

The growth-promoting action of the insulin-like growth factor family in the magnocellular neurosecretory system during axonal sprouting
by Xinrong Zhou

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biological Sciences
Montana State University
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Abstract:
The ability of uninjured magnocellular neurons to undergo collateral axonal sprouting was discovered in the early 1990s (Watt and Paden, 1991). Following unilateral destruction of the magnocellular neurosecretory system (MNS) by a hypothalamic knife-cut, a robust sprouting response occurred in the uninjured contralateral MNS neurons. What causes sprouting by magnocellular neurons following unilateral destruction of the MNS? Neurotrophins and other growth factors could play a role in axonal sprouting. The insulin-like growth factors (IGFs) are among those that have the potential to exert major growth-promoting actions that may be essential for sprouting in the MNS. This thesis is based on the hypothesis that IGF-I acts as a growth factor to initiate and/or support collateral sprouting of magnocellular axons. Several experiments were done to address this hypothesis. Immunoreactivity and mKNA expression of IGF-I, IGF-I receptor and insulin-like growth factor binding protein-2 (IGFBP-2) in the MNS were studied using immunocytochemistry and in situ hybridization. IGFBP-2 and its mKNA were found to be present in the pituicytes of the neural lobe. IGF-I receptor was detected in the magnocellular axons. The elevated expression of IGFBP-2 mKNA was detected at 10 days after the hypothalamic lesion. Upregulation of IGFBP-2 mKNA expression correlated with axonal sprouting spatially and temporally and therefore may act to promote collateral axonal sprouting in the neural lobe. Expression of IGF-I mKNA was detected in the magnocellular neurons, and following intraventricular injections of colchicine, IGF-I was also demonstrated to be present. Dual-label immunofluorescent staining showed that only a subset of oxytocinergic (OT) and vasopressinergic (VP) neurons contained IGF-I. Implantation of IGF-I in the hypothalamus was employed in an attempt to determine whether exogenous IGF-I was capable of stimulating axonal sprouting and/or neuronal survival. IGF-I implanted animals exhibited increased growth of OT axons compared to animals implanted with bovine serum albumin (BSA) or empty cannula. The growing nerve fibers were found along the IGF-I implantation track. In addition, growth of OT axons toward the ipsilateral anterior cerebral artery was present in all animals implanted with IGF-I. Thus, IGF-I in conjunction with other components of the IGF system may act to stimulate axonal sprouting in the MNS.

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ABSTRACT

The ability of uninjured magnocellular neurons to undergo collateral axonal sprouting was discovered in the early 1990s (Watt and Paden, 1991). Following unilateral destruction of the magnocellular neurosecretory system (MNS) by a hypothalamic knife-cut, a robust sprouting response occurred in the uninjured contralateral MNS neurons. What causes sprouting by magnocellular neurons following unilateral destruction of the MNS? Neurotrophins and other growth factors could play a role in axonal sprouting. The insulin-like growth factors (IGFs) are among those that have the potential to exert major growth-promoting actions that may be essential for sprouting in the MNS. This thesis is based on the hypothesis that IGF-I acts as a growth factor to initiate and/or support collateral sprouting of magnocellular axons. Several experiments were done to address this hypothesis. Immunoreactivity and mRNA expression of IGF-I, IGF-I receptor and insulin-like growth factor binding protein-2 (IGFBP-2) in the MNS were studied using immunocytochemistry and in situ hybridization. IGFBP-2 and its mRNA were found to be present in the pituicytes of the neural lobe. IGF-I receptor was detected in the magnocellular axons. The elevated expression of IGFBP-2 mRNA was detected at 10 days after the hypothalamic lesion. Upregulation of IGFBP-2 mRNA expression correlated with axonal sprouting spatially and temporally and therefore may act to promote collateral axonal sprouting in the neural lobe. Expression of IGF-I mRNA was detected in the magnocellular neurons, and following intraventricular injections of colchicine, IGF-I was also demonstrated to be present. Dual-label immunofluorescent staining showed that only a subset of oxytocinergic (OT) and vasopressinergic (VP) neurons contained IGF-I. Implantation of IGF-I in the hypothalamus was employed in an attempt to determine whether exogenous IGF-I was capable of stimulating axonal sprouting and/or neuronal survival. IGF-I implanted animals exhibited increased growth of OT axons compared to animals implanted with bovine serum albumin (BSA) or empty cannula. The growing nerve fibers were found along the IGF-I implantation track. In addition, growth of OT axons toward the ipsilateral anterior cerebral artery was present in all animals implanted with IGF-I. Thus, IGF-I in conjunction with other components of the IGF system may act to stimulate axonal sprouting in the MNS.

CHAPTER 1

INTRODUCTION

Statement of Purpose

The purpose of this project was to investigate the possibility that insulin-like growth factor I (IGF-I) and insulin-like growth factor binding protein-2 (IGFBP-2) as well as insulin-like growth factor I receptor (IGF-I receptor) are involved in the compensatory collateral sprouting of magnocellular neurosecretory axons following a unilateral hypothalamic lesion. Three research projects were undertaken to fulfill this goal: 1) localization of IGF-I, IGFBP-2 and IGF-I receptor in the magnocellular neurosecretory system, 2) detection of changes in expression, in particular, of possible up-regulation of IGF-I, IGFBP-2 and IGF-I receptor corresponding to the compensatory collateral sprouting, 3) investigation of the possibility that IGF-I stimulates growth of axons in the magnocellular neurosecretory system (MNS) by implantation of IGF-I in the hypothalamus.

To address the first goal, immunocytochemistry and in situ hybridization were used to localize IGF-I, IGFBP-2 and IGF-I receptor expression in the hypothalamus and the neural lobe of the pituitary. For IGF-I immunocytochemical staining, intraventricular

injections of colchicine were used to enhance the IGF-I immunoreactivity in cell bodies of magnocellular neurons in the supraotic nuclei (SON) and the paraventricular nuclei (PVN). To address the second goal, changes in IGF-I and IGFBP-2 as well as IGF-I receptor immunoreactivity were estimated visually and changes in the corresponding mRNAs were measured by densitometry of film autoradiograms from rats in both control groups (intact and sham) and lesion groups. To address the question of whether IGF-I stimulates growth of the magnocellular axons, IGF-I was implanted in the hypothalamus and released slowly from a polymer matrix. The density and distribution of magnocellular axons stained with anti-oxytocin antibody were compared in IGF-I implantation groups and controls (BSA implantation or implantation with empty cannula).

Plasticity in the Mammalian CNS

Plasticity in neuroscience usually refers to the capacity of the nervous system to change or modify synaptic activity at the anatomic, neurotransmitter or metabolic levels. Anatomical accounts emphasize the ability of the CNS to modify synaptic connections by axon sprouting or regeneration in response to CNS injury. Both forms of growth can reinnervate and restore function to denervated cells. The development of the new outgrowths appears to be controlled partially by the denervated tissues which can stimulate nerve growth and accept innervation, and by the availability of suitable pathways for the sprouting or regenerating axons to follow.

In the peripheral nervous system of nearly all species and the central nervous system of many lower forms, regeneration of severed axons from the proximal stump can occur, and the new outgrowths can be guided back to their original target cells by the structures in the degenerating distal nerve segments (Cajal, 1928; Grimm, 1971; Friede and Bischhausen, 1980). For example, following peripheral crush or cold injury, most regenerating axons remained within the Schwann cell basal membrane and then reinnervated their original target cells (Sunderland, 1978; Bolin and Shooter, 1994). The rate of regeneration was initially slow, but after a few days, the nerve grew up to 8mm per day.

In contrast to the PNS, regeneration is uncommon in the CNS of mammals. Although successful regeneration typically does not occur spontaneously within the mammalian CNS, the presence of abortive sprouting suggests that adult axons and neurons retain a growth potential or can upregulate their growth machinery in response to lesions. A variety of experimental results support this hypothesis. For example, implants of Schwann cells or of embryonic spinal cord tissue or injection of some neurotrophic factors greatly enhanced corticospinal tract sprouting (Schnell, et al., 1994; Li and Raisman, 1994). Transitory axotomy-induced sprouting accompanied by a transitory upregulation of the growth associated protein GAP-43 (a membrane phosphoprotein related to axonal growth and synaptic remodeling in the nervous system) has been observed in retinal ganglion cells (Schaden, et al.,1994). The sprouting of cerebellar Purkinje cells in response to axotomy has been observed after a delay of several weeks (Dusart and Sotelo, 1994). The sprouting of cholinergic

septohippocampal fibers was increased very extensively if fimbria-fornix lesions were fused with an implant containing nerve growth factor (He, et al.,1992).

In contrast to the failure of regenerative sprouting in the CNS, target denervation can induce collateral sprouting of intact adult axons. Collateral sprouting means that undamaged axons innervate the denervated areas by developing new outgrowths. Reactive collateral sprouting has been observed from intact axons into these denervated areas in several regions of the CNS. In the septum denervated by fimbria-fornix lesions, extensive aberrant sprouting of serotonergic axons was observed (Morrow, et al., 1983). In the hippocampus, perforant path lesions led to sprouting of several types of axons (Gasser and Dravid, 1987). Partial destruction of the inferior olive neurons and their climbing fibers to the cerebellum induced sprouting of surviving climbing fibers which innervated the denervated Purkinje cells (Wiklund, et al., 1990). In the adult spinal cord, dorsal root lesions induced a small amount of collateral sprouting from primary afferent fibers of neighboring intact roots (Janig, et al., 1996).

The Magnocellular Neurosecretory System

The magnocellular neurosecretory system (MNS) consists of large neurons which are concentrated in two paired structures, the supraoptic (SON) and paraventricular (PVN) nuclei. These cells are also found in other hypothalamic nuclei. Most of their axons project through the ventromedial hypothalamus, median eminence and infundibular stalk to the neural lobe of the pituitary where they form synaptoid contacts with the outer basement membrane of the capillary plexus. Their axon terminals contain

oxytocin (OT) or vasopressin (VP), peptide hormones that are synthesized in the cell somata and then transported down the axons into swellings, the largest of which are called "Herring bodies", and into the terminals where they are stored and secreted (Boersma, et al.,1993; Yukitake, et al., 1977). These two neurohypophysial hormones are transported to distant targets via the systemic circulation. Vasopressin acts as a antidiuretic hormone at the renal tubules and also acts as a vasoconstrictor in the circulatory system (Hofbauer, et al., 1984). Oxytocin induces milk ejection and contractions of the uterus (Jenkins and Nussey, 1991).

Organization of the SON and PVN

In the rat, the two SON are located at the ventral surface of the brain near the optic chiasm. They are divided by the optic tract into the principal part and the retrochiasmatic part. The principal part has been the subject of most experimental investigations. It begins with loosely packed neurons just dorsal and lateral to the optic chiasm and extends caudally into the posterior hypothalamus as a dense cluster of large cells adjacent to the optic chiasm (Dierickx and Vandesande,1979; Armstrong and Hatton, 1980). The ratio of vasopressinergic neurons to oxytocinergic neurons in the SON differs considerably among species. The ratio of VP:OT neurons in male rats is about 5:3 (Rhodes, et al. 1981; Dierickx and Vandesande, 1979). Oxytocinergic neurons predominate in the rostral and dorsal part of the SON, while vasopressinergic neurons are more numerous in the caudal and ventral regions (Rhodes, et al. 1981; Dierickx and Vandesande, et al., 1979).

The heart-shaped PVN lies on either side of the dorsal third ventricle. The PVN is divided into three subnuclei; the medial, lateral and posterior. Oxytocinergic neurons predominate in the medial subnucleus, while the lateral subnucleus consists of a core of vasopressinergic neurons with a few oxytocinergic neurons forming a rim around the vasopressin cells (Rhodes, et al. 1981; Dierickx and Vandesande, 1979). The neurons of the medial and lateral subnuclei are closely packed together and nearly round, while the neurons of the posterior PVN are loosely packed and spindle shaped with their long axis running in a medio-lateral direction. The ratio of oxytocinergic neurons to vasopressinergic neurons in the PVN is similar to that in the SON (Rhodes, et al. 1981; Swaab, et al 1975)..

By combining immunocytochemistry for the neurohypophysial peptides with injections of a retrograde tracer into the neural lobe, it was concluded that virtually all SON neurons that transported horseradish peroxidase from the neural lobe also immuno-stained for either OT or VP (Armstrong and Hatton, 1980; Hosoya and Matsushita, 1979). The SON neurons appear to project their axons exclusively to the neural lobe with those axons containing either VP and OT. Autoradiographic studies have shown that the axons from each SON spread throughout the entire neurohypophysis but are most dense in the central region of the organ (Fisher, et al 1979). In contrast to the limited distribution of the SON neural axon projections, those of the PVN are much wider. PVN magnocellular axons project to the external zone of the median eminance, to the brain stem and to the spinal cord caudally as far as the 6th lumbar segment as well as to the neural lobe (Fisher, et al., 1979; Hosoya and Matsushita, 1979).

The Fine Structure of the Neural Lobe

The neural lobe consists of neurosecretory axons, axonal swellings, and both non-neurosecretory and neurosecretory axon terminals in intimate contact with the pituicytes in addition to perivascular microglial cells. The vascular endothelia are fenestrated, and the vascular and neural compartments are separated by the endothelial and parenchymal basement membranes.

There are two kinds of glial cells, pituicytes and microglia, in the neural lobe. Pituicytes are fibrous glial cells which correspond to astrocytes in the brain. Pituicytes may be involved in the regulation of oxytocin and vasopressin secretion. Both neurosecretory endings and pituicytes abut the basal lamina that separates the neural from the perivascular compartment (Tweedl, et al., 1989). Under basal conditions pituicytes interpose their processes between the axon terminal and the basal lamina (Dellmann and Sikora, 1981; Hatton, 1990; Beagley and Hatton, 1992). At times of increased hormonal secretion, pituicytes will retract their processes. This allows axonal terminals to contact the basement membrane directly and to release hormones into the circulation (Dellmann, et al., 1988). In addition to undergoing morphological plasticity, pituicytes may proliferate during prolonged periods of intensive neurosecretory activity (Marzban et al., 1992; Beagley and Hatton, 1994).

Microglia constitute the other type of glial cells in the neural lobe. They are morphologically, immunophenotypically and functionally related to cells of the macrophage lineage (Dellmann and Sikora,1981; Hatton, 1997; Mander and Morris, 1996). Microglia may play a role in degradation and remodeling of the terminal architecture. Because microglia appear to be constantly engulfing axonal endings that

have invaded the perivascular space, it has been suggested that the degradation and formation of terminals may be a normal phenomenon in the neural lobe (Dellmann and Sikora,1981; Tweedle, et al., 1989).

Regeneration in the Magnocellular Neurosecretory System

The MNS exhibits a high degree of structural plasticity and recovery of neuroendocrine function following different kinds of neuroanatomical and physiological manipulations. Billingstein and Leveque (1955) were the first investigators to report spontaneous regeneration of neurosecretory axons in the severed hypophysial stalk and its reorganization into a neural lobe-like structure. This finding was confirmed by Adams (Adams, et al., 1969) and other investigators (Moll and DeWied, 1962). Since then, a wealth of literature has supported the contention that the magnocellular system, unlike most other parts of the brain, is a reliable model to investigate the processes of plasticity, regeneration, sprouting and recovery of function in the mammalian CNS. With the exception of the olfactory system, the magnocellular neurosecretory system is a unique structure with respect to its robust regenerative and sprouting capacities (Dellmann, et al., 1989; Kawamoto and Kawashima, 1987; Dellmann, et al.,1987).

Transection of magnocellular axons in the hypophyseal stalk or in the median eminence could induce magnocellular axonal regeneration (Dellmann, et al.,1988). New neurovascular contacts were established between regenerated axon terminals and the fenestrated capillaries in either of those regions, forming structures resembling miniature neural lobes (Wu, et al., 1989). Investigations employing immuno-electron microscopy further confirmed the regeneration of magnocellular axons following hypophysectomy.

By 4 weeks after hypophysectomy, a large number of magnocellular neurites grew into all zones of the median eminence, particularly the zona externa, in juxaposition to fenestrated capillaries of the hypophyseal portal system. Moreover, this process of neuritic regeneration was observed to occur in the lumen of the adjacent third ventricle (Scott, et al., 1995). These phenomena might represent a progressive compensatory physiological response to injury of the neurohypophyseal system, since release into the cerebrospinal fluid might allow entry of VP and OT into the systemic vascular circulation of hypophysectomized adult rats.

In contrast, very little axonal regeneration occurred when the site of transection was in the hypothalamus, although a few neurosecretory axons could regenerate into nearby perivascular areas (Marciano, et al., 1989). However, transected neurosecretory axons regenerated more abundantly into grafts of the neural lobe placed near the proximal tips of these axons, and also into the leptomeninges when the ventral surface of the hypothalamus was lesioned simultaneously with graft placement (Dellmann, et al., 1988). Light microscopic observations showed that neurovascular contact regions developed within the grafted tissues. Neurosecretory axons usually coursed individually through the grafts, but they occasionally formed bundles. Most frequently, axons had a tortuous, irregular arrangement, but sometimes they had a straight, almost parallel course (Dellmann, et al., 1989). When reaching blood vessels such as arterioles, venules and capillaries, they always formed plexuses ranging in density from a few single axons to numerous densely woven ones forming perivascular sleeves. At the fine structure level, neurosecretory axons penetrating grafts were consistently accompanied by astrocytes or astrocytic processes. Within the grafts, glial cells and their lamellopodia

ensheathed neurosecretory axons fully or partially. Processes of astrocytes also extended between terminals and the perivascular surface, frequently intervened between terminals and basal lamina. These fine structures were similar to typical arrangements of pituicytes and neuronal elements seen in intact neural lobes (Gash and Scott, 1980; Scott and Sherman, 1984).

Collateral Sprouting in the Magnocellular Neurosecretory System

The ability of uninjured magnocellular neurons to undergo collateral axonal sprouting was discovered in the early 1990s (Watt and Paden, 1991) and is currently the primary research topic within our laboratory. Following unilateral destruction of the MNS by a hypothalamic knife-cut, a vigorous sprouting response occurred in the uninjured contralateral MNS neurons. This sprouting response restored the axonal population of the partially denervated neural lobe to 76 percent of normal 32 days after the lesion and complete restoration was seen at 90 days. Using in situ hybridization, we were also able to demonstrate that sustained and coordinated increases in levels of mRNA for the axonal growth-associated α1 and βII-tubulin isotypes accompanied collateral axonal sprouting by uninjured SON neurons (Paden et al., 1995).

What causes magnocellular neuron sprouting following unilateral destruction of the MNS? It is unlikely that any single element or mechanism can account for such a complex process as axonal sprouting. Axonal sprouting can be regulated on at least two levels: in the cell body by an upregulation of the components of neural growth and at the level of the axon by signals that cause formation and elongation of growth cones. Neurotrophins and other growth factors can act on both of these levels and they could

play a role in axonal sprouting. A variety of studies indicate that specific growth factors can act to stimulate axonal growth in particular populations of neurons. These will be reviewed in the next section, including those studies suggesting that specific growth factors, such as nerve growth factor (NGF), basic fibroblast growth factor (bFGF) and insulin-like growth factor I and II (IGF-I and IGF-II), may act on neurons in the MNS.

Growth Factors in Neuronal Regeneration and Sprouting

At the turn of the century, Ramon y Cajal observed that regenerating axons preferentially grew towards the deafferented distal stump of a transected axon (Cajal, 1928). It was suggested that there were some soluble nerve-derived neurotrophic factors and growth factors in the damaged area. Since then many different neurotrophic factors and growth factors have been found such as NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), ciliary neurotrophic factor (CNTF), bFGF and the IGFs. These factors enter the lesion site from the blood and are also expressed and released into the local environment of the lesion site or deafferented area by the damaged tissue itself (Haynes, 1988; Scalapino, 1996). They could be produced by either non-neuronal cells or nerve cells.

The neurotrophin family consists of four peptides: NGF, BDNF, NT-3 and neurotrophin-4/5 (NT-4/5). Each individual neurotrophin facilitates survival, sprouting and regeneration of distinct neuronal populations. For example, it has been demonstrated that NGF and BDNF exert striking neurotrophic actions on cholinergic neurons, while in contrast, NT-3, but not NGF, supports the survival of the dopaminergic neurons of the substantia nigra (Hyman, et al., 1991).

Many studies have shown that NGF might play a role in axonal sprouting. In the hippocampal formation, there is a strong correlation between the regions innervated by sprouting axons and the distribution of NGF immunoreactivity following destruction of septal afferents by a fornix-fimbria lesion. Thus availability of NGF may stimulate sprouting by NGF-sensitive axons (Conner, et al., 1994). It has also been found that axotomy of cholinergic afferents to the hippocampus by a fornix-fimbria lesion induced an elevation of NGF content in the hippocampus. Elevated levels of NGF are important not only for the survival of the adult cholinergic neurons, but can also stimulate sprouting of the damaged fibers (Conner, et al., 1994). These findings indicate that endogenous NGF may play a crucial role in controlling axonal sprouting and reinnervation by septal cholinergic axons, and that NGF and possibly other endogenous signals may stimulate local terminal sprouting as well. These findings are further supported by experiments using infusion of NGF into the lateral ventricle of the mature rat brain. Exogenous NGF induced sprouting from axons associated with the intradural segment of the internal carotid artery. The total number of perivascular axons was increased three fold (Isacson, et al., 1992). Moreover, in a model of temporal lobe epilepsy, kindling has been reported to induce synthesis of NGF and also elicits mossy fiber sprouting and functional synaptogenesis in the hippocampus. Intraventricular administration of antibodies to NGF could block the kindling-induced mossy fiber sprouting into the stratum oriens of the CA3 region of the hippocampus (Van der Zee, et al., 1995).

However, NGF is ineffective in stimulating sprouting in other types of neurons. For example, BDNF could promote the sprouting of mature, uninjured serotonergic axons

and dramatically enhanced the survival and sprouting of 5-HT axons damaged by the serotonergic neurotoxin p-chloroamphetamine, while infusion of NGF did not have these effects (Mamounas, et al., 1995). Furthermore, when the dentate granule neurons were cultured in vitro with different neurotrophins for determining their effectiveness on axonal sprouting (Patel and McNamara, 1995), the rank order of effectiveness of axonal sprouting was: bFGF > BDNF > NT-4/5 > NT-3; NGF was ineffective.

