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

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.