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Regeneration of neurosecretory axons into intrahypothalamic grafts of neural lobe, sciatic nerve, and optic nerve: a neurophysin-immunohistochemical, HRP-histochemical, and fine structural study

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Iowa State University, 1992

Regeneration of neurosecretory axons into intrahypothalamic

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by

Mohammed Ouassat

A Dissertation Submitted to the

Graduate Faculty in Partial Fulfillment of the

Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Department: Veterinary Anatomy
Major: Veterinary Anatomy

Approved:

In Charge of Major Work

For the Major Department

For the Graduate College

Iowa State University
Ames, Iowa

1992

TABLE OF CONTENTS

v

GLOSSARY FOR ABBREVIATIONS

AVP: Arginine-vasopressin

BBB: Blood-brain barrier

BNB: Blood-nerve barrier

CNS: Central nervous system

DI: Diabetes insipidus

FGF: Fibroblast growth factor

HNT: Hypothalamo-neurohypophysial tract

HNS: Hypothalamo-neurohypophysial system

HRP: Horseradish peroxidase

NCAM: Neural adhesion molecule

NP: Neurophysin

NGF: Nerve growth factor

NGV: Neurosecretory granulated vesicle

OX :   Oxytocin

PNS: Peripheral nervous system

PVN: Paraventricular nucleus

RBC: Red blood cell

SON: Supraoptic nucleus

INTRODUCTION

Analysis of the regenerative events following axotomy has
revealed important and fundamental differences between the
peripheral (PNS) and central (CNS) nervous systems. Following
transection of PNS axons, the proximal stumps regenerate into,
and grow through, the distal segment of the severed nerve, and
may even reinnervate the original target tissue or organ,
thereby restoring partial or total function (Guth, 1956;
Sunderland, 1968). Within the CNS, however, the regenerative
response of transected axons is, with few exceptions,
abortive, and restoration of the original synaptic contacts
does not occur (Cajal, 1928; Puchala and Windle, 1977;
Björklund and Stenevi, 1979).

The hypothalamic magnocellular neurosecretory system is
one such exception. Transection of neurosecretory axons in
the median eminence, the hypophysial stalk or the neural lobe
is followed by spontaneous regeneration, leading to the
formation of a miniature neural lobe-like structure and
functional recovery from post-transectional diabetes insipidus
(Dellmann, 1973; Raisman, 1973; Polenov et al., 1974). These
transection sites are characterized by (i) their location
outside the blood-brain barrier (BBB), (ii) the presence of
pituicytes and (iii) wide pericapillary connective tissue

spaces. If the same neurons are transected more proximally within the hypothalamus, there is only limited spontaneous axonal regeneration without any noticeable functional recovery (Danilova and Polenov, 1977; Nagy et al., 1983). Frequently, however, these neurosecretory axons will regenerate into adjacent perivascular spaces (Antunes et al., 1979; Dellmann et al., 1987b). When the ventral hypothalamic surface is severed simultaneously with the hypothalamo-neurohypophysial tract, neurosecretory axons regenerate abundantly into the leptomeninges (Dellmann et al., 1988b).

Within the intact hypothalamus, the BBB is present, astrocytes are the predominant glial cell type, and there are no pericapillary spaces. In these intrahypothalamic areas and in the leptomeninges where neurosecretory axon regeneration is reported, the microenvironment is characterized by wide perivascular connective tissue spaces and by the presence of cerebro-spinal fluid (CSF) in the Virchow-Robin and the arachnoid spaces. Thus, blood-derived substances are present where regeneration of neurosecretory axons has been observed. Additionally, in all instances where this regeneration has been reported, there is a close association between regenerating neurosecretory axons and glial cells (Dellmann et al., 1987a, 1988b, 1989). These observations have provided the basis for the working hypothesis that regeneration and restoration of function require a specific vascular

their ability to support regeneration of transected
neurosecretory axons; (4) to monitor recovery from diabetes
insipidus (restoration of arginin-vasopressin, AVP, function)
by measuring pre- and post-operative water intake.

LITERATURE REVIEW

The Hypothalamo-Neurohypophysial System (HNS)

Microscopic anatomy of the HNS in rats

The HNS consists of magnocellular hypothalamic neurons whose axons project to the perivascular spaces of fenestrated capillaries of the neural lobe. Perikarya of these neurons are clustered in two large bilateral cell groups, the paraventricular nuclei (PVN) and the supraoptic nuclei (SON), and in smaller accessory cell groups in the rostral hypothalamus.

Magnocellular hypothalamic neurons. The magnocellular neurons of the hypothalamus can be identified by their large size, extensive Nissl substance, Golgi complexes, and their affinity for Gomori and related stains (Rhodes et al., 1981). Using immunohistochemical techniques, they can be stained with antisera directed against either their neuropeptides or their associated neurophysins (Sofroniew et al., 1984).

The paraventricular nucleus (PVN) represents what is classically described as the magnocellular PVN. It is situated in the dorso-medial hypothalamus, adjacent to the third ventricle (Swanson and Kuypers, 1980). This nucleus contains both oxytocin- and vasopressin-synthesizing neurons

(Swanson and Kuypers, 1980; Rhodes et al., 1981).

The supraoptic nucleus (SON) is situated rostrally in the hypothalamus, capping the optic tract as it leaves the chiasm and extending caudally along the base of the brain, immediately dorsal and lateral to the optic tract (Ambach and Palkovits, 1979). The SON is subdivided into a principal locus which contains 80% of the neurons, and a retrochiasmatic locus (Ambach and Palkovits, 1979; Rhodes et al., 1981). Oxytocin-producing neurons are located in the rostro-dorsal portion of the principal part. The retrochiasmatic SON is composed primarily of vasopressin-containing neurons (Rhodes et al., 1981; Swanson et al., 1987).

The hypothalamo-neurohypophysial tract (HNT). The HNT is composed of axons originating from the PVN and SON. About one third of these predominantly nonmyelinated axons arise in the PVN; the remaining two-thirds originate from the SON and accessory magnocellular nuclei (Fisher et al., 1979; Rhodes et al., 1981). The axons of the PVN leave the nucleus laterally, curve around the fornix, and, after a short run in a caudoventral direction, join the supraoptic axons at the caudolateral edge of the optic chiasm. This "gate", where most HNT fibers enter the medio-basal hypothalamus, is termed the lateral retrochiasmatic area (Palkovits, 1984). After entering the median eminence, the HNT continues through the

infundibular stalk and branches into the neural lobe. SON
axons terminate predominantly in the central portion of the
neural lobe, while PVN terminals occupy a more peripheral
location (Alonso and Assenmacher, 1981).

The neurohypophysis.   The neurohypophysis consists of
the median eminence, the infundibular stalk and the neural
lobe.   It contains an intrinsic population of pituicytes and
microglia, magnocellular neurosecretory axons, axon terminals
and blood vessels including fenestrated capillaries with their
adventitial structures.

Pituicytes.   Pituicytes are the predominant
non-neuronal cells of the neural lobe.   They lie in close
association with the neurosecretory endings and make up 25-30%
of the volume of the neural lobe (Nordmann, 1977; Pow et al.,
1989).   These cells resemble astrocytes in neuroectodermal
origin, configuration, antigenic properties (mainly GFAP) and
the presence of gap junctions (Wittkowski, 1986; Pow et al.,
1989).

Pituicytes are characterized by pale ovoid nuclei, a
peripheral rim of condensed chromatin and occasional nucleoli
(Takei et al., 1980).   Smooth endoplasmic reticulum with
tubules, vesicles and cisterns are distributed throughout the
perikarya, but rough endoplasmic reticulum is poorly
developed.   Mitochondria, lysosomes, and multivesicular bodies

are also present (Wittkowski, 1986). In addition to these
organelles, rat pituicytes contain lipid droplets.

Pituicytes extend processes among neurosecretory axons
and to parenchymal basal laminae of pericapillary spaces where
they terminate together with the neurosecretory axon terminals
(Tweedle, 1987). In the rat, between 26% (Carithers et al.,
1981) and 48% (Tweedle et al., 1985; Wittkowski, 1986) of the
pericapillary contact zone is covered by pituicyte endfeet and
the remainder by neurosecretory axon terminals. Synaptoid
contacts between neurosecretory, catecholaminergic and opioid
axons and pituicytes are frequent (Wittkowski, 1986).

The functional significance of pituicytes is still
debated. Hypothesized functions include: regulation of ionic
environment (Wittkowski, 1986), regulation of hormone release
by modifying the contact of neurosecretory processes with the
hormone release site at the basal lamina (Hatton et al., 1984;
Tweedle, 1987; Hatton, 1990) and production of trophic factors
for developing (Galabov and Schiebler, 1978) and regenerating
neurosecretory neurons (Kiernan, 1971; Dellmann and Sikora,
1981; Dellmann et al., 1987a).

Microglia. Microglial cells are a second cell
type present within the neurohypophysis. The majority of
these cells occupies a pericapillary position in the resting
gland. Microglial cells have small irregularly shaped
heterochromatic nuclei and are bipolar or multipolar cells

with finely branching cytoplasmic processes (Pow et al.,

1989). They are characterized by extensive rough endoplasmic

reticulum and numerous lysosomes. In adult animals,

microglial cells endocytose parts of apparently normal

neurosecretory axons (Pow et al., 1989), thus participating in

the remodelling of the terminal arborizations of

neurosecretory axons and in the processing or degradation of

hormones and peptides they contain. Recently, Yan et al.

(1990) demonstrated that microglial cells are the only

component of the rat neural lobe that possess nerve growth

factor (NGF) receptors. This suggests an as yet unknown role

for NGF and/or NGF receptors in microglial function.

Neurosecretory axons. The neurosecretory axons

of the neural lobe contain typical axonal organelles, as well

as groups of 100-200 nm dense core vesicles referred to as

neurosecretory granulated vesicles (NGVs). Regional axonal

enlargements or dilatations, may occur along the course of

these axons or at their terminals. These dilatations have

been classified as either "swellings", which do not abut basal

laminae, or "endings", which abut the perivascular basal

laminae and contain many microvesicles (Nordman, 1977). Axon

terminals occupy about 42% of the total neural lobe (Tweedle

and Hatton, 1985). They contain NGVs and 40-60 nm

electron-lucent dispersed or clustered microvesicles (Tweedle,

1987). Microvesicle-laden terminal contacts with basal

laminae are considered markers of sites of the most active hormone release (Hatton, 1990). Recent experiments have provided evidence of exocytosis of NGVs not only from HNT axon terminals, but also from swellings as well as undilated axons (Buma and Nieuwenhuys, 1987; Morris et al., 1988). The ratio of the total number of vesicles exocytosed to the number in each compartment (swelling or ending) was three times higher for the endings than for large swellings, thus indicating a higher turnover in the endings (Morris et al., 1988).

Capillaries and pericapillary structures. The neurohypophysis, like the hypothalamic magnocellular nuclei, is highly vascularized (Sposito and Gross, 1987), but unlike the latter, its capillaries are lined by a fenestrated endothelium with fenestrae of 30-50 nm in diameter (Takey et al., 1984). The endothelial wall is surrounded by a perivascular connective tissue space bounded by luminal and abluminal basal laminae (Scott, 1968). Pericytes, histiocytes, mast cells, fibrocytes and lymphocytes lie within this wide perivascular space.

Physiology of the HNS

Magnocellular neurosecretory neurons synthesize and release the nonapeptide hormones oxytocin (OX) and arginin-vasopressin (AVP), along with their associated neurophysins (NPs). OX, AVP and NPs are released by exocytosis from axon

results in initial regrowth from the injured proximal stump,
but regenerating axons soon cease growing and degenerate
(Cajal, 1928; Puchala and Windle, 1977; Björklund and Stenevi,
1977). Even since this puzzling observation was first
published by Cajal (1928), it has haunted neuroscientists
throughout the world, and still does to this day, even though
our knowledge of the events underlying regeneration in the
PNS, and of those preventing regeneration in the CNS has
advanced dramatically, especially during the past decade.
Strategies have been devised and continuously are developed to
induce, promote and sustain functional regeneration in the
CNS. They are too numerous for detailed discussion in this
chapter. Therefore, the discussion will be confined
essentially to those points relevant to the present research
project.

Regeneration in the mammalian PNS

When a peripheral nerve is cut or crushed, both the axons
and myelin sheaths degenerate distal to the site of injury,
and their remnants are removed by macrophages and
neurolemmocytes (Richardson et al., 1983). Concurrently the
neurolemmocytes proliferate within the remaining connective
tissue sleeves, giving rise to columns of cells surrounded by
basal lamina tubes, called the bands of Büngner (Sunderland,
1968; Aguayo et al, 1981). Induction of proliferation of

neurolemmocytes during reinnervation of severed peripheral nerves may depend on the close proximity of these cells to the growing tips of regenerating axons (Pellegrino and Spencer, 1985). Myelin debris may also act as a signal for neurolemmocyte multiplication (Hall and Gregson, 1975).

Regenerating axons from the proximal segment grow towards and along the bands of Büngner, which serve as guides to the sprouting axons. Recent evidence that neurolemmocytes deprived of axonal contact in the distal stump produce NGF and express NGF receptors on their surface membranes suggests that neurotrophic factors are important during peripheral nerve regeneration (Finn et al., 1986; Korshing et al., 1986; Johnson et al., 1988). Synthesis of NGF by neurolemmocytes in vitro can be induced by the addition of macrophages to the cultures (Lindholm et al., 1987). The basal lamina produced by and surrounding neurolemmocytes apparently participates substantially in the regenerative process, since axonal sprouts regenerate along basal lamina tubes even in the absence of their parent neurolemmocytes (Ide et al., 1983; Hall, 1986; Gulati, 1988; Ide et al., 1990). Since the axons proximal to the growing sprouts are associated with neurolemmocytes, it appears that the concerted action of both neurolemmocytes and basal lamina is required for successful regeneration. The contribution of other PNS components such as endoneurium, perineurium or the integrity of the blood-

nerve barrier (BNB) in sustaining and/or promoting PNS axon regeneration is unknown.

Regeneration in the mammalian CNS

There are few exceptions to the rule that spontaneous regeneration of transected CNS axons does not occur or is a temporarily abortive event. One such exception are the neurosecretory axons the HNS, in which spontaneous post-transectional regeneration was first described by Stutinsky in 1951. Other CNS axons in which regeneration has been observed include the monoaminergic (Björklund et al., 1971, 1973; Moore et al., 1971), and cholinergic (Svendgaard et al., 1976) systems, olfactory (Barber, 1981, 1982) and retinal (McConnel and Berry, 1982a,b) neurons, and neurons in the fetal and neonate brain (Kalil and Rech, 1979, 1982). The following literature review will focus only on the regenerative events in the mammalian HNS.

Regeneration in the HNS. The magnocellular neurosecretory system is one of the first sites in the CNS in which regeneration of severed axons was demonstrated in mammals (Fisher, 1939, cited by Dellmann, 1973; Stutinsky, 1951; Billenstein and Leveque, 1955; Moll and DeWied, 1962). Subsequent studies have confirmed that, following hypophysectomy or neurohypophysectomy, the transected

neurosecretory axons regenerate and form new neurohemal contacts at both the proximal end of the transected infundibular stalk (Moll and De Wied, 1962; Raisman, 1973; Kawamoto and Kawashima, 1987) and within the external zone of the median eminence, respectively (Raisman, 1973; Kawamoto and Kawashima, 1987). Regeneration of neurosecretory axons into the neural lobe across the severed infundibular stalk (Adams et al., 1969; Kiernan, 1971) and stalk compression (Dohanics et al., 1992) have also been described. Apparently, regeneration in these locations is spontaneous, i.e., it occurs at the site of severance and in the extrahypothalamic neurohypophysial portion of the HNS. Some intrahypothalamically transected axons also regenerate spontaneously into perivascular spaces (Antunes et al, 1979; Dellmann et al., 1987b) and into leptomeninges ventral to the hypothalamus (Dellmann et al., 1988b). In addition, regeneration of transected neurosecretory axons into intrahypothalamic grafts of neural lobe (Dellmann et al., 1987a), sciatic nerve (Dellmann et al., 1986), and optic nerve tissues (Dellmann et al., 1989) have been demonstrated. Regeneration into each of these grafts is not spontaneous, since it does not occur in their absence, but is only permitted by the presence of transplanted tissues.

Spontaneous regeneration of the HNS. The description by Fisher et al. (1938, cited by Dellmann, 1973)

of a "peculiar scar" formation in the median eminence and

stalk region after hypophysial stalk transection was the first

indication of regenerative capacity of mammalian HNS.

Following hypophysectomy, neurosecretory material rapidly

accumulates in the proximal stumps of severed neurosecretory

neurons, secondary to the continuous synthesis of

neuropeptides in their perikarya. A prominent swelling of the

proximal stump appears (Stutinsky, 1951; Billenstein and

Leveque, 1955; Moll and De Wied, 1962; Raisman, 1973). This

phase is characterized by symptoms of diabetes insipidus

(Raisman, 1973; Dohanics, 1992). The regenerating

neurosecretory axons within the swollen proximal stumps of the

median eminence and the hypophysial stalk were located almost

exclusively in the periphery of these two regions, in close

proximity to a rich vascular plexus (Dellmann, 1973; Raisman,

1973). Pituicytes mitotically divide and hypertrophy

(Dellmann, 1973). Lysosomes and lipid inclusions of

pituicytes increase in number (Murakami et al., 1969) and

rough endoplasmic reticulum and Golgi complexes become more

extensive (Dellmann, 1973). Macrophage-like cells were also

observed in the process of phagocytosis (Dellmann, 1973).

Vascular changes and capillary proliferation after

hypophysectomy were noted between the 6th and 9th post-

operative days, and the establishment of a dense vascular bed

preceded the appearance of neurosecretory material (Moll and

DeWied, 1962). Dellmann (1973) found that the neurosecretory axons and axon terminals contained NGVs long before they contacted the pericapillary space. Cessation of polyuria and polydipsia following hypophysectomy, however, coincides temporally with the establishment of neurohemal contacts (Raisman, 1973). Therefore, Raisman (1973) suggested that proliferation and hypertrophy of the fenestrated capillary vasculature in the median eminence may act as a stimulus for regeneration of the severed neurosecretory axons.

In addition to this spontaneous extrahypothalamic regeneration, spontaneous intrahypothalamic regeneration of neurosecretory axons also occurs in specific areas of the hypothalamus after experimental isolation of the medial basal hypothalamus (Danilova and Polenov, 1977; Antunes et al., 1979; Nagy et al., 1983; Dellmann et al., 1987b, 1988a,b). In these investigations, the authors described "muffs" of regenerating neurosecretory axons around the blood vessels proximal to the lesion. Some of these axons entered the isolated area and the scar tissue, while others surrounded meningeal vessels in the ventral hypothalamus and the mantal plexus area in the median eminence. New neurohemal contacts are established on "newly formed vessels" within the scar (Antunes et al., 1979), as well as on preexisting small hypothalamic vessels of the size of arterioles or venules (Antunes et al., 1979; Dellmann et al., 1987b, 1988). Nagy et

al. (1983) suggested that, in spite of such sporadic
regeneration, complete deafferentation of the hypothalamus
(medio-basal hypothalamus) constituted a permanent barrier to
regrowth of neurosecretory axons. It is noteworthy that
functional recovery was not demonstrated in these instances.

A relevant feature of both intra- and extrahypothalamic
sites is the close physical relationship between regenerating
neurosecretory axons and the vascular bed. In the median
eminence, the hypophysial stalk and the neural lobe, there is
normally no BBB to circulating proteins. Pituicytes and wide
pericapillary connective tissue spaces are present. Within
the areas of intrahypothalamic lesions, the BBB is temporarily
disrupted. Thus, in both sites, regenerating neurosecretory
axons are exposed to an extracellular microenvironment
containing blood-derived substances, and they grow and sustain
their regeneration as long as this microenvironment is
available.

Regeneration into intrahypothalamic grafts.   As
stated above, the spontaneous structural and functional
plasticity following injury at the level of the median
eminence, the hypophysial stalk and the neural lobe is not
reproducible when the neurosecretory axons are severed within
the hypothalamus (Nagy et al., 1983). Only limited
regeneration within the hypothalamus was observed by Danilova
and Polenov (1977), Antunes et al. (1979), and Dellmann et al.

(1987b, 1988a). When, however, a suitable microenvironment is presented to the proximal ends of the intrahypothalamically-lesioned neurosecretory axons, regeneration occurs to varying extents (Dellmann et al., 1985, 1986, 1987a, 1989).

In a series of experiments designed to establish the intrahypothalamic graft microenvironment that is best suited to initiate and sustain neurosecretory axons regeneration, Dellmann et al. have shown that a variety of neural and non-neural tissues, such as neural lobe (Dellmann et al., 1987a), sciatic nerve (Dellmann et al., 1986), optic nerve (Dellmann et al., 1989), and subcutaneous connective tissue grafts (Dellmann et al., 1985) support to varying extents the regeneration of neurosecretory axons severed within the hypothalamus. These investigations demonstrated that neurosecretory axon regeneration is sustainable in a variety of graft microenvironments. Regenerating neurosecretory axons were associated with respective graft tissue and/or with perivascular glia. Apparently, non-neuronal structures exerted tropic and/or trophic influences upon severed axons, since regenerating neurosecretory axons were always associated with the vascular bed and the glial cells of the grafts and host tissue (Dellmann et al., 1986, 1987a,b). In fact, the most densely vascularized grafts (neural lobes) contained the largest numbers of neurosecretory axons, while grafts with the fewest number of capillaries (optic nerves) sustained

considerably fewer regenerating neurosecretory axons (Dellmann et al., 1985). The role of viable glial cells for sustaining neurosecretory axon regeneration was recently substantiated in a study using intrahypothalamically cryotreated neural lobe explants (Carithers and Dellmann, 1992). Virtually no neurosecretory axon regeneration was observed into the transplanted neural lobe explants up to 15 days post-transection and grafting. It was hypothesized that the absence of pituicytes is responsible for this lack of neurosecretory axon regeneration (Carithers and Dellmann, 1992). When neural lobes are explanted, however, only a few capillaries survive, and those that do gradually lose their fenestrae over time (Dellmann et al., 1991). Thus, when grafting cryotreated explants, both the vascular and the glial microenvironments necessary for regeneration are absent. Consequently, neurosecretory axon regeneration is abortive.

Regenerating neurosecretory axons within intrahypothalamic grafts were considered to be axon terminals, based on morphological criteria (presence of clusters of microvesicles). Whether or not these terminals were releasing physiological amounts of hormones capable of restoring fluid homeostasis was not determined in these studies. Moreover, it was not established if graft vessels were permeable to HRP, and the relationships between the presence or absence of the BBB and neurosecretory axons regeneration was not addressed.

Likewise, the reason neural lobe grafts offer the best
microenvironment to transected neurosecretory axons is
unknown.

Regeneration in the CNS (exclusive of the HNS)

Transplantation into the CNS (exclusive of the HNS).

Transplantation of tissue into the adult CNS is of interest
because of the possibility that it may be useful in
reconstitution of neuronal connections and function following
trauma or injury to the brain. It also offers the possibility
of addressing the role of both neuronal and non-neuronal
tissue substitutes in stimulating regeneration of cut axons
(Björklund and Stenevi, 1979, 1984; Wallace and Das 1983; Gash
and Sladek, 1984; Raisman et al., 1985; Kruger et al., 1986).
Research on neural transplantation may lead to the
amelioration and/or the treatment of CNS injuries.
Neurological deficits such as Parkinson's disease (Perlow et
al, 1979), Huntington's disease (Isacson et al., 1984),
neuroendocrine disorders (Gash et al., 1980a,b; Krieger et
al., 1982), and cognitive deficits (Low et al., 1982; Dunnett
et al., 1982) may become treatable. To shed light on these
severe conditions, the last decade has witnessed an enormous
growth of transplantation techniques designed to study the
development and regeneration of neuronal connections following
experimentally-induced damage to the mammalian CNS.

Successful grafting in the CNS implies establishment of appropriate patterns of neuronal connections, which can conceivably be achieved through two different (but not mutually exclusive) strategies. The first approach involves transplantation of nervous tissue, which may receive synaptic inputs from the host and through outgrowing axons that reinnervate/innervate host tissues, forming a "neural bridge" (Björklund and Stenevi, 1984). The second approach involves transplantation of tissues (such as sciatic nerve) or compounds (biological materials) which form a "non-neuronal bridge" (Björklund and Stenevi, 1984; David and Aguayo, 1981; Kromer et al., 1981). Host neurons may grow along this bridge to reinnervate denervated areas, or the grafted tissue may serve as a chemical generator within the BBB and influence axonal regeneration by delivering trophic molecules or by secreting a neurotransmitter or neurohormone which diffuses into the surrounding host parenchyma and affects host target cells (Gash, 1987; Gage and Fisher, 1991).

Peripheral nerve transplants. Attempts to use transplants to promote regeneration in the CNS date back to Tello (1911) (cited by Clemente, 1964) and Cajal (1928), who grafted pieces of peripheral nerve in an effort to bridge brain lesions and to stimulate regrowth of lesioned CNS axons. These authors noted extensive growth of central fibres into the graft within two weeks. Recently, the viability of this

approach has been substantiated (Aguayo et al., 1978, 1982;

Richardson et al., 1980, 1982; David and Aguayo, 1981, 1985;

Benefey and Aguayo, 1982) in experiments showing that severed

axons from intrinsic nerve cells in the brain and spinal cord

of adult rat can regrow into a microenvironment of non-neural

PNS components (David and Aguayo, 1985). The regenerative

stimuli are unknown, but it has been suggested that they are

provided either by growth and trophic factors secreted by

neurolemmocytes, by cell surface adhesion molecules, and/or by

extracellular matrix components. For spinal cord axons

growing into PNS grafts, David and Aguayo (1985) reported that

about 14% of CNS neurons are capable of axonal growth into

grafts after injury. Despite this low percentage, the authors

suggest that severed CNS axons may regrow when they are

transected in an environment where they are able to interact

with non-neuronal components of the PNS. The exact nature of

such interactions remains unknown (David and Aguayo, 1985) but

could involve changes in the neuronal perikaryon triggered by

signals received from the growth cones, or from molecular cues

either secreted by ensheathing cells or present on cell

surfaces and/or the extracellular matrix (David and Aguayo,

1985). When sciatic nerve is grafted between the ventrobasal

thalamic nucleus and the somatosensory cortex of the rat, few

axons grow toward the cortex (Cossu et al., 1987). This

evidence that, at least for some neurons, peripheral nerve

grafts constitute an insufficient stimulus for axonal
regeneration indicates that intrinsic properties of neurons
also exercise an influence on the extent of axonal
regeneration.

Reasons for failure of CNS regeneration

For assessment of regenerative response in the mammalian
CNS, it is important to define conditions that permit
regeneration, and to identify precisely the factor(s)
that play a role or that may be capable of modifying that
response. It is, however, equally important to identify the
reasons underlying the failures of CNS regeneration. Many
attempts have been made in this latter direction. In the
following chapter, the most widely accepted hypotheses or
those of historical interest, will be reviewed.

Intrinsic inability of CNS neurons to regenerate.
Cajal's (1928) visionary perception that CNS neurons could
regenerate provided they were exposed to a conducive
environment, had unfortunately been either widely ignored or
remained unknown to most neuroscientists. It is now
considered likely that the demonstrated capacity for
regeneration of a limited number of neurons is shared by many,
and perhaps even all, CNS neurons. Thus, Le Gros Clark's
(1942, 1943) conclusion that there is an intrinsic inability

of CNS neurons to regenerate after axotomy, which was based on
the observation that CNS axons failed to grow into a graft of
peripheral nerve, is merely of historical interest. With
modern histological techniques (i.e., in semithin sections and
electron microscopy) he would probably have visualized some
regenerated axons in almost any of his preparations.

Formation of inappropriate synapses. In contrast to
PNS axons, which are unlikely to synapse until a target organ
is reached, most injured CNS axons that form synapses do so by
forming inappropriate synapses near the lesion site (Bernstein
and Bernstein, 1971). This rapid synaptogenesis by injured
CNS neurons in early stages of regeneration might remove a
stimulus for further axonal regeneration. Kiernan (1978)
stated that it is not known whether the formation of
inappropriate synapses in the mammalian CNS is the root cause
of the failure of axonal regeneration, or is rather a
consequence of the fact that regeneration has already failed
for other reasons. The failure of axonal regeneration in the
transected mammalian optic nerve (Berry, 1982) argues against
a role for inappropriate synaptogenesis, since there are no
neurons with which appropriate synapses can be formed in this
location (Kiernan, 1978).

Autoimmune inhibition of regeneration. Damage to the brain or spinal cord causes an opening of the BBB, thus exposing CNS autoantigens, normally sequestered by the BBB, to immune surveillance (Berry and Riches, 1974). Humoral and cell-bound antibodies would therefore gain access to these antigens at the lesion site. Thereafter, according to this theory antibody-antigen complexes could preclude successful regeneration by masking molecular pathways along which axons grow, by altering growth cone mechanisms or by interfering with protein synthesis in neuronal somata after retrograde transport from the lesion site (Berry and Riches, 1974). The implication that the immune response may contribute to unsuccessful regeneration is not supported by Willenborg et al. (1977), who found no cell-mediated immunity to myelin or to whole brain tissue after experimental spinal cord injury.

Necrosis at the lesion site and formation of cystic cavities. Guth et al. (1981, 1983) implicated progressive necrosis of nervous tissue and the development of cystic cavities at the site of injury in the inability of CNS neurons to regenerate. Lesioned monoaminergic and cholinergic neurons are reported to sprout into but not across the necrotic area of a substantial lesion (Björklund et al., 1971), but are able to cross a narrow necrotic zone into an implanted target, such as the iris or the embryonic hippocampus (Svendegaard et al.,

1976; Kromer et al., 1981). It seems, therefore, that tissue
necrosis per se is unlikely to be a primary barrier for
regenerating axons but large areas of necrosis may create a
gap that should be bridged successfully by regenerative
outgrowth (Kromer et al., 1981a).

Neuroglial scar. Astrocytes respond to injury in
essentially 3 ways. They (i) participate in the phagocytosis
and removal of cellular debris (Cook and Wisniewski, 1973;
Mattews, 1977; Nathaniel and Nathaniel, 1977), (ii) fill the
extracellular space left by the degeneration of neuronal and
glial elements (Cook and Wisniewski, 1973; Nathaniel and
Nathaniel, 1977; Raisman, 1977), and (iii) reconstitute a
glial limiting membrane along all aspects of the CNS that have
become exposed to non-CNS environments (Berry, 1979; Reier et
al., 1983).

It has been proposed that the regenerative potential of
CNS neurons may be hindered by the neuroglial scar that forms
at the lesion site (Cajal, 1928; Windle, 1956; Puchala et al.,
1977; Berry, 1979; Kiernan, 1979; Reier et al., 1983; Lindsay,
1986; Reier, 1986). This concept was based on the finding
that most of the transected axons terminate within the scar
region. Several observations, however, indicate failure of
neuronal regeneration, even in the absence of gliosis. In
lesions of the rat spinal cord, an early stage of fiber

28

outgrowth has been noted, which is then followed by axonal
involution. This latter event occurred even though the
extracellular space was enlarged and no glial scar had formed
(Gilson et Stensaas, 1974).

The postulated obstruction of regenerative axonal growth.
by scars does not explain the regeneration by "detours" around
the injured site in the adult rat brain (Foerster, 1982). The
regeneration of neurosecretory axons (Dellmann, 1973; Dellmann
et al., 1988a) is not consonant with a physical barrier
hypothesis since it has been shown unequivocally that the
glial scar in the hypothalamus does not constitute an
impediment to regenerating neurosecretory axons.

