11]. The subsequent decrease in both immunoreactivities is subtle when induced by CISL and more pronounced when induced by 2 % NaCl and is a reflection of altered transport and release rates; the return to control levels 3 days after cessation of CISL indicates recovery from the osmotic challenge. CISL had no effect on oxytocin immunoreactivity in the external zone of the median eminence, but caused an increase in vasopressin immunoreactivity identical to that observed in previous experiments [39]. Subtle increases in tubulin immunoreactivity early during CISL were not quantifiable, and the obvious later change is a reflection of increased axonal transport of NGVs in the internal zone, and sprouting in the subependymal and external zones. Results from our laboratory as well as from other laboratories support the view that parvocellular vasopressinergic neurons, whose terminals are located in the external zone, are not involved in hydromineral regulation [6,18,69]. Indeed, electron microscopy does reveal magnocellular neurosecretory axons deep

in the external zone at 2 dps, which provide an explanation for the increase in vasopressin immunoreactivity in the external layer. Vasopressinergic axons respond to the CISL stimulus by sprouting into the subependymal layer and toward the primary capillaries. The concomitant increase in tubulin immunoreactivity is a reflection of that sprouting activity. There is general agreement that after hypophysectomy, stalk transection, stalk compression or neural lobectomy, transected neurosecretory axons massively regenerate within the median eminence [1,5,15,37,39,50,83,101].

While regenerating neurosecretory axons have been identified by means of conventional staining methods for neurosecretory axons prior to the introduction of immunohistochemical methods, the use of antibodies against vasopressin and oxytocin has provided insights into differences in the capacity for regeneration between vasopressin and oxytocin axons.

The present results show that both types of regenerating neurosecretory axons are detectable in the external zone as early as 2 dps, confirming previous reports [83,101]. Only a few of them reach primary capillaries, and therefore it is not surprising that we could not find pericapillary magnocellular axon terminals at the fine structural level. At 2 dps, magnocellular neurosecretory axons --identified by their characteristic large NGVs-- are present in the external zone alongside glial cell processes that are oriented perpendicular to the ventral surface of the median eminence. They undoubtedly are axon collaterals sprouting from neurosecretory axons in the internal zone. These collaterals apparently use the glial cell processes as guiding structures to the short capillary loops in the external zone. This confirms previous reports of close relationships between regenerating neurosecretory axons

and glial cells [15,16,20,85], that have recently been identified as tanycytes [9]. This finding supports the concept that glial cells are indeed an important factor in, or may be even required for neurosecretory axon regeneration [16]. While there is no difference in the regenerative capacity of both types of neurosecretory axons between animals subjected to CISL or drinking water at 2dps, CISL distinctly causes a greater number and more intensely immunoreactive vasopressinergic axons to regenerate at all subsequent time periods; oxytocinergic axons are virtually unresponsive to CISL, and only at 18 dps did CISL appear to have a slight stimulatory effect. The gradual outgrowth of axons toward the long capillary loops in the internal zone, and from the internal zone to the external zone, as well as the increasing immunoreactivity around the primary capillaries of the median eminence strongly implies a vascular affinity of the regenerating axons. Many neurovascular contacts are definitely established at 5 dps, and the presence of clusters of microvesicles within them is a clear indication of their capacity to secrete [33]. Interestingly, there is a close parallelism in the staining intensity and topographic distribution in the median eminence between vasopressin-positive and tubulin-positive axons. This is actually not surprising because microtubules are required for axonal transport of NGVs, and since CISL-stimulated neurons transport more NGVs than euhydrated ones, they can be expected to also possess more microtubules. Furthermore, microtubules are considered to be indispensable for successful regeneration [75,76,80,98,99] . Their presence in greater numbers and in more axons of CISL-stimulated neurons indicates that these neurons have a greater capacity to regenerate. The present results support the conclusion of previous reports [37,68,101,106] that there is essentially no difference in the regenerative capacities of vasopressinergic and oxytocinergic

axons. The present results are, however, at odds with other investigations that reported a distinctly more vigorous regeneration of OT axons [2,25]. The drastic decrease in vasopressinergic axons that Alonso et al. [2] observed with increasing time after stalk transection is particularly puzzling. It is possible, though unlikely, that the different rat strains used in the experiments react differently to stalk transection.

Neurosecretory axon regeneration is particularly robust immediately proximal to the transection site [1,15,39]. Not surprisingly, our experiments revealed that at the lesion site in the hypophysial stalk regenerating neurosecretory axons invade the external zone and establish perivascular contacts earlier than more proximally in the median eminence. Thus, at 2 dps, neurovascular terminals were readily detectable at the electron microscopic level. A likely reason for this may be the proximity of the proximal stump of transected axons to the capillaries. Conceivably, that proximal stump has a greater ability to elongate than the axon in the median eminence to develop an axon collateral. This would explain why at the transection site regeneration is more pronounced than more proximally in the median eminence. The proliferation of capillaries in reaction to their severance by the stalk transection is more extensive than in the median eminence; this is probably another factor that contributes to the robust regeneration of neurosecretory axons. The stimulating effect of the salt-loading is clearly shown by the distinctly faster regeneration in the ST + CISL group than in the ST + water group as evidenced by the presence of more growth cones in perivascular spaces at 2 dps, and the greater number of neurovascular contacts at 10 dps.

4.8. Conclusions

Results from previous investigations in this laboratory [16,17,19-22,82] have led to the suggestion that neurosecretory axon regeneration is critically dependent on two essential elements: the presence of glial cells and of a permeable microvascular environment, i.e., capillaries without a blood-brain barrier.

For successful axonal regeneration to occur, the damaged neuron has to survive the injury, then the transected axon has to sprout and grow toward the target and finally establish functional contact, i.e., a synapse, with the target. Results of the current experiments clearly show that in animals subjected to CISL, survival and regeneration of vasopressinergic supraoptic neurons is vastly enhanced, and their functional capacity is completely restored.

The mechanism through which CISL accomplishes this greatly improved posttransectional recovery is envisioned as follows: Hypophysial stalk transection provides a strong initial stimulus for vasopressin synthesis now that vasopressin is no longer released from the disconnected axon terminals in the neural lobe. During the first 24 hours after transection, this stimulus is identical in ST + CISL and in ST + water animals; this is because CISL does not begin until 24 hours after stalk transection. In the following interphase, during which water consumption is normalized (because of release of vasopressin from the neurosecretory axon terminals in the neural lobe), vasopressinergic neurons in the ST + water group are not stimulated above normal levels to synthesize AVP. Vasopressinergic neurons in the ST + CISL group do, however, increase the hormone synthesis that is stimulated by the hyperosmotic challenge of salt-loading, known to increase vasopressin biosynthesis [27,28,67,93,94,95,104]. Concurrently, these cells express Fos; Fos in combination with

Jun, which is expressed subsequent to stalk transection, acts on target genes and consequently vasopressinergic cells increase their protein synthesis in general [63,64,77], and that of microtubules (as shown by an increased immunoreactivity for tubulin at 2 dps in the ST + CISL group), in particular [63,77]. Increased vasopressin synthesis generates an elevated number of NGVs and their anterograde axonal transport requires more microtubules [80]. Microtubules are likewise needed for retrograde transport to the neuronal cell body. Thus, in the CISL groups, earlier than in ST + water animals, the increased number of microtubules permits trophic factors originating in the median eminence to be transported back to the neuronal cell bodies in large quantities. Possible sources of factors are pituicytes, tanycytes, and blood [9,21,52]. These factors could conceivably be required [100] to sustain the increased level of metabolic activity induced by CISL [31]. Consequently, in the CISL groups more neurons are surviving than in the water groups. The stimulated neural cell bodies are now capable of sustaining axonal sprouting, for which microtubules are likewise necessary [80] and obviously available. A sprouting thrust must occur in the ST + CISL group between 2 dps and 5 dps; this is because at 2 dps there is no appreciable difference in the number of vasopressinergic and tubulin-positive axons in the external zone of the median eminence between ST + water and ST + CISL groups, but at 5 dps there are more axons present in the ST + CISL group. Neurons which have axons that have established pericapillary contact have reached their target, and consequently they downregulate the expression of Jun [14,35,59], and that is reflected in the significantly lower number of vasopressinergic cells expressing Jun at 5 dps in the ST + CISL group. In the ST + water group, on the contrary, fewer axons have reached the capillaries at 5 dps, and therefore a

much higher percentage of vasopressinergic cells expresses Jun. The earlier establishment of more neurovascular contacts in the ST + CISL group is probably a decisive event and accounts for the survival of more neurosecretory neurons in the ST + CISL group than in the ST + water group. It is conceivable that there is a critical time in which neurosecretory neurons have to establish neurovascular contacts, or at least come close to perivascular spaces, in order to survive; if they fail to do so, they degenerate. The comparison of the number of surviving vasopressinergic cells (Fig. 3) with the percentage of these cells expressing Jun (Fig. 9), and with the immunohistochemical data in the median eminence (Fig. 15), would put that critical time between 5 and 10 days after transection.

Acknowledgements. We thank A. Frankfurter, H. Gainer and A.J. Silverman for the donation of antibodies, and L. Wolins and M. Shelley for the statistical analysis of the data. The excellent technical assistance of R. DenAdel and C. Martens is gratefully acknowledged.

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Table 1. Immunostaining intensity for βIII-tubulin and number of positive cells in the SON, in sham-operated , stalk-transected and CISL-treated animals at different time periods.

	2 dps		5 dps		10 dps		15 dps		18 dps	
	inten.	cell no.	inten.	cell no.	inten.	cell no.	inten.	cell no .	inten.	cell no.
Sh+water	±	-	±	-	±	-	±	-	±	-
Sh+CISL	±	-	+	*	+	*	++	**	±	-
ST+water	±	-	++	**	+	*	±	-	±	-
ST+CISL	+	*	+++	***	+	**	++	**	±	-

Staining intensity: ± indicates basal level; +, ++, +++, designates increase above basal level; + (moderate), ++ (intense), +++ (pronounced).

Cell number: - indicates most cells in the SON; *, **, ***, designates number of cells with immunoreactivity above basal level; * (few), ** (moderate), *** (many).

Fig. 1. This graph depicts daily fluid consumption following sham operation or stalk transection (ST) at time 0, and chronic intermittent salt loading (CISL) for 14 days beginning 1 day post surgery (dps). Notice the fast decline of water consumption after cessation of CISL at 15 dps in the ST + CISL group to the level where there is no significant difference ($P>0.05$), at 18 dps. However, water consumption in the ST + water group still remains at a significantly higher level ($P<0.05$). All values represent mean ± S.E M.

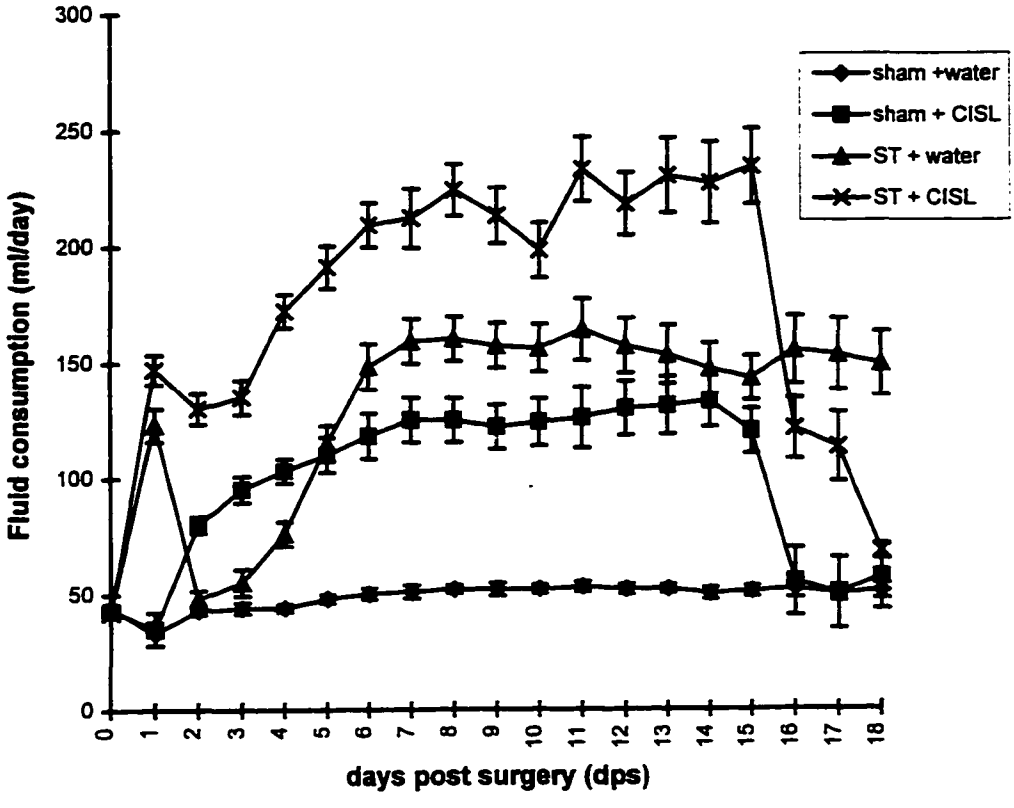

300
250
200
150
100
50
0
Fluid consumption (ml/day)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
days post surgery (dps)
sham +water
sham + CISL
ST + water
ST + CISL

Fig. 2. Sections of the supraoptic nucleus, dual immunostained for AVP and Jun, from ST + water (A, C) and ST + CISL (B,D) group at 5 dps (A,B) and 18 dps (C,D). Notice Jun-positive AVP cells are only present at 5 dps (A,B); at 18 dps, surviving AVP cells in the ST + CISL group (D) are more numerous than in the ST + water group (C). X 90.