Basic FGF also has well documented effects on axonal sprouting by a variety of cells, including PC12 cells, chromaffin cells and neurons of the CNS (Chalmers, et al., 1995; Miyamoto, et al., 1993). Basic FGF has also been found to be a neurite-promoting factor for cerebellar granule cells. This factor strongly enhanced axonal sprouting and neurite-outgrowth of granule neurons in adult cerebellum (Guthrie, et al., 1995). Moreover, investigations in vivo have demonstrated that bFGF also promoted axonal sprouting and regrowth of dopaminergic neurons. When bFGF was released via a gel foam implant in the 1-methyl-4-phenyl-1,2,3,6-tetra-hydropyridine (MPTP) lesioned nigrostriatal dopaminergic system of the adult mouse, it induced the sprouting and regrowth of striatal dopaminergic fibers (Park and Mytilineou, 1992). Moreover, it has been reported that the combination of bFGF and heparin could stimulate outgrowth of fetal mesencephalic dopaminergic neurons implanted in the denervated rat caudate-putamen (Zeng, et al., 1996).

Promotion of neurite outgrowth and axonal sprouting by IGF-I and IGF-II has also been well documented. In vitro, IGFs promoted neurite outgrowth and also selectively increased α- and β- tubulin mRNA expression in cultured human neuroblastoma SH-SY5Y cells (Fernyhough, et al., 1989). IGF-I increased survival and stimulated

outgrowth of neurites in cultured central and peripheral neurons, including mesencephalic dopaminergic nerve cells, forebrain cholinergic neurons, and spinal cord motor neurons (Recio-Pinto, et al., 1986; Caroni and Grandes, 1990). Using serum-free culture of lumbosacral sympathetic ganglia, both IGF-I and IGF-II were shown to stimulate neurite outgrowth (Knusel and Hefti, 1991).

Moreover, the IGFs have been shown to be potent stimulators of neuron regeneration and sprouting in vivo. Both IGF-I immunoreactivity and its mRNA level were increased within the injured areas in different injury models, including sciatic nerve crush (Pu, et al., 1995), the hippocampus deafferented by an entorhinal cortex lesion (Guthrie et al., 1995), hypoxic-ischaemic injury (Lee and Bondy, 1993b) and brain trauma (Walter, et al., 1997). IGFBP-2 is one of six insulin-like growth factor binding proteins (IGFBPs) which can bind to IGFs or regulate interaction between IGFs and their receptors. Both IGFBP-2 immunoreactivity and its mRNA level were also elevated in these injury models. Different members of the IGF family increased their mRNA expression at different times which could indicate coordination of their different functions in repair of damage during neuronal regeneration. This hypothesis was further supported by the finding that administration of IGF-I into the injured areas or systemic administration of IGF-I could prevent neurons from death and promote axonal sprouting and neurite growth (Conteras, et al., 1995; Guan, et al., 1993; Gluckman, et al., 1992; Akahhori and Horie, 1997).

Evidence for Specific Growth Factors in the MNS

There is some evidence for potential actions of specific growth factors in the MNS, including NGF, bFGF and IGF-I.

NGF-like immunoreactivity was seen in the hypothalamus following colchicine injection into the brain. Moderate immunostaining was detected in most magnocellular neurons of the PVN and SON (Conner and Varon, 1992). The term "NGF-like" is used here since the possibility exists that the antibody may cross react with other neurotrophins sharing a high degree of sequence homology with β–NGF.

Basic FGF and its high affinity receptor have been found in the hypothalamic-pituitary axis by immunocytochemistry and their mRNAs localized by in situ hybridization (Gonzalez, et al., 1994). In the neural lobe, bFGF immunoreactivity was detected in the basal lamina and axonal Herring bodies from projecting magnocellular neurons, while pituicytes exhibited intensive staining for FGF receptor. No mRNA for bFGF was found in the pituitary. In contrast, both the anterior and neural lobes contained FGF receptor mRNA. In the hypothalamus, bFGF and its receptor were associated with different structures. Basic FGF was detected immunocytochemically in the neuronal perikarya of some magnocellular neurons in both the PVN and SON, while its receptor was located in MNS axons. Magnocellular neurons of the PVN and SON also expressed mRNA for bFGF and its receptor by in situ hybridization (Wanaka, et al., 1990).

Both immunoreactivity and mRNA of IGF family members have been detected in the MNS (see Table.1). In the PVN and SON, the magnocellular neurons showed IGF-I immunoreactivity (Aguado, et al., 1992). The IGF-I staining was also found in their

axons and dendritic processes. The most intensive immunoreactivity of IGF-I was seen in the neural lobe, while the anterior lobe showed faint staining. By in situ hybridization, mRNA for IGF-I receptor was found to be widely distributed throughout the brain and pituitary (Aguado, et al., 1993). A high level of IGF-I receptor mRNA was detected in the neural lobe, while the SON and PVN also expressed IGF-I receptor mRNA. Another report showed that pituicytes expressed mRNAs of IGFBP-2, 4 and 5 by in situ hybridization, while the microglia could express IGFBP-3 mRNA. Among these IGFBPs in the neural lobe, IGFBP-2 was predominant (Bach and Bondy, et al., 1992; Marks, et al., 1991). In addition, both IGF-I and IGF-II mRNA were expressed in the neural lobe.

Components of the IGF system have also been detected in other brain regions which continue to show a high degree of neuronal plasticity in adulthood, including the olfactory bulb, hippocampus, and cerebellum (Bondy, et al., 1992; De Keyser, et al., 1994; Garcia-Segura, et al., 1997). These findings further suggest that the IGF system may be involved in neuronal plasticity.

Table 1. Distribution of the IGF System in the MNS

Location	Immunocytochemistry	in situ Hybridization	References
PVN, SON		IGF-I receptor	Aguado, 1993
	IGF-I		Aguado,1992
	IGF-I		Garcia-Segura,1991
Neural Lobe		IGF-I receptor	Aguado, 1993
	IGF-I		Aguado,1992
		IGF-I, IGF-II, IGFBP2-5	Bach, 1992
Median Eminence	IGF-I		Aguado,1992

Taken together, these data suggest that the IGF system has the potential to play a significant role in axonal sprouting within the MNS. It is therefore necessary to review the IGF system and its biological functions in neural injury and axonal sprouting in detail.

Insulin-Like Growth Factor (IGF) System

The insulin-like growth factor system consists of two growth factors which are IGF-I and IGF-II (Leroith, et al., 1993a), six IGF binding proteins (IGFBPs) (Clemmons, et al, 1993a; 1993b) and two receptors (IGF-I receptor and IGF-II receptor) (Leroith, et al., 1995). Although circulating IGFs are synthesized primarily in the liver and act in an endocrine mode, most other tissues also synthesize IGFs which can act locally in an autocrine or paracrine manner. IGFs are multifunctional polypeptides which participate in regulating development, support the growth and differentiation of fetal neurons, stimulate neuronal protein synthesis, increase neurite outgrowth and enhance the survival of injured neurons. IGFs act through the IGF-I receptor and IGF-II receptor which are trans-membrane bound tyrosine kinase receptors (Leroith, et al., 1995). Both IGF-I and IGF-II are present in the extracellular space bound mainly to specific, high affinity IGF-binding proteins which modulate the interaction of IGFs with the IGF receptors (Leroith, et al., 1993a; 1993b). In the rat, IGF-II is thought to have a primarily embryonic role and IGF-I has a postnatal role (Ayer-le Lievre, et al., 1991; Bondy, et al., 1992; Stylianopoulou, et al., 1988). This

review will therefore focus on the action of IGF-I and its receptor in the postnatal nervous system, as well as on IGFBP-2, the predominant IGFBP among six IGFBPs in the developing and adult brain (Sullivan and Feldman, 1994; Logan, et al., 1994).

Insulin-Like Growth Factor (IGF-I)

IGF-I is a basic, single-chain polypeptide of 70 amino acids which contains three disulfide bridges. IGF-I shares a high degree of sequence homology with insulin. This polypeptide, originally referred to as " somatomedin C" , was first identified as a serum protein which was believed to act physiologically as an endocrine mediator of the actions of growth hormone (Cohick and Clemmons, review, 1993). The majority of circulating IGF-I is made in the liver. IGF-I can also be synthesized in a variety of tissues, including CNS and muscle (Bondy and Lee, 1993; Cohick and Clemmons, 1993). Inside the brain, IGF-I is believed primarily to function in a paracrine or autocrine mode at the site of synthesis by binding to IGF-I receptors with high affinity (Leroith et al.,1995; Daughaday and Retwein, 1989).

Under normal physiological conditions, IGF-I immunoreactivity and mRNA are expressed in discrete areas of the nervous system including brain, spinal cord motor neurons, and autonomic ganglia as well as in skeletal muscle. Within or associated with these specific tissues, astrocytes, microglia, skeletal muscle and Schwann cells have been demonstrated to contain IGF-I immunoreactivity and/or mRNA (Han, et al., 1987; Bondy, et al., 1992; Werther, et al., 1990). In the developing rat brain, IGF-I mRNA is highly expressed by neurons in central sensory and cerebellar pathways during periods of dendritic maturation and synaptogenesis (Ayer-le Lievre, et al., 1991; Andersson, et al.,

1988). A highly focal neuroanatomical distribution of IGF-I mRNA as well as a dramatic developmental decline in levels was observed in the Purkinje cell layer of the cerebellum, and in specific areas of brainstem, midbrain and thalamus (Andersson, et al., 1988). In situ hybridization studies reported the presence of IGF-I mRNA in some areas of the adult CNS, including the olfactory bulb, piriform cortex, lateral geniculate body, hippocampus, cerebellum and retina (Garcia-Segura, et al., 1991). As a general trend, IGF-I mRNA is mainly present within the principal projection neurons in specific sites of sensory and cerebellar systems, but can be also found in the local circuit interneurons. For example, IGF-I mRNA was detected in medium to large pyramidal neurons in the cerebral cortex and hippocampus, whereas IGF-I mRNA was highly expressed in the mitral and tufted cells of the main olfactory bulb (Bondy, et al., 1992). These latter cells are virtually unique among central neurons in that they receive new primary sensory afferents and undergo continued dendritic and synaptic remodeling throughout adulthood. The presence of high IGF-I mRNA levels both in the developing brain and in the adult olfactory system suggests that IGF-I may play a role in the trophic support of afferent outgrowth and synaptogenesis.

IGF-I mRNA was not detected during early developmental stages except for a weak signal in the olfactory bulb (Bondy, et al., 1991). The expression of IGF-I mRNA became evident later in development and reached a peak in early postnatal life. IGF-I mRNA is found predominately in neurons. It generally exhibits its highest expression at times when neurons that express IGF-I are undergoing proliferation, such as in the cerebellum, olfactory bulb, hypothalamus and hippocampus (Lee, et al., 1992; Bondy, et al., 1991). For example, in the hippocampus, IGF-I expression is high in several

proliferative areas in the first 2 weeks of postnatal life, but reaches a peak at one month in the dentate gyrus where neuronal proliferation is more prolonged (Aguado, et al., 1992; Torres-Aleman, et al., 1994).

Targeted mutagenesis of the genes encoding IGF-I and the IGF type I receptor provides additional support for the important involvement of IGF-I during development. *Igf-1* (-/-) mutant mouse embryos grew at a normal rate before E-12.5. The growth deficiency of the mutants became evident at E-13.5 when the size of the mutant embryos was about 90% of normal. After that, the mutant embryos continued to grow at a slower rate than controls so that the mutant size only reached 60% of normal at birth. After birth, the mutant mice continued to grow at a retarded rate in comparison with control animals and eventually the weight of mutant adults was only 30% that of normal adults (Baker and Liu, 1993). IGF-I gene disruption resulted in reduced brain size, CNS hypomyelination, and loss of hippocampal and striatal parvalbumin-containing neurons (Beck et al., 1995). Those mice that survive to adulthood have very small brain (60% normal size) that is morphologically normal. These brains are characterized by a paucity of white matter owing to markly decreased myelination and an apparent decrease in the number of axons.

The growth-promoting effects of IGF-I on a variety of neuronal populations have been well documented. IGF-I stimulates sciatic nerve regeneration and sprouting after injury or lesion (Contreras, et al., 1995). Administration of IGF-I to either the dorsal root ganglion or locally to the crush site could stimulate regeneration of sciatic nerve after the crush Regeneration of these axons was dramatically increased after 3 to 4 days of superfusion compared to control groups.. Spontaneous regeneration was inhibited

when the nerve was treated with specific antibodies to IGF-1 (Near, et al., 1992). Another investigator reported that IGF-1 accumulated in the rat sciatic nerve after transection, and that IGF-1 could be involved in nerve regeneration processes (Pu, et al., 1995). It therefore appears that endogenous extracellular IGF-I plays an important role during regeneration of the sciatic nerve after damage. IGFs are presently the only neurotrophic factors endogenous to nerve that are known to increase the rate of nerve regeneration.

IGF-I is also a motor neuron sprouting factor. Axonal sprouting of spinal cord motor neurons is a compensatory process by which axons become more branched, enabling them to control more muscle fibers. IGF-I has been reported to increase intramuscular nerve sprouting 10-fold when administered subcutaneously to normal adult rats (Caroni and Grandes, 1990). This finding indicates that IGF-I has growth-promoting effects on motor neurons after peripheral administration. IGF-I administration also resulted in increased neuromuscular junction endplate size. The sprouting effects are likely mediated by accumulation of exogenously administered IGF-I at motor endplates, where this factor can then elicit a retrogradely mediated effect in motor neurons.

The up-regulation in expression of IGF-I and IGFBP-2 during motor neuron regeneration was observed in the facial nerve following transection (Gehrmann, et al., 1994). Hypertrophic astrocytes which surrounded the injured neurons expressed the mRNA of IGF-I and IGFBP-2. The gene expression of IGF-I and IGFBP-2 dramatically increased 4 days after lesion and reached peak levels at 7 days. The astrocytes continued to express the mRNA of IGF-I and IGFBP-2 for more than three weeks. The up-

regulation of astrocytic IGF-I and IGFBP-2 expression was prompt, prolonged and concentrated around injured neurons which exhibited increased expression of type-I IGF receptor mRNA. This injury-induced gene expression of IGF-I, IGFBP2 and type-I IGF receptor could promote regeneration of injured neurons and their axons by a paracrine pattern of action.

Deafferentation of the dentate gyrus induces a dramatic increase in IGF-I mRNA expression by reactive microglia that exhibits both temporal and spatial correspondence with axonal sprouting (Guthrie, et al., 1995). Following a lesion of entorhinal cortex, IGF-I mRNA in the outer molecular layer of the ipsilateral dentate gyrus became detectable at 2 days postlesion and reached peak levels at 4 days postlesion when the density of mRNA hybridization was 650% that of the contralateral side. After that, the expression declined and decreased back to near-normal levels at 10 days post-lesion. The large increase in IGF-I mRNA expression occurred just prior to and during the period of sprouting by surviving afferents to the dentate gyrus. In the deafferented hippocampus, the distribution of cells expressing IGF-I mRNA corresponded precisely with the area of axonal degeneration in the molecular layer of the dentate gyrus, and was also spatially restricted to fields in which sprouting occurred.

The most extensively studied model of IGF's role in injury of the CNS is the hypoxic-ischemic injury model. The expression of IGF-I, type I IGF receptor, IGFBP-2 and IGFBP-5 mRNAs was increased at different times following unilateral carotid artery ligation followed by exposure to 8% oxygen (Gluckman, et al., 1992; Klempt, et al., 1992). More interesting, hypoxic-ischemic injury could increase the movement of IGF-I into the injured areas from the CSF and circulatory system (Guan, et al., 1996).

Increased local IGF-I concentration could help repair damaged neurons and prevent them from death, while elevated local IGFBP-2 could enhance the movement of IGF-I.

IGF-I promotes neurite outgrowth and increases neurofilament and tubulin mRNA content in vitro (Fernyhough, et al., 1989). Tubulins form heterodimers that assemble to form microtubules. Microtubules are the major structural proteins of axons and also serve to support axonal transport. Tubulin mRNA increases during rapid neurite growth in rat brain. We reported coordinated up-regulation of α1- and βII-tubulin mRNA levels during collateral axonal sprouting (Paden and Zhou, et al., 1995). The mRNA levels of both α1- and βII- tubulin significantly increased in the contralateral (sprouting) SON 10 days after the hypothalamic lesion, immediately prior to the period of most rapid axonal sprouting in the neural lobe which occurs from 10 to 30 days post-lesion. IGF-I could increase both α- and β-tubulin mRNAs during neurite growth. Neurofilaments are the other major structural proteins of neurites and are assembled from light, medium and heavy subunits. IGF-I has been shown to increase mRNA of light and heavy subunits in vitro (Wang, et al., 1990).

Insulin-Like Growth Factor Binding Protein-2 (IGFBP-2)

The insulin-like growth factor binding proteins are a group of soluble proteins which have high affinity to bind IGFs. According to molecular weight and other characteristics, six insulin-like growth factor binding proteins have been found. IGFBP-2 mRNA expression begins as early as E7 during rat embryogenesis. The highest level of IGFBP-2 mRNA was found in the choroid plexus, pituicytes of the neural lobe, glia of the cerebellum and retina, and olfactory epithelia (Wood, et al., 1990; Sullivan and

Feldman, 1994; Lee, et al., 1992). IGFBP-2 may act to modulate effects of IGFs on neuronal and glial growth and differentiation during development.

IGFBP-2 mRNA continues to be expressed at a high level in some locations of the nervous system such as the choroid plexus, leptomeninges and pituicytes of the neural lobe during postnatal development and adulthood. The presence of IGF-I, IGF-II and their receptors has also been reported in these areas (Logan, et al., 1994). This correlation suggests that IGFBP-2 could modulate effects of IGF-I and IGF-II locally (Bach and Bondy, 1992). IGFBP-2 might modulate potential effects of the IGFs on the function of the MNS. High levels of IGFBP-2 and other members of the IGF system in the MNS suggests potential involvement in the morphological plasticity of this system. As reviewed in a previous chapter, mature magnocellular neurons exhibit unusually robust axonal growth in a variety of situations, including regeneration following hypothalamic lesion or transection of the pituitary stalk, collateral sprouting of uninjured axons following partial denervation of the neural lobe, and outgrowth of axons into intra-hypothalamic tissue grafts.

High expression of IGFBP-2 was observed in the infarct area following 90 minutes of hypoxia-ischemia in the rat (Klempt, et al., 1992). Three days after hypoxia-ischemia, expression of IGFBP-2 mRNA was found in the cerebral cortex, thalamus and hippocampus on the ligated side surrounding the sites of major cell loss. The level of mRNA peaked between 5 to 10 days post-ischemia. IGFBP-2 might have a unique function in injury. It is possible that intensive expression of IGFBP-2 mRNA is necessary following injury to transport or localize IGFs to the damaged area. Elevated IGFBP-2 could bind already existing IGFs present in tissue, CSF or the circulatory

system and be in place to mediate actions of IGFs subsequently induced in the injured area (Sandberg Nordqvist, et al., 1994; Walter, et al., 1997). Because IGFBP-2 contains an RGD sequence at the carboxyterminal end which is found in many extracellular matrix proteins (Clemmons, et al., 1993a; 1993b) and often serves as a recognition sequence for cell surface integrin receptors, IGFBP-2 might mediate migration of cells into the damaged site by binding to the cell surface.

Insulin-Like Growth Factor I Receptor (IGF-I receptor)

The IGF-I receptor mediates most of the biological actions of IGF-I and IGF-II. The affinity of the IGF-I receptor for IGF-I is approximately ten-fold higher than for IGF-II and 100 times that for insulin (Leroith, et al., 1993b). The IGF receptor is a tetramer composed of two α and two β subunits linked by disulphide bonds. The α subunit contains the ligand binding site, and the β subunit consists of a transmembrane domain and a cytoplasmic tyrosine kinase domain (Leroith, et al., 1993b).

Messenger RNA of the IGF-I receptor was found to be widely distributed in fetal tissue and was most prominent in the developing nervous system (Bondy, et al., 1991). IGF-I receptor mRNA was most abundant in the olfactory bulb and cerebral cortex during development. It was also highly expressed in the mesenchymal support structures of the brain, including the meninges, choroid plexus and vascular adventitia. IGF-I receptor mRNA decreased during the postnatal maturation of the cerebral cortex whereas IGF-I receptor mRNA remained abundant in the adult olfactory bulb (Kar, et al., 1993). The highest levels of IGF-I receptor in human brain as measured by autoradiographic localization of radioligand binding were found in the pituitary gland

and choroid plexus, olfactory bulb and cerebellar cortex (Torres-Aleman, et al., 1994). In the pituitary, IGF-I receptor binding was predominantly found in the anterior lobe, while an intermediate density was present in the neural lobe. Moderate densities of IGF-I receptors were also found in the cerebral cortex, amygdala, thalamus and the substantia nigra (Lesniak, et al., 1988; Werther, et al., 1989). Another study found that IGF-I receptor mRNA was present in the suprachiasmatic nuclei, SON and PVN (Aguado, et al., 1993). More interesting was that dehydration could induce up-regulation of IGF-I receptor expression in the SON but was without effect in the PVN. It was suggested that up-regulation of the IGF-I receptor could be related to the morphological plasticity of magnocellular neurons which is induced by osmotic stimulation during dehydration.

An increase in IGF-I receptor mRNA was detected in both the proximal and distal stump following sciatic nerve transection (Cheng, et al., 1996). IGF-I receptor expression was also observed in Schwann cells (Schumacher, et al., 1993). These findings supported the concept that one role of growth factor receptors on Schwann cell surfaces is to present growth factors to regenerating axons or sprouting axons. IGF-I binding to the IGF-I receptor is one means by which Schwann cells could increase the local bioavailability of IGF-I to regenerating or sprouting axons.

In contrast, no change of IGF-I receptor mRNA was found in the damaged cerebral cortex by ribonuclease protection assay or in situ hybridization after brain trauma although IGF-I mRNA and IGFBP-2 mRNA levels were significantly increased (Walter, et al., 1997). Altered IGF-I receptor mRNA transcription may not be a prime determinant of IGF-I bioactivity within brain wounds. It was suggested that IGF-I receptor turnover on target cells was sufficient to meet the needs of both physiological

and pathological responses. Transient increases in IGF-I and IGFBP-2 within the wound area suggested that ligand expression and bioavailability may be prime determinants of IGF-I bioactivity.

In summary, the strong growth-promoting effects of IGFs on sprouting in other systems and the unique distribution of the essential components of the IGF system within the MNS support the hypothesis that IGF-I may play a critical role in magnocellular neuron axonal sprouting following unilateral hypothalamic lesion.

IGF-I Polymer Implantation in the Hypothalamus

The growth-promoting effects of IGF-I on a variety of neuronal populations have been reviewed above. The IGFs have been demonstrated to be potent stimulators of motor neuron sprouting and nerve regeneration. In vivo, subcutaneous administration of IGF-I increased intramuscular nerve sprouting by ten times. Furthermore, administration of exogenous IGF-I stimulated axonal growth and increased the length of axons (Contreras et al., 1995).