Absence of neurolemmocytes in the CNS.   In the PNS,
the arrangement of neurolemmocytes and their basal laminae as
parallel strands in the degenerated distal segment is
considered to provide the basic guiding and orientating
substrate for regeneration of nerve fibers. Cajal (1928)
regarded the absence of a guiding system in the CNS as being
responsible for the regenerative differences between CNS and
PNS. In addition, he considered that the success of
peripheral nerve regeneration resulted from the existence of a
neurohumor (probably emanating form the neurolemmocytes) which
influenced positively fiber outgrowth. Aguayo et al. (1978,
1982) demonstrated that neurolemmocytes are more conducive to

axonal regeneration than are central glial cells. Obvious

candidates for producing a guiding substrate are the

neurolemmocytes within the PNS grafts, since the basal lamina

produced by these cells contains laminin which is thought to

play an important role in cell adhesion, migration,

differentiation, and neurite outgrowth in vitro (Varon et al.,

1983; Liesi, 1990).

Absence of growth factors. According to this

hypothesis, CNS axons would regenerate in the presence of

specific growth promoting factors (Kiernan, 1979; Lund, 1978;

Varon and Bunge, 1978; Berry, 1979; Levi-Montalcini, 1987;

Barde, 1988). The only well-documented neurite growth factor

is nerve growth factor (NGF), which exerts remarkable growth

effects on developing sympathetic and sensory neurons, as well

as on regenerating monoaminergic neurons (Bjerre et al.,

1973). The inability of central neuronal systems to

regenerate could conceivably be related to the absence or

unavailability of such specific factors either alone or in

combination with other non specific factors. Berry (1979)

suggested that growth factors, comparable to NGF, exist for

all types of neurons and that growth factors are taken up by

the growing axonal tips and retrogradely transported to the

perikaryon, where their actions on protein synthesis are

effected.

Inhibition of axonal growth by post-injury myelin

breakdown products. The non-permissive microenvironment in the CNS might result not only from the absence of appropriate substrate and/or growth factor but also from the presence of inhibitors of neuronal outgrowth. Khalil and Rech (1979, 1982) demonstrated in infant hamsters, that myelination starts at the seventh post-partum day, and continues at a slow rate until the third postnatal week, when the number of myelinated fibers increases rapidly. In discussing these findings, Berry (1982) proposed that failure of CNS neuron regeneration in mature animals is associated with the release of a growth inhibitory factor derived from myelin proteolysis. Successful axonal growth of severed corticospinal axons demonstrated by Kalil and Rech (1979, 1982) was correlated by Berry (1982) to the absence of myelin in immature mammals. Moreover, neurons in cell culture do not extend neurites into substrates containing myelin, presented either as cultured oligodendrocytes or as extracts of white matter tracts (Ffrench-Constant, 1991). Barron (1983) contended, however, that regrowth of mammalian CNS axons into implants occurs despite considerable degeneration of myelinated CNS fibres in the vicinity of the experimental surgery during grafting procedures.

Presence of BBB. The occurrence of regenerating axons in skin autotransplanted to the brain has led to the suggestion that plasma-derived proteins in the extracellular fluid around the growth cones of these axons may be responsible for regeneration (Heinicke and Kiernan, 1978). According to this proposal, axons of any type will regenerate if their growing tips have access to serum-derived growth promoting proteins. In the adult CNS, blood-derived proteins are generally not available to axons, due to the BBB (Houthoff et al., 1982; Broadwell et al., 1983). Regenerating axons would, therefore, need to grow into regions where the BBB has been breached in order to assimilate these proteins from the extracellular fluid (Kiernan, 1979).

Following injury, the BBB is breached temporarily (Kiernan, 1979), and regenerative axonal growth takes place (Kiernan, 1979). Re-establishment of the BBB seems to correlate with degeneration of the newly formed axonal sprouts (Kiernan, 1979) which has been noted about one month after axotomy by Cajal (1928) and Lampert and Cressman (1964). This degeneration of neurites in the absence of serum may suggest that blood-derived proteins are needed for the maintenance of axonal regenerative growth in the mammalian CNS. With respect to this proposal, it seems that all tissues that can support neurosecretory axon regeneration lack a BBB (Kiernan, 1971; Dellmann et al., 1987b; Dellmann and Carithers, 1992b).

Alteration of the BBB within the hypothalamus over a prolonged
period of time (thus increasing the availability of blood-
derived substances) coupled with morphological and functional
assessment of the neurosecretory axonal response could be used
as a basis to further test this hypothesis.

With respect to the hypotheses which are relevant to the
present work, three are worthy of consideration: the presence
of the BBB, the absence of specific glial cells, and the
absence of growth factors. Any one of these three hypotheses
is unlikely to account exclusively for the regenerative
response of neurosecretory axons. Indeed, the growth factors
(neuronotrophic or neurite-promoting factors) could be blood-
derived. Moreover, glial cells such as astrocytes,
neurolemmocytes or pituicytes, may attract regenerating fibers
by releasing neuronotropic factors or by serving as guidance
channels.

Growth and neurotrophic factors

Growth and neurotrophic factors, as well as specific
constituents of extracellular matrix and cell membranes, have
been identified as substrates for growing fibers, and these
probably act together in a complex manner during embryogenesis
(Heath and Rees, 1985) and repair. Growth factors are
molecules that contribute significantly to the regulation of

both hypertrophic and hyperplastic responses in eukaryotic
cells. Neuronotrophic factors are a subset of growth factors
that act on neurons of the PNS and CNS, to enhance primarily
neurite outgrowth and maintain cell viability (Snider and
Johnson, 1989).

Cells and extracellular matrix molecules

Basal lamina constituents. An important
component of the peripheral nerve is the basal lamina, which
surrounds each axon-neurolemmocyte unit. The basal lamina is
comprised of type IV collagen, laminin, fibronectin and
proteoglycans (Ide et al., 1983). Evaluation of these
elements has shown the highest neurite-promoting competence
for the glycoprotein laminin (Varon et al., 1989). During
axonal degeneration, the basal lamina tube is retained, and it
constrains neurolemmocytes to a linear array. Since
regenerating PNS axons grow along the inner aspect of this
basal lamina (Ide et al., 1983; Hopkins et al., 1985) and
adjacent to the contained neurolemmocytes (Berry et al.,
1986), it is not clear whether the regrowing axons primarily
find footing for their growth on neurolemmocyte surfaces, the
inner aspect of the retained basal lamina, or on both these
structures (Bunge, 1987). In peripheral nerves that have been
cryotreated, thus killing the neurolemmocytes, axons have been
observed to grow directly within basal lamina tubes that

contain no living neurolemmocytes (Ide et al., 1983; Hall,
1986). These axons become associated with neurolemmocytes
that apparently migrate from the proximal stump and
subsequently adhere to and wrap around the regenerating axons,
resulting in the reforming of complete nerve fibers.

In the CNS, basal laminae are found beneath ependymal and
meningeal linings and around blood vessel walls (Alitalo et
al., 1982; Liesi, 1985a). During various stages of neuronal
maturation and axonal growth, a punctate form of laminin
exists in the brain parenchyma in several regions (Liesi,
1985b, 1990; Zhou and Azmitia, 1988) but disappears in
adulthood. This form of laminin has been shown to be a
growth stimulatory substrate of neurons in vivo (Liesi, 1990).

Following brain injury by kainic acid, production of
laminin by reactive astrocytes was related directly to the
extent of local cellular damage (Liesi et al., 1984). This
induction of laminin might well be involved in scar formation.
Moreover, the ability of laminin to promote neurite outgrowth
and neuronal sprouting supports the view that laminin might be
needed specifically during early phases of brain tissue repair
and axonal reconnection (Liesi et al., 1984). In fetal
neurons transplanted into the adult brain, laminin induced
neuronal migration and fiber growth, thus leading to the
formation of nerve bundle-like structures (Zhou and Azmitia,
1988). Laminin also seems to be necessary for axonal growth

into glial scars. Human and rat amnion membrane matrix (a
rich source of laminin) is a good substrate for regenerating
cholinergic fibers into target hippocampus as well as other
peripheral and central neuronal tissues (Davis et al., 1987;
Danielsen et al., 1988).

Neural cell adhesion molecules (NCAMs). Cell
adhesion molecules are ligands that participate in cell-cell
recognition during the formation of tissue structures. In the
nervous system, several membrane glycoproteins have been
identified as NCAMs (Rutishauser et al., 1988). Although NCAM
is expressed transiently in many embryonic structures, its
occurrence becomes reduced as development progresses. In the
nervous system, NCAM is found at the earliest stage of neural
development (neural tube stage) but is less abundant in the
adult mammalian brain (Theodosis et al., 1991). It has been
proposed that NCAM may also be expressed in the adult nervous
system, where it may influence neuronal plasticity and cell
reshaping. Recently, Theodosis et al. (1991) demonstrated the
presence of NCAM-immunoreactivity in both the PVN and SON of
the hypothalamus and in the neural lobe. This
immunoreactivity was seen in dendrites, axons, axon terminals,
and in associated glial cells (astrocytes and pituicytes), but
not in perikarya of magnocellular hypothalamic neurons.
Theodosis proposed that the continued expression of NCAM
confers to magnocellular neurons and their glia the ability to

reversibly change their morphology in adulthood.

The regenerative response of transected neurosecretory axons is probably a reflection of the plasticity with which neurosecretory axons are endowed, even in the adult CNS (Theodosis et al., 1991). It would be interesting to determine to what extent regenerating neurosecretory axons and the various cell types with which they may be associated during the regenerative process express NCAMs, and to assess whether there are any temporal changes in NCAM levels as repair is completed.

Neurolemmocytes. The importance of neurolemmocytes (that retain residence within the amputated nerve stumps) in providing both tropic and trophic guidance for peripheral nerve regrowth has been emphasized since the time of Cajal (1928). Neurolemmocytes respond to the loss of axon contact by proliferating and establishing channels called the Bungner bands (Sunderland, 1978; Aguayo et al., 1981; Richardson et al., 1983). These channels provide an oriented environment along which the regenerating axons grow towards their targets. Regenerating peripheral nerve axons are regularly associated with neurolemmocytes (Anderson et al., 1983; Ide et al., 1983). There is evidence that axons cannot emerge from the proximal stump of severed peripheral nerves if proliferation of neurolemmocytes is inhibited (Hall, 1986). The requirement for living neurolemmocytes supporting PNS

suggests the production by these cells of growth factors (such as NGF) that sustain the survival of lesioned neurons. Synthesis of NGF by rat neurolemmocytes was reported by Korshing et al. (1986) and Johnson et al. (1988) in the distal segment of lesioned sciatic nerve supports this hypothesis.

Neurotrophic factors

Nerve growth factor (NGF).   The discovery of NGF (Levi-Montalcini and Hamburger, 1951), and its purification (Cohen et al., 1960) were major achievements in the history of developmental neurobiology. NGF is produced in peripheral tissues and organs and is endocytosed by axon terminals and transported retrogradely to the corresponding neuronal perikarya. Impedance of the transport system results in neuronal death (Stoeckel et al., 1975; Aloe and Levi-Montalcini, 1979).

Several investigations suggest that NGF available to sympathetic and dorsal root ganglion cells is synthesized primarily by peripheral target tissues. Nontarget cells, such as neurolemmocytes or neurolemmocyte-like cells, also secrete NGF (Liu et al., 1979). Moreover, tissue culture studies have shown that NGF is secreted by fibroblasts (Young et al., 1974), glioma cells (Longo and Penhoet, 1974), and astrocytes (Lindsay, 1979).

Evidence suggests that NGF regulates a variety of

cellular processes which are important for neuronal function. Administration of NGF to rodents results in striking increases in sympathetic ganglion cell size (Angeletti et al., 1971), in axonal branching in the periphery, and in dendritic arborization (Snider, 1988). NGF is also essential for regulation of the expression of specific properties of peripheral sympathetic and sensory neurons, including catecholamine synthesizing enzymes (tyrosine hydroxylase and dopamine α-hydroxylase) and specific peptides (substance P and somatostatin) (Thoenen et al., 1985).

Although NGF was initially believed to govern only PNS neurons, recent studies have demonstrated a role for NGF in many CNS neurons in both the developing and adult brain. Cholinergic neurons are responsive to exogenous NGF and may depend on endogenous NGF as one of their neurotrophic factors. Indeed, both NGF-mRNA and its NGF protein product were found in cholinergic innervation loci (hippocampal formation and cerebral cortex) (Korshing et al., 1986; Shelton and Reichardt, 1986) which express specific receptors for NGF (Taniuchi et al., 1986). Recent binding studies raise the possibility that the actions of NGF are not confined to cholinergic neurons, but may also influence non-cholinergic cell populations in the developing and adult brain (Raivich and Kreutzberg, 1987; Yan and Johnson, 1989; Yan et al., 1990). NGF receptors are expressed by perivascular microglia

of the neural lobe, suggesting a possible role for NGF and/or
NGF receptors in microglial function (Yan et al., 1990). This
diversity in populations of cells displaying NGF binding sites
suggests that the actions of NGF in the developing and mature
brain are more extensive than previously believed.

Fibroblast growth factor (FGF). FGF is
recognized as a trophic factor for a variety of cells
including neurolemmocytes, astrocytes, oligodendrocytes, and
endothelial cells (Ludwin, 1985; Gospodarowicz, 1987; Folkman
and Klagsbrun, 1987; Baird and Bohlen, 1990). It is present
in two forms: acidic FGF (aFGF) and basic FGF (bFGF) (Walicke
and Baird, 1988); the latter is more potent.

bFGF is found mainly in neuronal cell bodies and their
processes and is never associated with vesicular or other
membrane-bounded structures (Janet et al., 1987). This
suggests that bFGF may reach the extracellular space upon
disintegration of the neurons that synthesize it (Barde,
1988). This possibility is plausible when considering bFGF as
only wound-related factor, but not as a target-derived
neurotrophic factor regulating survival during development.
bFGF immunoreactivity was found in activated macrophages by
Baird et al. (1985), who named it macrophage-derived growth
factor. After CNS injury, the level of immunoreactive and
biologically active bFGF increases at the lesion site
(Finklestein et al., 1988; Frautschy et al., 1991), and many

bFGF-immunopositive cells are of monocyte-macrophage lineage
(Frautschy et al., 1991). These cells may either synthesize
bFGF or acquire it from phagocytosed neuronal debris. A
similar paradigm has been proposed for interleukin-1 (IL-1), a
growth factor synthesized by macrophages, which bears
structural homology with bFGF (Gage et al., 1988). bFGF has
also been identified in microglia, reactive astrocytes
(Frautschy et al., 1991), and in endothelial cells of brain
capillaries of the hypothalamus (Maciag et al., 1979). bFGF
also appears to possess chemotactic and mitogenic activity for
vascular endothelial cells, and appears to represent a source
of angiogenic activity in a variety of normal and tumor cells
(Folkman and Klagsburn, 1987) as well as intracerebral grafts
(Giacobini et al., 1991). The question which now arises is
whether the trophic effects that bFGF exerts on these tissues,
particularly on axonal regeneration in intracerebral grafts,
may be a secondary effect of the enhanced vascularization of
the grafts.

Brain-derived neuronotrophic factor (BDNF). BDNF
is a neuronotrophic factor that is found mainly in the spinal
cord and the superior colliculus (Lindsay et al., 1985). The
amount of BDNF-mRNA in the brain is considerably larger than
that of NGF-mRNA (Leibrock et al., 1989). BDNF supports
neuronal survival and stimulates neurite outgrowth in vitro of
primary sensory neurons, including trigeminal, nodose, and

vestibular (Lindsay et al., 1985; Barde et al., 1987).

NGF-like molecules. Few intrinsic chemically well-defined and physiologically important factors enhancing neurite outgrowth are known (Recio-Pinto and Ishii, 1984). One of these is insulin, which has NGF-like effects on a limited number of PNS and CNS neurons, both in vivo (Havranoka et al., 1978) and in vitro (Bhat, 1983; Recio-Pinto and Ischii, 1984). Gangliosides, also belong to this category of molecules capable of promoting axonal regeneration of cerebral (Massarelli et al., 1985) and cholinergic neurons (Wojcik et al., 1982).

BBB and regeneration

In the CNS, the BBB regulates the exchange of hydrophilic substances between the cerebral extracellular fluid and plasma (Rapoport, 1976). In the PNS, regulation is controlled by a similar barrier, the blood-nerve barrier (BNB), which limits the entry of blood-borne water-soluble substances into the endoneurial compartment. The morphological correlates of the BNB are the tight junctions between the cells of the endoneurial endothelium and the inner layers of the perineurium (Karnovsky, 1967; Bell and Weddell, 1984).

In the PNS, tension at suture lines in nerve grafts diminishes the blood supply and prevents axonal regeneration.

Fibers adjacent to blood vessels regenerate faster than those situated at a distance, and there is a direct correlation between the rate of axon regeneration and vascular permeability (Mellick and Cavanagh, 1968). In addition, Werasuria et al. (1980), studying perineurial permeability during Wallerian degeneration, suggests that restoration of a regulated endoneurial microenvironment is crucial for peripheral nerve repair.

The precise roles of the BBB in regeneration, as well as that of the graft vasculature's permeability, remain a matter of controversy. Several hypotheses involving the BBB have been advanced for the limited capacity of central axons to regenerate in the CNS. Autoimmune inhibition proposed by Berry and Riches (1974) was tested by Heinicke and Kiernan (1978), who examined axonal regeneration and vascular permeability in pieces of skin grafted into the brain. Results demonstrated that central axons regenerate only into regions where protein can pass freely from the plasma into the extracellular space. This suggests that the sustained presence of plasma proteins in the extracellular fluid around the tips of axons is necessary for axonal regeneration. This is supported by observations subsequent to optic nerve lesion in the goldfish that regeneration occurs in the absence of an intact BBB during the period of the study (4 weeks) (Kiernan and Contestabile, 1980), while in the rat, in which increased

vascular permeability is short-lived (2 weeks), regeneration
is abortive (Kiernan, 1985). In support of Kiernan's
hypothesis is the observation that axonal sprouts, which
develop following traumatic lesions in the CNS retract within
a month (Heinicke and Kiernan, 1978; Risling et al., 1989).
Retraction coincides temporally with the restoration of the
BBB, but it remains to be established whether these two events
are causally related. Furthermore, ingrowth of sensory axons
into the spinal cord following dorsal root lesion is possible
in neonate rats, when the BBB is immature, but not in one
week-old rats (Carlsdelt et al., 1987) by which age the BBB
has attained maturity (Lossinsky et al., 1986).

The potential of CNS tissue to recover from injury
depends on the substances and processes that support neuronal
survival, promote neurite outgrowth, and guide the neurites to
their target. Neurite outgrowth is considered to result from
a continuation of perikaryon protein synthesis (Thoenen et
al., 1983), and the absence of blood-derived proteins
necessary to induce the synthesis of proteins may be the
mechanism underlying abortive regeneration into the CNS.

The Blood-Brain Barrier (BBB)

The earliest experimental studies on vascular
permeability were made with synthetic dyes, and it has been

known since these reports, that intravenously injected acidic
dyes (such as trypan blue) stain all the tissues of the body
except the brain, spinal cord and peripheral nerves (Ehrlich,
1885). These findings demonstrated impedance to the passage
of a circulating tracer into the brain. Lewandowsky in 1900
was the first to suggest that brain capillaries were the site
of this barrier. The precise location of the barrier was not
possible, however, until the tracer horseradish peroxidase
(HRP), used in conjunction with electron microscopy, became
available.

Circumventricular organs (i.e., choroid plexus, vascular
organ of the lamina terminalis, subfornical organ, pineal
body, area postrema, median eminence and neural lobe) possess
fenestrated capillaries and lie outside the BBB (Brightman et
al., 1975; Broadwell and Brightman, 1976). The wide
perivascular space with connective tissue cells in
circumventricular organs is separated from the extracellular
space of the rest of the brain and from the cerebro-spinal
fluid by a sheath of cells (ependymal and choroid epithelial
cells) connected by tight junctions (Brightman et al., 1975).
Under normal conditions, peripherally administered blood-borne
HRP was shown to leak from vessels supplying the dura mater
and circumventricular organs. Peroxidase reaction product was
also observed on the ventricular and pial surfaces of the
brain, within the parenchyma adjacent to the circumventricular

organs and within the Virchow-Robin spaces of large vessels penetrating the pial surface (Balin et al., 1986).

Structural substrates of the BBB

Shortly after the introduction of HRP as a tracer for light and electron microscopic studies of vascular permeability, Reese and Karnovsky (1967) showed at the fine structural level that the BBB is located in the cerebral endothelium. After intravenous injection of HRP into mice, the tracer from the capillary lumen enters the narrow spaces between endothelial cells as far as the intercellular junctions, but the endothelial tight junctions prevent any further passage of HRP into the pericapillary space in the CNS. In many tissues, such as skeletal muscle, HRP passes readily between capillary endothelial cells to the pericapillary space. However, cerebral endothelial tight junctions prevent any extravasation of HRP via the intercellular space. Tight junctions in the CNS differ from those in the capillaries of other organs in that they are continuous, leaving no gaps between adjacent endothelial cells (Brightman and Reese, 1969). Muscle endothelial cells also contain a large number of transcellular transport vesicles (Simionescu et al., 1978). By contrast, in brain capillaries, the number of such vesicles is very small, and tracer studies showed no evidence of transcellular transport (Reese and

Karnovsky, 1967; Broadwell and Salcman, 1981; Broadwell,

1989). These CNS endothelial vesicles are destined to merge

with lysosomes and are not involved in transcytosis. The few

HRP-containing vesicles in endothelial cells found after

tracer perfusion of the normal brain did not appear to be

released at the abluminal surface of capillaries (Hirano et

al., 1969; Broadwell and Salcman, 1981). The morphological

basis of the BBB is therefore attributed principally to two

characteristic features of brain capillaries: the presence of

tight junctions and the paucity of endothelial vesicles. In

addition, the cerebral microvascular bed also possesses

periendothelial structural components, such as glial foot

processes, pericytes, and endothelial basal lamina, that act

as a secondary barrier, contributing to the BBB phenomenon.

An indication of their significance was provided by Stewart

and Wiley (1981), who demonstrated that the ability of CNS

endothelial cells to form a BBB is not intrinsic to these

cells but, is instead induced by the pericapillary

microenvironment. It is, however, still debated which

structure(s) in the CNS is/are responsible for inducing

endothelial cells to form tight junctions characteristic of

the BBB.

Glial foot processes. Although astrocyte endfeet form

a continuous sheath around cerebral capillaries, they do not

preclude macromolecular diffusion, because they are not

connected by tight junctions (Brightman and Reese, 1969).

Astrocytes may contribute to the synthesis of the endothelial

basal lamina (Bar and Wolf, 1972; Krum et al., 1991), and may

also selectively secrete and absorb solutes such as potassium

(Bradbury, 1979). In vitro studies demonstrated that the

expression of brain- specific features by endothelial cells

may be determined by signals released by the glial cells into

the basal lamina (DeBault and Cancilla, 1980; Fred et al.,

1987). The view that astroglia are actively involved in the

functional establishment of the BBB has became increasingly

credible (Tao-cheng et al., 1987; Janzer and Raff, 1987;

Bouchaud et al., 1989). The presence of extensive close

appositions between astrocyte processes and tight capillaries

contributes greatly to this hypothesis (Dermietzel and Krause,

1991).

Pericytes. These ramified cells of mesodermal origin

reside upon or within the basal lamina of capillaries or

venules (Van Deurs, 1976; Sims, 1986). Pericytes are able to

endocytose a large variety of tracers such as ferritin, HRP,

and microperoxidase, and to incorporate tracers into lysosomes

(Brightman, 1965; Cancilla et al., 1972; Van Deurs, 1976).

Since proteins are prevented from crossing the BBB in either

direction, an important role of pericytes may be to prevent

accumulation of proteins or other substances in the

extracellular space (Cancilla et al., 1972). Van Deurs

(1976), studying the BBB in hypertensive rats, suggested that pericytes play an important role in the periendothelial portion of the barrier.

The BBB in immature animals is less efficient than in adults. Following injection of HRP into the brain of suckling mice, pericytes endocytose extravasated HRP to a considerable extent, while no tracer reaches the neuropil (Kristensen and Olson, 1973). In light of these observations, it seems reasonable to assume that phagocytic pericytes play an important role in maintaining the BBB, at least temporarily as the animal develops and matures. Broadwell et al. (1989) considered intracerebral perivascular phagocytes (i.e., pericytes and macrophages) as additional cellular constituents of the BBB apart from the cerebral endothelium.

Basal lamina. The basal lamina is a specialized extracellular matrix that separates endothelial cells and pericytes from the surrounding extracellular space. In addition to a mechanically protective role, the basal lamina may function as a selective filter to particulate tracers (Brightman, 1965) or may bind selected compounds electrostatically (Kefalides, 1978).

Alteration of the BBB

An early event occurring a few seconds after CNS trauma in the spinal cord is the appearance of small "flame

hemorrhages" in grey matter and in pia-arachnoid membranes (De
La Torre, 1984). A hemorrhage first seen in the grey matter
spreads to the white matter within minutes (Griffiths and
Miller, 1974). Blood vessels in the wound rapidly thrombose
and fill with serotonin-laden platelets, and norepinephrine
metabolism of the tissue is altered (Rapoport, 1976). The
resulting vasospasm induces ischemic-hemorrhagic necrosis,
edema and lactocidosis (Osterholm and Mathews, 1972).
Capillaries undergo wall thickening, and there is
extravasation of blood and plasma into the adjacent nervous
tissue (Rapoport, 1976). Damaged endothelial cells often
separate from their neighbors at junctional regions, thus
forming gaps in the capillary lining, and pinocytosis
increases in the remaining cells (Rapoport, 1976). Tissue
edema may develop into progressive necrosis, characterized by
cysts and cavities, which are partially sealed off by a dense
network of astrocyte processes, the glial scar (Guth et al.,
1985).

After a cerebral stab wound, the BBB's permeability to
HRP is altered. The number of peroxidase-filled pinocytotic
vesicles at both luminal and abluminal surfaces and within the
endothelial cells increases (Hirano et al., 1969). HRP is
also observed between adjacent endothelial cells diffusing
from the lumen to the basal lamina, a consequence of opened
endothelial tight junctions (Hirano et al., 1969). Following

compression injury of the spinal cord, the degree of capillary

leakage of blood-borne HRP decreases with increasing distance

from the site of injury, but was correlated directly to the

intensity of vesicular transport activity (Beggs and Waggener,

1976). Similar observations have been reported after

mechanical brain stem injury. As early as three minutes after

injury, HRP-labeled abluminal vesicles were seen, apparently

discharging their contents towards the basal lamina, while the

tight junctions remained occluded (Povlishock et al., 1978).

Noble and Maxwell (1983) and Noble and Wrathall (1988) showed

that endothelial transcytosis is enhanced for as long as 7

days after injury. The concept of a vesicular

transendothelial transport (of macromolecules) as a structural

basis for extravasation into the brain under pathological

conditions is still subject to criticism by some researchers.

However, convincing information has accumulated supporting

such a mechanism. This in no way precludes the possibility

that opening of tight junctions is also involved.

In summary, homeostasis in the brain is partly maintained

by the particular anatomical features of its capillary bed.

Brain endothelial cells are connected by tight junctions, lack

fenestrations, and contain only a few vesicles that could be

involved in transport. These features ensure that in the CNS,

in contrast to other organs, blood-borne macromolecules cannot

pass through to enter the parenchyma. Under experimental challenges as transplantation or lesions, there are at least 2 possible routes for extravasation of exogenous proteins : (1) tight junctions may become patent and/or drastically damaged, (2) vesicular transport may be enhanced.

Angiogenesis

Neural transplantation within the mammalian CNS has been used to investigate aspects of brain development and neural plasticity. The capacity of transplanted tissues to integrate within the host CNS is critical for the survival of transplants. Angiogenesis, the formation of new blood vessels from preexisting vessels (Rissau et al., 1988; Klagsbrun and Folkman, 1990), is essential for survival of grafted tissues and must occur quickly to preclude irreparable ischemic damage. While the mechanism and process of transplant vascularization have been well documented in non-neural transplant models (Ausprunk et al., 1975; Ausprunk and Folkman, 1977), only a few investigators have described some vascular interrelationships between neural grafts and host CNS tissues in various paradigms (Scott et al., 1984; Rosenstein and Brightman, 1986; Broadwell et al., 1987; Wiegand and Gash, 1987; Rosenstein, 1987).

Microvascular endothelial cells normally exhibit extremely long turnover rates (years), and rarely undergo

mitosis (Folkman and Cotran, 1976). However, stimuli such as wound healing, inflammation, and tumor growth increase endothelial replication and result in the formation of new capillaries (Schoefel, 1963; Ausprunk and Folkman, 1977). The elongation of new vessels results from two processes, mitosis and migration of endothelial cells (Schoefel, 1963). These phenomena occur in different endothelial cells, with migration predominating at the tip of the growing capillary and mitosis occurring several cells proximal to the tip (Yamagami, 1970). Movement of host endothelial cells out of existing vessels, with consequent loosening of cell-to-cell junctions, may provide the stimulus for cell mitosis. Proliferation apparently occurs only when the continuity between adjacent endothelial cells has been disturbed (Folkman and Cotran, 1976; Ausprunk and Folkman, 1977).

Angiogenesis in lesioned CNS

Transplantation of neural tissues into the CNS involves the insertion of a needle device to cut a defined tract system and/or to inject tissue within a defined area of the CNS. The trauma associated with these procedures results in hemorrhage and edema immediately surrounding the path of the needle and at the site of deposition of the transplant.

Associated vascular changes within lesions and along the path of the needle carrying the transplants have not been

53

reported. There have been, however, numerous anatomical studies describing HRP extravasation after spinal cord contusion in rat (Noble and Wrathall, 1988), spinal cord lesion (Risling et al., 1989; Broadwell et al., 1991), cerebral freeze injury (Cancilla et al., 1979), and excitotoxic lesion with kainic acid (Dusart et al., 1989). The earliest evidence of endothelial regeneration within a lesion comes from the study of freeze injuries to the cerebral cortex of mice (Cancilla et al., 1979). As early as 3 days after lesioning, regenerating endothelium consists of cells or cell processes within the vascular lumen overlaying the necrotic endothelium. The basal lamina in damaged vessels undergoing re-endothelization is frequently folded and multilamellated, suggesting that the vessel was of a larger caliber before necrosis (Cancilla et al., 1979). Cells surrounding the vessel consist of macrophages filled with necrotic debris and astrocyte processes containing delicate bundles of filaments. By 5 days after lesioning, the endothelial cells at the site of injury are larger than normal and contain many ribosomes and mitochondria, and extensive rough endoplasmic reticulum . The abluminal surface of the endothelium is closely applied to the basal lamina, but astrocytes or their processes are either absent or form an incomplete layer. Tight junctions are present but the leakage of HRP is prominent (Cancilla et al., 1979). Between 15 and

35 days post-injury, vascular integrity to HRP is
reconstituted. The endothelium is flat and its organelles are
less prominent. These observations on the integrity of BBB
components after cerebral freeze injury correspond to those
observed after a penetrating intracerebral lesion (Risling et
al., 1989) and spinal cord contusive injury (Noble and
Wrathall, 1989). Broadwell et al. (1991), however, reported
that resolution of the needle track and re-establishment of
the BBB to circulating HRP are effected within 7-10 days.
After excitotoxic spinal cord lesion, the BBB function is
impaired for a much longer period (3-7 months) (Dusart et al.,
1989).

Angiogenesis in transplants

The functional effectiveness of a transplant depends
either on the neurons of the transplant making connections
with those of the host, or on the transplant providing a
bridge along which host neurons can grow. Before any
connections can occur, however, it is necessary for the
transplant to survive. Survival is crucially dependent on the
revascularization of the graft or inoscultation of the graft's
indigenous microvasculature. Although the process of
transplant vascularization has been well documented in
nonneural transplants such as tumors, muscle, skin and thyroid
(Ausprunk et al., 1975), it is not clear which stimulus

initiates the process of vascularization. Neither is it
understood fully what the relative contribution is from host
or transplant tissues, nor how the final vasculature is
structured. Since neural transplantation experiments
encompass a variety of tissues and techniques, it seems likely
that the process of (re)vascularization may vary with the
graft tissue source, the site of transplantation, the age of
both the host and the donor, and the transplantation
techniques.

