ADVANCES IN
NEURAL SCIENCE

Editor: SUDARSHAN K. MALHOTRA

Volume 2 1995

ADVANCES IN NEURAL SCIENCE

Volume 2 • 1995

DEDICATION

The second volume in the series on Advances in Neural Science is dedicated to Dr. Gopal D. Das who passed away suddenly on December 27, 1991. Many of the contributors in the volume were personal friends of Gopal and we knew him as a hard-working, conscientious scientific worker, who was always available and ready to help. He will be missed dearly.

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VOLUME 2 • 1995

JAI PRESS INC.

Greenwich, Connecticut *London, England*

55 Old Post Road, No. 2
Greenwich, Connecticut 06836

JAI PRESS LTD.
The Courtyard
28 High Street
Hampton Hill, Middlesex TW12 1PD
England

ISBN: 1-55938-625-8

Manufactured in the United States of America

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ACKNOWLEDGMENTS

Mrs. Arlene Cowan has been meticulous in handling the secretarial tasks for which I am most grateful. Mrs. Kamini Malhotra has provided valuable editorial assistance.

It has been a pleasure to work with the production staff, particularly, Mr. Fred Verhoeven, Production Editor, at JAI Press.

Sudarshan Malhotra

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EARLY APPEARANCE OF NEUROPEPTIDES DURING DEVELOPMENT:
NEUROMODULATORY AND NEUROTROPHIC ROLES

Susan Kentroti and Antonia Vernadakis

Advances in Neural Science,
Volume 2, pages 1–46.

ISBN: 1-55938-625-8

ABSTRACT

The reviewed literature presented here establishes that most if not all neuropeptides are present during early neuroembryogenesis. Moreover, the early appearance of receptors for these neuropeptides leads to the interpretation that these substances, in addition to their presumptive neurotransmitter function, have a neuromodulatory role in the developing nervous system. Although there is considerable evidence for the colocalization of neuropeptides, and neuropeptides with classical neurotransmitters in the adult, these attributes during early development are only recently attracting attention. However, the evidence that exogenous application of neuropeptides influences neuronal survival, phenotypic expression, and differentiation demonstrates that these substances play an important role in regulating neuronal plasticity during early neuroembryogenesis.

I. INTRODUCTION

It has been established that various neurotransmitter substances including acetylcholine (Ach), gamma-aminobutyric acid (GABA), 5-hydroxytryptamine (5-HT, serotonin) are present early in neurogenesis and play a role in early neuronal differentiation and maturation. An extensive and elegant review on this field has been recently published by Buznikov (1990). Recent findings have established that various neuropeptides including those originally thought to play only a hypothalamic-pituitary role are present in both the central and peripheral nervous systems and have been attributed with neurotransmitter properties. In this review we attempt to present information on the presence of neuropeptides

during neuroembryogenesis and offer the possibility that their colocalization with classical neurotransmitters makes them active partners in neuronal phenotypic expression and neuronal growth. Patterns of developmental expression of brain peptides delineate three major categories. Neuropeptides in the first category appear early in embryogenesis, undergo a marked increase around birth, and achieve maximal expression in the young animal. Neuropeptides in the second category first appear late in gestation and increase markedly after birth. In the third category, neuropeptides appear perinatally, increase to a maximum early in the postnatal period, but then decrease dramatically toward maturity. In the last category somatostatin (SRIF), vasoactive intestinal peptide (VIP), substance P (SP), neuropeptide Y (NPY), and neurotensin (NT) are primary examples. The transient appearance of these chemical messengers has led many investigators, including us, to suggest that they may play a leading role in the organization of the developing brain. The last section in this review will focus primarily on the trophic role of neuropeptides during early neuroembryogenesis.

Although there are several neuropeptides now implicated in CNS function, there are only a few that have been studied systematically throughout ontogenesis. In this review we chose to discuss only those for which a reasonable amount of data exists. Since the neuropeptide field is developing very rapidly, however, we expect that additional publications have appeared by the time of publication. In the interest of thoroughness, we have given a brief historical account of each of the neuropeptides reviewed. Finally, we have attempted to review information derived from various animal species including, whenever available, humans. Such interspecies comparison has provided information on the broad spectrum of neuropeptides ranging from neurotransmitter function to providing neurotrophic signals.

II. ONTOGENESIS

A. Somatostatin

A factor that inhibited the synthesis and release of growth hormone was first reported by Krulich and co-workers (1968) and was called growth hormone inhibiting factor. Subsequently this factor was isolated from sheep hypothalamic extracts, purified and sequenced, and named somatatropin-release inhibitory factor or SRIF (Vale et al., 1972; Brazeau et al., 1973; Burgus et al., 1973). Lauber and colleagues (1979), using gel chromatography and recombinant DNA, demonstrated that the tetradecapeptide form of SRIF was synthesized from large-molecular-weight precursors via various intermediate forms. SRIF has been localized in the stomach, intestine, and pancreas (Arimura et al., 1975; Dubois, 1976) and in various areas of the CNS of several species, including humans, with particularly high concentrations occurring in the brainstem, hypothalamus, and neocortex (Bugnon et al., 1977; Hökfelt et al., 1974; Brownstein et al., 1975; Kobayashi et al., 1977; Bennett-Clarke et al., 1980; Finley et al., 1981; Cooper et al., 1981; Shiosaka et al.,

1981a; Morrison et al., 1983; Johansson et al., 1984; Braak et al., 1985; Schoenen et al., 1985; Sorensen, 1982; Vincent et al., 1982; Mizukawa et al., 1987; Bouras et al., 1987; Amaral et al., 1988; Carlos Davila et al., 1988).

Cerebrum

Recently, Yamashita et al. (1989) have reported an immunohistochemical study on the ontogeny of SRIF in the cerebral cortex of the macaque monkey. Embryonic days 120 (E120) and E140 and newborn and postnatal day 60 (P60) were examined. SRIF-immunoreactive cells are observed from E120; the cell number increases between E120 and E140 and decreases until P60. The higher distribution of SRIF-immunoreactive cells at the early developmental stages than in the adult is reported in most areas of the cortex examined. As will also be discussed in a later section, the early presence and high number of these peptidergic neurons support the overwhelming view that peptides play a significant role in early cell–cell interactions that are important during development.

Extensive studies have been reported on the ontogeny of SRIF-containing neurons in the rat brain (Shiosaka et al., 1981a,b, 1982; Hoffman, 1980; Laemle et al., 1982). Parnavelas and colleagues (Parnavelas, 1986; Cavanagh and Parnavelas, 1988; Eadie et al., 1987) have published cellular and ultrastructural features of SRIF-immunoreactive neurons in the rat between E17 and P32. SRIF-immunoreactive neurons are observed in the rat occipital cortex at E17 with virtually no neurons being produced by E21. During the last stages of gestation and in early postnatal life SRIF-labeled cells are concentrated in the subplate region in the border of the marginal zone and cortical plate. Already at this stage, some cells exhibit considerable accumulation of organelles in the cytoplasm, including endoplasmic reticulum, which is organized in parallel arrays. Of interest is the observation that stained neurons in the superficial cortical layer are not present in this region after the perinatal period and during most of the first two postnatal weeks. Transient populations of peptide-positive neurons in the rat neocortex have also been reported (Chun et al., 1987; Foster and Schultzberg, 1984; Woodhams et al., 1985). There is some disagreement in the literature concerning the postnatal development of SRIF. Some radioimmunoassay (Ghirlanda et al., 1978) and immunohistochemical (McDonald et al., 1982a,b) studies suggest a continuous increase in cortical SRIF-immunoreactivity in the rat until adult levels are reached. Other studies (McGregor et al., 1982; Shiosaka et al., 1982) have found a rapid increase in SRIF-immunoreactivity during the first two postnatal weeks, with adult levels exceeded in the third postnatal week. A gradual decline in SRIF-labeled cells after reaching a peak at P10 days has also been reported in the rabbit visual cortex (Ramon et al., 1985). Similar findings have been reported in the rat dorsal hippocampus and dentate gyrus using *in situ* hybridization and immunohistochemistry (Naus et al., 1988a,b).

In more recent studies Forloni et al. (1990) and Bendotti et al. (1990) describe in detail the developmental expression of SRIF in the BALB/c mouse using both immunocytochemistry and *in situ* hybridization technology. SRIF-immunoreactiv-

ity is first discernible in the hypothalamus at P3 and reaches the adult level by P30. In the limbic system, SRIF-immunoreactivity is detectable at birth and in all other structures of the forebrain distribution, density, and morphology of immunoreactive neurons evolved over the following 2–3 weeks. By using *in situ* hybridization, preprosomatostatin mRNA can be detected at day E14 and E17 and is highest in neurons of the pyriform cortex, amygdala, and entopeduncular nucleus, whereas the signal is low in cells of the neocortex and hippocampal formation. In the diencephalon, high signal expression occurs by P21, whereas in the hypothalamus, high grain densities are detected as early as postnatal week 1. Based on these recent findings, Bendotti et al. suggest that the ontogenic expression of preprosomatostatin in the striatum could indicate that the message is not quantitatively processed into peptide or that ontogenic changes occur in the SRIF peptide in the BALB/c mouse. Further, studies from our laboratory have demonstrated the presence of pluripotential neurons in cultures derived from chick embryos at a very early stage of development (embryonic day 3) in which SRIF immunoreactivity is colocalized with markers for cholinergic, catecholaminergic and GABAergic neurons (Kentroti and Vernadakis, 1995). All studies agree with the view that the decline in SRIF levels by adult age suggests that SRIF plays an important role during early neuronal maturation.

Studies in the reeler mouse by Naus and Bloom (1988) further establish that SRIF is fundamental in the neuronal circuitry. In the developing reeler mouse neocortex, the equivalent of the normal layer I (external plexiform zone) is present as the intermediate plexiform zone between the upper and lower tiers of the cortical plate (Caviness, 1977). Despite the absence of a definite layer I in the mouse, a dense population of SRIF-immunoreactive fibers is still present immediately below the pial surface. However, the SRIF system does not merely develop in an inverted pattern; it also appears that SRIF neurons are less developed than in normal cortex.

Immunofluorescence studies of dissociated cell culture of 15-day-old rat fetal cerebral hemispheres have demonstrated the presence of SRIF in an exclusive neuronal population (Delfs et al., 1980). In addition, Louis et al. (1983) have reported that in neuron-enriched cultures derived from 8-day-old chick embryo cerebral hemispheres large quantities of SRIF-immunoreactive substance are produced and secreted into the medium. These findings suggest that neurons of the mammalian cerebrum can produce SRIF completely independently of the hypothalamus.

Cerebellum

Early appearance of SRIF-containing neurons has been reported in the cerebellum of rats (Naus, 1990; Inagaki et al., 1989) and primates (Yamashita et al., 1989, 1990a,b). *In situ* hybridization histochemistry has revealed numerous hybridizing neurons for SRIF mRNA from P1 to 9 days in the rat (Inagaki et al., 1989). Although the intensity of the signals for SRIF mRNA in the cerebellum decreases with age (Inagaki et al., 1989; Naus, 1990), the cerebellum of adult rats still contains a significant number of labeled cells in the granular cell layer that appear to correspond to Golgi cells. Since Golgi cells have been thought to contain GABA, the possibility that SRIF coexists with GABA (and other neuropeptides) in the Golgi

cells is raised and further suggests a neurotransmitter modulatory role of this neuropeptide. The coexistence of neuropeptides and neurotransmitters is discussed in more detail in a later section.

In the primate cerebellum somatstatin-like immunoreactivity (SLI) has been detected at E120 (Yamashita et al., 1989). SLI Purkinje cells, Golgi cells, and a few cells in the molecular layer have been detected during the early embryonic stages, up to E140. At the newborn stage and up to P60, immunoreactivity decreases in the external granular layer and the number of immunoreactive fibers increases in the white matter. As in other species, very little neuropeptide-immunoreactivity is observed in the cerebellum of the adult primates. In a subsequent study, the same authors reported that the expression of SRIF mRNA was highest at E120, a time at which the external granule cells are still migrating (Hayashi et al., 1990).

Olfactory Bulb

SLI has been reported to be present in the rat olfactory bulb at P1 (Matsutani et al., 1988). The number of these neurons increases slightly with age and reaches an adult-like distribution by day 15. The presence of SLI has also been reported in the aged human olfactory bulb (Cooper et al., 1981; Bouras et al., 1986; Ohm et al., 1988a,b).

Hypothalamus

The ontogenic development of the hypothalamic neuroendocrine system has been the subject of a number of detailed studies. Different parameters have been evaluated, such as axonal outgrowth, morphological differentiation of the neurons, and the appearance of specific secretory peptides. The ontogeny of hypothalamic SRIF gene expression has been recently reported in the rat (Almazan et al., 1989). SRIF mRNA is detectable at E14 and reaches 40% of adult levels by E18. The ontogeny of SRIF mRNA expression appears to coincide with the final mitotic events in the generation of diencephalic SRIF cells, which occur during the period from E12 to E16, with the bulk of SRIF neurons being formed at days E14 and E15 (Hoffman, 1980). An analysis of SRIF cells in the periventricular hypothalamus in 4- to 6-month-old rats that had received ^{3}H-thymidine on fetal days 12, 13, 14, 15, or 16 reveals that some neurons appear on each of these days, with the greatest number of labeled cells seen in those animals injected on fetal days 14 and 15. The developmental expression of the SRIF gene has also been reported in rat brain and stomach by Zingg et al. (1984). A single band of mRNA that hybridized specifically to the rat SRIF cDNA has been detected in both tissues examined throughout ontogenesis, suggesting that the same SRIF gene is expressed in these tissues in the developing as well as in the adult rat. Whereas SRIF mRNA is undetectable in fetal stomach and rises gradually only after birth, brain SRIF mRNA is already detectable by E7 and reaches concentrations corresponding to those in the adult brain by E20. This difference in the SRIF mRNA profiles may be related to the onset of functional activity in these two organs. An early report by Aubert et al. (1977) shows

immunoreactive SRIF to be present in the human fetal hypothalamus and cerebral cortex in the 10th to 22nd week gestational period, although hypothalamic immunoreactivity appears to be weak. Based on adult concentrations, it has been estimated that the hypothalamus of the human embryo contains 30-fold less SRIF than the adult.

Spinal Cord

The localization and distribution of SLI have been studied in postmortem human fetus and infant spinal cord and dorsal root ganglia (Charnay et al., 1987). Immunoreactivity is detected as early as E9, with a progressive increase in the number of SLI fibers within most of the gray area from the early to the late fetal and infant stages. Although the distribution patterns of SRIF-immunoreactive structures appear similar throughout the entire spinal cord, the highest density of immunolabeled fibers is seen at the lumbosacral level. Thus, based on these findings SRIF-containing neurons in the spinal cord appear earlier than those in the hypothalamus (see references above), again emphasizing the neuromodulatory role of this peptide during early CNS development.

Detailed studies on the ontogeny of somatostatinergic neurons in the spinal cord and spinal ganglia have been reported by Senba et al. (1982) and Ho (1988). SRIF-immunoreactivity is detected as early as E12 and appears to correspond with the birth dates of cells in those regions. Again, the observation that SRIF expression decreases during later development (Ho, 1988) supports the notion of a neuromodulatory role for this peptide early in development.

Of interest are recent reports on neuropeptide expression in intraocular spinal cord grafts (Henschen et al., 1988). Intraocular transplantation is a versatile approach in studying nervous system development and regeneration processes. Growth and development of defined areas of isolated CNS tissue can be followed by removing fetal brain or spinal cord from its normal milieu and placing it in the interim chamber of the eye. In the study reported by Henschen et al. (1988), fetal rat spinal cord was grafted to the anterior chamber of the eye in adult rats. After intraocular maturation for 2–3 months the amount and distribution of several neuropeptides, including SRIF, NPY, SP, enkephalin, VIP, peptide histidine-isoleucine, calcitonin gene-related peptide, and cholecystokinin immunoreactive terminals, and cell bodies were analyzed. Whereas all of these peptides are detected, the degree in the expression of some peptides is disturbed compared to *in situ* spinal cord, suggesting a lack of normal spinal connections or that target input is an important factor in peptide expression.

Spinal, Sensory, and Sympathetic Ganglia

In the rat spinal ganglion, SRIF-positive cell bodies appear at E15 and increase in number after E18, with 15% of the ganglion cells showing immunoreactivity on the P7–P10. Only a few SRIF-positive cells are detected in the adult rat (Senba et al., 1982).

During embryogenesis, neural crest cells migrate along definite pathways and differentiate into various types of neuronal and non-neuronal tissues, including most of the peripheral nervous system (Le Douarin, 1980, 1982). Various neuropeptides are found to be synthesized by neurons derived from the neural crest. Studies by Garcia-Arraras et al. (1984) and Maxwell et al. (1984) have shown that in quail embryos, SLI appears between days 3 and 4 of incubation in sympathetic ganglia immediately following ganglion formation and between days 4 and 5 of incubation in the adrenal gland, soon after the adrenal gland primordium first appears. Thus the appearance of SLI parallels the differentiation of neural crest into autonomic sympathetic ganglion cells. It is important to note that the amount of SLI in the adrenal gland increases while the amount of SLI in the sympathetic ganglia decreases. Moreover, this time of SLI appearance coincides with the appearance of catecholamines (Kirby and Gilmore, 1976) and raises the possibility that some of the same environmental cues may affect the regulation of both traits or those expressed in the same cells (see later section on regulatory mechanisms).

Retina

The retina has been a site of study of neurotransmitters and neuropeptides because of its accessibility and relatively simple anatomy. Tornquist et al. (1982) have described SRIF (and vasopressin (VP)) neurons in several species. In the rat, SRIF-immunoreactivity is present as early as E15, and by E16 levels are 10 times higher than in the adult retina. SRIF mRNA is also reportedly high at E16 in the rat retina and progressively declines by P12 (Ferriero et al., 1990). In the chick embryo SLI can first be detected around day 7 *in ovo*, increases rapidly between days 9 to 11 (a period during which ganglion cells, displaced amacrine cells, bipolar cells, and amacrine cells elaborate their dendrites) and again between day 18 to 19, a period of final development of photoreceptor elements (Morgan et al., 1983). SLI in the guinea pig retina has first been detected during the 6th week of gestation (38 days), declining between days 38 and 50 of gestation and rising again between days 50 and 68 (birth) (Spira et al., 1984). During the late fetal stages, photoreceptors have well-developed outer segments, and amacrine cell synapse formation is at its peak (Fry and Spira, 1982). SLI has also been reported in the adult human retina (Rostad, 1980).

B. Vasoactive Intestinal Polypeptide

VIP is a peptide that was isolated from porcine intestinal mucosa by Said and Mutt in 1970. It is widely distributed in mammals, not only in the gastro-enteric tract (Polak et al., 1974; Said, 1985) but also in the pancreas (Polak and Bloom, 1980), the nervous system (Larsson et al., 1976; Loren et al., 1979), the urogenital tract (Fahrenkrug, 1985), and the lung (Said, 1985). Very few data are available up to the present concerning the time of appearance and differentiation of VIP neurons during embryonic development.

Cerebrum

In early studies Emson et al. (1979) and McGregor et al. (1982) described the development of VIP in the cerebral cortex of rats during the first 3 weeks after birth (0, 7, 14, and 21 days), a period of active development of various forms of nonpyramidal neurons in the visual cortex of the rat (Parnavelas et al., 1978). McDonald et al. (1982a,b) have subsequently studied in more detail the morphology and distribution of VIP-containing neurons between the first postnatal day and adulthood. VIP-immunoreactive cells are first observed in layers V and VI at day 4, and by day 8 labeled cells are predominantly in layers II and III. Although the distribution of immunoreactive cells at this time resembles that of adult animals, their morphology displays immature features. The size and extent of their dendritic branching increase considerably during the second and third weeks and morphological maturation is attained by the middle of the fourth postnatal week.

Significant differences have been observed in the ontogeny of VIP neurons in the rat occipital cortex compared to SIRF (Cavanagh and Parnavelas, 1989). Most VIP neurons are generated at E19. Although a decline in VIP-immunoreactive neuronal somata is observed after the third week postnatally, VIP-immunoreactive dendrites are still visible, suggesting a reduction in VIP levels in the cell bodies and not a loss of VIP neurons. Thus, it appears that VIP neuron production increases as SRIF neuron production declines. It has been suggested that there may be a population of nonpyramidal neuron precursors that at first produce SRIF neurons, followed by VIP neurons with a very small number of cells that contain both peptides (Papadopoulos et al., 1987) being generated at the switchover (Cavanagh and Parnavelas, 1989). More recently, Hajos et al. (1990) have also reported VIP-containing cells in the developing rat occipital hemisphere. In the subventricular zone the VIP cell population is markedly reduced by P8, but isolated clusters of VIP cells persist into adulthood. Parallel to the reduction in number of the subventricular VIP cells an increasing number of VIP cells appear in the neo- and allocortex, developing, by P12, all of the features of the mature cortical bipolar fusiform neurons. The authors suggest that cells from the subventricular zone may migrate to the cortex, the subventricular VIP cells being the precursors of all cortical VIP neurons. VIP-containing neurons have also been described in organotypic slice cultures from rat visual cortex (Gotz and Bolz, 1989). The first VIP-positive cells are present after 5 days *in vitro*, gradually increase in number up to the middle of the second postnatal week, and show morphological maturation until about the fourth week in culture. These findings suggest that postnatal expression of VIP occurs in a culture system without thalamocortical afferents thought to play a role in the final differentiation of cortical neurons (Hamre et al., 1989).

Spinal Cord

The ontogeny of VIP-containing neurons has also been examined in dissociated cultures of embryonic mouse spinal cord and dorsal root ganglia (Eiden et al.,

1988). VIP levels rise from less than 1 to 30 pg/culture over 26 days and reach the half-maximal level by day 9. VIP immunoreactivity is visualized both in neurons with extensively branched processes and in bipolar cells, some of which appear to be dorsal root ganglion cells.

Gut

The fact that the intrinsic neurons of the gut are the site of various neuropeptide immunoreactivities was first established in 1978 by Schultzberg et al. using cultures of the intestinal primordium of mouse embryos. In a series of studies Le Douarin and colleagues have shown that quail neuroblasts implanted into chick embryos (and vice versa) develop adrenergic and cholinergic properties (Le Douarin and Teillet, 1974; Le Douarin et al., 1975). This group, using this quail-chick chimeric embryo (Fontaine-Perus et al., 1982), have found more recently that when the adrenomodullary crest cells are transplanted into the gut, the distribution of SP- and VIP-positive neurons occurs according to the normal gut pattern (i.e., VIP and SP are present in neurons belonging to the same ganglia). Moreover, they found that SP- and VIP-immunoreactivity occurs in all enteric ganglia and is detected as early as 9 days in the foregut.

The presence, distribution, and development of VIP-like immunoreactivity has also been investigated in the gastro-entero-pancreatic system of a cartilaginous fish, *Scyhorhinus stellaris* (Tagliafierro et al., 1988). Intestinal VIP-like immunoreactive endocrine cells are already present in the first developmental stage considered (embryos aged 4 months). They grow in number and before birth reach a frequency higher than in adults. In contrast, nerves and cell bodies showing VIP-like immunoreactivity appear later, before birth in this fish, and only after birth are their distribution and frequency similar to those found in adults. Similar findings have been reported by El-Salhy (1984) in the gastro-enteric tract of *Squalus acanthias*. On the other hand, Holmgren and Nilsson (1983), using a C-terminal VIP anti-serum, could only detect immunoreactive nerve fibers and nerve cell bodies, but no immunoreactive endocrine cells. The authors suggest that at least two VIP-like substances are present in the gastro-entero-pancreatic system of *S. stellaris*. It has further been suggested that VIP-like substance acts as a neurotransmitter on the non-VIP-immunoreactive ganglion cells localized in the gut wall.

C. Substance P

SP was originally detected in equine brain and intestine by Von Euler and Gaddum in 1931. This endecapeptide was isolated from the hypothalamus and characterized by Chang and Leeman in 1970–1971. Immunocytochemical studies have demonstrated an SP-containing neuronal system in the hypothalamus (Pernow, 1983; Langevin and Emson, 1982), and the peptide has been localized to cells of the anterior pituitary gland as well (DePalatis et al., 1984; Morel et al., 1982).

Cerebrum

The distribution and ontogeny of SP-immunoreactive structures has recently been investigated in various areas of macaque monkey cerebrum at E120, E140, newborn and P30, P60, and adult stages using immunocytochemistry (Yamashita et al., 1990b). SP-immunoreactivity is detected at E120, and the cell number increases until the newborn stage. After the newborn stage the number of immunoreactive structures decreases, and by P60 the distribution patterns of SP-immunoreactive structures has reached adult levels. The highest density of immunoreactivity is observed in striate cortex and precentral motor cortex fibers and terminals, in spite of low numbers of cell somata. While in the association areas (prefrontal cortex, superior temporal cortex, and parietal association cortex), a larger number of cells expressing immunoreactivity is noted up to the newborn stage followed by a decrease by P60. Among the association cortices the temporal cortex exhibits the largest number of cells in layer V. It is assumed that these SP-immunoreactive cells are developing pyramidal cells. As discussed repeatedly in this review for other peptides, the transient presence of high SP is thought to play a role in early neuronal development.

The fetal ontogeny of SP-containing neurons has been described in rats both morphologically (Inagaki et al., 1982; Pickel et al., 1982; Sakanaka et al., 1982) and biochemically (Gilbert and Emson, 1979). Immunohistochemical studies in rats have defined the initial appearance of SP-like immunoreactivity (SPLI) in both epithalamus and isolated areas of the lower brainstem at E14. The development and localization of SP have been reported in the nucleus of tractus solitarius in the medulla oblongata of human fetuses (Yew et al., 1990). SP neurons are localized in the commissural and medial subnuclei at a gestation age of about 13 weeks. Positive SP fibers appear even earlier, around 11 weeks of gestation in many subnuclei, notably the medial intermediate, ventral, ventrolateral, and dorsolateral subnuclei. Radioimmunoassay data have confirmed that SP is present in measurable amounts in the 15-day-old fetal rat brainstem (Gilbert and Emson, 1979; McGregor et al., 1982). SPLI is first detected in central perikarya at E15 and by E18 in peripheral sensory neurons (Pickel et al., 1982).

In the mouse brain initial expression of SPLI has been detected at E12 as reported in a detailed study by Ni and Jonakait (1988a,b). SPLI has been routinely detected 1 to 4 days earlier in murine brain nuclei than in rat, including the amygdala, bed nucleus of the stria terminalis, nucleus of the diagonal band, septal region, hippocampus, hypothalamus, brachium of the inferior colliculus, the interpeduncular nucleus, tegmental nuclei, central gray, vestibular nuclei, medullary raphe, and spinal cord. In contrast, SP perikarya in the nucleus accumbens, habenula, and spinal trigeminal nucleus make only a late and relatively feeble appearance in mice. Most noteworthy again is the decline postnatally in perikaryal staining in several groups, including those in the basal forebrain, hypothalamus, and medulla. As will also be discussed in a later section, the decline in peptide content appears to coincide

with the loss of SP receptors, as reported in the spinal cord by Charlton and Helke (1986).

The ontogeny of peptides in the chick has not been systematically investigated. The presence of SRIF, enkephalins, and SP has been detected in cultured neurons derived from 8-day-old chick embryo cerebral hemispheres (Louis et al., 1983). The development of SPLI has also been studied in the *Xenopus* embryonic nervous system (Gallagher and Moody, 1987). In general, the populations of the frog embryos that exhibit SPLI appear to be associated with sensory systems, especially visceral and somatosensory ganglia of the head and trunk. Within the embryonic brain only two populations of SPLI neurons are observed: hypothalamic cells and large plate neurons in the rhombencephalon. At spinal levels only Rohon-Beard neurons, interneurons, and extramedullary neurons exhibit SPLI. Of importance is the finding that in the Rohon-Beard population, the cells display SPLI several hours after initial axon growth and shortly after target contact (skin covering).

Spinal Cord

An extensive body of literature exists on the localization of SP in the spinal cord in several animal species, including humans. In the human, SP varicosities are apparent as early as the fifth week at the dorsolateral mantle and marginal layers of the spinal cord (Luo et al., 1988). Thus, SP positivity is already present at a time when the neural tube initially closes and the dorsal ganglion is just formed by the crest cells. This observation is also confirmed in the rat, where the presence of SP has been detected in the dorsal horn by gestation days 15 to 18 (Pickel et al., 1982).

Again, as also shown for other CNS areas, SP-immunoreactive cell bodies that are detected prenatally in the spinal cord of the rat decrease drastically at birth, but SP-positive fibers continue to increase postnatally (Senba et al., 1982). The decrease in content is also reflected in decreased SP binding sites (receptors) as a function of age (Charlton and Helke, 1986).

SPLI neurons have been detected in dissociated cell cultures prepared from E13 to E15 mouse spinal cords and dorsal root ganglia (Neale et al., 1982). SPLI appears to be localized within a small percentage of rounded or multipolar neuronal somata and in varicose processes: multipolar neurons derive probably from spinal cord, whereas the small rounded neurons are primarily of ganglion origin. In contrast, large dorsal root ganglion neurons are not reactive. This culture model system provides the opportunity to study some pharmacological and electrophysiological aspects of peptidergic neurons and their relation to neurotransmitter function.

Sensory and Sympathetic Ganglia

In the dorsal root ganglia (DRG), the population of primary sensory neurons derive from the neural crest. Neural crest cells migrate early to and proliferate in the DRG anlage at defined stages of embryonic development (Hamburger and Levi-Montalcini, 1949; Le Douarin, 1982). In the chick DRG, various subpopulations of primary sensory neurons have been identified either *in situ* (Fontaine-Perus

et al., 1985; Philippe et al., 1985) or in culture (Barakat et al., 1986) by combining cytological, immunocytochemical, and cytoenzymatic characteristics. SP-immunoreactive sensory neurons have been reported in dissociated cultures of chick DRG (Barakat and Droz, 1987). As will also be discussed in another section, during differentiation of neural crest-derived neurons the environment can play a critical role in determining neurotransmitter choice (see the review in Patterson, 1978; Le Douarin and Teillet, 1974). As for sensory neurons, the question is also raised as to whether the SP-positive and SP-negative phenotypes could be regulated to a certain extent by environmental factors.

In the rat spinal ganglion, SP-positive cells first appear on E17 and gradually increase in number, especially after E20, reaching the minimum number on postnatal days 5–7. After P10, SP-positive cells in the spinal ganglia gradually decrease in number, and none or only a few can be detected in adult rats (Senba et al., 1982).

D. Neurotensin

NT is a tridecapeptide widely and heterogeneously distributed in the adult mammalian central nervous system (Jennes et al., 1982; Mai et al., 1987; Makino et al., 1987). The role of this peptide as a neurotransmitter has been demonstrated in several regions of the brain (Pinnock, 1985; Herbison et al., 1986; Behbehani et al., 1987).

Cerebrum

Recently the distribution of NT in the human hippocampus with age has been reported by Lotstra et al. (1989). They observed that different neuronal structures containing NT are detected immunocytochemically during postnatal brain growth from birth to 4 years and include pyramidal cells of the subiculum and presubiculum, granular cells of the hilus, and varicosities of the mossy fibers among the pyramidal cells of the CA3 and CA2 subfields of the Ammon's horn. Of importance is the observation that after 4 years of age only the varicosities in the Ammon's horn exhibit NT immunoreactivity. This diminution with age of the density of NT-containing neurons appears to be a common phenomenon with peptidergic neurons, and as is discussed later in this review, it supports the neuromodulatory role of peptides during early development.

Spinal Cord

In contrast to the detection of several other neuropeptides (SRIF, SP) in the fetal rat spinal cord, no NT positive cells have been detected in the fetal rat; they first appear in the newborn (Senba et al., 1982), increase in number, and reach a maximum during P7–P10. After 10 days NT-positive cells decrease slightly in number, but a considerable number of positive cells are identified in the adult rat.

Olfactory Bulb

NT immunoreactive neurons have been detected in the primordium of the external plexiform or mitral cell layers of the olfactory bulb in rat at E16 to E18 (Matsutani et al., 1988). The number and immunoreactivity of these neurons are greatest by P1 and then decrease with age. Thus as in other brain regions, the function of NT appears to be specific to the development of the nervous system.