Polymeric implants can release precisely controlled quantities of active macromolecules, such as NGF, IGF and dopamine (Powell, et al., 1990; Freese, et al., 1989). While infusion systems have been used to deliver NGF and other growth factors directly to brain tissue in animals models of CNS regeneration (Kromer, 1987), all infusion systems require that drugs are maintained in an aqueous reservoir prior to delivery. Drugs which are not stable in aqueous solution, such as NGF or IGF-I, can not be delivered by these methods. Implant material for NGF controlled release in the brain

has been developed from a biocompatible polymer, ethylene-vinyl acetate copolymer (EVAc) which has been approved by the FDA for drug delivery applications (Powell, et al., 1990). EVAc polymer is inexpensive. EVAc matrices have successfully released growth factors and inhibitors in animals and in culture. Dopamine-releasing EVAc implants have been designed and tested in the brain of rats (During, et al., 1989). NGF was released at a slow pace and the release persisted for over 3 months (Kromer, 1987).

Justification, Hypothesis and Specific Objectives

The MNS exhibits vigorous regenerative and compensatory capabilities. This unusual plasticity has been confirmed by many investigators. It has been observed in situations ranging from axonal regeneration induced by hypothalamic lesions or transection of the pituitary stalk to outgrowth of axons into intrahypothalamic grafts of different tissues. Our laboratory has developed a model in which the intact contralateral magnocellular neurons undergo robust compensatory sprouting following unilateral destruction of the hypothalamo-neurohypophysial tract at the level of the hypothalamus (Watt and Paden, 1991). Compared to infundibular stalk section, hypophysectomy or neurolobectomy, a unilateral hypothalamic lesion permits the sprouting response of uninjured MNS neurons to be studied without the complications arising from inflammation, hemorrhage, scar formation or damage to the anterior pituitary. The simplified organization of the MNS provides a unique opportunity to investigate axonal plasticity both at the molecular and cellular levels. The long-term goal of our laboratory

is to determine what special characteristics of the MNS system underlie the sprouting response.

What factors are responsible for initiating and sustaining axonal sprouting by uninjured MNS neurons? While it is unlikely that any single mechanism or factor can account for such a complex process as axonal sprouting, the IGF system has the potential to exert major growth-promoting actions that may be essential for sprouting in the MNS. In addition to the evidence reviewed above for strong growth-promoting effects of IGFs on neurite growth and sprouting, the distribution of IGFs in certain specific areas of the postnatal CNS that exhibit a high degree of plasticity, such as the MNS, the olfactory bulb, hippocampus and cerebellum, strongly suggests that IGFs exert growth-promoting effects supporting neural plasticity.

As reviewed above, there is also some evidence that NGF and bFGF could act as trophic factors in the MNS. However, pilot experiments conducted in our laboratory failed to support a role for these agents. A monoclonal NGF antibody (UBI) was used for immunocytochemical staining in brain following colchicine injection in our initial study. No NGF immunoreactivity was detected in the PVN and SON, even in the lesioned animals. We could not replicate the finding of Conner and Varon (1994), and therefore did not further investigate the potential role of NGF in the MNS. To determine whether bFGF might act to stimulate collateral sprouting following unilateral destruction of the MNS, we searched for any lesion-induced changes in immunoreactivity of bFGF and its high affinity receptor. No difference in staining for bFGF or its receptor was found between lesion and control groups. In addition, we were unable to detect mRNA for bFGF or its receptor in either the SON and PVN or the

neural lobe. Thus, based on our laboratory's initial results, NGF and bFGF were excluded from further study as potential factors mediating collateral sprouting in the MNS.

This thesis is therefore based on the hypothesis that IGF-I acts as a trophic factor to initiate and/or support collateral sprouting of magnocellular axons following a unilateral hypothalamic lesion. To test this hypothesis, I have formulated the following specific objectives:

(a) to determine distributions of IGF-I, IGFBP-2 and the IGF-I receptor in the magnocellular neurosecretory system by immunocytochemical methods, and to find out which kind of cells contain IGF-I, IGFBP-2 and IGF-I receptor.

(b) to determine whether IGF-1 peptide undergoes retrograde or anterograde transport inside of magnocellular neurosecretory neurons by combining immunocytochemistry with in situ hybridization following intraventricular injections of colchicine.

(c) to determine where IGF-I, IGFBP-2 and IGF-I receptor are synthesized in the MNS using in situ hybridization.

(d) to determine if any changes in mRNA expression or immunoreactivity of IGF-I, IGFBP-2 and IGF-I receptor occur following unilateral hypothalamic lesions of the MNS and to determine their temporal and spatial patterns .

(e) to directly test the possibility that IGF-I stimulates collateral sprouting of axons in the MNS by implantation of human recombinant IGF-I peptide in the hypothalamus in both lesioned and intact animals.

CHAPTER 2

METHODS

Animals

All studies were conducted in accordance with the National Institutes of Health (NIH) Guidelines for Care and Use of Experimental Animals. All protocols were approved by the Montana State University (MSU) Institutional Animal Care and Use Committee. Male Holtzman rats were bred at the MSU Animal Resource Center from stocks obtained from Harlan Laboratories. Rats were housed under a 12-hour-light/12-hour-dark cycle with ad libitum access to laboratory chow and tap water. Unilateral hypothalamic lesions and other surgeries were performed at age 35 to 40 days post-parturition.

Unilateral Hypothalamic Lesion

The unilateral hypothalamic lesion was done in a manner nearly identical to what was developed in our laboratories (Watt and Paden, 1991). Each animal received an intramuscular injection of a ketamine cocktail at a dose of 0.55 ml/kg. The anesthesia cocktail was made of 26 mg ketamine, 5 mg xylazine and 0.8 mg acepromazine per kg

body weight. After loss of consciousness, the rat heads were shaved, washed with surgical scrub and 75% ethanol and fixed in the stereotaxic apparatus (David Kopf Instruments). A 2 cm incision along the midline was made on the dorsal surface of the cranium. The skin was retracted, any other connective tissue was removed, and the skull was exposed. The tip of the stereotaxic knife blade (the wire knife was made from 22-gauge stainless steel cannula/wire) was pointed at bregma, the lesion coordinates were calculated (AP -3 mm to +5mm and ML -0.7 mm), and then the marks were made on the surface of the skull. A #3 carbide burr was driven by a flexible drill (Dremel) to make a slot through the cranium. The underlying dura mater was cut by a sterile 27 gauge hypodermic needle. Any debris was cleared out and any blood was absorbed with a cotton tip swab. The wire knife was lowered into the brain at -2mm caudal to bregma. When the wire knife touched the ventral surface of the skull, the wire flexed slightly. Following that, the wire knife was retracted about 0.5mm and moved to the anterior end of the cranial slot. Then, the wire knife was pulled out, and any blood and debris were cleared from the slot. The cranial slot was covered by a small piece of Gelfoam (Upjohn), antibiotic nitrofurazone power was added around the wound site to prevent infection, and the skin was closed with scalp sutures (Davis & Geck #3-0 Dexon S). After surgery, the animal was put in a new cage with a lowered water bottle so that the animal could more easily access drinking water. Sham control animals received the same surgery except that the wire knife was lowered only 5mm from the dorsal surface of the skull. The wire knife at this depth caused damage only in the cortex and hippocampus, but the hypothalamus was intact.

Microinjection of Colchicine into the Brain

The purpose of injection of colchicine into the third ventricle is to enhance immunoreactivity of IGF-I in magnocellular nerve cell bodies and to determine the direction of transportation of IGF-I peptide inside magnocellular axons. Three groups of animals were given colchicine: lesion, sham and intact. Animals were anesthetized in the same manner which is described as the unilateral hypothalamic lesion. All animals in this group received intraventricular microinjection of colchicine 24 hours before they were sacrificed for study. The animal head was fixed in a stereotaxic instrument fitted with a 10 microliter (μl) Hamilton microsyringe. An incision was made along the midline of the dorsal surface of the skull and the skin was retracted. The tip of the microsyringe needle was positioned at bregma and then hole coordinates were calculated at AP -1.6mm and ML -1.2mm. The hole was made through the cranium with a #3 carbide burr driven by a flexible drill. The microsyringe needle was fixed at 9.5 degrees from vertical in the lateral to medial direction to prevent bleeding from the superior sagittal sinus and to direct the needle into the upper part of the third ventricle which is located in the midline of the brain. Each animal received a dosage of 35 μg colchicine per 100g body weight (Conner, et al., 1994). The colchicine was dissolved in normal saline (0.9% NaCl in distilled water) at the concentration of 18.2mg per ml. Each animal received 4μl to 7 μl of the colchicine solution. The microsyringe needle was lowered from 9.5 to 10mm from the dura at an angle of 9.5 degrees so that the tip of the microsyringe needle entered at the upper part of the third ventricle close to the PVN. The injection of colchicine was done very slowly (over about 30 seconds) and gently. At the completion of the injection, the injection needle was left in place for 2-3 minutes so

that the injected colchicine solution was completely released into the surrounding tissue or cerebrospinal fluid (CSF). The rest of procedure was the same as the surgery described in the previous chapter.

Implantation of IGF-I Peptide in the Hypothalamus

IGF-I Polymer Rod

A polymeric implant was employed to produce continuous release of IGF-I. These implants have been shown to release NGF for more than 30 days inside the brain (Kromer, et al.,1987).

Ethylene-vinyl acetate (EVAc) (Elvax 40W, Dupont Chemical) was washed for 24 hours with 10 changes of distilled water and then washed for 24 hours with 10 changes of 95% ethanol. The EVAc was dissolved in methylene chloride at 10% weight/volume overnight.

Twenty five μg human recombinant IGF-I (UBI) was mixed well with 1 mg bovine serum albumin (BSA), then the mixture was dissolved in 300 μl PBS in a 500 μl centrifuge tube. The tube was frozen in liquid nitrogen and then lyophilized for 24 hours to obtain a homogeneous powder. The resulting powder was crushed. A BSA control without IGF-I was prepared in an identical manner.

Using a glass capillary micropipette (VWR) , 30 μl EVAc (10% w/v) was added to a 100 μl centrifuge tube. 1 mg of the IGF-I and BSA mixture was added and stirred to form a homogenous mixture. The solution was quickly sucked into a 10 μl glass capillary micropipette with a mini pump and frozen in liquid nitrogen for 10 minutes.

With a 0.38mm cold stainless steel wire, the frozen IGF-I polymer rod was pushed out from the glass capillary micropipette onto a -40 °C glass plate. The polymer rod was allowed to dry at -20°C for 48 hours, then lyophilized overnight. The dried polymer rod was then cut into 4mm lengths. A total of 12 rods were made from 25 μg human recombinant IGF-I. Each rod contained about 2 μg IGF-I and was kept at -20°C until implantation. The pure BSA polymer rods for the negative control group were made in exactly the same manner except that the IGF-I was omitted.

Animals were anesthetized as described above. A 1 cm incision in the scalp was made in a sagittal direction and the dorsal surface of the cranium was exposed. A sterilized stainless steel cannula/wire (22 Gauge, Small Parts Inc.) was fixed in the stereotaxic instrument. The tip of the cannula was pointed at bregma, then coordinates calculated at AP -1.4mm, ML -2.1mm. A hole was made in the same manner described in the colchicine injection procedure. The IGF-I polymer rod was loaded inside the cannula at one end, and a stainless steel wire was fitted into the cannula at the other end, then slowly lowered until it touched the IGF-I polymer rod within the cannula. This prevented the polymer rod from being pushed up the cannula when it was being lowered. The cannula loaded with the polymer rod was lowered until it touched the ventral surface of the skull. Then the wire was clamped at that point and the outer cannula was retracted slowly out of the brain. After the cannula was retracted, the wire was pulled out from the brain. Thus the IGF-I polymer rod was left inside of the hypothalamus lateral to the SON. A small piece of Gelfoam was placed on the hole and antibiotic nitrofurazone was applied. Finally, the skin was sutured. The BSA control group was implanted in an identical manner except that a pure BSA polymer rod was used instead

of the IGF polymer rod. An additional control group underwent the entire implantation procedure using an empty cannula. All animals that received the implantation surgery were allowed to live 8 days or 14 days before sacrifice.

Tissue Preparations

The brains and pituitaries were collected from animals of the experimental groups sacrificed between 2 days and 35 days post-surgery (PS) as well as from animals of age-matched sham-operated and intact control groups. Animals to be used for immunocytochemical analysis for detecting immunoreactivity of IGF-I , IGFBP-2, IGF-I receptor, OT and vasopressin-neurophysin were anesthetized with ether. While unconscious, each animal was first perfused intracardially with 0.01 M phosphate buffered normal (0.9%) saline pH 7.4 (PBS) for one minute to push the blood out of the circulatory system, and then perfused with 500 ml of a modified Nakane's periodate-lysine-paraformaldehyde (PLP) fixative solution. The PLP fixative solution containing 2% paraformaldehyde, 0.075M lysine and 0.01M sodium periodate in 0.1M $NaPO_4$ (pH 7.4) was prepared immediately before use. Following perfusion, the brains and pituitaries were removed and postfixed in the same fixative solution for about 7 hours.

Depending on the antigen to be localized, tissue was prepared for sectioning either with a cryostat or Vibratome. Following postfixation, the tissues to be cryosectioned were moved into a cryoprotectant solution made of 30% sucrose in PBS for 24-48 hours at 4°C. The tissue was placed in aluminum foil molds filled with OCT, a commercial embedding compound (VWR) and frozen in liquid nitrogen. The tissues were kept at

–0°C until sectioned. The tissue to be used for Vibratome sectioning was directly loaded into a plastic mold filled with an egg yolk : 12 % gelatin mixture (2:1 ratio) and then fixed in 4% paraformaldehyde for about 24 to 48 hours. The tissues were kept at 4 °C until sectioned. The Vibratome was used to cut thick sections of egg-gel embedded tissue. Coronal sections of whole pituitaries and blocks of brain tissue containing the entire SON were cut at 40 µm and stored in cryoprotectant at -20 °C until used for immunocytochemical analysis.

Animals to be used for analysis of mRNA by in situ hybridization were sacrificed by decapitation under ether anesthesia. Brains and pituitaries were rapidly removed and frozen in powdered dry ice. The tissues were then stored at -80 °C until sectioned on a cryostat.

Coronal sections of fresh frozen whole pituitaries and brain blocks including the entire length of the SON for in situ hybridization as well as some fixed whole pituitaries and brains for immunocytochemical analysis were sectioned at 10 µm on a cryostat and sections were thaw-mounted on Superfrost slides (Fisher), and every fifth section throughout the rostal-caudal extent of the lesion was stained with cresyl violet and examined to confirm the complete unilateral cut of the hypothalamo-neurohypophysial tract in each animal. Tissue sections from animals with confirmed lesions were then kept at -80 °C until used for experiments.

Immunocytochemistry

All solutions were made in PBS and 3 X 10-min rinses with PBS were performed between all incubations in each of the following immunoperoxidase staining protocols. Triton X-100 (0.1%) was added to the PBS for all steps except the avidin-biotin-peroxidase complex (ABC) and diaminobenzidine (DAB) incubations.

1) IGFBP-2

Tissue sections to be used for immunoperoxidase staining of IGFBP-2 were pretreated with 0.1% H_2O_2 for 25 min to quench endogenous peroxidase activity and incubated in 2% normal goat serum for 1 hour to reduce nonspecific staining. Sections were then incubated sequentially in rabbit anti-human IGFBP-2 antibody overnight at 4 °C (1:500; UBI), biotinylated goat anti-rabbit IgG for 2 hours (1:200; Vector), and ABC for 2 hours (Vector Elite). The peroxidase reaction was carried out using 0.25mg/ml DAB (Sigma) as chromogen with generation of H_2O_2 by the glucose oxidase method using type-VII enzyme (Sigma). Control sections were incubated in PBS in place of the primary antibody.

Dual-label immunofluorescent localization of IGFBP-2 and protein S-100, a pituicyte marker, was performed using rabbit anti-human IGFBP-2 (UBI) in combination with rabbit anti-bovine S-100 (Accurate) as primary antisera. Pituitary sections were incubated sequentially in 2% normal goat serum for 1 hour (Vector), rabbit anti-human IGFBP-2 overnight at 4 °C (1:500), Lissamine-rhodamine (LRSC)-conjugated Fab fragment of goat anti-rabbit IgG for 2 hours (1:25, Jackson Immunoresearch), rabbit

anti-bovine S-100 for 6 hours (1:200), and fluorescein-isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (1:200, Jackson). Use of a Fab fragment as the LRSC-conjugated secondary antibody sufficiently saturated the binding sites on the primary anti-IGFBP-2 that any cross-reaction with the FITC-conjugated secondary was undetectable. Following final rinses, sections were mounted on slides and coverslips attached with Mowiol (Calbiochem). Confocal microscopy was performed using a BioRad DVC250 instrument equipped with an argon-krypton laser and a cooled integrating CCD camera. Controls for cross-reactivity and nonspecific binding of second antibodies were performed as described, except sections were incubated only with anti-human IGFBP-2 or only with anti-bovine S-100 instead of with both antisera, or primary antisera were omitted altogether. Analysis of control sections confirmed that cross-reaction of either the LRSC- or the FITC-conjugated secondary antibody with the inappropriate primary antibody was not detectable at the level of sensitivity employed to capture confocal images.

Dual-label immunofluorescent localization of IGFBP-2 and the C3bi complement receptor, a microglia marker, was performed using rabbit anti-human IGFBP-2 (UBI) in combination with mouse anti-rat C3bi (Serotec, Ox42). Pituitary sections were incubated sequentially in 1% bovine serum albumin for one hour, a mixture of rabbit anti-human IGFBP-2 (1:500) and mouse anti-rat C3bi (1:500) overnight at 4 °C, biotinylated horse anti-mouse IgG in 2% normal horse serum for 2 hours (1:200, Vector), and a mixture of FITC-conjugated goat anti-rabbit IgG (1:200, Jackson) and LRSC-avidin (1:200, Jackson) for 2 hours. Following final rinses, sections were mounted on slides and coverslips attached with Mowiol. Controls for cross-reactivity of

secondary antibodies and spillover of fluorescent markers were performed as described, except sections were incubated only with rabbit anti-human IGFBP-2 or only with mouse anti-rat C3bi instead of with both antisera, or primary antisera were omitted altogether.

2) IGF-I

Vibratome sections of brains and pituitaries from groups with or without colchicine injection to be used for immunocytochemistry of IGF-1 were pretreated with 0.3% H_2O_2 for 25 min to diminish endogenous peroxidase activity and then incubated with 4% normal horse serum to reduce nonspecific staining. The sections were sequentially incubated in mouse anti-human IGF-I (1:2000; UBI) diluted in 2% normal horse serum with 0.1% Triton X-100 in PBS, biotinylated horse anti-mouse IgG (1:200; Vector) in the same solution for 1.5 hour, and ABC in PBS for 1 hour (Vector Elite). The peroxidase reaction was carried out as described above. Two control groups were included. For the first, sections were incubated in PBS instead of the primary antibody. For the second, the sections were incubated in primary antibody solution that had been preadsorbed with human recombinant IGF-I peptide (UBI). This peptide was expressed in yeast and purified by HPLC, then lyophilized from 100 µl 0.1 M acetic acid. The preadsorbtion was done by mixing 50µl anti-IGF-I antibody (8µg/100 µl) and 64 µl IGF-I peptide (25µg/100 µl). The mixture was stored at 4 °C overnight, then centrifuged for 10 minutes at 13,000 rpm in a microfuge and the supernatant withdrawn.

Dual-label immunofluorescent localization of IGF-I and the magnocellular neuron markers OT or vasopressin-neurophysin was performed as follows. PBS was used as

the rinse solution. The sections were incubated in 4% normal goat serum for 1 hour, mouse anti-human IGF-I (1:500; UBI) diluted in 2% normal goat serum with 0.1% Triton X-100 in PBS overnight, goat anti-rabbit oxytocin (1:1000; Chemicon) or goat anti-rabbit vasopressin-neurophysin (1:5000; gift of Dr. Allan Robinson) diluted in 2% normal goat serum in PBS with 0.1% Triton X-100 overnight. A mixture of two secondary antibodies, FITC-conjugated goat anti-rabbit IgG (1:200; Jackson) and indocarbocyanine (Cy3)-conjugated goat anti-mouse IgG (1:200; Jackson) diluted in 2% normal goat serum was applied for 2 hours. The sections were mounted on the slides and coverslips attached with Mowiol (Calbiochem), and slides were stored in the dark at 4 °C until viewed on the confocal microscope. Confocal microscopy was performed in the same manner as described above. Negative control groups were treated identically except that either one primary antibody or both primary antibodies were omitted.

3) IGF-I receptor

Immunocytochemical staining for the IGF-I receptor was performed using the same protocol described above for IGF-I. Monoclonal mouse anti-human IGF-I receptor (10 μg/ml; Oncogene Science) was used as the primary antibody.

4) Oxytocin

A peroxidase immunocytochemical stain for OT was used to detect any growth by oxytocinergic magnocellular neurons following implantation of IGF-I into the hypothalamus. PBS with 0.1% Triton X-100 was used for all dilution and rinses. The Vibratome sections of brain were pretreated with 0.1% H_2O_2 for 25 minutes to inhibit

endogenous peroxidase activity and then incubated with 4% normal goat serum for 1 hour to reduce nonspecific staining. The sections were sequentially incubated in goat anti-rabbit OT (1:1000) diluted in 2% normal goat serum overnight, washed for 6 hours, and incubated in biotinylated goat anti-rabbit IgG (1:200) in 2% normal goat serum for 2 hours. The rest of the procedure was the same as that described above for IGFBP-2 immunocytochemical staining.

In situ Hybridization

1) IGFBP-2

Probe Labeling

The IGFBP-2 probe was generated in the laboratory of Dr. James Herman at the University of Kentucky from a cDNA kindly provided by Dr. M.M. Rechler of the NIH. The cRNA probe was synthesized from a 585-bp cDNA, corresponding to bases 502-1087 of rat IGFBP-2 mRNA. The IGFBP-2 construct was linearized with Eco RI or HIndIII and transcribed with SP6 or T7 RNA polymerase to generate sense and antisense probes, respectively. The labeling reaction consisted of 80 mCi [^{33}P]UTP (specific activity 2,942 Ci/mmol), 1 X SP6 transcription buffer (Boehringer-Mannheim), 15 μM DTT, 250 μM GTP, CTP and ATP, 10 μM cold UTP, 40 U placental RNAse inhibitor (40 U/μl, Boehringer-Mannheim), 1 μg linearized plasmid DNA, and 20 U of appropriate RNA polymerase (SP6 or T7, Boehringer-Mannheim). Reactions were incubated at 37 °C for 90 min. Subsequently, 12 U of RNAse-free DNAse I (Boehringer-Mannheim) was added to digest the DNA template, and after 5 min at 37 °C, the

reaction mixture was diluted to 100 µl with diethlpyrocarbonate-treated water and ethanol precipitated with 7.5 M ammonium acetate.