Histological analysis of the initial events of
intracerebral graft vascularization is extremely difficult.
For the first few days, the host-graft interface is greatly
disorganized by bleeding, necrosis, "opening up" of
perivascular spaces around the damaged host vessels, and
invasion by hematogenous cells (erythrocytes, polymorphs,
macrophages, and platelets) (Lawrence et al., 1984; Raisman et
al., 1985; Broadwell et al., 1991). The response of the host
brain to this expansion of perivascular space is to wall it
off with a layer of reactive astrocytic endfeet, which are
invariably covered by a basal lamina. This rapidly
reconstructed astrocytic basal lamina seals off the host
tissues from mesenchymal elements (connective tissue, vascular
wall and hematogenous cells), with the exception of
macrophages, which may remain in the neuropil around the
transplant site for some time before disappearing or convert

to resting microglia (Murabe and Sano, 1983).

The earliest vessels seen at the host-graft interface make up a marginal system of interconnecting capillaries that encircle the intracerebral transplant (Lawrence et al., 1984; Raisman et al., 1985; Krum and Rosenstein, 1987). These tortuous vessels are characterized by thin wall, wide diameter, and expanded perivascular spaces which are infiltrated to various degrees by fibroblasts and macrophages. The essential feature of these reactive blood vessels is a perivascular space bounded internally by the basal lamina of the endothelial cells, and externally by astrocyte basal lamina (Lawrence et al., 1984). In some places, the outer basal lamina extends into the intercellular spaces between perivascular astrocytic processes. These basal laminae outgrowths are called spikes or streamers (Lawrence et al., 1984). Basal lamina streamers are prominent during the initial vascularization period but gradually disappear (Lawrence et al., 1984). It is speculated that these streamers represent stages in the formation and remodeling of the scaffolds along which the endothelial cells of the definitive circulatory system grow (Cancilla et al., 1979; Schor, 1980). From the wide marginal vessels, the first fine capillary sprouts invade the transplant by 24 post-operative hours (Lawrence et al., 1984; Raisman et al., 1985; Zhou et al., 1986; Krum and Rosenstein, 1987). Collagen type IV

immunohistochemistry shows that the growing tips of these
sprouts emit spike-like streamers of uniform diameter. By 1-3
weeks post-transplantation, the overall pattern of the
intracerebral graft vasculature consists of large diameter
vessels (with associated adventitial cells and macrophages).
Over this period, the thin capillary sprouts, derived from the
marginal wide vessels, gradually extend deeper into the
transplant. Once these wide diameter vessels reach the center
of the transplant, the formation of narrow capillary sprouts
commences (Raisman et al., 1985). The establishment of
vascularity equal in density and capillary diameter to that of
the host, is gradual and does not reach completion until 4
weeks posttransplantation (Svendgaard et al., 1975; Lawrence
et al., 1984; Raisman et al., 1985). At this time, the wide
diameter vessels have largely disappeared (Raisman et al.,
1985). This vascular regression has also been observed in the
early development of cerebral vasculature (Marin-Padilla,
1985).

The pattern of graft neovascularization described above
is still debated. It has been suggested that the initial
vascularization of the grafted tissue in the brain occurs by
formation of connections between the existing vascular bed of
the graft host vessels (Raisman et al., 1985; Rosenstein and
Brightman, 1986). Krum and Rosenstein (1987) have suggested
that endothelial cells resident in the grafted tissue

anastomose with the host endothelial cells to form the new circulation because prelabeled graft endothelial cells are found in the graft vasculature. This line of thought is supported also by Lindsay and Raisman (1984), Nakano et al. (1989), and Broadwell et al. (1991). On the other hand, transplanted suspensions of PC 12 cells are vascularized by fenestrated vessels that must originate from host tissue because no endothelial cells are introduced with the cell suspension (Pappas and Sagen, 1986; Jaeger, 1987).

Blood-brain barrier in intracerebral grafts

The presence or absence of a BBB within various grafts is also a matter of controversy. Stewart and Wiley (1981) reported that avascular grafts from 3-day old quail brain are capable of inducing a competent BBB within blood vessels of noncerebral origin when transplanted into the coelomic cavity of chick embryos. Conversely, when avascular grafts of quail coelomic tissue are transplanted into embryonic chick brain, the invading vessels originating from chick brain do not have BBB characteristics. From these data a concept has emerged that the BBB properties of the vasculature of a graft are determined by the source of the graft and not by the vessel origin. Thus, capillaries in grafted CNS would always have BBB properties and be impermeable to circulating tracers. That the substrate for a BBB exists in brain grafts is

supported by studies of Broadwell et al. (1987, 1988, 1989,

1990a,b). These authors confirmed that morphological and

permeability properties of blood vessels supplying solid CNS

tissue or cell suspension grafts are graft-determined. A

solid CNS graft or a suspension of specific CNS cells (e.g.,

neurons, oligodendrocytes, astrocytes) is supplied with blood

vessels manifesting barrier properties identical to those of

host CNS vasculature (Janzer and Raff, 1987; Ebert et al.,

1989; Broadwell et al., 1991). Solid tissue or cell

suspension grafts of peripheral origin, such as the

adenohypophysis, are not supplied with barrier vessels, even

though the vessels supplying the graft are derived from the

host CNS (Broadwell et al., 1991).

Capillaries within intracerebral autogenic muscle and

skin grafts show morphology and permeability to HRP similar

to those of muscle and skin in situ. However, when all muscle

or skin cells have degenerated and are completely replaced by

fibroblasts and collagen, the graft capillaries are of the

cerebral type (Wakai et al., 1986; Heinike and Kiernan, 1978).

When all the muscle and skin tissues have been phagocytosed,

diffusible factors from the surrounding host CNS tissue may

reach the fibroblastic-collagenous scar and induce formation

of the BBB. In contrast, in intracerebral allografts of the

cranial cervical ganglion, the ganglion cells degenerate with

time, while fibroblasts, collagen, neurolemmocytes and blood

vessels persist. The graft capillaries, like those of intact cranial cervical ganglion remain permeable to HRP (Wakai et al.,1986; Krum and Rosenstein, 1987). It appears that in this case the characteristics of the graft vasculature are determined by the neurolemmocyte-fibroblasts microenvironment rather than by the neuronal one. Recently, Rosenstein (1991), studying the permeability of intraventricular solid fetal CNS grafts, demonstrated that endogenous or exogenous proteins infiltrate the grafts both at short (2 days) and long-term (20 months) survival times. Other researchers examining vessel permeability in intraventricular CNS grafts have argued that a BBB does not exist, based on the observations that although intrinsic blood vessels in solid grafts appear CNS-like, protein tracer observed in the grafts emanates from host median eminence (Wiegand and Gash, 1987; Broadwell et al., 1989, 1991). Thus, one cannot generalize that all CNS grafts have a BBB. The significance of all these studies is that the extent of permeability in CNS grafts is a variable phenomenon and that juxtaposition and placement are of major importance. The age of the transplant, the type of tracer used as well as the techniques of grafting may be factors in these differences. In spite of the discrepancies, one must assume that the inductive or suppressive events which generate or inhibit the BBB phenotype of the blood vessels of the graft depend on the microenvironmental conditions prevailing at the

graft site. It appears that if the structural components of the grafted tissue survive after transplantation, then the blood vessels phenotype is source-determined. If, however, these structural components degenerate, the blood vessels' phenotype is host-determined. Whatever the fate of the grafted tissue, the most interesting issue is what structural component(s) induce or maintain the BBB. It may be useful to use additional criteria for evaluating the BBB rather than relying on a single criterium, the tightness of capillary endothelial cells to protein tracers.

Angiogenic factors

The growth and development of new capillary blood vessels, or angiogenesis, is a critical process in embryogenesis, wound healing, and tissue transplantation. The induction and continuous growth of new capillaries appear to be the result of several cellular activities regulated by a number of interacting factors. These angiogenic factors affect various endothelial functions, including proliferation, migration, basal lamina degradation, production and deposition (Polverini and Leibovich, 1984).

Folkman and Klagsbrun (1987) proposed two classes of angiogenic factors, direct and indirect ones. Direct angiogenic factors stimulate locomotion or mitosis of vascular endothelial cells in vitro, and have the vascular endothelium

as their targets in vivo. This class includes acidic and
basic FGFs, platelet-derived endothelial growth factor (PD-
ECGF), vascular endothelial growth factor or vascular
permeability factor (VEGF/VPF), and tumor necrosis factor
alpha (TNF-α) (Folkman and Klagsbrun, 1987; Klagsbrun and
D'Amore, 1991). Indirect angiogenic factors have no effect on
vascular endothelial cells in vitro. It is speculated that
they act by mobilizing macrophages and activating them to
secrete direct angiogenic factors (Beck et al., 1983; Baird et
al., 1985; Folkman and Klagsbrun, 1987; Leung et al., 1989).
This class includes epidermal growth factor/transforming
growth factor alpha (EGF/TGF-α), angiogenin, and prostaglandin
E2 (Fett et al., 1985; Roberts et al., 1986; Leibovich et al.,
1987; Klagsbrun and D'Amore, 1991). In addition to these
natural mediators, many chemical substances are known for
their angiogenic activity. Heparin has been tested for its
ability to enhance endothelial cell migration and for its role
as a mediator secreted by mastocytes (which are known for
their facilitating role in angiogenic activity) (Folkman et
al., 1983; Mc Auslan et al., 1983).

MATERIALS AND METHODS

The following experiments were designed to test the hypothesis that regeneration of neurosecretory axons and restoration of their function require specific vascular and supportive glial microenvironments. To test this working hypothesis, isografts of neural lobe, or optic nerve or autologous sciatic nerve grafts were placed into bilaterally transected HNT of rats. Light microscopic and fine structural characteristics of these grafts and surrounding CNS tissues were evaluated at various post-transplantation time periods.

Materials

Adult male rats of the Holtzman strain served as transplant recipients. Donor tissues for transplantation were obtained from adult males of the same strain. All animals were maintained under the same condition of controlled temperature (21°C) and illumination (12 hours). Food and water were available ad libitum. Animals were individually housed in Nalgene cages throughout the experimental periods.

Methods

Stereotaxic lesioning

Recipient animals (250-274 g B.W) were anesthetized with a mixture of ketamine (50mg/ml) and xylazine (10mg/ml) at a dose of 50mg/100g B.W, ip). The HNT was then transected bilaterally in the lateral retrochiasmatic area with a U- or L- shaped wire knife (1.5 mm wide). The stereotaxic coordinates were: anteroposterior= -1.7, lateromedial= ±0.8 and dorsoventral= floor of the cranial cavity, with bregma as zero. To transect the HNT, the lesion knife was lowered into the brain until it touched the ventral surface of the cranial cavity, then it was raised approximately 0.5 mm and slowly moved up and down (3 times) to complete the lesion.

Preparation of the grafted tissues and stereotaxic transplantation

Neural lobes of male Holtzman rats (125-149g B.W) were separated from the adenohypophysis, including the pars intermedia, sliced into halves, drawn into a 19-gauge spinal needle, and placed stereotaxically into each lesion site at the lateral retrochiasmatic area in recipient rats (n=7 for each of 5 time periods).

Small pieces (0.25mm^3) of isografts optic nerve, stripped of its meninges, were grafted as described above (n=7

for each of 5 time periods).

Small pieces (0.25mm^3) of autologous *sciatic nerve*, stripped of its epineurium, were grafted stereotaxically as described above (n=7 for each of 5 time periods).

In control animals (n=3 for each of 5 time periods), bilateral *lesions* were made as described above, without graft insertion.

Horseradish peroxidase injection

Two transplanted recipients per treatment and time period were assigned randomly to receive intravenous injection of HRP in order to assess the permeability of the graft vasculature. A total of 40 animals received intravenous horseradish peroxidase injections (HRP, type II, Sigma Co., St. Louis, MO.). One hour before perfusion fixation, Ketamine-Xylazine-anesthetized animals were injected manually into the jugular vein with 20 mg of HRP /100g B.W., dissolved in 0.5 ml of physiological saline over a period of 3-5 min, immediately following withdrawal of an equivalent volume of blood.

Tissue preparation

Animals were sacrificed at the following post-lesion-transplantation times: 18 hours (h), 36h, 10 days (d), 30d, and 80d. The initial time period was selected because the

first connections between host and intraparenchymal CNS tissue graft vasculature have been reported to occur between 16h and 20h after lesioning (Zhou et al., 1986; Krum et al., 1987). The subsequent time periods were selected based on the reported time course of graft revascularization (Krum et al., 1987) and magnocellular neurosecretory axon regeneration in the CNS (Dellmann et al., 1987). Twenty four animals were sacrified at each of the five time periods (N=24x5=120), and their brains were removed, dissected, and processed for light microscopy (HRP-histochemistry and neurophysin-immunohistochemistry) and electron microscopy.

Fixation for HRP. One hour after HRP injection, animals were perfused transcardially with phosphate-buffered (0.1M, pH=7.2) 5% glutaraldehyde solution containing 0.025% of $CaCl_2$ for combined HRP-histochemistry, neurophysin-immunohistochemistry and electron microscopy analyses. Brains were removed and post-fixed for an additional 6-12 hours in the perfusion fluid, then cut into $4mm^3$ blocks, and stored at 4°C in 0.1M phosphate-buffer prior to processing. Animals which did not receive HRP injection were fixed by transcardiac perfusion with phosphate-buffered (0.1M, pH= 7.2) 2% glutaraldehyde-3% paraformaldehyde. The removed brains were postfixed in the same solution for an additional 6-12 hours.

Isolated hypothalami were stored at 4°C in 0.1M phosphate buffer prior to processing.

Gelatin embedding. To avoid damage to the delicate host-graft vascular connections and loss of the transplanted tissues during sectioning and processing, hypothalamic blocks obtained from animal groups surviving 12 and 36 hours were embedded into gelatin according to Gurusinghe and Ehrlich (1986). Briefly, this method consists of (i) block wash in tap water for 12 hours, (ii) infiltration of the block with 5% gelatin solution at 37°C for 24 hours, (iii) infiltration with 12% gelatin solution at 37°C for 24 hours, (iv) storage at 4°C for 24 hours, (v) adjunction of phosphate-buffered 5% formalin to harden the blocks for 24 hours at 4°C.

All hypothalami were cut using a vibratome into 50 um-thick horizontal sections (36 sections/block). Alternate serial sections were used for neurophysin-immunohistochemistry, HRP- histochemistry (HRP-injected specimens) and electron microscopy.

Neurophysin-immunohistochemistry. Polyclonal antibody against neurophysin was used on sections in order to evaluate the extent of magnocellular neurosecretory axon regeneration and its relationship to the vasculature. The immunohistochemical procedure was as follows: (i) 3 washes in

phosphate buffered saline (PBS), 15 min each, (ii) inhibition
of endogenous peroxidase in a 10% methanol/3% H2O2 in PBS, 15
min, (iii) incubation in bovine serum albumin for 2 hours at
room temperature, (iv) incubation in Sofroniew's neurophysin
antiserum (which identifies both vasopressin- and oxytocin-
associated neurophysins; Sofroniew et al., 1979) diluted at
1:1000, overnight at room temperature, (v) 3 washes in PBS, 15
min each, (vi) incubation in goat anti-rabbit antiserum (1:40)
for 2 hours at room temperature, (vii) 3 washes in PBS, 15 min
each, (viii) reaction with peroxidase-antiperoxidase complex
(1:50) for 2 hours at room temperature, (ix) 2 washes in PBS
and 1 wash in Tris-buffer for 15 min each, (x) reaction with
0.025% diaminobenzidine (DAB) containing 0.002% H2O2 for 8-15
min in the dark and at room temperature, (xi) 3 washes in PBS,
5 min each followed by a wash in distilled water. The
sections were then mounted on gelatin-coated slides, dried,
dehydrated, and coverslipped.

HRP-histochemistry. Sections from HRP-injected
animals were incubated for histochemical localization of HRP
reaction product in Fahimi medium using diaminodenzidine (DAB)
as chromagen (Fahimi, 1970) : 10 mg of DAB, 10 ml of 0.1M
phosphate buffer and 0.2ml of H2O2 with a final pH of 7.1-7.4
for 10-20 min. Sections for light microscopy were mounted on
gelatin-coated slides, dried, dehydrated, and coverslipped.

Electron microscopy procedures. The series
of sections for electron microscopy were postfixed in 1% OsO4
containing 0.75% potassium ferricyanide for 1 hour, stained
with 2% uranyl acetate for 2 hours, dehydrated in graded
alcohols and embedded in an Epon-Araldite mixture. Sections
from HRP-injected animals were incubated in Fahimi medium and
processed for electron microscopy as described but not stained
with uranyl acetate. Thin sections were stained with lead
citrate and examined at 50 KV in a Zeiss EM9S electron
microscope.

Light microscopic morphometry

Tissue sampling. For the morphometric analysis two
semithin sections, 10 μm apart, were obtained from each of the
two 50 μm-thick vibratome sections from which subsequently
thin sections were used for the fine structural analysis.
These sections were stained with 1% toluidine blue.

Delimitation of the analyzed zones. Morphometric
analyses were carried out on photomicrographs of the semithin
sections at a final magnification of 800X or 1,200X. In these
micrographs, three zones were identified starting at the
apposition of hypothalamus and graft as the starting line: (i)
the *host-graft interface zone,* which includes fifteen

micrometers on either side of this line; (ii) the *hypothalamic zone*, a thirty-micrometer wide zone peripheral to the previous one; (iii) the *graft zone*, i.e., the entire region circumscribed by the other two zones (Fig. 1). In the lesion group only two zones were delimited: (i) a *scar zone* (consisting of astrocytes and their processes), and (ii) an adjacent thirty micrometer wide *hypothalamic zone* (Fig. 2).

Capillary sampling. Capillaries were selected for analyses according to the following criteria: (i) microvessel walls were composed of endothelial cells and pericytes; (ii) microvessel profiles were either approximately circular or slightly oval in shape; (iii) microvessel diameters were 15 μm or less.

The parameters recorded with a digitizer -GTCO- connected to an IBM computer using a statistical analysis program (developed by H. Guglia, University of Vienna, Austria) were the surface area measured on the micrograph and the perimeter of the capillaries. For each animal the statistical analysis program provided the total tissue area measured, the number of capillaries in that area, the capillary mean diameter, and the capillary volume (i.e., the area occupied by the capillary profiles expressed as a percent of the total tissue area). From these measurements the capillary density (i.e., the

number of capillaries per mm^2 of tissue area) was calculated.

Statistical analysis. The morphometric data were evaluated by analysis of variance. All analyses were conducted at a 1% level of significance.

Metabolic analysis

To determine whether neurosecretory axon regeneration into various grafts was extensive enough to alleviate the post-lesioning induced diabetes insipidus (DI), measurements of water consumption were initiated one week prior to the transplantation and continued for the duration of the experiment. Ten and 30 days after surgery, 4 animals/treatment, and at 80 days three animals/treatment, had diabetes insipidus. Data obtained from these animals were analyzed using one way analysis of variance (ANOVA1), followed by testing of difference in daily group means using the STATITCF program and the Newman-Keuls test. All analyses were conducted at the 5% level of significance.

Fig. 1. Horizontal sections of lesioned and optic nerve grafted hypopthalami showing the approximate levels of the zones delimitated for morphometric analysis of the capillary beds. Toluidine blue. X300.

A. In the lesion group, the scar zone is labelled S and the hypothalamic zone H.

B. In the grafts, three zones are delimited: the hypothalamic zone (H), the host-graft interface zone (HG), and the graft zone (G).
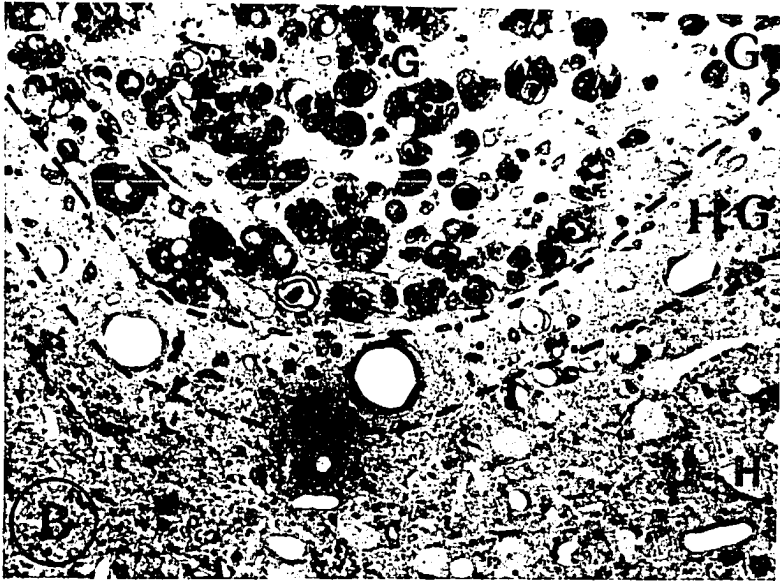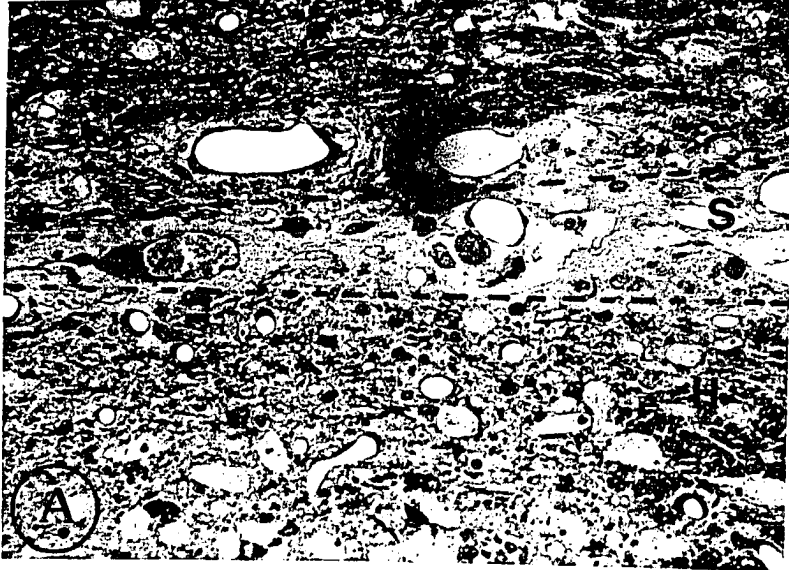
RESULTS

Morphological Findings

Control animals

The axons of neurons of the paraventricular nuclei run mostly laterad and ventrad in the hypothalamus, and when they reach the optic chiasm they join the fibers from the supraoptic nuclei and form a compact tract. This tract is located immediately caudal to the optic chiasm and extends caudally to a vertical plane through the beginning of the median eminence: the lateral retrochiasmatic area. It is in this location that the tract was transected bilaterally, 0.8 mm lateral to the midline. This tract of delicately beaded neurosecretory axons runs through the median eminence (Fig. 2A) and the hypophyseal stalk, and terminates on the fenestrated capillaries of the neural lobe. Neurosecretory axons do not make vascular contacts with the blood vessels of the hypothalamus.

Within one hour of i.v. administration of HRP, the median eminence, the ventricular surface of the ventral hypothalamus, and the adjacent neuropil reacted positively for HRP (Fig. 2B). In addition, random loci of HRP-reaction product were seen frequently throughout the hypothalamus. These loci were generally associated with vessels of the size of arterioles or

venules (Fig. 2C). Occasionally, neuronal perikarya of the arcuate, paraventricular, and supraoptic nuclei were faintly labeled with particulate HRP-positive granules. HRP reaction product was also localized in perivascular granulated cells in the median eminence and periventricular hypothalamus, and in a few granulated cells scattered throughout the nearby neuropil. Perivascular cells contained aggregates of fine HRP-positive granules in their perikarya and processes (Fig. 2D). These processes were of irregular shape and extended around or along blood vessels. The scattered cells were spherical, devoid of processes, and less intensely stained than the perivascular ones. At the fine structural level, HRP reaction product within the hypothalamus was confined to the lumen and/or occasional pinocytotic vesicles in some endothelial cells and to perivascular and scattered cells in the hypothalamus adjacent to the median eminence and ventral part of the III ventricle.

Experimental animals

18 and 36 hours survival times. No appreciable morphological differences were observed between these two time periods. Therefore, they will be described together.

Fig. 2. Neurophysin-immunohistochemistry and HRP-histochemistry of control animals.

A. Horizontal section, through a control hypothalamus at the level of the retrochiasmatic area. Neurophysin-immunostained axons of the HNT course in caudomedial direction from the supraoptic nucleus (son) to the median eminence (me). Arrows indicate the direction and extent of the transection of the tract. X30.

B. HRP leaked from the fenestrated capillaries of the median eminence into the adjacent neuropil, the III ventricle surface (arrowheads) and the ependymal surface (arrows). X120.

C. Extravasation of blood-borne peroxidase (large arrows) is associated with some arterioles or venules scattered throughout the cerebral parenchyma. X270.

D. High magnification of the square in figure 2B showing perivascular granulated cells (arrows) which are commonly seen along large caliber vessels in regions of HRP extravasation. X120.
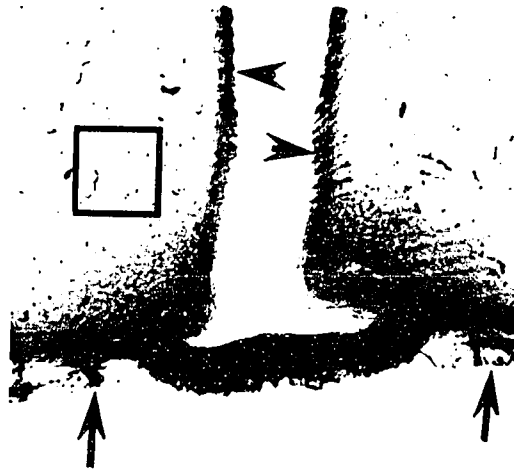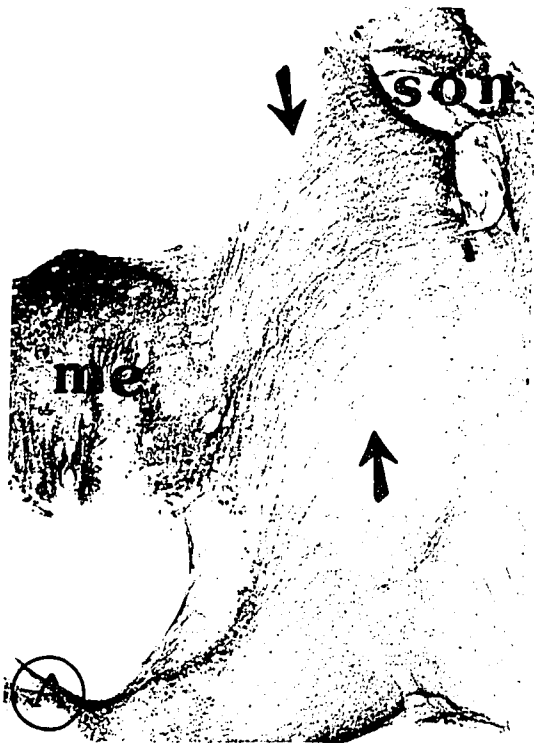
<u>Light microscopic observations</u>.   The grafts[1] were
almost completely surrounded by extravasated blood.   The
lesion[1] site was filled by a hemorrhagic clot (Fig. 3A).   It
was not uncommon to find free erythrocytes within the adjacent
hypothalamus.   In addition to HRP-positive areas corresponding
to those observed in control animals, the lesion site and the
blood clot surrounding the grafts contained HRP reaction
product.   This reaction product gradually diminished in
intensity as it extended radially into the surrounding
hypothalamic tissue.   Intense HRP extravasation was frequently
observed around some large caliber vessels of the size of
arterioles or venules in the immediate vicinity of the lesion
or the grafts (Fig. 3C) and also beyond the area of diffuse
HRP in the hypothalamus.   Grafts were devoid of HRP reaction
product, except for a narrow peripheral and faintly stained
zone in some grafts.   There was no vascular connection between
host and grafts at these times.   Cells containing fine HRP-
positive granules were present around blood vessels and less
frequently throughout the neuropil in the vicinity of the
lesion.

Neurophysin-immunohistochemistry revealed neurosecretory
material that had accumulated in the severed axons proximal

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[1]Lesion refers to lesion of the HNT without graft;
   graft refers to lesion and graft

and distal to the lesion (Fig. 3B), and the grafts (Fig. 3F) and filled ovoid or spherical dilatations in the immediate vicinity of lesions or grafts. Axons did not cross the lesion and did not invade any of the grafts. In the neural lobe grafts, however, a strong homogeneous neurophysin-positive reaction, similar to that observed in situ, was present at 18 hours (Fig. 3F) and diminished in staining intensity by 36 hours. Additionally, proximal axons had a tortuous course and were enlarged to varying extents. In the adjacent hypothalamus proximal to the lesion, neurosecretory axons formed single-layered and lightly stained perivascular plexuses around some of the leaky vessels observed in HRP-reacted specimens (Fig. 3D). This is the first time that neurosecretory perivascular plexuses have been reported in areas where HRP is leaking (Table 1).

Electron microscopic observations. Because gelatin embedding was used to avoid damage of the presumptive delicate host-graft vascular connections during sectioning and processing, all details appeared blurred due to the homogeneous background staining. Consequently, membranes were indistinct and it was difficult to focus sharply.

In all experimental animals, the hypothalamus adjacent to the lesion or the grafts was edematous, and many blood cells, fibrin, and macrophages containing numerous phagolysosomes

Fig. 3. 18 and 36 hours survival times

A. Extravasated RBCs (hemorrhage) are seen along the lesion track. Notice the HRP labelling on both sides of the lesion (arrowheads) and the blood vessel in the adjacent hypothalamus (square). 36 hours. X40.

B. Neurophysin-immunostained horizontal section of a lesioned hypothalamus. Proximal (P) to the lesion site which is filled with RBCs (H) neurosecretory axons have a tortuous course. Distally (D), numerous retraction balls are seen (arrows). 36 hours. X60.

C. Extravasation of blood-borne peroxidase around a large caliber vessels in the adjacent hypothalamus of a lesion as shown in the square of figure 3A. 36 hours. X210.

D. Neurosecretory axon regeneration around a large caliber vessel (arrow) at the proximal side of lesion. 36 hours. X210.

E. Extravasation into adjacent hypothalamus of HRP (arrowheads) around a neural lobe graft. The hemorrhagic clot completely encircles the graft. 18 hours. X110.