E. Vasopressin

VP is synthesized and released by the hypothalamo-neurohypophyseal system, and its peripheral actions have been well described (Pickering et al., 1975). The presence of VP in the adult brain has also been reported (Buijs, 1978; Buijs and Swaab, 1979). More recently, VP has been localized in the developing brain. Radioimmunoassayable VP is found in the rat brain as early as E14 (Sinding et al., 1980a,b). The presence of VP (Boer et al., 1980) appears to coincide with the generation of the neurons of the VP-synthesizing nuclei (supraoptic, paraventricular, and suprachiasmatic) (Anderson, 1978; Ifft, 1972). The levels of hypothalamic VP mRNA rise during gestation and lactation in the rat (Zingg and Lefebvre, 1988). Recent studies by Bloch et al. (1990) using *in situ* hybridization show that VP mRNA can be detected as early as day 16 of rat fetal life with the radioactive probe and day 17 with the biotinylated probe. The first reactive neurons are expressed in the supraoptic nucleus at E16 and in the paraventricular nucleus at E17, and by P3 the staining of neurons in the two nuclei appears similar. The mRNA is located in the cytoplasm of neuronal cell bodies but also in processes both in adult and during ontogeny.

F. Growth Hormone-Releasing Hormone

Although there is considerable information on the distribution of GHRH in the adult human, monkey, and rat (Bloch et al., 1983, 1984; Jacobowitz et al., 1983; Merchanthaler et al., 1984), little information has been reported on the ontogeny of the GHRH neuronal system.

The first appearance of hypothalamic GHRH immunoreactive structures in the human fetus is between 18 and 29 weeks of gestation (Bloch et al., 1984; Bresson et al., 1984). In this context it is of interest that the appearance of growth hormone-producing cells and the presence of growth hormone in blood have been detected at the eighth week of fetal life (Kaplan et al., 1976). In the rat, immunoreactive nerve terminals, but not perikarya, have first been detected in rat hypothalamus at E20 (Gennaro et al., 1986). The number of immunoreactive nerve terminals increases markedly by P5, with a moderate increase at P10 and P20. Age-related changes in GHRH in the rat hypothalamus have also been reported by Morimoto et al. (1988). Rodier et al. (1990) have more precisely determined when GHRH cells are formed in the rat hypothalamus. They report peak proliferation of cells

between E13 and E15 from anterior to posterior hypothalamus, with no birthdays noted by E17.

G. Gastrin-Releasing Peptide/Bombesin

GRP with 27 amino acid residues has been isolated from the porcine gastrointestinal tract (McDonald et al., 1978), and the carboxy terminal portion of this peptide has been found to be identical to the tetradecaptide bombesin, which was originally isolated from the skin of the European amphibian *Bombina* (Anastasi et al., 1971). The presence of bombesin in brain synaptosomes was reported early in 1979 (Brown and Vale, 1979). Yanaihara et al. reported in 1981 a widespread distribution of GRP-like immunoreactivity in the gastrointestinal tract and brain of the pig. GRP/bombesin-like immunoreactivity has been reported to be present in the gastrointestinal tract of the guinea pig (Roth et al., 1983), rat (Dockray et al., 1979), and human (Price et al., 1984). A characteristic regional distribution of GRP/bombesin immunoreactivity in discrete nuclei of the hypothalamus of the rabbit has been reported recently by Kita et al. (1986). Moreover, concurrent localization of GRP/bombesin-like immunoreactivity and SRIF in some parts of the hypothalamus has been interpreted as a possible interaction of these peptides in these areas under various physiological and pathological status.

The regional distribution of GRP/bombesin (along with SP, VIP, and SRIF—see appropriate sections) in the developing rat brain has been reported by McGregor et al. (1982). In contrast to the other neuropeptides (SP, SRIF, and VIP) GRP/bombesin has not been detected in the brainstem at PO and in the cerebral cortex and diencephalon by P7.

GRP immunoreactivity has been reported to be present in the rat retina (McKillop et al., 1988). The chromatographic characterization shows GRP_{10} and GRP_{14}, whereas GRP_{27} represents only a small percentage. It has been speculated that GRP may be colocalized with choline acetyltransferase (ChAT) in the amacrine cells in the retina.

H. Neuropeptide Y

NPY is a 36 amino acid peptide that is widely distributed in the central and peripheral nervous systems. NPY was isolated initially from porcine brain and is homologous to polypeptide YY and other members of the pancreatic polypeptide family (Tatemoto, 1982). NPY is most concentrated in the nervous system and the adrenal gland (Lundberg et al., 1984a,b; Lukinius et al., 1986; Miyachi et al., 1986). A very detailed analysis of the distribution and localization of NPY cells has been described by de Quidt and Emson (1986a,b) in rat brain and spinal cord. In other mammals, NPY neurons have been detected in the medulla oblongata of rabbits (Blessing et al., 1986), the hypothalamus of cats (Leger et al., 1987), the olfactory bulb and peduncle of cats and rats (Matsutani et al., 1988; Sanides-Kohlrausch and Wahle, 1990), the neocortex (Hendry et al., 1984a,b) and basal forebrain (Smith et

al., 1985) of monkeys, and in the brain of the crested newt, *Triturus cristatus carnifex* (Perroteau et al., 1988). Anatomical and physiological studies suggest multiple and important functions for NPY in mammalian nervous system (Gray and Morely, 1986). In addition to behavioral and neuroendocrine functions, a role of NPY in central autonomic regulation has been suggested (Harfstrand et al., 1986). Colocalizations of NPY and norepinephrine have been documented in peripheral and CNS tissues (Hökfelt et al., 1983a,b).

The ontogenesis of the NPY system has been extensively studied in the olfactory bulb of the rat (Matsutani et al., 1988) and cat (Sanides-Kohlrausch and Wahle, 1990). The neurogenesis of the olfactory bulb has been studied in mice by Hinds (1968) and rats by Altman (1969) and Bayer (1983). They have shown that the projecting neurons are formed prenatally, whereas most interneurons, such as granule and periglomerular cells, are formed postnatally. NPY immunoreactive neurons appear in newborn rats, and this is in contrast to SP, which is detected in the tufted cells of E18 rats; tufted cells appear at E16–E17. The number and intensity of immunostaining of these neurons continue to increase with age until the adult pattern is obtained.

The development of NPY has also been reported by Sanides-Kohlrausch and Wahle in the cat (1990). The NPY neuronal system develops postnatally. Maximum neuron number is reached during the third postnatal week. The appearance of NPY-immunoreactive neurons slightly precedes the formation of the terminal fields and of the fiber projection in the internal olfactory tract.

III. REGULATION OF SYNTHESIS AND RELEASE OF NEUROPEPTIDES

As we have already discussed, neural culture systems have been very useful tools for studying neuronal and glial properties and interactions, and the role of microenvironment in neuronal and glial phenotypic expression and function. Thus several of the mechanisms involved in the synthesis and release of neuropeptides from peptidergic neurons and interactions of neuropeptides have derived from studies in culture.

A. Somatostatin

It has been reported that VIP stimulates the release of SRIF in primary monolayer cultures derived from fetal rat telencephalon or diencephalon in a dose-dependent fashion. This property is also exhibited but to a lesser degree by the related peptide PHI (peptide-histidine-isoleucine-27) but not by secretin, growth hormone releasing hormone (GRH_{1-44}–NH_2), or cholecystokinin (Tapia-Arancibia and Reichlin, 1985). The same group (Tapia-Arancibia et al., 1988) also found that under the same conditions VIP causes a large increase in intracellular cyclic AMP. Both the release of SRIF and the increase in cyclic AMP elicited by VIP require exogenous

calcium, can be blocked by cobalt ion, and can be qualitatively mimicked by depolarizing concentrations of exogenous potassium ion. Direct activation of adenylate cyclase by forskolin does not induce SRIF release. The authors suggest that Ca^{2+} and cyclic AMP could act as synarchic coordinate messengers in the control of SRIF secretion having distinct temporal roles. The Ca^{2+} messenger system would be responsible for the fast exocytolic responses and the cyclic AMP pathway would regulate SRIF synthesis. In another study Tapia-Arancibia and Astier (1988) reported that increasing concentrations of glutamate in primary cultures prepared from fetal rat (day 7) diencephalon stimulate SRIF release in a dose-dependent manner. Moreover, they found that excitatory amino acid agonists evoke SRIF release in the following order of potency: quisqulate > glutamate = *N*-methyl-D-aspartate (NMDA) kainate. The increase in SRIF release elicited by glutamate or NMDA is selectively antagonized by D,L-2-amino-5-phosphono-valeric acid and by thyenyl-phencyclidine, two specific antagonists of NMDA receptors. It appears from these findings that glutamate exerts its stimulatory action on SRIF release essentially through NMDA-type receptor sites. Finally, factors from non-neuronal cells (i.e., glial cells) have been implicated in SRIF release. Pares-Herbuté et al. (1988) report that primary cultures prepared from cortex of 17-day-old rat embryos consisting of both neurons and glial cells contain a higher amount of SRIF than neuron-enriched cultures. In addition, conditioned media from glial-enriched cultures prepared from newborn rat induce SRIF release from neuron-enriched cultures. The glial factors responsible for the SRIF secretion remain to be investigated. The influence of non-neuronal factors on SP and SRIF metabolism has been extensively studied in sympathetic and sensory neurons in culture (Kessler, 1984). They found that non-neuronal cells exert a profound influence on the sympathetic expression of these peptides. Sympathetic neurons cultured without associated non-neuronal cells contain virtually no SP but do express SRIF, whereas neurons grown in the presence of ganglion non-neuronal cells contain substantial levels of both peptides. In addition, they observed that SP responds to ganglion non-neuronal cells in a parallel fashion with respect to ChAT expression (cholinergic), whereas SRIF responds similarly with respect to tyrosine hydroxylase (TH) expression (catecholaminergic), thus suggesting a linkage between peptides and neurotransmitters.

B. Substance P

The culture systems primarily used to study regulatory mechanisms of SP have been sensory, dorsal root, and sympathetic ganglia. Using cultures of sympathetic ganglia prepared from neonatal rat superior cervical ganglion, Hart et al. (1991) have reported increased SP in cultures containing interleukin 1β (IL-1β), and this increase appears to be secondary to an increase in mRNA coding for the preprotachykinin precursor of SP. Nuclear transcription assays detect an early increase in preprotachykinin-specific nascent transcripts, suggesting that the ultimate effect of

IL-1 is on transcription itself. Depolarizing agents, γ-interferon, glucorticoid hormones, and prostaglandin synthesis inhibitors (indomethacin) all diminish the induction of SP and preprotachykinin mRNA by IL-1. In another study Jonakait and Schotland (1990) found that factor(s) released from Con A-stimulated splenocytes raises SP levels in cultured sympathetic ganglion cells. Thus they suggest, based on these two studies, that IL-1 may be one molecule active in the medium conditioned by splenocytes. The authors finally conclude that since SP has stimulatory effects on the immune system (several references in this article), the IL-1-induced increase in ganglionic SP may be one means by which the nerves and immune systems interact during an acute ganglionic injury.

The effects of nerve growth factor on early neuronal phenomena have been extensively reviewed since the early work of Levi-Montalcini (see Levi-Montalcini, 1987). Its role in neuropeptide release is not surprising. In an early study Schwartz and Costa (1979) showed that incubation of DRG from 9-day-old chick embryos in growth medium containing fetal calf serum produces a 2–3-fold increase of SP content by 20 hours. Incubation of DRG with β-nerve growth factor results in an increase in the content of SP by about 60%. Of interest is their finding that in DRG removed from 15-day-old chick embryos, nerve growth factor fails to cause a further increase. MacLean et al. (1988), using dissociated cultures of rat nodose ganglia, found that whereas nerve growth factor does not alter neuronal survival, SP content is doubled in the presence of nerve growth factor. In subsequent studies, plating of neurons onto previously dissociated rat atrocytes (for target cells) increased survival by 50% but did not alter SP content in surviving neurons. Thus, nerve growth factor appears to regulate SP control in nodose ganglion neurons. Nerve growth factor appears to also play a role in the regulation of the levels of SP in mature dorsal root ganglion neurons in culture, as has been shown by Lindsay et al. (1989). Kessler and Black (1981) have reported that SP and SRIF immunoreactivities develop synchronously in the rat DRG and spinal cord. Moreover, nerve growth factor treatment results in similar increases in both peptides. They further suggest that the commonality of responses raises the possibility that these two peptides may coexist in the same neurons.

C. Vasopressin

A considerable number of studies have focused on the regulation of VP in the hypothalamus. Studies by Zingg et al. (1986) have shown that osmotic stimulation results in a long-lasting elevation of hypothalamic VP mRNA. During rehydration, these elevated mRNA levels direct VP biosynthesis at a rate that surpasses secretory demands. Also, VP mRNA accumulation does not appear to be directly regulated by either pituitary or hypothalamic VP. The same authors (Zingg and Lefebvre, 1988) examined hypothalamic oxytocin and VP gene expression during gestation and lactation in rats. They conclude that in late pregnancy and lactation the expression of both neuropeptide genes is stimulated in parallel by mechanisms

operating at a pretranslational level, involving increased gene transcription or mRNA stabilization or both. It has been shown that glucorticoid levels regulate expression of VP mRNA in a subset of VP neurons in the paraventricular nucleus of the hypothalamus (Wolfson et al., 1985; Davis et al., 1986). A decrease in this particular class of steroid (after adrenalectomy) appears to increase the potential for altering the expression of the propressophysin gene within this nucleus. In a subsequent study the same group (Baldino et al., 1988) found that adrenalectomy-induced plasticity in VP expression is further enhanced by ablation of a specific afferent pathway. The authors suggest that synaptic events may interact with the hormonal environment to regulate neuronal phenotypes.

D. Vasoactive Intestinal Polypeptide

Using dissociated cultures of embryonic rat spinal cord and dorsal root ganglia, Eiden et al. (1984) studied the ontogeny and release of enkephalin- and VIP-containing neurons. They reported spontaneous release of both enkephalin and VIP from cultured neurons to be high, comprising 10–20% of total cellular enkephalin content and up to 20% of total cellular VIP content per day. This spontaneous release appears to be dependent on neuronal electrical activity, since more than 70% of spontaneous peptide release is inhibited by tetrodotoxin (TTX), which blocks sodium channels and inhibits spontaneous electrical activity. Based on the view that neuronal survival in culture appears to be dependent at least in part on spontaneous neuronal activity, these authors characterized the behavior of VIP and enkephalin neurons in cultures treated chronically with TTX to cause electrical blockade. The levels of both peptides are reduced after such chronic treatment and may reflect the death of these peptidergic neurons. On the other hand, TTX may exert its effects at the level of biosynthesis.

Factors that regulate VIP content and release have also been examined in the bovine fetal adrenal glands by Cheung (1988). Cheung and Hotzwarth (1986) have also reported that VIP is present in the adrenal of the near-term bovine fetus and that VIP can stimulate catecholamine release from fetal adrenomedullary cells *in vitro*. Adrenal VIP content, as measured by radioimmunoassay, is low at 70 and 80 days of gestation. This is followed by a rapid increase in VIP content from 80 to 110 days, reaching a plateau between 110 and 130 days at levels comparable to that in the adult. Release of VIP from fetal adrenocortical cells maintained in culture for 2 days is significantly elevated by angiotensin II, whereas adrenocorticotrophic hormone (ACTH) has no effect. In addition, Ach and high potassium stimulate fetal adrenal VIP release, whereas norepinephrine does not. The possible colocalization of VIP in cholinergic neurons is discussed in another section.

Synthesis and secretion of VIP have also been studied in dissociated cultures prepared from fetal rat (E17) cerebral cortex or hypothalamus (Lorenzo et al., 1989). Time course studies of the total content (media and cells) of VIP present in the culture at different times after plating indicate that the VIP present in the culture

after the first week originates from *de novo* synthesis during the culture period. VIP release in these cultures can be stimulated by Na^+ channel activation using the drug veratridine, and this effect can be blocked by tetrodoxin. Of interest is the finding that rat GHRH stimulates VIP release and the simultaneous addition of anti-rat GHRH IgG blocks this effect. These findings suggest that VIP secretion is regulated by the same membrane-active factors involved in other brain peptides and neurotransmitters. In rat pituitary cells in culture, glucocorticoids at physiological concentrations rapidly inhibit the cAMP production and prolactin release induced by VIP (Rotsztejn et al., 1981). It is suggested that specific VIP receptors in normal rat pituitary are coupled with the activation of cAMP, and this effect is regulated by steroids. Earlier studies have also suggested a VIP-glucocorticoid interaction (Rotsztejn et al., 1975).

E. Neuropeptide Y

The influence of neurogenic and humoral factors on the levels of mRNA encoding for NPY in rat adrenal medulla *in vivo* has been reported recently (Fischer-Colbrie et al., 1988). Twenty-four hours after insulin injection, levels of mRNAs encoding NPY (and also enkephalin) increase by 6.5-fold, and bilateral transection of the splanchnic nerves completely prevents this increase. In contrast, hypophysectomy does not change NPY mRNA levels. This phenomenon has been termed "stimulus-secretion-synthesis coupling."

IV. EARLY PRESENCE OF NEUROPEPTIDE RECEPTORS DURING ONTOGENESIS

A. Somatostatin

There is an explosive amount of research focusing on SRIF receptors in various species ranging from *Xenopus* oocytes to human brain. In mammals, SRIF-binding sites are particularly abundant in the deep layers of the cortex, in all components of the limbic system, and in the locus coeruleus (Leroux and Pelletier, 1984; Tran et al., 1984; Epelbaum et al., 1985; Reubi et al., 1986; McCarty and Plunkett, 1987). Two classes of receptors can be distinguished in the brain and pituitary on the basis of their relative affinities for SRIF-14 (S14) and SRIF-28 (S28) and synthetic analogs (Srikant and Patel, 1981a,b; Heiman et al., 1987). In a recent study, Krantic et al. (1990) describe in detail the heterogeneity of SRIF receptors in rat extrahypothalamic brain. In contrast, in the brain of a nonmammalian vertebrate, the frog *Rana ridibunda*, a single class of high-affinity ^{125}I-Tyr^0, D-Trp^8-S14 binding sites is found (Laquerriere et al., 1989). The K_D value for the radioligand binding is 7-fold higher in frog than in rat brain.

Studies both *in vivo* and in culture of mouse hypothalamus have shown that SRIF-binding sites are measurable as early as day 1 after birth and after 6 days *in*

vitro in neuronal cultures derived from fetal hypothalamus at E16. *In vivo* binding site number reaches a maximum at 14 days and does not increase further with age. Of interest is the observation that SRIF-binding sites are also detectable in glial cell cultures (Heided et al., 1990). The presence of binding sites is in agreement with the presence of SRIF as well as SRIF gene expression at E14 in rat (Almazan et al., 1989; McGregor et al., 1982) and mouse (Bendotti et al., 1990). Heided et al. also report a difference between SRIF binding on neurons and glial cells, the neurons having both low- and high-affinity binding sites, whereas glial cells have only low-affinity sites. However, SRIF has not been detected in glial cells (Heided et al., 1990; Kentroti and Vernadakis, 1995). SRIF binding sites have also been reported on rat diencephalic astrocytes in cultures derived from 2-day-old rat and in frozen tissue sections of 3–34-month-old rats (Krisch et al., 1991). It appears that the most prominent labeling of astrocytes is in (1) large subependymal glia in the ventricular wall, particularly in a position that corresponds to that of SRIF-producing perikarya and (2) perivascular astrocytes in the same regions. The significance of SRIF receptors on astrocytes is a matter of speculation. It is proposed (Krisch et al., 1991) that astrocytes may be involved in the removal of the peptide from the intercellular cleft or the target cell; on the other hand, glial cells may be the primary target of SRIF. The subependymal astrocytes are suggested to represent a particular population of astrocytes (Leonhardt et al., 1984) controlling the transport between ventricular and intercellular cerebrospinal fluid compartments (Krisch and Leonhardt, 1984).

The ontogeny of SRIF receptors has also been reported in the rat visual system (Bodenant et al., 1991). The binding sites show high affinity for SRIF (^{125}I-Tyr^0, D-Trp^8-SRIF-14) and SRIF analogues (SRIF-28) and is regulated by GTP as early as day 16 of fetal age, indicating that they represent functional SRIF receptors. During fetal life, SRIF receptors are observed in the retina, optic nerve, optic chiasma, optic tract, and lateral geniculate nucleus. The highest densities of SRIF receptors are found between E16 and E18 in the retina and primary optic pathways. The expression of SRIF receptors appears to be transitional and to vary among the various areas of the optic system: the density of SRIF receptors decreases dramatically in the retina and disappears by P21 in both optic pathways and dorsal lateral geniculate nucleus. This transient appearance of SRIF receptors has been interpreted to suggest that SRIF plays a trophic role during early development in the visual system. It is suggested by Bodenant et al. (1991) that in the retina a large proportion of SRIF receptors are expressed by immature ganglion cells.

Gonzalez et al. (1988) have also shown that the rat cerebellum displays transient expression of SRIF-binding sites. Specific SRIF receptors are first detected at E15, reach a maximum at P13, and gradually decline between P13 and P23. Virtually no SRIF receptors can be detected in the adult rat cerebellum. In a subsequent study the same group of authors (Gonzalez et al., 1990) characterized the SRIF receptors in the immature (13-day-old) rat cerebellum using membrane enriched preparations and autoradiography for binding of two ligands, ^{125}I-Tyr^0, D-Trp^8 SRIF-14 and ^{125}I-SMS 204–090. The pharmacological profiles observed are similar to that of

adult rat cortex. Again, the physiological significance of the transient expression of SRIF receptors in the cerebellum can only be speculated on at present. At the microscopic level, SRIF receptors are associated with the external granule layer and thus may play a role in the proliferation of the stem cells of the granule cells, and their disappearance appears to coincide with the migration of the neuroblasts. The hypothesis that SRIF may act as a trophic factor involved in the regulation of cell division and/or migration in the external granular layer is supported by the absence of synaptic contacts in this layer, thus suggesting that SRIF receptors in this layer are not involved in neurotransmission.

Najimi et al. (1991) report a regional distribution of SRIF-binding sites in postmortem human hypothalamus both in infants and adults using *in vitro* autoradiography. Guanosine triphosphate pretreatment, before incubation, allowed them to detect higher ^{125}I-Tyr^0-D-Trp^8 SRIF-14 binding site densities in hypothalamic structures such as preoptic and anterior hypothalamic areas and ventromedial and dorsomedial nuclei but not in other hypothalamic regions. This regional effect was apparent in both infant and adult hypothalamus. Scatchard analysis using ^{125}I-Tyr^0-D-Trp^8 SRIF-14 reveals no differences in the binding sites between adult and infant hypothalamus. SRIF has been shown to produce differential effects on adenylate cyclase in different brain regions (Markstein et al., 1989). In homogenates of hippocampus, SRIF both inhibits forskolin-stimulated adenylate cyclase activity in the nanomolar range and stimulates basal adenylate cyclase in the micromolar range. In the substantia nigra only a stimulation of basal adenylate cyclase is seen, whereas in the striatum only inhibition of stimulated cyclase is observed in the presence of SRIF. The authors attempted to correlate these variable effects of SRIF with the various SRIF receptor populations in these brain areas. Inhibitory effects of SRIF adenylate cyclase activity have also been reported in non-neuronal tissues (considered SRIF target tissues) (Heisler and Srikant, 1985; Ray et al., 1986). Miyoshi et al. (1989), using hippocampal neuronal cultures prepared from 18-day rat embryos, found that SRIF increases intracellular Ca^{2+} concentration in a dose-dependent manner. Since this increase appears to be inhibited by ω-conotoxin GVIA, a neurotoxic peptide from the venom of a marine snail, it is suggested that SRIF receptors may be coupled with N-type volgate-sensitive Ca^{2+} channels in cultured hippocampal neurons.

B. Vasoactive Intestinal Polypeptide

Early studies by Taylor and Pert (1979) showed specific binding of VIP to adult rat brain membranes. Later Besson et al. (1984) described the autoradiographic distribution of VIP-binding sites in the rat CNS using ^{125}I-labeled VIP. High densities of VIP-binding sites are observed in the granular layer of the dorsal dentate gyrus of the hippocampus, the basolateral amygdaloid nucleus, the dorsolateral and median geniculate nuclei of the thalamus, as well as the ventral part of the hypothalamic dorsomedial nucleus. In an earlier study Pearlmutter et al. (1983)

characterized VIP-binding sites in particulate preparations of rat brain and found that the cellular location of binding of VIP in the hippocampus and septum differs from that in other brain regions. These brain areas have been notably implicated in the maintenance and acquisition of memory (Van Ree et al., 1978). A detailed distribution of VIP-binding sites in the rat brain has recently been described by Martin et al. (1987) using *in vitro* autoradiography on slide-mounted sections. High densities of binding sites are reported in olfactory bulb, cerebral cortex and dentate gyrus, subiculum, various thalamic and hypothalamic nuclei, superior colliculus, locus coeruleus, area postrema, subependymal layer, and pineal gland. Intermediate densities are found in amygdala, nucleus accumbens, caudate-putamen, septum, bed nucleus of the stria terminalis, hippocampus, and central gray. Of note is the observation that the presence of VIP receptor binding sites is not necessarily accompanied by VIP immunoreactivity, as in the cerebellum, for example, which is devoid of VIP immunoreactivity but contains a low density of VIP recognition sites.

Studies on the ontogeny of VIP receptors are few. The postnatal development of VIP-binding sites in rat forebrain and hindbrain has been examined by Roth and Beinfeld (1985). In both regions of the brain, VIP binding is detectable by P2, rises markedly between P7 and P17, and correlates with VIP immunoreactivity (discussed in another section). An early study by Robberecht et al. (1979) showed that VIP maximally activates adenylate cyclase during this age period and thus correlates with the expression of VIP receptors. In other systems in the adult these investigators (Christophe et al., 1984) have suggested that the VIP effects are mediated by adenylate cyclase activation.

As we discussed earlier, considerable information concerning ontogenetic aspects of neuropeptide receptors has derived from in culture studies of CNS tissue. Hosli and Hosli (1989) have described binding sites for VIP using autoradiography in explant cultures of rat spinal cord, brainstem, cerebellum, and cortex. In addition to neurons, binding sites are also observed on astrocytes. Labeling of astrocytes is more intense in spinal cord and brainstem than in cultures of cerebellum and cortex, suggesting a different functional role of astrocytes in the different CNS areas.

The molecular mechanisms of VIP actions has been studied in human lymphocytes (O'Dorisio et al., 1985). Dose-dependent VIP-induced stimulation of adenylate cyclase was observed in Molt 4b lymphoblasts. Accumulation of intracellular cAMP occurs in the presence of VIP or forskolin. Of interest is the observation that pancreatic GHRH competes for ^{125}I-VIP binding sites. However, GHRH exhibits no effect on cAMP. cAMP-dependent protein kinase II is predominantly demonstrated in these cells, and increased phosphorylation of a specific M_r 41,000 protein occurs after addition of forskolin or VIP, whereas, again, GHRH does not affect protein phosphorylation. These findings demonstrate the presence of a functional VIP receptor that is linked to the G protein–adenylate cyclase complex. The cAMP-dependent protein kinase and the VIP-mediated protein phosphorylation in Molt 4b lymphoblasts provides evidence on a molecular level for neuropeptide modulation, at least of human lymphocyte function.

C. Substance P

As discussed earlier, the intermediolateral column of the spinal cord contains a relatively high amount of SPLI. Takano and Loewy (1984) studied ^{3}H SP binding in the intermediolateral column and striatum in the rat using slide-mounted sections. Both the intermediolateral column and striatum exhibit a single high-affinity binding component. In addition, using various SP structurally related tachykinins in displaying ^{3}H SP binding, the rank of potencies is similar in both regions: substance P > physalaemin > substance K = eledoisin = kassinin > (D-Pro^{4}, D-Trp^{7,9}) SP (4–11). On the basis of rank orders of potency two SP receptor subtypes have been proposed by Lee et al. (1982). A detailed autoradiographic localization and characterization of spinal cord SP-binding sites by Charlton and Helke (1985) shows high densities in discrete localization of sensory, autonomic, phrenic, and Onuf's motor nuclei. A close correlation between the binding sites for SP and the presence of cholinesterase-stained neurons suggests that the SP receptors are located on or proximal to cholinergic neurons. Again, the differential sensitivity of the SP receptors to unlabeled SP suggests that there are heterogeneous receptors for SP in the spinal cord, which may be relevant to the role of SP in different spinal cord functions. Reiser and Hamprecht (1988) and Reiser et al. (1982), using neuroblastoma x glioma hybrid cells, found that SP stimulates the uptake of guanidinium. In addition, the SP site on the hybrid cells that activates cation permeability has a low affinity. This site, according to Reiser and Hamprecht, does not fit into the usual classification of tachykinin receptors but rather resembles the site that modulates nicotinic acetylcholine receptors on chromaffin cells.

The ontogeny of SP receptors in the developing brain has not been systematically examined, except in the spinal cord (Charlton and Helke, 1986). Light microscopic autoradiography and membrane homogenate binding of ^{125}I-Bolton-Hunter-SP was used to study SP binding sites in the spinal cord of rats of different ages. In pups up to 15 days old, binding sites are diffusely distributed over the grey matter and become progressively more defined in specific nuclei as the rats age. High densities of binding sites have been noted at P1 in a nucleus located in the ventrolateral ventral horn of caudal segments; whereas in the phrenic motor nucleus and in the dorsal horn binding sites are expressed after P8. Of importance, again, is the finding that SP receptors decrease with age. This decrease is in contrast to other neuropeptides such as VIP (Roth and Beinfeld, 1985) and opiates (Geladopoulos et al., 1987).

As discussed throughout this chapter, culture studies have provided evidence of the cellular localization of neuropeptides and their receptors. Hosli and Hosli (1985) have contributed an extensive amount of information in this field. Using organotypic cultures of rat spinal cord and brainstem and autoradiography, they found binding sites for SP on many interneurons located in the dorsal horn, whereas in the ventral horn, mainly large neurons (probably motoneurons) are labeled. Binding sites are detected in the brainstem neurons. In contrast, glial cells in these cultures do not reveal binding sites for SP. However, Mantyh et al. (1989) found that

SP-binding sites are expressed by glial cells *in vivo*. They examined the glial scar in the transected optic nerve of the albino rabbit by quantitative receptor autoradiography. Ligand displacement and saturation experiments indicate that SP and receptor binding sites expressed by the glial scar have pharmacological characteristics similar to those of SP receptors in the rabbit striatum, rat brain, and rat and canine gut. It is suggested that SP may play a role in regulating the glial response to injury in the CNS. The possible origin of SP that could occupy the SP receptors expressed by the glial cells after injury can only be speculated on, but circulating leukocytes have been reported to synthesize SP (Weinstock et al., 1988).

D. Neurotensin

Although several studies have been reported on NT-binding sites and their distribution in the adult CNS (Szigethy and Beaudet, 1989; Dilts and Kalivas, 1989; Moyse et al., 1987; Kohler et al., 1987; Quirion et al., 1982, 1987; Kitabgi et al., 1987), very little is known ontogenetically.

In the adult rat CNS NT-binding sites are found to be partly associated with dopaminergic nerve terminals in the mesolimbic and nigrostriatal systems (Goedert et al., 1984; Palacios and Kuhar, 1981). Two distinct NT-binding sites have been identified in the rat forebrain: the NT_1 acceptor site (levobastine-sensitive) and the NT_2 receptor site (Schotte and Laduron, 1987). In the rat brain, NT_2 receptors are present at birth, reach maximum level on P10, and plateau at a lower lever in the adult. NT_1 receptors are not detected before P10 and reach maximum levels at P30. Again, the high expression of NT_1 receptors early in the postnatal period may be associated with neuronal maturation.