In situ Hybridization

Sections from rats sacrificed at each age examined were processed simultaneously to facilitate qualitative comparisons of hybridization intensity. Sections were fixed for 10 min in 4% buffered paraformaldehyde and washed in 2 changes of 0.1 M diethypyocarbonate treated potassium PBS (5 minutes), 2 changes of potassium PBS containing 0.2% glycine (5 minutes), and 2 changes of potassium PBS (5 minutes). Slides were then washed in 0.1 M triethanolamine (pH 8.0, 2 minutes) and subsequently acetylated in triethanolamine containing 0.25% acetic anhydride (10 minutes). Slides were then rinsed in potassium PBS and dehydrated in graded alcohols. The labeled probe was added to a hybridization buffer containing 50% formamide (Amresco) and 20 mM DTT, and 50 µl ($1X10^6$ cpm) of diluted probe applied to each slide. Subsequently, the slides were coverslipped, placed in moistened chambers, and incubated overnight at 55 °C. Following hybridization, coverslips were removed and the slides rinsed two times in fresh standard sodium citrate (SSC) for 10 minutes. Slides were treated with RNAse A (100 mg/ml) for 30 minutes at 37 °C and transferred to fresh 2X SSC, then rinsed 3 X in 0.2 X SSC at 10 min per wash, followed by a 1 hour wash in 0.2 X SSC at 65 °C. Slides were dehydrated in graded alcohols, dried at room temperature, exposed for 5 days to Kodak BioMAX film and subsequently dipped in Kodak NTB2 emulsion and exposed for 28 days. Following development, slides were counterstained with cresyl violet and coverslipped with DPX mountant (Fluka).

2) IGF-I and IGF-I receptor

The protocol for making probes and the procedures for in situ hybridization of IGF-I mRNA and IGF-I receptor mRNA were identical to these described above for the IGFBP-2 experiment. The cRNA IGF-I probe was synthesized from a 690 bp cDNA corresponding to the 5' end of the rat IGF-I mRNA (courtesy D. LeRoith, NIH). The IGF-I receptor probe was a 265 bp fragment corresponding to the 5' untranslated region, signal sequence and alpha subunit of the rat IGF-I receptor mRNA (courtesy D. LeRoith, NIH).

Quantification of Grain Densities

Quantification of relative mRNA levels was accomplished by computerized image analysis. Calibrations were checked prior to each sampling session against a known standard sample. High magnification images were captured with a CCD camera (Sierra Scientific) and a MCID/M4 microcomputer imaging device (Imaging Research) directly from film autoradiographs. The image of the neural lobe of each animal was digitized and the background image was taken from the plain film adjacent to the neural lobe. The value used for statistical analysis was the film density of the neural lobe minus that of the adjacent film background. At least 6 sections from each animal were measured and each group contained at least 5 animals.

Statistical Analysis

One-way analysis of variance (ANOVA) was performed using the AV1W program in the MSUSTAT statistical package which was developed by Dr. R. Lund of MSU. The MSUSTAT program COMPARE and the Tukey-Kramer multiple comparisons test were used for testing the significance of differences in group means. In all cases, if the P value was less than 0.05, the differences in group means were regarded as statistically significant.

CHAPTER 3

RESULTS

Insulin-like Growth Factor Binding Protein-2

Immunocytochemical Identification of IGFBP-2-Containing Cells in the Neural Lobe of Intact Animals

Heavy immunoreactivity for IGFBP-2 was present in cells throughout the neural lobe during postnatal development at each age examined from postnatal day 1 (P1) through postnatal day 37 (P37)(fig.1). These cells possessed the characteristic size and morphology of pituicytes, including large spherical nuclei. Dual-label immunofluorescence was performed for IGFBP-2 and S-100 which is an astrocyte marker protein. It was found that all cells that expressed IGFBP-2 also showed immunoreactivity for the astrocyte marker S-100 (fig.2A), confirming that they were pituicytes. This was found to be true at each of three ages examined: P1 (370 IGFBP-2-positive cells examined), P11 (650 IGFBP-2-positive cells examined) and P37 (630 IGFBP-2-positive cells examined). Only rare S-100 positive cells failed to express detectable IGFBP-2. Overall, 92% of S-100 positive pituicytes were also positive for IGFBP-2 (fig.3).

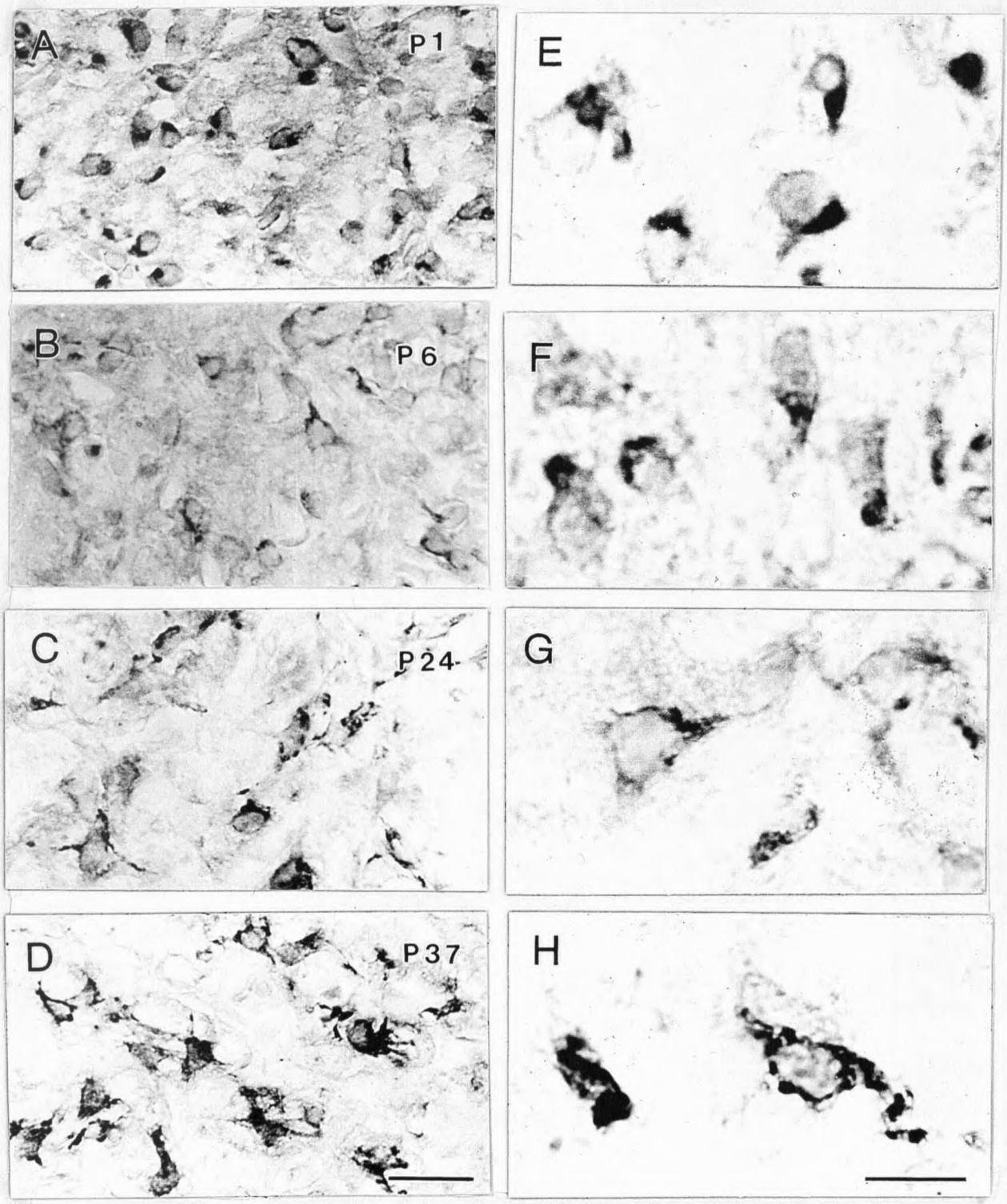


Fig. 1. IGFBP-2 immunoreactivity in the neural lobe during postnatal development. Staining was typically confined to one pole of the perinuclear cytoplasm in immature pituicytes during the first postnatal week, as seen at postnatal day 1 (A, B) and postnatal day 6 (C, D). As pituicytes matured, IGFBP-2 staining became more punctate and was often found associated with perikarya, as seen at postnatal day 24 (E, F) and postnatal day 37 (G, H). Scale bar = 20 μm (A, B, C, D) and 10 μm (E, F, G, H).

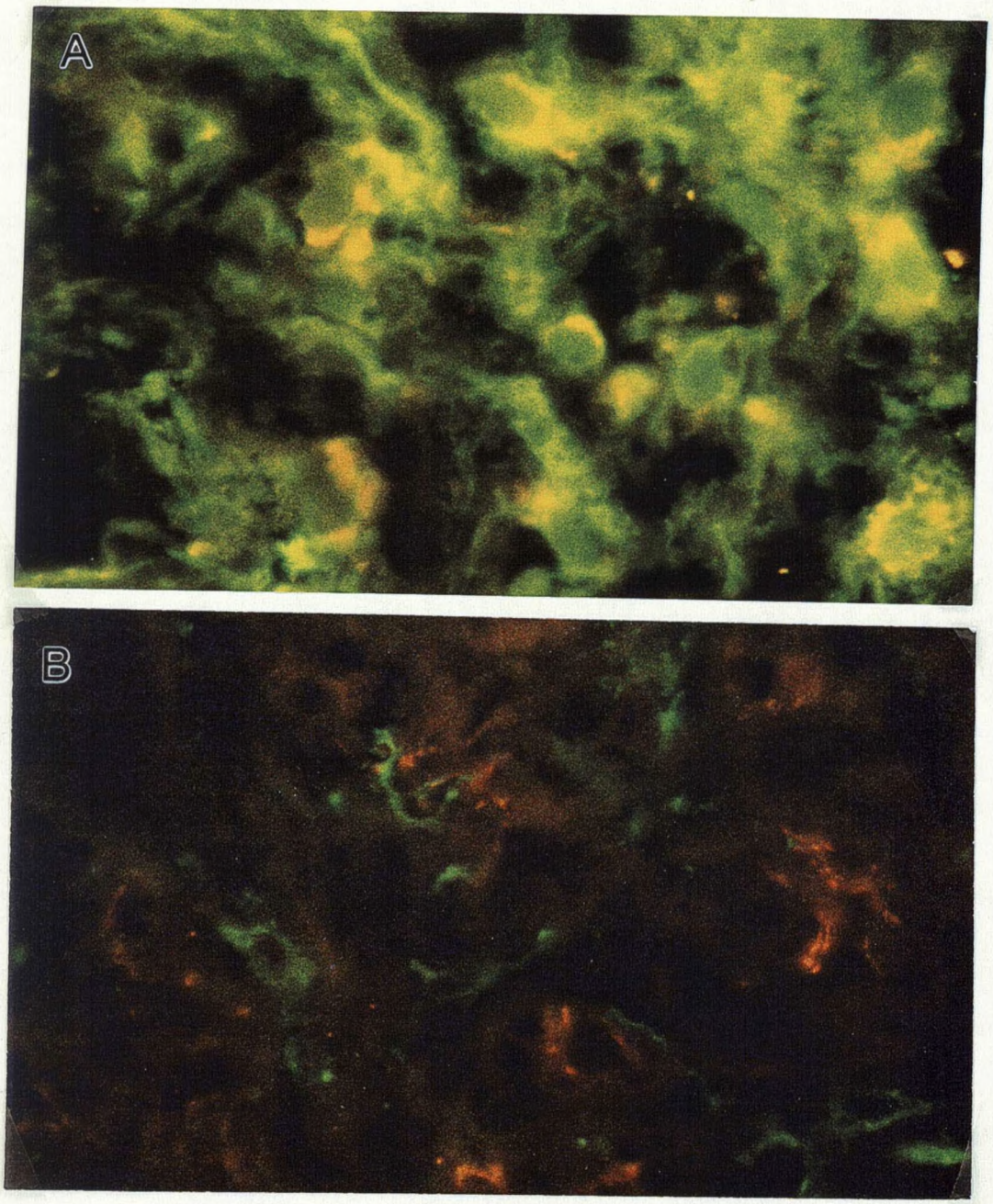


Fig. 2. Photomicrographs of dual-label staining for IGFBP-2 and S-100 (A) or IGFBP-2 and C3bi (B) at postnatal day 37 in the neural lobe using confocal immunofluorescence microscopy. In fig. A, IGFBP-2 is present only in pituicytes as revealed by colocalization (yellow) of IGFBP-2 (red) and S-100 protein (green) in the same cells. IGFBP-2 staining shows association with the cell membrane, as compared to the diffuse labeling for the cytoplasmic S-100. In contrast, no examples of colocalization of IGFBP-2 (red) and the microglia marker C3bi (green) were observed (B).

In contrast, IGFBP-2 was not found to be present in microglial cells of the neural lobe. No examples of colocalization of IGFBP-2 and a microglial marker, the C3bi complement receptor, were observed among 550 C3bi-positive cells examined at P37 (fig.3). Dual label studies for C3bi and IGFBP-2 were also performed at P1 and P11 to determine whether transient expression of IGFBP-2 might occur in microglia during development. IGFBP-2 was not detected in C3bi-positive microglia at either P1 or P11. Furthermore, the total number of microglia in the P1 NL was very low, with only 10 microglia found in sections that contained over 800 IGFBP-2-positive pituicytes.

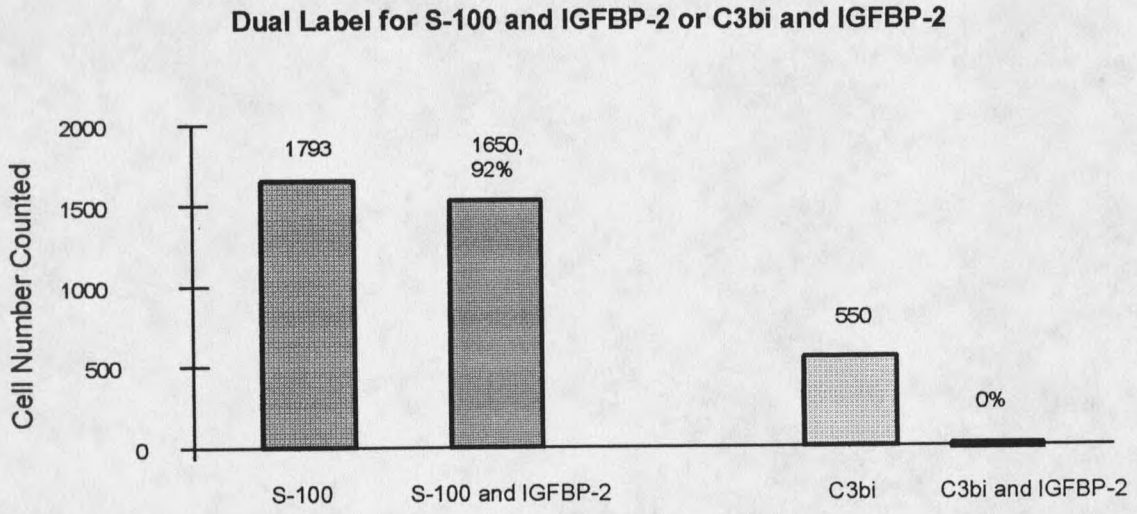


Fig.3. Number of cells in the neural lobe immunoreactive for S-100 and IGFBP-2 or C3bi and IGFBP-2. Dual-label immunofluorescence revealed that all IGFBP-2 positive cells contain the astrocyte marker protein S-100. Overall, 92% of 1793 pituicytes (S-100 positive) examined from P1 to P37 were also positive for IGFBP-2. In contrast, no microglia (C3bi positive) showed immunoreactivity for IGFBP-2 among 550 C3bi positive cells examined at P37.

Intracellular Distribution of IGFBP-2 in Pituicytes

IGFBP-2 immunoreactivity at P1 and P6 appeared as an intense spot in the perinuclear cytoplasm of immature pituicytes, where it was almost always confined to one pole of the cell (fig.1A, B). Only occasional cells possessed immunoreactive processes when stained for either IGFBP-2 or S-100, and those that were visible were short and straight. By P11, IGFBP-2 immunoreactivity began to be more widely distributed in the perikarya of pituicytes, and an increasing number of short cell processes were intensely stained (fig.1C,D). However, this pattern changed by P24, when one (occasionally two) processes with intense IGFBP-2 staining were visible on many pituicytes (fig.1E.F). By P37, pituicytes had developed mature morphology with multiple and more extensive processes. These were intensely stained for IGFBP-2, which also persisted in the perikarya (fig.1G, H). A similar pattern of IGFBP-2 immunoreactivity was observed in the P65 group, indicating no changes occurred in the distribution of IGFBP-2 immunoreactivity within pituicytes after P37.

There were two differences in the intracellular staining patterns between IGFBP-2 and the S-100 protein in the pituicytes. First, nuclear staining for IGFBP-2 was never observed, while both nuclear and cytoplasmic staining were present for S-100. Second, while both antigens were present in pituicyte processes and perikarya, immunoreactivity for IGFBP-2 was much more punctate, suggesting a possible association with the cell membrane, while that of S-100 was diffuse at all ages examined.

In situ Hybridization of IGFBP-2 mRNA in the Neural Lobe

Heavy expression of IGFBP-2 mRNA was found in the neural lobe at each age examined (P6, P37 and P65) (fig.4 A,B,C). Staining of sections with cresyl violet revealed that grains were localized over pituicytes, consistent with the immunocytochemical results presented above. Intense signal was observed in the neural lobe at all ages, but the density of grains over individual pituicytes appeared to increase somewhat between P6 and P37, and remained unchanged at P65.

IGFBP-2 mRNA Expression and IGFBP-2 Immunoreactivity in the Brain

Coronal sections of P37 rat brain were stained for IGFBP-2 to determine whether astrocytes other than pituicytes of the neural lobe were immunoreactive. No IGFBP-2 staining was found in astrocytes, neurons or other cells within the brain parenchyma. This was true even in the subfornical organ and median eminence, indicating that the absence of a blood-brain barrier does not account for the selective expression of IGFBP-2 in pituicytes (fig.5 A,B). In contrast to the lack of reactivity in brain astrocytes, IGFBP-2 staining was present in epithelial cells in the choroid plexus and meningeal cells on the same sections (fig.5 C,D).

The distribution of IGFBP-2 mRNA in the brain at P37 was consistent with localization of IGFBP-2 staining by immunocytochemistry (fig.5 E,F). Grains for IGFBP-2 mRNA were only observed over epithelia in the choroid plexus and meningeal cells on coronal brain sections. However, the level of the IGFBP-2 signal was lower than that seen over pituicytes from the same animals (fig.4 B). No signal above background was observed elsewhere on the brain sections.

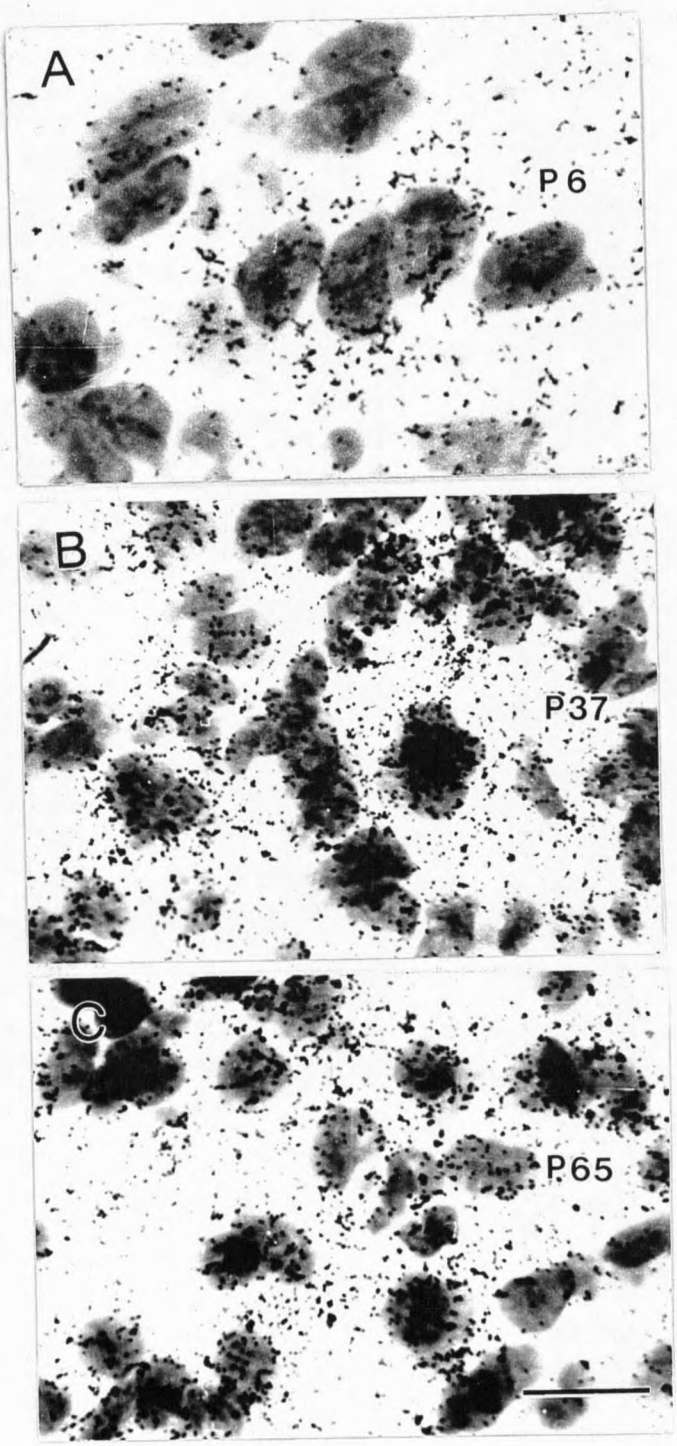


Fig. 4. Localization of IGFBP-2 mRNA by in situ hybridization in the neural lobe at different postnatal ages: P6 (A), P37 (B), P65 (C). Cell nuclei are stained with cresyl violet. Extremely high labeling is present over pituicytes at every age. Note that the grain density over pituicytes at P37 is substantially greater than seen over cells in the choroid plexus and leptomeninges (fig.5 E, F). All figures are to the same scale; bar = 10 μm.