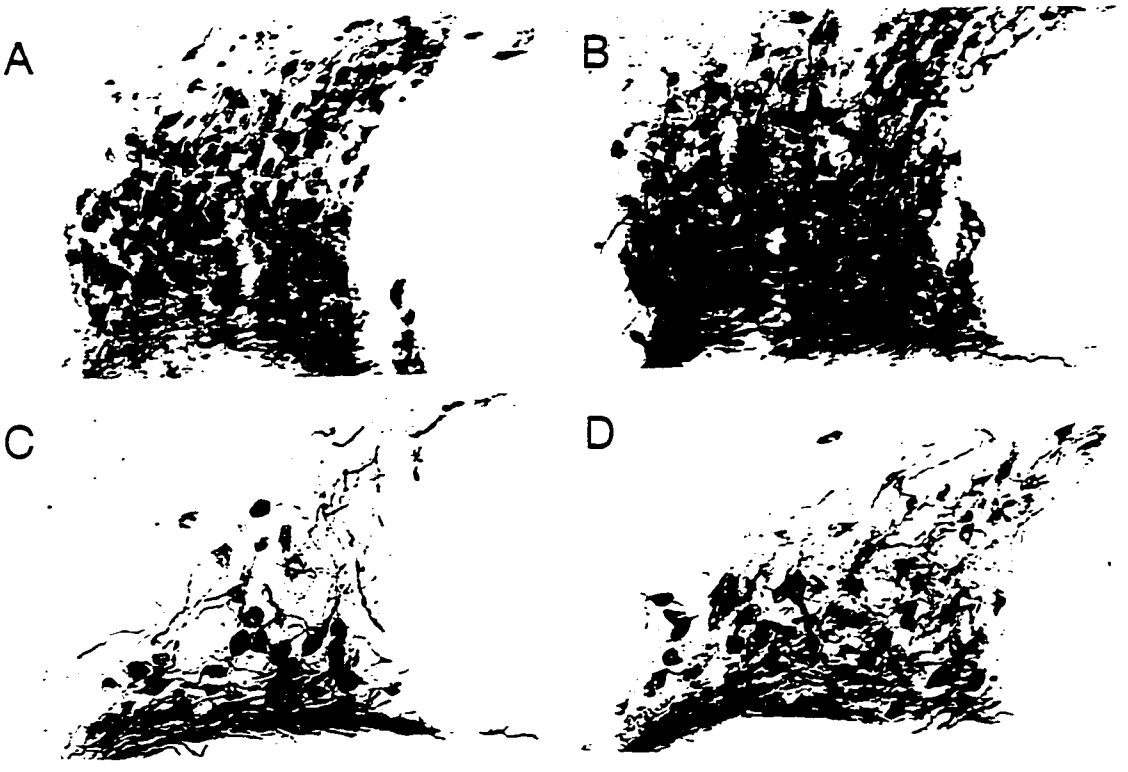

A
B
C
D

Fig. 3. This graph depicts the number of surviving vasopressinergic neurons at various time periods within the supraoptic nuclei in sham-operated and stalk-transected (ST) groups on water and CISL. Notice from 10 dps on the number in the ST + CISL group is significantly higher than that in the ST + water group ($P<0.05$). Values represent mean ± S.E.M.

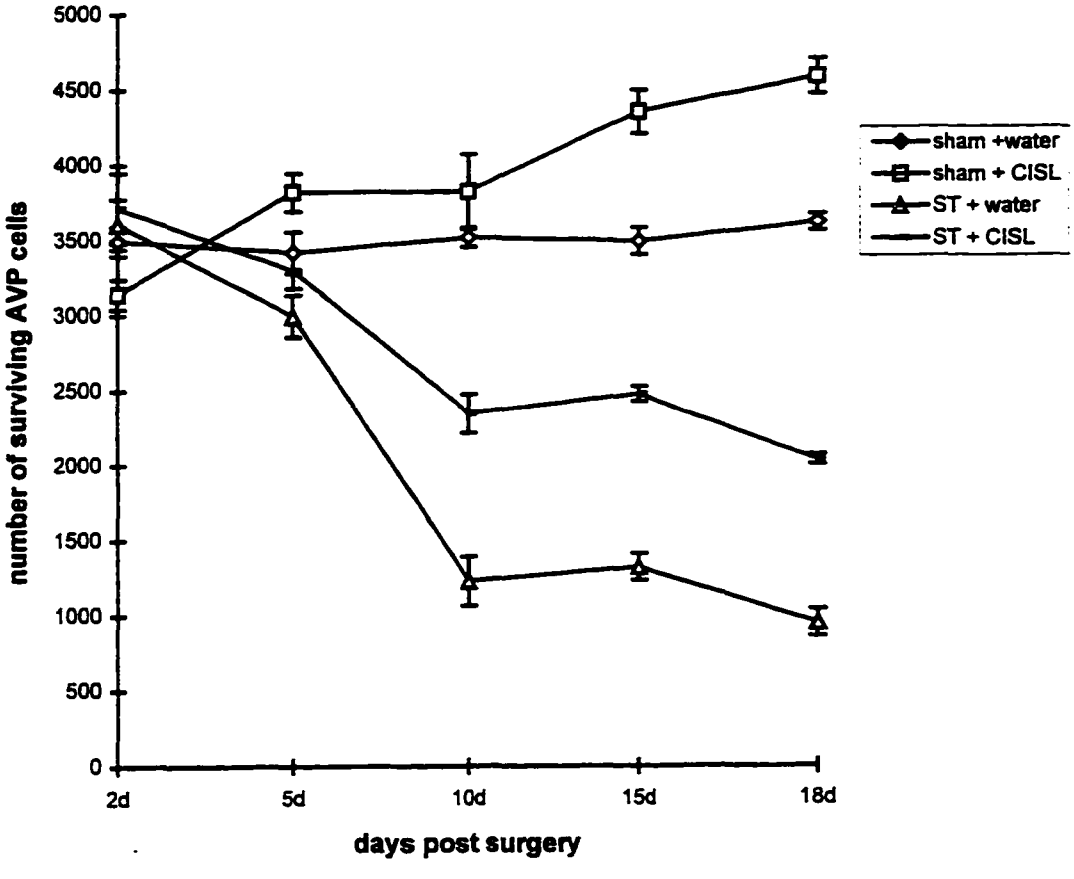

5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
number of surviving AVP cells
2d
5d
10d
15d
18d
days post surgery
sham +water
sham + CISL
ST + water
ST + CISL

Fig. 4. Sections of the supraoptic nucleus, dual immunostained for OT and Jun. A: ST + CISL, 2 dps; B: ST + water, 5 dps; C: ST + water, 18 dps; D: ST + CISL, 18 dps. Jun-positive cells are only present at 2 dps (A) and 5 dps (B); also at 18 dps more OT-positive cells survive in the ST + CISL group (D) than in that ST + water group (C). X 90.

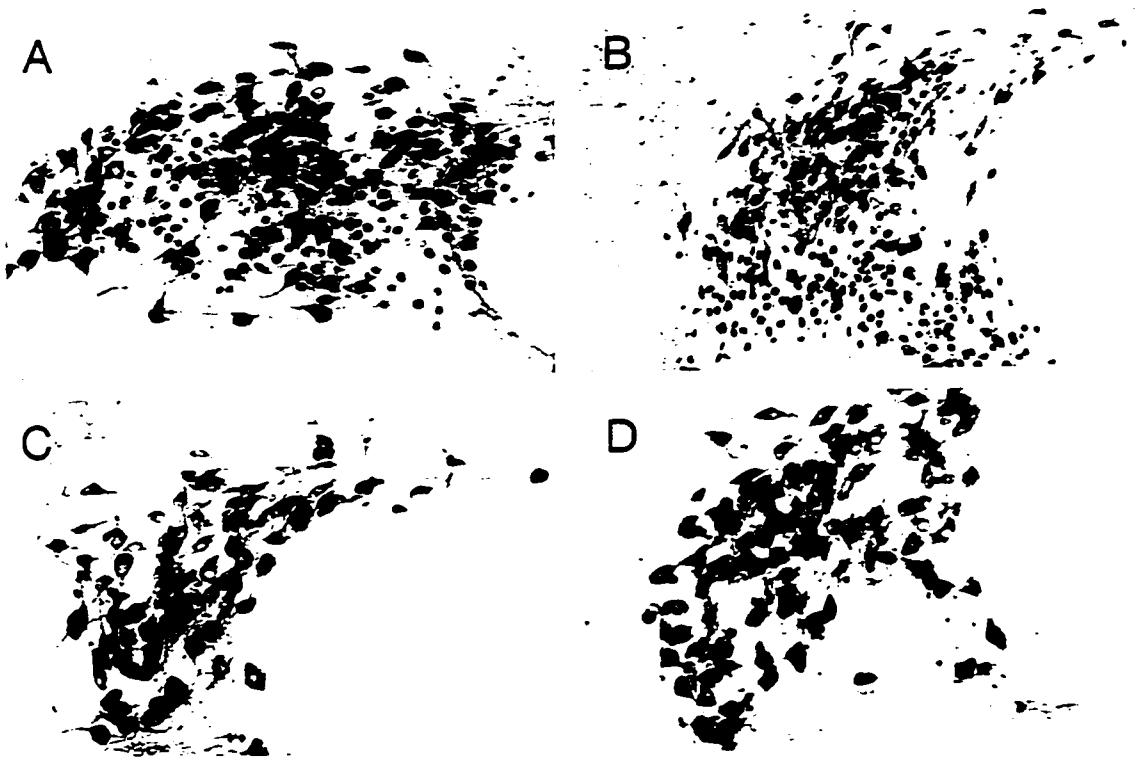

A
B
C
D

Fig. 5. This graph depicts the number of surviving oxytocinergic neurons at various time periods within the supraoptic nuclei in sham-operated and stalk-transected (ST) groups on water and CISL. At 10 and 18 dps, the number of cells in the ST + CISL groups is significantly higher than that in the ST + water groups ($P<0.05$), but there is no significant difference between ST + CISL and ST + water group at 15 dps ($P>0.05$). Values represent mean ± S.E.M.

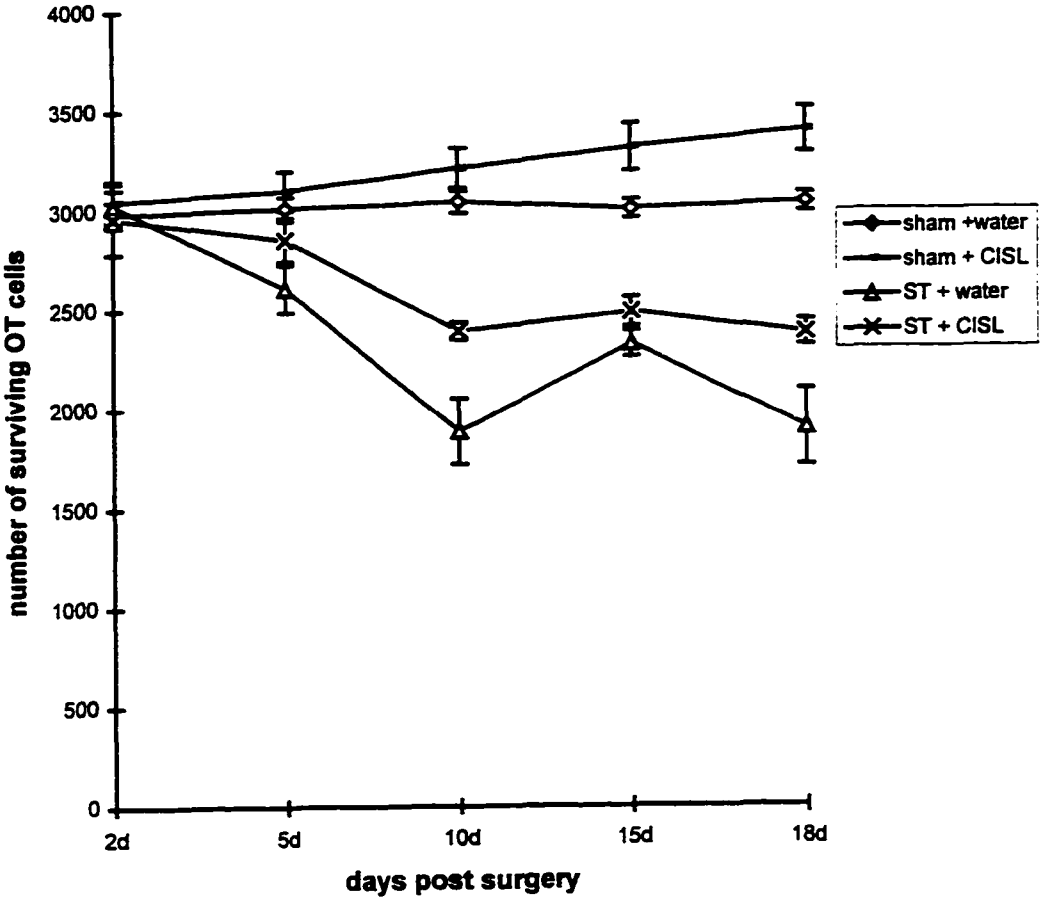

4000
3500
3000
2500
2000
1500
1000
500
0
number of surviving OT cells
2d
5d
10d
15d
18d
days post surgery
sham +water
sham + CISL
ST + water
ST + CISL

Fig. 6. βm-tubulin-immunoreactive cells in the supraoptic nucleus of A: sham + water, 10 dps; B: sham + CISL, 5 dps; C: sham + CISL, 15 dps; D: sham + CISL, 18 dps; E: ST + water, 2 dps; F: ST + CISL, 2 dps. All sections are from the same level. Notice the increase in the number of positive cells in sham + CISL animals at 5 dps (B) and especially at 15 dps (C), and the decline at 18 dps (D). In stalk-transected animals at 2 dps, there was no change in the ST + water group (E) but there was an increase in the number of the positive cells and axons in the ST + CISL group (F). X160.