Most cortical areas, including the visual areas, have generally shown low density of NT receptor binding sites (Quirion et al., 1982). Lobo and Parnavelas (1988) have examined the ontogeny of NT-binding sites in the rat visual cortex using *in situ* receptor autoradiography. The distribution of NT receptor binding sites undergoes considerable change during ontogeny. Moderate densities of binding sites are present in the occipital cortex as early as E14 and E18. The density of NT-binding sites is extremely high in the occipital region at the time of birth, with a gradual decline starting in the second postnatal week to very low levels in the adult. The role of NT receptors in early mitogenic events is again suggested by these reports. Recently it has been reported (Bozou et al., 1989) that NT and SRIF (and bradykinin) inhibit, in a time- and concentration-dependent manner, prostaglandin E1 or forskolin-stimulated cAMP production in neuroblastoma N1E115 cells (Bozou et al., 1989). Cell treatment with pertussis toxin reverses the inhibition elicited by these peptides after short incubation periods, but alone, pertussis toxin has no effect. Protein kinase C activation with phorbol ester does not inhibit basal or stimulated cAMP production. These findings are interpreted to indicate the existence of both pertussis toxin-sensitive and -insensitive mechanisms of neuropeptide-mediated inhibition of cAMP formation in N1E115 neuroblastoma

cells. The authors propose several hypotheses for the peptide-induced inhibition of cAMP, including a theory suggesting that inhibition occurs through a G_i independent mechanism.

E. Growth Hormone-Releasing Hormone

As is the case for other neuropeptides, some information concerning receptors for GHRH has derived from culture studies of various cells and tissues. Binding sites have been found in cultured granulosa cells, and their expression appears to be induced by follicle-stimulating hormone and to be associated with increased cAMP production (Bagnato et al., 1991). It is suggested that GHRH, which is also formed in the ovary, could exert a positive autoregulatory action to accelerate follicular maturation by amplifying the granulosa cell response of follicle-stimulating hormone. GHRH-binding sites have also been found in cultures of the anterior pituitary and explain the desensitization after chronic stimulation with GHRH; a 48% loss of binding sites occurs after a 2-hour pretreatment with GHRH (0.5 nM), with a maximum loss occurring after 8 hours (Bilezikjian et al., 1986).

As discussed in a later section, interactions and colocalization of neuropeptides occur in several tissues, including nervous tissues. Of interest is the report by Laburthe et al. (1983) that GHRH inhibits competitively the binding of VIP to human and rat intestinal epithelial membranes. The affinity of GHRH for VIP receptor is 700–800 times lower than that of VIP in both humans and rats. GHRH stimulates adenylate cyclase through its interaction with VIP receptors in rat and human membranes. It is noted, however, that GHRH behaves as a VIP agonist in human tissue, and it is a partial agonist/antagonist of VIP in the rat. The specificity in GHRH interaction with VIP receptors has been further tested in rat and human GHRH and VIP receptors (Laburthe et al., 1986). It is observed that rat GHRH has a much higher affinity than human GHRH for both human and rat VIP-binding sites. Studies of adenylate cyclase stimulation reveal that rat GHRH and the analog His1 of GHRH are full VIP agonists in humans and rats, whereas human GHRH and its other analogs (Ac-Tyr1) behave as partial agonists in both humans and rats. This is the first example of a competitive VIP antagonist by a GHRH analog.

The interaction with GHRH and VIP receptors and coupling to adenylate cyclase has been examined in membranes from rat anterior pituitary, liver, and pancreas (Robberecht et al., 1985). In the anterior pituitary, GHRH and analogs interact with specific GHRH receptors, whereas in liver and pancreatic membranes they interact with VIP receptors.

F. Gastrin-Releasing Peptide/Bombesin

An elegant review has been reported by Battey and Wada (1991) on the two distinct receptor subtypes for mammalian bombesin-like peptides, GRP and neuromedin B. *In situ* hybridization studies using either GRP-R or neruomedin-R probes have shown that GRP-R expression is most prominent in the hypothalamus,

particularly in the suprachiasmatic and supraoptic nuclei, whereas neruomedin-R expression is most prominent in the olfactory regions and the thalamus. Regional distribution of high-affinity binding sites and second messenger studies (Hollingsworth, 1989) reveal that the areas with the greatest amount of ^{125}I-GRP specific binding also show the most robust stimulation of phosphatidylinositol breakdown by bombesin. Receptor distribution, in parallel with second messenger production, supports the postulation of a physiological relevance to this binding site. Battey and Wada suggest from their studies that GRP produced by neurons may be released locally and bind to high-affinity, GRP-preferring bombesin receptors, thereby mediating a biological affect.

Xenopus oocytes have been a useful model for studying cellular and molecular properties of neuropeptides, including early receptor expression. Oocytes can be used as an mRNA translation system whereby mammalian brain receptors can be transplanted from the brain into a simple cell preparation (Dascal, 1987). This system has been used effectively to study the electrophysiology, biochemistry, pharmacology, and molecular biology of brain receptors (Dascal, 1987; Lübbert et al., 1987; Mishina et al., 1985; Sumikawa et al., 1981; Van Renterghem et al., 1987).

Functional expression of brain bombesin/GRP receptors has been reported in *Xenopus* oocytes (Moriarty et al., 1988). Total RNA was extracted from 15-day-old whole rat brains. Microinjection of the RNA into *Xenopus laevis* oocytes induces electrophysiological responsiveness to bombesin by depolarization of the cell membrane, which is characteristic of receptor-mediated polyphosphoinositide breakdown. The depolarization is caused by an influx of Cl^- ions. Similar findings have been reported by Meyerhof et al. (1988), also using *Xenopus laevis* oocytes. Studies by Hari Prasad and Wood (1989) suggest that bombesin-like peptide receptors may utilize phosphotidyl inositol as a second messenger. They report that bombesin and GRP significantly stimulate inositol-1-phosphate (IP_1) production in frontal cortex slices, but bombesin had no effect on cAMP or cGMP levels. Moreover, they observed that the density of bombesin receptors in various brain regions correlates with the ability of bombesin to stimulate phosphotidyl inositol turnover; IP_1 levels are elevated in those brain regions enriched in bombesin receptors such as the olfactory bulb, hippocampus, striatum, thalamus, and frontal cortex, whereas IP_1 levels are not increased in areas that have a low density of bombesin receptors such as the cerebellum, medulla/pons, and midbrain.

G. Neuropeptide Y

As with GRP, a single class of high-affinity binding sites for NPY has been reported in brain based on binding of ^{125}I-NPY (Unden et al., 1984; Savia et al., 1985) and ^{125}I-*N*-succinimidyl-3-(4-hydroxy-5-iodophenyl)propionate-NPY (^{125}I-Bolton-Hunter NPY) (Chang et al., 1985). Two binding sites for ^{125}I-PYY (peptide YY) recently have been reported in brain (Inui et al., 1988). Lynch et al. (1989) have reported a detailed localization of NPY receptors in rat brain using both

^{125}NPY and ^{125}J-polypeptide YY as ligands for *in vitro* autoradiography and provide evidence for differential distribution of the high- and moderate-affinity sites. These authors, however, suggest that the endogenous ligand for the majority of sites is most likely NPY, since relatively little authentic polypeptide YY is found in the brain (Broome et al., 1985; Ekman et al., 1986). Of interest is the finding of low levels of receptors in the hypothalamus, which contrasts with the high levels of immunoreactivity (discussed in another section). One speculation is that the levels of NPY and its receptors are differentially regulated, depending on the use of various neuronal pathways and the influence of modulating systems (Lynch et al., 1989).

V. NEUROREGULATORY MECHANISMS

A. Interactions Between Neuropeptide Systems

Colocalization of Neuropeptides and Neurotransmitters

Somatostatin. Early studies have shown that SRIF and norepinephrine are colocalized in the peripheral sympathetic neurons (Hökfelt et al., 1977). In the CNS SRIF has been shown in several species to coexist with GABA (Somogyi et al., 1984; Hendry et al., 1984b; Oertel et al., 1983; see references in Epelbaum, 1986). The majority of cortical SRIF-positive neurons found in layers II–III and V–VI are also glutamic acid decarboxylase (GAD) positive. In the hippocampus they are located in the stratum oriens and in the hilus of the dentate gyrus. In contrast, SRIF and Ach do not appear to be colocalized in the CNS (Eckenstein and Baughman, 1984; Vincent and Johansson, 1983). However, SRIF immunoreactivity coexists with acetylcholinesterase (AChE) in cultured rat cortical neurons (Delfs et al., 1984) as well as in rat neocortex and hippocampus (Zhu et al., 1984). It is known that not all AChE-containing neurons also contain ChAT, the cholinergic neuronal marker. Thus it appears that SRIF could be present in cholinoceptive cortical neurons (Delfs et al., 1984). More recently, Van der Zee et al. (1991) have reported colocalization of muscarinic Ach receptors and SRIF in nonpyramidal neurons of the rat dorsal hippocampus. This finding provides additional evidence for a direct cholinergic influence upon somatostatinergic nonpyramidal neurons and defines the coexistence of SRIF and cholinoceptive neurons in the dorsal hippocampus. Forloni et al. (1989) also examined the distribution of AChE staining and SLI in mouse hippocampus during postnatal development. Colocalization was assessed by a double-staining method. A large percentage of the neurons staining for one of these markers also stained for the other. In the stratum oriens, the number of SLI neurons expressing AChE is high up to P2 and begins a slight decline by adulthood. Again, the expression of AChE by SRIF neurons reflects their cholinergic innervation. Moreover, the transient expression of AChE in SRIF neurons may be related to a trophic noncholinergic role proposed for AChE in other structures (see references in Buznikov, 1990). More recently, studies from our laboratory have demonstrated the colocalization of SRIF with markers for three classical neuronal

phenotypes: cholinergic, catecholaminergic and GABAergic. Coexpression of SRIF with other neuronal phenotypes occurs in pluripotential neuroblasts in cultures derived from chick embryos at a very early stage of development (embryonic day 3) (Kentroti and Vernadakis, 1995) and infers a modulatory role for somatostatin during early neural development.

Vasoactive Intestinal Polypeptide. Eckenstein and Baughman (1984) have reported that in the adult rat cerebral cortex 80% of the bipolar neurons positive for ChAT immunoreactivity also express VIP immunoreactivity. On the other hand, the ChAT-positive cells in the basal forebrain, which are another major source of cholinergic innervation of the cortex, contain no detectable VIP immunoreactivity. Thus a large portion of the cortical intrinsic cholinergic neurons are also able to synthesize VIP. Hara et al. (1985) found that within the neurons of the sphenopalatine ganglion VIP and AChE coexist. Coexistence of VIP and AChE has also been demonstrated within sympathetic and parasympathetic neurons in the cat (Lundberg et al., 1979). Thus cholinoceptive neurons appear to contain VIP, as also observed with other neuropeptides (i.e., SRIF).

Substance P. The ontogeny of serotonergic neurons in the medullary raphe that colocalize SP has been extensively studied (see the review in Jonakait et al., 1991). Although both 5-hydroxytryptamine (5-HT) and SP immunoreactivities are initially detected in the developing mouse rhombencephalon at E12, there is no initial overlap in their areas of distribution. SPLI is not found within cells of the medullary raphe unit at E13 (Ni and Jonakait, 1988a,b). However, immediately upon its detection, it is co-localized with 5-HT. Double-label immunohistochemistry applied to CNS tissue at later stages suggests that colocalization is an enduring feature of these cells: more than 80% of 5-HT-positive neurons contain SPLI throughout gestation and into adulthood (Ni and Jonakait, 1988b). In contrast, in the bed nucleus of the stria terminalis and the central nucleus of the amygdala SP-containing cells transiently express TH (Verney et al., 1988). Appearing at E17, this catecholaminergic marker declines after the seventh postnatal week. Walker et al. (1990) have further examined the relationship between 5-HT and SP colocalization and determined whether inhibition of 5-HT synthesis during development would affect the developmental profile of colocalized SP. They found that although 5-HT and SP may appear synchronously, the initial expression of SP is not dependent on serotonin expression, or, if it is, a very low level of 5-HT expression is sufficient to trigger the expression of SP. Schmechel et al. (1984) have reported the coexistence of GAD immunoreactivity and SRIF in many nonpyramidal neurons of rat, cat, and monkey cerebral cortex. Also of interest is a recent report by Kachidian et al. (1991) on the coexistence of SP, thyrotropin-releasing hormone, GABA, met-enkephalin, and leu-enkephalin in the serotonergic neurons of the caudal raphe nuclei of the rat. The proportion of each coexisting peptide with 5-HT appears in decreasing order as TRH < SP < MET-ENK = LEU-ENK < GABA. Given the evidence that these raphe nuclei possibly project to the spinal cord, these data constitute an anatomical substrate for the several distinct physiological functions

presumably subserved by 5-HT in spinal cord, namely, the modulation of nociception, motor, and autonomic functions.

Neuropeptide Y. In the periphery, NPY is found with noradrenergic sympathetic neurons supplying many different tissues (Lundberg and Hökfelt, 1986). Considerable evidence suggests that NPY coexists (Lundberg et al., 1982) and is coreleased with norepinephrine from sympathetic nerves upon electrical stimulation (Lundberg et al., 1984a,b, 1986a, 1989) or reflexogenic activation (Lundberg et al., 1985, 1986b; Pernow et al., 1986). Immunoreactivities for TH and NPY have recently been studied in developing sympathetic neurons of bullfrog tadpoles and adults (Stofer and Horn, 1990). Whereas at stage III nearly all ganglion cells are positive for TH, NPY first appears in a few principal cells of the paraventricular ganglia at stage XI. Thus the development of NPY expression begins long after the onset of adrenergic differentiation and ganglionic synapse formation. In addition, Stofer and Horn found that cellular levels of TH and NPY can be independently altered, and they suggest that the onset of NPY expression is not linked to maturation of peripheral targets, but rather to some global event operating synchronously along the rostro-caudal axis.

Neuropeptide Colocalization

There is considerable literature describing the coexistence of neuropeptides within neurons in both the peripheral and central nervous systems. In view of the lack of information on neuropeptide colocalization during ontogeny, a very brief review of neuropeptide colocalization in adults will be presented here.

The colocalization of SRIF and NPY has been reported in individual subfields of the rat hippocampal region (Kohler et al., 1987). Colocalization of SRIF and NPY has also been found in cortical and subcortical cells of the rodent (Chronwall et al., 1984; Vincent et al., 1982; Vincent and Johansson, 1983) and primate brain (Chronwall et al., 1984; Hendry et al., 1984b). Studies in the rat and the primate brain seem to indicate that regional differences in the degree of colocalization may exist. Chronwall et al. (1984) have reported a high incidence of colocalization of SRIF and NPY in the rat and human neocortex, whereas few cells in the arcuate nucleus contain both peptides. McDonald (1989) found extensive colocalization of SRIF and NPY in all of the amygdaloid nuclei, with the exception of the intercalated nucleus and the lateral subdivision of the central nucleus. In contrast, he found no colocalization of SRIF and NPY.

Coexistence of NPY and VIP has been found in the guinea pig in non-noradrenergic axons innervating cerebral arteries (Gibbins and Morris, 1988). The coexistence of vasodilator (i.e., VIP) and vasoconstriction (i.e., NPY) neuropeptides in axons innervating the cerebral circulation suggests a strong regulatory role of these peptides in cerebral function.

Colocalization of VIP- and SP-containing nerves has been reported in cat bronchi (Dey et al., 1988). VIP- and SP-containing nerve fibers are colocalized in bronchial smooth muscle, in the walls of pulmonary and bronchial arteries, and around submucosal glands. These peptides are also found colocalized in nerve cell bodies

that comprise the intrinsic airway ganglia. The colocalization of VIP and SP in the same nerve fibers suggests that airway and pulmonary vascular function may be partially regulated by the simultaneous or sequential release of VIP and SP from the same nerve fibers (Dey et al., 1988).

Although in this review we have not discussed neurokinin B, another peptide of the tachykinin family (Maggio, 1988), it is of interest to note the coexpression of neurokinin B and SP genes in a subset of neurons in the rat habenula (Burgunder and Young, 1989); moreover, these peptides are also colocalized with ChAT.

There is extensive literature on the interrelationship between SP and opioid peptides in the CNS, including colocalization (Neal et al., 1989), induction of opioid release by SP (Del Rio et al., 1983; Tang et al., 1983), and receptor expression of both peptides (Herkenham and Pert, 1980; O'Donohue et al., 1987). Again, no studies are yet available on such interrelationships during ontogenesis.

B. Neurotrophic Role of Neuropeptides

As we have discussed throughout this review, the presence of neuropeptides and their receptors during early embryogenesis, and their colocalization with neurotransmitter substances, at least as documented in the adult CNS, are supportive evidence that these endogenous substances may be another set of growth factors with several of the functions attributed to the classical growth factors, including neuronal survival, neuronal growth, and neuronal phenotypic expression. In this section, we will briefly review some of the neurotrophic functions of some neuropeptides. We will use our prerogative to present some of our findings in more detail.

Neuronal Survival

Cell death is a prominent as well as a normal aspect of neurogenesis (Hamburger and Oppenheim, 1982; Oppenheim, 1985). Neurons are overproduced during early embryogenesis and their subsequent death has been shown to coincide with the onset of electrical activity (Oppenheim and Nunez, 1982; Pittman and Oppenheim, 1978). Within the nervous system cell death or, conversely, neuronal survival is regulated by intricate relationships that occur at various cellular levels. Growth factors and neurotransmitters comprise two major classes of endogenous intracellular signals that affect neuronal survival and thus establish the neuroarchitecture (for a review see Mattson, 1988). Neuropeptides represent a third closely related group of endogenous substances that have been most recently shown to influence the survival of developing neurons.

Early studies have shown neuropeptides to be important mediators of brain development. For example, studies by Boer and colleagues (1980, 1985) on the Brattleboro rat model have shown that the genetic deficiency of VP results not only in the spontaneous onset of diabetes insipidus but also in a significant decrease in brain weight and cell number, especially in cerebellum, which persists into adult-

hood. Further studies by this group have shown that only prenatal treatment of embryos with VP prevents these developmental brain anomalies.

Other neuropeptides as well have been shown to affect the survival of developing neurons. The responses are specific for each neuropeptide and each neuronal type. Brenneman and colleagues have pioneered work showing that neuropeptides may directly influence survival in populations of developing neurons (Brenneman and Siegel, 1986; Brenneman et al., 1985). Their studies have shown that VIP increases the survival of dissociated embryonic spinal cord neurons *in vitro*, an effect that can be blocked with TTX. In contrast, GHRH significantly decreases neuronal survival in these same cells in culture (Brenneman and Foster, 1987). These findings demonstrate that the survival-promoting effects of VIP are confined to a critical period of neuronal growth in culture. In this study, the critical period occurs between 7 and 21 days after plating and is significant because it relates to the period of normally occurring cell death. The mechanism of action for the survival-promoting effects of VIP have not been fully elucidated; however, the presence of astrocytes in these spinal cord-enriched cultures is essential (Brenneman et al., 1987, 1990).

In the case of SRIF, studies from our laboratory (Kentroti and Vernadakis, 1991a) have shown that although this neuropeptide has no direct survival-promoting effect in neuron-enriched cultures derived from 3-day-old whole chick embryo (cultures consisting of both neural tube and neural crest elements), it was found to counteract the effects of *in ovo* administration of ethanol in this system. Ethanol, when administered to chick embryos very early during embryogenesis (days 1 and 2 of development), results in a significant enhancement in cell death when neurons are cultured at E3. Addition of SRIF to the growth medium of these cells in culture partially restores the normal profile of neuronal survival. We suggest that SRIF may act as a regulatory substance for neuronal survival during development.

Studies by Clos and Gabrion (1989) have described a synergistic effect of the neuropeptide VP with 3,3′,5-triiodo-L-thyronine (T_3) in promoting survival of hippocampal cells dissociated from 5-day-old rat. As observed with SRIF, AVP alone has no direct effect on survival of hippocampal neurons in culture; however, in concert with T_3, AVP prevents cell death in developing neurons.

Neurotransmitter Expression

The early events of neural development are influenced by numerous genetic and endogenous soluble factors (Aizenman et al., 1987; Anderson, 1988). Among the list of soluble effectors we can now include the endogenous neuropeptides. As described earlier in this review, the ontogenesis of most peptidergic neurons in the CNS occurs early in development and coincides with the period of neural crest migration. The evidence supporting the notion of a peptide influence on neuronal phenotypic expression is accumulating as researchers pursue the exact identity of these peptide factors. McManaman and co-workers have reported cholinotrophic effects of a small-molecular-weight peptide derived from skeletal muscle extract (McManaman et al., 1985). Dal Toso et al. (1988) have identified a basic neurono-

trophic protein of 14 kDa derived from the striatum. Howard and Bronner-Fraser (1986) have reported the presence of neural tube-derived factors that promote the development of adrenergic and inhibit development of cholinergic characteristics in neural crest cells. During the early stages of development of the primary phenotypes (catecholaminergic, cholinergic, and GABAergic) neuroblasts have been shown to possess a high degree of plasticity with respect to neurotransmitter phenotype (Le Douarin and Teillet, 1974). Neuronal balance is established under the influence of endogenous factors, which may shift expression from one neurotransmitter phenotype to another. The studies reported below provide evidence that neuropeptides can regulate neuronal phenotypic expression by influencing neuronal survival, shifting neuronal expression, and/or promoting neuronal maturation of a specific phenotype.

Catecholaminergic Expression. Several investigators have examined the development of catecholaminergic expression in the embryonic brain. Allen and Newgreen (1977) reported the first appearance of catecholamine histofluorescence at stage 21 (3.5 days) in the chick embryo. We have found catecholaminergic expression as early as E4 in the avian model system, using TH activity as a biochemical marker for catecholaminergic neurons (Kentroti and Vernadakis, 1989). Recently we have found that the critical period for neuroblasts to respond to GHRH is between E1 and E3 in the chick, a period characterized by active neuronal proliferation and differentiation (Bennett and DiLullo, 1985a,b). As reported, most chick brain neurons proliferate and become postmitotic between E4 and E8 both *in vivo* (Bennett and DiLullo, 1985b; Fujita, 1964; Tsai et al., 1981) and in culture (Aizenman et al., 1986; Barakat et al., 1982). Embryos treated with GHRH *in ovo* on embryonic days 1, 3, 5, and 7 exhibit a significant increase in TH activity. Similar results are obtained when GHRH is administered in a single dose on day 1 or day 3 but not on day 5 of development. The influence of GHRH on catecholalminergic neuronal expression appears to be GHRH-mediated and not through its influence on growth hormone (GH), since growth hormone administration during the same critical period produces no changes in TH activity (Kentroti and Vernadakis, 1989). The critical period for the GHRH effect observed in our study is not unique to this neuropeptide. The requirement for NGF during maturation of sensory and sympathetic neurons exhibits an absolute critical period response: sensory neurons require NGF early in fetal life, whereas survival of sympathetic neurons is dependent on NGF exposure during later development (Black, 1986). Thus, different neuronal phenotypes require the same trophic molecule at distinctly different stages of development. A possible mechanism by which GHRH may influence catecholaminergic neuronal phenotypic expression would be to promote differentiation of catecholaminergic neurons from neuroblasts. However, since both sympathoblasts and primordial peptidergic neurons arise concomitantly from differentiating neural crest cells in the avian embryo (Fontaine-Perus et al., 1982; Le Douarin and Teillet, 1974), the possibility that GHRH is involved in the differentiation of neuroblasts to catecholaminergic neurons is not very likely. In addition, studies in the rat

embryo have shown that the ontogenesis of both TH and catecholamine immunoreactivity occurs at 12.5 days of gestation (36–37 somite stage) (Cochard et al., 1978), whereas GHRH immunoreactivity appears around E18 (see earlier section). These findings suggest that differentiation of catecholaminergic neurons precedes that of peptidergic neurons containing GHRH.

Another mechanism by which GHRH may affect catecholaminergic expression is to increase survival of catecholaminergic neurons in the chick brain. A major component of neurogenesis involves cellular degeneration and death (Hamburger and Levi-Montalcini, 1949; Cowan et al., 1984). As discussed earlier in this section, neuronal survival in culture can be regulated by neuropeptides (Brenneman and Nelson, 1987; Cowan et al., 1984). Nerve growth factor and insulin-like growth factor II enhance significantly the survival of embryonic sensory and sympathetic neurons at various stages of development (Barde et al., 1980; Recio-Pinto et al., 1986). Bombesin and epidermal growth factor have been shown to promote mitogenic activity in chick otic vesicle neurons in culture (Represa et al., 1988). On the other hand, Brenneman and Foster (1987) have reported that GHRH significantly reduces the survival of spinal cord neurons in culture. In contrast, our data suggest that the effect of GHRH administered to chick embryos during a critical period of neuroembryogenesis, from E1 to E3, may enhance the survival of catecholaminergic neurons in the brain, the increased viability being reflected as an increase in TH activity. The apparent discrepancy between our findings and those of Brenneman and Foster may reflect the difference between the *in vivo* and *in vitro* systems.

The role of GHRH as a neuromodulator has only recently been investigated with the report of Rivier et al. (1982) that GHRH acts along the hypothalamic-hypophysial axis as a potent releasing factor for SRIF. Within the hypothalamus, GHRH regulates homeostasis with respect to growth hormone release by both stimulating the release of SRIF (Aguila and McCann, 1983) and suppressing its own release via an ultrashort loop feedback mechanism (Lumpkin et al., 1985; Stachura et al., 1988). Based on our findings (Kentroti and Vernadakis, 1989), GHRH may be a neuromodulator involved in the maturation of catecholaminergic neurons. Supplemental treatment of embryos with GHRH during the critical period enhances the activity of TH to a level comparable to that observed at a later stage of development (i.e., 8-day-old embryos exhibited a level of TH activity equal to that in 10-day-old embryos). The possibility that GHRH may shift the time course of neuronal maturation warrants consideration. Endogenous peptides have been shown to mediate the time course of developmental events. For example, Hayashi et al. (1985) have shown that NGF accelerates the normally occurring changes in neuronal SRIF and SP content and in TH activity in the developing chick embryo. We suggest that GHRH acts to increase the survival of catecholaminergic neurons. Early reports of Le Douarin and Teillet (1974) have shown that all cells of the neural crest can potentially give rise to catecholaminergic or cholinergic phenotypes. In the normally occurring sequence of events cholinergic traits appear earlier in

ontogeny than catecholaminergic traits in primordial neurons of the neural crest (Fauquet et al., 1981; Kellogg et al., 1971). Moreover, the events culminating in adrenergic or cholinergic expression can be modified by soluble factors elaborated by non-crest cells (Vernadakis, 1981), including the neural tube (Howard and Bronner-Fraser, 1986). Neuropeptides in general and GHRH in particular may be examples of neurohumoral factors that regulate catecholaminergic expression in the developing chick embryo.

Cholinergic Expression. As presented earlier, GHRH enhances expression of the catecholaminergic phenotype when administered *in ovo* during a critical period of neuroembryogenesis (Kentroti and Vernadakis, 1990b). We have also tested GHRH and its functional antagonist, SRIF, on expression of the cholinergic phenotype, using the activity of ChAT as a biochemical marker. Neuroblasts of the neural crest and tube give rise to the cholinergic system very early during embryogenesis (Fauquet et al., 1981; Le Douarin, 1980). The ontogenesis of cholinergic neurons is reflected by the profile of ChAT activity in the embryonic brain during development (Kentroti and Vernadakis, 1990a; Mangoura et al., 1988; Vernadakis and Arnold, 1980). Enzyme activity is detected as early as 2 days of embryonic age (Vernadakis et al., 1986; Mangoura et al., 1988; Kentroti and Vernadakis, 1991a) and increases steadily through 12 days of embryonic age in the chick. The increase in enzyme activity slows through 18 days, the period corresponding to normal neuronal death (Kentroti and Vernadakis, 1991d; Hamburger and Oppenheim, 1982), followed by a sharp increase around the time of hatching (20 days). When chick embryos receive GHRH or SRIF *in ovo* on days 1, 3, 5, and 7 a significant increase in the activity of ChAT is observed when embryos are sacrificed at E8 (Kentroti and Vernadakis, 1990a). Similar findings are obtained when GHRH or SRIF is administered in a single dose on day 2 or 3 of development. However, embryos receiving the same dose of GHRH on day 1 or 5 exhibit no significant difference in ChAT activity. Thus, embryonic days 2–3 are the critical developmental window for presumptive cholinergic neuroblasts to respond to GHRH. Again, that this effect is not mediated through growth hormone is demonstrated by the finding that when growth hormone is administered during the same critical period (day 3) no difference is observed in ChAT activity as compared to controls. Thus, the effects of GHRH on enzyme activity do not appear to be mediated through growth hormone (Kentroti and Vernadakis, 1990a). We interpret the increase in ChAT activity to represent an enhancement in cholinergic neuronal maturation rather than an effect of GHRH on the enzyme.

The trophic potential of neuropeptides in modulating the events of cholinergic neuronal maturation is rapidly gaining acceptance. GHRH and SRIF may be examples of these endogenous neuropeptides that possess cholinotrophic properties. When these peptides are tested directly on neuron-enriched cultures derived from 3- or 6-day-old chick embryos, the critical period of sensitivity to GHRH and SRIF remains intact. In cultures derived from 3-day-old whole chick embryo, consisting primarily of neuroblasts, GHRH or SRIF elicits a dose-dependent

increase in ChAT activity, whereas no response is observed in cultures derived from 6-day-old chick embryo whole brain, consisting primarily of differentiated neurons (Kentroti and Vernadakis, 1990a). These findings not only substantiate the *in ovo* observations but indicate that GHRH and SRIF may elicit their effects on proliferating neuroblasts (E3) and not on more mature differentiated neurons (E6).

GABAergic Expression. The ontogenesis of the GABAergic system in the CNS has been described by many investigators (Kentroti and Vernadakis, 1991c; Mangoura and Vernadakis, 1988; Vernadakis et al., 1986; Jong et al., 1986; Lauder et al., 1986). GABAergic neuronal elements, verified both biochemically and immunocytochemically, are present by 3 days of embryonic development in the chick embryo (Mangoura and Vernadakis, 1988). As discussed earlier, neuroblasts at this embryonic stage of development are actively proliferating and are characterized by a high degree of plasticity, as demonstrated in both cholinergic and catecholaminergic populations (Kentroti and Vernadakis, 1989, 1990a). The plasticity of early developing neuroblasts is further demonstrated in the response of presumptive GABAergic neuroblasts to GHRH or SRIF. When chick embryos are administered GHRH or SRIF *in ovo* on embryonic days 1, 3, 5, and 7 and sacrificed at day 8, GABAergic neuronal expression is retarded, as shown by reduced GAD activity. Similar results are obtained when GHRH is administered in a single dose at day 1 or 3 or when SRIF is administered in a single dose at day 3. In contrast, embryos treated with either GHRH or SRIF on day 5 of development show no changes in GABAergic neuronal expression (Kentroti and Vernadakis, 1991b). These findings together with the previous observations that GHRH and SRIF enhance cholinergic and catecholaminergic neuronal phenotypic expression (Kentroti and Vernadakis, 1989; 1990a) support the existence of a mechanism whereby homeostatic balance is innately maintained in developing neuronal systems. As previously described, embryos exhibit a discrete critical period of sensitivity to the effects of neuropeptide administration with an optimum efficacy occurring between E1 and E3. This developmental window (E1–E3) is common for excitatory cholinergic and catecholaminergic elements as well as inhibitory GABAergic elements.

VI. CONCLUSIONS

Considerable evidence is now available concerning the early presence of several neuropeptides during early neuroembryogenesis. The high but transient endogenous levels of some peptides such as SRIF, VIP, SP, NPY, and NT has led to the conclusion that these peptides are involved in early mechanisms of neuronal differentiation and growth. Although the research on the ontogeny of neuropeptide receptors is not extensive, the decline in receptor binding sites from development to adulthood is further supportive evidence that neuropeptides have neuromodulatory actions during early neuroembryogenesis. Finally, recent research attempting to explore the neuromodulatory actions of neuropeptides during early brain development clearly demonstrates that neuropeptides are actively involved in (*a*) neuro-

nal homeostatic balance via their influence on neuronal survival and (*b*) the establishment of neuronal phenotypes by influencing neuronal plasticity during early neuronal differentiation.

This field of neuropeptide research is actively pursued, and it can be projected that further research in neuropeptides will reveal that these substances may eventually be used as therapeutic agents in specific developmental brain disabilities.

ADDENDUM

A delay in publication and not oversight accounts exclusion of the most recent references.