F. Indigenous neurosecretory axons and axon-terminals of neural lobe graft (NL) are strongly neurophysin-immunoreactive. Neurophysin-positive axons (arrow) are confined to the adjacent hypothalamus at the proximal side of the graft. The gap between the hypothalamus and graft is due to the loss of RBCs during processing. 18 hours. X80.
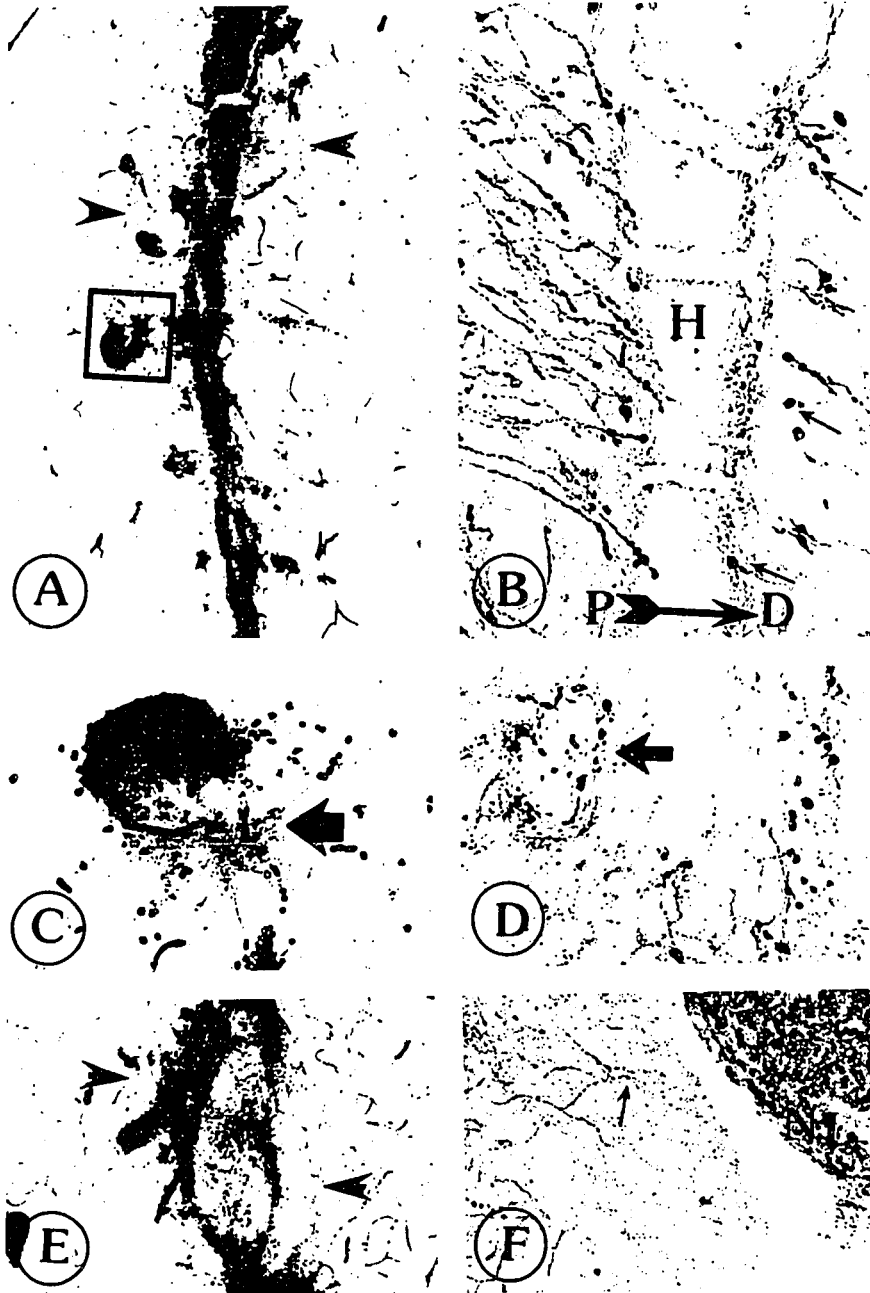
Table 1. Frequency of neurosecretory perivascular plexuses at the proximal hypothalamus adjacent to lesion or grafts

Time period	Lesion	Neural lobe	Sciatic nerve	Optic nerve
36 hrs	+ (*L)	+ (*L)	+ (*L)	+ (*L)
10 days	++ (*L)	+++ (*L)	+++ (*L)	+ (*L)
30 days	++ (**S)	++++ (**S)	++++ (**S)	++ (**S)
80 days	+ (*L)	++ (*L)	++ (**L)	+ (*L)

Symbols: ++++ indicates the largest number of perivascular plexuses, + the smallest number; * = one layer of neurosecretory axons, ** = two or more layers; L = light neurophysin-immunostaining, S = strong neurophysin-immunostaining.

were present in the enlarged intercellular spaces (Fig. 4A).

Most of the capillaries had the same morphological

characteristics as those found in undamaged brain tissue.

Some, however, had features characteristic of capillaries

undergoing angiogenesis, called stimulated capillaries[2], i.e.,

having an irregular shape, high endothelium with many free

ribosomes and elongated profiles of rough endoplasmic

reticulum, numerous mitochondria as well as abluminal

cytoplasmic projections and a discontinuous basal lamina (Fig.

4B, C). The capillaries at the host interface were filled

with erythrocytes, and consequently they were not flushed

during perfusion-fixation. A few HRP-filled vesicles were

identified within the cytoplasm of some endothelial cells near

the edge of the cut. Profiles of axons and dendrites were

often swollen. Neurosecretory axons proximal to the lesion

usually contained many NGVs, and some had developed growth

cones. Retraction balls were seen distal to the lesion. No

signs of glial scar formation were observed.

In neural lobe transplants only minor changes were

observed in comparison to control neural lobes. Thus,

neurosecretory axons frequently contained clustered and

enlarged NGVs of varying electron density and sizes. In a few

axons the axolemma had disappeared and axonal organelles

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[2]This terminology is preferred to high-endothelium or
sprouting capillary and will be subsequently utilized.

merged. Pituicytes differed from those in control neural lobes by the apparent retraction of their processes. They were still recognizable by their lipid inclusions, but cell limits were difficult to detect (Fig. 5A). Microglial cells, however, were not affected and were engaged in phagocytosis of neurosecretory axons. Most capillary endothelial cells were fragmented but the surrounding basal laminae remained intact. In a few capillaries the endothelium was intact but its cytoplasm was more electron dense than in control neural lobes.

In sciatic nerve grafts early signs of degeneration were apparent, including: vacuolated axoplasm, swollen mitochondria, dilated cisternae of endoplasmic reticulum, swollen neurolemmocytes with granulated cytoplasm, and disintegrated myelin (Fig. 5B, D). Collapsed capillaries, mostly lined by intact but thickened endothelial cells or, occasionally, by fragmented cells within an intact basal lamina were frequently observed. After 18 hours, a few macrophages were present at the host-graft interface, and by 36 hours they were more numerous, and were occasionally observed within the grafts.

In optic nerve grafts, numerous axons possessed a vesiculated axoplasm accompanied by loosening of the myelin lamellae. Numerous elongated myelin sheaths occurred around atrophied axons. Interaxonal spaces were enlarged and filled

with vesiculated astrocytes which contained small electron
dense fine granular aggregates and swollen mitochondria (Fig.
5C).

This is the first report of the fine structural
characteristics of lesion and grafts within the hypothalamus
at early time periods.

10 days survival time

Light microscopic observations.   At the lesion
site, the hemorrhagic clot had virtually disappeared; a glial
scar had formed and the BBB to circulating HRP was restored
(Fig. 6A). Faint HRP-reaction product was observed, however,
in macrophages located in the scar, in perivascular cells and
in some scattered cells throughout the adjacent neuropil. The
latter two cell types were less numerous than at the previous
observation periods.

In the hypothalamus surrounding the grafts, a more
pronounced staining reaction for HRP was noticed than at the
previous time periods, with a gradual centrifugal decrease in
intensity (Fig. 6C). The larger vessels at the host-graft
interfaces and/or in the vicinity of the grafts were
frequently leaky, as evidenced by HRP extravasation.
Occasionally, because of incomplete perfusion, erythrocytes
which stained positively for endogenous peroxidase, remained
in some vessels of the microvasculature. Some were seen to

Fig. 4. 36 hours lesion

A. Electron micrograph of the lesion and adjacent hypothalamus. The lesion site is filled with RBCs and the adjacent hypothalamus is edematous. Profiles of axons and dendrites are swollen. The capillary (cap) within this damaged area is of the continuous type and appears normal. X5400.

B. Ultrastructure of a stimulated capillary at the host interface. The endothelium is of varying heights, and contains numerous microvesicles. Notice the large perivascular space (pvs) containing collagen fibrils (col). One endothelial cell has a pseudopod-like process (P) projecting away from the vascular lumen. X14,400.

C. Fenestrated capillary (arrows) within an optic nerve graft showing a pseudopod-like process (P) guided apparently by pericyte (Per) processes. X28,500.
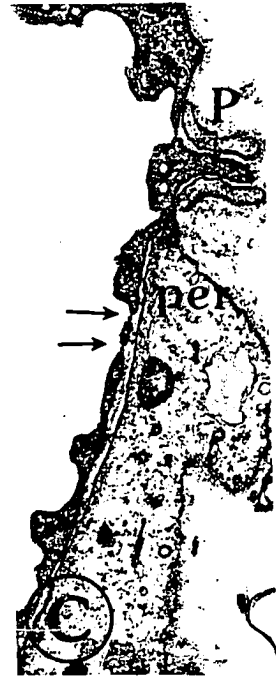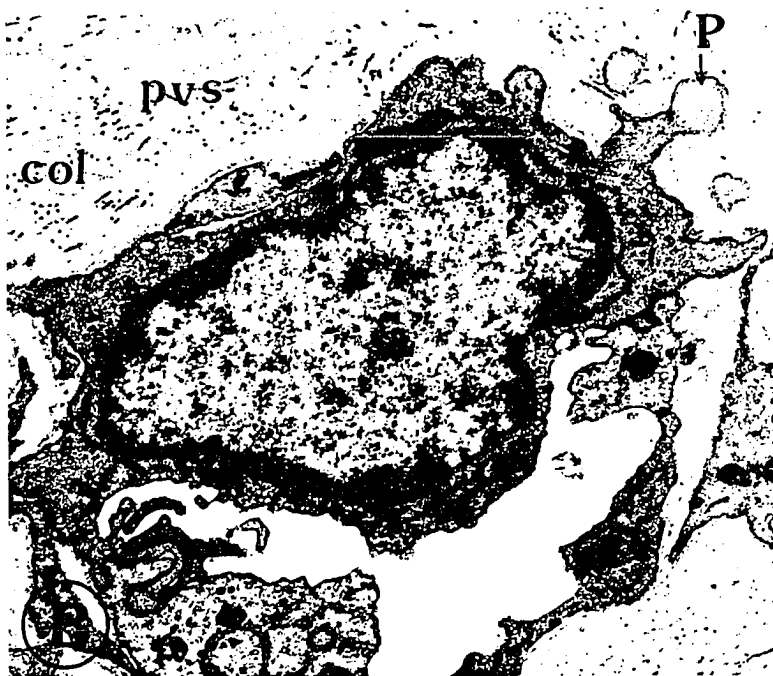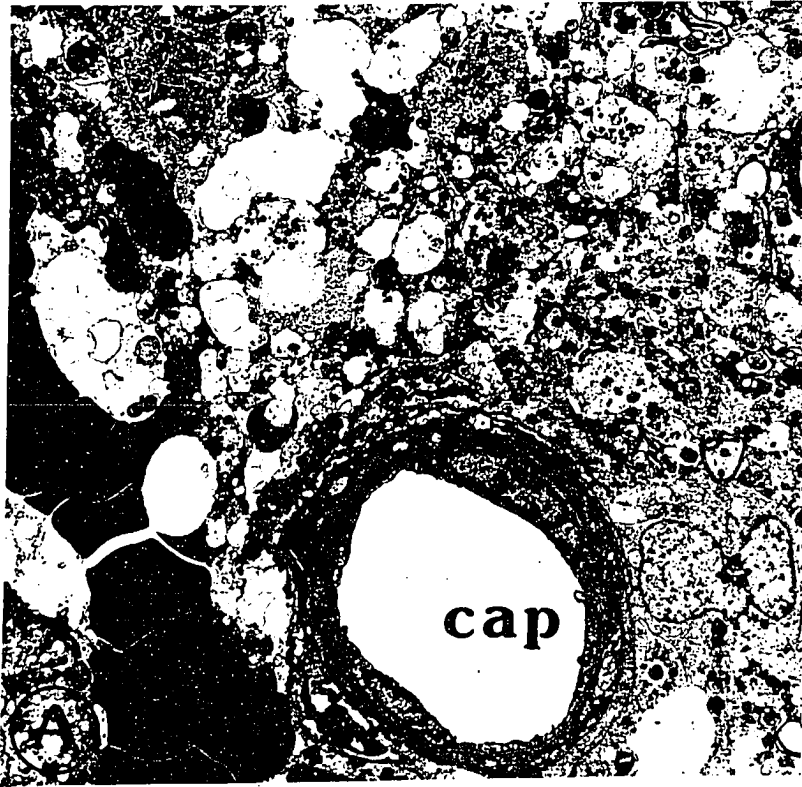
Fig. 5. 36 hours grafts

A. Pituicytes (Pit) of the transplanted neural lobe with numerous lipid droplets (L) and some phagosomes (ph). In contrast to the normal neural lobe, these pituicytes are characterized by the apparent retraction of their cytoplasmic processes. Neurosecretory axons (ns) between these pituicytes appear intact. X4,500.

B. Early signs of myelin disintegration are evident in this sciatic nerve graft. Neurolemmocyte-associated basal laminae (bl) remain closely apposed to the cell plasmalemma. X7,500.

C. Optic nerve graft showing axon and myelin degeneration. A vesiculated astrocyte (ast) is seen at the top of the picture. X4,500.

D. A neurolemmocyte (nlc) in a sciatic nerve graft, with distended rER, and a granulated appearance. X7,500.
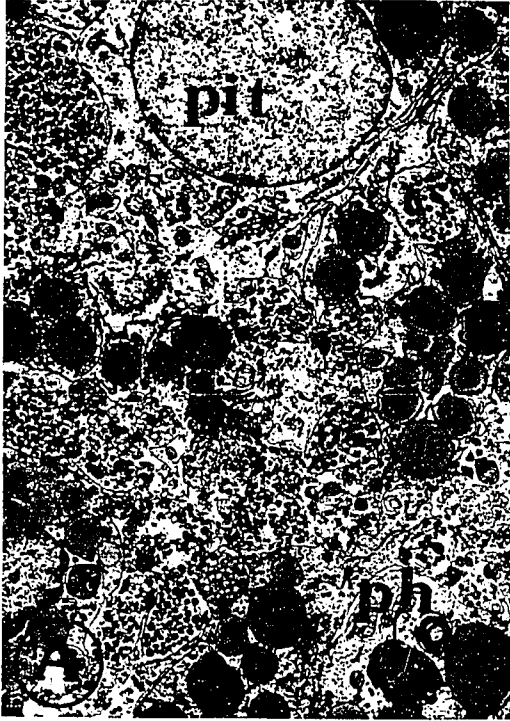
cross the host-graft interface at random sites, revealing that vascular anastomosis of the host and the grafts had already occurred (Fig. 6C).

Neural lobe grafts did not possess a BBB to HRP, and extensive leakage of HRP occurred from their rich capillary network (Fig. 6C). Perivascular cells containing HRP-positive granules were observed in the grafts.

The extravasation of HRP within sciatic and optic nerve grafts was not homogeneous, and it ranged from a very intense reaction in some areas, frequently in the graft periphery, to a faint one in others (Fig. 6E, G). Invariably the large vessels of the microvasculature were leaky. A few perivascular cells containing granular reaction product were present within sciatic nerve grafts, while in optic nerve grafts numerous reactive perivascular cells and scattered cells were present within the graft and the nearby hypothalamus.

The results of the neurophysin-immunohistochemical staining in the adjacent hypothalamus were identical in all experimental groups. Neurosecretory perivascular plexuses in the hypothalamus adjacent to the lesion or grafts were as numerous as at 18 and 36 hours, but their neurophysin-immunostaining was more pronounced (Fig. 12C, D; Table 1). Proximal to the lesion, neurophysin-positive axon dilatations were larger than at previous time periods. A few delicate,

beaded neurosecretory axons were present in the scar tissue, which obliterated the lesion site (Fig. 6B). Distal to the lesion, mostly spherical accumulations of neurophysin-positive material were observed. In some lesioned animals cavities had formed that were either empty or partially filled with erythrocytes or loose connective tissue. In the first animals, neurophysin-positive axons remained confined to the hypothalamus. In other animals, however, neurosecretory axons had penetrated the connective tissue within which they formed dense perivascular plexuses.

Most grafted pieces had coalesced obliterating any noticeable line of demarcation. Invasion of the grafts by neurosecretory axons was conspicuous in all types of grafts, but the distribution of regenerating axons varied with the graft type. Within neural lobe grafts, thin and finely beaded neurosecretory axons and variably sized spherical aggregates were present (Fig. 6D). Neurophysin-negative areas were also observed. Local variations in the density of neurosecretory axons were found to correlate with capillary density. Areas of high capillary density contained the largest accumulations of neurophysin-positive material.

Within sciatic nerve grafts, a large number of regenerating neurosecretory axons was concentrated at the graft periphery. Occasionally, neurosecretory axons with numerous swellings penetrated deeply into the graft (Fig. 6F).

In optic nerve grafts, individually coursing neurosecretory axons were either fine or coarse with dilatations reaching the size of those seen in the normal neural lobes (Fig. 6H). Around the microvasculature of the graft periphery, neurosecretory axons formed dense plexuses.

These findings confirm and expand previous observations by Dellmann et al. (1985; 1986; 1987a,b).

Electron microscopic observations. The hypothalamus in the vicinity of the cut or the grafts was still damaged, although less so than at previous time periods (Fig. 7A). The morphology of damaged hypothalamic tissue proximal and distal to the lesion or grafts varied within wide limits between individual animals. In some rats, damage was minimal and only indicated by slightly enlarged intercellular spaces, some degenerating neurites, and phagocytic microglial cells. Macrophages containing large phagolysosomes were also present within that tissue; frequently they occurred around the wall of arterioles or venules. In others, extracellular spaces were wide, and degenerating neurites were numerous. Large profiles of neurosecretory axons and many growth cones were observed proximal to the cut, many of them in the vicinity of and even within the scar. Large rounded axonal profiles containing mainly NGVs and lysosomes, representing "retraction balls" observed with the light microscope, dominated the picture distal to the lesion.

Fig. 6. 10 days survival time

A. The BBB is reestablished at the lesion which is only visible due non-specific stained macrophages (arrows). X80.

B. Following the resorption of the hemorrhagic clot, proximal (P) and distal (D) stumps are separated by a thin glial scar. Neurosecretory axons are proximal to the lesion and retraction balls (arrows) distal to it. X50.

C. Reaction product of blood-borne HRP inundates the entire neural lobe graft and to a lesser extent the adjacent hypothalamus (arrows). Notice the vascular connections between the graft and the host hypothalamus (curved arrows). X60.

D. Neurophysin-immunoreactive axons have penetrated and permeated the neural lobe graft. X80.

E. Sciatic nerve graft showing an inhomogeneous HRP staining. Some areas around blood vessels are strongly reactive (arrows). X80.

F. Neurophysin-positive axons have regenerated into sciatic nerve graft. Retraction balls are seen distal (D) to the graft. Notice that the graft is not homogeneously permeated by regenerating neurosecretory axons. X60.

G. This optic nerve graft is inundated by blood-borne HRP which is inhomogeneously distributed. X60.

H. Delicate neurophysin-positive axons (arrows) have invaded inhomogeneously this optic nerve graft. X60.

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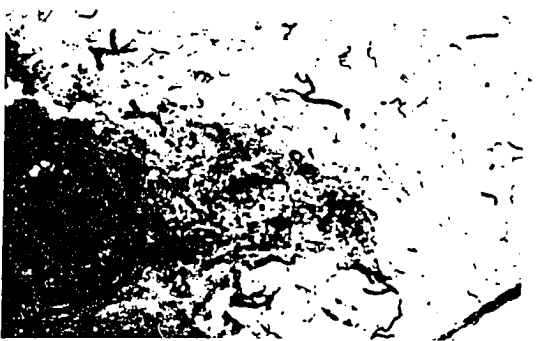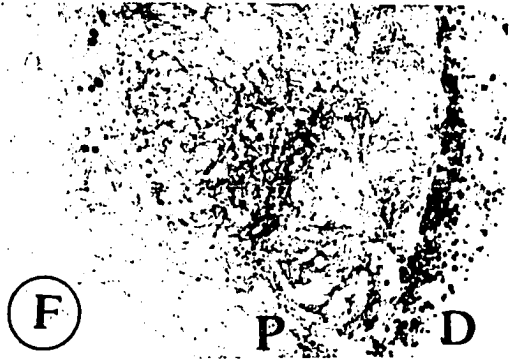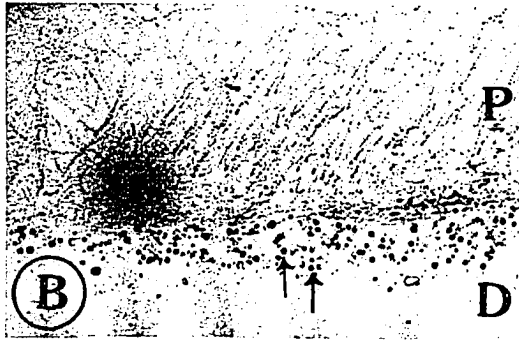
Neurosecretory perivascular plexuses were present around
arterioles, venules and some capillaries. They consisted
mainly of one layer, and rarely of several layers of
neurosecretory axonal profiles containing NGVs, mitochondria
and clustered microvesicles; the latter were concentrated at
points of contact between axon terminals and basal laminae.
These axonal profiles were associated with neurolemmocyte-like
cells and/or astrocyte processes containing bundles of
intermediate filaments. The perivascular spaces within the
lesion site and the host interface were wider than in
controls. Stimulated capillaries were frequently seen both
within the glial scar and in the immediate vicinity of the
lesion site. These capillaries were of the continuous type
with tight junctions between endothelial cells.

Irrespective of the graft type, the host-graft interface
consisted of several layers of thin and long astrocyte
processes containing densely packed glial filaments, or of one
or several layers of thicker astrocyte processes and/or
astrocyte cell bodies (Figs. 7C and 9B). The astrocyte layer
facing the graft was covered by a basal lamina. Frequently,
irregularly shaped and branching astrocyte processes
penetrated into the connective tissue of the graft for short
distances, occasionally even deeply into the grafts. In some
instances, these astrocyte processes were associated with
axonal growth cones or neurosecretory axons.

Fig. 7. 10 days survival time

A. In the hypothalamus surrounding the lesion. Astrocyte (ast) processes and axonal and dendritic profiles are present within wide intercellular spaces. Notice the continuous type of capillary (cap) with this area. X5,400.

B. Pituicyte (pit) with a large phagosome (ph) containing remnant of NGVs and a myelin figure. Pituicyte cytoplasmic processes envelop a regenerating neurosecretory axon (ns). X15,000.

C. Basal lamina (bl)-surrounded astrocyte processes accompanying regenerating neurosecretory axons penetrate the neural lobe graft (G). X12,000.

97
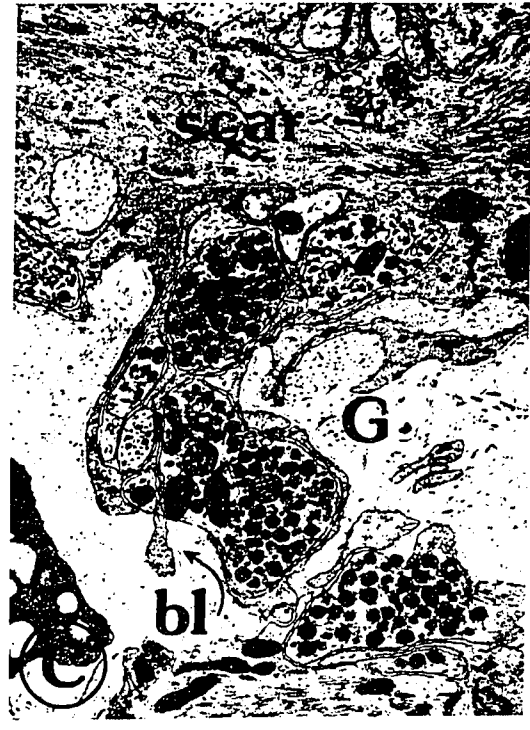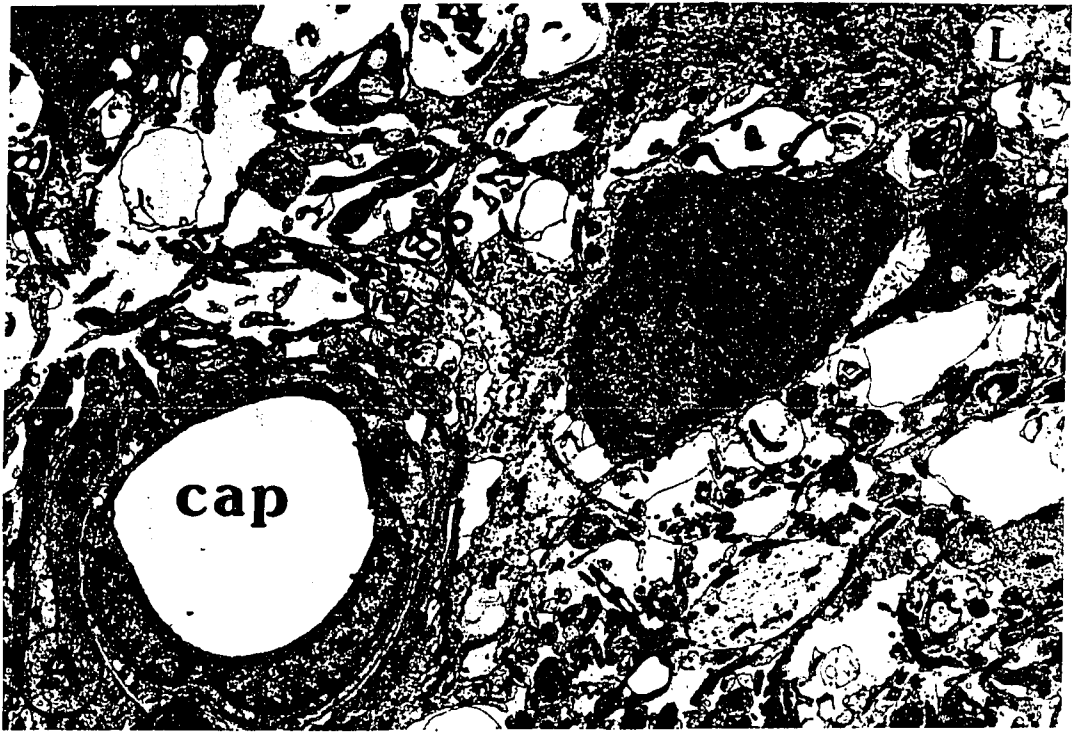
Within the neural lobe transplants a striking increase,
as compared to intact neural lobes, was observed in the number
of delicate collagen fibrils and associated fibroblasts.
Pituicytes were readily identified by their lipid inclusions
of varying density and frequency, and by occasional dense
bodies representing remnants of phagocytosed axons (Fig. 7B).
In many pituicytes, rough endoplasmic reticulum and especially
Golgi complexes and associated vesicles were numerous (Fig.
8A). Pituicyte cell bodies and, more frequently, pituicyte
cytoplasmic processes were in close contact with regenerating
neurosecretory axons surrounded by either straight or crenated
basal lamina tubes. These basal lamina tubes frequently
enclosed large spaces containing electron dense granular
material which was also found free in the extracellular matrix
intermingled with collagen fibrils. Neurosecretory axonal
profiles were both terminals (i.e., containing numerous
microvesicles, and in contact with a basal lamina) and
preterminals (i.e., usually without or only a few
microvesicles, and separated from the basal lamina by
pituicyte processes). Most capillaries of neural lobe grafts
were fenestrated, but continuous capillaries were likewise
present, especially in the graft periphery. Macrophages were
rare and occurred mainly at the host-graft interface. They
contained many homogeneous lipid droplets together with
phagolysosomes. Profiles of stimulated capillaries were seen

at the graft periphery.

Sciatic nerve grafts contained numerous macrophages that were in the process of disposal of degenerating axons and myelin sheaths. The endoneurial connective tissue was more abundant than in control animals. It consisted of fibroblasts with often elongated thin cytoplasmic processes, large amounts of collagen fibrils, and abundant amorphous ground substance. Within this connective tissue, several basal lamina-bounded neurolemmocytes or profiles of empty scalloped basal lamina scaffolds were present (Fig. 8B). Associated with these neurolemmocytes were varying numbers of growth cones and axons, many of which were identified as neurosecretory. Most neurosecretory axons were located within cytoplasmic invaginations of the neurolemmocyte cell body, others were surrounded or partially ensheathed by their cytoplasmic processes (Fig. 8D). Three types of capillaries were discerned in the grafts. Continuous capillaries with tight junctions between endothelial cells, characteristic of intact sciatic nerve, predominated. Small-diameter stimulated capillaries and fenestrated capillaries were less frequent. In the last, only occasional fenestrae were observed.

Optic nerve grafts were dominated by densely packed macrophages containing degenerating axons (Fig. 9A), myelin sheaths and numerous lipid inclusions. Astrocytes and oligodendrocytes were not easily identified (Fig. 8C). Optic

Fig. 8. 10 days survival time

A. Cytoplasm of a pituicyte containing several Golgi complexes (arrows) with numerous associated vesicles as an indication of secretory activity. X10,000.

B. Folded basal lamina scaffolds (bls) with several profiles of regenerating axons accompanied by neurolemmocyte processes in sciatic nerve graft. A fibroblast (F) is laying in the dense collagen matrix. X14,400.

C. In the adjacent hypothalamus of an optic nerve graft with numerous retraction balls (rb) are present. A macrophage (M) filled with lipid inclusions is seen at the host-graft interface. X4,000.