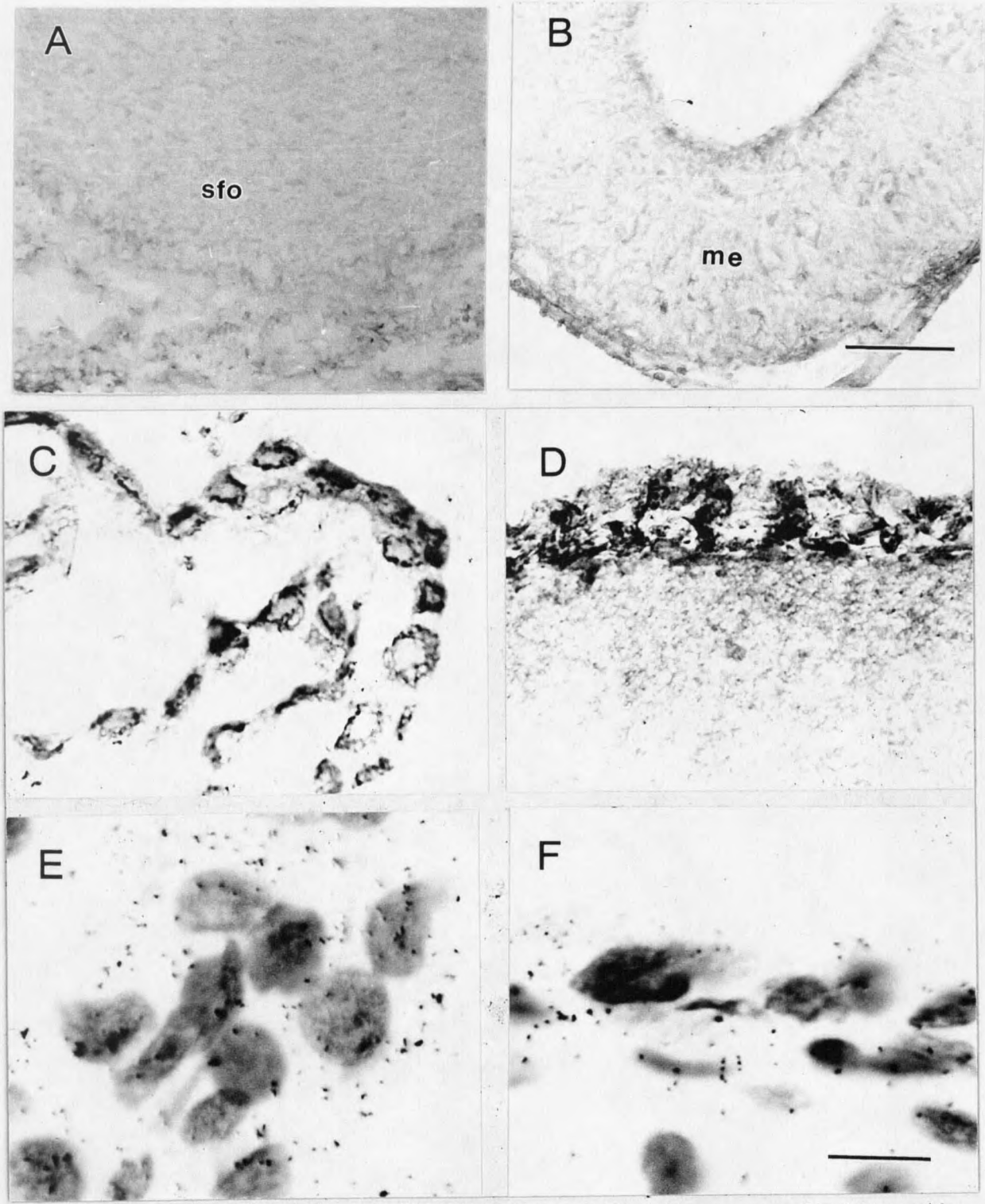


Fig. 5. Expression of IGFBP-2 in the P37 brain localized by immunocytochemistry and in situ hybridization. No immunoreactive glia are found in the brain, even in the regions lacking a blood-brain barrier such as the subfornical organ (A) and median eminence (B). However, intensive immunoreactivity is present in cells of the choroid plexus (C) and meninges (D) on the same sections. Expression of IGFBP-2 mRNA shows a similar pattern, with signal above background present only over the choroid plexus (E) and meninges (F). SFO = subfornical organ; ME = median eminence. Scale bar = 100 μm (A, B). and 10 μm (C-F).

Upregulation of IGFBP-2 mRNA Level in the Neural Lobe during Collateral Sprouting

The ipsilateral destruction of both the SON and PVN was performed by the knife-cut in the hypothalamus. The lesion severed the hypothalamohypophysial tract, eliminating approximately one half of the neurosecretory axons which normally project to the neural lobe of the pituitary. A marked recovery of axon number is seen in the neural lobe between 10 and 30 days post-lesion and this increase occurs as the result of robust collateral sprouting by the uninjured contralateral magnocellular neurons of the SON and PVN (Watt and Paden, 1991). Because robust collateral sprouting occurs between 10 and 30 days after the lesion, we chose 2 , 5, 10 and 30 days post-lesion as time points to look for any changes in the level of IGFBP-2 mRNA or IGFBP-2 immunoreactivity which might occur prior to or during the period of collateral sprouting.

Preliminary studies were performed to determine the optimal concentration of anti-IGFBP-2 IgG and to look for any obvious changes in IGFBP-2 immunoreactivity after the lesion compared to intact or sham control groups. Due to very intensive immunoreactivity for IGFBP-2, we found that immunocytochemistry was not sensitive enough to determine whether or not changes in IGFBP-2 immunoreactivity in the neural lobe occurred during collateral sprouting even when a highly diluted (1:5000) primary antibody was used. Therefore, a more sensitive method, in situ hybridization, was used to determine whether any changes in the level of IGFBP-2 mRNA occurred after the lesion and whether or not they were temporally correlated with collateral sprouting. Messenger RNA levels were estimated by densitometry of film autoradiographs of 6-12 coronal pituitary sections (10 μm) for each animal. A trend toward increased intensity of hybridization of IGFBP-2 mRNA was observed only in lesioned groups at both 5 and

10 days post-lesion (Fig. 6). IGFBP-2 mRNA level was increased by 25% at 5 days post-lesion compared to that of either the intact or sham-operated controls, although this difference was not significant. At 10 days post-lesion, the mean hybridization intensity for IGFBP-2 mRNA in the lesion group was 43% higher than that of either the intact or sham operated control groups ($p<0.05$; one-way ANOVA; Fig.6). At 30 days post-lesion, the mean level of IGFBP-2 mRNA had decreased to almost the basal level. This difference was not significant. There were also no significant differences in the level of IGFBP-2 mRNA between sham and age-matched intact groups.

This transient increase of IGFBP-2 mRNA expression in the pituitary was further confirmed by examining grain density in the emulsion coated sections for IGFBP-2 mRNA. Photos taken from emulsion sections that were counterstained with cresyl violet showed increased grain density over pituicytes at 10 days post-lesion compared to intact or sham animals (Fig.7A, B). This grain density increase was consistent with the results of densitometric analysis as discussed above. Representative film autoradiographs are shown in Fig 7C, D.

In addition to the significant increase in the neural lobe at 10 days after the lesion, a moderate level of IGFBP-2 mRNA was expressed near the lesion track in damaged areas of the brain at each time point in all lesion animals. This expression was also seen in the traumatic injury caused by the sham operation in all sham groups. However, no IGFBP-2 mRNA was detected in the SON or PVN in either lesion, sham or intact animals.

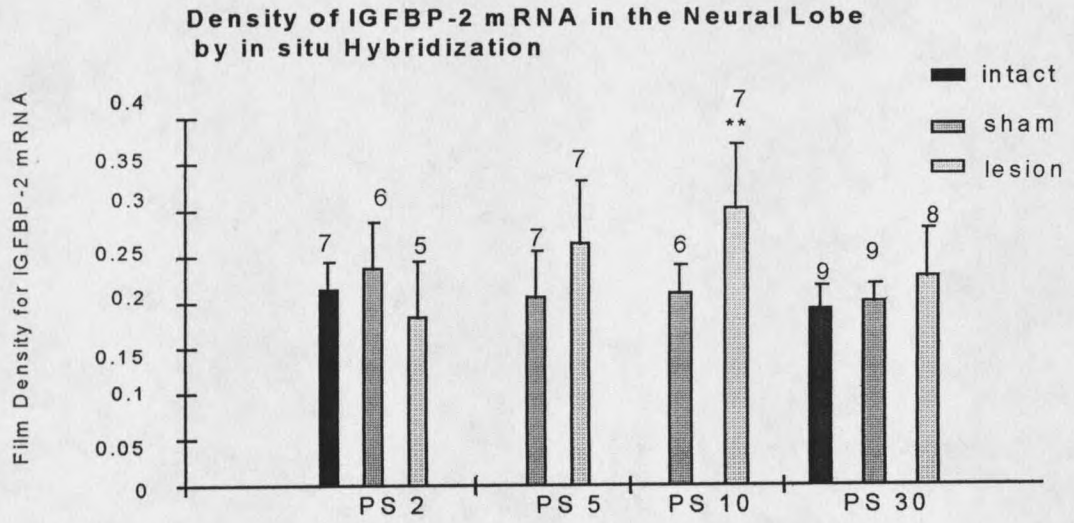


Fig. 6. The time course of expression of IGFBP-2 mRNA in the neural lobe by in situ hybridization at different time points following the unilateral hypothalamic lesion. The level of IGFBP-2 mRNA was measured at 2, 5, 10 and 30 days post-lesion (PS2, PS5, PS10, PS30). The number of animals in each group is shown at the top of each column. Values represent the group mean $\pm$ standard deviation of the mean. The mean hybridization intensity for IGFBP-2 mRNA was increased by 25% at 5 days post-lesion compared to that of either the intact or sham-operated controls, although this difference was not significant. At 10 days post-lesion, the value of the lesion group was 43% higher than that of sham operated control group (**$p<0.05$, one-way ANOVA).

Insulin-like Growth Factor I

IGF-I Immunoreactivity in the MNS

A monoclonal antibody to IGF-I was used to localize IGF-I immunoreactivity in the MNS and to detect any possible changes in immunoreactivity after the unilateral hypothalamic lesion. The IGF-I immunoreactivity was very faint in magnocellular cells of the PVN and SON in all animals examined (fig.8A). No increase in IGF-I immunoreactivity was found in lesion groups at any time point (2, 5, 10 and 30 days after the lesion) in comparison with age-matched intact or sham groups. Each group

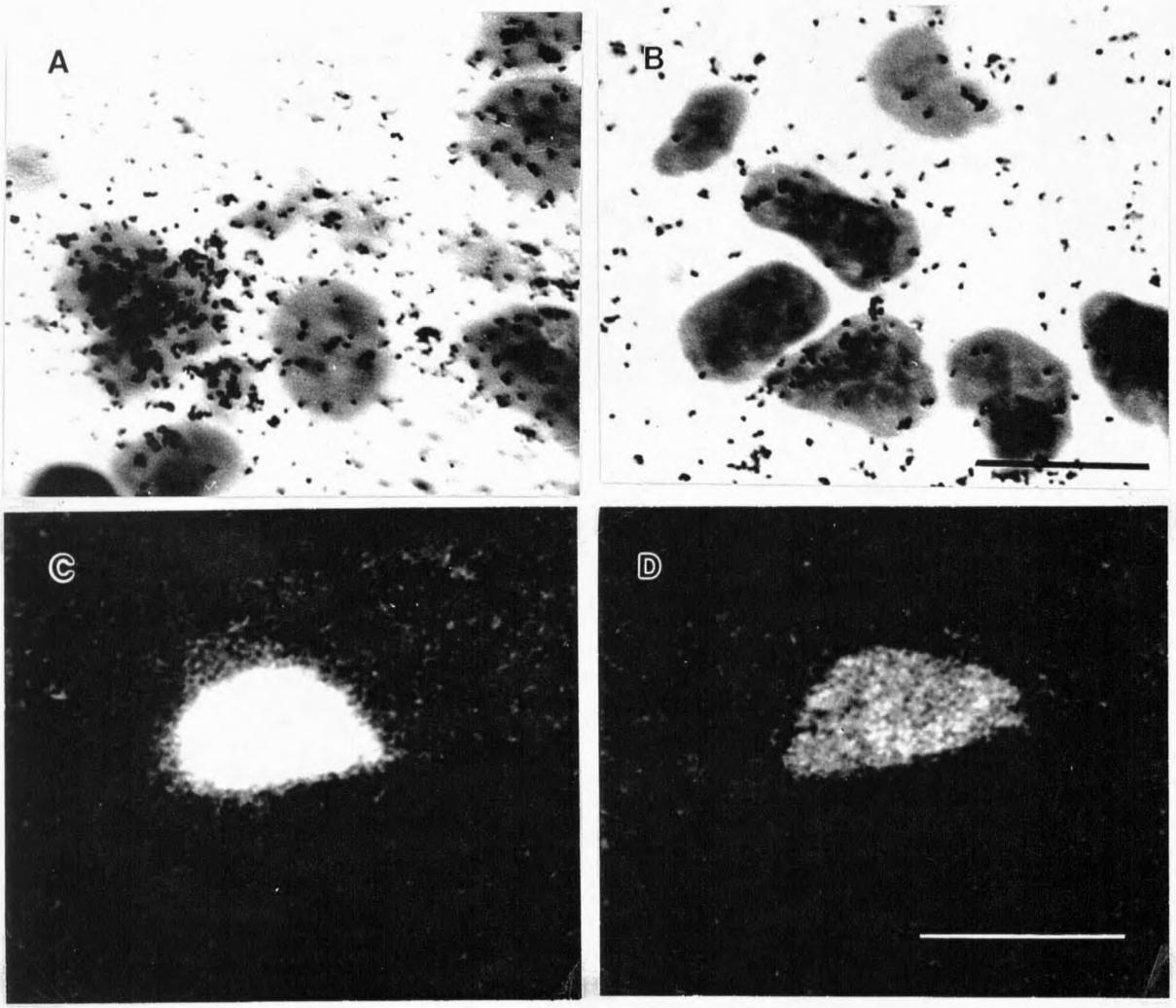


Fig. 7. (A, B) Emulsion autoradiographs of IGFBP-2 mRNA expression in the neural lobe at 10 days post-lesion by in situ hybridization. Sections are counterstained with cresyl violet. The section from a lesioned animal (A) shows increased grain density over pituicytes compared to that of the sham animal (B). Figure C and D are film autoradiographs of IGFBP-2 mRNA in the neural lobe of a lesioned (C) and a sham (D) animal at 10 days post-lesion. Scale bar = 10 μm (A, B). and 100 μm (C, D).

had more than four animals. In contrast, IGF immunoreactivity was detected in the damaged brain tissue along the lesion track at all time points examined. The morphology of these positive cells suggested that they were glia.

In order to increase IGF-I concentrations in magnocellular neurons, colchicine was injected into the third ventricle to inhibit axonal transport. Twenty-four hours following injection of colchicine into the third ventricle, IGF-I immunoreactivity was greatly increased in the magnocellular cell bodies of the SON and PVN (fig.8B). IGF-I immunoreactivity was also found in the magnocellular accessory nuclei. Within the magnocellular cell bodies, staining displayed a punctate or rod-like pattern. Very intensive immunoreactivity for IGF-I was also found in neuronal processes (fig.8C). While the colchicine dramatically enhanced IGF-I immunoreactivity in the cell somata and processes, the neuroanatomical distribution of IGF-I immunoreactivity was similar to that seen in intact animals.

Because colchicine inhibited axonal transport so dramatically, the IGF-I accumulation was very fast and resulted in very intense immunoreactivity for IGF-I in magnocellular cell bodies. The intensity of the immunocytochemical stain for IGF-I obscured any possible changes in IGF-I levels which may have occurred in the magnocellular neurons of the SON and PVN following unilateral destruction of the hypothalamohypophysial tract. No apparent differences in immunoreactivity for IGF-I were found at 2, 5, 10, or 30 days after the lesion either between lesion and intact groups or between lesion and age-matched sham-operated groups after treatment with colchicine.

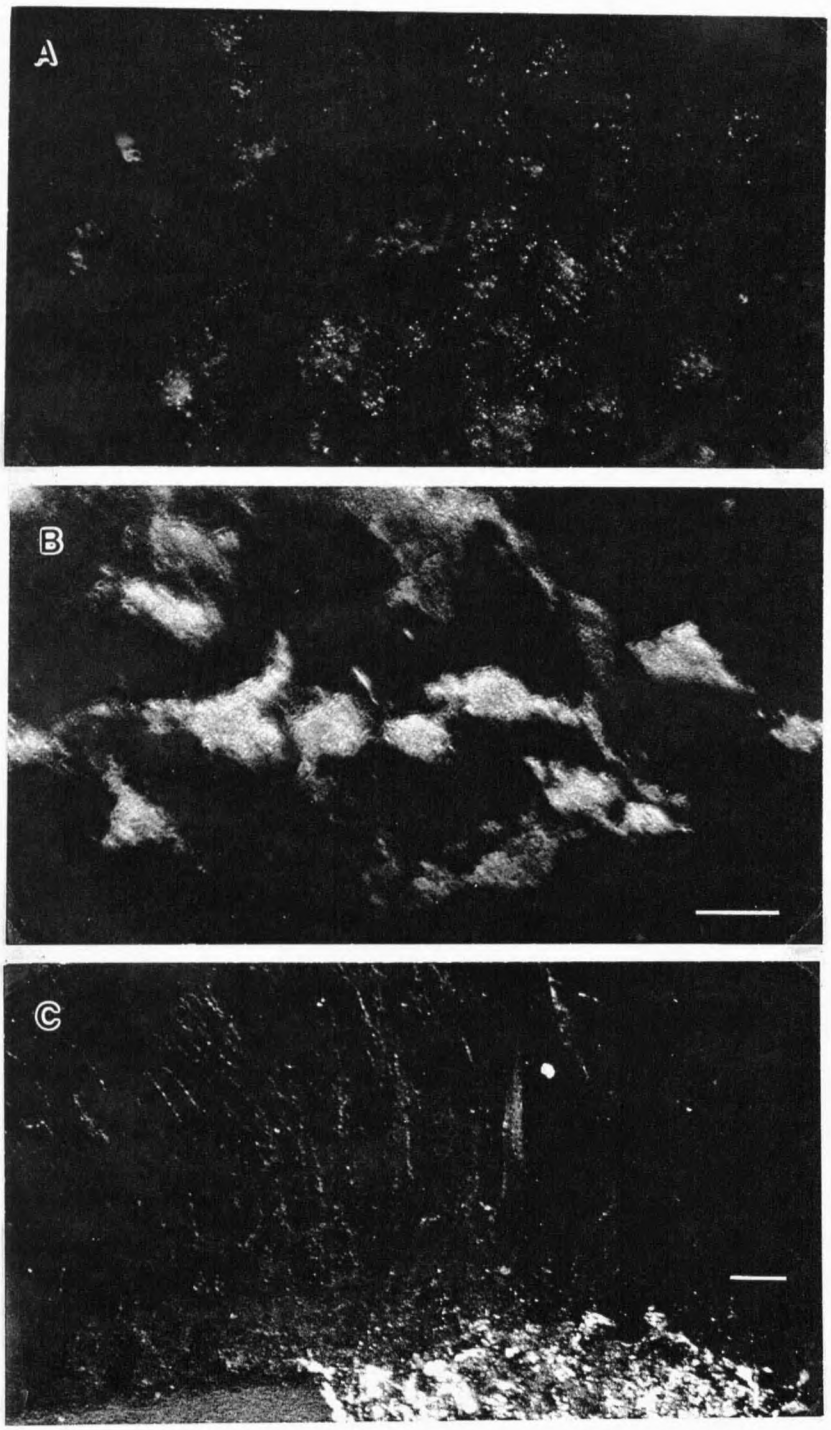


Fig. 8. IGF-I immunoreactivity in magnocellular neurons of the MNS. The IGF-I immunoreactivity is very faint in magnocellular cells of the PVN and SON in all animals examined (A). No increase in IGF-I immunoreactivity is noted in lesion groups at any time point (2, 5, 10 and 30 days after the lesion) in comparison with age-matched intact or sham groups. Twenty-four hours following injection of colchicine into the third ventricle, IGF-I immunoreactivity is greatly increased in the magnocellular cell bodies of the SON and PVN, and staining displays a punctate or rod-like pattern (B). Very intense immunoreactivity for IGF-I is also found in neuronal processes (C). Scale bar = 10 μm (A, B) and 100 μm (C).

The heavy IGF-I immunoreactivity that resulted from colchicine treatment could be decreased by diluting the primary monoclonal antibody in PBS. Anti-IGF-I IgG at diluted concentrations ranging from 1:1K to 1:128K was applied to sections of colchicine injected brains to determine if staining disappeared at different antibody dilutions in animals from different experimental groups. No consistent differences were apparent between lesion and control groups at any time point. Dilution of the primary antibody below 1:32K concentration began to decrease staining intensity in all groups, and in most animals IGF-I immunoreactivity in the PVN and SON disappeared at a dilution of 1:128K. In addition, a high degree of variability in IGF-I immunoreactivity in the PVN and SON was noted among animals within all groups, possible due to variations in the effectiveness of the colchicine treatment.

In addition to the colchicine injection experiment, salt-loaded animals were used in an attempt to detect any relationship between the activity of magnocellular neurons and the level of IGF-I in these cells. Salt-loading is a simple and effective procedure to increase the firing rate of magnocellular neurons. Five animals without colchicine injection were given 2% sodium chloride to drink for 8 days. No change in IGF-I immunoreactivity was observed in the SON or PVN between salt-loaded and normal animals.

To test the specificity of the anti-IGF-I antibody, the monoclonal anti-IGF-I antibody solution was preadsorbed with a three fold excess of purified IGF-I antigen. When the preadsorbed antibody was employed at dilutions between 1:4K and 1:32K, no IGF-I immunoreactivity was detected in sections of the SON and PVN from colchicine treated animals.

Co-localization of IGF-I with Oxytocin and Vasopressin in the SON and PVN

In order to determine if there are any differences in the content of IGF-I between oxytocinergic or vasopressinergic neurons, and to determine the proportion of each kind of neuron containing IGF-I, Vibratome brain sections of five colchicine injected intact animals at 37 days old were used for dual-labeling of IGF-I and OT immunoreactivity or IGF-I and VP immunoreactivity. Three to six brain sections through the SON and/or PVN were randomly selected from each animal. Dual-label immunofluorescence confirmed that both vasopressinergic and oxytocinergic neurons contained IGF-I (fig.9). In general, vasopressinergic neurons had more intensive IGF-I immunoreactivity than oxytocinergic neurons did. It was also true that a much higher proportion of vasopressinergic neurons were IGF-I positive than were oxytocinergic neurons. In the PVN, a total of 983 vasopressin positive neurons were examined and 58% of these were positive for IGF-I immunoreactivity, while only 24% of 830 oxytocinergic neurons examined were positive for IGF-I. Similarly, in the SON, 1050 vasopressinergic neurons were examined and 43% showed IGF-I immunoreactivity, while 742 oxytocinergic neurons were examined and only 19% of these contained IGF-I (fig.10).