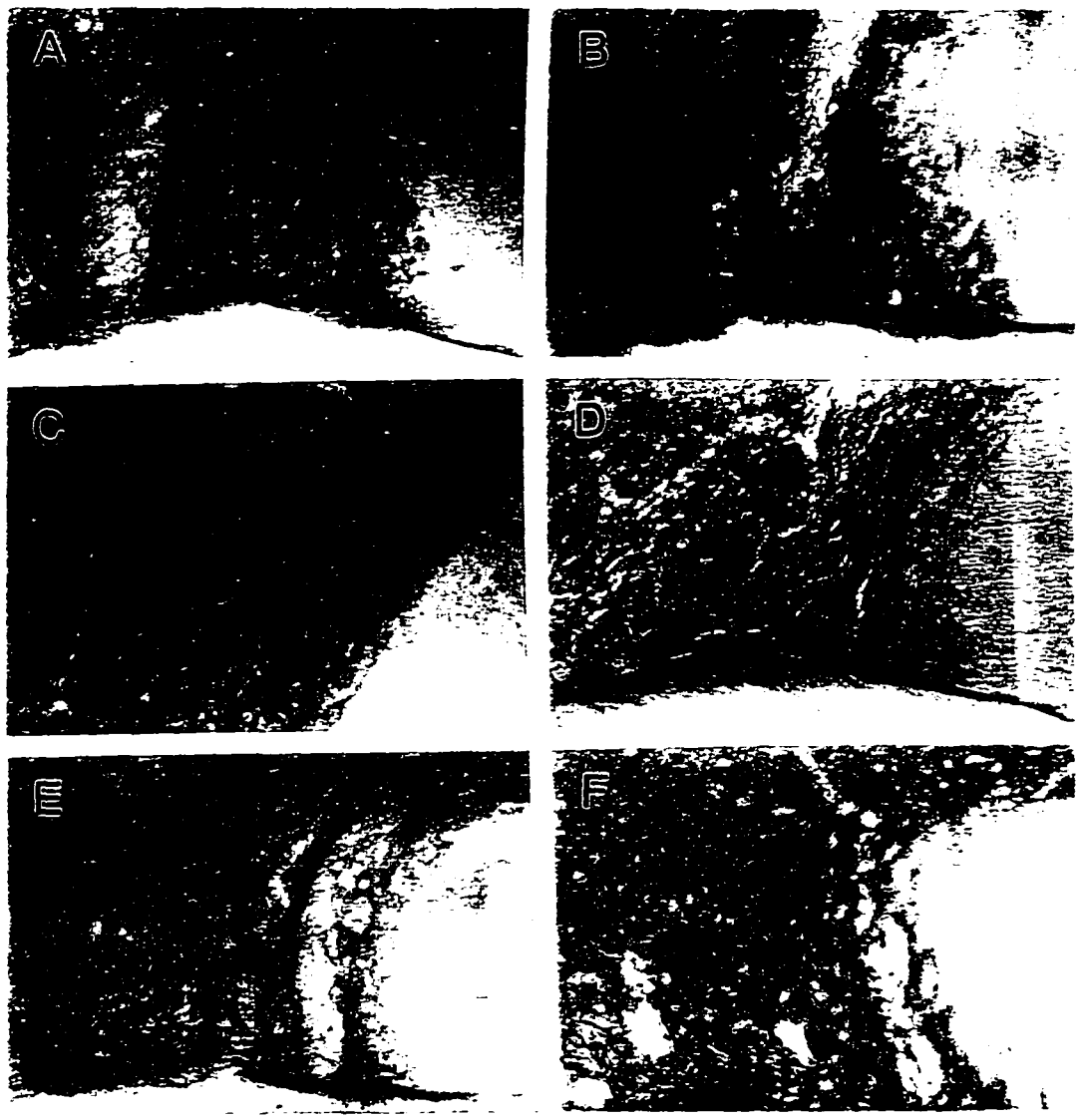

A
B
C
D
E
F

Fig. 7. βIII-tubulin-immunoreactive cells in the supraoptic nucleus of ST + water (A, C, E and G) and ST + CISL groups (B, D, F and H) at 5 dps (A, B), 10 dps (C, D), 15 dps (E, F) and 18 dps (G, H). More positive cells are present in the ST + CISL (B, D, F) than in the ST + water group (A, C, E) at 5, 10 and 15 dps. X160.

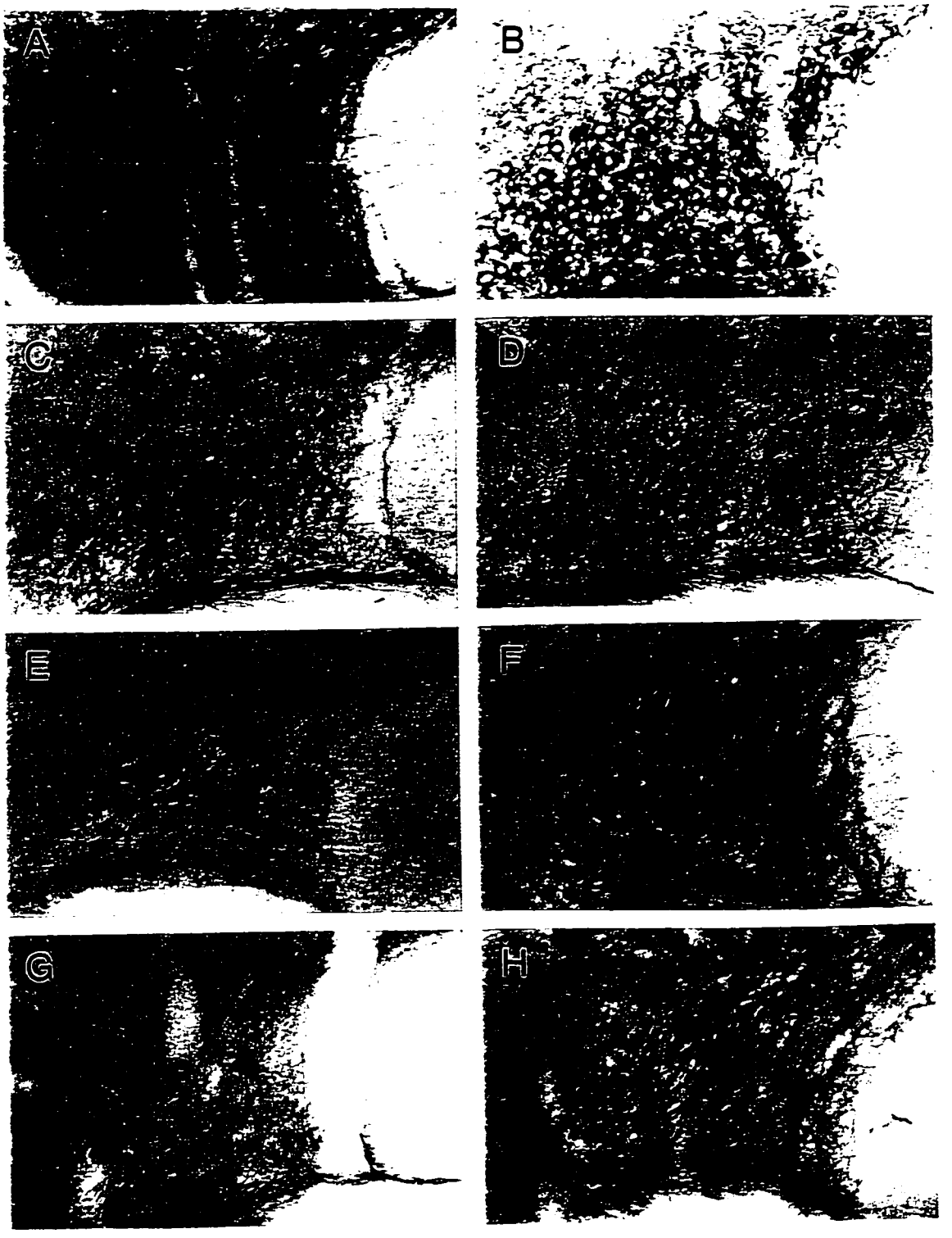

A
B
C
D
E
F
G
H

Fig. 8. Histogram depicting the number of βIII-tubulin positive cells within the supraoptic nucleus in stalk-transected (ST) animals on CISL or water at 5 and 10 days post surgery (dps). The number of positive cells in the ST + CISL group is significantly higher than in the ST + water group at either 5 or 10 dps. Also, the number of positive cells in both groups at 10 dps is significantly lower than at 5 dps. Values represent mean ± S.E.M. * $P<0.05$.

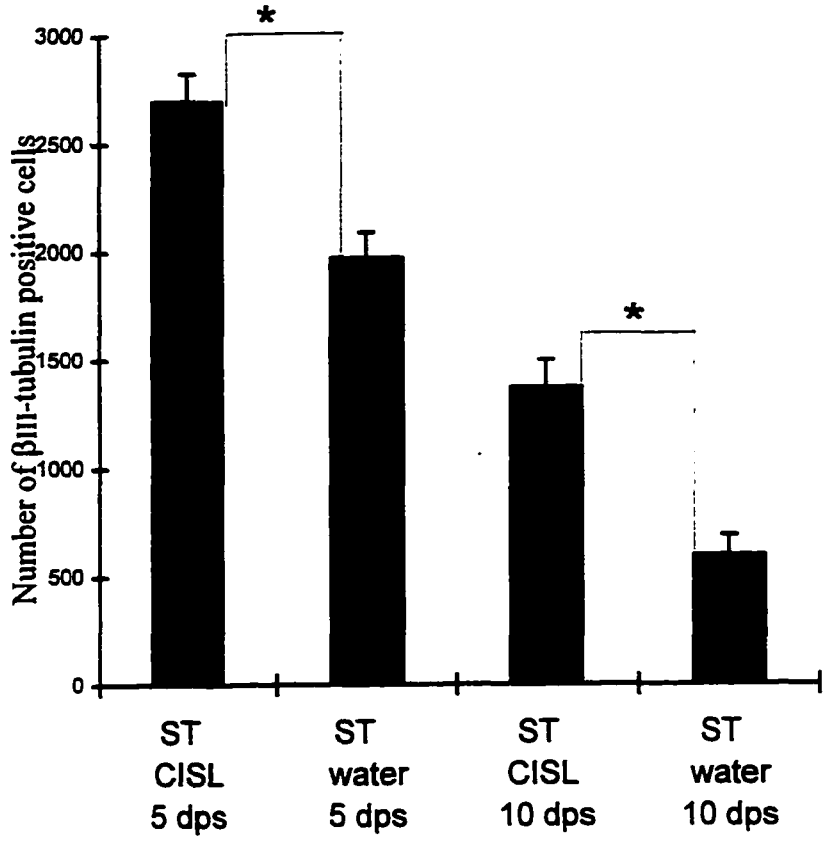

*
*
3000
2500
2000
1500
1000
500
0
Number of βIII-tubulin positive cells
ST CISL 5 dps
ST water 5 dps
ST CISL 10 dps
ST water 10 dps

Fig. 9. This graph represents the percentage of Jun-positive cells in the surviving vasopressinergic neurons of the SON at various postsurgery times in stalk-transected animals. Notice, at 5 dps, the percentage in the ST + CISL group decreases, while that in the ST water group increases. There is a significant difference ($P<0.05$) between these two groups. Values represent mean ± S.E.M.

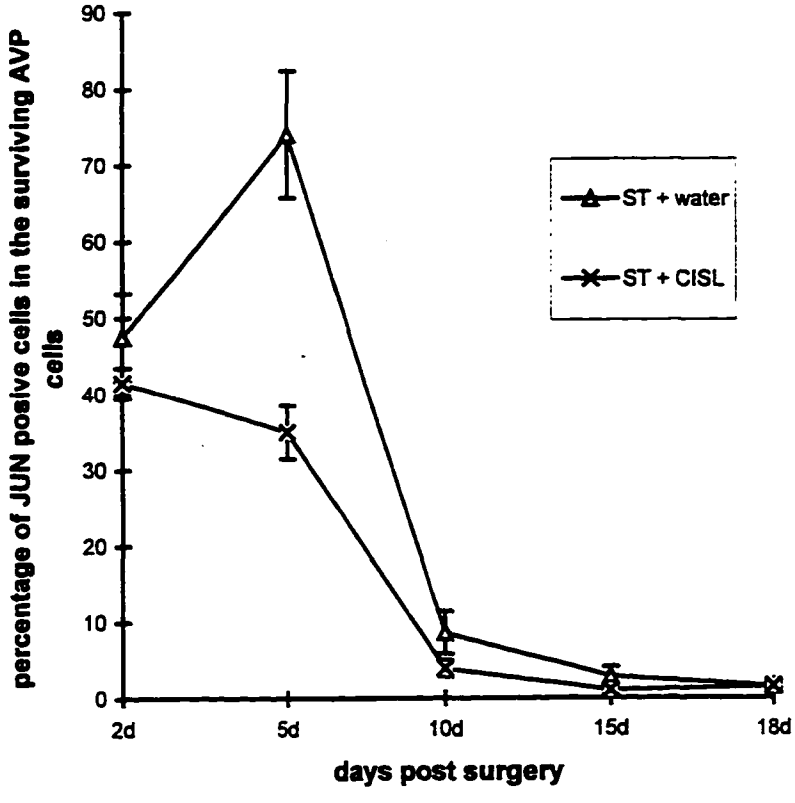

percentage of JUN posive cells in the surviving AVP cells
90
80
70
60
50
40
30
20
10
0
2d
5d
10d
15d
18d
days post surgery
ST + water
ST + CISL

Fig. 10. This graph depicts the percentage of Jun-positive cells in the surviving oxytocinergic neurons of the SON at various postsurgery times in stalk-transected animals. The percentage in the ST + CISL group is significantly lower than that in the ST + water group at 2 and 10 dps. The percentage in both groups at 15 and 18 dps is less than 1 %. Values represent mean ± S.E.M.