D. Profiles of regenerating neurosecretory axons are surrounded by neurolemmocytes (nlc) and fibroblasts (F). X5,400.
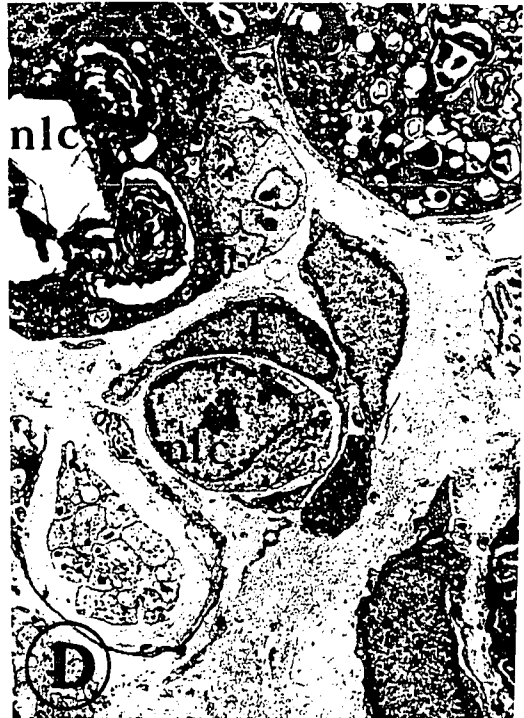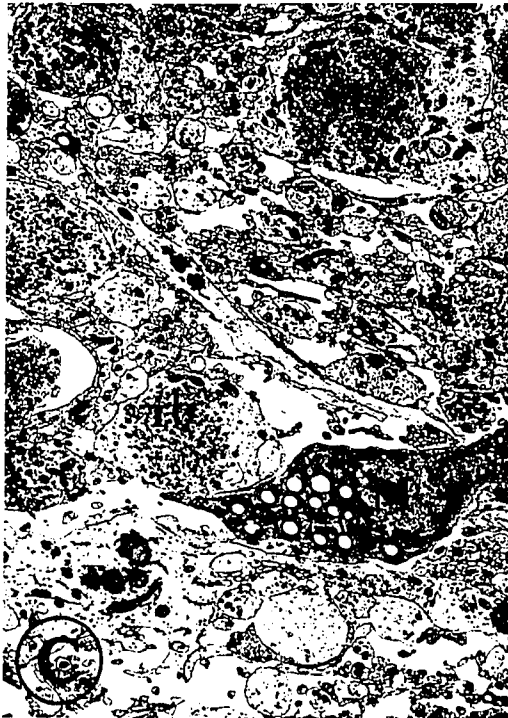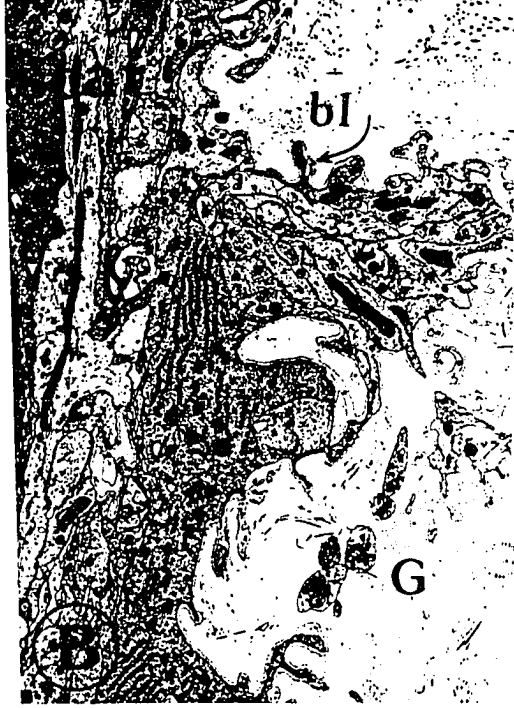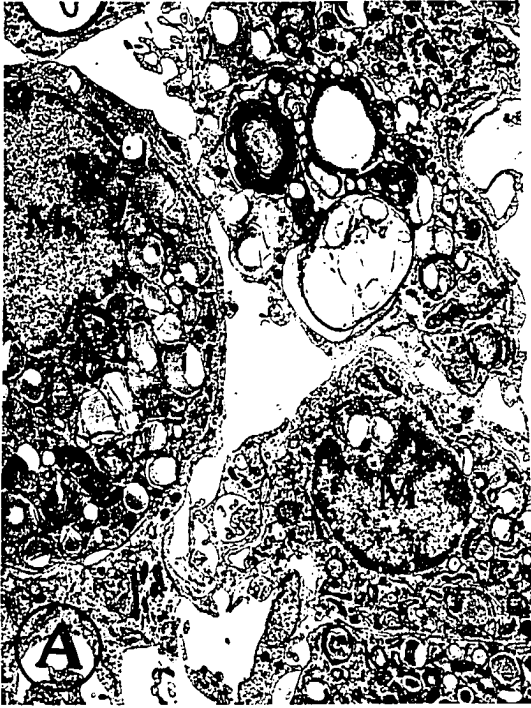
Fig. 9. 10 days survival time ·

A. Disposal of myelin remnants by macrophages (M) in an optic nerve graft. X4,500.

B. Astrocyte projections from the scar at the host-optic nerve graft interface extend into the graft (G). These astrocyte processes are covered by a basal lamina (bl). X4,750.

nerve pieces were partially surrounded by connective tissue areas of varying extent, within which fenestrated capillaries and numerous stimulated capillaries were found. The occurrence of fenestrated capillaries is an unexpected finding because fenestrated capillaries are not present in optic nerve in situ. The periphery of the graft was most densely invaded by neurosecretory axons, which were rarely present within the areas with numerous phagocytes. Perivascular neurosecretory axons were always associated intimately with basal lamina-enclosed neurolemmocyte-like cells, either invaginated into the cytoplasm or surrounded by thin processes. Thus, the light microscopic observation of perivascular plexuses was confirmed.

The fine structure of HRP reaction product in all permeable grafts and at all times was similar. Some blood vessels were densely filled with HRP reaction product, whereas others contained very little or even no HRP reaction product. Within endothelial cells, as well as in perivascular granulated and scattered granulated cells, the tracer was associated with vesicles, multivesicular bodies and tubular profiles (Fig. 10A, C and D). It was also detected in endothelial basal laminae in adjacent perivascular spaces (Fig. 10B, E). Occasionally neurosecretory swellings and terminals contained some HRP-filled vesicles. HRP reaction product was also seen in some perineurial cells of sciatic

nerve grafts (Fig. 10F).

30 days survival time

Light microscopic observations. The pattern of HRP extravasation in the grafts and surrounding hypothalami was similar to that reported at 10 days, but the amount of HRP leakage in the adjacent hypothalami had declined as compared to the previous time periods (Fig. 11C, E and G). Consistent HRP extravasation was associated with large caliber vessels both proximal and distal to the lesions or the grafts (Fig. 11A, C). Optic nerve grafts showed regional variations in HRP reaction product depending upon graft location. HRP leakage was more visible and intense in grafts located in proximity to the ventral pial surface than in those situated more dorsally in the hypothalamus (Figs. 11G and 12A).

Neurosecretory perivascular plexuses around the microvasculature immediately proximal, and to a lesser extent, distal to the lesion or the grafts were more numerous and intensely stained than at 10 days survival time (Fig. 12D; Table 1). Within the glial scar of the lesion, neurosecretory perivascular plexuses surrounded some large caliber vessels (Fig. 11B). In some rats, cavities were present at the site of lesion. In the presence of such cavities, neurosecretory axons formed perivascular plexuses only proximal to the

Fig. 10. HRP-histochemistry at the fine structural level

A. HRP is present in a multivesicular body (large arrow) and cytoplasmic tubular profiles of an endothelial cell of a continuous capillary (cap) in a neural lobe graft. 10 days. X28,500.

B. HRP reaction product in a neural lobe graft is homogeneously distributed within the endothelial basal lamina and extends into the intercellular space adjacent to a capillary endothelial cell (ec). 30 days. X8,400.

C. A perivascular granulated cell process, located in the perivascular space of a capillary in an optic nerve graft, contains HRP dense bodies (arrows). 10 days. X14,400.

D. A scattered granulated cell within a 30-day optic nerve graft containing HRP dense bodies. X28,500.

E. Sciatic nerve graft with HRP reaction product filling the pericapillary (arrowheads) and interstitial (arrows) spaces. 30 days. X14,400.

F. HRP dense bodies (arrows) within cytoplasmic processes of a perineurial cell (pc) surrounding a bundle of regenerating axons. Sciatic nerve graft, 30 days. X28,500.
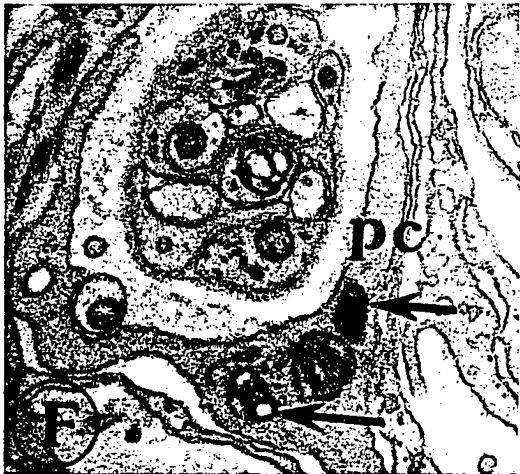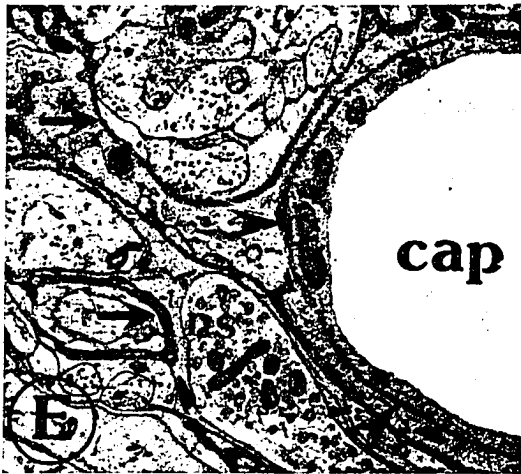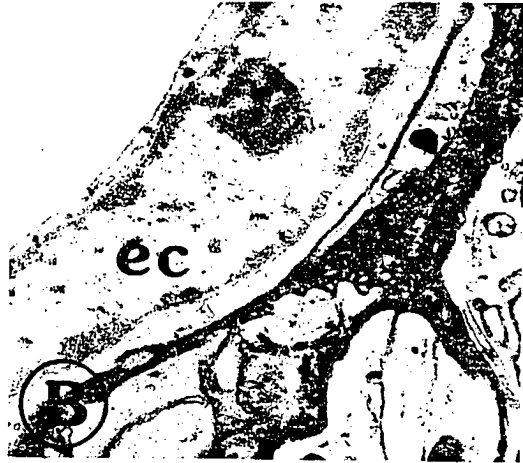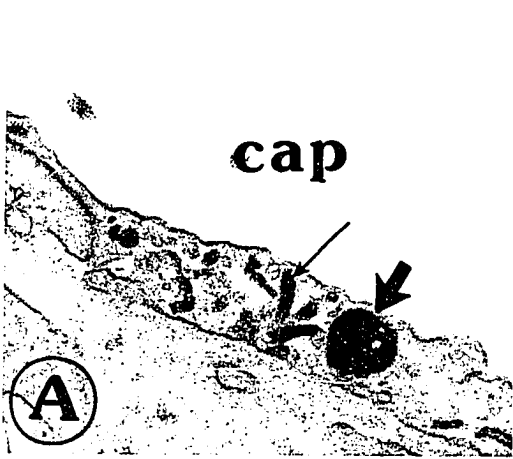
cavities and in the connective tissue, which fills partially these cavities.

In neural lobe grafts, neurophysin-negative areas were no longer present. The distribution of neurophysin immunoreactivity was similar to that seen in the normal neural lobe in situ, with some single, beaded neurosecretory axons coursing through the graft. Regenerating neurosecretory axons closely invested the rich capillary bed of the entire graft (Fig. 11D).

Within sciatic nerve grafts, neurosecretory axons were also more numerous, although fewer than in the neural lobe grafts, and they permeated virtually the entire graft (Fig. 11F). Most of these neurosecretory axons, however, were located in the graft periphery, where they were most heavily concentrated proximally. These axons were enlarged and possessed numerous swellings.

Only relatively few neurosecretory axons had invaded optic nerve grafts. These axons were mostly concentrated in richly vascularized areas of the graft, i.e., in grafts situated ventrally in the hypothalamus (Fig. 11H). Regenerating neurosecretory axons were usually single, but occasionally they formed bundles. Only very few regenerating neurosecretory axons were seen in grafts located dorsally in the hypothalamus (Fig. 12B).

Fig. 11. 30 days survival time

A. The lesion is barely visible (arrows). Some large caliber vessels (arrowheads) within and adjacent to the lesion are surrounded by HRP reaction product. X63.

B. Only some perivascular neurosecretory plexuses are visible proximal and distal to the lesion. X80.

C. Neural lobe graft with a dense capillary network which is leaky to HRP that diffuses into the adjacent host hypothalamus (arrows). X120.

D. Neurophysin-immunohistochemistry of neural lobe graft showing the extent of regenerating neurosecretory which closely invest the rich capillary bed of this neural lobe graft. X100.

E. Sciatic nerve graft entirely filled with HRP reaction product. Notice the faint leakage of HRP into the adjacent hypothalamic tissue (arrows). X40.

F. Regenerating neurosecretory axons are permeating the entire sciatic nerve graft. X140.

G. Optic nerve graft located at the ventral hypothalamus shows strong HRP extravasation as does the periventricular surface visible at the left hand side. X160.

H. This picture of an optic nerve graft, taken at a corresponding level of the one in Fig. 5G, shows vigorous neurosecretory axon regeneration. X200.
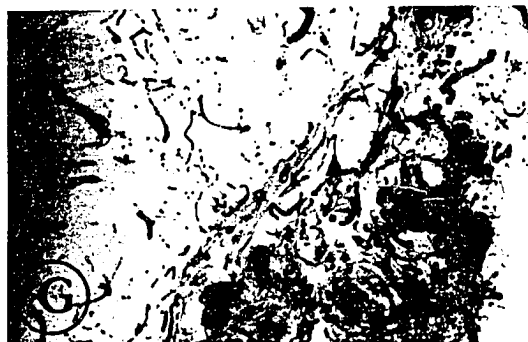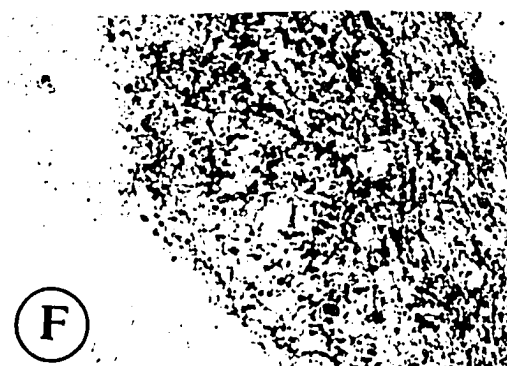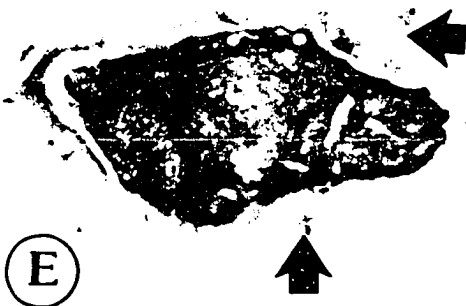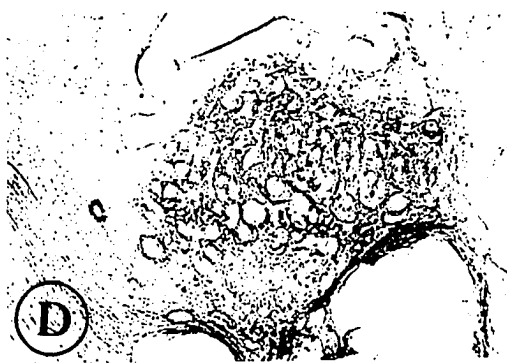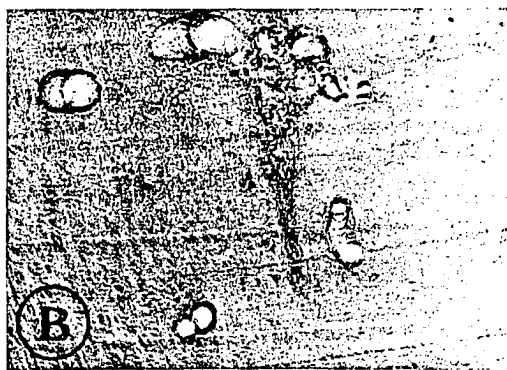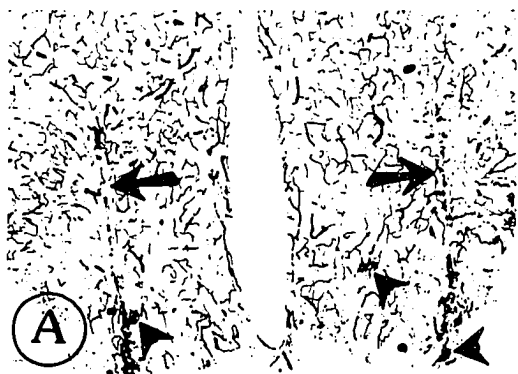
Fig. 12. 30 days survival time

A. HRP reaction product is absent in this optic nerve graft situated dorsally in the hypothalamus. X80.

B. There are only a few regenerating neurosecretory axons (arrows) in this optic nerve graft situated in the dorsal hypothalamus. X110.

C. In the vicinity of a 10-day sciatic nerve graft, neurophysin-positive axons forming a single layer around a large caliber vessel (square). X60.

D. Higher power of the hypothalamic perivascular neurosecretory plexus of the square in figure 6C. X150.

E. Dense perivascular neurosecretory plexuses within the hypothalamus adjacent to a 30-day neural lobe graft. Notice the increased number of neurosecretory axon layers and the intensity of staining. X120.
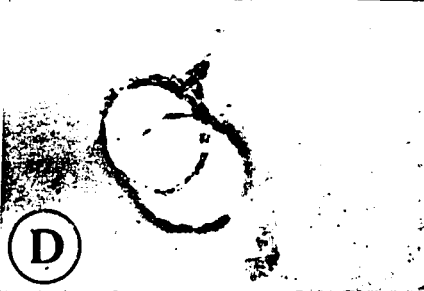
Electron microscopic observations.   Fine

structural analysis of 30-day lesioned specimens confirmed the

immunohistochemical light microscopic observations. The

astrocytic scar had almost completely replaced the damaged

hypothalamic tissue; it was wider than at 10 days, and was

composed of tightly packed lamellar processes which contained

numerous bundles of intermediate filaments (Fig. 13A).

Neurosecretory perivascular plexuses were present around

arterioles and venules (Figs. 13B and 14A), which were

surrounded by wide connective tissue spaces bounded

peripherally by a parenchymal (astrocytic) basal lamina.

These perivascular plexuses were composed of bundles of

neurosecretory axons wrapped by neurolemmocyte-like cells

(Fig. 14B). The ensheathing cells were surrounded by a basal

lamina and regularly embraced groups of neurosecretory axonal

profiles containing numerous NGVs and clusters of

microvesicles characteristic of axon terminals (Fig. 15A).

Axon bundles occurred in several layers. The innermost layer

was adjacent to the endothelial or muscular basal lamina.

Successive layers were separated by fibroblasts and/or their

thin cytoplasmic extensions, or by intercellular spaces

containing small bundles of collagen fibrils. Single

neurosecretory axons unrelated to the vascular bed were also

identified within the glial scar.

Stimulated capillaries were frequently observed within

the scar tissue and adjacent hypothalamus. These capillaries were sometimes accompanied by regenerating neurosecretory axons (Fig. 15B).

The glial scar at the host-graft interfaces consisted of only one or two layers of delicate astrocytic processes in few locations (Fig. 16A). At times, cytoplasmic projections of astrocytes processes into the grafts were observed in association with regenerating neurosecretory axons. In grafted neural lobes, neurosecretory axonal profiles were abundant throughout the entire grafts (Fig. 16B, C). Preterminal axons and axon terminals were in contact with each other and/or with pituicytes. Axon terminals formed palisades along perivascular basal laminae (Fig. 17B). At the point of contact between axolemma and the basal lamina, microvesicles formed clusters of varying extent and density (Fig. 17A). It was not unusual to observe axon terminal profiles that contained exclusively either dispersed or clustered microvesicles. Occasionally neurosecretory axons not enclosed by a basal lamina were present in the perivascular spaces. Pituicytes had fine structural features and spatial relationships with axon and axon terminals identical to those observed in control neural lobes. Some pituicytes possessed extensive Golgi complexes. Most capillaries were sinusoidal with fenestrated endothelia (Fig. 16C). The perivascular spaces were of varying widths, and frequently protruded with

Fig. 13. 30 days survival time

A. The glial scar at the lesion site consists of several layers of astrocyte (ast) cell bodies or processes. X5250.

B. Numerous neurosecretory axons (ns) and axon terminals (arrows) together with accompanying astrocyte processes (ast) are forming at least four layers within this wide perivascular space (pvs) in the scar. X7,500.
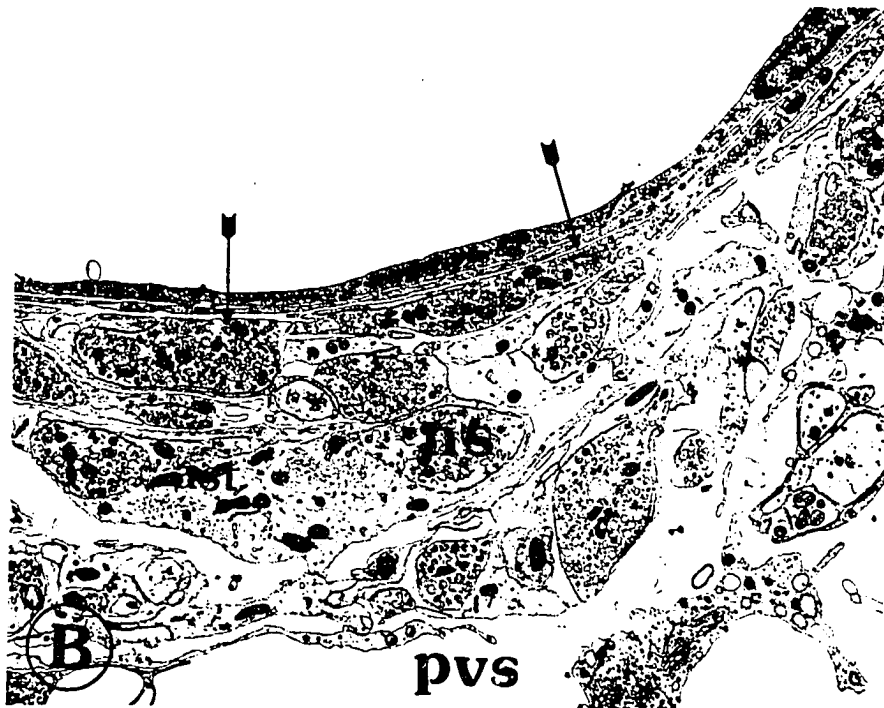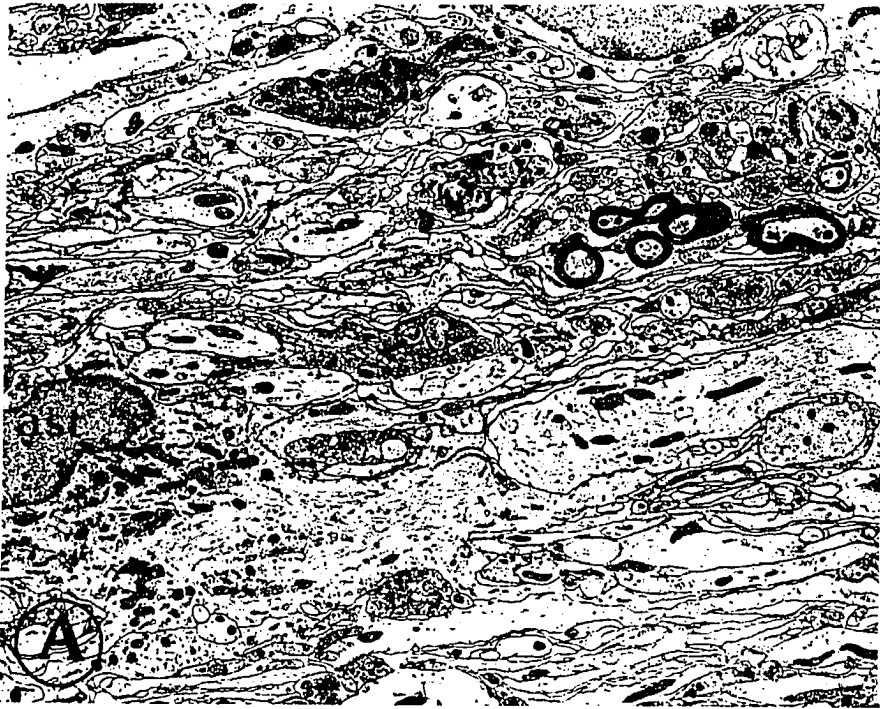
Fig. 14. 30 days survival time

A. Neurosecretory axon regeneration into a perivascular space of the hypothalamus adjacent to the lesion. This pericytic venule (ven) is partially surrounded by a multilayered perivascular neurosecretory plexus. Axonal profiles are ensheathed by neurolemmocyte-like cells (nlc). X2400.

B. Bundles of neurosecretory axons (ns) are wrapped by the processes of a neurolemmocyte-like cell (nlc) surrounded by a basal lamina (bl). X14,400.

ven
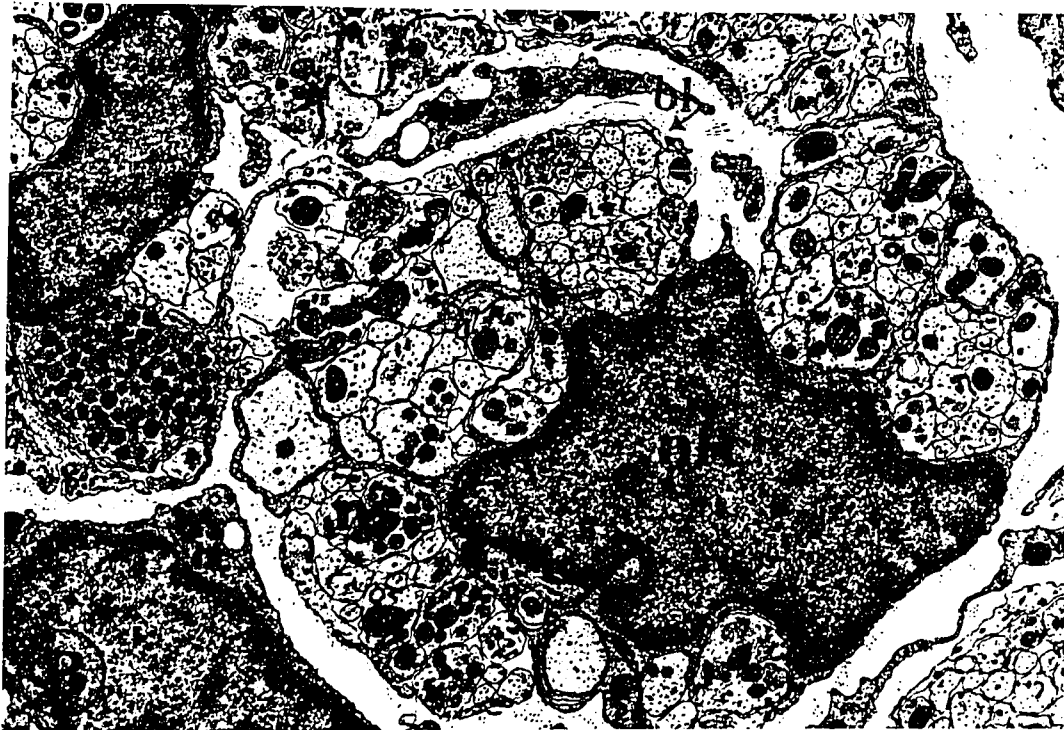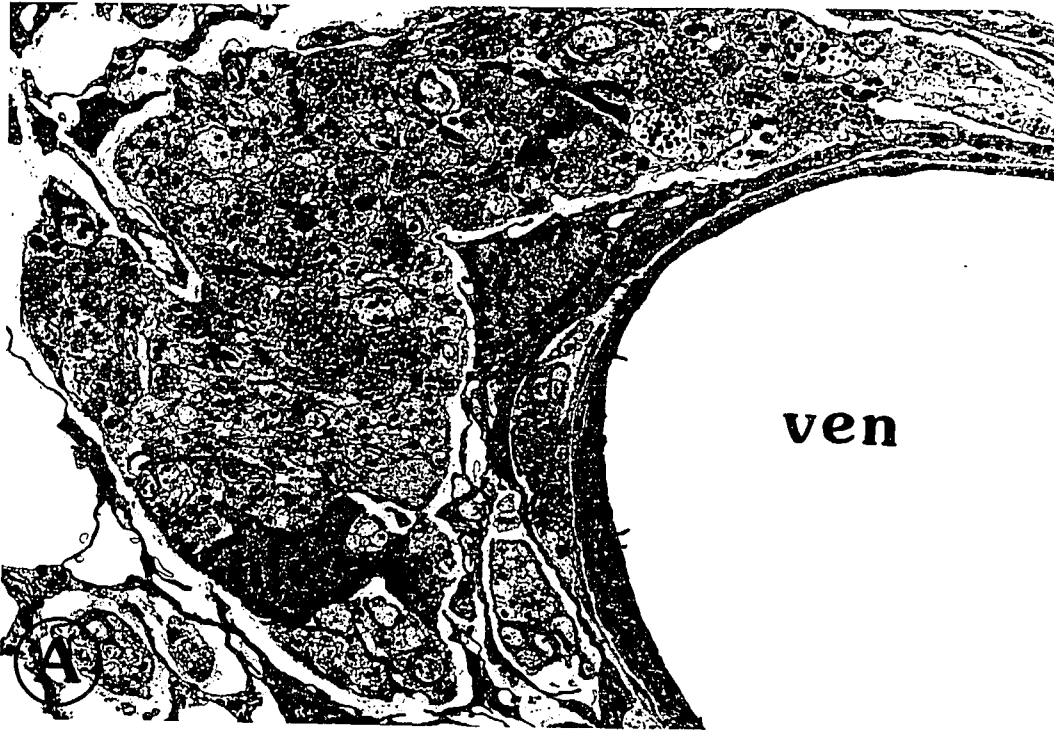
Fig. 15. 30 days survival time

A. Neurosecretory axon terminals abutting the perivascular space of an arteriole. These terminals contain NGVs (arrowheads) as well as clusters of microvesicles (mv). 30 days. X28,500.

B. Stimulated capillary, with a small lumen and high endothelial cell, surrounded by pericyte processes (per) and neurosecretory axon (ns). 30 days. X28,500.

narrow channel-like projections into the surrounding tissue
(Fig. 17A). Larger expansions of the perivascular space often
included folded pieces of basal laminae. At times, the
parenchymal and endothelial basal laminae were fused. A
consistent feature of all grafted neural lobes was the
presence of small-diameter non-fenestrated capillaries, some
of which were characteristic stimulated capillaries (Fig.
16B). These capillaries were surrounded by wide perivascular
spaces or only the fused parenchymal and endothelial basal
laminae.

The fine structural characteristics of sciatic nerve
grafts were essentially the same as those observed at 10 days.
The phagocytic disposal of axons and myelin, however, was more
advanced, and regenerating neurosecretory axons were
considerably more abundant and often occurred in bundles (Fig.
18A, B). At times, single neurosecretory axons were seen
within a basal lamina scaffold, not accompanied by
neurolemmocytes. The capillary types and the frequency of
their occurrence were the same as at 10 days.

In optic nerve grafts, phagocytosis of axons and myelin
sheaths was still in progress, although the number of
macrophages was greatly reduced. Astrocytes had undergone
reactive gliosis, virtually substituting the grafted tissue by
a glial scar (Fig. 19A). Regenerating neurosecretory axons
together with their ensheathing astrocytic processes were

Fig. 16. 30 days neural lobe graft

A. The glial scar around this neural lobe graft is formed by one layer of astrocyte processes. The capillaries are of continuous type and have a small diameter. The graft is dominated by numerous neurosecretory axons together with pituicytes (pit). X5,400.

B. The intercapillary neuropil is packed with neurosecretory axons. Notice the stimulated capillaries with small diameters. X5,400.