Distribution of IGF-I mRNA in the Brain

In situ hybridization was used to determine the distribution of IGF-I mRNA in the MNS and to examine whether changes in the level of IGF-I mRNA are correlated with collateral sprouting. Animals with and without colchicine injection were used in this experiment. Lesion, sham and intact groups were examined at 2, 5, 10 and 30 days

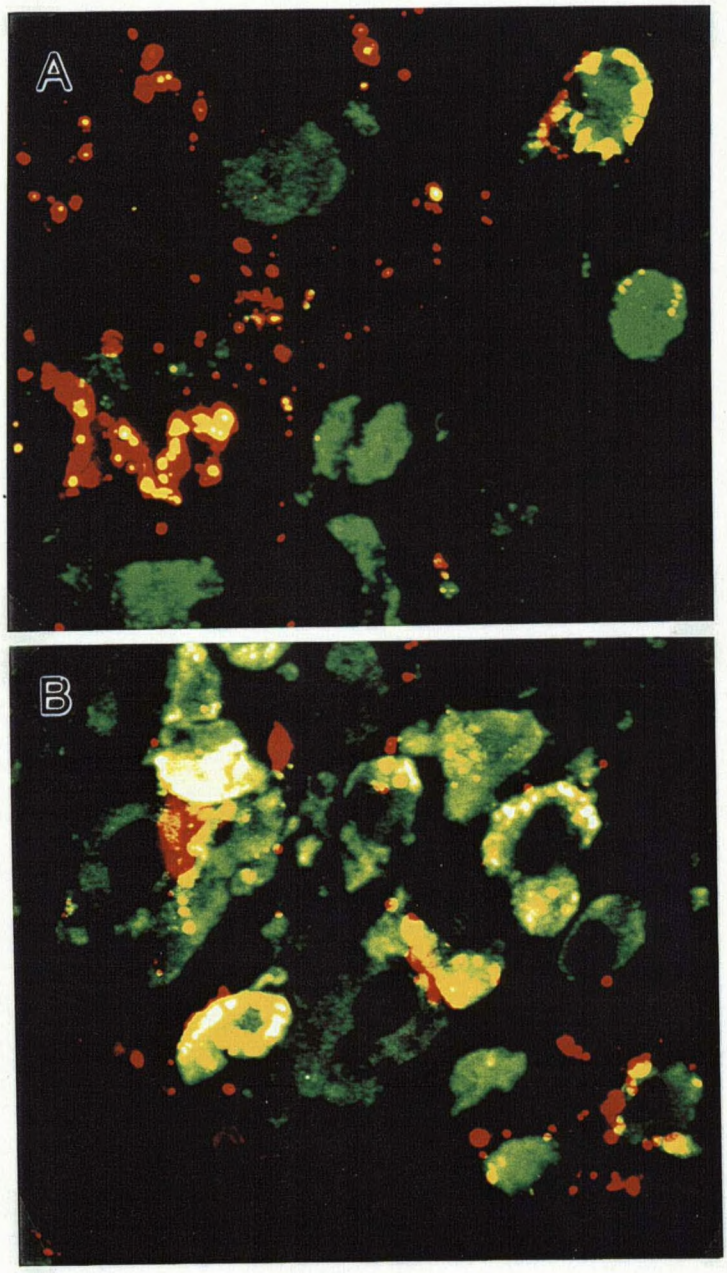


Fig. 9. Photomicrogaphs of dual-label staining for IGF-I and oxytocin or IGF-I and vasopressin in the SON and PVN at day 37 using confocal immunofluorescence microscopy. Fig. A shows that some oxytocinergic cells (green) contain IGF-I immunoreactivity (red). Some vasopressinergic cells (green) also contain IGF-I immunoreactivity (red) (B). IGF-I staining displays a punctate or rod-like pattern. In general, vasopressinergic neurons have more intense IGF-I immunoreactivity than oxytocinergic neurons do.

post-lesion. A weak to moderate hybridization signal for IGF-I mRNA was visible in the SON and PVN on film autographs (fig 11). The highest signal intensity for IGF-I mRNA was located in the hippocampus, while some weak hybridization signals were present elsewhere on the same brain sections. Colchicine injection did not stimulate upregulation of IGF mRNA compared to control animals. No changes in the level of IGF-I mRNA were apparent in the SON and PVN between either lesion and sham or intact control groups at any time point. IGF-I mRNA was upregulated in the injured brain tissue around the lesion track. This finding was consistent with the presence of IGF-I staining in the same areas by immunocytochemistry.

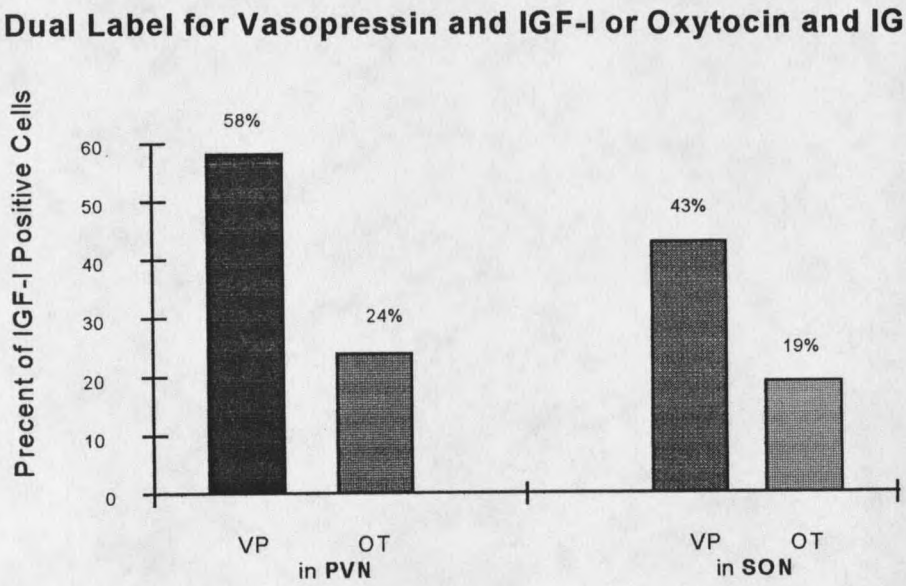


Fig. 10. Dual-label staining for IGF-I and oxytocin or IGF-I and vasopressin in the SON and PVN at day 37. Five intact animals were used following colchicine injection. In the PVN, IGF-I immunoreactivity was present in 58% of vasopressin positive neurons, while only 24% of oxytocinergic neurons are positive for IGF-I. Similarly in the SON, 43% of vasopressinergic neurons showed IGF-I immunoreactivity, while only 19% of oxytocinergic neurons contained IGF-I.

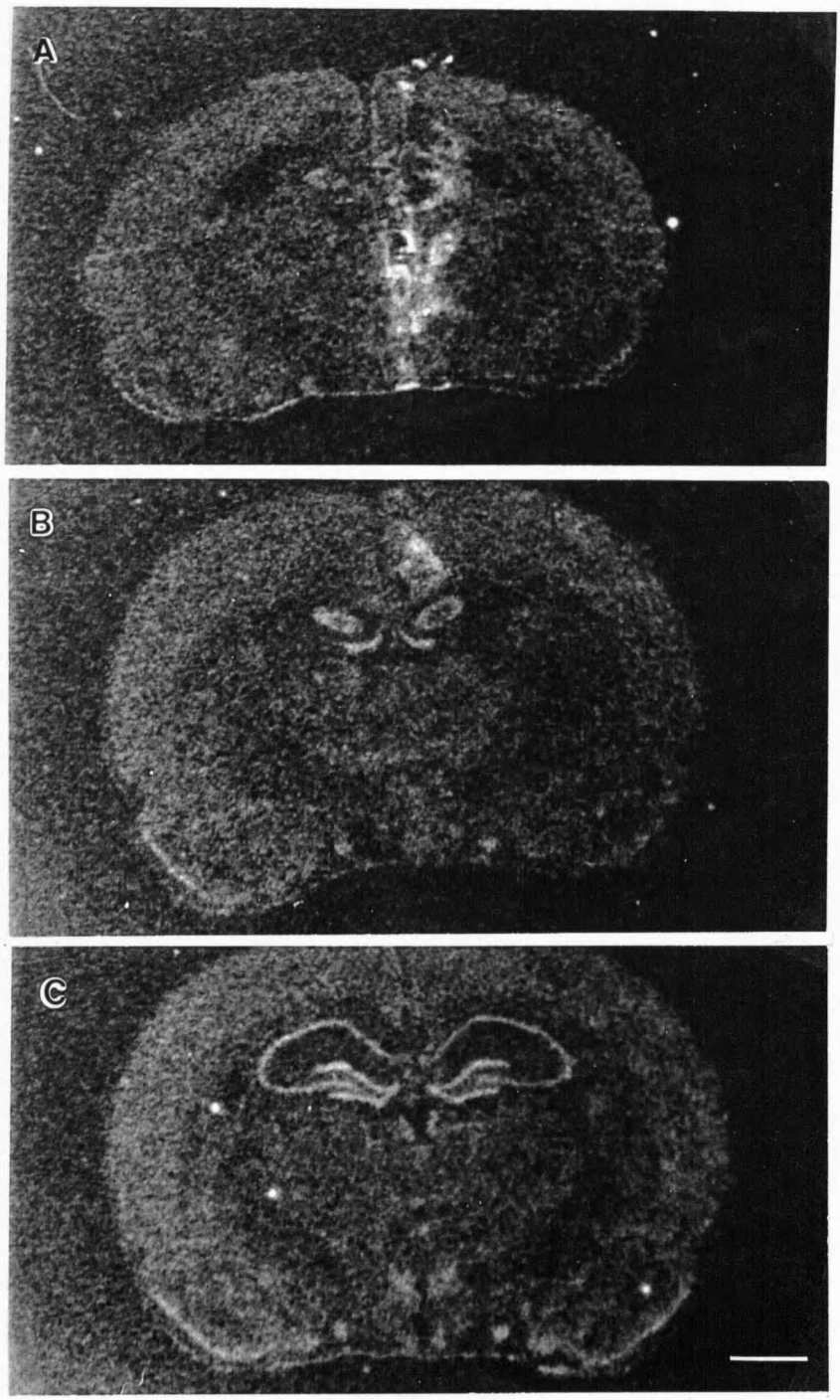


Fig.11. Distribution of IGF-I mRNA in the brain. Lesion (A), sham (B) and intact (C) groups were examined at 2, 5, 10 and 30 days post-lesion. A weak to moderate hybridization signal for IGF-I mRNA was visible in the SON and PVN on film autoradiographs (A, B, C). The highest signal intensity for IGF-I mRNA was located in the hippocampus, No changes in the level of IGF-I mRNA were apparent in the SON and PVN between either lesion and sham or intact control groups at any time point. However, IGF-I mRNA was upregulated in the injured brain tissue around the lesion track. Scale bar = 2 mm (A, B, C).

Insulin-like Growth Factor I Receptor

IGF-I Receptor Immunoreactivity in the MNS

Immunocytochemistry was performed to determine the distribution of the IGF-I receptor and to detect possible changes in IGF-I receptor immunoreactivity associated with collateral sprouting. At least four animals from each lesion, sham or intact group at 2, 5, 10 and 30 days post-lesion were examined. Only occasional animals showed IGF-I receptor immunoreactivity in the median eminence and the neural lobe. Nerve fibers stained with IGF-I receptor antibodies displayed a varicose morphology and were found mainly in the internal layer of the median eminence, while a few varicose fibers were also present in the external layer (fig.12A). In the neural lobe, immunoreactivity for IGF-I receptor was punctate, suggesting a possible association with neurosecretory axons (fig.12B). This occasional stain for IGF-I receptor in the median eminence and the neural lobe was randomly found in lesion, sham or intact groups. IGF-I receptor immunoreactivity was not correlated with neurosecretory axonal sprouting following the unilateral hypothalamic lesion. Furthermore, no immunoreactivity for IGF-I receptor was detected in the SON and PVN. We also tried very high concentrations of IGF-I receptor antibody for immunocytochemical staining. Dilutions ranging from 1:10 to 1:100 (1-10 μg/ml) of the primary antibody were used and the pattern of immunoreactivity was still the same.

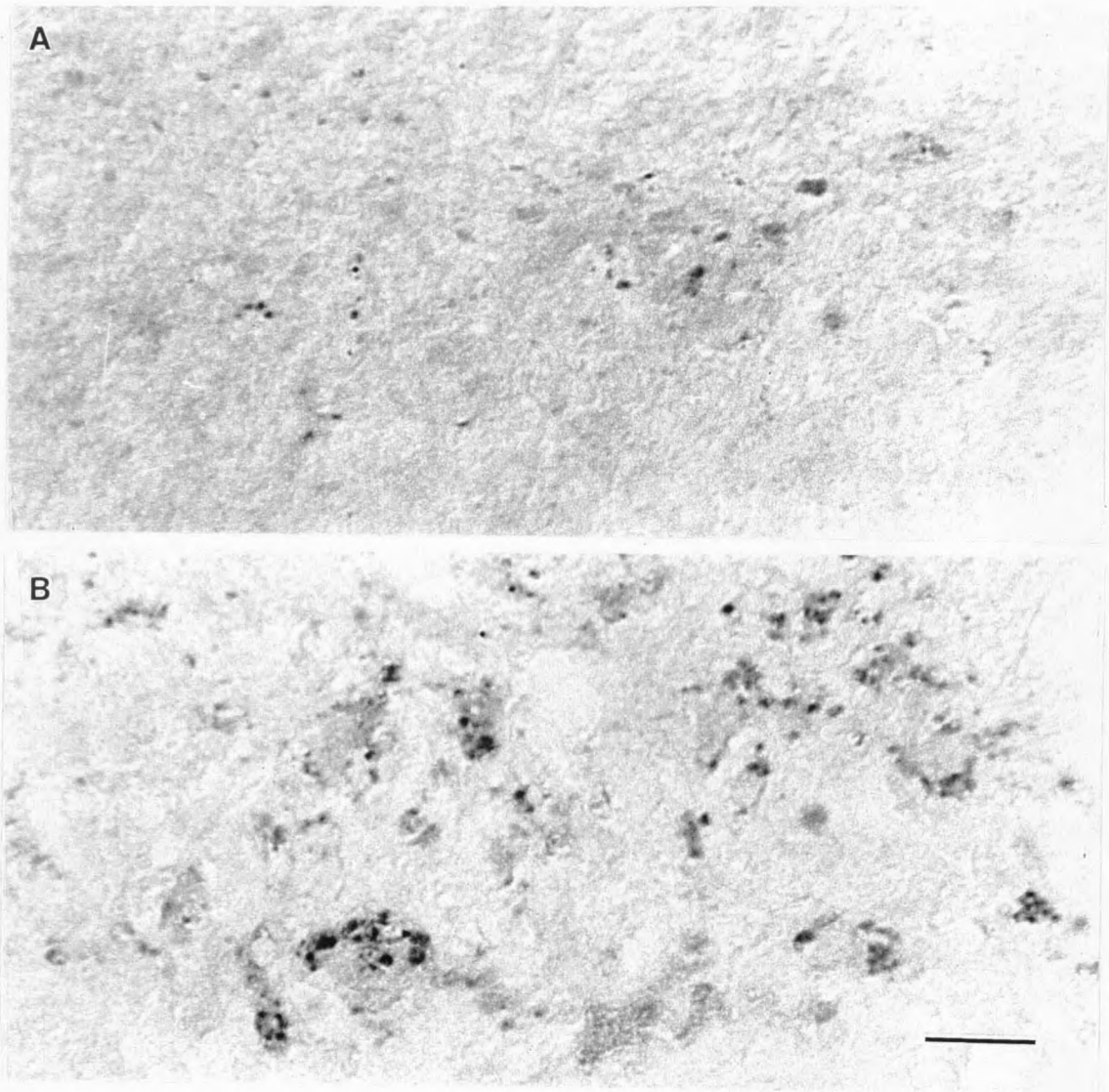


Fig.12. Photomicrographs of IGF-I receptor immunoreactivity in the MNS. Magnocellular nerve fibers stained with IGF-I receptor antibodies display a varicose morphology and are found mainly in the internal layer of the median eminence (A). In the neural lobe, immunoreactivity for IGF-I receptor is punctate, suggesting a possible association with neurosecretory axons (B). No immunoreactivity for IGF-I receptor was detected in the SON and PVN. Scale bar = 10 μm (A, B).

In situ Hybridization for IGF-I Receptor mRNA

Only low levels of IGF-I receptor mRNA were found in the brain and pituitary. In brain, the hippocampus showed the highest levels of hybridization for IGF-I receptor mRNA. In contrast to IGF-I mRNA distribution, IGF-I receptor mRNA was not detected in the SON and PVN of the hypothalamus. Moreover, no signal for IGF-I receptor mRNA was detected in the damaged brain tissue along the lesion track at 2, 5, 10 and 30 days after the lesion (fig.13). These results were consistent with the lack of IGF-I receptor immunoreactivity. However, a moderately high level of IGF-I receptor mRNA was present in the pituitary gland, predominately in the anterior lobe, and there was also a moderate signal in the neural lobe.

To determine whether IGF-I receptor mRNA expression was temporally correlated with magnocellular neurosecretory axonal sprouting after the unilateral hypothalamic lesion, in situ hybridization for IGF-I receptor mRNA was performed in lesion, sham and intact control groups at 2, 5, 10 and 30 days post-lesion. No changes in IGF-I receptor mRNA expression were apparent between lesion and sham or lesion and intact groups, and no changes in expression over time were seen.

Implantation of IGF-I Peptide in the Hypothalamus

To address whether exogenous IGF-I is capable of stimulating axonal regeneration or sprouting in the lesioned MNS, a rod of IGF-I polymer matrix was implanted lateral to the SON in the lesioned side of the brain immediately after lesion surgery (fig.14 A). IGF-I was continuously released from the EVAc polymer matrix

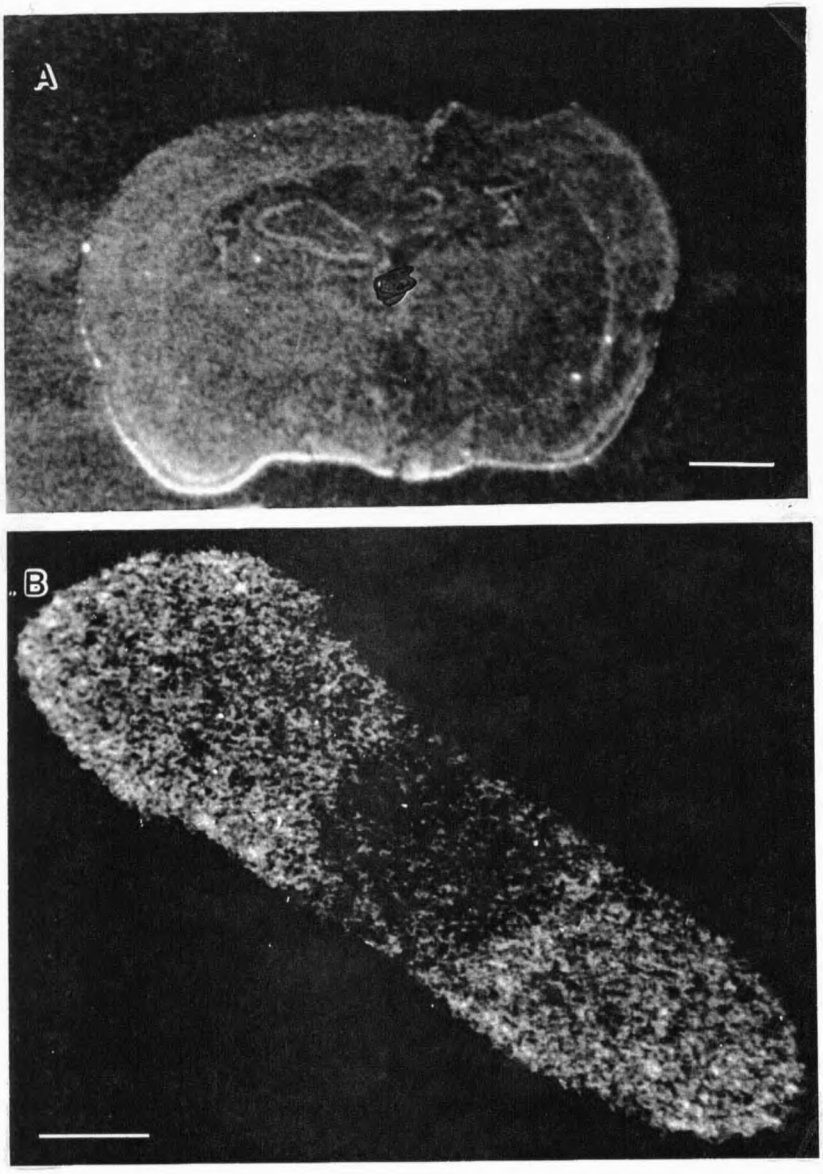


Fig. 13. Photomicrographs of IGF-I receptor mRNA expression in the brain (A) and pituitary (B) by in situ hybridization at 10 days post-lesion. (A) A moderate level of IGF-I receptor mRNA is noted in the hippocampus. IGF-I receptor mRNA is not present in the SON and PVN of the hypothalamus, nor was any signal for IGF-I receptor mRNA detected in the damaged brain tissue along the lesion track at 2, 5, 10 and 30 days after the lesion. (B) A moderately high level of IGF-I receptor mRNA is present in the pituitary gland, predominately in the anterior lobe, and there is also a moderate signal in the neural lobe. Scale bar = 2 mm (A) and 200 μm (B).