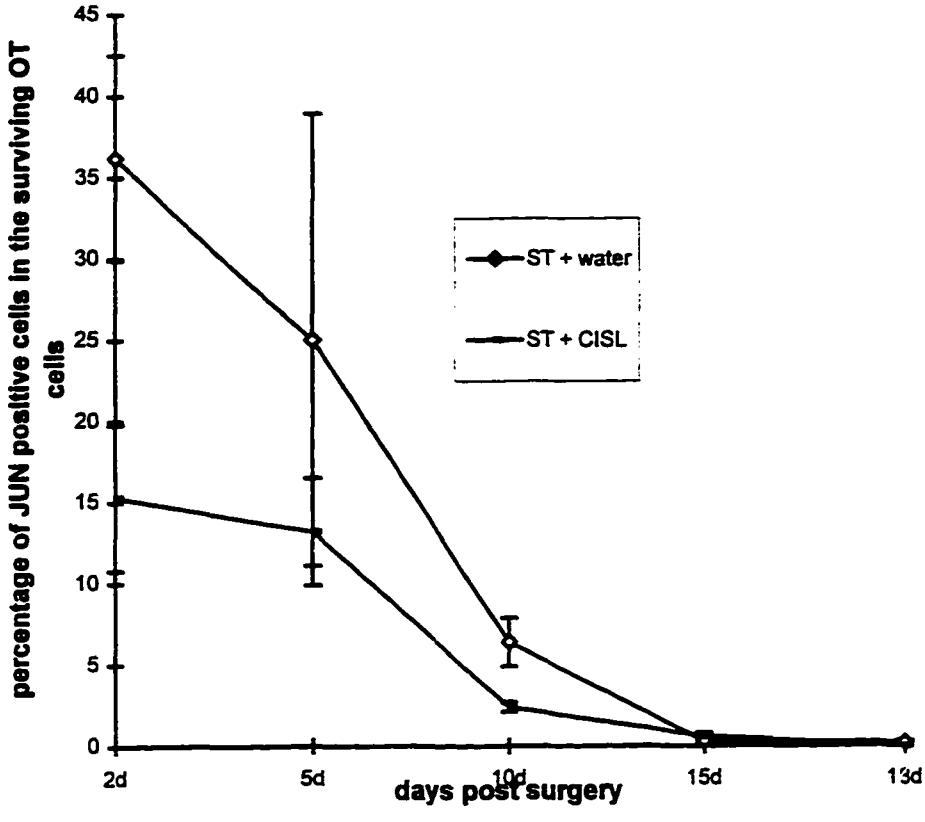

45
40
35
30
25
20
15
10
5
0
percentage of JUN positive cells in the surviving OT cells
ST + water
ST + CISL
2d
5d
10d
15d
13d
days post surgery

Fig. 11. This graph depicts the percentage of Fos-positive vasopressinergic cells within the supraoptic nuclei at various time periods in sham-operated and stalk-transected animals on CISL. The percentage at 5, 10 and 15 dps is significantly higher than that at 2 dps in both ST + CISL and sham + CISL groups, except that in the sham + CISL group, there is no significant difference between 2 and 10 dps. Also, there is no significant difference among 5, 10 and 15 dps in both groups. Values represent mean ± S.E.M.

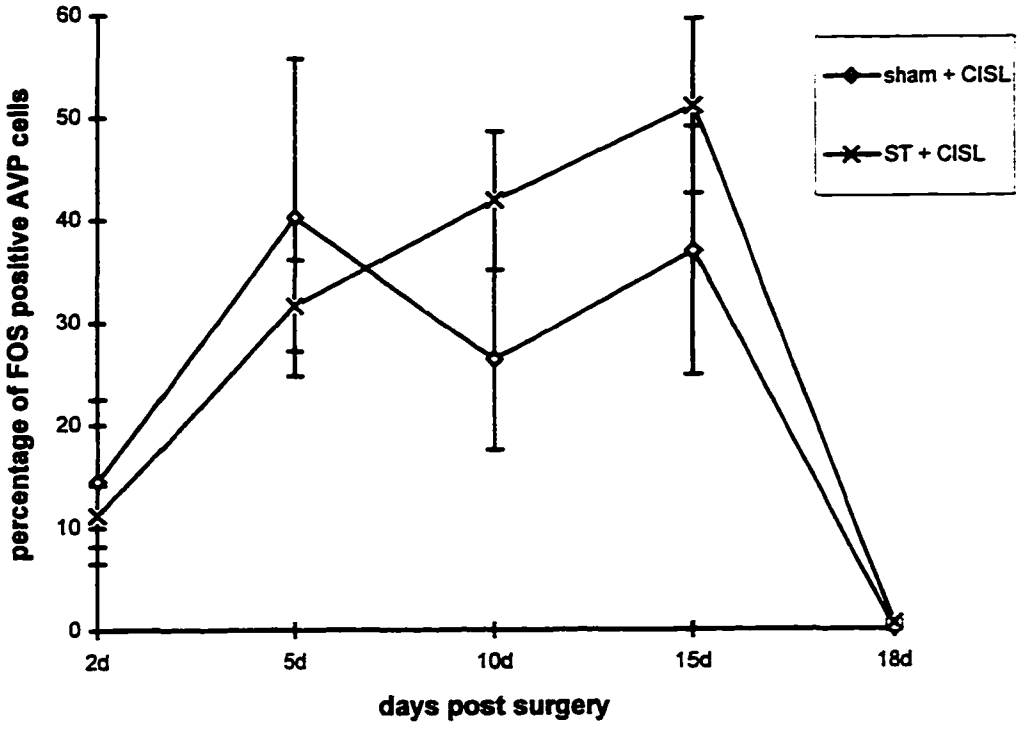

60
50
40
30
20
10
0
percentage of FOS positive AVP cells
2d
5d
10d
15d
18d
days post surgery
sham + CISL
ST + CISL

Fig. 12. This graph depicts the percentage of Fos-positive oxytocinergic cells within the supraoptic nuclei at various time periods in sham-operated and stalk-transected animals on CISL. Notice that there is no significant difference in the percentage of Fos-positive cells between ST + CISL and sham + CISL at various time periods, except at 5 dps. Values represent mean ± S.E.M.

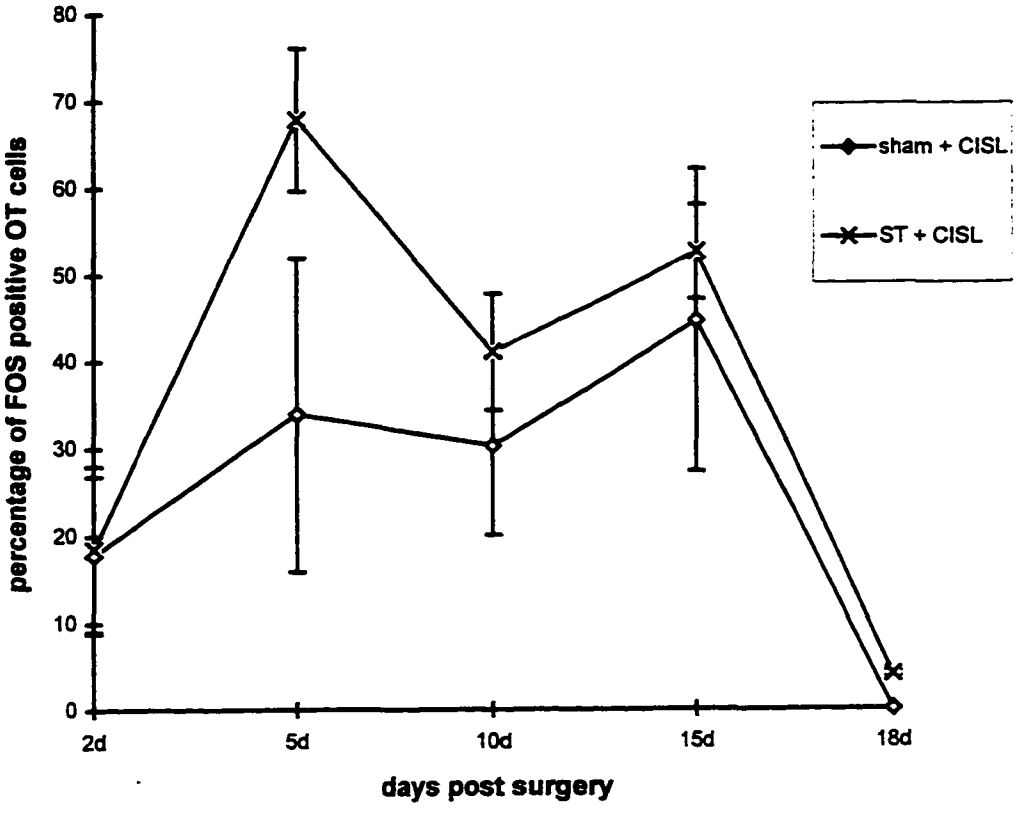

80
70
60
50
40
30
20
10
0
percentage of FOS positive OT cells
2d
5d
10d
15d
18d
days post surgery
sham + CISL
ST + CISL

Fig. 13. Fine structural morphology of neurons from the SON of A: sham + water, 10 dps; B: intact + CISL, 14 days; C: sham + CISL, 10 dps; D: sham + CISL, 18 dps. Notice the changes in the appearance of the rER, the number of immature and mature NGVs, and lysosomes, and the perinuclear area between groups. X 7500.

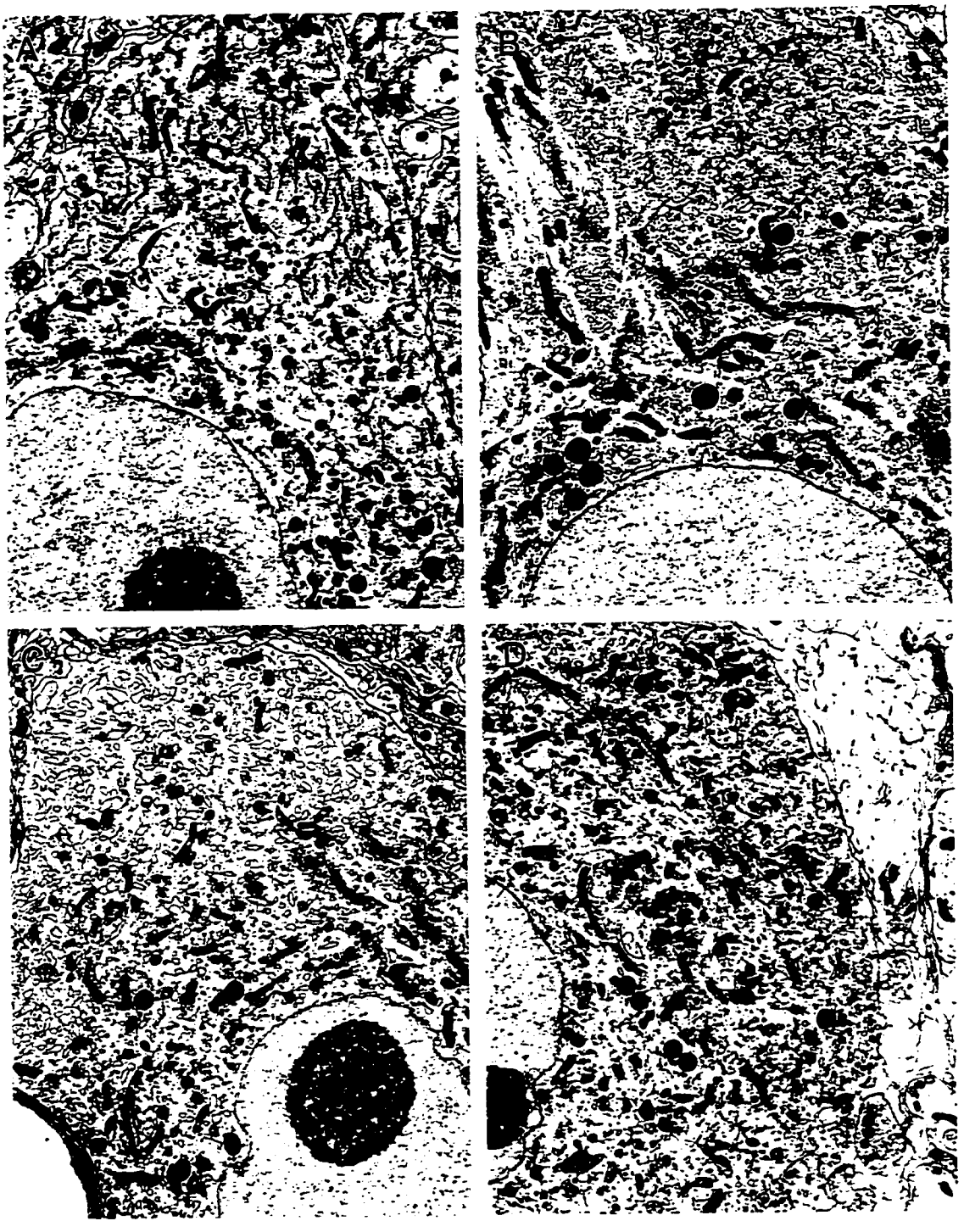

A
B
C
D

Fig. 14. Fine structural characteristics of neurosecretory neurons from the SON of A: ST + water, 10 dps; B: ST + CISL, 10dps; C: ST + water, 18 dps; D: ST + CISL, 18dps. In A, the extent of the perinuclear area occupied by the Golgi complex as well as the numerous immature NGVs are particularly striking. In B, notice the appearance of the rER typical neurons of animals on CISL (compare with Figs. 1 B and 1 C). Cells depicted in C and D, have recovered from the stalk transection (C) and additional CISL (D); notice morphologic signs of vigorous secretory activity in the cell in D. X 7500 (A, C, D); X 9000 (B).