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PHOSPHORYLATION OF NEUROFILAMENT PROTEINS

Michael G. Sacher, Eric S. Athlan, and Walter E. Mushynski

ABSTRACT

This chapter provides an overview of neurofilament structure and phosphorylation, with major emphasis on protein kinases and protein phosphatases involved in the modification of neurofilament proteins. Recent advances in the identification of protein kinases that act on neurofilaments can now be complemented by studies on protein phosphatases due to the availability of highly potent and specific phosphatase

Advances in Neural Science,
Volume 2, pages 47–65.

ISBN: 1-55938-625-8

inhibitors such as okadaic acid. We show that treatment of cultured dorsal root ganglion neurons with okadaic acid caused a shift in the electrophoretic mobilities of neurofilament subunits, signifying their increased phosphorylation. This was accompanied by the ordered conversion of neurofilament subunits to Triton X-100-soluble forms and by marked changes in their immunofluorescent staining patterns. These results indicate that neurofilaments are dynamic entities whose assembly and organization are modulated by the balanced addition and removal of phosphate moieties.

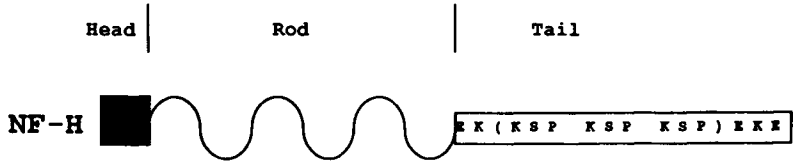
I. INTRODUCTION

Neurofilaments (NFs) belong to a class of cytoskeleton components known as intermediate filaments. Intermediate filaments have a diameter of approximately 10 nm, and their protein subunits can be divided into five subclasses on the basis of sequence comparisons (see Steinert and Roop, 1988, for a review). The expression of these different proteins is characterized by high levels of tissue specificity (Osborn and Weber, 1982). NFs represent the principal intermediate filament found in neurons. Other neuron-specific intermediate filament proteins such as peripherin (Portier et al., 1984; Parysek et al., 1988), α-internexin (Fleigner et al., 1990), and nestin (Lendahl et al., 1990) are either limited in distribution or appear transiently during development.

In common with other intermediate filament proteins, NF subunits contain a highly conserved α-helical "rod" domain, which is flanked on one side by the N-terminal "head" domain and on the other by the C-terminal "tail" domain (Geisler et al., 1983). This domain organization is illustrated for one of the NF subunits in Figure 1. The biochemical and antigenic differences between intermediate filament proteins from different subclasses are to a considerable degree due to the variable head and tail domains (Steinert and Roop, 1988). The formation of filaments of similar diameter and morphology by the different proteins reflects involvement of the highly conserved rod domain, which enables different intermediate filament subunits to co-assemble in cells transfected with heterologous cDNAs (Monteiro and Cleveland, 1989; Chin and Liem, 1990).

Mammalian NFs are composed of three subunits with apparent molecular masses of about 68 kDa (NF-L), 150 kDa (NF-M), and 200 kDa (NF-H), as determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Hoffman and Lasek, 1975). The latter size estimates were shown to be too high because of the anomalous behavior of NF proteins on SDS-PAGE (Kaufmann et al., 1984; Georges and Mushynski, 1987). Actual values for NF-L, NF-M, and NF-H, based on sequencing data, are about 62 kDa (Geisler et al., 1985), 100 kDa (Myers et al., 1987), and 115 kDa (Julien et al., 1988), respectively.

The size differences between the three NF subunits are largely due to variations in the length of their C-terminal tail domains, which are rich in charged amino acids (Geisler et al., 1983; Julien and Mushynski, 1983) and can be seen to project from



Source of NF-H	Number of KSP Repeats	Most Abundant Repeat Unit(s)	Reference
Human	43	KSPEKAKSP$^{V}_{A}$KEEA	Lees et al., 1988
Mouse	51	KSP$^{G}_{A}$EA	Julien et al., 1988
Rat	52	KSPAE$^{V}_{A}$	Chin and Liem, 1990

Figure 1. Domain structure of NF-H and consensus sequences of the most abundant repeated motifs in the tail. The amino acid sequences are listed using the single letter symbols: A, Ala; E, Glu; G, Gly; K, Lys; P, Pro; S, Ser; V, Val. The amino acid symbols depicted in the tail portion of the diagram of NF-H emphasize the high levels of these residues in the domain.

the filament axis in rotary shadowed samples of NFs (Hisanaga and Hirokawa, 1988). The tail domain of NF-H forms apparent cross-bridges between NFs and other structures (Hirokawa et al., 1984), but the nature and role of these apparent contacts are still unknown.

Transfection studies with variously deleted NF subunit-encoding cDNAs have indicated that sequences in the head domain are required for early steps in filament assembly. Deletions within the C-terminal region of the rod domain yield mutant proteins whose incorporation into intermediate filaments in small amounts cause their rapid disruption (Wong and Cleveland, 1990; Gill et al., 1990). The latter effect indicates that NFs are dynamic entities capable of rapid subunit exchange (Angelides et al., 1989; Steinert and Liem, 1990) rather than the stable, unchanging structures they were once thought to be (Lasek et al., 1983). The presence of soluble forms of NF subunits in cells may in part reflect this exchange process (Georges et al., 1987; Lindenbaum et al., 1987).

II. NEUROFILAMENT PHOSPHORYLATION

A. General Background

The NF subunits from rat myelinated axons contain, on average, about 3, 6, and 14 moles of phosphate per mole of NF-L, NF-M, and NF-H, respectively. These

numbers are lower than the initially reported values (Julien and Mushynski, 1982), because they have been calculated using the true molecular weights of NF subunits which became available more recently (see above). Subsequent reports showed similar levels of phosphate in bovine (Wong et al., 1984), porcine (Georges et al., 1986), and human NFs (Ksiezak-Reding and Yen, 1987), although bovine NFs have also been reported to contain much higher amounts of phosphate (Jones and Williams,1982; Ksiezak-Reding and Yen, 1987). Treatment of NFs with alkaline phosphatase causes an increase in the mobilities of NF-M and NF-H on SDS-PAGE (Julien and Mushynski, 1982). This effect of phosphorylation on the gel electrophoretic mobilities of NF-M and NF-H provides a simple means of assessing the approximate phosphorylation states of NF-M and NF-H, keeping in mind that the subunits from exhaustively dephosphorylated NFs still contain significant levels of phosphate (Georges et al., 1986; Ksiezak-Reding and Yen, 1987).

The analysis of proteolytic cleavage products of NFs showed that the phosphate moieties in NF-M and NF-H were for the most part located in the tail domain (Julien and Mushynski, 1983), and ^{31}P-NMR analysis verified that those in NF-H were clustered (Zimmerman and Schlaepfer, 1986). The sequence characteristics of the phosphorylated region were reported by two groups in 1987. Myers et al. (1987) showed that the tail domain of human NF-M contains six tandem repeats of a 13 amino acid sequence, each containing two copies of the sequence Lys-Ser-Pro (henceforth referred to as KSP, using the single letter abbreviations for these amino acids). Geisler et al. (1987) reported that the tail domains of porcine NF-M and NF-H contained degenerate repetitive sequences with the KSP motif. The Ser in this repeated triplet represents the major phosphorylation site in NF-M and NF-H (Geisler et al., 1987; Lee, V.M.-Y. et al., 1988; Xu et al., 1992). Mouse (Levy et al., 1987) and rat (Napolitano et al., 1987) NF-M each contain only five copies of the KSP sequence, and these are not clustered to the degree seen in human NF-M. As indicated in Figure 1, the KSP triplet is repeated 51 times in the tail domain of mouse NF-H (Julien et al., 1988), 52 times in rat NF-H (Chin and Liem, 1990), and 43 times in human NF-H (Lees et al., 1988).

The KSP repeats are highly conserved in large NF proteins (Lee et al., 1986; Mencarelli et al., 1991), although there is some interspecies variation in the length and sequence of the spacers that separate this triplet sequence (Fig. 1; also see Geisler et al., 1987). A survey of available protein sequences indicates that the KSP motif is comparatively rare. Next to the large NF subunits, KS/TP occurs most prominently in histone H1 (Suzuki, 1989). MAP-2 and tau each contain two KSP sequences (Lee, G., et al., 1988; Lewis et al., 1988), whereas nestin contains one (Lendahl et al., 1990). There appears to be a redundancy of KSP phosphorylation motifs in NF-H, as less than one-third of the sites are modified in highly phosphorylated NF-H from rat myelinated axons (Julien and Mushynski, 1982; Chin and Liem, 1990).

The discovery of monoclonal antibodies that could distinguish between phosphorylated and dephosphorylated forms of NF-H and NF-M (Sternberger and

Sternberger, 1983) represented a major breakthrough in NF research. These antibodies, which recognize KSP repeat domains (Lee, V. M.-Y., et al., 1988), provide a facile means of monitoring the phosphorylation states of NF-M and NF-H *in situ* or by Western blot analysis. Their use has shown that the NF-H and NF-M in axons are generally more highly phosphorylated than those in the perikaryon and dendrites, although certain pathological states are associated with increased phosphorylation of perikaryal NFs (Schlaepfer, 1987). The extensive library of monoclonal antibodies characterized by Lee and co-workers provides unprecedented levels of discrimination between a broad range of phosphorylation states (Carden et al., 1985; Lee et al., 1987). Some of the antibodies can also be used to estimate the spacing between phosphate moieties (Clark and Lee, 1991b).

The structural organization of NFs is generally regarded as consisting of a central, thread-like core composed of associated rod domains, with a peripheral zone of highly charged lateral projections representing the tail domains of NF subunits (Julien and Mushynski, 1983; Weber et al., 1983; Hisanaga and Hirokawa, 1988). The multiple phosphorylation sites in the tail domains are important elements of this charged peripheral zone. Their capacity to undergo cyclical phosphorylation/dephosphorylation (Nixon and Lewis, 1986) represents a potential mechanism for modulating conformational changes (Otvos et al., 1988) relevant to tail domain function(s). The enzymes that mediate these cyclical changes are therefore of utmost relevance to any discussion of NF phosphorylation.

B. Protein Kinases

A number of protein kinase activities copurify with mammalian NFs and/or phosphorylate NF subunits *in vitro* (see Table 1). Although some of these associations may be physiologically relevant, artefactual binding of kinases to the highly charged NF proteins might also occur. Additional information is therefore required to establish whether a protein kinase phosphorylates NFs *in vivo*.

The major activity copurifying with NFs is regulator-independent and shares some properties with casein kinase I (Julien et al., 1983). A similar protein kinase is associated with squid NFs (Floyd et al., 1991), indicating that the relationship is conserved in nerve cell evolution. The regulator-independent protein kinase was implicated in the *in vivo* phosphorylation of NFs by comparative mapping of phosphopeptides from *in vivo* and *in vitro* ^{32}P-labeled NF subunits (Julien and Mushynski, 1981).

Fractionation of the regulator-independent activity yielded a so-called NF kinase that also phosphorylated other proteins (Toru-Delbauffe et al., 1986) as well as a NF-specific kinase that phosphorylated the tail domain of NF-H exclusively (Wible et al., 1989). The latter enzyme did not act on dephosphorylated NF-H, indicating that prior phosphorylation of the substrate is required.

Protein kinase A and protein kinase C also copurify with NFs and have been shown to phosphorylate NF subunits *in vitro*. The preferred substrate for the two

Table 1. Protein Kinases That Phosphorylate Mammalian Neurofilament Proteins

Type Protein Kinase	*Copurifies with NFS*	*NF Phosphorylation*		*Preferred Substrate*	*References*
		In Vitro	*In Vivo*		
Regulator-independent	+	+	Mapping	M > L > H	Julien and Mushynski, 1981; Runge et al., 1981; Shecket and Lasek, 1982; Julien et al., 1983
NF kinase	+	+	ND	H > M > L	Toru-Delbauffe et al., 1986
	+	+	ND	H	Wible et al., 1989
	–	+	ND	KSP sites	Roder and Ingram, 1991
Protein kinase A	ND	+	–	M >> H, L	Leterrier et al., 1981
	+	ND	ND	Peptide	Caputo et al., 1989; Dosemeci et al., 1990; Dosemeci and Pant, 1992
	ND	+	Mapping	L, M head	Sihag and Nixon, 1989, 1990
Protein kinase C	ND	+	Mapping	M > L > H	Sihag et al., 1988
	+			M head	Sihag and Nixon, 1990
		ND	ND	Peptide	Dosemeci et al., 1990
	ND	ND	Phorbol	M > L > > H	Georges et al., 1989; Clark and Lee, 1991a
Ca^{2+}/Calmodulin kinase II	+	+	ND	M > L > H	Vallano et al., 1985
	+	+	Mapping	L	Sihag and Nixon, 1989
	+	ND	ND	Peptide	Caputo et al., 1989; Dosemeci et al., 1990
cdc2 kinase	ND	+	ND	KSP sites	Hisanaga et al., 1991; Guan et al., 1992
Protein kinase FA	ND	+	ND	M > L, H	Guan et al., 1991

Notes: "Copurifies with NFs" denotes protein kinases found in NF-enriched fractions (+). Involvement in the *in vivo* phosphorylation of NFs determined by comparative phosphopeptide mapping (mapping) of *in vivo* and *in vitro* ^{32}P-labeled NF subunits or by activation of protein kinase C (phorbol ester) in metabolically labeled cell cultures. Under the heading "preferred substrate," L, M, H refer to the NF subunits, "head" and "tail" refer to the N- and C-terminal domains, "peptide" refers to cases where kinase-specific peptide substrates were employed, "KSP sites" refers to the repeats in the tail domains of NF-M and NF-H. ND, not determined.

enzymes is NF-M (Table 1). Sihag and Nixon (1989, 1990) demonstrated by comparative phosphopeptide mapping that the two kinases phosphorylate sites in the head domain of NF-M and NF-L while the regulator-independent kinase phosphorylates sites in the tail domain. This work led to the proposal that head and tail domain phosphorylations are under separate regulation. The *in vivo* phosphorylation of NF subunits by protein kinase C has also received more direct support from studies showing that phorbol esters stimulate NF phosphorylation in cultured cells (Georges et al., 1989; Clark and Lee, 1991a).

The role of Ca^{2+}/calmodulin kinase II in NF phosphorylation is unclear at present, although comparative phosphopeptide mapping suggests that it may be involved

(Sihag and Nixon, 1989). The rationale for studying the *in vitro* phosphorylation of NF proteins by protein kinase F_A (Guan et al., 1991) is based on its involvement in the sequential phosphorylation of glycogen synthase. This process, also known as hierarchical phosphorylation (Roach, 1991), involves the creation of a phosphorylated recognition site by a protein kinase, followed by one or more phosphorylations by a second kinase that requires such phosphorylated sites in the substrate protein. Multiply phosphorylated proteins such as NF-M and NF-H are potential candidates for this type of interdependent phosphorylation. It is noteworthy that the NF kinase isolated by Wible et al. (1989) appeared to require prior phosphorylation of the tail domain, perhaps signifying the participation of a second enzyme in phosphorylation of the KSP repeats. A potential candidate for this role might be a protein kinase related to cdc2 kinase.

The cdc2 kinases from starfish oocytes (Hisanaga et al., 1991) and mammalian cells (Guan et al., 1992) were shown to phosphorylate the tail domain of dephosphorylated NF-H. The addition of only four phosphates per polypeptide to dephosphorylated bovine NF-H by the starfish enzyme was sufficient to return its electrophoretic mobility to the position of fully phosphorylated NF-H. Since it appeared that the less abundant KSPXK motif was phosphorylated by the starfish cdc2 kinase (Hisanaga et al., 1991), phosphorylation of the KSP repeat may involve a hierarchical type of mechanism.

The NF kinases purified by Roder and Ingram (1991) appear to phosphorylate the KSP repeats in exhaustively dephosphorylated NF-H and NF-M as they restore the phosphorylated epitopes recognized by the monoclonal antibody SMI 31 (Sternberger and Sternberger, 1983). However, one of these activities (PK40) phosphorylated NF-M at a much higher stoichiometry than NF-H, achieving only a partial shift in the mobility of the latter subunit. The second kinase (PK36) phosphorylated NF-H very poorly. These results suggest again that phosphorylation of the KSP repeats may involve more than one enzyme.

It is not known at present whether the enzyme(s) involved in phosphorylating KSP sites in NF-M and NF-H is (are) found exclusively in neurons. Transfected NF-H expressed in fibroblasts is not phosphorylated (Chin and Liem, 1990; Nash and Carden, 1991), whereas transfected NF-M is (Chin and Liem, 1990). Human NF-M, which contains multiple tandem repeats of the KSP sequence similar to but not as extensive as those seen in NF-H, is phosphorylated at these sites in transfected mouse L cells (Pleasure et al., 1990). Clearly, more work is required to explain these seemingly disparate results.

C. Protein Phosphatases

The preceding section has provided an overview of the extensive literature on NF phosphorylation by NF-associated and exogenous protein kinases. In marked contrast, little is known about the protein phosphatase(s) that act on NF proteins *in vivo*, as exemplified by the lack of information on this subject in reviews of NF

phosphorylation that have appeared within the past 5 years (Schlaepfer, 1987; Matus, 1988; Nixon and Sihag, 1991). Although phosphate turnover in NF proteins has been reported in a number of systems (e.g., Nixon and Lewis, 1986; Georges et al., 1987; Lindenbaum et al., 1987), a survey of the recent literature turned up only a few reports on NF-associated phosphatase activity (Guru et al., 1991; Shetty et al., 1992).

Phosphate turnover reflects the combined actions of protein kinases and phosphatases, the relative activities of the two types of enzyme determining the phosphorylation state of NF proteins. The recent report that okadaic acid (OA) increases vimentin phosphorylation in fibroblasts (Yatsunami et al., 1991) prompted us to study its effect on NF proteins. OA is a specific and highly potent inhibitor of protein phosphatase 1 (PP-1) and protein phosphatase 2A (PP-2A) (Bialojan and Takai, 1988). This toxin is a complex fatty acid derivative made by dinoflagellates, and its hydrophobicity allows it to be used on intact cells to identify physiological substrates of PP-1 and PP-2A (see Hardie et al., 1991, for a review).

The system we selected to study the effects of OA on NF phosphorylation consisted of primary cultures of dissociated dorsal root ganglia (DRG) from E15 rat embryos (Windebank et al., 1985) maintained in a defined medium (Bottenstein and Sato, 1979). Our previous work has shown that neurons in these cultures express high levels of all three NF subunits and that NF-M and NF-H are present in a broad range of phosphorylation states.

Different concentrations of OA were tested for their effects on the behavior of NF subunits on SDS-PAGE, as increased phosphorylation of the KSP repeats in the tail domains of NF-M and NF-H is known to reduce the electrophoretic mobilities of the subunits (Julien and Mushynski, 1982). Figure 2 shows that a reduction in the mobility of the most highly phosphorylated form of NF-H (pH) occurred after the DRG neurons were exposed to 10 nM OA for 6 hours. On the other hand, the mobility of the hypophosphorylated form of NF-H (H) did not shift noticeably until 100 nM OA and then continued to decrease at higher OA concentrations. The mobility shift of the highly phosphorylated form of NF-H at 10 nM OA was unexpected because the concentrations of PP-1 and PP-2A in most cells approach the micromolar range (Hardie et al., 1991). This result may indicate that phosphate turnover on highly phosphorylated NF-H is more rapid than on the hypophosphorylated form, or that more phosphates must be added to the latter to obtain an observable shift in mobility. However, *in vitro* studies with a cdc2 kinase have shown that the maximal shift in mobility of dephosphorylated NF-H is observed after phosphorylation of only four of the repeated KSP sequences (Hisanaga et al., 1991). This *in vivo* response to 10 nM OA indicates that PP-2A may be involved in dephosphorylation of the NF-H tail domain, because it is more sensitive to OA inhibition ($IC_{50} \approx 0.1$ nM) than is PP-1 ($IC_{50} \approx$ (10–15) nM) (Cohen, 1991). In addition, PP-2A has been shown to dephosphorylate the KS/TP sites in histone H1 that are phosphorylated by cdc2 kinase (Sola et al., 1991).

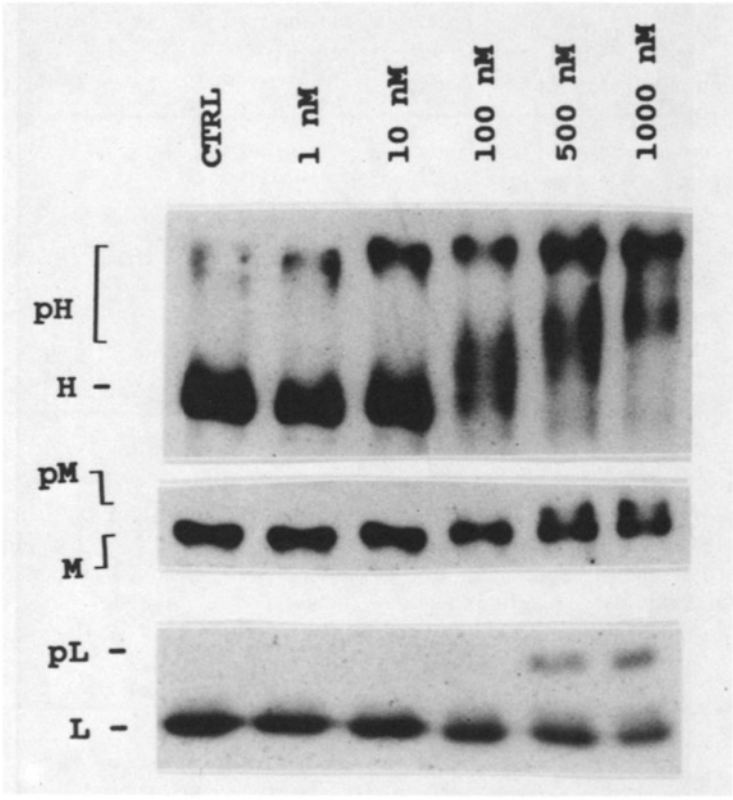


Figure 2. Effect of different okadaic acid concentrations on neurofilament subunits in dorsal root ganglion neurons. DRG cultures were treated for 6 hours with increasing concentrations of OA. Total protein was fractionated on a 5% SDS-polyacrylamide slab gel, transferred to polyvinylidene fluoride (PVDF) membrane, and detected using specific antibodies to the three NF subunits obtained from Sigma. H, M, and L refer to the NF subunits containing low phosphate levels, and pH, pM, and pL refer to the more highly phosphorylated forms of the subunits.

The appearance of slower migrating forms of NF-M (pM) and NF-L (pL) was observed at 500 nM OA and persisted at 1000 nM OA (Fig. 2). Similar to the situation for NF-H, the effect of phosphorylation on the gel electrophoretic mobility of NF-M could be due to modification of the KSP repeats in the tail domain. On the other hand, NF-L does not contain similar KSP sequences and the nature of other sites whose phosphorylation would reduce the mobility of the subunit is unknown. As shown by two-dimensional gel electrophoresis (Fig. 3), the mobility changes were accompanied by decreases in the isoelectric points of NF-M and NF-L to more acidic values, because of increased phosphorylation.

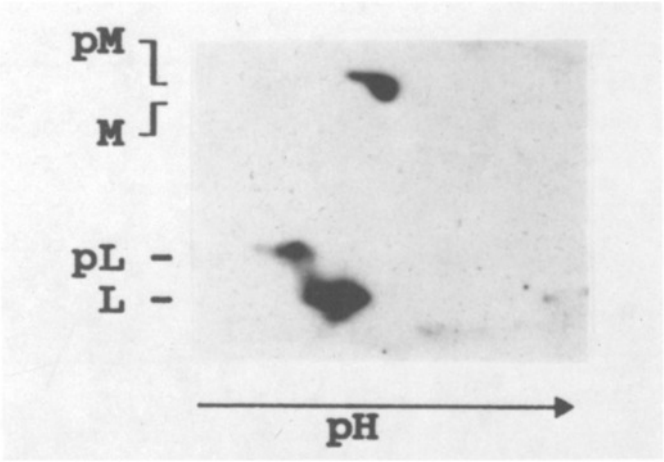


Figure 3. Two-dimensional gel electrophoresis of dorsal root ganglion cultures treated with okadaic acid. DRG cultures were treated with 1000 nM OA for 6 hours, harvested in 9.5 M urea, 2% Triton X-100, 5% β-mercaptoethanol, and ampholytes in the pH range 5–8, focused in tube gels in the first dimension; and fractionated on a 5% SDS-polyacrylamide slab gel in the second. Proteins were transferred to PVDF membrane and detected with antibodies against NF-L and NF-M. The subunits are designated as described in the legend to Figure 1.

We chose to use OA at 1000 nM in all subsequent experiments since this concentration has been recommended for studies with intact cells because of their high levels of PP-1 and PP-2A (Hardie et al., 1991). Since *in vitro* phosphorylation of the head domain of NF-L homopolymer has been shown to block its assembly and cause disruption of NF-L (Gonda et al., 1990; Hisanaga et al., 1990; Nakamura et al., 1990), we determined whether the change in phosphorylation state of NF proteins that accompanies OA treatment affected their state of assembly. This was accomplished by extracting OA-treated cells in Triton X-100-containing buffer and centrifuging to separate the detergent-insoluble cytoskeleton from soluble subunits. In control cells, all three NF subunits were found in the cytoskeleton fraction. Within half an hour almost all of the NF-H appeared in the supernatant fraction along with smaller proportions of NF-M and NF-L. By 3 hours all of the NF-M was redistributed to the supernatant fraction, whereas complete disappearance of NF-L from the pellet fraction was seen at 6 hours. The entire process was completely reversed within 24 hours of removing OA from the cultures. The order in which the NF subunits reassociated with the cytoskeletal fraction after removal of OA was the exact reverse of their order of dissociation (Sacher et al., 1992).

Under the centrifugation conditions we had used to separate Triton-soluble and -insoluble components, the soluble fraction has been shown to contain oligomeric NF proteins in addition to the subunits (Shea et al., 1988). Since the order of appearance of NF subunits in the Triton-soluble fraction progressed from NF-H to NF-M and finally NF-L, it is unlikely that the OA-induced conversion to soluble forms was due to the simple fragmentation of NFs. It is interesting to note that the dissociation pattern conforms with models of NF organization in which NF-L is proposed to form the filament core, whereas NF-M and NF-H have a peripheral localization (see Tokutake, 1990, for a review). This sequential solubilization of NF subunits should be accounted for in any model of heteropolymeric NF assembly, because it implies that the different subunits may be incorporated as separate units.

The shift of NF subunits to Triton X-100-soluble forms in OA-treated cultures indicated that the NF network was being disrupted. To verify this point, pairs of cultures were treated with OA for up to 6 hours, and one member of each pair was harvested for Western blot analysis while the other was fixed for immunofluores-

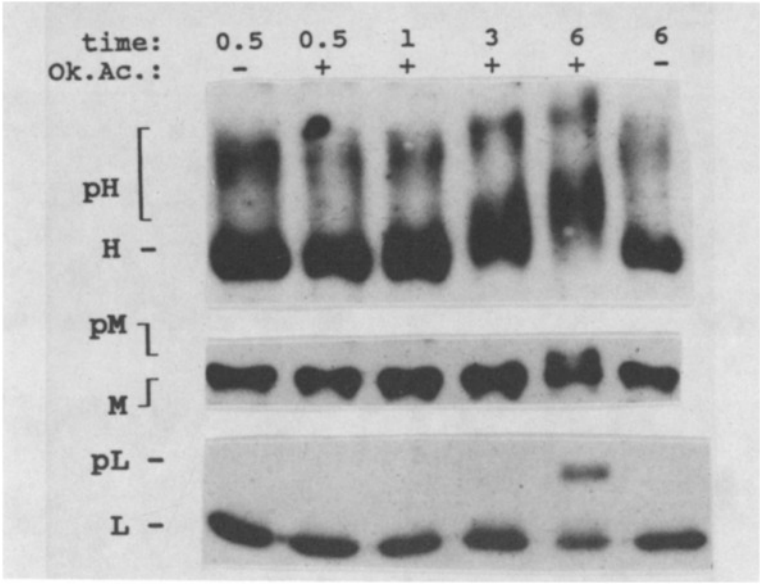


Figure 4. Time course of okadaic acid treatment of dorsal root ganglion cultures analyzed by Western blotting. DRG cultures were treated with 1000 nM OA (+) or without the inhibitor (–) for the stated amount of time. Total protein was fractionated on a 5% SDS-polyacrylamide gel, transferred to PVDF membrane, and detected using antibodies specific to each NF subunit. Sister cultures were used for immunofluorescence staining (see Fig. 5). The NF subunits are designated as described in the legend to Figure 1.

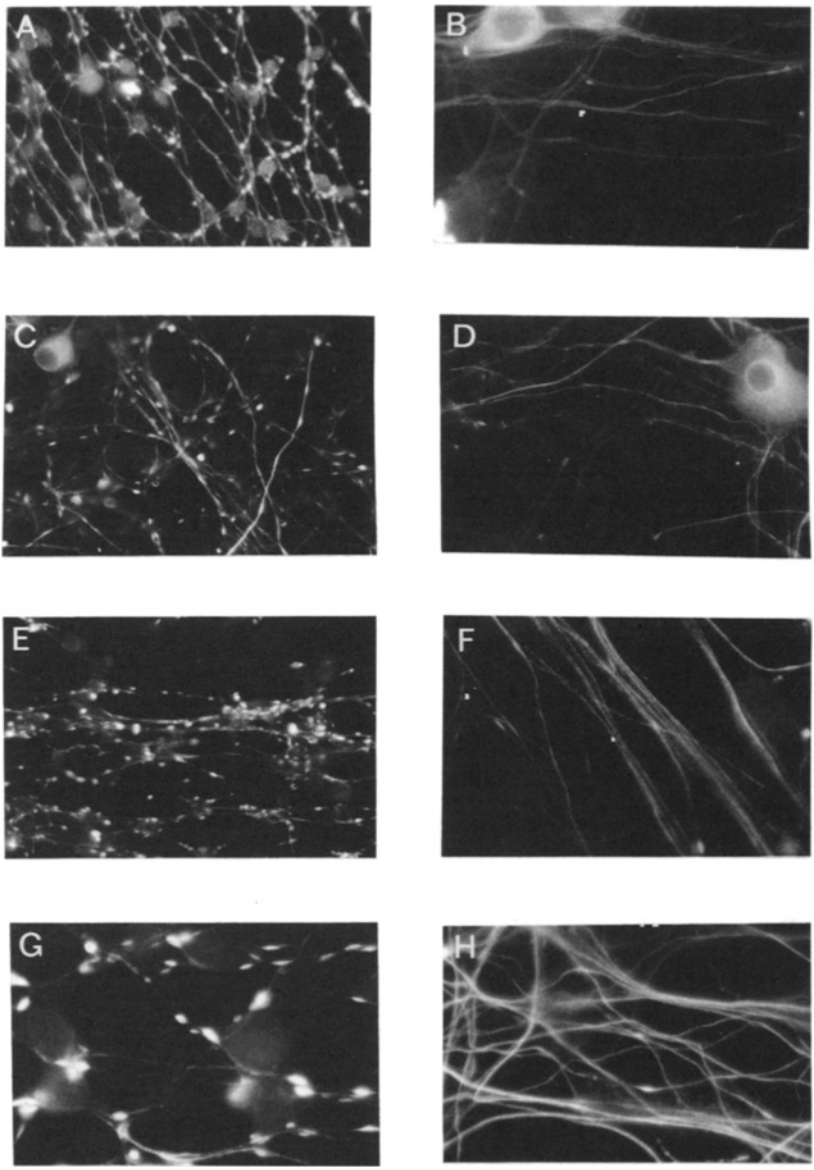


Figure 5. Time course of okadaic acid treatment of dorsal root ganglion cultures analyzed by immunofluorescence microscopy. DRG cultures were treated with OA for 0.5 hour (**A**, **C**, **E**, and **G**) or grown in its absence, fixed with ethanol:acetic acid (90:10), blocked, and stained with SMI31 (**A**, **B**), anti-H (**C**, **D**), anti-M (**E**, **F**), and anti-L (**G**, **H**) antibodies followed by Texas Red conjugated to an anti-mouse secondary antibody.

cence microscopy. As can be seen from the Western blot in Figure 4, NF-L and NF-M increased in apparent molecular weight only after 6 hours, whereas the faster migrating form of NF–H (H) began to shift within 1 hour of OA treatment. Although no changes in the mobilities of NF subunits were seen after 0.5 hours of OA treatment, antibodies to each NF subunit showed marked changes in the immunofluorescence staining pattern (Fig. 5, A, C, E, and G). The continuous axonal staining of control neurons differed markedly from the punctate staining of axons in the OA-treated cells. This punctate staining did not indicate the extent of NF disruption. Electron microscopy of cultures treated with OA for 30 minutes showed that intermediate filaments were still present in neurites, although they were somewhat scarcer than in controls (Fig. 6). The presence of visible intermediate filaments (some, no doubt, consisting of NFs) after 30 minutes of OA treatment is not surprising, considering the fact that significant amounts of only NF-H had been rendered Triton X-100-soluble at this time (Sacher et al., 1992).