C. Neurosecretory axons (ns) abutting perivascular connective tissue spaces and intimately associated with pituicytes (pit). The capillaries are sinusoidal and fenestrated (arrows). X8,000.
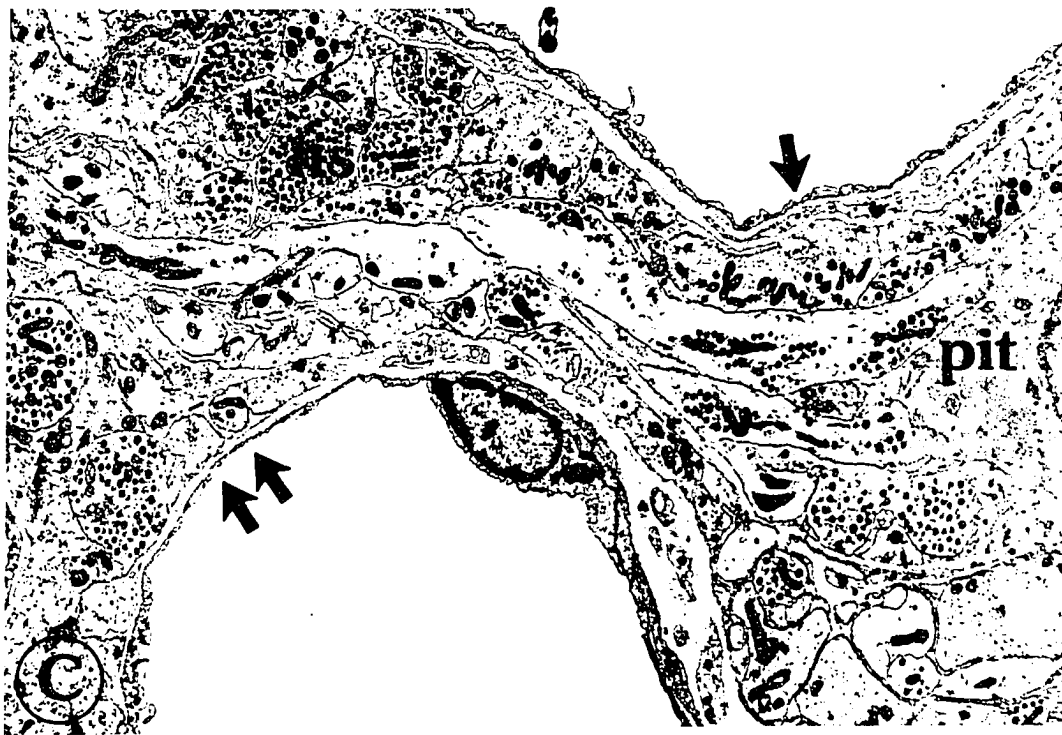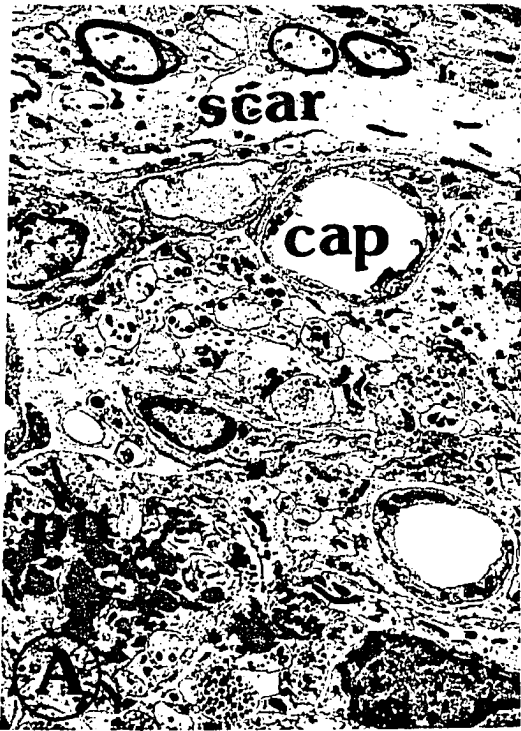
Fig. 17. 30 days neural lobe graft

A. Neurosecretory axon terminals are totally or partially ensheathed by pituicyte processes (pit) as in the control neural lobe. Secretory activity is evidenced by numerous clustered or dispersed microvesicles (mv) located at the vicinity of extensions of perivascular spaces protrusions (pvs). X28,500.

B. Axon terminals with clustered microvesicles (mv) form palisades at a pericapillary space of a fenestrated capillary (arrow). A pituicyte (pit) is ensheathing some neurosecretory axons and terminals in this field. X14,400.
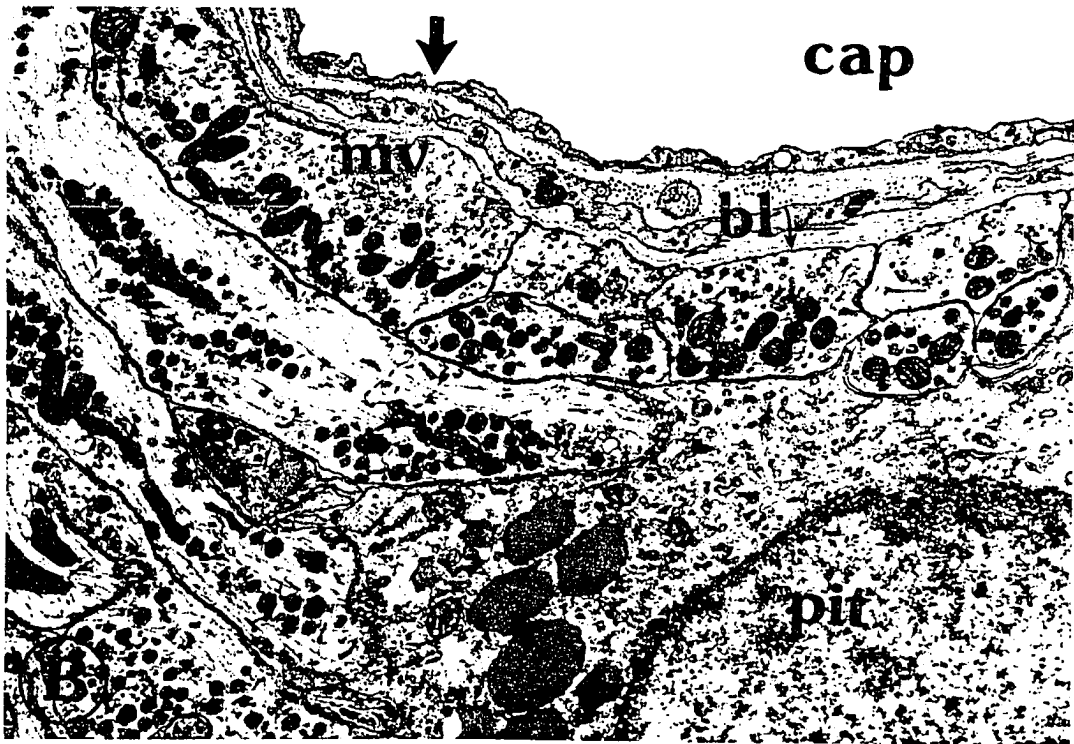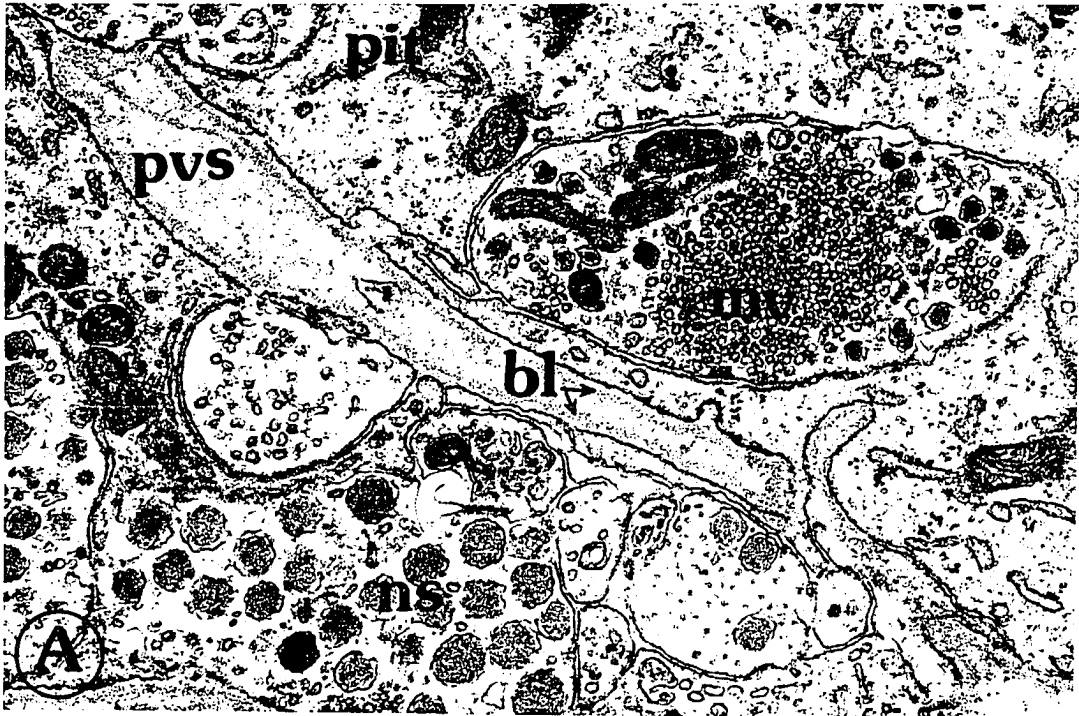
Fig. 18. 30 days sciatic nerve graft

A. Cross section of numerous neurolemmocyte-enclosed bundles of axonal profiles within a sciatic nerve graft. Some of these axonal profiles, neurosecretory (ns) in nature. All axons are ensheathed by neurolemmocyte (nlc) and/or their processes. X5,400.

B. Neurosecretory axons (ns) ensheathed by neurolemmocyte processes (nlc) are abutting the perivascular space (pvs) of a fenestrated capillary. X8,400.
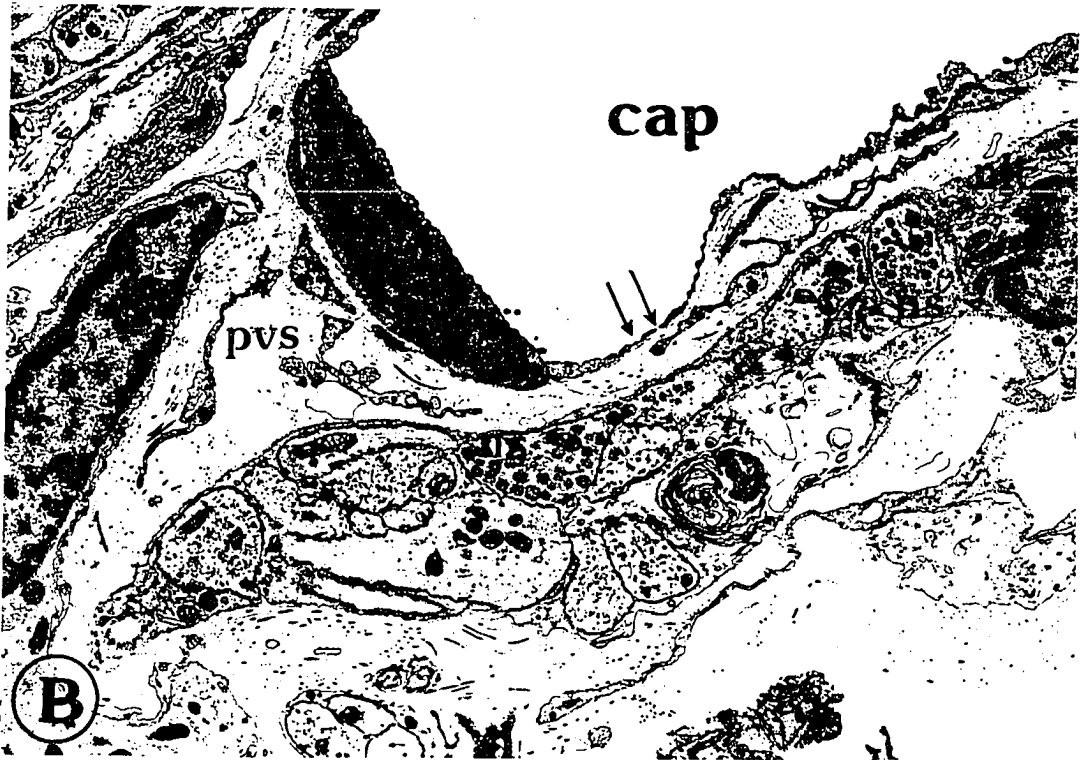
observed either singly or grouped into bundles in connective tissue spaces. Around venules and occasionally around capillaries, they were arranged in several layers (Fig. 19B, C). When neurosecretory axons abutted the basal laminae of these vessels, they contained NGVs and dispersed or clustered microvesicles (Fig. 19C). Capillaries located in this scar tissue were of the continuous type and were surrounded by a continuous basal lamina. Stimulated capillaries, some of which were fenestrated, were commonly seen within the connective tissue areas at the periphery of optic nerve grafts.

80 days survival time

Light microscopic observations. The only noticeable change in the lesion group as compared to previous time periods was the disappearance of HRP-positive perivascular and scattered cells at the lesion site (Fig. 20A).

HRP extravasation within neural lobe grafts was comparable to that observed at previous times, but it was less pronounced in the immediate vicinity of the graft (Fig. 20B). Phagocytic cells with HRP-positive granules were principally associated with large vessels of the microvasculature, both in the graft and at the host-graft interface. Their number, however, had conspicuously decreased as compared to previous

time periods.

In sciatic nerve grafts, the intensity of HRP labelling gradually decreased in ventro-dorsal direction as in the optic nerve at 30 days. While a strong reaction was observed in grafts located ventrally (Fig. 20E), reaction product was scarce in grafts located more dorsally in the hypothalamus (Fig. 20F). Perivascular and scattered cells with granular HRP-reaction product had the same topographic distribution as at 30 days. Their number decreased in parallel with the staining intensity of the graft.

Within optic nerve grafts, the distribution of HRP reaction product was the same as at the previous time period. In addition, numerous perivascular and scattered HRP-positive cells were still present within the grafted tissue and the surrounding hypothalamus.

In all animal groups, hypothalamic perivascular neurosecretory plexuses were less numerous and less intensely stained than at 30 days. In addition, the number of layers forming these perivascular neurosecretory plexuses also decreased (Table 1).

Neural lobe grafts were characterized by a neurophysin-immunoreactivity essentially identical to that of the normal neural lobes in situ (Fig. 20C, D).

Fig. 19. 30 days optic nerve graft

A. Neurosecretory axons (ns) between the astrocyte processes of an optic nerve graft. X15,000.

B. Neurosecretory axons (ns) together with ensheathing astrocyte processes (ast) are abutting a pericapillary space of a fenestrated (arrows) capillary in an optic nerve graft. X28,500.

C. Numerous neurosecretory axons (ns) with their enveloping astrocyte processes (arrows) around a large vessel in an optic nerve graft. X4,500.
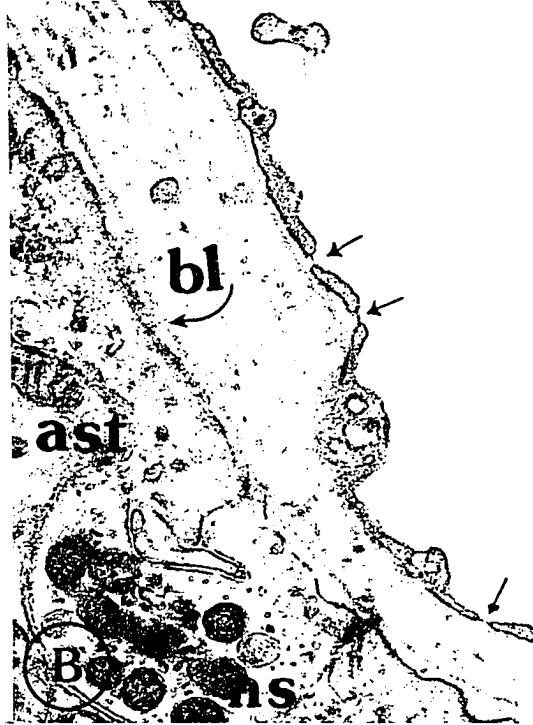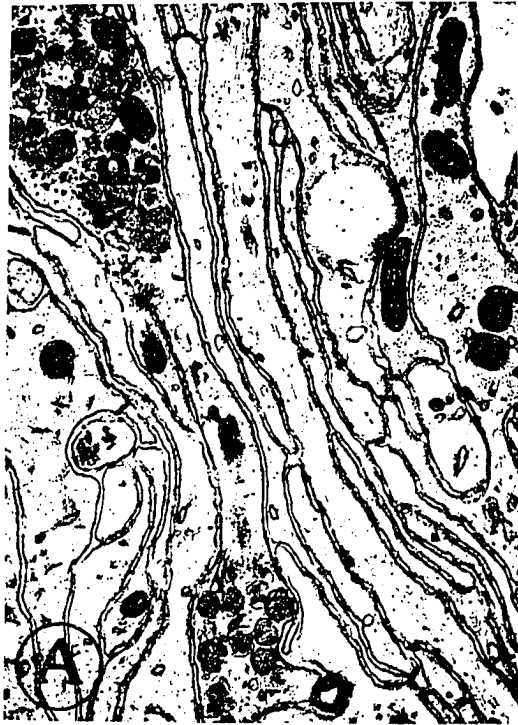
Within sciatic nerve grafts, neurosecretory axons were more numerous than at 30 days, particularly in sections located ventrally in the hypothalamus. At this level, neurosecretory axons were evenly distributed throughout the graft, while in the dorsal sections, only a few neurosecretory axons were seen (Fig. 20G).

In the periphery of optic nerve grafts, which had considerably diminished in size, regenerating neurosecretory axons formed densely woven perivascular plexuses that were less immunoreactive than at 30 days. A few single neurosecretory axons persisted throughout the grafted tissue.

Electron microscopy findings. The glial scar had replaced completely the damaged hypothalamic tissue (Fig. 21A). Neurosecretory perivascular plexuses were considerably less extensive than at 30 days. They consisted of one or two layers of neurosecretory axons occurring singly or in bundles (Fig. 21B). In addition, axon terminals contained mainly microvesicles and fewer NGVs.

The fine structural characteristics of neural lobe and optic nerve grafts (Fig. 23A, B) were the same as those reported at 30 days.

In sciatic nerve grafts, a few macrophages persisted and the connective tissue was less extensive than that observed at 30 days. There was a conspicuous increase in the number of regenerating neurosecretory axons in grafts located ventrally

Fig. 20. 80 days survival time

A. Neurosecretory axons are absent from the scar except in some perivascular plexuses (arrows). X80.

B. Neural lobe graft is leaky to blood-borne HRP; the intensity of leakage at the nearby hypothalamus has diminished. Notice vascular connections between the graft and host tissue (curved arrows). X60.

C. The density and pattern of regenerating neurosecretory axons are similar to those observed at 30 days. X50.

D. High magnification of the square in figure 20C showing the close investment of regenerating neurosecretory axons of the vascular bed. X120.

E. This sciatic nerve graft located in the ventral hypothalamus exhibits strong HRP reaction product. X30.

F. Within the dorsal hypothalamus, however, the sciatic nerve graft is not labelled by HRP reaction product. Only macrophages are faintly labelled with endogenous peroxidase. X70.

G. In sciatic nerve graft situated ventrally in the hypothalamus containing numerous finely beaded neurosecretory axons (arrows). X120.
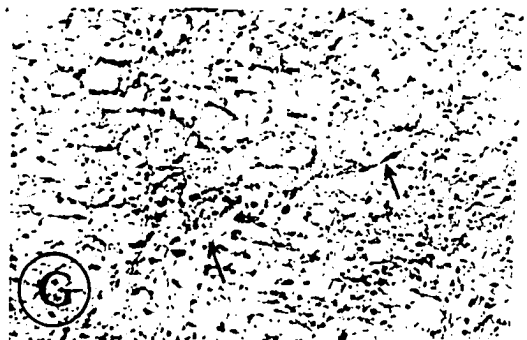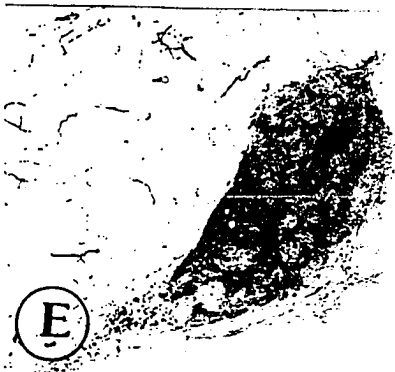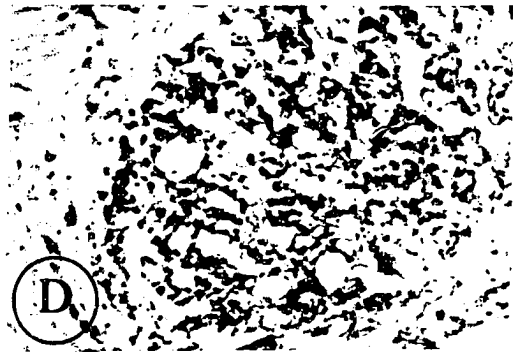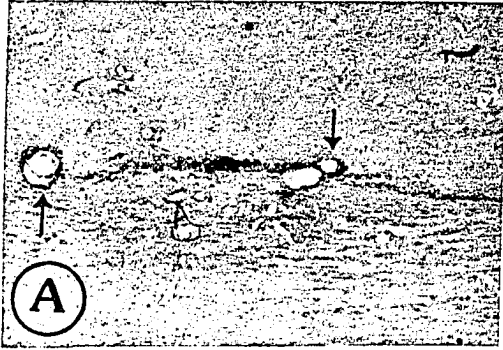
in the hypothalamus (Fig. 22A, B). Neurolemmocytes and their associated neurosecretory axons were usually enveloped by endoneurium-like cells. Fascicles of neurosecretory axons were surrounded by several layers of perineurium (Fig. 22C).

These fine structural findings confirm and expand those reported by Dellmann et al. (1985; 1986, 1987a,b, 1989)

Morphometrical Findings

In all analyzed tissues, the microvasculature was heterogeneous, except in neural lobe grafts where the vascular bed was uniformly distributed. Regional variations in the microvasculature within sciatic and optic nerve grafts were frequently observed in which case the entire graft zone was analyzed. Capillary density, mean diameter, and volume are presented according to groups and zones (Tables 2,3,4, and 5).

Grafts vs controls analysis

There were no significant differences in capillary density, mean diameter, or volume in time between the lesion and optic nerve grafts groups and their respective controls (i.e., hypothalamus and optic nerve). Comparison of neural lobe grafts with control neural lobes showed no difference in capillary densities with time. There was, however, a significant difference in capillary mean diameter and volume with time. Comparison of sciatic nerve grafts with their

Fig. 21. 80 days lesion

A. Dense glial scar at the lesion site with numerous interdigitating astrocyte processes. X5,400.

B. This venule (ven), located within the glial scar, is surrounded by one layer of neurosecretory axons (ns). The left hand side of this picture is the host hypothalamus. X5,400.

ven

ns

scar
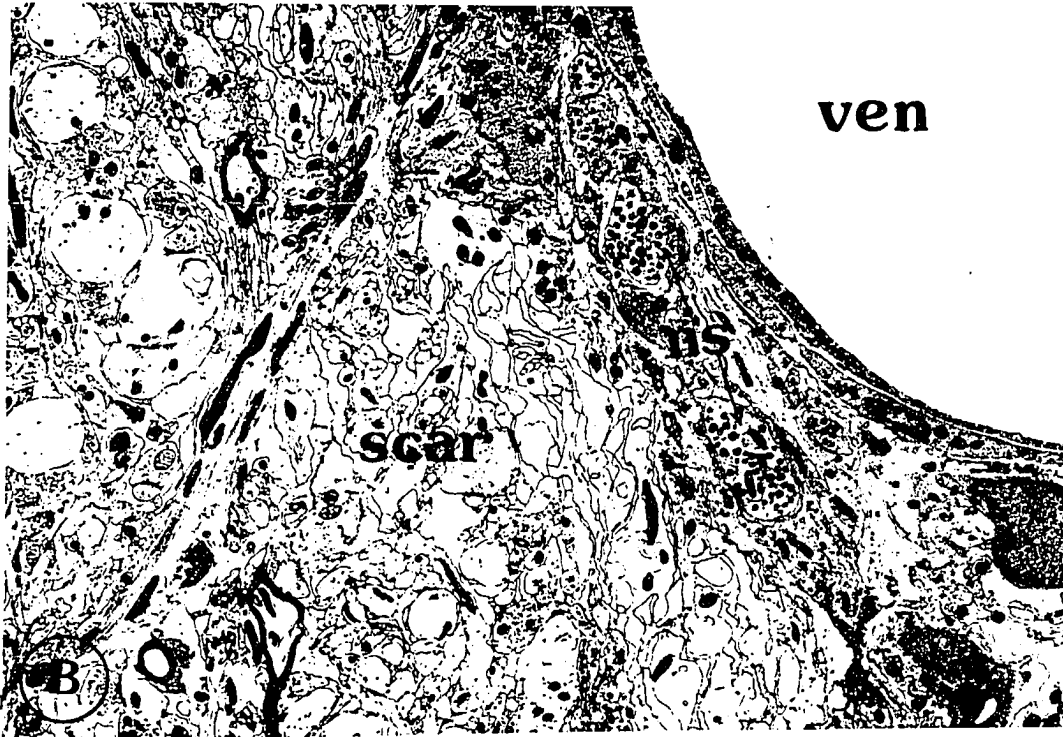
Fig. 22. 80 days sciatic nerve graft

A. Neurolemmocyte processes ensheathe bundles of regenerating axons, some of which are neurosecretory (ns). The axonal bundle together with the enveloping neurolemmocyte processes is covered by a basal lamina (bl). 80 days sciatic nerve graft. X5,400.

B. Fenestrated capillary within a sciatic nerve graft located ventrally in the hypothalamus. Notice the wide pericapillary space filled with collagen (col) fibrils. 80 days. X15,000.

C. Small bundle of regenerating neurosecretory axons (ns) running in parallel with a continuous capillary (cap) surrounded by neurolemmocyte processes. 80 days. X5,400.
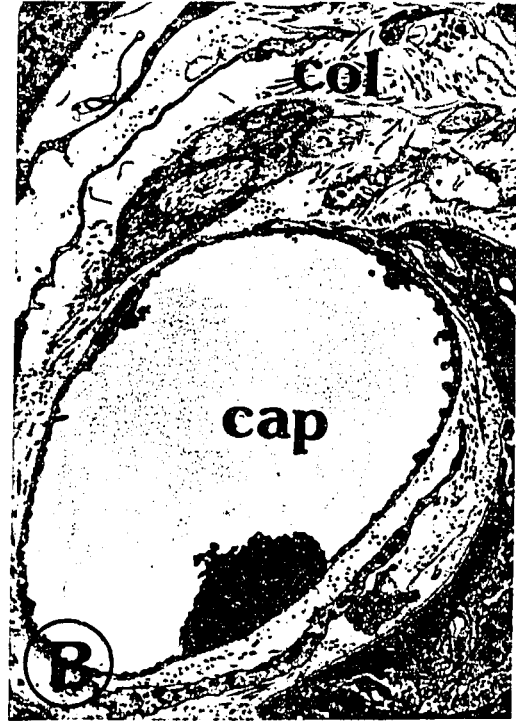
Fig. 23. 80 days optic nerve graft

A. At 80 days, the grafted optic nerve has been transformed into a glial scar. X14,400.

B. Neurosecretory axon-astrocyte processes within a wide perivascular connective tissue space of an arteriole (art). X8,400.

pvs

art.
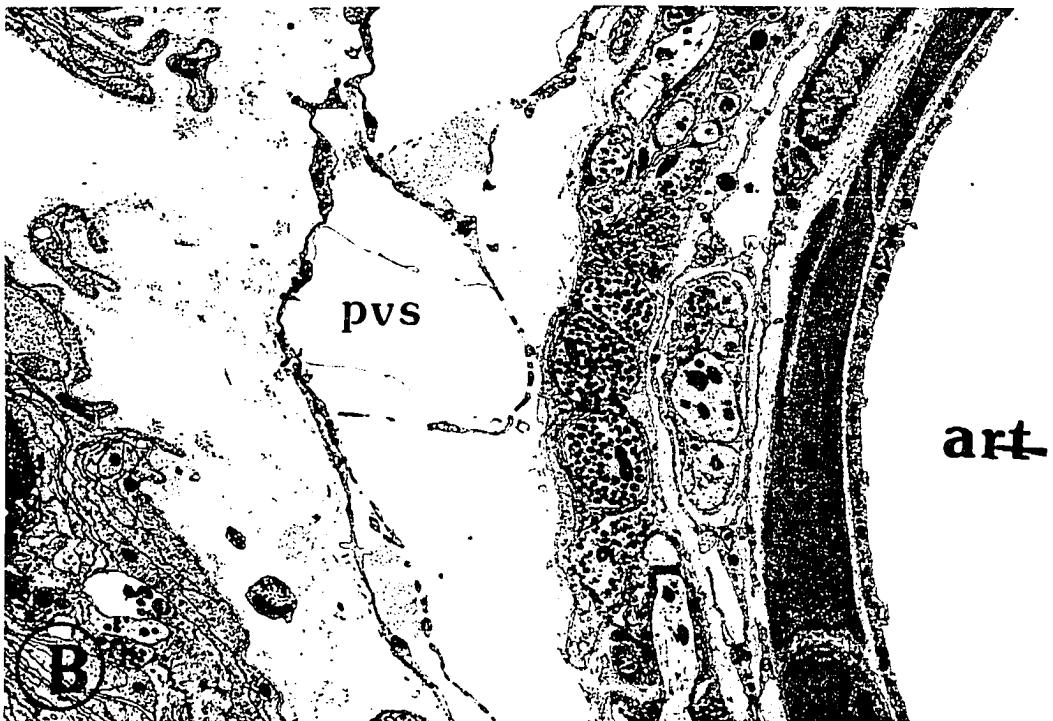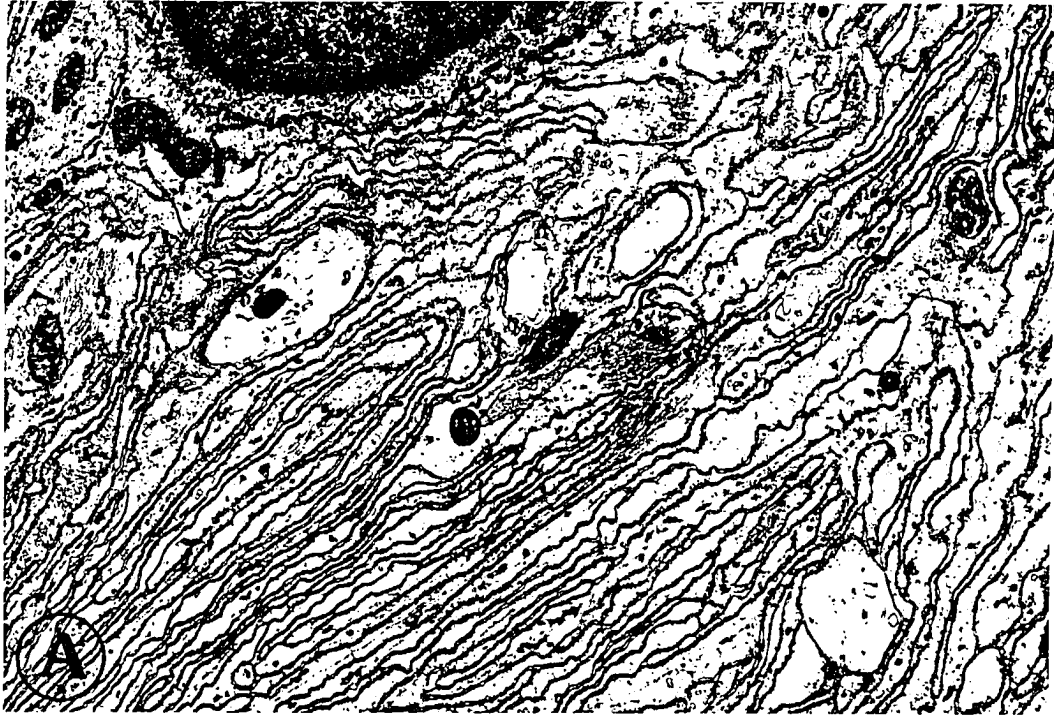
controls did not show any difference, except in capillary mean diameter.

Intergroup analysis

The capillary densities were significantly different between groups and zones. Capillary density was significantly higher in neural lobe graft zones as compared to sciatic and optic nerve graft zones (Fig. 24). Capillary diameters were always smaller at 36 hours in all groups and hypothalamic zones (Fig. 25). Those of the host-graft interface zones, however, were always significantly larger in all groups (Fig. 26). Capillary volumes were similar in all groups, except in neural lobe graft zone where the capillary volume was significantly higher than in other graft zones.

Intragroup analysis

The capillary density in the lesion group decreased significantly with time in all zones (Fig. 27). In the neural lobe group, the capillary density peaked at 30 days at the graft zone, while in the sciatic and optic nerve groups, the densities were similar throughout the different zones.