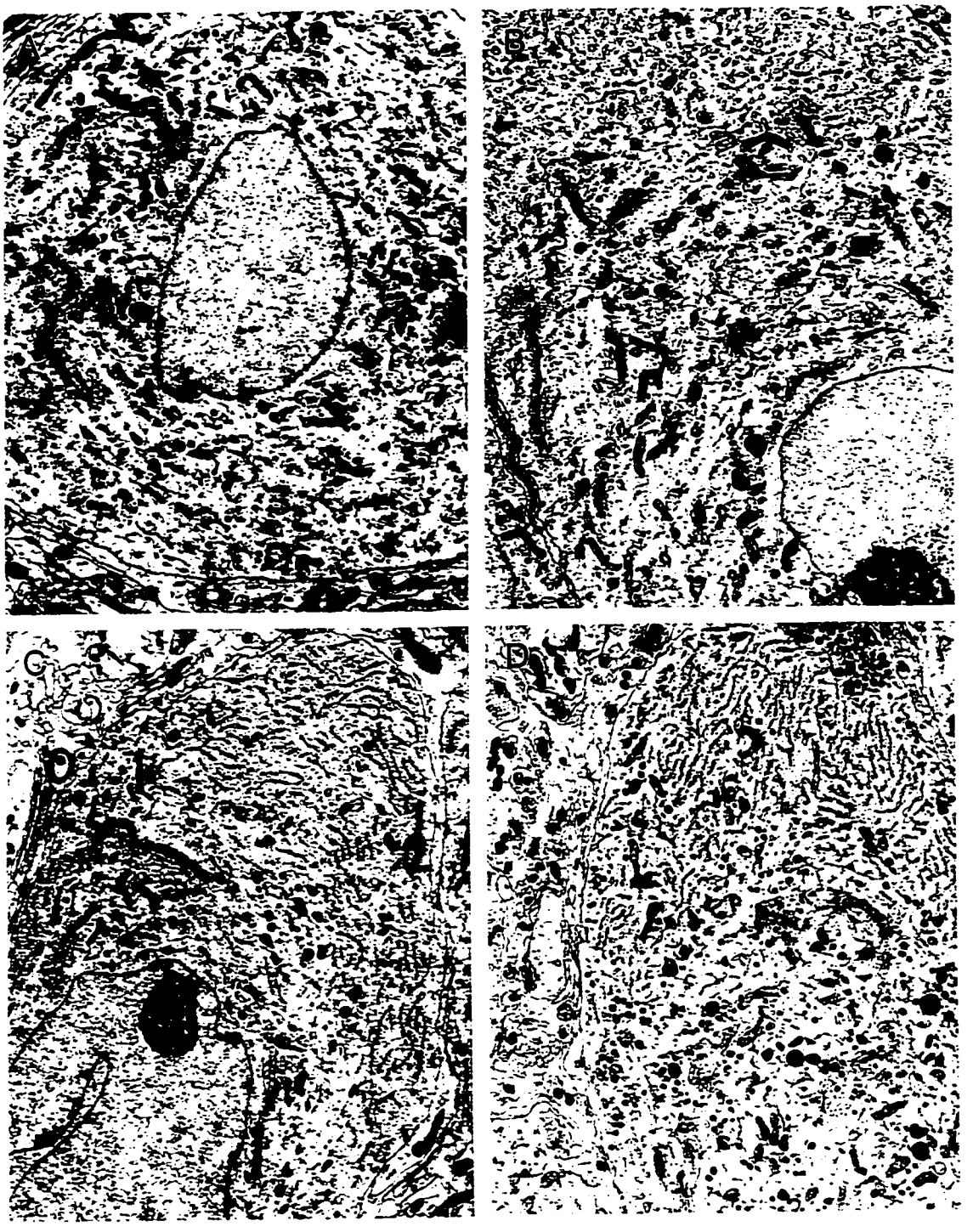

A
B
C
D

Fig. 15. Sections of the median eminence, immunostained for AVP, from A: sham + water, 10 dps; B: sham + CISL, 15 dps; C: ST + water, 5 dps; D: ST + CISL, 5 dps; E: ST + water, 18 dps; F: ST + CISL, 18 dps. AVP-positive axons are present in the external zone and subependymal layer in B. There are more axons in the external zone and subependymal layer in the ST + CISL groups (D, F) than in the ST + water groups (C, E) at respective time periods. X 90.

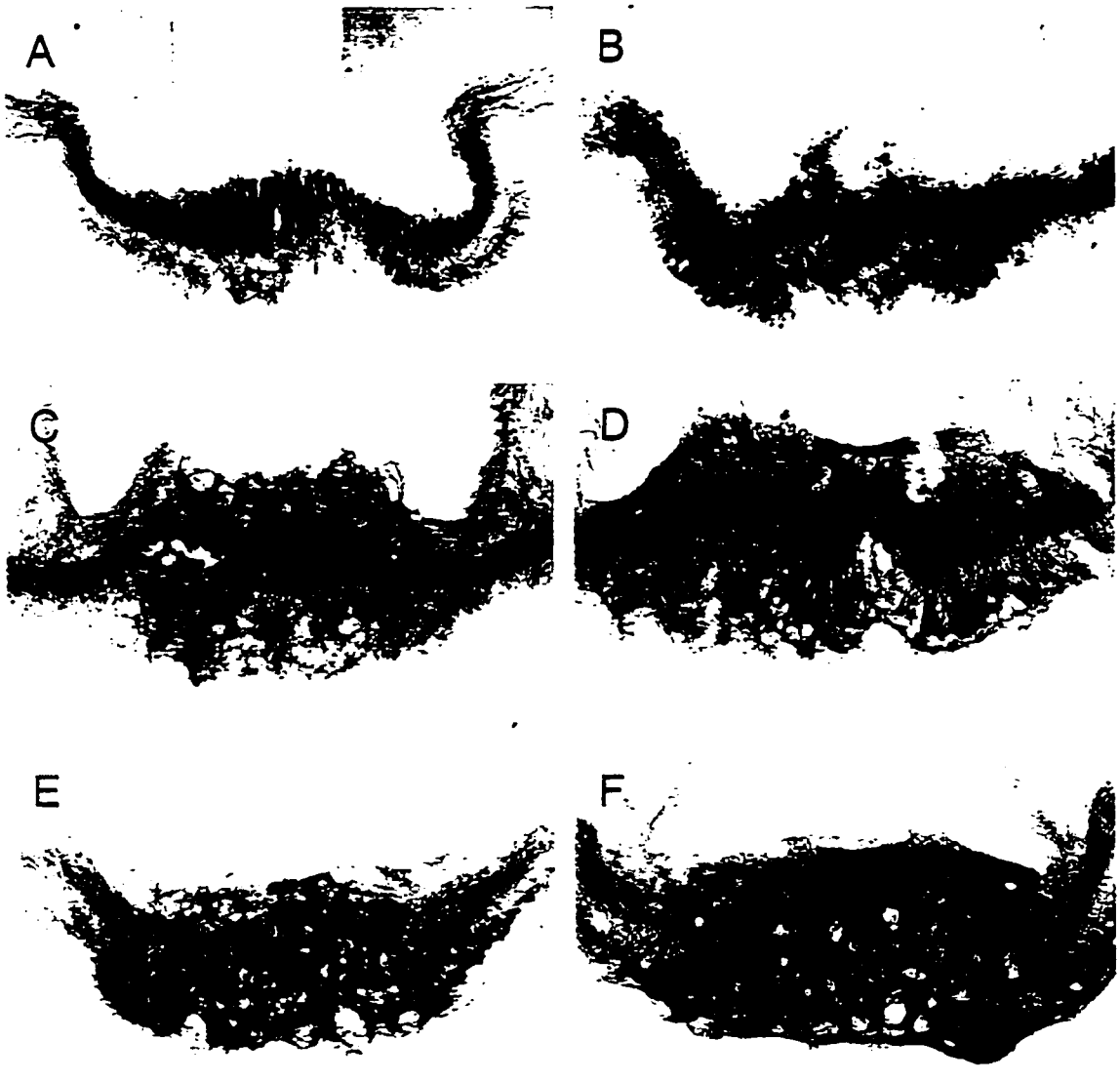

A
B
C
D
E
F

Fig. 16. Sections of the median eminence, immunostained for OT, from A: sham + water, 10 dps; B: ST + water, 18 dps; C: ST + CISL, 18 dps. There are more OT- positive axons in the external zone and subependymal layer in the ST + CISL (C) than in that ST + water group (B). X 90.

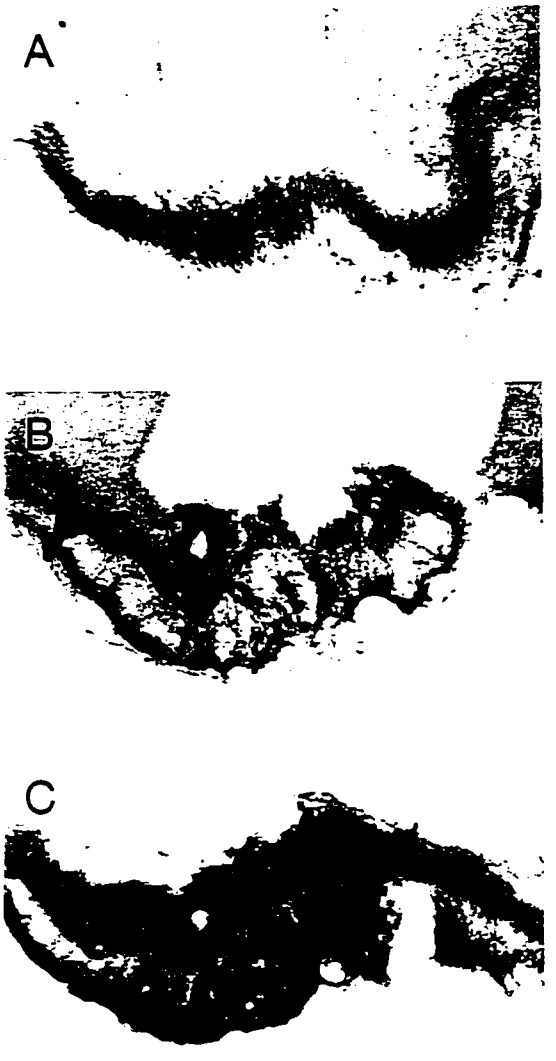

A
B
C

Fig. 17. Sections of median eminence, immunostained for βIII-tubulin. A: sham + water, 10 dps; B: sham + CISL, 10 dps; C: sham + CISL, 15 dps; D: sham + CISL, 18 dps. Notice positive axons in the subependymal layer at 10 dps (B) and 15 dps (C), and also the increase of staining intensity in the fiber layer at 15 dps (C) and the decline at 18 dps (D). X 160.

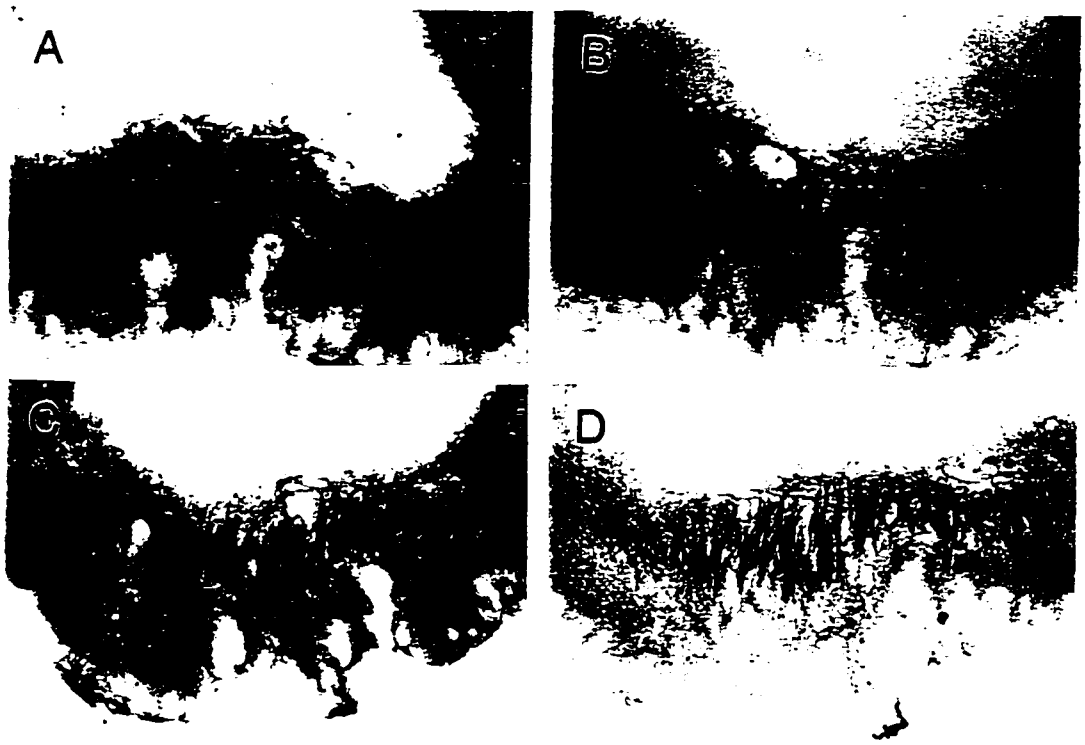

A
B
C
D

Fig. 18. Sections of the median eminence, immunostained for βIII-tubulin, from ST + water groups (A, C, E, G) and ST + CISL groups (B, D, F, H) at 5 dps (A, B), 10 dps (C, D), 15 dps (E, F) and 18 dps (G, H). In the external zone in the ST + CISL group positive axons are more numerous than in the ST + water group at respective time periods. Positive axons are particularly dense aroumd the blood vessels. X 160.