Since the preceding results indicated that OA was increasing the phosphorylation state of NF proteins, we wished to confirm that the toxin was affecting phosphate turnover. DRG cultures were metabolically labelled with $^{32}P_i$, washed, and placed in $^{32}P_i$-free medium. OA was added, and samples were harvested at various times and immunoprecipitated with antisera against NF-M and NF-H. Figure 7 shows that in control cells, ^{32}P turnover could be observed over the 6-hour chase period for NF-M and NF-H. The particular rabbit polyclonal anti-NF-H antiserum used in this experiment immunoprecipitated only the highly phosphorylated form of NF-H (pH, cf. Fig. 2). ^{32}P turnover in NF-M and NF-H was essentially halted in OA-treated cells. ^{32}P labeling of the subunits actually appeared to increase during the chase period, as indicated by both increased ^{32}P content and reduced mobility on SDS-PAGE (Fig. 7).

The continued labeling of NF-H in particular could indicate that OA is exerting an effect not only through inhibition of PP-2A and/or PP-1, but also by activating protein kinase(s) that phosphorylates the subunits. A type 2A phosphatase is known to negatively regulate cdc2 kinase (Yamashita et al., 1990; Félix et al., 1990), and *in vitro* studies suggest that a related type of protein kinase may be involved in phosphorylating the KSP repeats in the tail domains of NF-H and NF-M. (Hisanaga et al., 1991; Guan et al., 1992).

The present study indicates that phosphate turnover is taking place on both the head and tail domains of NF subunits. Inhibition of head domain dephosphorylation would account for the disassembly of NFs, whereas hyperphosphorylation of the tail domain would cause the reduced mobility of NF subunits on SDS-PAGE. Although evidence has been presented that head and tail domain phosphorylation are under separate regulation (Nixon and Sihag, 1991), dephosphorylation of the two domains may be mediated by the same enzyme, possibly PP-2A.

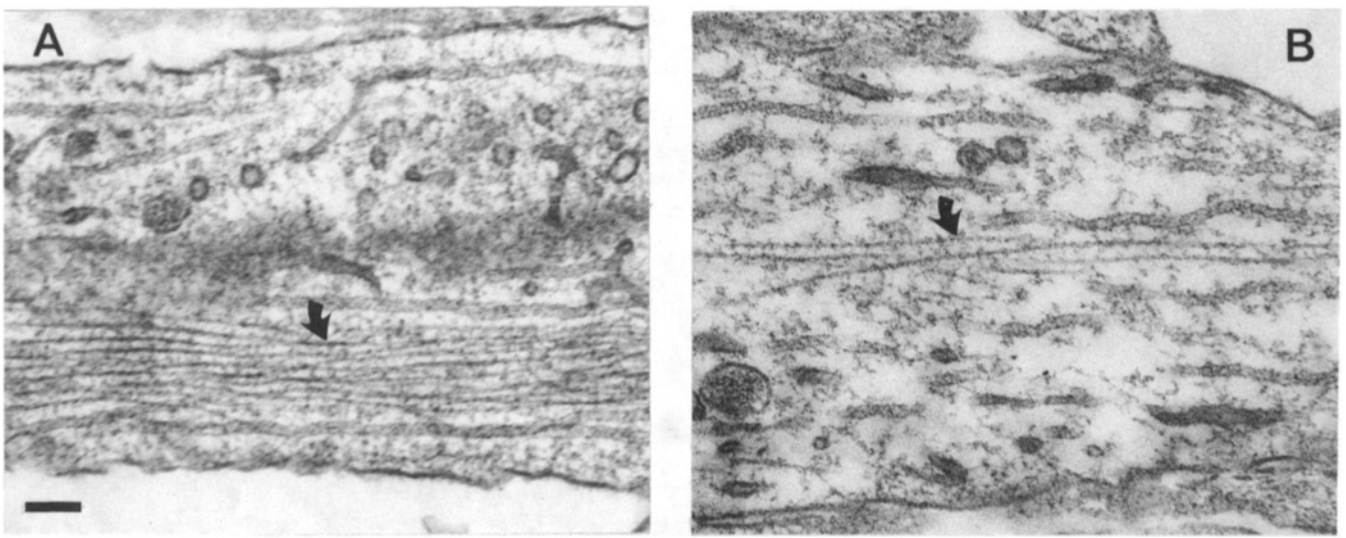


Figure 6. Electron micrographs of neurites from control (**A**) and okadaic acid-treated (**B**) cultures. The curved arrow points to intermediate filaments (presumably NFs) in the two samples. The intermediate filament bundles in neurites from cultures treated with OA for 30 minutes (**B**) were for the most part much sparser than those in controls (**A**). The bar represents 100 nm.

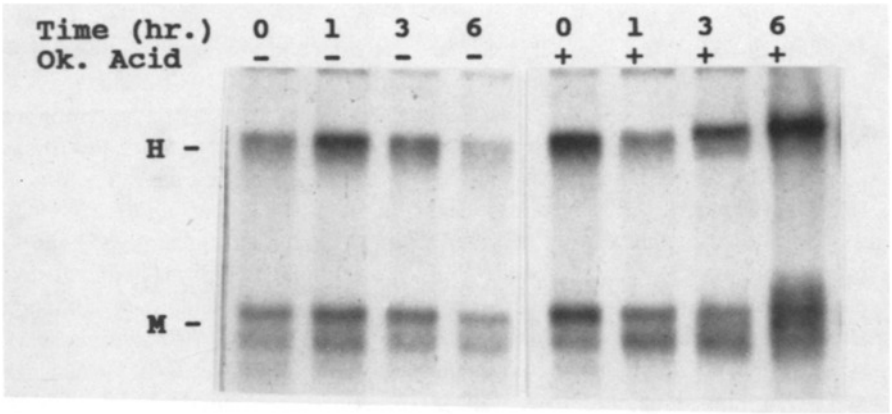


Figure 7. Immunoprecipitation of dorsal ganglion cultures labeled with $^{32}P_i$ and chased in the presence of okadaic acid. DRG cultures were labeled overnight with 500 μCi/ml carrier-free $^{32}P_i$, washed with unlabeled medium, and incubated with (+) or without (–) 1000 OA for the times shown prior to immunoprecipitation with antibodies specific for the three subunits. NF-H, NF-M, and NF-L refer to the locations of rat NF standards.

III. THE ROLE OF NEUROFILAMENT PHOSPHORYLATION

The finding that the head and tail domains of NF subunits are phosphorylated by different protein kinases (Sihag and Nixon, 1989, 1990) suggests that phosphorylation modulates several aspects of NF metabolism. The dephosphorylation of NF subunits by alkaline phosphatase does not affect their *in vitro* assembly (Georges et al., 1986), indicating that the phosphate moieties in isolated NFs are not involved in filament formation. These phosphates are located mainly in the tail domain of the subunits (Julien and Mushynski, 1983). On the other hand, *in vitro* phosphorylation of sites in the head domain of NF-L by protein kinase A or protein kinase C inhibits NF-L assembly and initiates filament disassembly (Gonda et al., 1990; Hisanaga et al., 1990; Nakamura et al., 1990). This effect of head domain phosphorylation has also been observed for other intermediate filament proteins and may represent a general mechanism for regulating their assembly (see Skali and Goldman, 1991, for a review). The disassembly of NFs to Triton-soluble components in OA-treated neurons (see above) suggests that this type of mechanism may operate *in vivo*, although the location of phosphorylation sites in the soluble subunits remains to be determined.

Another role for phosphorylation may be to stabilize NF proteins. *In vitro* studies have demonstrated that dephosphorylation renders NF subunits, and NF-H in particular, more susceptible to proteolysis (Goldstein et al., 1987; Pant, 1988). The increased phosphorylation of NF-H in nerve growth factor-treated PC12 cells might

explain how NF-H levels can increase in these cells without there being a change in the steady-state level of NF-H mRNA (Lindenbaum et al., 1987, 1988).

Phosphorylation has been shown to affect both homologous and heterologous NF interactions. The capacity of NFs to form a reticulated network *in vitro* is abolished by dephosphorylation and reestablished by protein kinase A treatment (Eyer and Leterrier, 1988). Dephosphorylation of NF-H has been reported to block its ability to stimulate tubulin polymerization (Minami and Sakai, 1985) and to promote its association with taxol-stabilized microtubules (Hisanaga and Hirokawa, 1990). The reason for these contradictory results is unknown, although Hisanaga and Hirokawa (1990) found that neither phosphorylated nor dephosphorylated NF-H had any effect on tubulin polymerization. More recently, Hisanaga et al. (1991) showed that inhibition of the binding of NF-H to microtubules could be restored when the dephosphorylated subunit was treated with cdc2 kinase. This indicates that the KSP repeat domain is involved in microtubule binding.

The effect of dephosphorylation on the gel electrophoretic mobilities of NF-H and MF-M (Julien and Mushynski, 1982) may be due at least partly to conformational changes. Studies with synthetic peptides corresponding to segments of the KSP repeat domain of human NF-M showed marked conformational differences between the phosphorylated and unphosphorylated species (Otvos et al., 1988). However, structural changes could not be detected in electron microscopic studies, which showed that inhibition of the phosphate moieties had no effect on the appearance of lateral projections, or on the ability of NFs to form cross-bridges *in vitro* (Hisanaga and Hirokawa, 1989).

The proposal that NFs are determinants of axonal caliber (Hoffman et al., 1985) prompted the suggestion that phosphorylation may enhance the space-filling properties of NFs (Carden et al., 1987). This would account for the higher level of NF phosphorylation in the axon as compared to the perikaryon (Sternberger and Sternberger, 1983). A correlation between NF phosphorylation state and axonal caliber has been noted in studies with Trembler mice (de Waegh et al., 1992). The same correlation has been established in cultured DRG neurons. Myelination increases the caliber of axons in these cultures (Windebank et al., 1985), and we have shown that this change is accompanied by an increase in the phosphorylation state of NF-H (Athlan, E., unpublished results). Similarly, axonal narrowing at the node of Ranvier is accompanied by a reduction in NF phosphorylation (Mata et al., 1992).

ACKNOWLEDGMENTS

This work was supported by a grant from the Medical Research Council of Canada. M.G.S. and E.S.A. were the recipients of studentships from the Fonds de la Recherche en Santé du Québec. The skilled technical assistance of Sylvia Levine and secretarial aid of Marlene Gilhooly are gratefully acknowledged.

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NEURONAL DEVELOPMENT IN EMBRYOS OF THE MOLLUSK, *HELISOMA TRIVOLVIS*: MULTIPLE ROLES OF SEROTONIN

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Advances in Neural Science,
Volume 2, pages 67–87.

ISBN: 1-55938-625-8

ABSTRACT

Our current understanding of the physiological mechanisms underlying cell excitability, synaptic transmission, learning, and neuroplasticity is based largely upon studies of identified neurons from adult mollusks. To determine how these mechanisms are expressed and function in the developing nervous system, complementary studies on embryonic molluscan neurons are required. However, such studies have been limited because of the small size of molluscan embryos and the planktonic larval stage of molluscan development. To circumvent these difficulties, we have been investigating embryos of *Helisoma trivolvis*, a freshwater pulmonate that develops from oocyte to juvenile entirely within an egg mass. Through a combination of *in vivo* and cell culture approaches, the developmental neurobiology of molluscan embryos is now becoming understood. This paper reviews the pioneering developmental studies on embryonic *Helisoma* neurons. Descriptive studies on embryonic stages, nervous system formation, and the expression of embryonic neurotransmitters are first discussed. These studies provided the foundation for experimental studies on the regulation of neuronal development. Specifically, complementary cell culture and *in vivo* studies on the multiple roles of the neurotransmitter serotonin in the developing embryo are presented. These studies revealed that serotonin is expressed early in embryogenesis and functions to regulate the neurite outgrowth, synapse formation, and physiological activity of specific target cells.

I. INTRODUCTION

Our current understanding of the mechanisms and strategies by which the nervous system forms and becomes functional during embryonic development has arisen through studies on a wide variety of species. Various invertebrate and lower vertebrate taxa, including members of the annelids (Keleher and Stent, 1990; Jellies and Kristan, 1991), insects (Harrelson and Goodman, 1988; O'Connor et al., 1990), nematodes (Chalfie, 1984), and teleosts (Eisen, 1991), have proved to be excellent model systems for investigating neural development. The particular experimental advantages offered by these simple systems include large embryonic size, simple nervous systems, identifiability and experimental accessibility of specific embryonic neurons, well-defined cell lineages, and suitability for genetic analysis. To date, relatively less information has arisen from studies on molluscan species, as molluscan embryos do not generally possess the experimental advantages listed above. In fact, small embryonic size and the free-swimming larval stage occurring in most molluscan species have historically made the study of neural development particularly difficult in this group of organisms.

The mature molluscan nervous system, on the other hand, is particularly well suited for experimental studies. As a result, enormous contributions have been made in areas such as synaptic physiology (Haydon et al., 1991), learning and memory (Cleary et al., 1991), and neuroplasticity (Bulloch, 1985). Given the rich body of information derived from experiments on adult mollusks, investigating nervous

system development in these animals could reveal whether the mechanisms employed during learning and other forms of neuroplasticity represent the re-expression of mechanisms utilized in the original formation of the nervous system. With this question in mind, approaches for studying molluscan neuronal development have been developed that take advantage of a great number of technological advances over the last decade. One of these approaches has been to study the changes in the nervous system that occur between the juvenile and mature stages of postlarval development in the sea slug *Aplysia californica* (Nolen et al., 1986; Cash and Carew, 1989; Hickmott and Carew, 1991). These studies focused specifically on the neural mechanisms underlying a set of defensive reflex behaviors and how these behaviors undergo specific adaptive changes according to the developmental context. Although these studies have provided insights about the development of learning, they did not address any of the early events concerning nervous system development.

Another avenue of research on nervous system development in mollusks has focused on the mechanisms underlying the metamorphic transformation from veliger to juvenile stages of development (see Marois and Carew, 1990, for a review). Although these studies have addressed certain aspects of neural control in larvae (Arkett et al., 1987; Arkett, 1988; Mackie et al., 1976), as well as anatomical and chemical components of the metamorphic response (Arkett et al., 1989; Morse, 1991; Chia et al., 1992), this line of investigation has provided little information about fundamental developmental mechanisms at the cellular level.

A different approach to studying neural development in mollusks, yet one that is more conducive to the investigation of fundamental developmental mechanisms, begins with selecting a species that undergoes direct development. In such species, eggs develop into juveniles entirely within an egg mass along a rigid developmental time course. Characterized by the absence of a prolonged free-swimming larval period, the availability of large numbers of sibling embryos, and the easy identification of specific developmental stages, directly developing molluscan species are particularly well suited for developmental studies. In addition, by employing complementary anatomical and physiological analyses on both *in vivo* and cell culture experimental systems, researchers can easily overcome previous limitations imposed by the small size of molluscan embryos. This approach has been implemented over the last 5 years in our studies of embryos of the freshwater pond snail *Helisoma trivolvis*. This species was chosen largely because of the wealth of knowledge about mechanisms of neuroplasticity obtained from studies of its mature neurons (Bulloch, 1985; Kater et al., 1988; Kater and Mills, 1991). Through such an integrated approach, we are beginning to understand some of the early regulatory events that occur in the development of a molluscan nervous system. Most importantly, these studies revealed that specific neurotransmitters expressed early in neuroembryogenesis play key roles in regulating the development and function of their target neurons. This chapter reviews the studies carried out on neural development and function in *Helisoma* embryos. This overview will examine how the

study of molluscan development has advanced the field of developmental neurobiology. In addition, it will reveal the value of this experimental system for understanding the relationship between embryonic neural development and adult neuroplasticity.

II. STAGES OF EMBRYONIC DEVELOPMENT

As a prerequisite for experimental analyses, a system defining the embryonic stages of *Helisoma* was constructed to provide a consistent means of quantifying, categorizing and distinguishing the many different phases of embryonic development (McKenney and Goldberg, 1989). The staging system for *Helisoma* was modeled after the system used by Bentley et al. (1979) in describing grasshopper development. Thus, embryonic stages are expressed as percentages of the total duration of embryogenesis. This is advantageous over a system in which actual temporal durations are used to label stages because of the strong temperature dependence of molluscan embryonic development (Morrill, 1982). By expressing each stage as a percentage of total embryonic development, this method ensures that embryos at the same stage will be equally developed regardless of their chronological ages.

The total duration of embryogenesis was taken as the period from the oocyte, designated as stage E0 (i.e., 0% of embryogenesis), to hatching out of the egg mass membrane, designated as stage E100 (McKenney and Goldberg, 1989). Analysis of 380 embryos revealed that under normal rearing conditions at 26.5°C, the median duration of embryogenesis is 216 hours (9 days). Embryonic stages are characterized mainly by the size, morphology, and behavior of embryos at specific time points over the 216 hours of development. These features can be easily assessed under a stereomicroscope, as *Helisoma* embryos develop within transparent, accessible egg masses (Goldberg et al., 1988). Table 1 summarizes some of the key

Table 1. Diagnostic Events for Identification of Embryonic Stages

Embryonic Stage (% of Embryogenesis)	*Onset of Behaviors*	*Morphological Features*
E0	—	Oocyte
E1	—	First cleavage
E15	Cilia-driven spinning	Motile cilia
E20	—	Pedal-abdominal furrow
E25	—	Shell gland
E30	—	Shell deposition
E35	Body wall contraction	—
E40	Cardiac beating; crawling	"Hippo" morphology
E50	Buccal mass contraction	Juvenile morphology
E65	Radular rasping	—
E70	—	Shell coiling

diagnostic events that occur during *Helisoma* embryogenesis and their corresponding embryonic stages. Taken together with simple rearing conditions and large numbers of sibling embryos per egg mass (Goldberg et al., 1988), the ease with which embryos are staged reinforces the usefulness of *Helisoma* for studies of embryonic development.

Lymnaea stagnalis is another molluscan species for which there exists an abundance of information regarding embryonic development. Species of the genus *Lymnaea* are of particular interest for a variety of reasons. Their embryology has been extensively studied (see Morrill, 1982, for a review). Furthermore, along with *Helisoma*, they are basommatophoran pulmonates and have been subjected to intense neurobiological investigation (see Bulloch, 1985, for a review). Finally, they exhibit postembryonic neural development similar to that described in *Helisoma* (Croll and Chiasson, 1989; Goldberg and Kater, 1989; Diefenbach and Goldberg, 1990). Our recent analysis of embryonic stages in *Helisoma* development has provided the opportunity to compare the development of these two species. The main differences between embryogenesis of *Helisoma* and *Lymnaea* involve the time of expression of specific features (Cumin, 1972; Morrill, 1982). In general, most of the postgastrulation development in *Lymnaea* occurs more slowly than in *Helisoma*. Differences in the gross morphology of embryos are less obvious than differences in relative developmental time courses. An exception to this, however, occurs during gastrulation, when *Lymnaea* embryos assume a flattened placode-like morphology (Morill, 1982). On the other hand, *Helisoma* embryos maintain a relatively spherical shape throughout gastrulation (McKenney and Goldberg, 1989). Examination of other planorbid and lymnaeid species may reveal whether these basic differences that have emerged are consistently expressed between these two pulmonate families.

III. FORMATION OF THE CENTRAL NERVOUS SYSTEM

In order for *Helisoma* to be an effective experimental animal in cellular studies of neural development, it is essential to know both the spatial and temporal patterns of nervous system formation. Without such fundamental information, it would be difficult to test specific hypotheses through well-designed, easily interpreted experiments. The central nervous system of *Helisoma* is composed of five bilateral pairs of ganglia located at the anterior region of the digestive tract. In mature *Helisoma*, only the cerebral, buccal, and pleural ganglia appear to have perfectly symmetrical right and left forms. In contrast, the left and right parietal ganglia are markedly different in size and shape. Furthermore, immunofluorescence studies revealed that the paired parietal and pedal ganglia are asymmetrical with respect to the complement and location of serotonin-immunoreactive neurons (Goldberg and Kater, 1989; Diefenbach and Goldberg, 1990). Finally, there is an unpaired visceral ganglion located posteriorly in the nervous system.

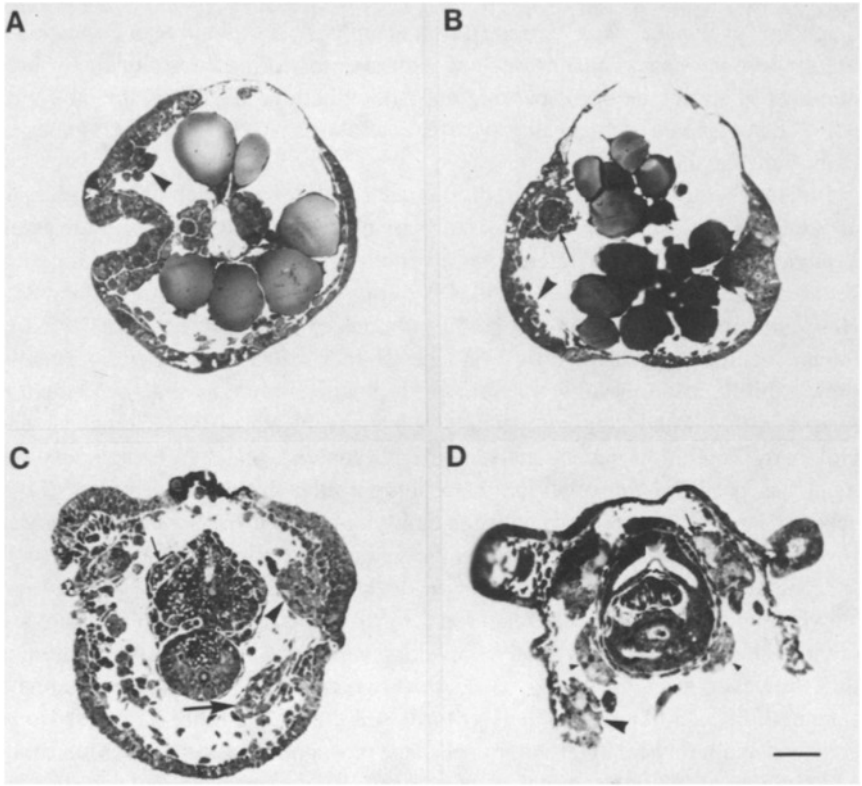

Figure 1. Early development of neuronal ganglia. (**A**) Longitudinal section of stage E20 embryo; anterior at left. Early cerebral ganglion is indicated (*arrowhead*). (**B**) Longitudinal section of stage E25 embryo; anterior at left. Cerebral ganglion (*arrow*) and early pedal ganglion (*arrowhead*) are indicated. (**C**) Cross section of stage E30 embryo. Cerebral (*arrowhead*), pedal (*large arrow*), and buccal ganglia (*small arrow*) are indicated. (**D**) Horizontal section of stage E45 embryo. Cerebral ganglion neuropile (*arrow*), buccal ganglia (*small arrowheads*), and pleural ganglion (*large arrowhead*) are indicated. Calibration: **A**, 30 μm; **B**, 40 μm; **C** and **D**; 32 μm.

To determine the embryonic stages at which the various central ganglia begin to form, we performed a combined analysis of stained histological sections and live embryos using bright-field (Fig. 1) and Nomarski differential-interference contrast (DIC) (Fig. 2) microscopy, respectively. In thin sections of embryos fixed at different stages of development, ganglia were identified by their characteristic cortex-neuropile organization and their relative locations (Fig. 1). Accordingly, the onset of gangliogenesis for each ganglion was determined by analyzing earlier stages for the first appearance of presumptive nerve cells in organized clumps at the appropriate sites. The onset of gangliogenesis spanned the middle stages of

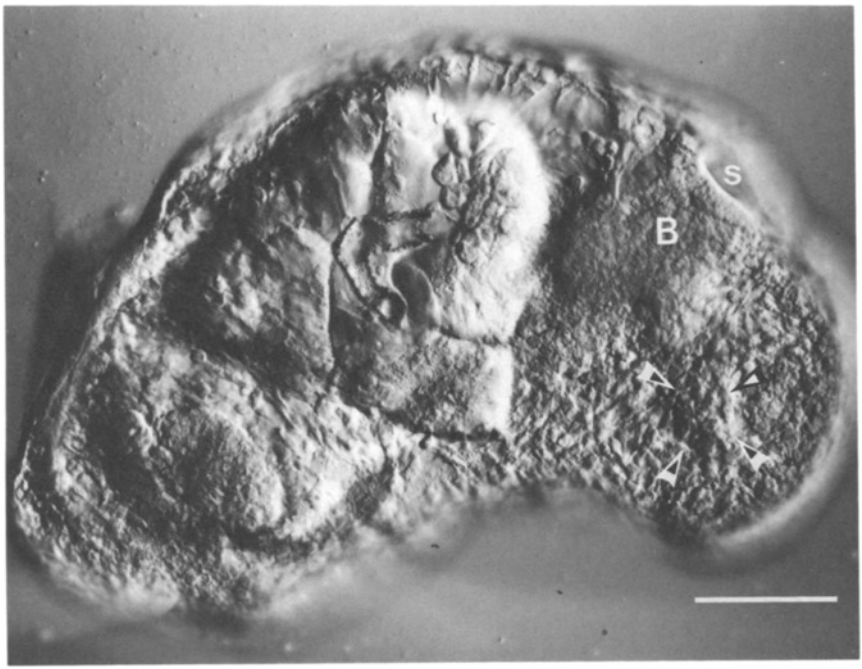

Figure 2. Nomarski DIC micrograph of stage E35 embryo. Development of individual neurons and whole ganglia can be followed in live embryos using DIC microscopy. *Arrows* indicate the right pedal ganglion, as seen in the right lateral aspect of the embryo. B, buccal mass; S, stomadeum. Calibration: 90 μm.

embryogenesis (Table 2). Ganglion formation proceeded in an anterior-posterior order in a manner similar to that previously observed in embryos of *Lymnaea stagnalis* (Cumin, 1972). The anteriorly located cerebral ganglia began to form at stage E20, whereas the most posterior ganglia, the parietal and visceral ganglia, began to form at stage E40. As seen below, this information on the time course of gangliogenesis provides a framework for utilizing *Helisoma* embryos in experimental studies of neural development.

The specific cellular events involved in ganglion formation are largely unknown. In studies on embryos and juveniles of the opisthobranch sea slug, *Aplysia californica*, this question was addressed by pinpointing the location and time of terminal cell divisions using tritiated thymidine labeling (Jacob, 1984; Hickmott and Carew, 1991). These studies indicated that ganglionic neurons are generated by a terminal cell division of neuronal precursors that are located in cellular placodes in specific regions of the body wall. Subsequent to the terminal cell division, cells migrate inward to join the forming ganglion and then begin to display specific characteristics of differentiated neurons. Our observations of the early development of an

Table 2. Onset of Gangliogenesis

Ganglion	*Embryonic Stage*
Cerebral	E20
Pedal	E25
Buccal	E30
Pleural	E35
Parietal	E40
Visceral	E40

identified *Helisoma* neuron, embryonic neuron C1 (ENC1), suggest that the cellular events observed in *Aplysia* may not represent a general pattern for all mollusks. ENC1 begins to express neuronal phenotypes such as serotonin immunoreactivity and prominent neurites while still located amid undifferentiated cells in the body wall, prior to an inward migration (Goldberg and Diefenbach, 1992). Furthermore, this neuron does not appear to arise from the placode-like structure described in *Aplysia*. However, since ENC1 is one of the first neurons to differentiate in the *Helisoma* nervous system, its sequence of development may not be representative of later forming neurons. Neuronal birth-dating studies using anti-bromodeoxyuridine labeling techniques are currently under way to better resolve the cellular events underlying ganglion formation in *Helisoma*.

IV. PRESENCE OF NEUROTRANSMITTERS IN *HELISOMA* EMBRYOS

The hypothesis that neurotransmitters may function in embryos to regulate the development of specific target cells was first prompted by studies on adult *Helisoma* neurons regenerating in culture. Haydon et al. (1984) demonstrated that the addition of serotonin to cultures of identified neurons resulted in the reversible inhibition of neurite outgrowth in select adult neurons. Further work showed that other neurotransmitters, such as dopamine (McCobb et al., 1988a) and glutamate (Jones and Bulloch, 1988), regulate neurite outgrowth, with each neurotransmitter acting upon specific but overlapping subsets of identified neurons. Finally, these studies showed that combinations of certain neurotransmitters can act in concert to produce specific actions on neurite outgrowth (McCobb et al., 1988b). Taken together, these results demonstrated that neurotransmitters can be important regulators of neuronal morphology in regenerating adult neurons. Moreover, they gave rise to the hypothesis that neurotransmitters play key roles during embryogenesis to influence the morphological development of embryonic neurons. Since the formation of synapses with specific target cells is a direct function of a cell's cytoarchitecture (Haydon et al., 1984), a corollary of this hypothesis is that the morphogenetic actions of neurotransmitters in embryos help shape functional neural circuits.

As a first step in testing these predictions, it is necessary to confirm that specific neurotransmitters are indeed expressed in neurons during nervous system development. Studies have shown that several neurotransmitters are expressed during various stages of embryogenesis in mollusks (Kempf et al., 1987; Marois et al., 1987; Goldberg and Kater, 1989). A consistent finding from these studies is that serotonin appears early in embryogenesis, often prior to the expression of other neurotransmitters. This finding is particularly striking in *Helisoma* embryos, where serotonin-like immunoreactivity in neurons precedes the initial appearance of other major neurotransmitters such as dopamine (Goldberg and Kater, 1989; Fig. 3) and the molluscan peptides FMRFamide and SCPB (Goldberg and Kater, unpublished observations). This trend of relatively early development of the serotonin system is also seen in other taxa, including arthropods (Kravitz et al., 1985; Valles and White, 1990) and mammals (Lauder et al., 1982). Given such early developmental expression, it is not surprising that much of the work on the developmental roles of neurotransmitters has focused on serotonin.

In *Helisoma*, serotonin is first expressed in a pair of identified neurons, ENC1s, between stages E15 and E20 (Goldberg and Kater, 1989; Goldberg and Diefenbach, 1992; Fig. 3A). This expression represents the earliest episode of neuronal differentiation known to occur during development of the *Helisoma* nervous system. It occurs at a stage where ganglion formation has yet to commence and the ENC1 cell bodies still reside among the undifferentiated ectodermal cells of the body wall. By stage E25, these cells are electrically excitable (Diefenbach et al., 1991a), have extensive neurite outgrowth, and have innervated their target cells (Diefenbach et al., 1991b). Since a large majority of neural development in *Helisoma* occurs subsequent to these early events, the precocial ENC1s may play key regulatory roles in guiding the development of other embryonic neurons. Evidence in support of this hypothesis will be discussed below.