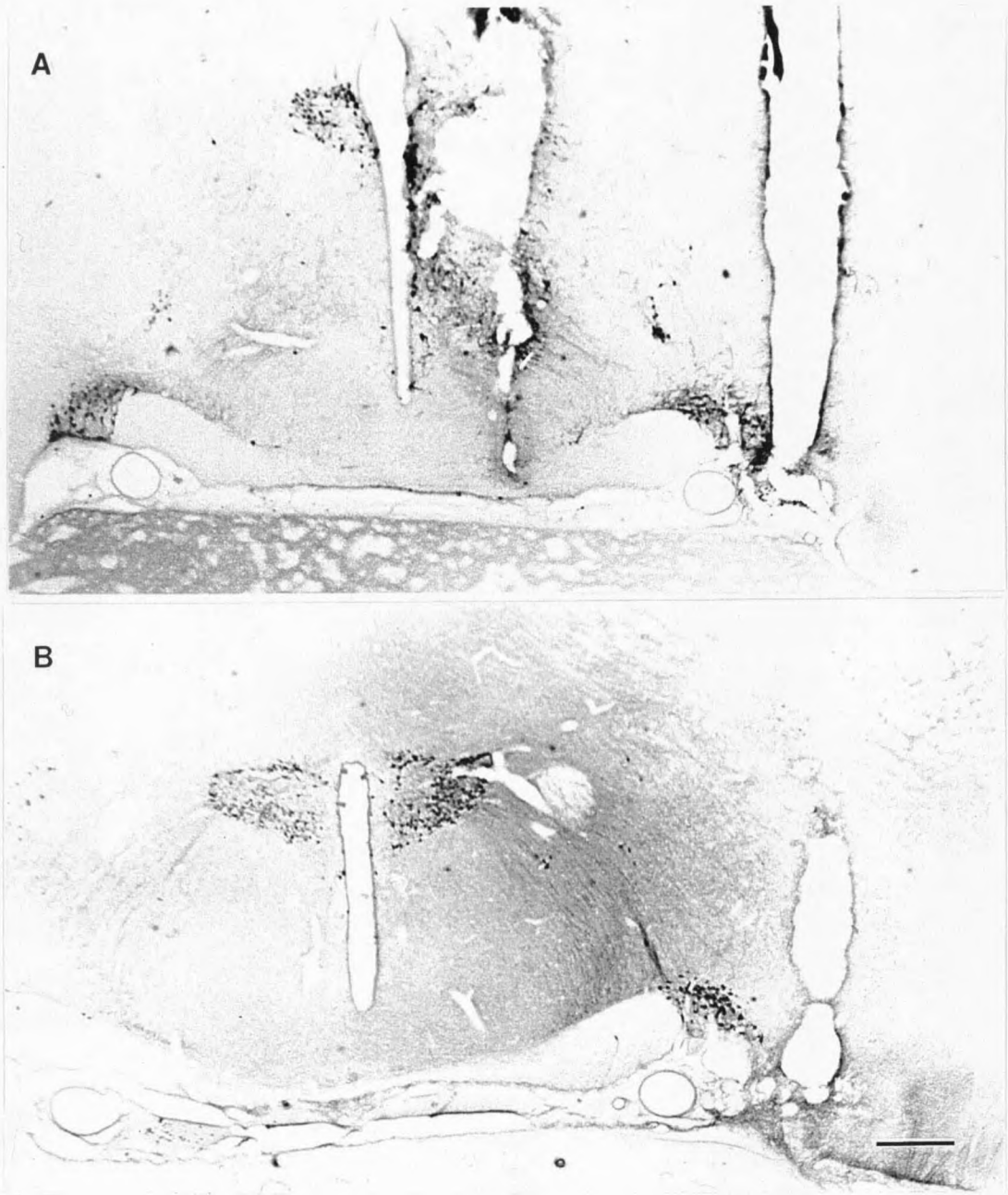


Fig. 14. Implantation of IGF-I polymer in the hypothalamus. Figure A shows implantation with a rod of IGF-I polymer matrix lateral to the SON on the lesioned side of the brain immediately after lesion surgery. The implantation site is in the hypothalamus lateral to the SON where there are few oxytocinergic axons, a site chosen to facilitate visualization of axonal sprouting. (B) Implantation with IGF-I in an intact animal by the same procedure. Oxytocinergic perikarya and axons have been immunocytochemically stained in both figures. Scale bar = 500 μm (A, B).

after implanting it into the hypothalamus according to other growth factor implantation experiments (Kromer, 1987). The negative control animals were implanted with a BSA polymer rod or an empty implantation cannula using the exact procedure as for the IGF-I implantation. The lesion surgery often induced a cerebral hemorrhage resulting in varying degrees of cavitation surrounding the knife tract. This cavitation extended from the dorsal surface to varying depths of the brain in most lesion animals. Immunocytochemical staining with anti-oxytocin antibody showed that the cavitation typically compressed the ipsilateral PVN, resulting in death of most OT neurons in the PVN. Only rare PVN neurons appeared to be spared. An extensive invasion of macrophages, monocytes and other blood borne elements was observed.

At least five animals in each of the IGF-I implanted or BSA implanted groups were examined by immunocytochemical staining with anti-oxytocin IgG at 8 to 14 days after implantation. In IGF-I implanted animals, it was observed that there were a variable number of neurons in either the ipsilateral PVN or SON and a varying degree of axonal regeneration in the hypothalamus as well as some axonal growth toward the ipsilateral anterior cerebral artery. Some surviving neurons and axons in the ipsilateral PVN, but no axonal growth around the ipsilateral cerebral anterior artery were detected in BSA implanted control groups. However, it was not possible to quantify group differences in the number of surviving neurons or in the extent of axonal growth. There were several factors that contributed to this problem. First, each individual animal had a varying degree of cavitation which resulted in a highly variable extent of neuronal death. It was impossible to differentiate the potential effects of the IGF-I peptide from the effects of cavitation in contributing to the number of surviving neurons. The same problem was

encountered with regard to quantifying the extent of axonal growth. Second, the distribution of oxytocinergic neurons is uneven throughout the SON and PVN, and slight differences in the position of the knife tract resulted in large differences in the number of surviving neurons, and in the number of regenerating axons. In an attempt to circumvent these problems, intact animals were implanted with IGF-I EVAc polymer to detect any effects of IGF-I on magnocellular axonal sprouting. Implantation of BSA polymer or insertion of an empty cannula in the same position were used as controls .

IGF-I Stimulated Magnocellular Axon Growth in Intact Animals

The implantation site in this experiment was in the hypothalamus lateral to the SON where there are not very many oxytocinergic axons, a site chosen to facilitate visualization of axonal sprouting (fig.14B). Six animals were implanted with IGF-I polymer, while 10 animals were implanted with BSA polymer and 3 animals were damaged in the same location by implantation with an empty cannula as controls. After 8 or 14 days post-implantation, axonal sprouting was examined immunocytochemically with anti-oxytocin antibody. Four of ten BSA implanted animals and one of six animals implanted with IGF-I were found to have extensive necrosis around the implantation site. Because invasion of necrotic tissue by immune cells will produce numerous factors, some of which could affect axonal growth, these animals were excluded from the experimental analysis.

We chose two landmarks to judge axonal growth. One was the number of oxytocinergic nerve fibers around the implantation track. Typically there are not many magnocellular nerve fibers in this region, although some rare axons were observed which

were straight and smooth in shape (Fig.15B). In contrast, axons which grew toward the implantation site or cannula track usually followed a tortuous, irregular pathway (Fig.15A). They appeared to be newly growing axons. The relative number of these tortuous, irregular axons are indicated by a rating from – to + + + (Table 2). The other measure was the number of oxytocinergic axons which grew toward or even directly touched the ipsilateral branch of the cerebral anterior artery. It was found that many axons grew toward the ipsilateral anterior cerebral artery, and some axons directly touched this blood vessel or surrounded it in animals of the IGF-I implantation group. The density of these fibers was also scored as – to + + +. Almost all axons which surrounded the artery had tortuous, irregular shapes (Fig. 16A). In contrast, no oxytocinergic axons were found around the artery in normal animals. Furthermore, we never detected any oxytocinergic axons around the contralateral cerebral anterior artery in either IGF-I implanted or control animals (Fig. 16B). In addition, we also observed some axons growing into the meninges below the SON. These results indicated that the density of the axons around the ipsilateral cerebral anterior artery could be a reliable landmark for stimulated growth of magnocellular axons.

The results are presented in Table 2. Each of five intact animals implanted with IGF-I peptide polymer matrix showed magnocellular axon growth. Regeneration or sprouting of oxytocin positive processes occurred in all animals before 8 days or 14 days following IGF-I implantation. All these results suggest that IGF-I was released from the polymer matrix and diffused into nearby tissue. The elevated IGF-I concentration appeared to stimulate axonal growth toward the IGF-I implantation track where IGF-I

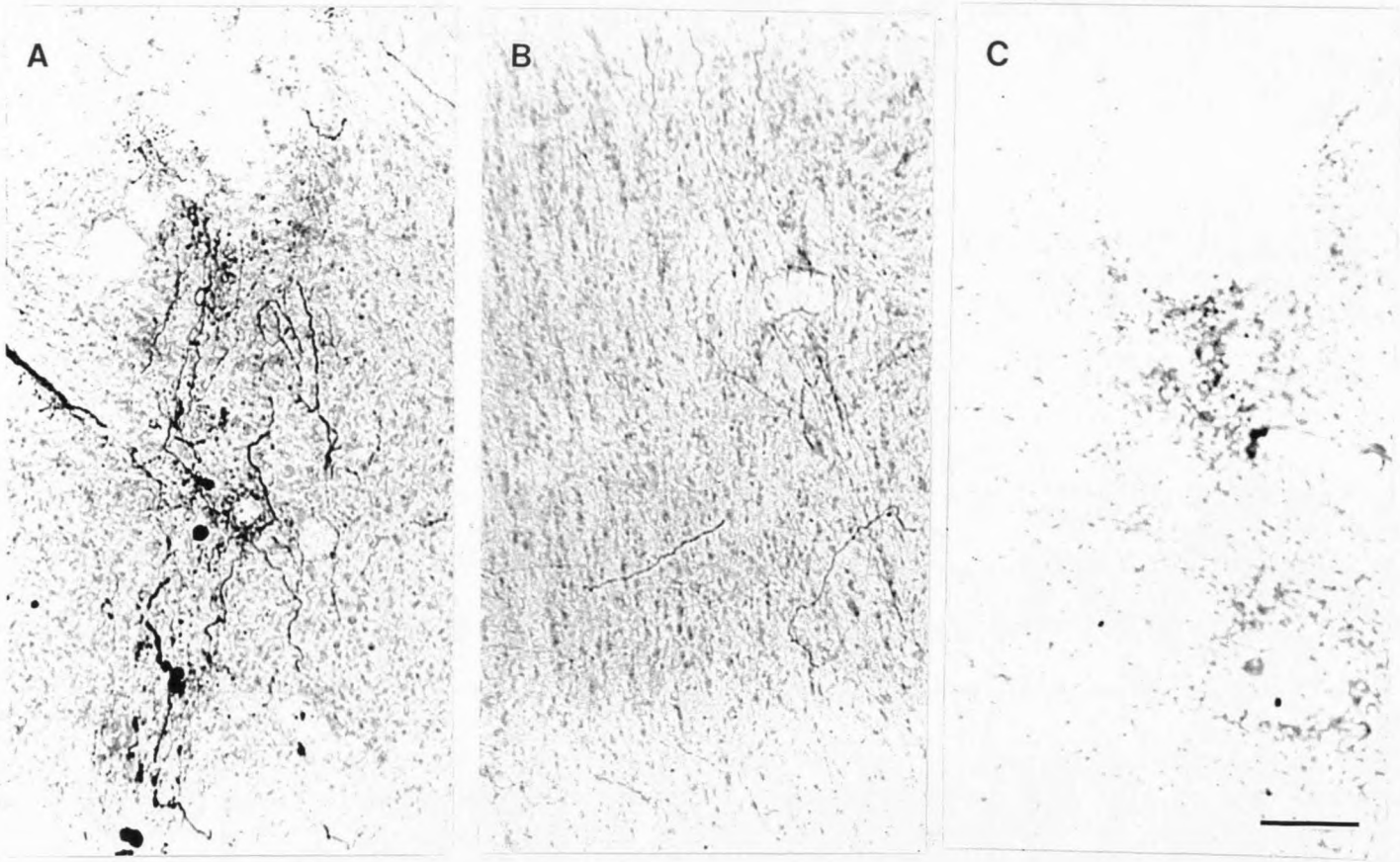


Fig. 15. IGF-I stimulates magnocellular axonal growth around the implantation site in intact animals at 8 days after implantation. Immunocytochemical staining for oxytocin shows that new oxytocinergic axons grew toward the implantation site in a tortuous, irregular shape (A). Figure B shows the same location on the contralateral side of the hypothalamus. There are not many magnocellular nerve fibers in this region. Negative control animals implanted with BSA polymer matrix or empty cannula do not show axonal growth (C). Scale bar = 100 μm (A, B, C).

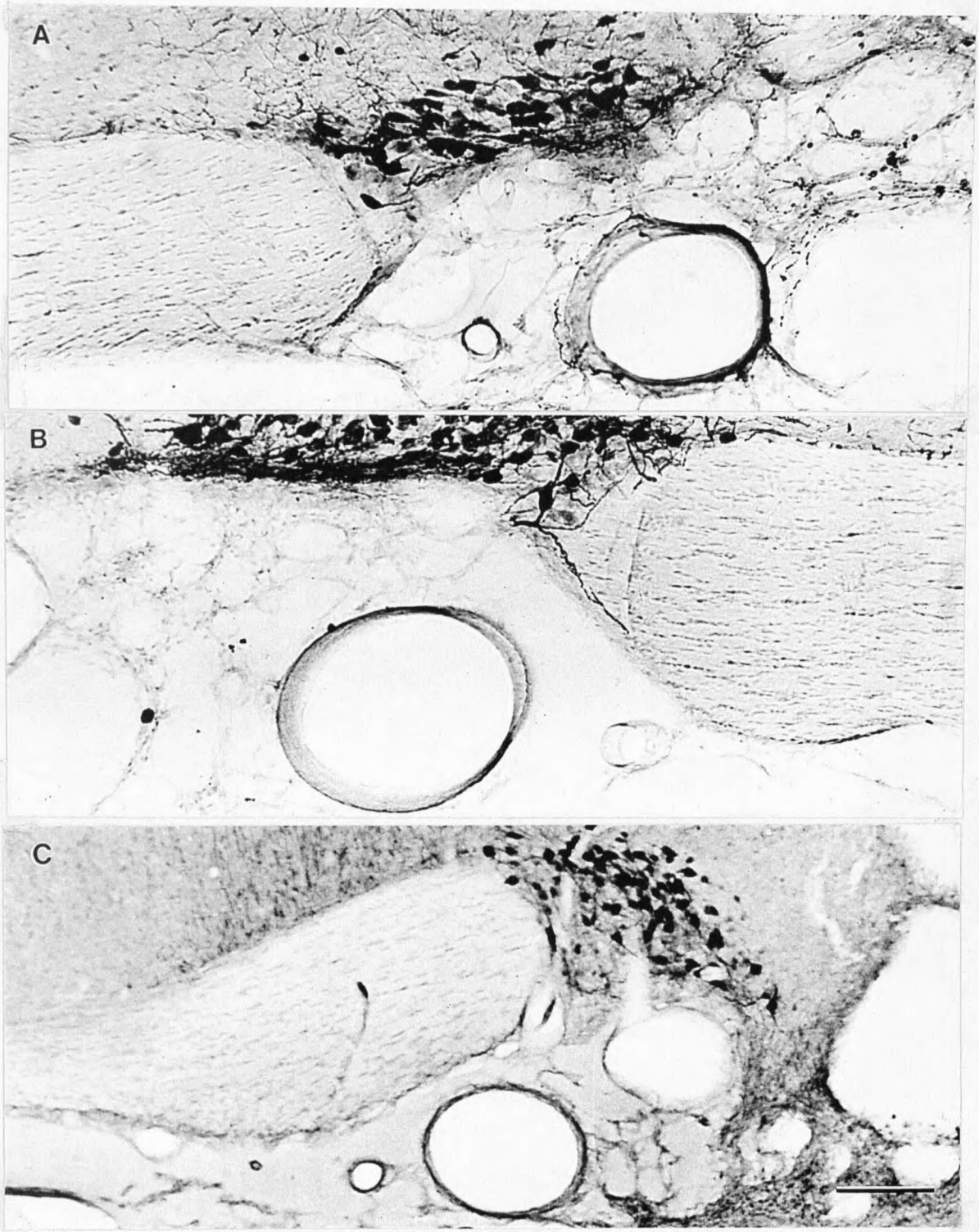


Fig. 16. IGF-I stimulates magnocellular axonal growth toward and around the ipsilateral anterior cerebral artery. Oxytocinergic axons grew toward or even directly touched the ipsilateral branch of the cerebral anterior artery at 8 or 14 days after IGF-I implantation (A). Almost all axons which surround the artery have a tortuous, irregular shape. No oxytocinergic axons are noted around the contralateral cerebral anterior artery in either IGF-I implanted (B) or control animals. No oxytocinergic processes are seen around the anterior cerebral artery on the same side as the BSA implant (C). Scale bar = 100 μm (A, B, C).

concentration was presumably the greatest, and to promote axon growth toward the ipsilateral anterior cerebral artery.

Table 2: Oxytocinergic Axon Growth in the Hypothalamus Implanted with IGF-I Polymer

Animal #	Implantation type	Relative Axon Density		
		Implantation track	Ipsilateral A.C.A*.	Contralateral A.C.A.
1	IGF-I (PS8)	+++	+++	–
2	IGF-I (PS8)	++	++	–
3	IGF-I (PS8)	+++	+++	–
4	IGF-I (PS14)	+++	+++	–
5	IGF-I (PS14)	++	++	–
1	BSA (PS8)	–	–	–
2	BSA(PS8)	–	–	–
3	BSA (PS8)	–	–	–
4	BSA (PS8)	–	–	–
5	BSA (PS14)	–	–	–
6	BSA (PS14)	+	+	–
1	empty cannula (PS8)	–	–	–
2	empty cannula (PS8)	–	–	–
3	empty cannula (PS8)	–	–	–

* A.C.A. = anterior cerebral artery

In contrast, negative control animals implanted with BSA polymer matrix or an empty cannula did not show axonal growth (Fig. 15C; Fig. 16C) except for one BSA implanted animal, where only a few processes grew toward the implant site. No magnocellular processes were detected around the anterior cerebral artery on the same side as the BSA implant in five animals, but one animal showed a few processes around the ipsilateral anterior cerebral artery, which was the same animal that had some axonal growth along the BSA implantation tract. Furthermore, no growing axons were detected either near the cannula track or around the ipsilateral anterior cerebral artery in

any of the animals implanted with an empty cannula. As was the case for the IGF-I implanted animals, no oxytocin positive fibers were observed growing around or toward the contralateral anterior cerebral artery.

CHAPTER 4

DISCUSSION

Research in a variety of neuronal systems has demonstrated that deafferenting lesions induce growth or sprouting by surviving afferents, but the signals that initiate and sustain the sprouting response have remained a mystery. Our unilateral hypothalamic lesion model has similar characteristics with deafferenting lesions, although the magnocellular neural axons do not have a synaptic connection with post synaptic neurons but directly connect with blood vessels or pituicytes in the neural lobe (Dellmann, et al., 1988). It is possible that loss of half of the axons simply removes competitive inhibition and allows intact collateral neuronal axonal sprouting to occur. Alternatively, degenerating neural elements might initiate the release of diffusible chemical signals or may establish a chemical gradient in the neuropil that both initiates the growth response and stimulates axon sprouting in the neural lobe. Such a chemical signal could arise from degenerating neuronal elements or from local glia cells.

In this dissertation, I have examined the possibility that the IGF system might play a role in the compensatory collateral sprouting of magnocellular neurosecretory axons following a unilateral hypothalamic lesion. Several members of the IGF system have been detected in the MNS. The discussion is divided into four sections: IGF-I, IGFBP-2, IGF-I receptor and IGF-I implantation.

IGF-I

Present results showed that IGF-I immunoreactivity was detectable in the MNS. The IGF-I immunoreactivity was very faint in magnocellular cells of the PVN and SON in all animals examined. These results are partially consistent with previous reports that IGF-I immunoreactivity was detected in neurons of the SON and PVN in normal intact animals (Aguado, et al., 1992; Garcia-Segura, et al, 1991). However, in contrast to these reports, no IGF-I immunoreactivity was found in the neural lobe. The cause of this discrepancy is not known. It might be related to variations in tissue preparation or the primary antibody used. Another report showed IGF-I distribution in the tissue and extracellular compartment in different regions of rat brain, in which the tissue content of IGF-I was the highest in pituitary among eight different regions examined (Yamaguchi, et al, 1990). The result was obtained by radioimmunoassay (RIA) of extracted IGF-I from fresh tissues. There was a high correlation ($r= +0.935$, $p<0.01$) between tissue content and extracellular IGF-I level determined by intracerebral microdialysis. It was suggested that IGF-I might exist extracellularly. PLP fixation used by our laboratory for immunocytochemistry might not be efficient to retain this soluble IGF-I. Thus it is possible that the pituitary might have high concentrations of IGF-I that are undetectable by immunocytochemistry.

A similar distribution of IGF-I immunoreactivity in the MNS was observed following colchicine injection into the third ventricle. Twenty-four hours following injection of colchicine, IGF-I immunoreactivity was dramatically increased in the

magnocellular cell bodies of the PVN and SON. Magnocellular accessory groups also showed IGF-I immunoreactivity. Moreover, the magnocellular fibers showed intensive punctate immunoreactivity for IGF-I. IGF-I immunostaining in the MNS of colchicine treated rats was probably not an artifact since it was abolished by pre-absorption with recombinant human IGF-I peptide.

The robust increase in IGF immunoreactivity in magnocellular neuronal somata following colchicine treatment suggested that IGF-I peptide is synthesized in magnocellular cell bodies and then transported down to the neural lobe. Hypothalamic-hypophyseal transport of IGF-I is also supported by another experiment. It has been reported that cycloheximide, an inhibitor of protein synthesis, dramatically decreased the concentration of IGF-I released into culture media from the hypothalamus, but not from the intermediate lobe (Binoux, et al., 1981).

The synthesis of IGF-I in the magnocellular neuronal somata was further confirmed by in situ hybridization for IGF-I mRNA. Our results showed that IGF-I mRNA was detected in the PVN and SON where the magnocellular neuronal somata are located. This finding is consistent with another report which showed high concentrations of IGF-I mRNA in the hypothalamus (Rotwein, et al., 1988).

Moreover, dual label immunocytochemical staining for IGF-I and OT or IGF-I and VP further supported the conclusion that magnocellular neurons synthesize IGF-I. Fifty-eight percent of vasopressinergic neurons showed IGF-I immunoreactivity in the PVN, while 24% of oxytocinergic neurons were positive for IGF-I. A similar pattern was seen in the SON, where 43% of vasopressinergic neurons and 19% of oxytocinergic neurons exhibited IGF-I immunoreactivity. In addition, vasopressinergic neurons exhibited more

intensive IGF-I reactivity than oxytocinergic neurons did. To my knowledge, this is the first demonstration that IGF-I is colocalized with both VP and OT in the magnocellular neurons of the PVN and SON. It is unclear why only a subset of magnocellular neurons exhibited IGF-I immunoreactivity, and why a greater percentage of vasopressinergic neurons than oxytocinergic neurons showed IGF-I immunoreactivity.