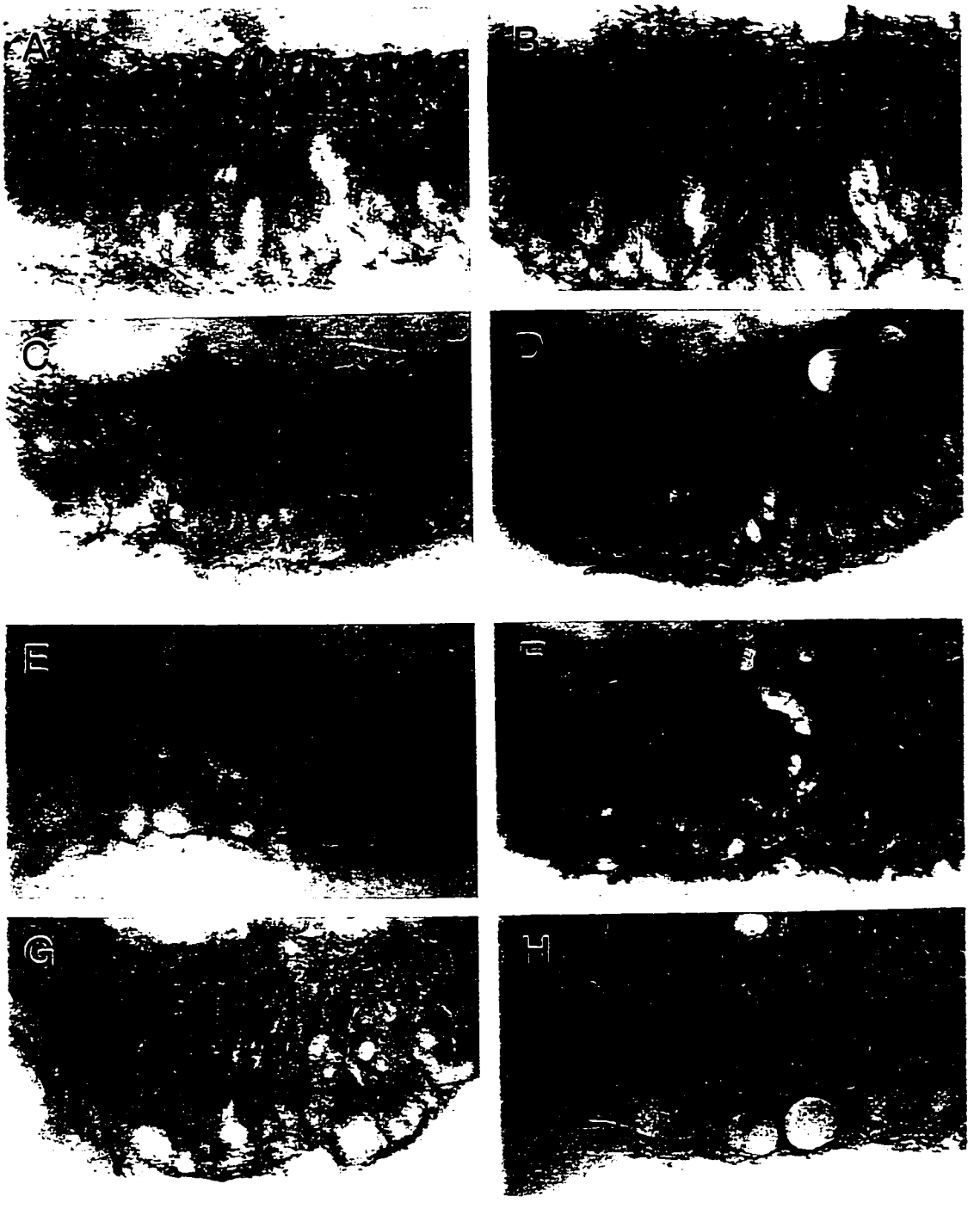

A
B
C
D
E
F
G
H

Fig. 19. All micrographs are from the external zone of the median eminence. A: Axon terminals containing small dense core vesicles and microvesicles abut a pericapillary space (sham + water, 10 dps); B: No changes are observed at 2 dps in the axon terminals of the ST + CISL group; C: A magnocellular neurosecretory axon courses along a tanycyte process in the deep layer of the external zone. X 8000 (A); X 10000 (B); X 21000 (C).

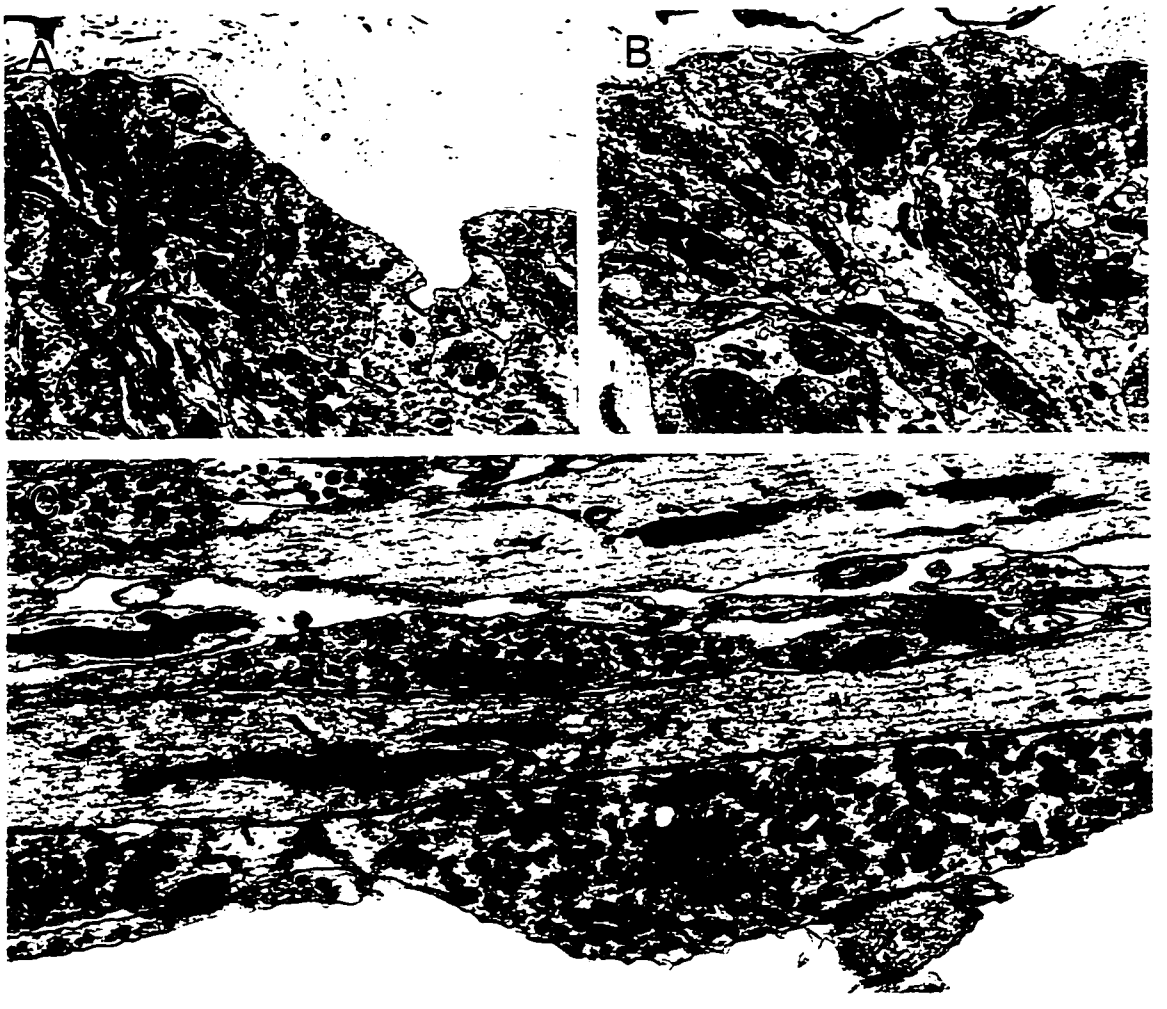

A
B

Fig. 20. All micrographs are from the external zone of the median eminence. A: Numerous growth cones and magnocellular axon profiles had invaded pericapillary spaces (ST + CISL, 5 dps); B: A magnocellular neurosecretory axon is present in a pericapillary space, others are terminating at it or close to it (ST + CISL, 5 dps); C: Large magnocellular neurosecretory axon profiles are accompanied by pituicytes or pituicyte processes in the vicinity of pericapillary spaces (ST + CISL, 18 dps); D: A pericapillary space is bordered by magnocellular neurosecretory axon terminals, whereas those containing small dense core vesicles are no longer in contact with it (ST + CISL, 18 dps). X 9000 (A); X 12000 (B); X 6000 (C); X 15000 (D).

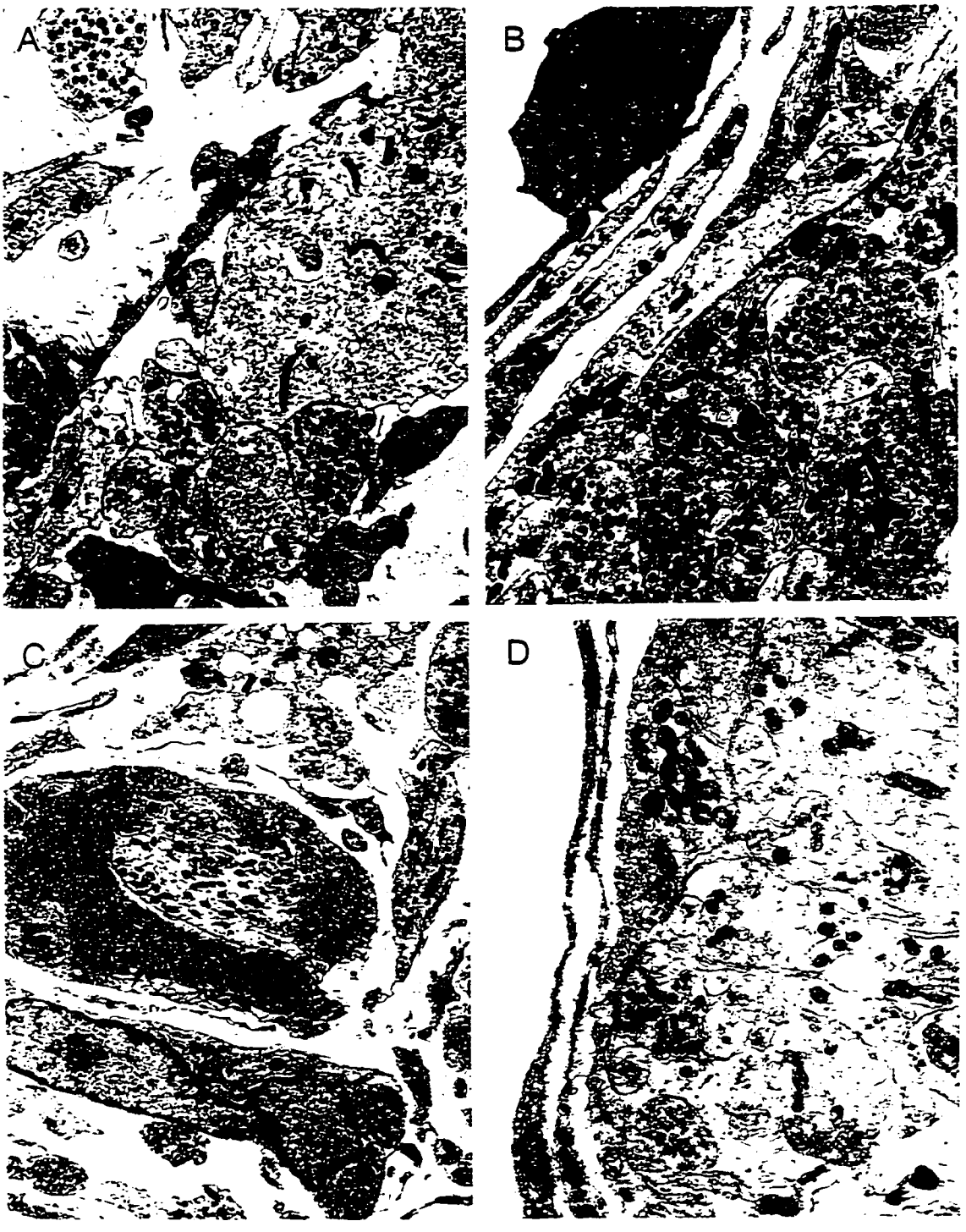

A
B
C
D

Fig. 21. Longitudinal sections of the hypophysial stalk at the lesion area, immunostained for AVP. A: sham + water, 10 dps; B: ST + CISL, 5 dps; C: ST + water, 10 dps; D: ST + CISL, 10 dps; E: ST + water, 18 dps; F: ST + CISL, 18 dps. Notice AVP-positive axons pass through the center of the hypophysial stalk. The number of positive axons increases at longer survival times, but it is higher in the ST + CISL groups (D, F) than in the ST + water groups (C, E) at respective time periods. X 90.