Following the early expression of serotonin in specific neurons, there is extensive development of serotonergic neurons throughout all the latter stages of embryogenesis (Goldberg and Kater, 1989). Furthermore, the number of serotonergic neurons continues to rise throughout postembryonic development, even during adult stages of *Helisoma* development (Goldberg and Kater, 1989; Diefenbach and Goldberg, 1990). While this characteristic of postembryonic neuronal development is contrary to the axiom that most neuronal development occurs embryonically, it may be a common feature of development in gastropod mollusks. To date, postembryonic development of the serotonin system has been observed in *Aplysia* (Nolen et al., 1986), *Lymnaea* (Croll and Chiasson, 1989), *Melampus* (May et al., 1987), and *Helisoma* (Goldberg and Kater, 1989; Diefenbach and Goldberg, 1990). Evidence from studies on *Aplysia* (Hickmott and Carew, 1991) and *Helisoma* (Cavers and Goldberg, 1993) indicate that this postembryonic increase in serotonergic neurons is a result of the addition of new cells, rather than phenotype switching or the latent differentiation of dormant cells. However, further studies are required to confirm this interpretation. In any case, a consequence of the extensive embry-

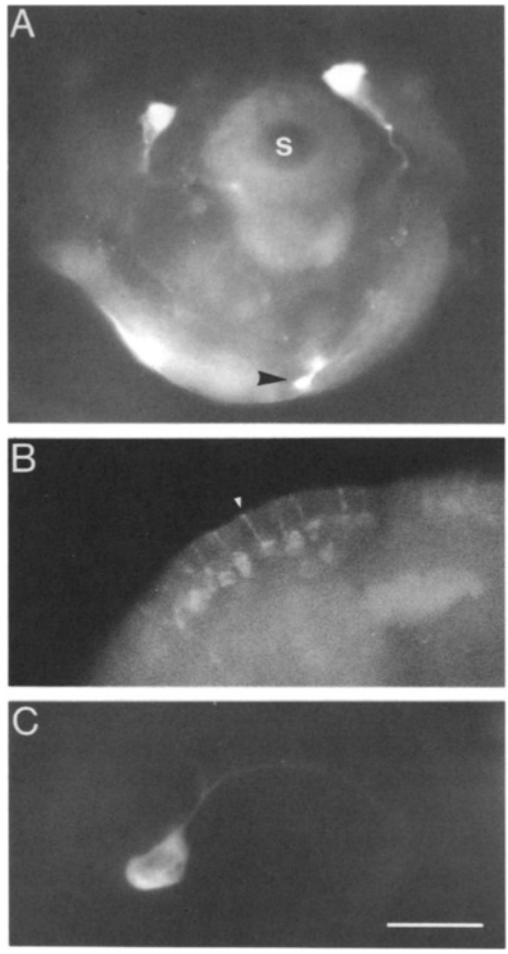

Figure 3. Expression of neurotransmitters in *Helisoma* embryos. (**A**) Serotonin immunoreactivity in stage E25 embryos. A single pair of neurons, ENC1, expresses the serotonin phenotype at stage E25. These neurons extend primary neurites ventrally toward their target, a band of ciliated epithelial cells (not shown). The immunoreactivity is seen in the ventral region in conjunction with distal projections of ENC1 neurites (**arrow**). The stomadeum (s) is also visible in this anterior view. (**B**) Tyrosine hydroxylase immunoreactivity is first seen at stage E40 in a group of cells lining the anterior edge of the developing foot. The extension of immunoreactive processes to the embryonic surface (*arrow*) suggests a sensory role for these cells. (**C**) Tyrosine hydroxylase immunoreactivity is first seen in the CNS at stage E50 in an unpaired left pedal ganglion neuron. This neuron, tentatively identified as neuron P1 of adult *Helisoma* (McCobb et al., 1988a), is shown at stage E70. Calibration: **A**, 35 μm; **B**, 14 μm; **C**, 10 μm.

onic and postembryonic development of the serotonin system is that this neurotransmitter clearly stands out as one of the most important and highly expressed neurotransmitters in the entire *Helisoma* nervous system.

In addition to serotonin, the prominent molluscan neurotransmitters dopamine, FMRFamide, and SCPB are expressed in *Helisoma* embryos. Dopamine expression, as indicated by tyrosine hydroxylase (TH) immunoreactivity, first appears at stage E40 in peripherally located somata (Fig. 3B). It is not known whether these cells differentiate before migrating to their ganglionic locations, as is the case for ENC1, or whether they remain in the periphery and function as sensory neurons, as suggested by their morphology. Slightly later in embryonic development, at stage E50, TH-immunoreactive neurons are seen centrally, with the appearance of the left pedal ganglion neuron P1 (Fig. 3C). Also occurring at stage E50 is the first appearance of FMRFamide-immunoreactive and SCPB-immunoreactive neurons (Goldberg and Kater, unpublished observations). Embryonic expression of SCPB immunoreactivity was also demonstrated in embryos and larvae of the nudibranch *Tritonia diomedea* (Kempf et al., 1987). Therefore, the presence of these various neurotransmitters in embryos of *Helisoma* and other gastropod mollusks suggests that any or all of them may function as regulators of neuronal development.

V. ROLES OF NEUROTRANSMITTERS IN THE REGULATION OF NEURONAL DEVELOPMENT

The inhibition of neurite outgrowth by specific neurotransmitters in adult *Helisoma* cells in culture suggested that similar responses could occur during nervous system development (Haydon et al., 1984). We have taken two complementary experimental approaches to test this idea. The first approach was to assess whether neurotransmitters can exert on neurite outgrowth of cultured embryonic neurons regulatory actions similar to those seen in adult cells. Such experiments were designed to indicate whether embryonic neurons have the capability of responding. In addition, examination of isolated neurons in the highly controlled cell culture milieu allows one to address the mechanism of neurotransmitter action. In contrast to the reductionist *in vitro* approach, the experimental perturbation of whole embryos and ganglia is necessary to determine the relative roles of neurotransmitters in the highly complex, interactive environment of the intact nervous system. By combining these two approaches, we have found that serotonin, and possibly other neurotransmitters, acts during embryogenesis to regulate the development of specific target cells.

A. Cell Culture Studies

One of the main reasons that so little is known about developing molluscan neurons is the small size of molluscan embryos. One way to circumvent the experimental difficulties associated with size limitations is to dissociate whole embryos and culture the resulting cell suspension *in vitro*. The outcome of this

procedure should be the survival, attachment, and development of several distinct cell types, including neurons. Recently this approach has been successfully employed to gain experimental access to live, developing neurons from stage E25 *Helisoma* embryos (Goldberg et al., 1988). Dissociated-embryo cell cultures have already proved to be a valuable model system for studying neuronal development (Goldberg et al., 1991, 1992). Furthermore, since cultures of mixed neuronal populations and identified neurons are well established for adult *Helisoma* neurons, the embryonic cell cultures are also useful for comparative *in vitro* analyses between developing and regenerating neurons (Goldberg et al., 1991, 1992).

Since neurons are one of many cell types that survive in culture, several experiments were carried out to ensure the correct identification of neurons (Goldberg et al., 1988; Goldberg and Price, 1991). The strategy employed to confirm neuronal phenotype was to determine whether cells displaying typical neuronal morphology also expressed other neuron-specific characteristics. For example, transmission electron microscopy carried out on morphologically identified neurons verified that these cells had ultrastructural features typical of neurons (Goldberg et al., 1988). In addition, analysis of serotonin immunofluorescence in embryonic cell cultures revealed the expression of serotonin immunoreactivity specifically in cells with neuronal morphology (Goldberg et al., 1988). Finally, in a comprehensive electrophysiological study, cells with neuronal morphology displayed electrical properties common to neurons, including action potentials and voltage-gated sodium, potassium, and calcium currents (Goldberg and Price, 1991). As expected from differentiated neurons, virtually all embryonic neurons expressed the basic currents necessary for the generation of regenerative action potentials. In contrast, only some of the neurons displayed fast, early potassium currents that modulate electrical excitability. This profile of ion current expression is typical of that seen in populations of differentiated neurons. Several lines of evidence thus confirmed that embryonic cells displaying neuronal morphology in culture were indeed differentiated neurons.

One of the prime objectives in developing an embryonic neuronal culture system was to investigate neurotransmitter actions on embryonic neurite outgrowth. To lay the foundation for these studies, neurons were examined repeatedly over the course of several hours to establish the normal pattern of neurite outgrowth (Goldberg et al., 1991; Fig. 4). As neurons tended to elongate in an incremental rather than continuous fashion, the rate of neurite elongation was not a useful parameter for experimental analyses. On the other hand, analysis of the pattern of stepwise increments in elongation at 2-hour intervals revealed a useful probabilistic approach to studying the effects of specific compounds. The analysis revealed that two consecutive periods of elongation were highly likely to be followed by a third period of elongation (Table 3). Likewise, there was a high probability that consecutive periods without elongation would be followed by a third period without elongation. Thus, compounds can be added to cultures after recording outgrowth

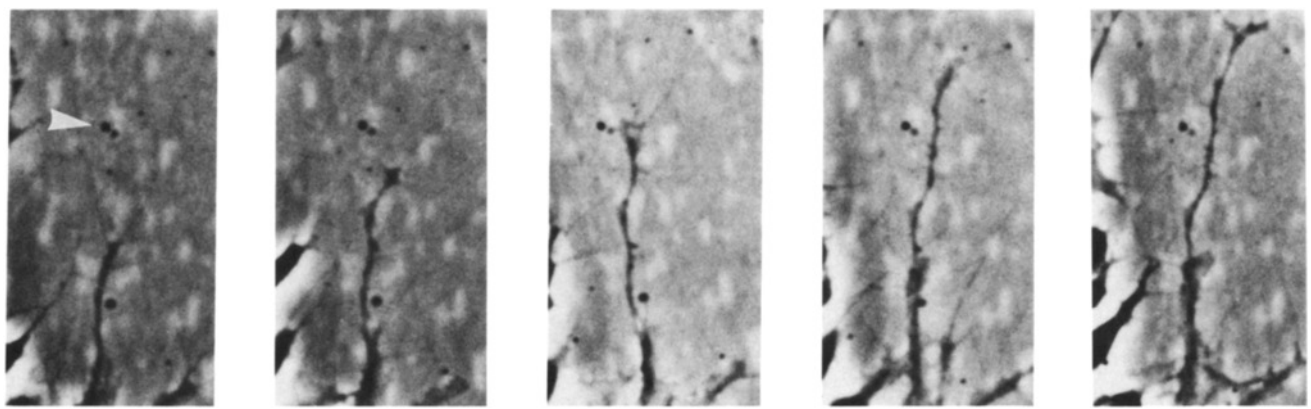

Figure 4. Measurement of neurite outgrowth in cultured embryonic neurons. Growth cone advances are recorded at 2-hour intervals (left to right). Continuous neurite elongation, as seen in the example shown, is observed only in a minority of embryonic neurites (Table 3). Stationary markings on the culture plate are indicated in the first panel (*arrow*).

for two consecutive periods to determine whether they will reverse the predicted state of outgrowth during the third period (Fig. 5).

Using this experimental paradigm, it was determined that specific neurotransmitters, such as serotonin and dopamine, can regulate neurite outgrowth in significant percentages of embryonic *Helisoma* neurons. To date, most of the work has focused on the actions of serotonin. We found that serotonin exerts a dose-dependent inhibition of neurite outgrowth in approximately 50% of the elongating embryonic neurons (Goldberg et al., 1991). Surprisingly, analysis of nonelongating neurons revealed a dose-dependent reinitiation of neurite outgrowth in a similar percentage of neurons. Therefore, a large percentage of the embryonic neuronal population is capable of responding to the neurotransmitter serotonin by a reversal in the state of outgrowth.

An obvious question stemming from the above results is whether the inhibitory and stimulatory actions of serotonin occur on the same subset of embryonic neurons that differ only in their state of outgrowth. On the other hand, there may be two populations of embryonic neurons, each with the capability of only one type of response. Unlike embryonic neurons, adult *Helisoma* neurons display only inhibitory outgrowth responses to serotonin (Mattson et al., 1988a; Goldberg et al., 1991). Since the inhibition was shown to be mediated through an increase in intracellular cytosolic calcium (Cohan et al., 1987; Mattson and Kater, 1987), examination of intracellular calcium concentration in cultured embryonic neurons may help determine why two opposing outgrowth responses are seen in embryonic neurons. Using the single cell Fura 2 calcium-imaging technique to measure intracellular calcium, it was found that populations of embryonic and adult *Helisoma* neurons responded identically to the addition of serotonin (Goldberg et al., 1992). Serotonin-induced increases in calcium concentration were observed in a large number of neurons, whereas serotonin-induced decreases were not detected. These results suggest that the outgrowth-stimulatory actions of serotonin may be mediated by an increase in

Table 3. Characteristics of Embryonic Neurite Outgrowth

Outgrowth Pattern (2-Hour Periods)	*% of Neurites*	*n*
+ → ++++	32.2*	59
+ → +	67.7	96
++ → +	87.5	48
– → – – – –	19.4	31
– → –	52.7	55
– – → –	78.4	37

Notes: *32.2% of the neurites that grew during the first period also grew during each of four subsequent periods

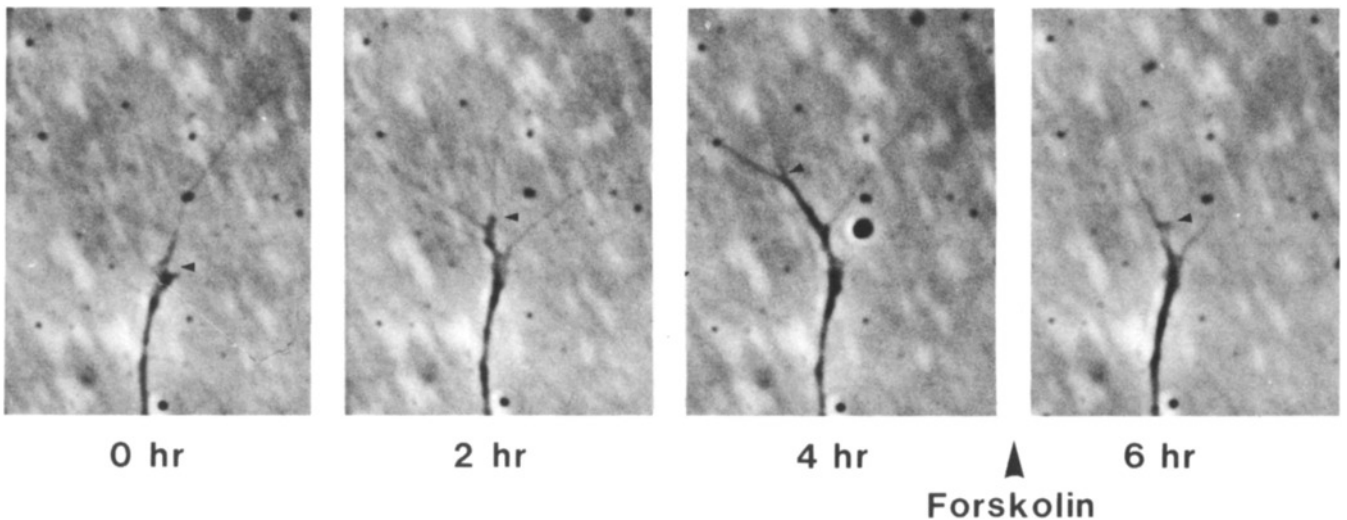

Figure 5. Inhibition of neurite outgrowth by forskolin. Forskolin (1.0 μ), an activator of adenylate cyclase, inhibited neurite outgrowth in a cultured embryonic neuron. These responses were seen in approximately 50% of the neurons examined. Since serotonin inhibited neurite outgrowth in a similar percentage of embryonic neurons, the response to serotonin likely involves an increase in cyclic AMP. *Arrows* indicate the leading edge of the growth cone at each time point.

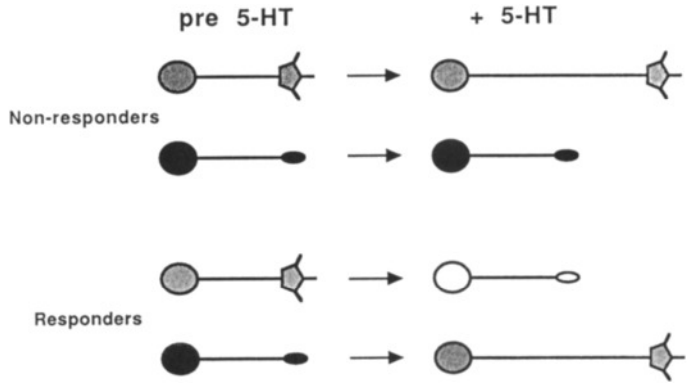

Figure 6. Model accounting for the effects of serotonin on neurite outgrowth and intracellular calcium. Neurite elongation occurs only when the intracellular calcium concentration is within a specific range (*shaded*). If the calcium concentration is either above this range (*white*) or below this range (*black*), outgrowth is arrested. Approximately 50% of the neuronal population is equipped with the receptors, second messengers, and ion channels (*stippled cell outlines*, responders) involved in mediating serotonin responses. In elongating neurons, serotonin elevates the calcium concentration beyond the range that is permissive for outgrowth, and outgrowth is arrested. In stable neurons, serotonin elevates the calcium concentration into the permissive range, and outgrowth is reinitiated. In nonresponding neurons, neither the intracellular calcium level nor the state of outgrowth is affected by serotonin.

calcium, as is the case for outgrowth inhibition. Alternatively, intracellular calcium may not be involved in the outgrowth-reinitiation responses.

At present, the working hypothesis is that both outgrowth inhibition and stimulation involve increased levels of intracellular calcium and occur in the same subset of the neuronal population. This interpretation is consistent with the model that neurite outgrowth proceeds when the intracellular calcium concentration is within a particular range of permissive calcium levels (see Kater and Mills, 1991, for a review; Fig. 6). Since intracellular calcium is higher in growing neurites than in stable neurites (Cohan et al., 1987), serotonin inhibition of outgrowth would occur by raising intracellular calcium beyond the range that is permissive for outgrowth (Mattson and Kater, 1987; Cohan et al., 1987). In contrast, serotonin stimulation of neurite outgrowth would occur by raising the intracellular calcium concentration from a suboptimal level into the range that is permissive for outgrowth. Thus, while the primary action of serotonin is to increase intracellular calcium, the resulting effect on neurite outgrowth may be inhibitory or stimulatory, depending on what level of intracellular calcium is achieved. Clearly, confirmation of this model awaits the simultaneous measurement of neurite outgrowth and intracellular calcium in cultured embryonic neurons.

The signal transduction pathway mediating serotonin's actions on the outgrowth of identified adult neurons has been largely determined. Serotonin activates receptors that have pharmacological characteristics of both 5-HT1-type and 5-HT2-type receptors (Price and Goldberg, 1993a,b). Receptor activation leads to increased cytoplasmic levels of cyclic AMP (Mattson et al., 1988a), which in turn results in the activation of a sodium conductance and the inactivation of a potassium conductance (Price and Goldberg, 1993a). The resulting depolarization causes the activation of voltage-gated calcium channels, leading to the increase in intracellular calcium described above. Intracellular calcium then binds to calmodulin, and through the action of a calcium/calmodulin protein kinase on an unknown intracellular target (Polak et al., 1991), outgrowth is arrested. Studies on chick dorsal root ganglion cells suggest that actin filaments are one of the likely targets for calcium or calcium/calmodulin protein kinase to act upon in the regulation of neurite outgrowth (Lankford and Letourneau, 1991).

Specific components of this signal transduction cascade have been examined in cultured embryonic neurons to determine whether the mechanisms employed by select adult neurons are common to both embryonic and adult cells. These experiments have produced somewhat surprising results. As expected, the serotonin-induced inhibition of outgrowth appears to involve the same serotonin receptor (Goldberg, unpublished observations) and cyclic AMP-dependent pathway (Fig. 5). Conversely, forskolin, an activator of adenylate cyclase, and cyclic AMP analogues were unable to mimic the serotonin-induced reinitiation of outgrowth in stable embryonic neurites. Although these results are not consistent with the "calcium hypothesis" as presented above, it is possible that direct stimulation of the cyclic AMP system leads to larger increases in intracellular calcium than indirect stimulation through activation of serotonin receptors. The resulting high level of intracellular calcium would therefore be beyond the permissible level for outgrowth, thus precluding stimulatory responses. Taken together, the experimental results still favor the model that the outgrowth inhibition and stimulation induced by serotonin occur in the same subset of neurons and that the response depends on the final concentration of intracellular calcium (Fig. 6). Only *in vivo* experiments can resolve the question of whether serotonin does in fact regulate neurite outgrowth during normal embryonic development. If so, are both inhibitory and stimulatory classes of outgrowth responses expressed in the developing nervous system?

B. *In Vivo* Studies

Helisoma embryos, although smaller than many model embryonic systems, can still be extremely useful for *in vivo* experimental studies, given the large array of technical resources currently available. Furthermore, these embryos possess certain advantageous traits for developmental analyses, including simple maintenance and staging procedures, a convenient time course of embryogenesis, availability of a

large number of sibling embryos from a single egg mass, transparency and relative permeability of egg mass membranes, and a reasonably short generation time (Goldberg et al., 1988; McKenney and Goldberg, 1989). Since specific neurotransmitters are expressed in neurons very early in *Helisoma* embryogenesis (Goldberg and Kater, 1989; see above), this system is particularly useful for addressing questions regarding the signaling roles played by neurotransmitters in the developing nervous system.

In the first *in vivo* study to address the developmental actions of neurotransmitters in *Helisoma* (Goldberg and Kater, 1989), embryos were treated with 5,7-dihydroxytryptamine, a serotonin analogue that specifically acts upon serotonergic neurons to render them incapable of releasing neurotransmitter (Gadotti et al., 1986). This treatment resulted in a transient depletion of embryonic serotonin content during the latter half of embryogenesis (Goldberg and Kater, 1989). Treated embryos were then raised into juvenile snails, at which stage morphological and physiological assays could be carried out on identified neurons. These experiments revealed that specific nonserotonergic neurons developed abnormally after the transient depletion of embryonic serotonin content. The developmental aberrations included instances of aberrant neurite outgrowth and enhancement of electrical synapse formation, as determined by dye injection and electrophysiological assays. Since electrical synapse formation in *Helisoma* is a direct function of active, overlapping neurite outgrowth (Hadley et al., 1983), the enhanced synaptogenesis may have been secondary to the enhanced neurite outgrowth occurring in serotonin-depleted embryos (Goldberg and Kater, 1989). Conversely, serotonin depletion may also have direct effects on the formation of electrical synapses.

The experimental paradigm employed in this first study does not determine whether the developmental aberrations resulted directly from the depletion of serotonin during development. However, when taken together with the cell culture studies carried out on adult and embryonic *Helisoma* neurons, it is highly likely that the direct actions of serotonin were involved. First, the specific neurons that were affected by serotonin depletion in the *in vivo* study (Goldberg and Kater, 1989) were the same identified neurons that responded directly to serotonin in culture (Haydon et al., 1984; McCobb et al., 1988a). Second, results of both cell culture and *in vivo* experiments were consistent with outgrowth inhibition being the primary action of serotonin. Finally, studies on cultured embryonic neurons showed that select embryonic neurons are indeed capable of responding to serotonin (Goldberg et al., 1991). Therefore, the complementary findings of cell culture and *in vivo* experiments lead to the conclusion that the development of specific neurons is normally regulated by endogenous serotonin during embryonic development.

In a second set of *in vivo* experiments, a slightly different experimental approach was taken so that neuronal development could be assayed directly in embryos (Diefenbach et al., 1995). Embryos were treated with *p*-chlorophenylalanine, a pharmacological inhibitor of serotonin synthesis, at a stage when serotonergic neurons ENC1 begin to extend neurites. Anti-serotonin immunofluorescence re-

vealed that in response to serotonin depletion, ENC1 developed significantly more neuritic branches. Since ENC1 are the only serotonergic neurons that are differentiated at these early stages of embryonic development, these results indicate that serotonin normally released from ENC1 acts in an autoregulatory manner to restrict the amount of neurite branches produced by this same neuron. Furthermore, these results reinforce the notion that outgrowth inhibition is a primary role of serotonin during embryonic development. More work is required, however, before ruling out the possibility that serotonin also acts to stimulate neurite outgrowth in some phases of neuronal development, as suggested by cell culture studies (Goldberg et al., 1991).

A final set of *in vivo* experiments were undertaken to determine if serotonin released from ENC1 acts exclusively to regulate neuronal development, in lieu of any classical neurotransmitter actions. At stage E25, ENC1 neurites project to motile ciliated cells that are involved in embryo locomotion within the egg capsule. By examining the effect of serotonin and serotonin antagonists on cilia-driven locomotion, it was determined that a specific component of this behavior is generated by the release of serotonin from ENC1 (Diefenbach et al., 1991b). Thus, serotonergic ENC1 is a multifunctional embryonic neuron that regulates both the development and physiological activity of various target cells in the developing embryo.

VI. CONCLUSION

In the last ten years there has been an enormous shift toward cell culture experiments to address specific aspects of neuronal development. Only when cell culture studies are complemented by experimental analyses of intact systems can one appreciate the relative roles of specific developmental signals in the complex, interactive environment of the developing nervous system. For example, although outgrowth-regulating effects of neurotransmitters have been established in cell culture studies on mammalian neurons (Whitaker-Azmitia and Azmitia, 1986; Mattson et al., 1988b), there is little information on how these potential mechanisms are manifested during nervous system development. In contrast, the complementary cell culture–*in vivo* experimental approach implemented in studies of embryonic *Helisoma* neurons has enabled the developmental roles of neurotransmitters to be examined from extreme viewpoints such as mechanism of action and developmental context. Further implementation of this combined approach will undoubtedly reveal more valuable insights on the diverse actions of neurotransmitters in the developing nervous system. In addition, given recent advances in visualizing, identifying, and manipulating embryonic neurons in live, intact embryos (Goldberg and Diefenbach, 1992), many other aspects of neuronal development can be addressed using this model system. Thus, our studies on *Helisoma* not only have revealed an initial understanding of early neuronal development in mollusks; they

provide a solid foundation for the investigation of developmental principles that may apply across various taxa.

ACKNOWLEDGMENTS

The author is grateful to Kimberley Christopher, Kenneth Cavers, Rafe Garofalo, and Thomas Diefenbach for their editorial and photographic assistance. In addition, I would like to thank Rafe Garofalo and Thomas Diefenbach for assistance with the DIC and tyrosine hydroxylase studies, respectively. This research was supported by the Natural Sciences and Engineering Research Council of Canada.

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OPIOID GROWTH FACTOR AND RETINAL MORPHOGENESIS

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Advances in Neural Science,
Volume 2, pages 89–105.

ISBN: 1-55938-625-8

ABSTRACT

An endogenous opioid system, consisting of opioid growth factor [Met^5]-enkephalin and the zeta (ζ) opioid receptor, is known to play an important role in the proliferation, differentiation, and survival of cells in the nervous system. In the developing mammalian retina [Met^5]-enkephalin inhibits DNA synthesis, and its action is mediated by the ζ opioid receptor. This opioid peptide is tonically active, since blockade of opioid–receptor interaction by a potent opioid antagonist stimulates DNA synthesis. [Met^5]-enkephalin and the ζ receptor are present in the neonatal but not the adult retina, suggesting a temporal and spatial expression consistent with a function in growth. [Met^5]-enkephalin is derived from preproenkephalin A, and *in situ* hybridization studies have suggested that this growth factor is produced in an autocrine (i.e., neuroblasts) and paracrine (i.e., ganglion cells) manner. The potential relationship of opioid growth factor to the etiology and pathogenesis of retinal abnormalities is discussed.

I. DEVELOPMENT OF THE VERTEBRATE RETINA

The pattern of retinal development is remarkably similar in all vertebrates (Ramon y Cajal, 1972), and retinal ontogeny has been studied extensively using morphologic (Meller, 1964; Weidman and Kuwabara, 1969; Braekevelt and Hollenberg, 1970; Morest, 1970; Pei and Rhodin, 1970; Hinds and Hinds, 1974, 1978, 1983; Spira, 1975; Greiner and Weidman, 1978, 1980; Rhodes, 1979; Calley et al., 1988; Spira and Marotte, 1989; Nordquist and McLoon, 1991; Reichenbach et al., 1991), autoradiographic (Sidman, 1961, 1970; Fujita, 1962; Denham, 1967; Straznicky and Gaze, 1971; Kahn, 1974; Blanks and Bok, 1977; Carter-Dawson and LaVail, 1979; Young, 1985a,b; Harman and Beazley, 1989; Spence and Robson, 1989; Zimmerman et al., 1988; LaVail et al., 1991; Prada et al., 1991; Reichenbach et al., 1991; Reese and Collelo, 1992), electrophysiological (Meister et al., 1991), and gene transfer and tissue culture (Vollmer and Layer, 1986; Turner and Cepko, 1987; Adler and Hatlee, 1989; Caffe et al., 1989; Reh and Kljavin, 1989; Layer et al., 1990) techniques. A number of reviews on the subject have been published (Raedler and Sievers, 1975; Grun, 1982; Young, 1983; Meller, 1984; Barnstable, 1987; Finlay and Sengelaub, 1989; Harris and Holt, 1990). During ontogeny, the retina develops as an extension of the diencephalic part of the neural tube (Jackson, 1976; Silver and Robb, 1979), and its pattern of cytogenesis resembles that found in other regions of the central nervous system (CNS). Cells in the mitotic cycle form a compact layer that abuts the outer limiting membrane. This has been commonly termed the "neuroblast layer" (also the "cytoblast layer" by Robinson et al., 1985). Initially, the developing retina is composed of a single layer of neuroblasts, which have long processes with attachments at both the inner and outer limiting membranes (Ramon y Cajal, 1972; Hinds and Hinds, 1974). Cell bodies

engaged in DNA synthesis and prophase are located in the proximal portion of this germinative layer. Cells that have completed a cycle of DNA synthesis lose their attachment at the inner limiting membrane, and their cell bodies migrate to the outer surface of the retina, where mitosis takes place (Hinds and Hinds, 1974). After cell division, the daughter cells may either undergo another round of DNA synthesis by once again extending processes to the proximal retina and migrating, or commence differentiation, but both daughter cells appear to follow the same destination. A developmental wave exists, inasmuch as the central retina develops much earlier than the peripheral retina. This "to-and-fro" movement of the nucleus during each mitotic cycle was proposed by Sauer (1936), who termed this phenomenon "interkinetic nuclear migration." The region in which this migration occurs was termed the "ventricular zone" by Angevine et al. (1970). Thus, the neuroblast layer of the retina corresponds to the ventricular zone of the neural tube. Although neurogenesis in the retina is similar in most mammals, the time course and sequence of events may vary from species to species and are dependent on the length of gestation of the animal (Polley et al., 1989).

The development of the retina of rodents has been examined extensively (Sidman, 1961; Denham, 1967; Weidman and Kuwabara, 1969; Braekevelt and Hollenberg, 1970; Morest, 1970; Pei and Rhodin, 1970; Hinds and Hinds, 1974, 1978; Raedler and Sievers, 1975; Blanks and Bok, 1977; Grun, 1982; Young, 1983, 1985a,b; Turner and Cepko, 1987; Calley et al., 1988; Caffe et al., 1989; Reh and Kljavin, 1989; Reese and Colello, 1992). The newborn rat retina is still very immature, with only a well-established ganglion cell layer, developing inner plexiform layer, and nerve fiber layer corresponding to the structures of the adult retina. Ganglion cells in the rat are first generated on embryonic day 11 and appear as rounded cells at the proximal margin of proliferating neuroblasts on embryonic day 15; ganglion cell precursors terminate proliferation before birth. The generation of other cell types, including amacrine and horizontal cells and photoreceptors, all overlap in their development and extend into the first postnatal week. On postnatal day 1, the bulk of the retina is still a germinative layer of neuroblasts, with some cells undergoing DNA synthesis, some in transition, and others in the process of mitosis. Differentiated cells in the neuroblast layer are characterized by circular cell bodies containing pale, rounded nuclei. These differentiating neurons form a layer of two or three amacrine cells at the inner margin of the proliferating cells and a single layer of rounded cell bodies located in the center of the germinative layer that corresponds to differentiating horizontal cells or photoreceptors. DNA synthesis occurs within the inner half of the remaining undifferentiated neuroblasts, and mitosis occurs at the outer margin of the immature retina. Toward the end of the first postnatal week, cell proliferation ceases in the central retina; mitosis continues at the periphery of the developing retina until postnatal day 10.