This is the first quantitative report on the microvasculature of intracerebral grafts. The main findings of this morphometrical analysis are: (1) capillary diameters are significantly lower at 36 hours in all groups and zones,

(2) the significantly higher capillary density in neural lobe graft zones as compared to other graft zones, (3) decrease in lesion group of the capillary density with time, (4) the significantly higher capillary diameter of the host-graft interface zone in all groups and at all times, (5) the quantitative similarity of the capillary bed between the grafts and their respective controls.

Functional Findings

I explored the possibility of lesion- or graft-induced changes in circulating AVP indirectly by measuring daily water consumption. Daily water intake in all experimental rats increased remarkably during the first 24 hours following surgery. During the next two days, water consumption declined in all animals. Following a second peak 3-4 days later, a gradual decrease in water consumption was observed in all animals. This decrease was more substantial in neural lobe-grafted rats (Fig. 28). Starting at 30 days after surgery, it was significantly different (at $p \leq 0.05$) from that in all other animals. Only neural lobe-grafted animals showed some recovery from diabetes insipidus. This recovery, however, did not reach normal values observed before surgery. These findings support the morphological observations that regenerated axons within neural lobe grafts are functional and that the released AVP hormone reaches the vascular system.

Table 2. Morphometry of the capillary bed in the hypothalamic and scar zones of the lesion group. Density = number of capillaries/mm^2, diameter (μm), and volume (%)

	Control	1.5 days	10 days	30 days	80 days
A. Hypothalamic zone					
density	248±21[a]	573±12	170±20	458±106	335±72
diameter	8.09±0.48	6.35±0.50	7.92±0.52	7.98±0.28	6.2±0.70
volume	1.48±0.11	13.9±6.44	1.33±0.32	7.21±1.09	6.4±2.40
B. Scar zone					
density	248±21	282±50	333±96	253±65	310±32
diameter	8.09±0.50	6.65±10	9.24±0.70	8.97±0.07	8.47±0.07
volume	1.48±0.11	5.39±2.7	3.69±0.54	2.04±0.23	3.07±0.66

Table 3. Morphometry of the capillary bed in hypothalamic, host-graft interface and graft zones of the neural lobe group.

	Control	1.5 days	5 days	10 days	30 days	80 days
A. Hypothalmic zone						
density	248±21[a]	189±54	283±27	234±29	276±22	332±56
diameter	8.09±0.48	4.63±0.17	7.63±0.36	8.5±0.57	7.71±0.78	7.86±0.24
volume	1.48±0.11	0.35±0.07	1.38±0.03	1.77±0.08	1.49±0.58	2.73±1.28
B. Host-graft interface zone						
density	248±21	520±232	470±56	187±28	409±95	423±104
diameter	8.09±0.48	6.41±0.90	10.3±0.45	10.7±0.47	8.83±0.15	8.73±0.55
volume	1.48±0.11	1.92±0.57	6.6±0.30	4.11±0.33	3.83±0.61	4.41±0.58
C. Graft zone						
density	1248±776	325±186	588±191	379±125	930±443	437±85
diameter	7.35±0.73	5.76±1.98	9.66±0.37	8.71±0.50	7.61±0.40	8.6±0.50
volume	4.35±0.36	1.15±0.55	7.75±1.66	2.59±0.63	4.76±1.6	3.6±1.00

[a] Means ± standard error for means for three animals.

Table 4. Morphometry of the capilary bed in the hypothalamic, host-graft interface and graft zones of the sciatic nerve group

Control		1.5 days	5 days	10 days	30 days	80 days
		A. Hypothalamic zone				
density	248±21	350±56	146±27	308±08	266±49	237±53
diameter	8.09±0.48	5.51±0.37	8.32±0.22	7.69±0.10	7.92±0.29	8.35±0.20
volume	1.48±0.11	0.93±0.05	1.48±0.45	1.92±0.30	2.12±0.56	1.72±0.44
		B. Host-graft interface zone				
density	248±36[a]	245±108	256±48	203±33	279±29	303±40
diameter	8.09±0.83	7.46±1.80	10.43±0.34	9.51±0.46	8.47±0.44	8.83±0.40
volume	1.48±0.19	1.35±0.41	4.79±0.48	3.56±0.58	3.56±0.58	4.64±1.40
		C. Graft zone				
density	122±24	387±210	235±146	178±39	170±37	289±35
diameter	8.01±0.71	5.27±0.70	9.29±0.27	9.04±0.31	8.79±0.25	8.65±0.50
volume	1.11±0.08	0.74±0.22	2.61±0.66	1.74±0.21	1.98±0.54	2.61±0.60

Table 5. Morphometry of the capillary bed in the hypothalamic, host-graft interface, and graft zones of the optic nerve group

	Control	1.5 days	5 days	10 days	30 days	80 days
		A. Hypothalmic zone				
density	248±21[a]	402±163	252±54	307±29	205±32	268±29
diameter	8.09±0.50	4.6±0.23	7.17±0.65	8.22±1.12	7.44±0.21	7.19±0.60
volume	1.48±0.11	1.07±0.40	1.8±0.23	1.68±0.28	3.35±1.80	1.47±0.20
		B. Host-graft interface zone				
density	248±21	437±188	372±101	376±47	253±48	282±36
diameter	8.09±0.50	5.23±0.41	9.81±0.45	8.17±0.52	8.48±0.10	9.1±0.46
volume	1.48±0.11	2.3±0.23	5.62±0.63	4.32±0.31	2.7±0.60	3.57±0.50
		C. Graft zone				
density	332±10	356±73	136±26	179±36	224±56	246±45
diameter	7.83±0.70	4.02±0.80	8.9±0.44	8.44±0.75	9.16±0.56	9.53±0.50
volume	1.75±0.03	0.9±0.33	2.24±0.52	1.9±0.25	2.85±0.50	2.7±0.45

[a] Means ± standard error for means for three animals.
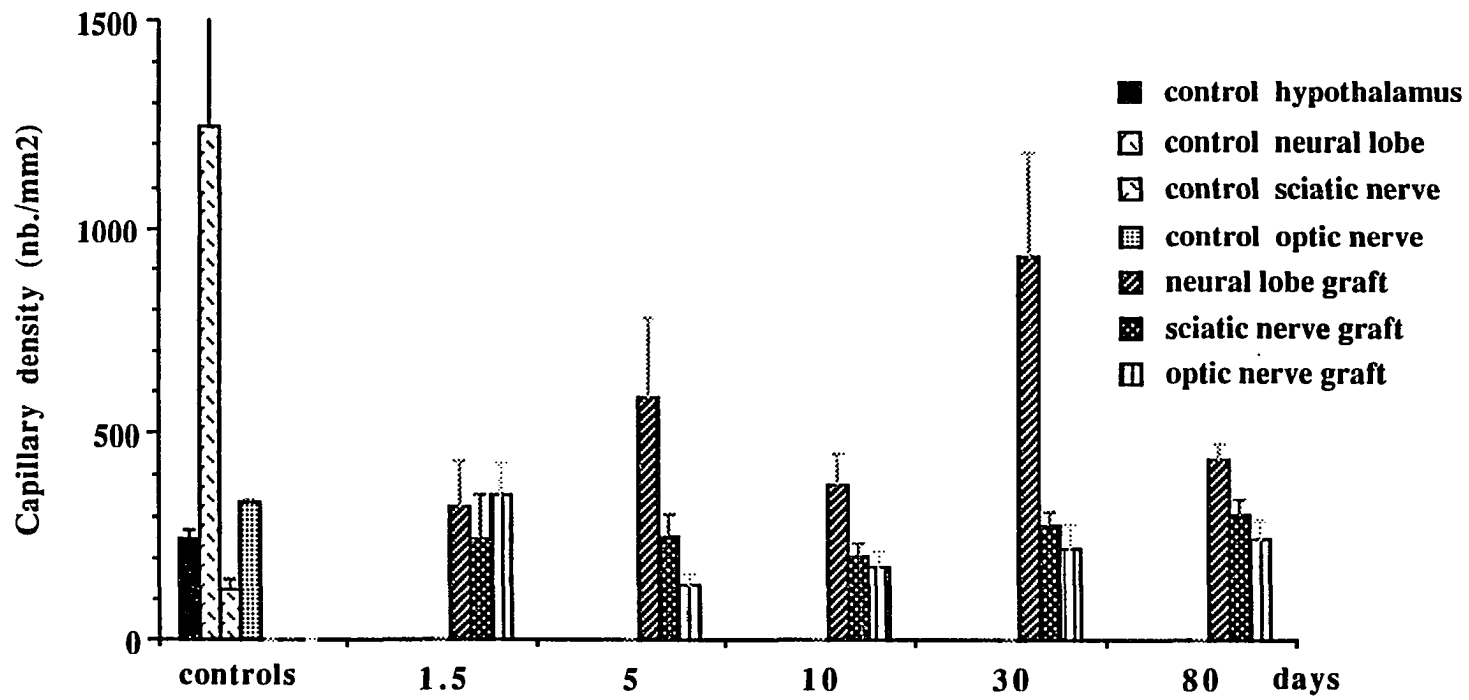
Fig. 24. Capillary densities in controls and all grafts zones.

146
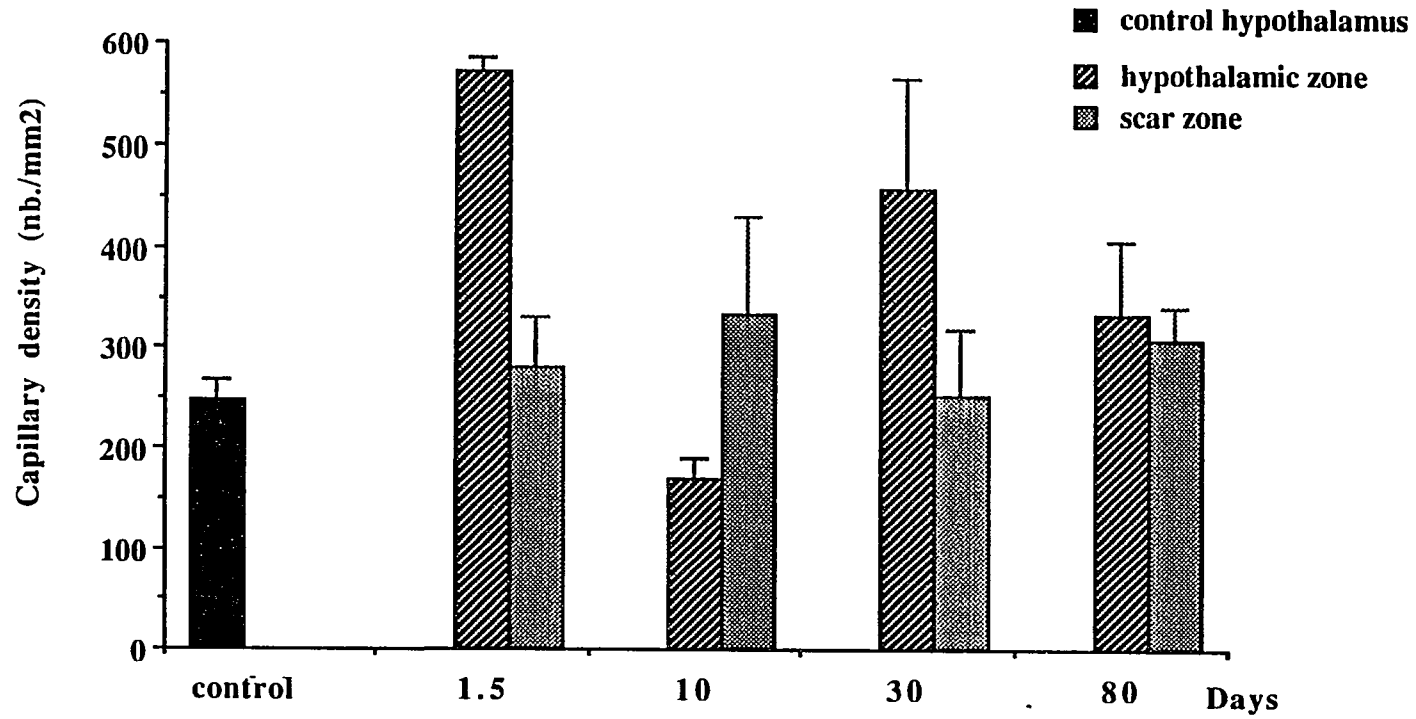
Fig. 25. Capillary densities in the hypothalamic and scar zones of the lesion group.
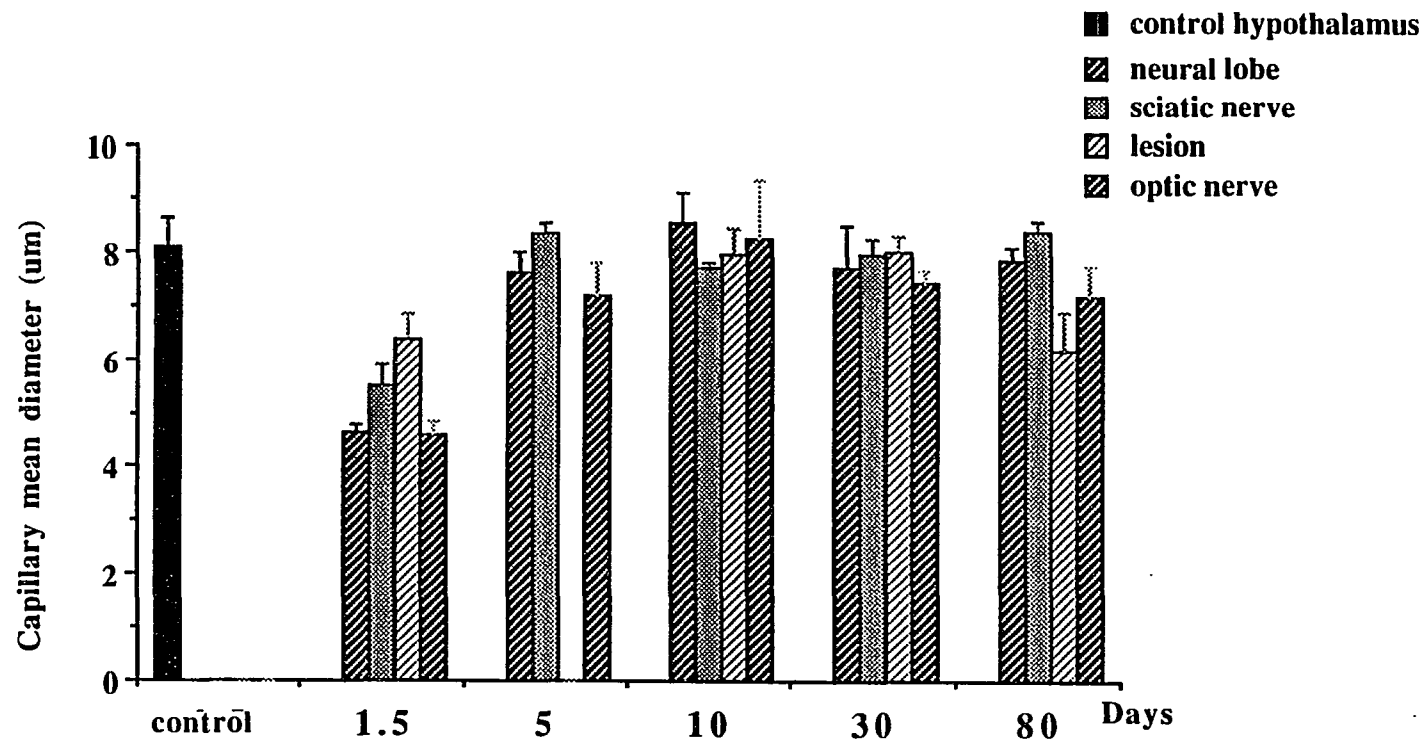
Fig. 26. Capillary mean diameter (μm) in lesion scar zone and grafts zones.

capillary mean diameter (um)

control hypothalamus
sciatic nerve
neural lobe
optic nerve

12

10

8

6

4

2

0

control   1.5   5   10   30   80   days
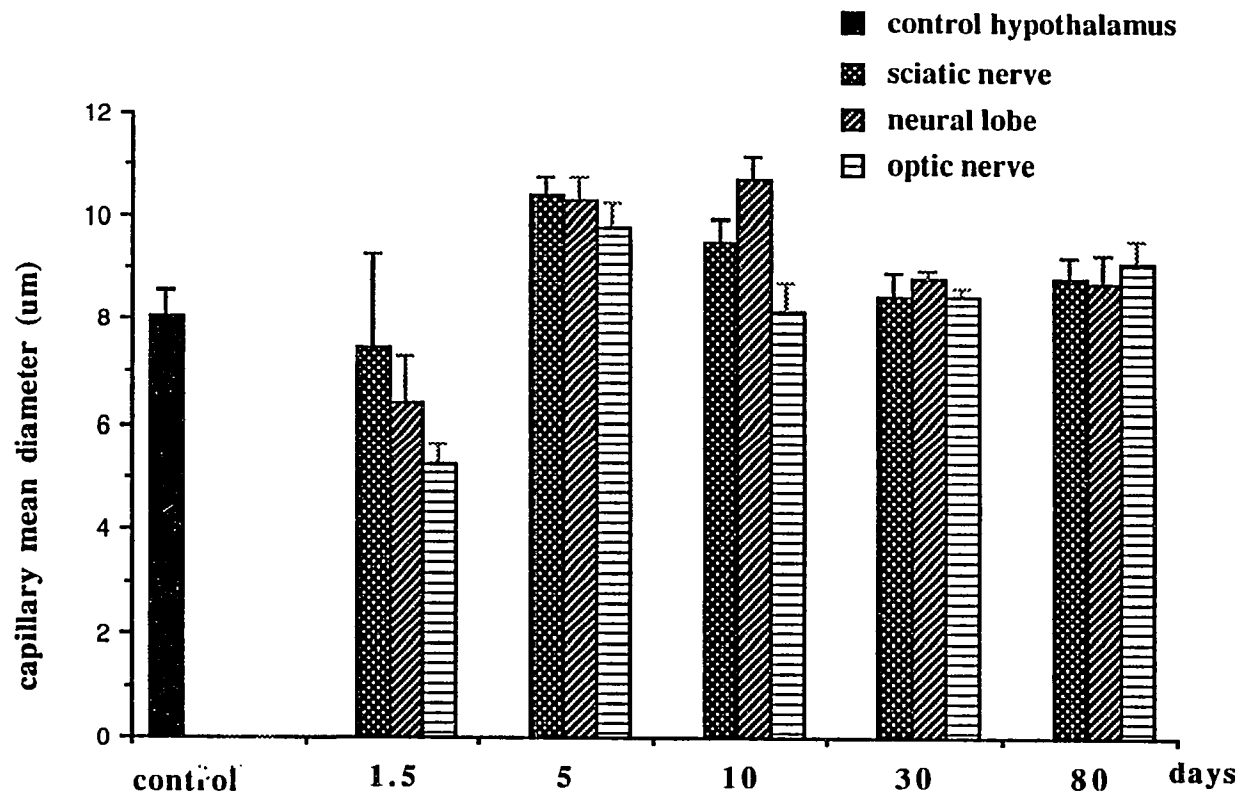
Fig. 27. Capillary mean diameter·(μm) in the host-interface zones.
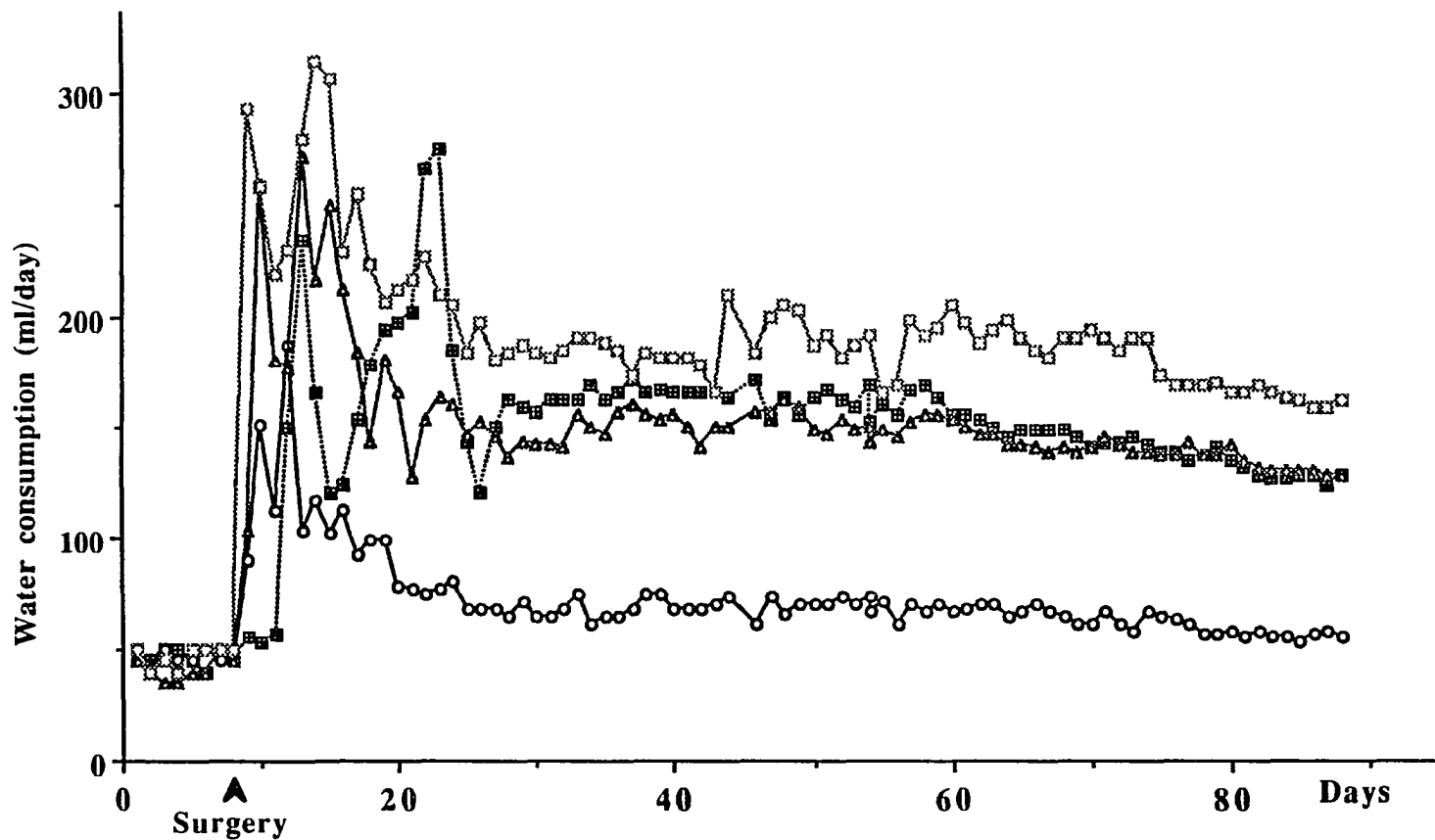
Fig. 28. Water intake of lesioned rats ▪▪▪▪ , and grafted neural lobe ◦◦◦◦ , sciatic nerve ▲▲▲▲ , and optic nerve ⊠⊠⊠ rats. Experimental days 1-18 (n=11), days 19-48 (n=7), and days 49-80 (n=3).

DISCUSSION

In a multifaceted study of neurosecretory axonal regeneration and vascularization of grafts placed at the transected hyopothalamo-neurohypophysial tract, I have shown that: (1) regeneration of neurosecretory axons occurs exclusively in areas of the brain where the BBB has been breached, (2) regenerating neurosecretory axons have an affinity for the microvasculature of grafts and/or adjacent hypothalamus, (3) permeability characteristics of the microvasculature vary with the grafted tissue, (4) temporal changes in hypothalamic perivascular neurosecretory plexuses are correlated with the permeability changes of their vasculature, (5) regenerating neurosecretory axons are closely associated with glial cells, (6) restitution of near normal values of water intake occurs only in animals in which the graft is characterized by the absence of the BBB and the presence of pituicytes, (7) grafts are reperfused, not revascularized, thus conserving their indigenous microvasculatures.

These findings seem to support the working hypothesis upon which the experimental design was based i.e., regeneration of neurosecretory axons and restoration of function require a specific vascular and supportive glial

microenvironment. The subsequent discussion will begin with a critical appraisal of the vascular microenvironment in which neurosecretory axon regeneration occurs, followed by a discussion of the glial microenvironment in various grafts, and finally a consideration of the functional correlates of these findings. Since a diversity of grafted tissues supports neurosecretory axon regeneration in the rat brain, the question arises whether a common denominator exists in all tissues or areas in which neurosecretory axonal regeneration occurs and, if so, what morphological characteristics could be responsible for the differences in the magnitude of the regenerative response in different tissues?

Morphological Analysis

Vascular permeability

The fine structure of grafted neural lobes is virtually indistinguishable from that of intact neural lobes, and their morphological and permeability characteristics did not change. Their vascular beds are composed primarily of fenestrated capillaries, and they are permeable to blood-borne HRP throughout the entire experimental period. Blood-borne HRP escapes from fenestrated capillaries, which are also permeable to protein tracers, in the neural lobe in situ (Brightman et al., 1976; Broadwell and Brightman, 1976). Thus, vascular permeability of neural lobe grafts remains identical to that

of the neural lobe in situ. In contrast, hypothalamic blood vessels are not permeable to HRP. This observation supports earlier evidence that the permeability characteristics of blood vessels in transplants depend upon the cellular nature of the transplant rather than upon that of the host (Stewart and Wiley, 1981). These findings are also consonant with observations made in intracerebral grafts of autonomic ganglia (Rosenstein and Brightman, 1983), muscle and skin (Wakai et al., 1986) and anterior pituitary (Broadwell et al., 1987, 1991).

Sciatic nerve grafts are similarly permeable to HRP throughout the period of study, with the exception of those situated dorsally in the hypothalamus, which were no longer leaky to HRP after 80 days. The microvasculature of the sciatic nerve grafts consisted of continuous as well as fenestrated capillaries. Fenestrated capillaries, though few in number, were an unexpected finding because the grafts used in this study were stripped of their epineurium, leaving only the endoneurial microvasculature and the perineurium. These vessels are not fenestrated. In fact, tight junctions between endothelial cells of endoneurial capillaries and between the perineurial cells are a component of the blood-nerve barrier function (Karnovsky, 1967; Bell and Weddell, 1984). The presence of fenestrations and leakage of HRP from these capillaries are indicative of a high degree of vascular

permeability. Since only a few fenestrated capillaries are

found, they cannot entirely account for the large amount of

HRP reaction product observed in sciatic nerve grafts. There

are two possible alternative sources for the HRP in these

grafts. The first one is by diffusion from hypothalamic blood

vessels and adjacent meningeal vessels, which are damaged

during HNT transection. Meningeal vessels are normally

permeable to circulating proteins (Broadwell et al., 1989;

Rosenstein, 1991). In support of this interpretation is the

observation that after 80 days only those grafts situated

ventrally in the hypothalamus contain HRP. The second

possible source is through host fenestrated capillaries

supplying the adjacent median eminence. Leakage of HRP into

the loose neuropil of adjacent grafts is possible. In view of

the rather puzzling observation of fenestrated capillaries, it

could be argued that these microvessels are epineurium-

derived. The preferential location of fenestrated capillaries

in the extensive connective tissue areas of the graft rather

than in former endoneurial compartments, which contain

continuous capillaries in the normal sciatic nerve, suggests

that capillary fenestrations are induced by the

microenvironment of proliferated epineurial remnants remaining

after the epineurium was stripped. Epineurium contains

fenestrated capillaries in intact sciatic nerves (Karnovsky,

1967; Bell and Weddell, 1984). Another factor that may induce

fenestration is the presence of neurosecretory axons. They may, through release of hormone, create a microenvironment that induces capillary fenestrations. A similar situation occurs in the neural lobe during development, where the first capillaries to develop are of the continuous type, and fenestrations are formed only upon arrival of the ingrowing neurosecretory axons (Dellmann, personal communication). It may be also speculated that the absence of astrocytes in sciatic nerve transplants favors the differentiation of fenestrated capillaries. Blood vessels supplying intracerebral tissue grafts that are free of astrocytes have been shown to lack BBB characteristics, and to be "leaky" to blood-borne macromolecules (Wakai et al., 1986; Rosenstein and Brightman, 1986; Rosenstein, 1987; Broadwell et al., 1987, 1988). Fenestrated capillaries have also been reported in injured and regenerating peripheral nerves (Anderson et al., 1991) and in peripheral nerve segments implanted into the rat parietal cortex (Mitchell et al., 1985), suggesting that the development of fenestrations may be injury-related.

Neurosecretory axon regeneration was least, overall, in optic nerve grafts; however, a clear correlation was displayed in these grafts between the degree of vascular permeability and the magnitude of neurosecretory axon regeneration. Neurosecretory axons grow preferentially into areas where the BBB is breached. HRP diffusion in optic nerve grafts was

observed at 10 days and only in grafts located ventrally in the hypothalamus at 30 and 80 days. Moreover, among the three graft types used in this study, the optic nerve graft is the least leaky to HRP, which correlates with the limited support of neurosecretory axon regeneration. Fenestrated capillaries were seen, however, only at 10 and 30 days. The question arises as to what induces capillaries in optic nerve grafts to change from continuous type at the time of transplantation to fenestrated type at 10 and 30 days after transplantation, and back to non-fenestrated ones at 80 days? Through 30 days, fenestrated capillaries were observed in the connective tissue areas of the graft. Fenestrations in these capillaries may be induced by the ingrowing neurosecretory axons (as discussed in sciatic nerve grafts). Another possibility could be related to the absence of astrocytic contacts with endothelial cells, which could delay or prevent the formation of continuous capillaries. At 80 days, optic nerve transplants resolve almost totally into an astrocytic scar (Dellmann et al., 1989, present study). Diffusible astrocytic factors may now overcome those of neurosecretory axons, thus causing the fenestrations to disappear.

The most convincing argument in support of neurosecretory axon regeneration in HRP-permeable areas of rat brain is provided by the observations in intrahypothalamic perivascular neurosecretory plexuses. Following hypothalamic lesioning or

grafting, adjacent hypothalamic tissues were HRP-positive after intravenous HRP administration, but the amount of reaction product decreased, both in intensity and extent, with advancing survival times. Since the microvascular bed of the hypothalamus is formed by continuous capillaries, how then does the blood-borne HRP reach the hypothalamus? On the basis of the observations reported here, there are three possibilities. Firstly, HNT transection interrupts hypothalamic blood vessels, allowing HRP to diffuse along their perivascular spaces. Such vascular damage is apparently minor and of short duration since in the lesion group, the hypothalamus is again impermeable to HRP after 10 days. In grafted animals, however, HRP leakage into the surrounding hypothalamus is long-lasting, being greatest in the presence of neural lobe grafts, less with sciatic nerve grafts, and least with optic nerve grafts. Most likely blood-borne HRP leaks out through permeable blood vessels in the grafts, diffuses along perivascular connective tissue spaces of connecting vessels at the host-graft interface into the adjacent hypothalamus. HRP diffusion in this manner would explain why reaction product diminishes centrifugally from the graft origin. A third possible source of HRP extravasation into the surrounding hypothalamic tissue is the large vessels in the spaces of Virchow-Robin, which are innately permeable to a variety of systemically injected tracers (Broadwell,

1989; Broadwell et al, 1991; Rosenstein, 1990). The difference in the frequency of perivascular neurosecretory plexuses between groups (highest in neural lobe and sciatic nerve groups and lowest in optic nerve and the lesion group) favors the second possibility since if only the vessels with Virchow-Robin spaces were leaky, there would be no difference in the magnitude of neurosecretory axon regeneration into perivascular spaces. Finally, transcellular vesicular transport through non-fenestrated endothelia has been claimed to be responsible for intracerebral extravasation of HRP under a variety of experimental conditions (Beggs and Waggener, 1976; Westergaard, 1977; Povlischock et al., 1978; Noble and Wrathall, 1988; Risling et al., 1989). There is, however, no convincing evidence for transendothelial transport in the CNS (Broadwell, 1989), and the fine structural observations reported here support the notion that, even under experimental conditions, endothelial cells within intrahypothalamic grafts exhibit only modest numbers of endothelial vesicles, which appear to be within the normal range. HRP injection is reported to cause an elevation in blood pressure that may affect endothelial permeability (Westergaard, 1977). Also perfusion-fixation has been shown to induce vascular leaks (Balin et al., 1986), and prolonged circulation time of HRP is said to promote vascular permeability (Broadwell, 1989). In the current experiments, however, adjacent hypothalamic

tissues were not influenced by the lesions and grafts demonstrated a well working BBB for intravascular HRP. Therefore, these artifacts can be excluded.