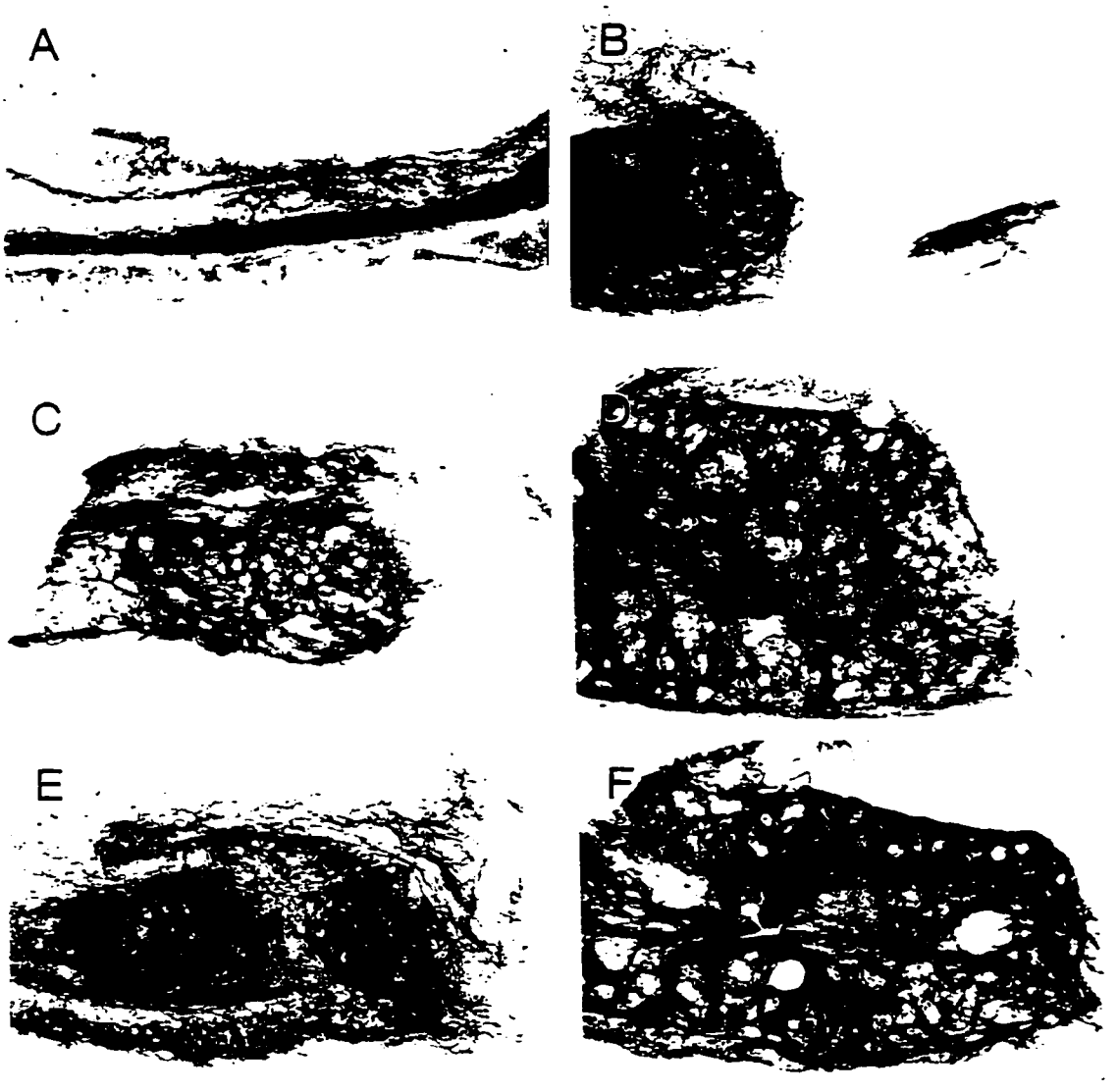

A
B
C
D
E
F

Fig. 22. Longitudinal sections of the hypophysial stalk at the lesion area, immunostained for OT. A: sham + water, 10 dps; B: ST + CISL, 5 dps; C: ST + water, 10 dps; D: ST + CISL, 10 dps; E: ST + water, 18 dps; F: ST + CISL, 18 dps. Most OT-positive axons pass through the center of the hypophysial stalk and only single axons extend to the the stalk periphery (A); the number of regenerating axons increases at longer survival times, and more axons are present in the ST + CISL groups (D, F) than in the ST + water groups (C, E) at 10 dps and 18 dps. X 90.

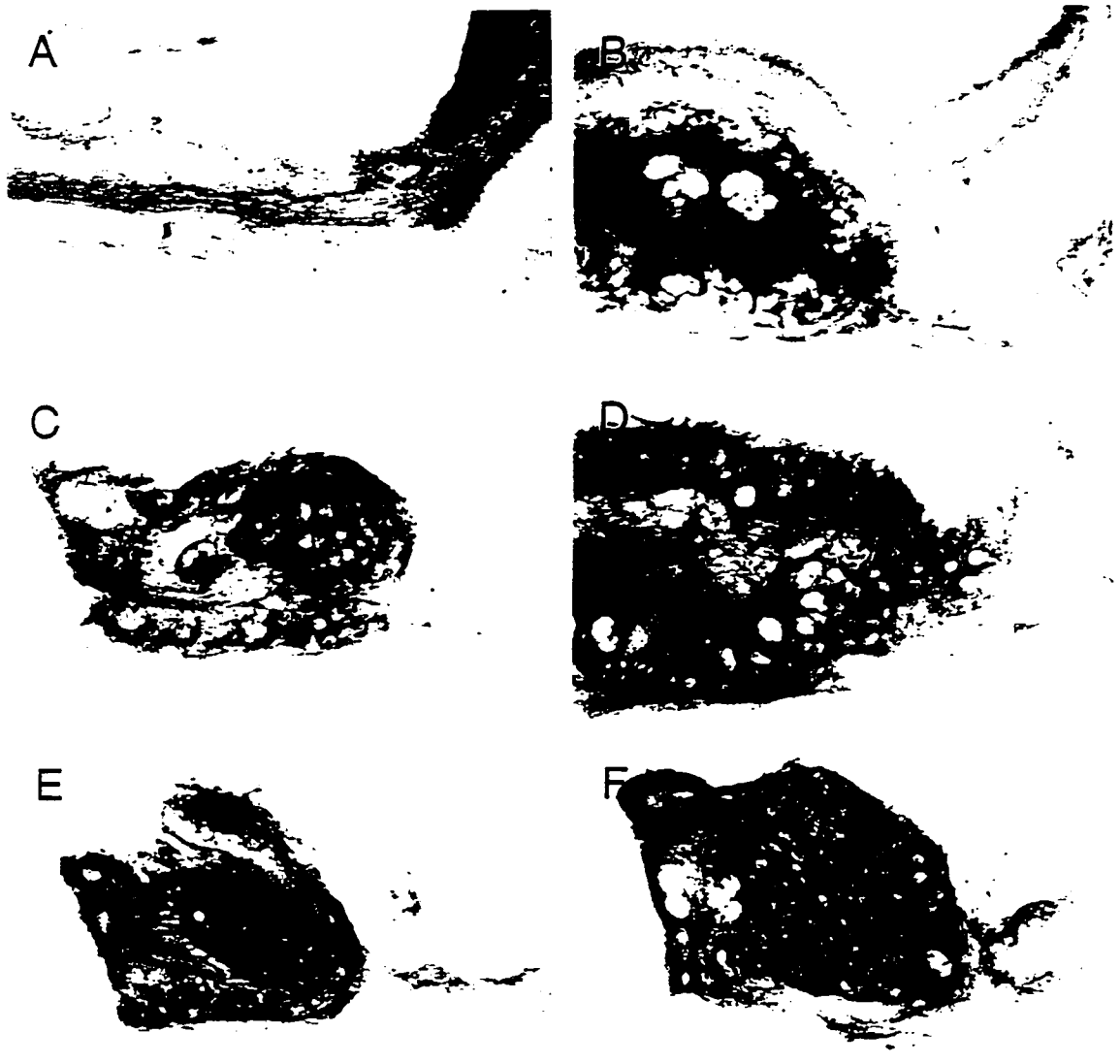

A
B
C
D
E
F

Fig. 23. Longitudinal sections of the hypophysial stalk at the lesion area, immunostained for βIII-tubulin. A: sham + water, 10 dps; B: ST + CISL, 2 dps. Notice βIII-tubulin-positive axons in the hypothalamo-neurohypophysial tract pass through the center of the stalk (A), and regenerating βIII-tubulin-positive axons have grown out near the lesion site at 2 dps. X 90.

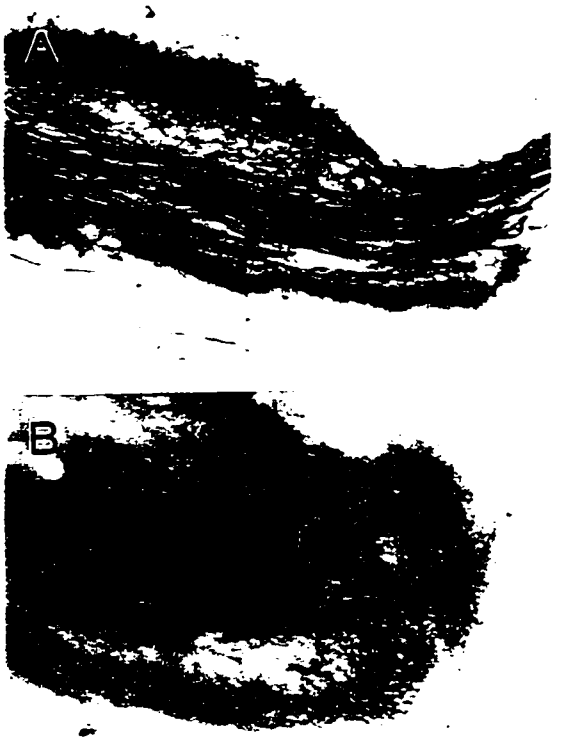

A
B

Fig. 24. Longitudinal sections of the hypophysial stalk at the lesion area, immunostained for βIII-tubulin, from ST + water group (A, C, E, G), and ST + CISL group (B, D, F, H), at 5 dps (A, B), 10 dps (C, D), 15 dps (E, F) and 18 dps (G, H). Note βIII-tubulin positive axons surround the blood vessels; the number of positive axons increases at longer survival times, and there are more axons in the ST + CISL groups than in the ST + water groups at respective time periods. X 160.

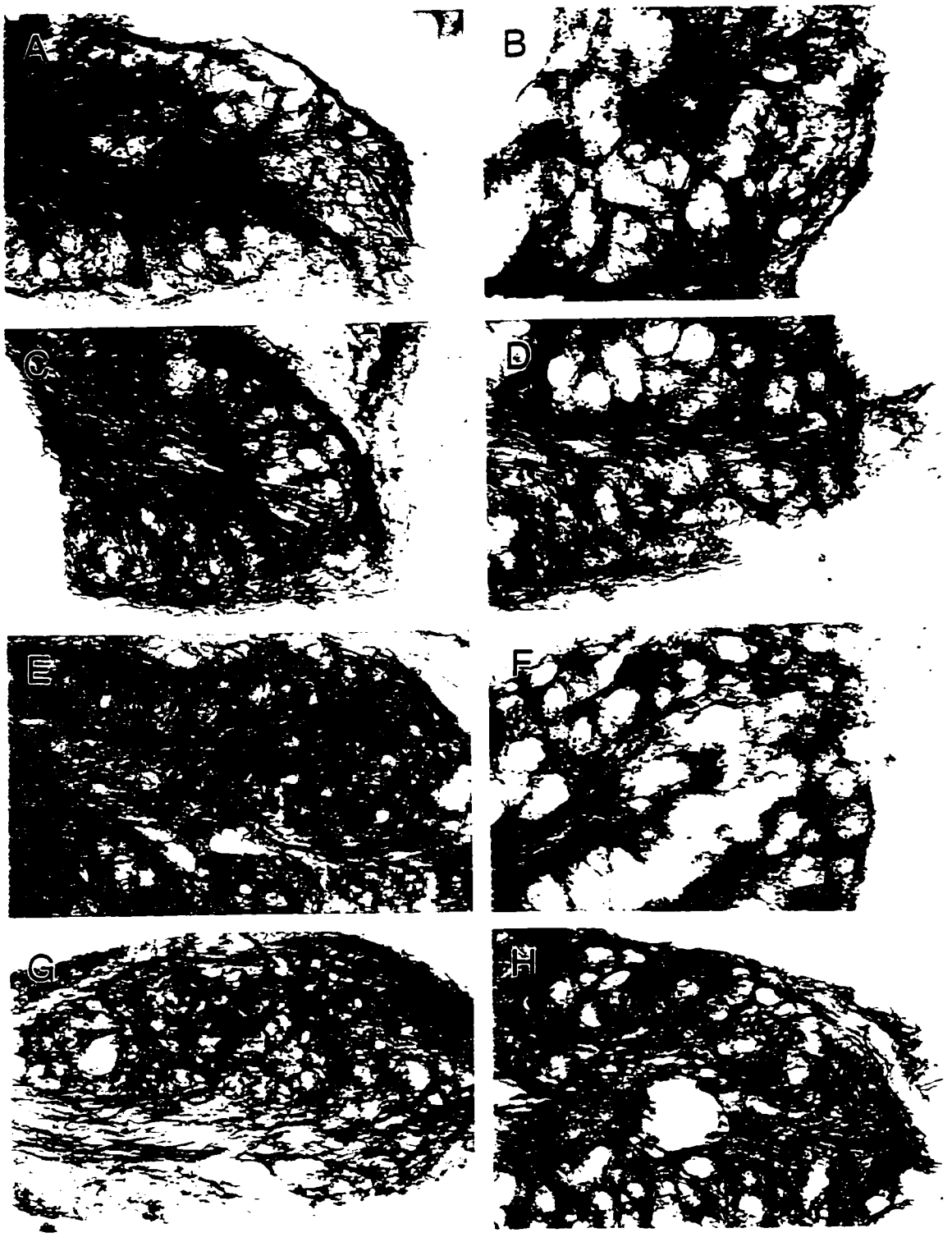

A
B
C
D
E
F
G
H

Fig. 25. Electron micrographs from the lesion area in the hypophysial stalk. A: Numerous NGVs have piled up in magnocellular axon profiles in the internal zone immediately proximal to the lesion (ST + water, 2 dps); B: Magnocellular axon terminals are abutting a perivascular space at the lesion site; one profile is greatly enlarged through accumulated NGVs; a few growth cones and profiles of degenerating magnocellular axons are also present (ST + water, 2 dps); C: In the ST + CISL group (2 dps) long stretches of axons often have growth cone characteristics; D: Growth cones and accompanying pituicyte processes have invaded a pericapillary space in the external zone of the lesion area (ST + CISL, 2 dps). X 12000 (A); X 7500 (B,C); X 15000 (D).