II. GROWTH FACTORS IN THE RETINA

There has been increasing interest in the role of growth factors in retinal development (Barnstable, 1987; Finlay and Sengelaub, 1989; Harris and Holt, 1990; Anchan et al., 1991; Tripathi et al., 1991). In particular, brain-derived neurotrophic factor (BDNF) is a 12 kDa protein that has been shown to enhance the survival of retinal ganglion cells in monolayer culture (Johnson et al., 1986; Rodriguez-Tebar et al., 1989). A second class of factors, isolated from the eye and most probably related to the class of acidic and basic fibroblast growth factors (FGFs) (Baird et al., 1985), has also been shown to have effects upon the survival and process outgrowth of a number of neuronal cell types (e.g., PC12 cells) (Wagner and D'Amore, 1986). Nerve growth factor (NGF) (Ebendal and Persson, 1988; Carmignoto et al., 1991; Henderson, 1991; Waldbillig et al., 1991) and its receptor and mRNA (Carmignoto et al., 1991); transferrin receptor (Cho and Hyndman, 1991), insulin and insulin-like growth factor 1 (IGF-1) (Hansson et al., 1989; Tesoriere et al., 1992) and receptors (Bassnet and Beebe, 1990) aFGF (Mascarelli et al., 1987), and bFGF (Mascarelli et al., 1987; Cirillio et al., 1990); FGF receptor (Fayein et al., 1990; Heuer et al., 1990); $pp60^{c-src}$ (Sorge et al., 1984); somatostatin receptors (Bodenant et al., 1991) and mRNA (Ferriero et al., 1990); transforming growth factor α (TGF-α) (Fassio et al., 1989); and N-*myc* and c-*myc* (Hirning et al., 1991) have been detected in the developing chick or rodent retina, but the function(s) of these elements has yet to be elucidated. Only recently studies have begun to explore growth factors related to cell proliferation in the retina. Spoerri (1988), using cultures of embryonic chick retina, found that 10^{-5} M GABA promoted the proliferation and differentiation of retinal neurons, and suggested that GABA functions as a trophic or regulatory factor of at least certain retinal cell types. Anchan and colleagues (Anchan et al., 1991), using dissociated cultures of the fetal and neonatal rat retina, discovered that EGF and TGF-α stimulated the proliferation of retinal neuroepithelial cells and that the receptor and mRNA for these factors were present in cultures of developing retinal cells. Tesoriere et al. (1992) localized insulin to embryonic chick retina and found that insulin administered to chick retina explants caused an increase in DNA synthesis; the excitatory function observed *in vitro* was not reproduced when insulin was applied *in ovo*.

III. OPIOIDS AND NEURAL DEVELOPMENT

In the past few years, endogenous opioid systems have been reported to participate in the development of the mammalian brain (see review by Zagon and McLaughlin, 1992). In experiments utilizing rats, investigators found that administration of naltrexone, a potent opioid antagonist, profoundly influenced somatic and neurobiological development (Zagon and McLaughlin, 1983). A blockade of opioid receptors resulted in animals that demonstrated marked increases in growth that were dependent on the duration of opioid receptor blockade (Zagon and McLaugh-

lin, 1984). Animals given opioid antagonists to completely block receptors for the entire period of potential interaction exhibit a greater number of neurons and glia, stimulation of cell proliferation, an increase in dendritic elaboration and synaptogenesis, and acceleration in the acquisition of physical and behavioral characteristics (Zagon and McLaughlin, 1983, 1985, 1986a,b, 1987, 1991; Hauser et al., 1987, 1989; Isayama et al., 1991). These results indicate that opioid antagonists block the interaction of endogenous opioid peptides from opioid receptor and that this interaction must be related to developmental events of the brain. Endogenous opioid(s), therefore, must be able to regulate growth by an inhibitory process, serving as a negative regulator of growth. Moreover, the endogenous opioid related to growth must be tonically active, since interference with opioid–receptor interaction produces growth alterations. The effects of opioid antagonists on growth were found to obey pharmacologic principles underlying classical opioid–receptor interactions, because the (–) isomer of antagonist was active and the (+) isomer was inactive (Zagon and McLaughlin, 1989). Further research revealed that the control of cell replication in the brain was the target for opioid action; endogenous opioids depressed DNA synthesis and mitosis in proliferating neural cells destined to become neurons and glia (Zagon and McLaughlin, 1987, 1991). Study of the opioid(s) involved with growth showed that the pentapeptide [Met^5]-enkephalin was one of the most potent peptides in regard to regulating neuronal and glial precursor cell replication (Zagon and McLaughlin, 1991) in the central nervous system. Concentrations as low as 100 μg/kg of [Met^5]-enkephalin resulted in a marked reduction in DNA synthesis of replicating cerebellar cells; administration of naloxone, an opioid antagonist, blocked the effect of [Met^5]-enkephalin. Opioids selective for receptors such as mu (μ), delta (δ), kappa (κ), epsilon (ε), and sigma (σ) did not alter DNA synthesis. Using antibodies to [Met^5]-enkephalin, researchers found enkephalin-like peptide to be associated with developing neural tissues, but not their adult, differentiated counterparts (Zagon et al., 1985; Zagon and McLaughlin, 1990). Observations with electron microscopy showed that enkephalin-like immunoreactivity was detected in the cortical cytoplasm of germinative neural cells and macroneurons (e.g., Purkinje cells), but not adult neural cells (Zagon and McLaughlin, 1990). Recent studies in which radiolabeled [Met^5]-enkephalin was utilized in receptor binding assays demonstrated specific and saturable binding with high affinity in homogenates of developing cerebellar tissues (Zagon et al., 1991). The binding affinity (K_d) was 2.2 nM and the binding capacity (B_{max}) was 22.3 fmol/mg protein. Competitive inhibition profiles showed that [Met^5]-enkephalin was the most avid compound displacing the radiolabeled ligand. Moreover, subcellular fractionation studies showed that the location of [Met^5]-enkephalin binding was nuclear rather than cytoplasmic/membranous, the putative location of other opioid receptors (Pert and Snyder, 1973). Given the particular function (i.e., regulation of cell proliferation) of this opioid peptide binding site, the receptor was named from the Greek word *zoe* (life): zeta (ζ), the sixth letter of the Greek alphabet.

IV. OPIOIDS, OPIOID RECEPTORS, AND THE RETINA

Although endogenous opioids such as enkephalin-like peptides have been localized to various vertebrate visual systems, including goldfish (Su et al., 1986), chicken (Su et al., 1985), and guinea pig (Altschuler et al., 1982), these peptides have not been detected in the adult rat retina (Howells et al., 1980; Altschuler et al., 1982; Hoffman, 1983). Opioid binding sites, however, have been reported in the adult rat retina. Medzihradsky (1976) described stereospecific binding of radiolabeled etorphine equaling 70% of that in retinal homogenates from 18- to 20-day-old rats. Howells et al. (1980) found that a variety of opioids exhibited high-affinity, saturable binding to retinal membrane preparations of the adult rat, as well as the cow, toad, and skate. Until recently, no studies have reported on opioids and opioid

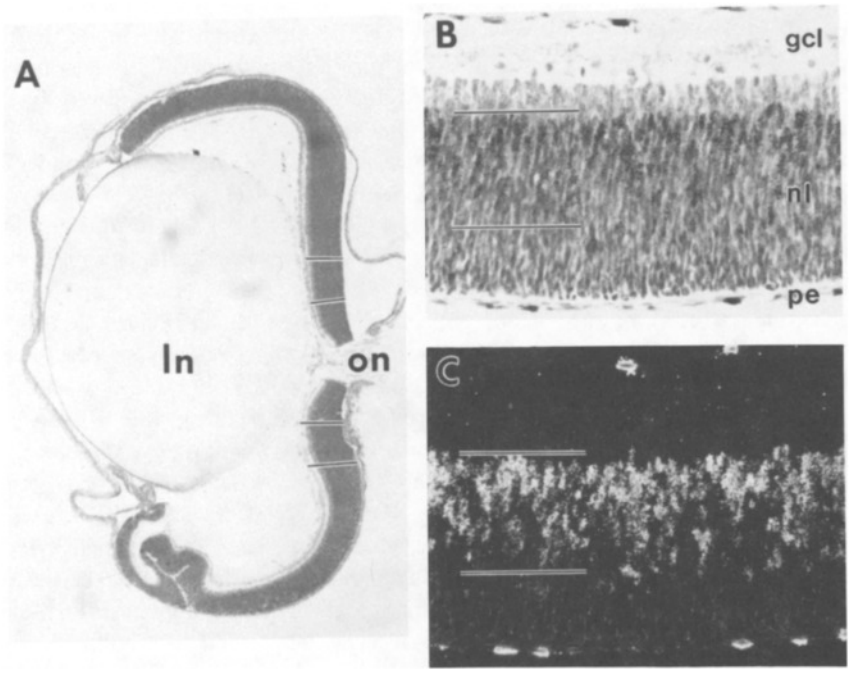

Figure 1. All photomicrographs are sagittal sections of the retina from 1-day-old rats. (**A**) The region evaluated for cell proliferation (see Fig. 2 for data) in autoradiographs (demarcated by brackets) extends 300 to 500 μm from the optic nerve. on, optic nerve; ln, lens. ×15. (**B** and **C**) Autoradiographic slides counterstained with cresyl violet and viewed by brightfield (**B**) and darkfield (**C**) optics; the region of the neuroblast layer assessed by quantitative analysis is indicated. gcl, ganglion cell layer; nl, neuroblast layer; pe, pigment epithelium. ×120. (Reproduced with permission from Isayama et al., *Brain Res.* **1991**, *544*, 79–85.)

receptors with respect to the ontogeny of the retina. It is interesting to note that human retinoblastoma, a tumor thought to arise from retinal germinal epithelial cells, has been reported to have immunoreactivity for [Met^5]-enkephalin and [Met^5, Arg, Phe]-enkephalin (Tarkkanen et al., 1984).

Despite a great deal of information about the development of the retina, our knowledge concerning the fundamental mechanisms involved with the proliferation and eventual differentiation of retinal cells is still unclear. We know that the microenvironment can influence mitosis and the number of neurons and glia produced (Anchan et al., 1991), but few candidate molecules have been shown to govern this process *in vivo*. A review of the literature (see Section II) shows that under *in vitro* conditions, EGF, TGF-α, GABA, and insulin stimulate retinal cell proliferation/DNA synthesis (Spoerri, 1988; Anchan et al., 1991; Tesoriere et al., 1992). Other studies have identified known growth factors and/or their receptors, but the functions of these substances in the developing retina are unknown.

Intrigued by the discovery of opioid growth factors in brain development, we addressed the question of whether opioid growth factors played a role in retinal development. A series of experiments were designed to determine if opioids regulate growth activities in the developing retina, and if so, whether opioids and/or opioid receptors were present in the developing retina. The 1-day-old rat retina was selected for study since at this early age an abundance of cell proliferation was still present in the germinative neuroblast layer (Fig. 1). Since the duration of cell replication in the postnatal rat retina is very short (less than 1 week), it permits the investigator to record growth-associated events over a circumscribed and limited period of time.

V. ENDOGENOUS OPIOID SYSTEMS AND RETINAL DEVELOPMENT

A. Opioids Regulate Cell Proliferation in the Developing Rat Retina

Initial experiments involved the administration of an opioid peptide, [Met^5]-enkephalin (100 μg/kg), to infant rats. [Met^5]-enkephalin was chosen because of previous literature showing this opioid peptide to be a potent growth factor (Zagon and McLaughlin, 1987, 1991). Animals were pulsed with ^{3}H-thymidine 30 minutes prior to sacrifice and killed 4 hours after drug exposure. Analysis of the labeling index (LI) showed that control animals had a LI of 35.8%, whereas [Met^5]-enkephalin-treated animals had a 10.6% decrease from control levels (Fig. 2). This decrease in LI was blocked by concomitant administration of a short-acting antagonist, naloxone (1 mg/kg), resulting in a LI of 35.1%; naloxone alone did not alter the LI. To determine whether endogenous opioids tonically regulated cell proliferation, the interaction between endogenous opioid peptides and opioid receptors was blocked by the long-acting, potent opioid antagonist naltrexone. Naltrexone was administered at 50 mg/kg, a dosage known to block opioid

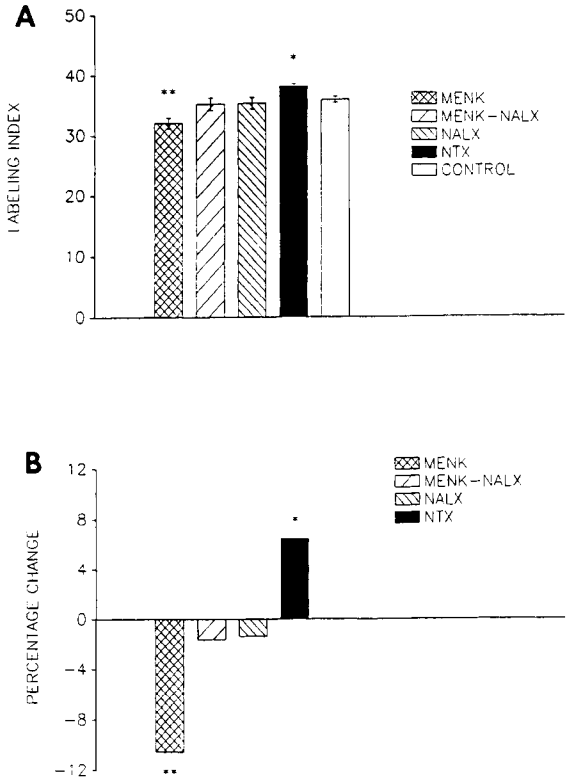

Figure 2. The labeling index of 1-day-old rat retina at 4 hours following administration of 100 µg/kg [Met^5]-enkephalin (MET), 100 µg/kg [Met^5]-enkephalin and 1 mg/kg naloxone (MET-NAL), 1 mg/kg naloxone (NAL), 50 mg/kg naltrexone (NTX), or sterile water (CTR). Both the labeling index (**A**) and percentage difference from the controls (**B**) are given; pulse time for [^{3}H]-thymidine was 30 minutes. Significant differences from controls at $^*P < 0.05$ or $^{**}P < 0.01$. (Adapted with permission from Isayama et al., *Brain Res.* **1991**, *544*, 79–85.)

receptors for 24 hours but was only 2% of the LD_{50} (Zagon and McLaughlin, 1984). The LI of the retina from naltrexone-treated animals (38%) was elevated significantly ($P < 0.05$) from control level. With the exception of the percentage of change, these studies in the retina provided results similar to those obtained earlier in the cerebellum, indicating that retinal cell proliferation is regulated by opioid growth factors. Whether [Met^5]-enkephalin is the most potent opioid growth factor in the retina is a question that remains to be addressed.

It was interesting that although marked and statistically significant, the alterations in ^{3}H-thymidine labeling in the retina were not as dramatic as seen previously in the cerebellum (Zagon and McLaughlin, 1987, 1991). A number of reasons could

account for the difference between opioid actions in the retina and the cerebellum. It may simply reflect differences in the development of various neural regions. Alternatively, the cell cycle of retinal neuroblasts is considerably longer (approximately 33%) than that of cerebellar external germinal cells. Moreover, the synthesis phase of proliferating cells in the retina occupies over 40% of the cell cycle (Denham, 1967), suggesting that a great number of cells in an actively dividing tissue could be synthesizing DNA. Hence, the LI in the normal retina was substantially higher than that in the cerebellum. This would have the effect of narrowing the number of cells available to be recruited into synthesis. In addition, the G_1 phase of retinal cells is much longer than that of cerebellar germinative cells, making it more difficult to compress or expand this phase in a short period. Since drug was injected for a total of 4 hours, and radiolabeling was allowed to take place for 30 minutes, these results in the retina show the potency of opioid action over a short period of time.

B. Localization of Opioid Growth Factor

To examine the presence of endogenous opioids in developing retina, immunocytochemistry studies were undertaken. One-day-old rat retinas were harvested and frozen sections were collected on slides. Tissue was stained with an antiserum to [Met^5]-enkephalin that was produced and characterized in our laboratory (Isayama et al., 1991). The results indicated that enkephalin-like immunoreactivity could be localized to several distinct areas in the developing retina (Fig. 3). In 1-day-old retina, immunoreactivity was present in the ganglion cell layer and in the germinative layer of neuroblasts. Higher-resolution microscopy indicated that enkephalin-like peptides appeared to be associated with the cortical cytoplasm and not the cell nucleus. Control sections treated with antiserum preabsorbed with excess (10^{-3} M) [Met^5]-enkephalin exhibited no staining. Retinas of adult rats also were processed with antiserum to [Met^5]-enkephalin and no immunoreactivity was noted. Immunocytochemistry studies performed subsequent to this earlier report have revealed that enkephalin-like immunoreactivity entirely disappears by 6 days of age (Isayama et al., unpublished observations).

C. Receptor Localization

To determine whether developing retina possesses opioid receptors, *in vitro* autoradiographic localization of the ζ receptor was performed using radiolabeled [Met^5]-enkephalin. Frozen sections of 1-day-old rat retina were incubated with 1.5 nM ^{125}I-[Met^5]-enkephalin and exposed to X-ray film. Control sections were incubated with the labeled compound in the presence of 10^{-6} M naloxone. After 2.5 days of exposure, autoradiograms indicated the presence of opioid receptors in developing retina (Fig. 4), but not in sections incubated with both [Met^5]-enkephalin and naloxone. No binding of the radiolabeled ligand was recorded in the adult retina. Although the receptor involved with retinal cell proliferation has

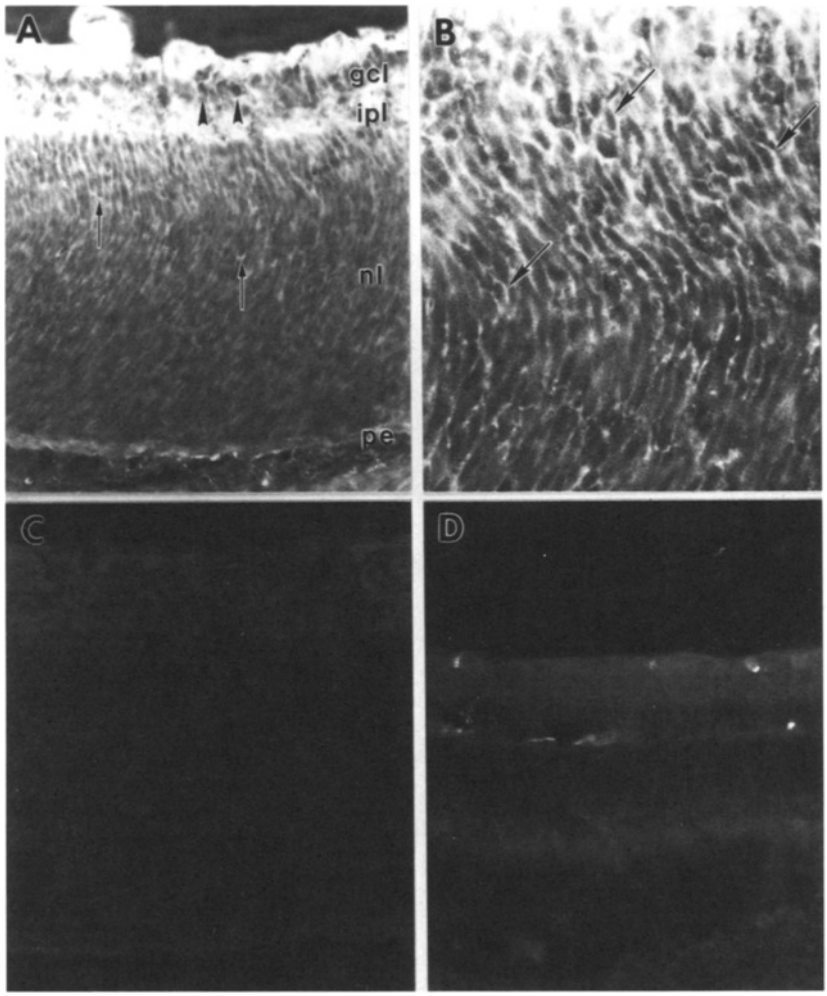

Figure 3. All photomicrographs are sections of the rat retina stained with anti-[Met^5]-enkephalin IgG and rhodamine-conjugated goat anti-rabbit IgG. (**A**) One-day-old retina showing immunoreactivity associated with the cortical cytoplasm of ganglion cells (*arrowhead*) and neuroblast cells (*arrow*), but not the cell nucleus. gcl, ganglion cell layer; ipl, inner plexiform layer; nl, neuroblast layer; pe, pigment epithelium. ×120. (**B**) High-magnification photomicrograph of the 1-day-old rat retina showing a distinctive honeycomb pattern of immunofluorescence associated with the cortical cytoplasm (*arrow*) of the neuroblast cells. ×290. (**C**) One-day-old rat retina of a control specimen processed with anti-[Met^5]-enkephalin IgG preabsorbed with [Met^5]-enkephalin. The magnification and time of photographic exposure are the same as in **A**. ×120. (**D**) Adult rat retina showing little immunoreactivity. ×150. (Reproduced with permission from Isayama et al., *Brain Res.* **1991**, *544*, 79–85.)

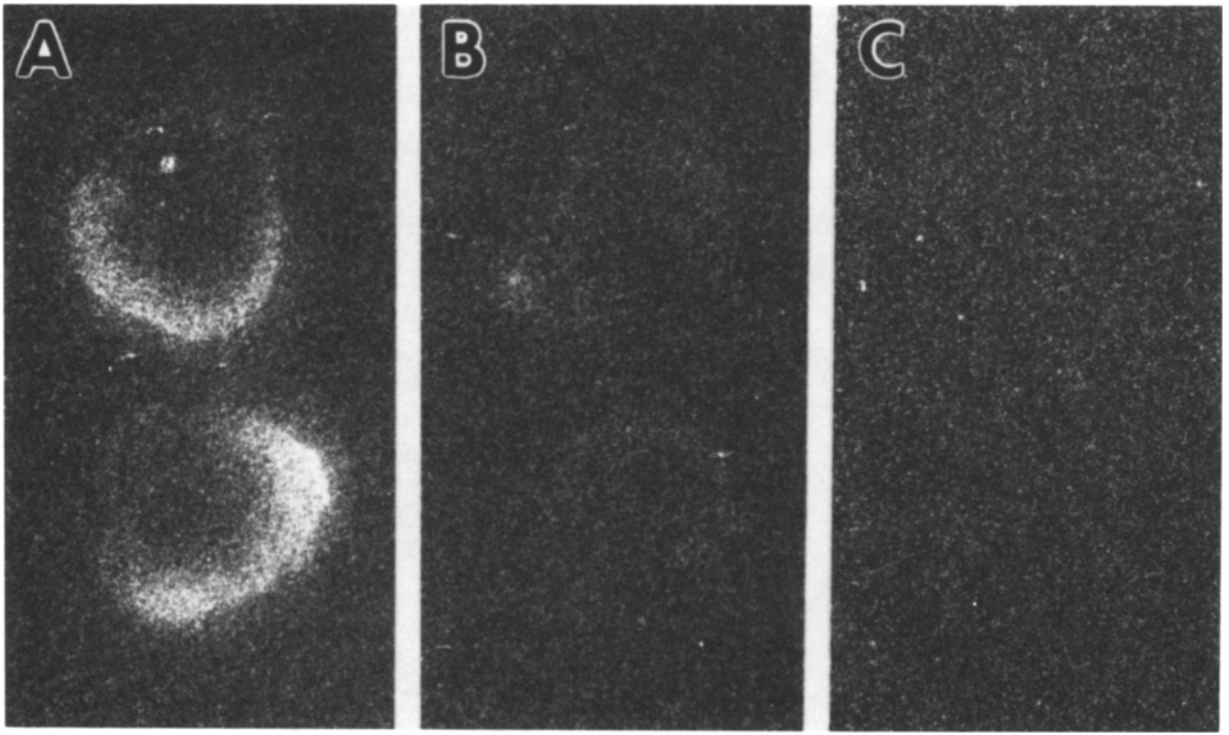

Figure 4. One-day-old (**A, B**) or adult rat retina incubated with either 1.5 nM ^{125}I-[Met^5]-enkephalin (**A, C**) or ^{125}I-[Met^5]-enkephalin and 1 μM naloxone (**B**). ×9. (**A**) Note that the radiolabel is associated with the retina. (**B**) Labeling with ^{125}I-[Met^5]-enkephalin is blocked by concomitant exposure to naloxone. (**C**) No labeling of the adult rat retina with ^{125}I-[Met^5]-enkephalin can be discerned. (Reproduced with permission from Isayama et al., *Brain Res.* **1991**, *544*, 79–85.)

yet to be fully established, the transient appearance of [Met^5]-enkephalin binding at nanomolar concentrations in the developing retina suggests that it is the ζ opioid receptor.

D. Gene Expression

The presumptive opioid growth factor [Met^5]-enkephalin is derived from the prohormone preproenkephalin A (PPE) (Comb et al., 1982). In experiments using an oligonucleotide probe to PPE, we (Isayama and Zagon, 1991) determined the presence and location of PPE mRNA in the neonatal rat retina using *in situ* hybridization. Our results indicated that PPE mRNA was present in the 1-day-old rat retina (Figs. 5 and 6). Prohormone message was localized in both the ganglion cell layer and the neuroblast layer (Fig. 6); control tissue that had been pretreated with RNase A revealed a lack of radiolabel. This result extended our immunocytochemistry findings by showing that the cells comprising the neonatal rat retina possess the message necessary to synthesize the opioid growth factor. Our findings also indicate that enkephalin-like peptides are produced in both an autocrine and paracrine manner by the neuroblasts and the ganglion cells, respectively. The apparent abundance of PPE mRNA in the ganglion cells is consistent with preliminary data, which indicates that these large macroneurons are the first to express the peptide and the prohormone mRNA (Isayama et al., unpublished observations).

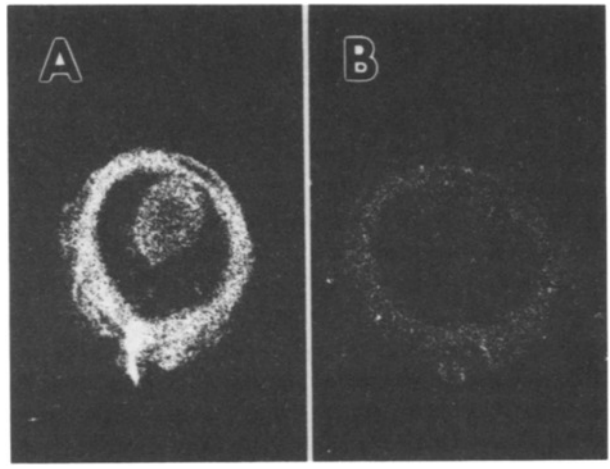

Figure 5. Autoradiographs of 1-day-old rat eyes labeled by *in situ* hybridization with ^{35}S-labeled PPE oligonucleotide probe. ×9. (**A**) PPE mRNA is localized to the areas corresponding to the developing retina and surrounding connective tissues. (**B**) RNase A-pretreated control shows no labeling with the ^{35}S-labeled oligonucleotide probe for PPE. (Reproduced with permission from Isayama and Zagon, *Brain Res. Bull.* **1991**, *27*, 805–808.)

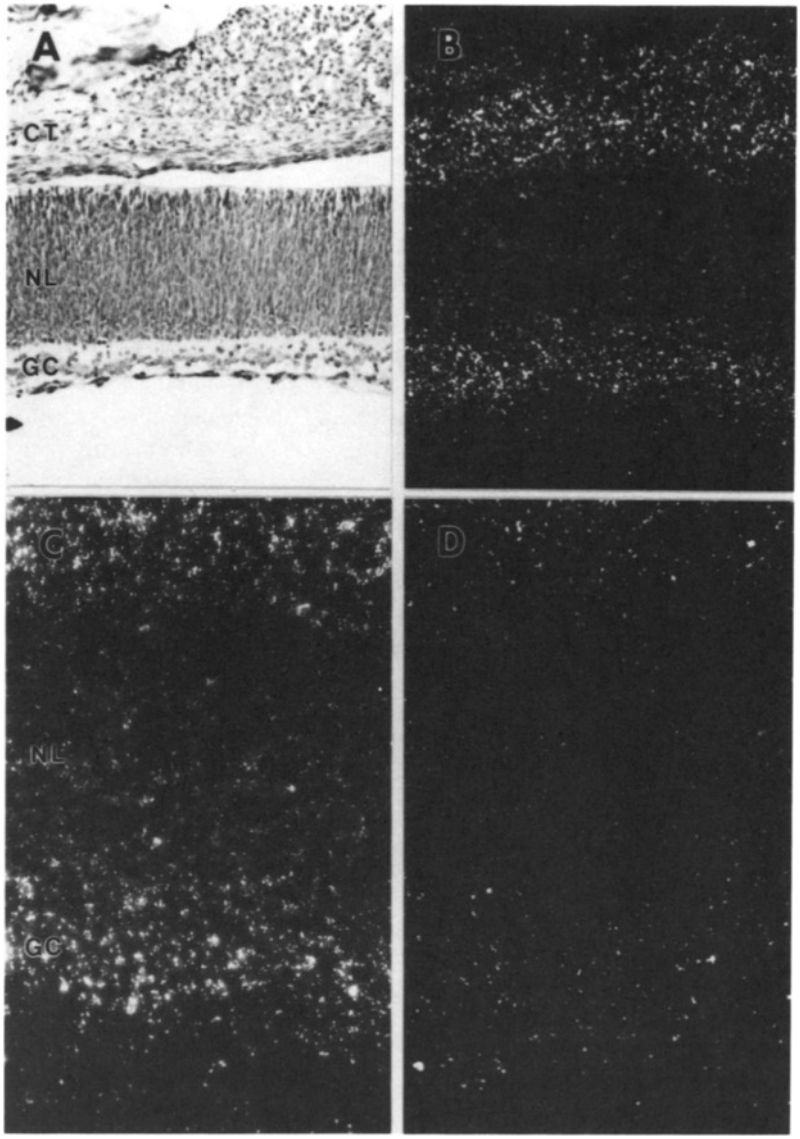

Figure 6. All micrographs are 1-day-old rat retina. (**A**) Bright-field photomicrograph indicating the different layers of the neonatal retina. ×180. GC, ganglion cell layer; NL, neuroblast layer; CT, connective tissue. (**B**) Dark-field photomicrograph of emulsion-coated autoradiograms showing the labeling in the GC, NL, and CT. Note that the concentration of PPE message found associated with the neuroblasts of the NL appears to be less abundant than that related to the ganglion cells. ×170. (**C**) Higher-magnification micrograph of the retina showing the abundance of PPE mRNA in the GC. ×315. (**D**) Dark-field micrograph of an RNase A-pretreated control eye displaying a lack of labeling. ×315. (Reproduced with permission from Isayama and Zagon, *Brain Res. Bull.* **1991**, *27*, 805–808.)

VI. SUMMARY AND CLINICAL IMPLICATIONS

Our studies have provided evidence that opioid peptides are functioning as growth factors during retinal histogenesis. These data are the first to establish, under *in vivo* conditions, that a neuropeptide system has a specific, growth-associated function in the developing retina. [Met^5]-enkephalin appears to be produced by both autocrine (i.e., neuroblast) and paracrine (i.e., ganglion cells) sources. This opioid peptide is a negative regulator of retinal growth and is tonically active. Moreover, the temporal and spatial expressions of [Met^5]-enkephalin and the ζ receptor are consistent with the role of this opioid growth factor in developmental neurobiology. It could be envisioned that opioid growth factor is critical to the cessation of cell production in the retina, perhaps counteracting stimulating signals from other growth peptides. Diminishment of cell proliferation would terminate this germinative phase of neural genesis and ensure an orderly progression toward subsequent stages in neuro-ontogeny (e.g., differentiation, migration/translocation, synaptogenesis).