At the lesion site, the few neurosecretory axons that regenerate were located predominantly around blood vessels of the size of arterioles and venules. This microenvironment is obviously suitable for neurosecretory axon regeneration. Because the BBB at the lesion site is restored after 10 days, the reason for a transient increase in neurophysin-immunoreactivity of the perivascular neurosecretory plexuses, observed at 30 days, is problematical. It may reflect a buildup of neurosecretory peptides within axon terminals due to increased hormone synthesis and transport in response to the effects of denervation of the host neural lobe. The time course of the BBB's recovery to circulating peroxidase coincides with that (7-10 days) reported by Broadwell et al. (1991) after a penetrating lesion. The rapid restoration of the BBB in lesioned animals is probably due to rapid ensheathment of the newly formed capillaries by astrocytes in the scar region. Transplantation studies have established that astrocytes play a significant role in the formation of the BBB; cerebral astrocytes transplanted into the iris induce barrier formation in normally leaky vessels (Janzer and Raff, 1987). The signals that induce endothelial cells of CNS capillaries to express the BBB phenotype are believed to

result from specific actions of the ensheathing perivascular astrocytes on the endothelial cells (DeBault and Cancilla, 1980; Beck et al., 1984; Janzer and Raff, 1987; Tao-Cheng et al., 1987).

In sum, in all areas where neurosecretory axon regeneration occurs the BBB is absent. How might the absence of the BBB support neurosecretory axon regeneration? In the intact HNS, neurosecretory axons terminate at pericapillary spaces of fenestrated capillaries. It is conceivable that normal function of neurosecretory neurons is dependent on their ability (i) to release their hormones into these spaces and ultimately into the capillary lumen, and (ii) to endocytose and convey to the parent soma by retrograde transport blood-borne substances to which the neural lobe is highly permeable. Blood-borne substances are available within leaky grafts, and could, therefore enhance not only graft survival, but also promote neurosecretory axon regeneration. These substances could act locally at the proximal tip of the transected axon to initiate axon elongation. Rapid posttransectional reestablishment of retrograde axonal transport (Bisby, 1984) would also enable these substances, following endocytosis, to reach the soma and to signal the existence of a microenvironment conducive to axonal regeneration, which then can proceed through the wide intercellular spaces of the various grafts. This scenario is

supported by the striking parallelism between the extent of
perivascular neurosecretory plexuses and the absence of BBB
characteristics. The reestablishment of the BBB to HRP within
lesion scars and in optic nerve grafts, as well as in the
adjacent hypothalami of all grafts, is associated with a
decrease in the density of perivascular neurosecretory
plexuses. The reestablishment of the BBB leads to the
unavailability of blood-borne substances to regenerating
neurosecretory axons, and consequently a decrease in their
numbers is observed. The decreased number of neurosecretory
axons is an indication that the continued availability of
blood-borne substances is vital for neurosecretory neuron
regeneration, and that in the absence of these substances,
axons and axon terminals and possibly entire neurons
degenerate. Survival of the somata of magnocellular
neurosecretory neurons has been considered a function of the
distance between the site of axotomy and the cell body. The
closer the axotomy is to the cell body the more likely the
cell will degenerate (Dellmann, 1973). If this degeneration
is actually due to the unavailability of blood-borne factors
to the proximal stump for retrograde transport to the parent
soma, then the availability of such factors in leaky grafts or
areas of such factors should reduce neuronal loss.
Determining the number and fine structural characteristics of
the PVN and SON neuronal somata surviving transection and

grafting over time could clarify this point.

Angiogenesis

Since neurons of the HNS normally release their secretory

products into the vascular system (i.e., into perivascular

spaces of fenestrated capillaries of the neural lobe), it is

evident that neurovascular contacts are essential. Axotomy of

neurosecretory neurons deprives them of this contact. For the

physical and physiological survival of these neurons, it may

be necessary, and for the restoration of function it is surely

imperative, to reestablish neurovascular contacts. The sooner

contact with the vascular system is reestablished after

lesioning or grafting, the better should be the chances of

neurosecretory neuron survival and axonal regeneration. The

establishment of the graft vascularity, a crucial process for

intracerebral graft survival and physiological integration

with the host, has received little attention, particularly at

the quantitative level. The present study addressed some of

the quantitative and ultrastructural changes in

vascularization within and surrounding these intrahypothalamic

grafts.

Short post-transplantation times (18 and 36 hours) were

selected because the first connections between the

microvasculatures of host and intracortical CNS graft have

been reported between 16 and 20 hours post-grafting (Zhou et

al., 1986; Krum and Rosenstein, 1987). At these times,
intrahypothalamic grafts are almost completely encircled by a
hemorrhagic clot. The extravasated blood, while isolating
grafts from the surrounding hypothalamus and impeding both
vascular connection and neurosecretory axon regeneration,
provides a source of nutrients for the grafted tissues prior
to vascularization. The observed delay for vascular
connection of intrahypothalamic grafts, as compared to
intracortical ones, most likely is due to the dense capillary
bed of the hypothalamus (Sposito and Gross, 1987) which, when
damaged, causes an extensive hemorrhage, thus delaying
vascular connections. Another significant factor could be
that whole ganglionic tissue was used for intracortical grafts
versus chopped grafted pieces in the current study. These
latter apparently need more time to establish vascular
connections. By 36 hours, some capillaries in the
hypothalamus immediately adjacent to the grafts had high
endothelial cells with fine structural features characteristic
of stimulated capillaries (Schoefel, 1963; Sholley et al.,
1984; Böck, personal communication). This is evidence that
the vascular bed has reacted to the inflicted injury by
stimulation of endothelial cells, which is the first step in
the complex sequence of angiogenesis events (Folkman, 1985;
Paku and Paweletz, 1991). Regardless of the type of
transplanted tissue, large diameter blood vessels crossing the

host-graft interface zone were identified at 10 days in this study. Blood vessels in this zone were always characterized by wide diameters compared to the host or graft zones. How these morphometric data relate to the cellular events of angiogenesis is unknown. One possibility is that the increase in capillary diameter reflects hypertrophy of endothelial cells, which is known to occur during angiogenesis (Cancilla et al., 1979; Sholley et al., 1984; Folkman, 1985).

Evidence that connections are established between the graft vasculature and that of the hypothalamus was the presence of empty and dilated capillaries in the grafts of perfusion-fixed specimens, and the presence of HRP reaction product in grafts after intravenous tracer administration to the host. Intrahypothalamic grafts of neural lobe, sciatic nerve, and optic nerve have been reported (based on perfusion-fixation criteria) to be connected to the hypothalamic vasculature already by 5 days post-grafting (Dellmann et al., 1986, 1987a, 1989). The dense vasculature of the surrounding hypothalamus, initially an impediment for vascular and neurosecretory axon regeneration presumably becomes an asset, at 5 days, by providing multiple vascular connections. Therefore connections are achieved appreciably earlier than the 7 days for solid CNS grafts reported by Broadwell et al. (1988, 1991). Time periods between 36 hours and 5 days would need to be investigated to determine the exact time course for

establishment of initial host-graft vascular connections.

To what extent are the grafts' vessels newly formed from host origins which have grown into the transplants (neovascularization), as opposed to reperfusion of surviving grafts vessels which become connected to the host circulation? All grafts in this study contained indigenous vessels at the time of transplantation. The absence of damaged endothelial cells within these grafts after 36 hours and complete perfusion of the grafts at 5 days support the hypothesis that graft vessels are indigenous and not host-derived. Statistical morphometrical analyses of the vascular beds substantiates this assumption, since no changes in capillary densities were detected between the grafts and their respective controls. This interpretation is in agreement with observations by other investigators (Raisman et al., 1985; Krum and Rosenstein, 1987; Nakano et al., 1989; Broadwell et al., 1991). Because pieces of chopped graft tissues could no longer be distinguished individually within the grafts after 10 days, vascular connections are probably reestablished between these pieces by this time.

It is not known how the indigenous graft vessels connect with those of the host. Possible explanations include (i) growth of capillary-like sprouts, which extend from the wall of existing wide capillaries and venules into the surrounding tissue (Schoefel, 1963; Sholley, 1984) or (ii) proliferation

by mitotic division and formation of new vessels (Folkman,
1985; D'Amore and thompson, 1987). The observation of
pseudopodia-like projections from the abluminal surface of
some endothelial cells in both the host and grafts supports
the first explanation. These endothelial processes may make
contact with similar processes from the opposite side to form
a sprout, thus connecting host vessels to those of grafts. In
this manner, a new vessel of dual origins could form by
migration of existing endothelial cells derived from both the
host and the grafts. Concerning the second possibility,
although mitotic figures in endothelial cells were not
observed in this study, time periods between 36 hours and 5
days would need to be investigated to determine if endothelial
cell proliferation via mitosis occurs. Whether endothelial
migration and proliferation are separable or interdependent
events during angiogenesis in vivo is unclear, although it is
known that endothelial cell migration alone is sufficient to
repair small defects in cultured monolayers (Sholley et al.,
1977) or the intima of larger vessels (Reidy and Schwartz,
1981).

A variety of polypeptide growth factors are capable of
inducing angiogenesis (Giulian, 1986; Folkman and Klagsbrun,
1987; Finklestein et al., 1988). Given the abundance of
macrophages within the lesion site or grafts, interleukin-1
(IL-1), a potent angiogenic factor released by activated

macrophages or by microglia (Giulian, 1988), may be involved.

Similarly, growth factors released from other cell types in

the injured hypothalamus and/or within the grafts could

participate. These include bFGF, PDGF, VPF, and TNF (Folkman

and Klagsbrun, 1987; Klagsbrun and D'Amore, 1991; Bjornsson et

al., 1991). An increase in bFGF has been shown to occur at

the site of focal brain wounds (Finklestein et al., 1988,

Logan et al., 1991). bFGF-mRNA is present in macrophages and

microglia soon after cortical lesioning (Frautschy et al.,

1991). Since the distribution and effects of these factors

are not yet known in detail, it is difficult to speculate on

their relative contributions to angiogenesis.

Role of glial cells

It is obvious from this study and other investigations

(Dellmann et al., 1986, 1987a, 1987b, 1988, 1989) that

wherever neurosecretory axons regenerate they are always

accompanied by glial cells. This close and consistent

association between basal lamina-enclosed glial cells and

neurosecretory axons and/or terminals within all grafts and

perivascular spaces as well as the apparent secretory activity

of some of glial cells, suggests that these glial cells play a

decisive role in the regeneration process. When regenerating

neurosecretory axons grow into these grafts, they encounter

and become closely associated with specific glial cells of the

respective transplants, i.e., pituicytes in the neural lobe,
neurolemmocytes in the sciatic nerve, neurolemmocyte-like
cells within hypothalamic and some graft perivascular spaces,
and astrocytes in the optic nerve grafts. What is the
significance of these glial cells in the neurosecretory axon
regenerative process?

Pituicytes. Present results confirmed those obtained
by Dellmann et al. (1985, 1987a) that considerably greater
numbers of neurosecretory axons regenerate into neural lobe
grafts than into sciatic or optic nerve grafts. The affinity
of regenerating neurosecretory axons for, and their close
spatial relationship to, pituicytes suggests that pituicytes
have an important role in neurosecretory axon regeneration.
Pituicytes apparently have several functions. Besides their
role in enclosure and release of neurosecretory axons and
homeostasis in the normal neural lobe (Wittkowski, 1986;
Hatton, 1990), they may serve as bridge-like template to guide
regenerating neurosecretory axons. They may also release
substances that promote survival of hypothalamic neurons in
the regeneration process (Dellmann et al., 1987a; Dellmann and
Carithers, 1992b). Based on studies on the development of the
magnocellular neurosecretory neuronal system, a secretory role
has been proposed for pituicytes, which would provide trophic
and tropic factors to the growing neurosecretory neurons
(Galabov and Schiebler, 1978; Dellmann and Sikora, 1981;

Wittkowski, 1986). Posttransectional hypertrophy of pituicytes in the median eminence (Dellmann, 1973; Kawamoto and Kawashima, 1985) and concurrent increases in the extent of rough endoplasmic reticulum and Golgi complexes (Dellmann et al., 1987a; present study) lend morphological support to the concept that these cells do, in fact, become actively secretory at a time one would expect trophic molecules to be directed to neurosecretory axons.

The characteristic palisade alignment of neurosecretory axons in the control neural lobe also occurs in neural lobe transplants, but not in sciatic and optic nerve transplants (Dellmann et al., 1987a; present study). This alignment may be induced by pituicytes or pituicyte secretions. The lack of neurosecretory axon regeneration into intrahypothalamic grafts of neural lobe in which pituicytes had been killed by cryotreatment (Carithers and Dellmann, 1992) further supports a role for pituicytes in neurosecretory axon regeneration. Because neurosecretory axons that regenerate into intrahypothalmic neural lobe grafts are entering their normal target organ, which should provide the most suitable microenvironment, it is not surprising that the most robust regeneration is into these grafts.

Neurolemmocytes. Within sciatic nerve grafts, the consistent association of regenerating neurosecretory axons with neurolemmocytes and their surrounding basal lamina

indicates the importance of these cells in the regeneration
process. In the PNS, regeneration of a variety of transected
axons occurs in the presence of basal lamina-ensheathed
neurolemmocytes (Ide et al., 1983; Hopkins et al., 1985; Berry
et al., 1986; Bunge et al., 1988). The peripheral nerve
microenvironment, when grafted into the CNS, also supports
axonal regeneration (Benefey et al., 1982; Aguayo et al.,
1982, 1983; Aguayo, 1985; David and Aguayo, 1981, 1985;
Mitchell et al., 1985), including that of neurosecretory axons
(Dellmann et al., 1986; present study). This proves that
regeneration of neurosecretory axons is not critically
dependent on the presence of pituicytes per se. Empty basal
lamina tubes, which persist after neurolemmocytes are killed
by cryotreatment, support axonal regeneration in the PNS (Ide
et al., 1983) but fail to do so in the CNS (Smith and
Stevenson, 1988), including the HNS (Dellmann and Carithers,
1992a). Neurosecretory axon regeneration into sciatic nerve
grafts is always associated with basal lamina-enclosed
neurolemmocytes. This close relationship is suggestive of a
guiding role. The factors responsible for the ability of
neurolemmocytes and their basal laminae to act as a preferred
substrate for axonal growth are likely to be complex and may
involve a combination of molecules. Neurolemmocytes have been
implicated in axotomy-induced biosynthesis of neurotrophic
factors (Varon et al., 1981; Politis et al., 1982; Dellmann et

al., 1986, 1988; Finn et al., 1986; Johnson et al., 1987). In
severed PNS axons, neurolemmocytes are known to have NGF
receptors (Taniuchi et al., 1986: Raivich and Kreutzberg,
1987) and also to secrete NGF (Varon et al., 1981; Politis et
al., 1982; Kromer and Cornbrooks, 1985; Finn et al., 1986;
Johnson et al., 1988). Since neurosecretory axons do not
possess NGF receptors (Yan et al., 1990), it is unlikely that
neurolemmocyte-derived NGF could act directly on
neurosecretory axons. However, this could be accomplished
indirectly by processes involving other supportive cells such
as microglia. Microglia possess NGF receptors (Yan et al.,
1990), and could react to locally released NGF by producing
unspecified molecules that are tropic and/or trophic for
neurosecretory axons.

Neurolemmocyte-like cells.   Damage to the HNT
stimulates neurosecretory axon regeneration into hypothalamic
perivascular spaces in the vicinity of the lesion or grafts
(Dellmann et al., 1987b, present study). In addition,
neurosecretory axon regeneration occurs in perivascular spaces
of neural lobe and optic nerve grafts (Dellmann et al., 1987a,
1989, present study), of leptomeninges (Dellmann et al.,
1988b), and in hypothalamic neurovascular contact zones
(Dellmann and Carithers, 1992b). Within these connective
tissue spaces, regenerating neurosecretory axons are
associated with cells whose fine structural characteristics

and relationships are similar to peripheral neurolemmocytes
(Peters et al., 1991). Because their identity and origin have
not been established with certainty, these cells have been
referred to as neurolemmocyte-like cells (Dellmann et al.,
1987b, 1988b). Their close spatial relationship to
neurosecretory axons suggests that they may have a guidance
role similar to that of pituicytes and neurolemmocytes. The
growing evidence for elaboration of neuronotrophic factor(s)
by neurolemmocytes (Politis et al., 1982; Richardson and
Ebendal, 1982; Kromer and Cornbrooks, 1985, 1987; Schwab and
Thoenen, 1985) as well as morphological signs of secretory
activity (Dellmann et al., 1987b) makes it seem likely that
the neurolemmocyte-like cells observed here may also produce
trophic factors that influence neurosecretory axonal growth.

Astrocytes.   These cells also appear to be preferred
substrate for regenerating neurosecretory axons. Regenerating
neurosecretory axons pierce the newly-formed glia limitans-
associated basal lamina, which separates host tissue from the
grafts, and invade, to varying extents, each of the grafted
tissues. At all graft peripheries, astrocytes accompanied
neurosecretory axons bridging the host-graft interface,
lending support to the bridge-like template for astrocytes.
Evidence for chemotropic guidance from astrocytes within the
CNS is increasing (Tessier-Lavigne and Placezek, 1991).
Neurite outgrowth on astrocytes is promoted by N-CAM, which is

the cell surface receptor for several extracellular matrix glycoproteins (Schachner, 1991). The idea that astrocytic scars act as mechanical and/or chemical barriers to neurite outgrowth (Cajal, 1928; Windle, 1956; Berry et al., 1977; Reier et al., 1983, 1989; Wells et al., 1985; Liuzzi and Lasek, 1987; Reier and Houle, 1988) is based on the finding that most transected axons terminate within the scar (Bernstein and Bernstein, 1971; Carlstedt, 1985). This barrier is, however, not absolute, because the glial scar around intrahypothalamic grafts is not an obstacle to neurosecretory axon regeneration (Dellmann et al, 1988a; present study). Apparently neurosecretory axons continue towards the permeable microenvironment beyond the scar. Wherever perivascular contact is established, astrocytes fully or partially ensheathe axons and axon terminals in a similar way to other glial cells. Considerable in vitro and in vivo evidence has shown that astrocytes synthesize and secrete neuronotrophic factors that support survival and/or axonal regeneration of both PNS and CNS neurons (Lindsay, 1979; Liesi et al., 1984; Nieto-Sampedro et al., 1985; Manthorpe et al., 1986; Furukawa et al., 1986; Lu et al., 1991; Yoshida and Gage, 1991). To date, the evidence supports the hypothesis that only reactive and/or proliferating astrocytes secrete trophic and tropic factors (Gage et al., 1988).

Phagocytic cells. A variety of cell types, endogenous as well as exogenous have been regarded as sources of phagocytes in injured CNS (Perry and Gordon, 1991). In the present study, three types of phagocytic cells were identified. The perivascular and scattered granulated cells were observed in all areas of HRP extravasation, including those of control specimens. The only morphological evidence for their role in phagocytosis is their labelling with HRP reacted granules. Their morphology, as well as their density, is consistent with that of microglia (perivascular monocytes and parenchymal microglia, Lassmann, personal communication). Phagocytic cells of a third type engaged simultaneously in both phagocytosis of cellular debris and extravasated HRP were identified as macrophages. The prevailing concept is that circulating blood monocytes are the precursors of microglia (Thomas, 1992). Recent evidence suggests that microglia activated by neuronal injury convert into active phagocytic macrophages (Thomas, 1992). The contribution of these phagocytic cells in the series of changes that follow hypothalamic lesioning or grafting, including angiogenesis, glial proliferation, and neurosecretory axon regeneration, is not clear, but may be profound. Cellular changes following CNS injury are probably mediated, in part, by the action of factors derived from these cells. Among these factors, IL-1 (Giulian, 1986), bFGF, NGF (Finklestein et al., 1988; Mallat

et al., 1989), and TNF (Leibovich et al., 1987) have potent
effects on endothelial cells, glia, and neurons (Finklestein
et al., 1988; Frautchy et al., 1991; Ishikawa et al., 1991).
In the CNS, macrophages are the predominant and persistent
cells associated with injury (Beck et al., 1983). These cells
contain a wide variety of growth factors, and they can
presumably deliver them at the lesion site. Giulian (1988,
1989) showed that IL-1 secreted by microglia and macrophages
stimulates astrogliosis and neovascularization. IL-1 is also
reported to regulate the synthesis of NGF in non-neuronal
cells in the damaged sciatic nerve (Lindholm et al., 1987).
The induction of astrocyte proliferation by IL-1 (Giulian,
1988, 1989) may also stimulate these cells to secrete NGF
which, in turn, attracts regenerating axons. This, however,
does not appear to apply to neurosecretory neurons, since no
neurosecretory axons are seen in the grafts during
phagocytosis and removal of cellular debris.

Extracellular matrix

In all grafts and perivascular spaces, neurosecretory
axon regeneration occurred within connective tissue spaces.
Connective tissue, therefore, appears to present a favorable
microenvironment for neurosecretory axon regeneration.
Extracellular matrix molecules such as type IV collagen,
laminin, and fibronectin (Ide et al., 1983), either alone or

in combination with other cellular molecules (Kromer and Cornbrooks, 1985), are known to promote axonal growth. Basal lamina scaffolds obtained from peripheral nerves (Ide et al., 1983; Smith and Stevenson, 1988) support axonal growth in the PNS, but fail to do so in the CNS (Smith and Stevenson, 1988, Dellmann and Carithers, 1992a). Characteristically, neurosecretory axons terminate upon basal laminae in both intact and grafted tissues, and thus differ from non-neurosecretory axons in the CNS. Therefore, neurosecretory axons may possess extracellular matrix receptors which attract them to basal laminae. This chemotropism, however, has been shown to be functional only in the presence of glial cells. Intrahypothalamic grafts in which the glial cells were killed by cryotreatment and the basal laminae were preserved were shown to be poor substrate for neurosecretory axon regeneration (Dellmann and Carithers, 1992a; Carithers and Dellmann, 1992).

Fibroblasts, although not seen in direct contact with regenerating neurosecretory axons in connective tissue spaces, could promote neurosecretory axonal growth and survival. Indeed, FGF has been shown to possess neuronotrophic activity in the adult CNS (Sievers et al., 1987). In vitro studies have also shown that fibroblasts represent a rich source of NGF (Young et al., 1974).

The question that then arises is, what is essential for neurosecretory axon regeneration, the permeable vascular bed or the glial microenvironment or both? The fact that neurosecretory regeneration occurs under all experimental conditions in which the BBB is breached, and always occurs in association with various functional glial cells indicates that several factors work in concert to permit the survival and growth of transected neurosecretory axons. The observations that neurosecretory axon regeneration did not occur into cryotreated neural lobe explants, in which pituicytes were killed, points towards an important, maybe pivotal, role of pituicytes (Carithers and Dellmann, 1992b). Conversely, since neural lobe explants are poorly vascularized and their capillaries are of the continuous type (Dellmann et al., 1991), the impermeable vascular bed in relation to neurosecretory axon regeneration may also have a significance in the results of this study.

The hypothesis that the presence of specific vascular and glial microenvironments is a prerequisite for successful regeneration of neurosecretory axons underscores the important interplay between vascular and glial microenvironments. Neural lobe, sciatic nerve, and optic nerve grafts, i.e., tissues that provide significantly different vascular and cellular components, are all capable of initiating and sustaining limited regeneration of intrahypothalamically

severed neurosecretory axons when placed into contact with them. Following lesioning and/or grafting, the BBB to circulating HRP becomes ineffective at and around the lesion site and the grafts. During this period of compromised BBB, neurosecretory axon regeneration was commonly seen in areas permeable to HRP. The timing of regressive neurosecretory axon regeneration coincides with the reestablishment of the BBB. Neurosecretory axons regenerating into these areas were associated with glia characteristic of the respective grafts. There are noticeable differences as to the magnitude of neurosecretory axon regeneration into different types of grafts; regeneration is most intensive into transplanted neural lobes, fewer neurosecretory axons regenerate into sciatic nerve grafts, and the least number of neurosecretory axons regenerate into optic nerve transplants. Therefore, glia apparently support neurosecretory axon regeneration, but with variable extent, with pituicytes providing the best glial microenvironment, followed by neurolemmocytes and neurolemmocyte-like cells, and then astrocytes. Even though there is morphological evidence for hormone release from axon terminals within all three types of graft, substantial recovery from transection-induced diabetes insipidus occurs only in neural lobe grafted animals.

What accounts for these differences ? What are the specific characteristics of the neural lobe microenvironment

that makes this graft the most successful one ? Which structural elements are present in the sciatic and optic nerve grafts that support neurosecretory axon regeneration ? Is there a relationship between capillary density and/or presence or absence of capillary fenestrations and the magnitude of neurosecretory axon regeneration ?

The present investigations confirm previous studies (Dellmann et al., 1987a) and extend them in that both morphology and function of the transplanted neural lobes are evaluated, with the following results. Three prominent features differentiate neural lobe grafts from other graft types: (1) the extent of their vascular permeability, (2) their capillary density and (3) the presence of pituicytes. The BBB was absent throughout the entire experimental periods only in neural lobe grafts. Morphometric data demonstrate that neural lobe grafts have capillary networks that are 112% denser than in sciatic nerve grafts, and 135% more dense than in optic nerve grafts. Thus, the neural lobe grafts, which contain the densest capillary network, are also the site of much greater neurosecretory regeneration than the sciatic and optic nerve grafts.

Functional analysis

Daily water intake measurements were undertaken with the expectation that deficits in AVP secretion following HNT-

transection might be reversed by subsequent neurosecretory axon regeneration into various grafts. In all animals, a consistent multiphasic pattern of water intake is observed. There is a marked polydipsia during the first 24 hours, followed by a short period of decreased water consumption. This is followed by a third period of increased water consumption for 2-3 days, and finally by a decrease for the rest of the experimental periods. There are two possible explanations for the brief postsurgery peak in water consumption. All animals were anesthetized with xylazine in combination with pentobarbital; and xylazine, a potent diuretic (Doherty, 1988), which may be responsible for the increase in water intake. The second reason is that because neuronal perikarya and axon terminals are disconnected in tract-transected animals, signals for AVP secretion in response to partial water loss are not transmitted, and increased hormone release is not initiated. Increased drinking may, therefore, be the animal's response to anesthesia-induced water loss in the absence of hormone release.

To date, plasma AVP values in intrahypothalamically-transected animals have not been reported in the literature, but one may reasonably assume that HNT transection completely blocks AVP release. Following the brief post-surgery polydipsia, a short period of decreased water consumption

occurs, which coincides with the time interval during which the disconnected axons in the neural lobe are disposed of by phagocytosis (Dellmann, 1973; Dohanics et al., 1991). No data have been reported in the literature on plasma AVP levels during this period. Neural lobe explants, in which phagocytosis proceeds as in vivo, but at a slower pace, release AVP into the culture medium (Dellmann, personal communication). It is likely that phagocytic activity also releases AVP in vivo. The slow rise in water consumption (2-3 days post-surgery) is considered to be the result of the absence of further AVP release from the degenerated neural lobe terminals. Such a pattern of water intake would not be expected in neurolobectomized rats (Moll and De Wied, 1962), since the neural lobe is removed. The subsequent drop in water intake (6-8 days) occurs during initial vigorous axonal regeneration into perivascular spaces of the adjacent hypothalamus and into the grafts (Dellmann et al., 1987a).

Comparison of water consumption curves and morphologic findings reveals a correlation only in neural lobe grafted animals. In these subjects, decreased water consumption began 6-8 days after surgery. This coincides temporally with morphological observations of significant neurosecretory axon regeneration into neural lobe grafts (Dellmann et al., 1987a, present study). The subsequent continuous decline in water intake coincides with a gradual increase in neurosecretory

axon regeneration in neural lobe grafts. Regeneration produced significant amelioration of the polydipsia following transplantation. In the responding animals, a complete restoration of normal water balance (i.e., identical to presurgery values) was almost reached. It is possible that a more complete study covering longer post-grafting observations will reveal the true extent to which functional recovery occurs.

Contrary to expectations, sciatic nerve-grafted animals with marked morphological signs of AVP release remain at all times at diabetic levels (more than 120 ml/day). This situation may by explained by a release of hormones which do not gain access to the general circulation due to the low capillary density and/or the predominance of non-fenestrated capillaries. Another possible explanation is that the number of regenerating neurosecretory axons is so small that the total amount of AVP released is insufficient to ameliorate diabetes insipidus. Finally, one cannot minimize the contribution to neurosecretory axon regeneration and functional recovery that may be unique to specific supportive cells, i.e., pituicytes. In this context, pituicytes are thought to influence hormone release in a number of ways discussed previously (Wittkowski, 1986; Hatton, 1990). This could explain why otherwise morphologically well organized terminal fields observed in sciatic nerve grafts do not

support functional recovery in the absence of pituicytes.
Animals receiving bilateral cuts or optic nerve grafts
remained diabetic at all times. This situation is consistent
with the morphological findings of low numbers of regenerating
neurosecretory axons in lesion scar or optic nerve grafts.

SUMMARY AND CONCLUSIONS

The results of the present investigations provide evidence in support of the hypothesis that regeneration of neurosecretory axons occurs only in the presence of specific vascular and glial microenvironments. Neurosecretory axon regeneration was initiated in a microenvironment lacking a BBB. The degree of vascular permeability to HRP in the various grafts was correlated with the magnitude of neurosecretory axon regeneration.

In neural lobe grafts, where the BBB is permanently absent, blood-borne substances were available throughout the experimental period and regeneration of neurosecretory axons was extensive as compared to other graft types. Sciatic nerve grafts were heterogeneously permeable to HRP throughout 80 days, and neurosecretory axon regeneration was most pronounced into areas of the grafts in which the BBB was lacking. In lesions and optic nerve grafts, where the BBB was restored after 10 and 30 days, respectively, only relatively few neurosecretory axons regenerated. Neurosecretory axon regeneration around intrahypothalamic perivascular spaces in the vicinity of all grafts appears to regress with post-transplantation time, suggesting that neurosecretory axon regeneration ceases when the reformed BBB at these sites

deprives them of blood-derived factors.

The contribution of glial cells, pituicytes, neurolemmocytes, neurolemmocyte-like cells, and astrocytes to neurosecretory axon regeneration and formation of terminal fields is also of great importance. The intimate and consistent association of these glial cells with regenerating neurosecretory axons suggests a significant role of these cells in influencing the regenerative and functional responses either by direct guidance and/or secretion of diffusible factors.

Substantial functional recovery was only observed in neural lobe-grafted animals. Restoration of near normal water intake occurred after 30 days at which time neural lobe grafts had structural characteristics similar to those of control neural lobes. Sciatic nerve grafts, in spite of their invasion by numerous regenerating neurosecretory axons failed to alleviate diabetes insipidus. It remains to be established whether fewer regenerating neurosecretory axons in the sciatic nerve grafts release insufficient amounts of AVP or whether released AVP fails to access the general circulation because of low capillary density and/or the predominance of non-fenestrated capillaries. It may also be that functional regeneration is only possible in the presence of pituicytes.

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188

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ACKNOWLEDGEMENTS