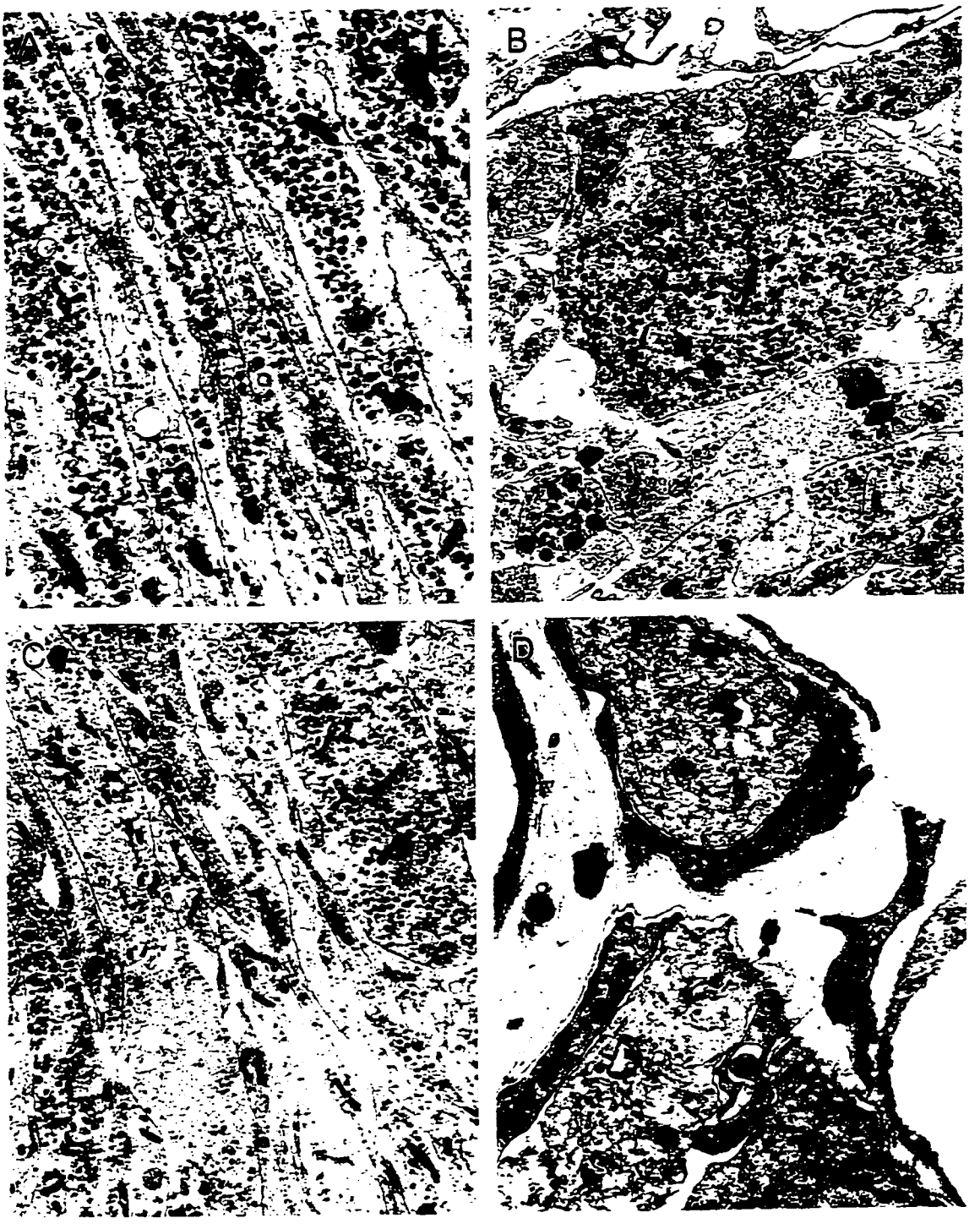
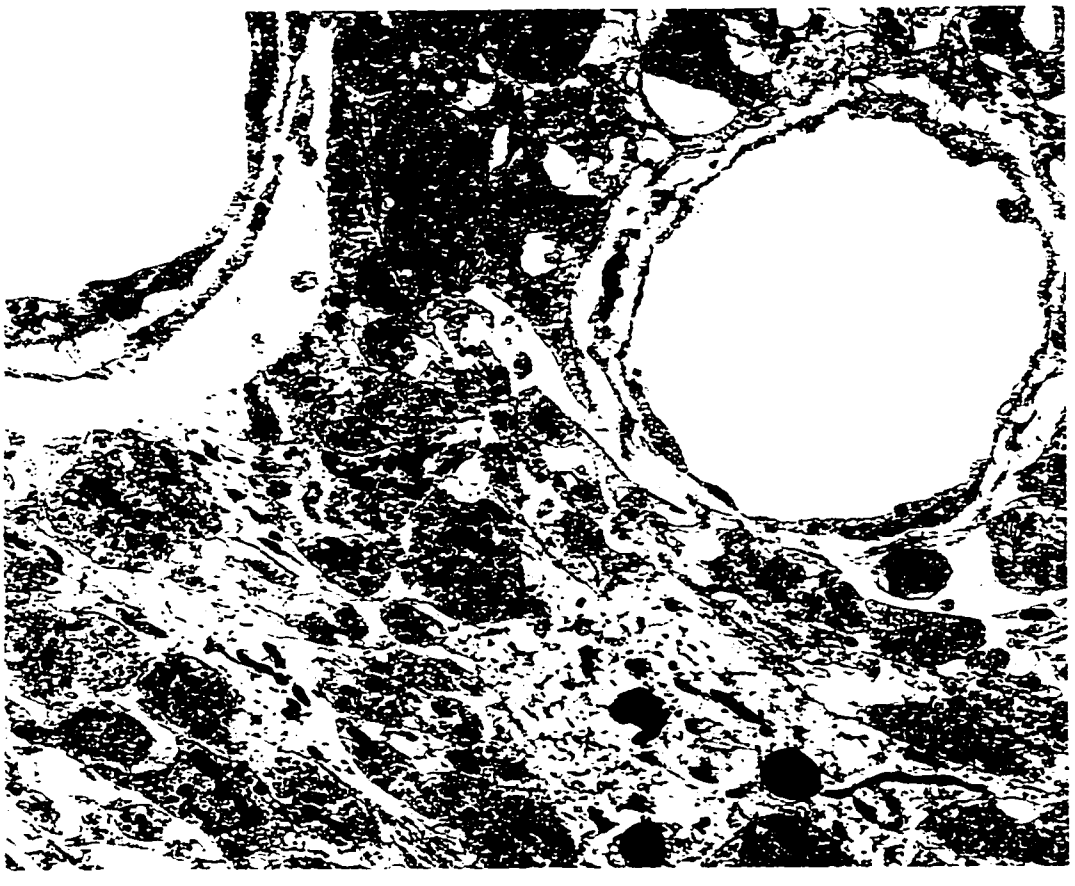

A
B
C
D

Fig. 26. Electron micrographs from the lesion area in the hypophysial stalk. At 18 dps, in the ST + CISL group, most of the tissue in the vicinity of the lesion has virtually all the fine structural characteristics of the normal neural lobe. X 8400.



GENERAL CONCLUSIONS

1. Summary

In the first paper, the working hypothesis that stimulation of vasopressin synthesis through oral administration of 2 % NaCl would increase the rate of postaxotomy neurosecretory neuronal survival and stimulate axonal sprouting was tested. Hypophysial stalk-transected (ST) and sham-operated animals either had ad lib access to drinking water or were subjected to a chronic intermittent salt loading regimen (CISL) for 14 days beginning 1 day post surgery (dps), and sacrificed at 15 and 36 dps. Three days after the termination of CISL, i.e., at 18, dps water consumption in the ST + CISL group decreased to the same level as that of sham-operated animals, while that in the ST + water group still remained at a significantly higher level. The number of surviving vasopressinergic neurons in the supraoptic nuclei (SON) in the ST + CISL group was significantly higher than that in the ST + water group. CISL also induced sprouting of vasopressinergic axons into the external zone of the median eminence (ME) and formation of subependymal perivascular plexus.

Experiments reported in the second paper were designed to test the hypothesis that CISL through stimulation of microtubular protein synthesis would accelerate neurosecretory axonal regeneration and re-establishment of perivascular contacts, permitting serum-derived and glia-derived factors to be retrogradely transported to the perikarya earlier and in a greater quantity, and consequently more neurons would be rescued to accomplish the complete recovery from polydipsia.

Stalk-transected and sham-operated animals either drank water ad lib or were subjected to CISL for various time periods and sacrificed at 2, 5, 10 and 15 dps, and at 18 dps, i.e., 3 days after cessation of CISL. We confirmed the previous observation that,at 18 dps, the water consumption in the ST + CISL group was not significantly different from that in the sham-operated group. From 10 dps on, the number of surviving vasopressinergic neurons in the SON in the ST + CISL group was significantly higher than that in the ST + water group. There was also a significant difference in the number of surviving oxytocinergic neurons between ST + CISL and ST + water group at 10 and 18 dps, but not at 15 dps, but the difference between these two groups was less pronounced than in vasopressinergic neurons.

CISL, but not stalk transection, induced expression of c-fos in both vasopressinergic and oxytocinergic neurons beginning at 2 dps and persisting throughout the period of salt loading. Stalk transection on the other hand, induced the expression of c-jun in both types of neurosecretory neurons. The earlier cessation of Jun expression in the ST + CISL group than in the ST + water group is interpreted as an indication of establishment of functional neurovascular contacts by regenerating neurosecretory axons. Both CISL and stalk transection induced increased expression of βIII-tubulin in the neurosecretory neurons. However, stalk transection in combination with CISL caused a shorter and earlier (2 dps) increase in tubulin immunoreactivity in neurosecretory neurons in the ST + CISL than in the ST + water group. From 5 dps on, CISL induced a noticeably higher number of vasopressin- and βIII-tubulin-positive axons to sprout into the external zone of the ME and lesion area, where they made perivascular contacts with the proliferating capillaries. Only at 18 dps, was

there a slightly higher number of oxytocinergic axons present in the ST + CISL than in the ST + water group.

In conclusion, the following explanation is proposed: ST induces c-jun expression, CISL induces additional expression of c-fos. Fos then enhances the binding of Jun to one of its presumptive target genes, the gene for βIII-tubulin, and promotes its expression. βIII-tubulin is present earlier and in larger quantities in the ST + CISL group and causes faster postaxotomy collateral sprouting and establishment of perivascular contacts, target derived factors are transported retrogradely to the neuronal perikarya earlier and in a greater quantities, and thus induces the postaxotomy survival of a significantly greater number of vasopressinergic neurons capable to achieve complete recovery from post-transectional polydipsia.

2. Prospective studies

Stalk-transected animals with free access to drinking water never recover from posttransectional diabetes insipidus, and continue to drink copious amounts of water. However, stalk-transected animals subjected to chronic intermittent salt-loading do achieve complete recovery from post-transectional polydipsia. This observation has potential for the design of treatment strategies for human patients to improve recovery from post-traumatic or post-surgical DI. Such patients are usually treated with a vasopressin analogue, DDAVP, to provide symptomatic relief from diabetes insipidus [56]. However, this therapy may in fact be detrimental to the survival of AVP- and OT-cells since administration of AVP or its

analogue to stalk-injured animals inhibits the survival and regeneration of the injured neurosecretory cells [56,57,92,93].

We found that after 10 dps neurosecretory cell loss in the SON is insignificant, and that at that time many regenerating axons have established perivascular contacts. Therefore, it seems plausible that even shorter periods of salt-loading could accomplish the same goal. Future investigations will be designed to find out the minimum time period of CISL capable to induce full recovery from polydipsia.

Since it is not pleasant for a patient to continually drink hypertonic saline, experiments will address the question whether injured neurosecretory neurons can be stimulated through intravenous injection of saline. Strategies, then, need to be devised for optimal treatment (e.g., optimal dosage, number of injections, time between injections).

We have shown that CISL enhances the survival of injured neurosecretory neurons and accelerates the regrowth of their axons; we speculate that this is mediated through the increase in βIII-tubulin, a component of microtubules. Microtubules are involved in anterograde axoplasmic transport of NGVs, and the retrograde transport of target-derived factors to the neuronal cell body. Since target-derived factors are presumably a key element in the regenerative response, blockade of the retrograde transport should in effect prevent regeneration. Low doses of intraventricular colchicine have been shown to reversibly block axoplasmic transport in neurosecretory neurons through inhibition of microtubule assembly, and do not affect the general overall condition of the animal [55]. When administered immediately after stalk transection, they are expected to prevent or at least considerably

diminish axon regeneration and survival of neurosecretory neurons in euhydrated and salt-loaded animals.

It would be of interest to obtain information about the expression of other tubulin genes after interruption of the hypophysial stalk. Studies of peripheral neurons have shown that not all tubulin gene isotypes are influenced to the same extent by axotomy [98,157]. The expression of α_{I}-, β_{II}- and β_{III}-tubulin is greatly increased after axotomy [98,112,157,170,171] while β_{I}-, β_{IV}- and α26-tubulin are much less affected [98,157]. Recent studies have shown that only two of five tubulin isotypes (II and III) are preferentially localized in newly growing neurites of PC-12 cells [113]. Effective axonal regrowth may depend on the ability of the cells expressing appropriate tubulin genes needed for insertion into axons. Future studies of neurosecretory neurons could be directed at determining the expression of other tubulin genes in stalk-transected euhydrated and salt-loaded animals.

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ACKNOWLEDGMENTS

I would like to express my sincere appreciation to my major professor, Dr. H. Dieter Dellmann, for his valuable guidance and help throughout my doctoral program at Iowa State University. My sincere gratitude goes to his wife, Renate Dellmann, for everything she did to make my family stay at Ames less stressful.

I also grateful to my committee members, Drs. Walter H. Hsu, Carol D. Jacobson, Don Sakagushi and Etsuro Uemura, for their help and support during the completion of my studies.

Thanks also go to Dr. L. Wollins and Dr. M. Shelley for the statistical analysis of the data. Special thanks also go to Dr. A. J. Silverman, H. Gainer and A. Frankfurter for the donation of antibodies.

I also would like to acknowledge and thank the Ministry of Education, Taiwan, R.O.C. for funds provided for this research project. My thanks also go to Dr. Mary Helen Greer and Dr. Carol Jacobson for their financial support.

I also want to thank my colleagues at the Department of Veterinary Medicine, NCHU, Taiwan, R.O.C. for their encouragement, help and understanding.

My sincere gratitude is also extended to Randy DenAdel and Cathy Martens for their excellent help and kind friendship.

I wish to express my gratitude to my parents, mother-in law, sisters and brothers for their love, encouragement and help.

I especially thank my wife, Ching-Shiang, and two daughters, Hong-Tzu (Sandy) and I-Chieh (Betty) for their infinite love, full support and understanding.