Increases in IGF-I immunoreactivity or IGF-I mRNA levels have been reported to occur in different kinds of cells following several types of CNS lesions. Following experimentally induced demyelination (Komoly, et al., 1992) or transient forebrain ischemia (Lee, et al., 1992), IGF-I expression was detected in GFAP-positive astrocytes. Similarly, following facial nerve axotomy, intensive IGF-I immunoreactivity appeared on the astrocytic perikarya and processes with an apparent staining peak between 4 and 7 days (Gehrmann, et al., 1994). These hypertrophic astrocytes often had IGF-I positive processes closely opposed to surfaces of axotomized neurons. In the deafferented hippocampus model, IGF-I mRNA expression was dramatically increased in microglia following destruction of the entorhinal cortex (Guthrie, et al., 1995). The spatiotemporal pattern of elevated IGF-I mRNA corresponded with that of axonal sprouting in the hippocampus.

In contrast to the above reports, no significant increase was observed in IGF-I immunoreactivity or its mRNA expression in the PNV or SON, or in the neural lobe following the unilateral hypothalamic lesion. There are several potential explanations for this discrepancy. First, collateral sprouting by the intact magnocellular neurons occurs in the neural lobe which is a highly vascularized tissue located outside of the blood brain barrier. This morphology provides the magnocellular axon terminals with direct

exposure to IGF-I and/or IGF-II present in the circulatory system. In contrast, increased IGF-I expression observed in other brain injury models may be necessary to prevent neuronal damage and to stimulate injured neurons to grow because there exists a blood brain barrier between brain tissue and the circulatory system. The movement of IGF-I into the brain parenchyma after hypoxic-ischemic injury has been demonstrated (Guan, et al., 1996). Furthermore, it is possible that IGF-I levels could be increased in the circulatory system following the unilateral hypothalamic lesion. This possibility is worthy of further investigation. Lastly, we observed increased IGFBP-2 mRNA levels in pituicytes of the neural lobe after the unilateral hypothalamic lesion. IGFBP-2 could mediate IGF-I bioavailibility by binding IGF-I from the circulatory system. This passive increase in the local extracellular concentration of IGF-I could increase binding to its receptor on axons of the magnocellular neurons. Following IGF-I binding to its receptor, it could be retrogradely transported to the cell body where it may alter gene expression so as to stimulate collateral axonal sprouting. For example, it has been demonstrated that IGF-I can stimulate α–I and β–II tubulin mRNA expression in vitro (Wang, et al., 1992). We have previously reported that expression of these cytoskeletal genes is upregulated in the SON during collateral axonal sprouting (Paden, et al. 1995). In short, the elevated level of IGFBP-2 in the neural lobe could be a prime determinant of IGF-I bioactivity during collateral sprouting in the absence of any increase in expression of IGF-I itself.

We also failed to observe an increase in IGF-I immunoreactivity in the PVN or SON after 8 days of oral saline treatment. Salt loading is a potent activator of the MNS, inducing numerous changes in the magnocellular neurons, including morphological

plasticity and upregulation of neuropeptide mRNA, and increased release of VP, OT and their co-existing peptides. One possible explanation for the lack of any increase in IGF-I immunoreactivity is simply that immunocytochemistry is not sensitive enough to detect a small increase in IGF-I level after salt loading treatment. A second possibility is that the normal level of IGF-I synthesis in the MNS is sufficient to mediate the response of magnocellular neurons to salt loading treatment. This hypothesis is supported by the observation that levels of IGF-I receptor mRNA exhibited a dramatic increase in the SON following salt loading treatment (Aguado, et al., 1993), suggesting that IGF-I receptor expression might act as a prime determinant of IGF-I bioactivity in salt loaded animals in the absence of any increase in the level of IGF-I .

We did observe increased expression of IGF-I mRNA along the lesion track in the brain. This observation is consistent with other studies of brain traumatic models (Guthrie, et al., 1995; Sandberg Nordqvist, et al., 1996). Microglia and astrocytes as well as invading macrophages could express IGF-I mRNA in the damaged area. This IGF-I mRNA expression could promote survival of damaged neurons and prevent them from death. It could also be involved in repair of brain damage. But it seems unlikely that this local IGF-I expression along the lesion track could stimulate axonal sprouting by intact magnocellular neurons, because of its distance from the contralateral PVN or SON, or the neural lobe.

IGFBP-2

Immunoreactive IGFBP-2 was found to be constitutively present in virtually all pituicytes of the rat neural lobe from birth into adulthood. No IGFBP-2 was detectable in microglia or endothelia within the neural lobe. The intensive immunostaining for IGFBP-2 protein in pituicytes is accompanied by an equally substantial level of IGFBP-2 mRNA in these cells at all ages examined. These findings are consistent with previous reports of IGFBP-2 mRNA expression in the rat neural lobe at P12 (Lee, et al., 1993) and in the adult (Bach and Bondy, 1992). In contrast to the high levels present in pituicytes, neither IGFBP-2 nor its mRNA were detectable in astrocytes elsewhere in the brain at P37, consistent with studies showing that transient developmental expression of IGFBP-2 mRNA by brain astrocytes has ceased by this age (Lee, et al., 1992).

Pituicytes appear to be unique among neuroglia with respect to constitutive production of IGFBP-2. This finding is consistent with the hypothesis that IGFBP-2 production by pituicytes could modulate effects of IGF-I or IGF-II on function of the MNS. Production of IGFBP-2 by pituicytes might be analogous to synthesis of the binding protein by astrocytes in the developing retina and cerebellum. In those regions, developing projection neurons synthesize IGF-I, while IGFBP-2 mRNA is expressed in a contiguous population of astrocytes, a pattern suggestive of an inductive effect of neuronal IGF-I on glial production of IGFBP-2. Expression of IGF-II is also linked with expression of IGFBP-2 in the choroid plexus and leptomeninges throughout the postnatal period and continuing into adulthood (Stylinopoulou, et al., 1988). Thus

expression of IGFBP-2 by pituicytes could be influenced by local production of IGF-II, given that IGF-II mRNA has been reported to be expressed by capillary endothelia in the adult neural lobe (Bach and Bondy, 1992). Our results demonstrate that it is not simply the absence of a blood-brain barrier that accounts for the constitutive expression of IGFBP-2 in the pituicytes, as neither IGFBP-2 nor its mRNA were detectable in the subfornical organ and median eminence. However, these findings do not exclude the possibility that circulating factors may act in combination with local production of IGFs to influence IGFBP-2 expression by pituicytes.

IGFBP-2 could also act locally within the neural lobe to promote pituicyte motility. IGFBP-2 contains an Arg-Gly-Asp (RGD) sequence, as does IGFBP-1, enabling these binding proteins to bind integrins and associate with the cell surface (Clemmons, et al., 1993). While we are unaware of any studies demonstrating the functional significance of IGFBP-2 binding to integrins, an intact RGD sequence is required for IGFBP-1 to mediate migration of smooth muscle cells in vitro (Clemmons, et al., 1993). Thus it is possible that IGFBP-2 could mediate cell motility in the neural lobe, where pituicytes undergo rapid morphological adaptations during periods of heightened neurosecretory activity, retracting processes surrounding axons to facilitate contact of neurosecretory terminals with capillaries (Hatton, et al., 1990). Pituicyte motility might also facilitate growth of new axon sprouts in the neural lobe in an analogous fashion.

There are several conceivable ways in which IGFBP-2 might modulate potential effects of the IGFs on function of the MNS. One such action could be to target either one or both of the IGFs to magnocellular neurons where they could serve to stimulate neuronal survival and axonal growth. The bioavailability of IGFs is believed to be

modulated by IGFBP-2 (Clemmons, et al., 1993) and documentation of the spatial and temporal response of IGFBP-2 within the neural lobe provides a first step in elucidating the potential function of the IGFs in the sprouting response. The elevated expression of IGFBP-2 mRNA seen at 10 days after the hypothalamic lesion might be necessary to promote collateral sprouting in the neural lobe. Increased IGFBP-2 could bind already existing IGFs in the neural lobe or in the circulatory system, thereby enhancing actions of the IGFs on target magnocellular neurons by increasing the half-life of the IGFs and/or by facilitating ligand presentation to the IGF-I receptor.

Moderate expression of IGFBP-2 mRNA along the lesion track was also detected following the unilateral hypothalamic lesion. This finding is consistent with results from other brain injury models. A significant, precise focal increase in IGFBP-2 protein and mRNA expression has been demonstrated after a penetrating brain injury (Walter, et al., 1997). This increase in IGFBP-2 mRNA was seen in the damaged neural parenchyma, predominately in astrocytes as defined by morphological criteria. Double immunolabeling experiments showed that IGFBP-2 and GFAP were colocalized in hypertrophic astrocytes after brain traumatic injury (Breese, et al., 1996). In a rat cerebral contusion model, IGFBP-2 mRNA expression was dramatically increased in cortical regions adjacent to the injury 24 hours after cerebral contusion, with the peak ten fold increase apparent in most of the ipsilateral cortex 2 and 3 days post-injury. By seven days following contusion, IGFBP-2 mRNA had decreased to moderate levels (Klempt, et al., 1992). In a hypoxia-ischemia rat model, elevated expression of IGFBP-2 mRNA was detected surrounding the infarcted regions three days following unilateral carotid ligation.

Cells types other than astrocytes are also involved in the response to brain injury. In response to hypoxia-ischemia injury, macrophages derived from either activation of microglia locally or by blood borne invasion move into the infarcted areas (Klempt, et al., 1992). Similarly, the IGFBP-2 expression along the lesion track in this study is correlated with activation of local microglia and macrophages invading from the blood following the hypothalamic traumatic injury. The IGFBP-2 might play a role in increasing the availability of IGF-I or IGF-II by recruiting IGFs from the circulatory system, thus enhancing neuronal survival and axonal growth. Moreover, IGFBP-2 could enhance movement of macrophages into injured areas by increasing macrophage motility. My speculation is that activated microglia and macrophages synthesize IGFBP-2 following the injury, although direct evidence is still lacking. However, other cell types known to invade the brain after injury, for example fibroblasts (Katz, et al.,1990), can not be excluded as possible sources of IGFBP-2. We also observed that IGFBP-2 mRNA was expressed in the choriod plexus epithelium. This is consistent with other reports (Logan, et al.,1994; Sullivan and Feldman, 1994). It was suggested that IGFBP-2 may be secreted into the CSF. Thus transport through ventricles and subarachnoid spaces to sites in the brain parenchyma is possible. After a traumatic injury, IGFBP-2 may therefore concentrate in the wound areas from distal as well as local sources (Walter, et al., 1997).

In summary, IGFBP-2 is unique among the IGFBPs in response to CNS injury. It has been reported that all six IGFBPs accumulated in penetrating CNS wounds, but IGFBP-2 predominated (Walter, et al., 1997). Moreover, CNS expression of IGFBP-2 was specifically induced by the cytokines CNTF and Interleukin-1 β (Wood, et al.,

1995), and release of these cytokines may be among the earliest responses to brain injury. No response to these cytokines was observed for other CNS expressed IGFBPs in the CNS, suggesting that IGFBP-2 may have a unique function in recovery from brain injury and in neuronal regeneration or sprouting.

IGF-I Receptor

IGF-I stimulates neural sprouting or increases neural survival via binding to IGF-I receptors on the target neurons (Leroith, et al., 1993a). Our results showed that IGF-I receptor mRNA was not detectable in the PVN and SON of intact animals. This result is different from the report on in situ hybridization for IGF-I receptor mRNA by Aguado (Aguado, et al., 1992). An autoradiographic study also showed that there was a very low level of binding sites for [^{125}I] IGF-I in hypothalamus (De Keyser, et al., 1994). One possible explanation for the difference between my result and Aguado's is the use of different cRNA probes in situ hybridization. Aguado generated a 200bp cRNA IGF-I receptor probe from a 750 bp cDNA representing the 5' untranslated end of the cDNA. The cRNA probe was labeled with ^{35}S-labeled UTP. The IGF-I receptor probe in my experiment was a 265 bp fragment corresponding to the 5' untranslated region, signal sequence and alpha subunit of the rat IGF-I receptor mRNA. The different cRNA probe could have altered the sensitivity of in situ hybridization.

IGF-I receptor immunoreactivity was observed in the neural lobe and median eminence in some animals. The morphology of this staining was consistent with possible localization to magnocellular axons. No change in expression of IGF-I receptor mRNA

or in IGF-I receptor immunoreactivity was detected following the unilateral hypothalamic lesion. Furthermore, in contrast to IGF-I mRNA expression, no IGF-I receptor mRNA expression was detected along the lesion track in the brain. These results were consistent with another report (Walter, et al., 1997) in which no significant change in IGF-I receptor mRNA expression was noted in the cerebral cortex following a stab wound. Apparently, the upregulation of IGF-I receptor mRNA expression may not be necessary following CNS trauma. Our results suggest that very low levels of IGF-I receptor expression in neurons of the MNS might be sufficient to mediate IGF-I actions under both physiological and pathological conditions.

A Model for Effects of the IGF-I System During Axonal Sprouting in the MNS

In situ hybridization studies reported that IGF-I mRNA was located in several areas of the adult CNS, such as the olfactory bulb, hippocampus and cerebellum. For example, IGF-I mRNA were found in the cell bodies of mature Purkinje neurons (Nieto-Bona, et al., 1993). No IGF-I immunoreactivity could be demonstrated in the normal adult cerebellum. Following colchicine injection, IGF-I immunoreactivity was found in cerebellar Purkinje cells but not in neuroglia cells (Andersson, et al., 1988). Our results are consistent with these reports. Based on the literature review and our present results, a model for effects of the IGF-I system during axonal sprouting in the MNS has been hypothesized (Fig. 17). In this model, normal magnocellular neurons synthesize IGF-I, and transport IGF-I inside of axons anterogradely down to axonal terminals and release it in the neural lobe. IGF-I can bind to IGF-I receptors present on

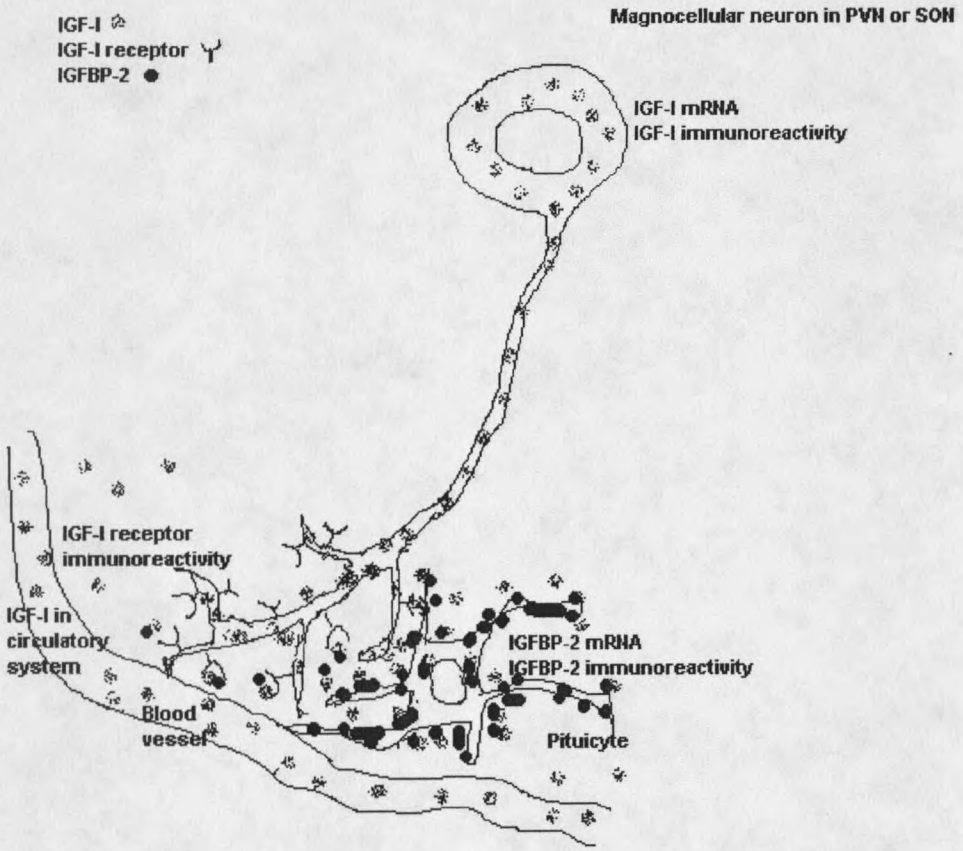


Fig. 17. A model for effects of the IGF-I system during axonal sprouting in the MNS. IGF-I was synthesized in the soma of magnocellular neurons in the PVN and SON, and transported inside of axons anterogradely down to axonal terminals and released in the neural lobe. IGF-I can bind to IGF-I receptors present on the magnocellular axons in the neural lobe. This interaction can be enhanced through IGFBP-2 synthesized in pituicytes of the neural lobe.

the magnocellular axons in the neural lobe. This interaction can be enhanced through IGFBP-2 synthesized by pituicytes of the neural lobe. This autocrine mode of IGF-I action in the magnocellular neurons may be necessary for the high degree of plasticity exhibited by magnocellular axons (Dellmann, et al 1988). Following colchicine injection

into the third ventricle, axoplasmic transport was blocked, causing an accumulation of IGF-I within soma of magnocellular neurons. Following the unilateral hypothalamic lesion, a significantly increased IGFBP-2 mRNA level was detected in the pituicytes. This increase in IGFBP-2 could enhance IGF-I bioavailability by binding IGF-I from the circulatory system or released from magnocellular axons. This passive increase in the local extracellular concentration of IGF-I could increase binding to its receptors on the magnocellular axons in the neural lobe. IGF-I may alter gene expression and stimulate axonal sprouting through signal transduction after binding to its receptors.

IGF-I Polymer Implantation in the Hypothalamus

Implantation of IGF-I in the hypothalamus was employed in an attempt to determine whether exogenous IGF-I was capable of stimulating axonal sprouting and/or neuronal survival in intact animals as well as following the unilateral hypothalamic lesion. The results from lesioned animals implanted with IGF-I polymer or with BSA control polymer were such that it was impossible to determine if IGF-I exerted growth-promoting effects on magnocellular neuron axons, because the hypothalamic lesion caused varying degrees of tissue damage and cavitation in the hypothalamus as a result of slight changes in the position of the knife track. The number of surviving magnocellular neurons was highly variable, and may have depended on the balance between the severity of tissue damage and any neurotrophic effects of IGF-I. The same problem was encountered when we attempted to count the number of magnocellular fibers.

Because of these problems, intact animals implanted with IGF-I, BSA or an empty cannula were employed in the second experiment to address the question of whether IGF-I can stimulate magnocellular axonal growth. Animals that had necrotic tissue around the implant site were excluded from the experimental analysis, because glia and non-neural cells invading necrotic tissue could make numerous factors that could affect axonal growth. We do not know why there were more animals with necrotic tissue in the BSA control group than in the IGF-I implanted group.

The results of this experiment showed that IGF-I implanted animals exhibited increased growth of OT axons compared to animals implanted with BSA or empty cannula. The growing nerve fibers were found along the IGF-I implant track, where nerve fibers exhibited an irregular and tortuous arrangement. This pattern is consistent with the report that new regenerating MNS axons were irregular and tortuous in shape within hypothalamic tissue grafts (Dellmann, et al., 1989). This similarity suggests that the irregular fibers in or around the IGF-I implant site are new growing magnocellular axons. In addition, growth of OT axons toward the ipsilateral anterior cerebral artery was present in all animals implanted with the IGF-I polymer. In contrast, there were no oxytocinergic axons around the contralateral anterior cerebral artery in all animals examined, providing a further demonstration of magnocellular axon growth in response to IGF-I.

These results strongly suggest that the polymer implant resulted in increased levels of IGF-I levels in the surrounding tissue. While it is not surprising that the elevated IGF-I concentration could stimulate axon growth toward the IGF-I implantation rod, it is less clear how the elevated IGF-I concentration released from the

polymer matrix stimulated magnocellular axon growth toward the anterior cerebral artery. Perhaps released IGF-I was bound to perivascular sites. Alternatively, the implant may have stimulated random growth of OT axons which were then attracted to the artery by endogenous growth signals.

The ability of IGF-I implants in the hypothalamus to increase magnocellular axon growth is consistent with the results of other studies employing either local or systematic infusion of IGF-I. It has been demonstrated that local administration of IGF-I, infused for one or two weeks dissolved in buffered saline, significantly increased the rate of regeneration of transected sciatic nerves (Sjoberg J., 1990). Intraventricular injection of IGF-I two hours after hypoxic-ischemic injury reduced neuronal loss and increased nerve fiber regrowth (Guan, et al.1993), and subcutaneous administration of IGF-I resulted in increased neuromuscular junction endplate size (Lewis, et al., 1993).

All negative control animals implanted with BSA polymer matrix or an empty cannula did not show growth of magnocellular processes except for one BSA implanted animal, in which a few processes were seen around the ipsilateral anterior cerebral artery and near the implantation track. This result suggests that tissue damage may have resulted in elevated levels of endogenous IGF-I or other growth factors, possibly secreted by damaged magnocellular neurons.

Concluding Statements

The present studies have described the distribution of IGF-I, IGFBP-2 and the IGF-I receptor in the MNS, and have also documented where mRNAs for these members of the IGF system are located. These results are partially consistent with other reports (Aguado et al, 1993; Aguado et al, 1992; Bach and Bondy, 1992). Increased expression of IGFBP-2 mRNA was shown to correspond with axonal sprouting spatially and temporally in the neural lobe. IGFBP-2 may modulate potential effects of the IGFs by targeting either one or both of the IGFs to magnocellular neurons where they could serve to stimulate axonal sprouting. In order to directly test the hypothesis that IGF-I stimulates collateral sprouting of axons in the MNS, implantation of IGF-I polymer in the hypothalamus was employed to examine the extent of axonal growth. The IGF-I implant did increase growth of magnocellular axons in the MNS. This result strongly suggests that IGF-I might act as an endogenous growth factor which acts to stimulate collateral axonal sprouting in the MNS following the unilateral hypothalamic lesion.

The mechanisms of collateral sprouting in the CNS are far from understood. The finding that IGF-I in conjunction with other members of the IGF system may be involved in axonal sprouting by magnocellular neurons provide additional insights into this process. These results also lay the foundation for further investigations designed to determine whether IGF-I plays an essential role in axonal sprouting in the MNS.

A greater understanding of IGF-I's effects on axonal sprouting has important implications for the potential of IGF-I as a therapeutic agent for treatment of

neurological diseases and brain injury. In the PNS, IGF-I has been used to slow the progression of amyotrophic lateral sclerosis (ALS), a chronic progressive disease that results in the selective destruction of spinal cord and cortical motor neurons (Dore et al,1997). In the CNS, IGF-I and its receptor are distributed in some specific regions (Bondy et al 1992. Garcia-Segura et al, 1991). Neurons in these regions may therefore be sensitive to the growth-promoting and neuroprotective effects of IGF-I. The results presented in this thesis add to the growing body of knowledge that may ultimately determine the potential usefulness of IGF-I in the treatment of brain trauma and neurodegenerative diseases such as Alzheimer's and Parkinson's diseases.

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