The information derived from these studies provides novel and important data on cues that guide retinal histogenesis. The findings should have bearing on clinical problems with respect to retinal dysplasia. For example, retinoblastoma is the most common intraoccular neoplasm in childhood, with a worldwide incidence of 1 in 15,000–25,000 live births (Vogel, 1979). It is unclear whether the tumor is neuronal in origin, or whether it is derived from primitive germinal neuroepithelia that is bipotential (or multipotential) (Kyritsis et al., 1984; Tsokos et al., 1986; Katsetos et al., 1991). Factors regulating the termination of cell proliferation are unknown. One report has documented the observation that retinoblastoma cells have enkephalin-like immunoreactivity, suggesting that this peptide originates and/or is utilized by these cells (Tarkkanen et al., 1984). Our hypothesis would be that this opioid may be a growth factor necessary in the autocrine regulation of cell proliferation. Working with human retinoblastoma Y79 cells, we have gathered preliminary evidence that [Met^5]-enkephalin binds to these cells in a specific and saturable fashion, with a K_d of 1.9 nM and a B_{max} of 21 fmol/mg protein. In addition, immunocytochemical staining of human retinoblastoma cells grown in culture have demonstrated enkephalin-like immunoreactivity. These data document the observation that human retinoblastoma cells grown *in vitro* have a growth-related opioid peptide and opioid receptors (putatively, the ζ receptor recognized in cerebellar and brain homogenates) and add support to the hypothesis that retinoblastomas may rely on opioids to control growth. Our studies also may cast light on the origin and course of such tumors. Defective ζ receptors and/or decreases in opioid growth factor could be postulated to interfere with the regulation of cancer cell growth and promote cell proliferation. Another example in which the present results could be of clinical significance concerns optic nerve hypoplasia (Lambert et al., 1987; Zeki and Dutton, 1990), a nonprogressive, congenital abnormality of one or both optic nerves associated with a diminished number of axons. Over the past decade optic

nerve hypoplasia has been cited as a major cause of visual loss in children (Lambert et al., 1987; Zeki and Dutton, 1990). The etiology of this disease is unknown (Lambert et al., 1987; Zeki and Dutton, 1990). A supranormal regression of optic nerve axons or a primary failure of differentiation has been hypothesized to be the factor responsible for optic nerve hypoplasia. Yet another reason may be the production of fewer retinal ganglion cells. It may be conjectured that a high level of opioid peptides *in utero* diminishes the number of ganglion cells. A third example in which data from our studies could be of interest is related to retinal detachment. Retinal detachment is often accompanied by Muller cell proliferation, and this active cell replication results in scar formation and prevention of therapeutic measures and reestablishment of connections (see Fisher et al., 1991). The relationship of opioid growth factor to Muller cell proliferation requires further exploration. Finally, a recent report by Fisher and colleagues (1991) points out that cell proliferation in the retina has received relatively little attention compared with that in the brain. These investigators discuss the importance of cell proliferation as a common feature of many retinal diseases and injuries, including diabetes, photocoagulation, massive retinal gliosis, branch retinal vein occlusion, proliferative vitreoretinopathy, and macular pucker. Studies in which endogenous opioid systems are explored in normal development may provide clues to understanding and providing an exciting substrate in which to pursue future research.

ACKNOWLEDGMENTS

The work conducted in this chapter was supported by NIH grant NS-20500.

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THE MOLECULAR BASES OF NERVE REGENERATION

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Advances in Neural Science,
Volume 2, pages 107–130.

ISBN: 1-55938-625-8

ABSTRACT

Some have surmised that our scientific knowledge of nerve regeneration consists of no more than disjointed pieces of an obscure puzzle. The main purpose of this chapter is to present an overview of the most salient data available. The chapter contains brief summaries of basic peripheral nerve anatomy, situations that result in nerve injuries and require surgical intervention for maximum recovery, and the conditions and biochemical affects of the outcomes. Specific cells and their established contributions to the process of nerve degeneration and regeneration are reviewed. Substrate and cell adhesion molecules, trophic and mechanical factors, and neurotransmitters are included. All of this leads to the conclusion that the phenomenon is complex, and many more investigations are needed to elucidate mechanisms that underlie the process. Continued study of the molecular bases of nerve regeneration is critical for at least two reasons. New data will allow us to develop a more detailed understanding of both central and peripheral nervous system function. In addition, they will lead to new intervention strategies for patients with brain damage or disease.

I. INTRODUCTION

The broad capacity of nerves to respond to various types of injury has been described for decades, and potential contributions to the process have been proposed for many substances. Because many cell substrates and growth mechanistic and trophic factors all appear to contribute to recovery of function in injured nerves, the regeneration process is very complex. In fact, the most critical elements (e.g., initiators and regulators) have not been defined yet. Therefore, this chapter is an attempt to compile and summarize the most salient published data in an updated fashion.

A. Normal Nerve Structure

Nerve trunks are composed of bundles of axons organized by connective tissues into fascicles. Nonneural glial (Schwann) cells, fibroblasts, and endothelial cells are also present. Nerves can contain motor, sensory, or mixed fibers, depending on their target and related function. Motor fibers conduct electrical impulses at a more rapid rate than sensory fibers. The presence of myelin profoundly enhances the velocity of impulse transmission by allowing saltatory conduction (daSilva et al., 1985). A myelinated fiber has a compact myelin sheath, a lipid and protein bilayer formed by a Schwann cell wrapped spirally around the axon. Individual Schwann cells meet along fibers at the nodes of Ranvier; here, small gaps in the myelin exist and the axon is surrounded only by the Schwann cell basal lamina. Small bundles of nonmyelinated axons can be encircled by a single Schwann cell, but no myelin is present (Kuczynski, 1980).

There are three layers of connective tissue within nerves: endoneurium, perineurium, and epineurium. All individual axons are covered by endoneurium; this

covering provides tensile strength to the nerve and promotes resistance to internal axonal pressure. Groups of nerve fibers form fasciculi that are encircled by perineurium. The perineurium can be sutured in order to anastomose severed nerves. Thick, outer connective tissue called epineurium covers the nerve trunk and has extensions that separate fasciculi and blend with the perineurium (see Gibson and Daniloff, 1989).

B. Injuries to Nerves

Severe nerve injuries can result from surgical removal of tumors, intramedullary pin placement in bones, or injections; nerve trauma via long bone fractures, bite wounds, bullet injuries, and lacerations with sharp instruments is also frequent (Gibson and Daniloff, 1989). Recovery is more likely to succeed if axons are simply compressed or have a very short (less than 5 mm) interstump gap to cross (McQuarrie, 1986; Lisney, 1989). Regeneration is more likely to fail if the interstump gap is greater than 1 cm, is associated with soft tissue damage, or occurs in older subjects (Gibson and Daniloff, 1989). Although reactive axonal sprouting is an intrinsic neuronal response to severe injury, the subsequent reorganization of these axonal sprouts is highly dependent upon the presence of Schwann cells (Aguayo and Bray, 1980; Lisney, 1989; and Ramon y Cajal, 1928b).

Transection of peripheral nerves also results in the breakdown of myelin in the distal stump. Macrophages are recruited to remove myelin debris from the distal stump. It has been suggested that these macrophages interact with Schwann cells and may be a source of a Schwann cell mitogen (Scheidt and Friede, 1987).

During the injury response, Schwann cells proliferate (Ramon y Cajal, 1928b). Cell replication begins within the first 24 hours after the injury and continues for approximately 8 days. If the distal stump is separated from the proximal stump, Schwann cells comigrate with regrowing axons, apparently responding to axonal cues. Through transient up-regulation or re-expression of molecules, Schwann cells provide a favorable environment for axonal extension and attract bundles of regrowing axons across interstump gaps up to 1 cm in length. The Schwann cells that proliferate are both myelin-forming and non-myelin-forming (Clemence et al., 1989). The environment through which axons respond to damage of the peripheral nervous system consists of Schwann cells and their basal laminae, fibroblasts, and collagen. During degeneration the environment contains axonal debris, degenerating myelin, and phagocytic cells, some of which are reactive Schwann cells (Ramon y Cajal, 1928b). Other contributing factors, include laminin, fibronectin, collagen, growth and trophic factors, and cell adhesion molecules (Fawcett and Keynes, 1990; Bunge et al., 1980b). External factors that influence regeneration include the effect of a conditioning lesion and the use of pulsed electromagnetic fields (Aebischer et al., 1987). In principle, functional restoration can be accomplished by either random outgrowth and secondary central adjustments of synaptic connections or directed outgrowth toward and reinnervation of the "original" target (McQuarrie,

1986). There are indications that specific factors are present in the distal stump of transected nerves which preferentially attract axons of the corresponding proximal stump into the distal nerve stump (Politis et al., 1982).

C. Nerve Degeneration

Nerve degeneration is a prerequisite for normal motor and sensory axon regeneration following an injury to a nerve (Bisby and Chen, 1990). It leads to the removal and recycling of axonal and myelin-derived material, the preparation of a sound environment through which regenerating axons regrow, and the initiation of nerve regeneration. Degeneration is influenced by the peripheral connection of the injured nerve and the distance the cut section is from the cell body (Gibson et al., 1989; Delgado-Lezama and Munoz-Martinez, 1990). Following axotomy, most surviving cell bodies undergo a variety of anatomical changes and modifications in gene expression and cellular metabolism. The main morphological event is chromatolysis: swelling of cell soma, a pyknotic nucleus, and the dispersal of Nissl substance due to the disintegration of large granular condensations of rough endoplasmic reticulum (Ramon y Cajal, 1928a,b).

Distal to the site of injury, the severed axon and myelin sheath undergo degeneration (Ramon y Cajal, 1928a). The axon, without the continuity of supporting structures and trophic substances from the cell soma, begins to degenerate within 12 hours (Stoll et al., 1989). The axon degenerates before the Schwann cell sheath and becomes irregularly swollen and beaded. The myelin sheath draws away from the axon and breaks apart. Both the axon and the myelin degenerate. Trophic factors accumulate that stimulate Schwann cells to replicate and move along the endoneurium (Ramon y Cajal, 1928b). One factor may have recently been isolated (see below, Ratner et al., 1988). When they reach the injury, Schwann cells fuse with one another to form columns or bands classically referred to as the bands of Büngner (Ramon y Cajal, 1928b). Some Schwann cells become phagocytic in response to injury and ingest extracellular fragments of the axon and myelin (Bunge, 1980a).

This degeneration continues distal to the synapse, where there is a progressive loss of synaptic vesicles and the nerve terminal is phagocytized away from the postsynaptic membrane. A similar process occurs in unmyelinated nerve fibers.

II. REGENERATION

To recover from injury, a neuron must undergo a series of molecular rearrangements. Initially, it must respond to injury by degenerating. Then the terminal membrane and cytoplasm reorganize to generate a motile structure, or nerve growth cone. Growth cones move in a directed manner to appropriate target cells and eventually form synaptic terminals. The neuron produces all the membrane, cyto-

plasmic, and cytoskeletal elements required by the elongating and expanding axonal sprout.

In adult mammals, damaged axons can regenerate for many centimeters within the peripheral nervous system (PNS). These regenerating axons are typically found within conduits of basement membrane, often in contact with Schwann cells. Like many other basement membranes, those in the PNS are comprised of laminin, fibronectin, and other extracellular matrix materials. *In vitro*, purified fibronectin, collagen, and laminin stimulate outgrowth as well as guide nerve processes. This growth requires binding of cell surface receptors to extracellular matrix adhesive proteins as well as binding of regenerating axonal sprouts to Schwann cells. There are multiple adhesive systems operating in the regenerating nerve; both cell–cell and cell–substrate adhesion molecules are present on neurons, Schwann cells, and fibroblasts.

A. Surgical Intervention

Nerve Repair

Surgical procedures have been developed to maximize the number of axons that sprout and grow through the injury site to reinnervate the correct targets (Millesi, 1990; Fields and Ellisman, 1986). Ultimately, the failure of axons to regenerate leads to poor sensory and motor recovery. This explains why such diverse surgical techniques are attempted and why so much study is underway in this field. These techniques are reviewed here because they are frequently adapted for scientific assays that investigate the contributions of various proteins to nerve regeneration.

The two most common methods of surgical repair for injured nerves are direct suturing of nerve stumps that approximate one another and implanting sensory nerve autografts (Gibson and Daniloff, 1989). Epineurium can be used to manipulate the nerve during repair and is the most frequent site of suture placement for repair of simple nerve transection when no gaps exist between nerve stumps (Sunderland, 1980; Braun, 1982). Autologous sensory nerve grafts are often used to bridge gaps in severely injured nerves (Gibson et al., 1989). Overall results include variable recovery of function and the irrevocable loss of sensation associated with the removal of a sensory nerve (Kline and Kahn, 1982).

Surgical Factors

Excessive tension on the nerve repair is believed to result in decreased regeneration and reduced overall function. Tension stretches the endoneurial tubes and results in physical disruption of nerve fibers, impairment of electrical conduction, and compromise of the blood supply to the proximal and distal stumps (Miyamoto, 1979).

Sutureless methods of nerve transection repair have been investigated. Some of these include the use of adhesives to join nerve ends, plasma clots to align nerve

stumps (Romano et al., 1991), and fibrin glue (Kuderna et al., 1979) and carbon dioxide lasers to weld nerve stumps together (Tupper, 1980).

Entubulization, the implantation of nerve cuffs or guide tubes, has been shown to be an alternative to direct suture techniques (Molander et al., 1983). The use of these nerve guide conduits is appropriate for injuries with significant nerve gaps (LeBeau et al., 1988). It is also pertinent for situations where direct realignment of nerve fascicles is impossible. Collagen-based nerve guide conduits are capable of supporting and maintaining axonal outgrowth, extension, and maturation *in vivo* (Archibald et al., 1991).

B. Adhesion Molecules

Substrate Adhesion Molecules

Laminin. A high-molecular-weight glycoprotein of MW 850,000 or greater that is composed of disulfide-bonded subunit chains of 400 and 200 kDa. Laminin is present in basement membrane throughout the body, including the glomerular basement membrane and vascular endothelial basement membrane, and underlies the epithelium of the skin (Foidart et al., 1980). It promotes the attachment of epithelial cells to Type IV collagen (Terranova et al., 1980) and can bind to glycosaminoglycans (Del Rosso et al., 1981). Laminin promotes axon extension by interacting with axonal glycoproteins that are members of the integrin family of receptors. Antibodies against integrins inhibit the extension of central and peripheral axons on laminin or extracellular matrix substrates (Tomaselli et al., 1988).

Fibronectin. A high-molecular-weight glycoprotein of 440 kDa with two apparently similar disulfide-bonded chains of approximately 220 kDa. It is found in plasma and is a major component of basal lamina, connective tissue, and the extracellular matrix produced by fibroblasts. It is involved in adhesion of cells including fibroblasts and platelets to collagen *in vitro* (Ruoslahti et al., 1981). In development, fibronectin may play a role in muscle morphogenesis and in the spatial organization of cells in the developing chick wing (Ruoslahti et al., 1981). Surfaces coated with laminin or fibronectin stimulate a more rapid extension of neuronal processes than do serum- or collagen-coated plastic (Pierce et al., 1988).

Results from an *in vitro* study of Schwann cell adhesion and proliferation showed that Schwann cells from injured nerves possess binding sites for laminin and fibronectin that permit Schwann cell adhesion to substrates *in vitro* (Komiyama et al., 1991). Another *in vitro* study showed that neurons induced the laminin mRNA levels of Schwann cells by releasing a diffusible signal into culture medium (Bunge et al., 1989). It is thought that regenerating axons induce the expression of laminin and possibly fibronectin receptors on Schwann cells that are responsible for the enhanced adhesiveness of Schwann cells *in vitro*. Laminin and fibronectin appear

to be similar in their ability to promote the adhesion of Schwann cells from injured nerves at different times post-crush or post-transection.

The presence of laminin but little fibronectin in the endoneurium of mouse sciatic nerve indicates that laminin occurs *in vivo* in a position where it may play a role in the regeneration and myelinization of injured axons (Foidart et al., 1980). Laminin is also present along pathways of axonal growth during development (Rogers et al., 1983) and regeneration (Hopkins et al., 1985). The presence of fibronectin in the perineurium (Foidart et al., 1980) indicates that it may play a role in Schwann cell–neuron connective interactions (Chiu et al., 1991; Martin and Timpl, 1987). Fibronectin is also found at nodes of Ranvier (Terranova et al., 1980).

Fibronectin and laminin are distinct in several ways. One study revealed that (1) both fibronectin and laminin are components of the mature peripheral nerve and are localized in different patterns; (2) antibodies to each protein differentially recognize Schwann cells (laminin) and fibroblasts (fibronectin) in tissue culture preparations; and (3) laminin is expressed by the Schwann cell prior to the development of a morphologically recognizable basal lamina (Cornbrooks et al., 1983). Data were also provided to demonstrate that normal Schwann cells were capable of synthesizing laminin *in vitro* but incapable of synthesizing fibronectin under the same conditions. In contrast, cultures of Schwann cell tumors were able to synthesize extracellular matrix components, including fibronectin and laminin (Palm and Furcht, 1983). Overall, the presence of laminin *in vitro* was reported to enhance Schwann cell attachment, cell growth, and elongation (McGarvey et al., 1984).

Cytotactin. An extracellular matrix protein, a molecule of widespread distribution in embryonic neural and non-neural tissues. It has been identified in embryonic brain tissue as three closely related polypeptides of M_r 220,000, 200,000, and 190,000 (Grumet et al., 1985a,b). Similar molecules have been called J1/tenascin (Chiquet-Ehrismann et al., 1986) or myotendenous antigen (Chiquet and Fambrough, 1984). Cytotactin binds to a chondroitin sulfate proteoglycan on neurons, as well as to fibronectin (Hoffman and Edelman, 1987), and appears to be involved in cell movement in the developing embryo (Chuong et al., 1987; Crossin et al., 1986; Rieger et al., 1986; Tan et al., 1987). Nerve damage results in modulation of cytotactin in both nerve and target muscles; cytotactin accumulates in interstitial spaces and near previous synaptic sites on muscle fibers. Although levels are elevated in endo-, epi-, and perineurial connective tissue encasements, antibody staining patterns return to normal by 30 days after injury, following reinnervation (Daniloff et al., 1989). The results suggested that an epigenetic signaling system between neurons and glia may regulate expression of adhesion molecules like cytotactin in coordination with other molecules (Daniloff et al., 1989).

Cell Adhesion Molecules

The Neural Cell Adhesion Molecule. A member of the immunoglobulin superfamily (Hemperly et al., 1986b). This group of proteins shares a homologous sequence of 100 amino acids bridged by a disulfide bond (Hoffman et al., 1982). The three polypeptides that make up the neural cell adhesion molecule (N-CAM) are encoded by different messenger RNAs and are produced by alternative splicing of a single gene (Friedlander et al., 1985; Hemperly et al., 1986a).

Approximately 98–99% of all brain N-CAM is membrane associated (Hoffman and Edelman, 1983). In muscle, however, a soluble form of N-CAM is synthesized as a primary translation product; its mRNA sequence distinguishes it from the membrane-associated isoforms of N-CAM (Walsh, 1988). At present, little is known about contributions of this soluble isoform to recovery of motor function, despite the observation that it is expressed by satellite cells that surround denervated muscles (Daniloff et al., 1986b).

Neurons adhere to other neurons by binding homophilically to the N-CAM on their respective surfaces (Brackenbury et al., 1977; Edelman, 1983; Rutishauser, 1983). In the embryo, N-CAM binding is important in neural induction (Chuong, 1990), neurite outgrowth (Rutishauser et al., 1978; Rutishauser and Jessell, 1988; Chang et al., 1987), formation of retinal layers (Buskirk et al., 1980), nerve–muscle interactions (Grumet and Edelman, 1984; Grumet et al., 1982), the ensheathment of axons (Rieger et al., 1988), and the formation of synapses (Fraser et al., 1984; Sanes, 1989). N-CAM is present from the blastoderm stage in chicken (Crossin et al., 1985) and is present on most cells in the brain with a relatively uniform distribution (Persohn and Schachner, 1987). N-CAM is present on all neurons examined in both the CNS and PNS (Thiery et al., 1982; Mirsky et al., 1986). In the PNS, N-CAM is present on neurons, nonmyelinating Schwann cells, and satellite cells of sympathetic and dorsal root ganglia (Mirsky et al., 1986; Daniloff et al., 1986a).

In the developing brain, N-CAM has been shown to mediate calcium-independent neuron–neuron, astrocyte–neuron, and astrocyte–astrocyte adhesion (Edelman, 1983; Seilheimer and Schachner, 1988; Keilhauer et al., 1985). The activity of N-CAM can be regulated epigenetically through modulatory events that alter local N-CAM affinity, prevalence, mobility, and distribution on the cell surface (Cunningham et al., 1987; Edelman, 1984; Crossin et al., 1985). In mature tissues, N-CAM may be a local signal for reformation of nerve–muscle contacts following peripheral nerve injury (Daniloff et al., 1986b; Rieger et al., 1988; Doherty et al., 1991). The embryonic (E) form of N-CAM, which is rich in polysialic acid (Edelman and Chuong, 1982b), appears in injured nerves during the early responses to injury; expression continues until target muscles are reinnervated. This suggests that the E form of N-CAM participates in modulating nerve regeneration (Daniloff et al., 1986b; Landmesser et al., 1990).

The expression of N-CAM was found to increase in response to either minor (e.g., nerve compression) or severe (e.g., transection) injuries. This enhanced expression was found to be greater in nerve stumps on the proximal (i.e., on the nervous system side) of the injury. It is the E form, not the A form, that predominates during the nerve's response to injury (Daniloff et al., 1986b). Immunohistological localization indicated that both Schwann cells and axons were positive for N-CAM, suggesting that they both participate in the process (Daniloff et al., 1986b).

Abnormally low expression of N-CAM has been observed in several strains of mutant mice with demyelination and dysmyelination syndromes (Bhat and Silberberg, 1988a,b, 1990). These results support the possibility that N-CAM contributes to remyelination of regenerated axons.

D-2 Protein, D-2-CAM. A developmentally regulated cell surface sialoglycoprotein (Meier et al., 1984) related to, if not identical with, N-CAM. It comprises three related proteins of M_r 180,000–200,000, 135,000–140,000, and 115,000–120,000 (Hansen et al., 1985). Results following local application of N-CAM antibodies to injured nerves (Remsen et al., 1990) and the intraventricular injection of D-2-CAM in adult rodent brains (Nolan et al., 1987) suggest these proteins are involved in both axon guidance and synapse stabilization. These occur in regenerating nerves (Daniloff et al., 1986b) and at the neuromuscular junction in developing nerves (Rieger et al., 1985).

Nerve Growth Factor-Inducible Large External Glycoprotein. The nerve growth factor-inducible large external glycoprotein (NILE) was originally isolated from cultures of the transformed neural cell line PC12 and consists of a single 230 kDa protein (Greene and Tischler, 1976). Subsequent studies have shown that NILE occurs on all nervous system derivatives (Akeson and Hsu, 1978; Salton et al., 1983), including Schwann cells (Stallcup et al., 1983; Salton et al., 1983). NILE regulates neurite–neurite interaction in embryonic brain cultures (Stallcup and Beasley, 1985). It has been suggested that NILE and the transiently expressed axonal glycoprotein (TAG-1) work in concert to produce orderly neurite outgrowth during nerve development and possibly nerve regeneration (Jessell, 1988).

Neural Cadherin. Cadherins are transmembrane proteins, and their cytoplasmic domain is highly conserved among different members of this molecular family. They are a molecular family that is essential for the calcium-dependent process of cell–cell adhesion (Takeichi, 1988). Their mature form consists of 723 to 748 amino acids and has a single transmembrane domain that divides the molecules into the amino-terminal extracellular and the carboxy-terminal cytoplasmic domain (Shimoyama et al., 1989). It was observed that cells expressing one type of cadherin when mixed with cells expressing another type of cadherin and cultured in suspension tended to aggregate separately (Takeichi et al., 1985). Cadherins interact with cadherins only in a homophilic manner (Takeichi, 1990). Cadherin-mediated

adhesion is temperature dependent, whereas the Ig superfamily-mediated adhesion is not (Takeichi et al., 1985). Neural cadherin (N-cadherin) mediates the attachment of neurites to cells on the substratum. In cell cultures of N-cadherin-transfected cells, a vigorous extension of optic axons took place. The growth cones of the axons attached only to the surface of transfected cells, and not to a culture dish (Tomaselli et al., 1988).

N-cadherin has a major role in the initial contacts of nerve growth cones with Schwann cells (Takeichi, 1988). Sensory neuronal growth cones on a laminin substratum were inhibited from migrating onto the upper surfaces of Schwann cells by soluble antibodies to N-cadherin. This behavior was blocked reversibly when N-cadherin was inactivated by lowering the calcium concentration of the culture medium from 1 to 0.1 mM (Volk and Geiger, 1986). Retinal growth cones were also blocked from moving onto Schwann cells by lowered calcium levels. Electron microscope immunocytochemistry indicated that N-cadherin is abundant on growth cones, on Schwann cells, and at points of growth cone–Schwann cell contact (Letourneau et al., 1990). The above results indicate that N-cadherin has a significant role in growth cone migration onto Schwann cells, since lowered calcium and anti-N-cadherin reduced growth cone migration onto Schwann cells. It is important in the initial migration of growth cones onto the surfaces of Schwann cells.

The Neuron-Glia Cell Adhesion Molecule. Ng-CAM contains three polypeptides; components of chicken Ng-CAM migrate at M_r 200,000, 135,000, and 85,000 on SDS polyacrylamide gels (Grumet et al., 1985a). The 135,000 M_r component predominates in both developing (Grumet and Edelman, 1984) and regenerating (Daniloff et al., 1986b) nervous systems. N-CAM and Ng-CAM share at least one antigenic determinant (Grumet et al., 1985a,b). Developmental expression of this molecule occurs in concert with the movement of neurons along glia, neurite fasciculation, and tract formation (Grumet and Edelman, 1984; Grumet et al., 1984, 1985a,b; Daniloff et al., 1986a,b). Localization in injured nerves suggests similar contributions: Schwann cell movement, nerve regeneration, fasciculation, and tract reformation (Daniloff et al., 1986b). The localization and timing of Ng-CAM expression in damaged nerves also suggest that Ng-CAM, although clearly distinct from N-CAM, may interact with it in response to nerve injuries.

C. Trophic and Mechanistic Factors

Our current understanding of this area is neither homogeneous nor circumscribed. Trophic factors and their receptors are extracellular signals involved in the regulation of normal and injury-induced regenerative nerve cell growth. It has been implied that peripheral effector organs produce limiting amounts of specific neurotrophic factors to ensure proper innervation by appropriate neurites (Korsching and Thoenen, 1983). These factors are believed to be necessary for maintaining the normal, functional, and chemical status of the axon. In fact, data

presented over a half a century ago suggested that such factors also contribute to the normal status of striated muscles (Tower, 1937).

Nerve Growth Factor

Nerve growth factor (NGF) is the only defined molecule for which a chemotropic role has been postulated. *In vitro* the growth cones of sensory neurons orient toward a source of NGF (Gundersen and Barrett, 1979). There are changes in the quantities of NGF and its receptor in regenerating nerves. Very little NGF or NGF receptor is found in a normal peripheral nerve. However, if the nerve is cut or crushed, the level of both molecules and their respective mRNAs in the region distal to the injury increases enormously. The expression of NGF receptor by Schwann cells is probably controlled by axonal contact and decreases when axonal contact is restored (Taniuchi et al., 1986).

When Schwann cells are released from axonal contact, they express NGF receptors on their surface and secrete NGF. This NGF receptor expression and NGF secretion by Schwann cells also extensively occur in the nerves undergoing active degeneration and subside again when nerve regeneration is completed. NGF is actually a potent modulator for increasing the neurite sprouts from the adult rat dorsal root ganglion (DRG) in culture.

β Nerve Growth Factor

β nerve growth factor (β-NGF) supports the differentiation, maturation, and survival of sympathetic and primary sensory neurons (Thoenen and Barde, 1980; Raivich et al., 1989). It is bound by NGF receptors on the surface of neurites, internalized, and then transported to the neural perikarya, where it exerts its neurotrophic effects. Injury to a peripheral nerve results in a biphasic increase in the endoneural mRNA coding for β-NGF, leading to an increase in the local synthesis of ß-NGF protein. However, peripheral axotomy leads to a highly reproducible disappearance of neuronal β-NGF receptors (Taniuchi et al., 1986). Following transection or crushing of the sciatic nerve, β-NGF receptors disappear from the neuronal perikarya in the DRG by 6 days after injury. This decrease is greater after transection than after a nerve crush. There is a concomitant decrease in retrograde axonal transport of endogenous NGF during sciatic nerve regeneration, but the decrease stabilizes at a level that is 33% of normal control values (Raivich et al., 1989). This decrease in retrograde transport corresponds to a decrease in β-NGF in the proximal part of the regenerating sciatic nerve. With peripheral reinnervation there is a gradual increase in the axonal expression of β-NGF receptors and β-NGF retrograde transport. Both of these values reach normal levels 30–40 days later (Korsching and Thoenen, 1983).

Insulin-like Growth Factor I

IGF-I has growth-stimulating activity and is believed to mediate some of the actions of growth hormone. It has been found that IGF-I promotes neurite out-

growth in cultured sensory and sympathetic neurons (Recio-Pinto et al., 1986), spinal cord (Ishii et al., 1989), and cloned human neuroblastoma cells (Recio-Pinto and Ishii, 1984, 1988). IGF-I also stimulates proliferation and growth associated with nerve regeneration (Froesch et al., 1985; Kanje et al., 1989; Sjöberg and Kanje 1989; Hansson et al., 1986). Physiological concentrations of IGF-II can also support the long-term survival of peripheral neurons in culture (Recio-Pinto et al., 1986). One hypothesis is that IGF-I produced by Schwann cells in the distal nerve segment is taken up by the regenerating nerve fibers. Internalized IGF-I is then transferred to the nerve cell body by retrograde axonal transport. In the cell body, IGF-I initiates or enhances the regenerative process, possibly by stimulating lipid and protein synthesis (Kanje et al., 1990).

Growth Associated Protein, B-50/GAP-43

Also known as F1, pp46, and calmodulin binding protein P-57 (Skene, 1989), B-50/GAP-43 is one of a small subset of cellular proteins selectively transported by a neuron to its terminals. Its enrichment in growth cones and its increased levels in developing or regenerating neurons suggest that it has importance in neurite growth. In the adult rat, crush lesion of the sciatic nerve results in a rapid expression of B-50/GAP-43 mRNA followed by synthesis of B-50/GAP-43 protein in DRG and transport into the newly formed sprouts (Gispen et al., 1990). During reinnervation following damage, neuromuscular junctions contain abundant amounts of GAP-43. These levels return to normal after completion of reinnervation (Gispen et al., 1990).

The precise function or mechanism of action by B-50/GAP-43 in developing and regenerating neurons and in adult peripheral and central nervous system neurons is still not clear. Recent data indicated that it is a substrate for protein kinase C and that phosphorylation reduces its ability to bind calmodulin. This suggests that B-50/GAP-43 acts as a transmembrane signal transducer (Gispen et al., 1990). In the regenerating neuron, the axonal growth cone is enriched in B-50/GAP-43 and may need this protein either as a buffer of calmodulin or as a regulator in the polyphosphoinositide response to external signals that guide motility (Gispen et al., 1990). These data suggest that the most likely function of B-50/GAP-43 involves a membrane-associated process like growth cone extension. Furthermore, modulation of B-50/GAP-43 phosphorylation in the adult presynaptic terminal may alter its synaptic plasticity (Benowitz and Routtenberg, 1987).

Ciliary Neurotrophic Factor

The large quantities of ciliary neurotrophic factor (CNTF) present in the sciatic nerve of adult rats suggest that it functions as a "lesion factor," preventing motoneuron degeneration after nerve lesion (Manthorpe et al., 1986; Stockli et al., 1989). This is supported by the following observations: (1) transection of the sciatic nerve in adult rats results in chromatolysis in the motoneuron cell bodies. However, negligible degeneration of facial motoneurons occurs within 1 week (Seniuk et al.,

1992); (2) transection of the facial nerve in newborn animals results in a degeneration of all neurons (Stockli et al., 1989). The extent of degeneration decreases rapidly in the postnatal period and parallels the increase in levels of CNTF in the peripheral nerves (Stockli et al., 1989). The fact that the local administration of CNTF in newborn animals can almost completely prevent the degeneration of the corresponding motoneuron cell bodies supports the validity of a causal relationship between the extent of degeneration and the levels of CNTF in lesioned nerves (Seniuk et al., 1992).

A current study showed that CNTF had a survival effect on embryonic chick motoneurons at the developmental stage where physiological cell death occurs (Arakawa et al., 1990). It was found that CNTF supported maximally 64% of initially plated spinal motoneurons after 3 days in culture and 53% after 6 days.

D. Mechanical Factors

Conditioning Lesions

A conditioning lesion is a mild nerve injury, usually a crush, that is applied prior to a second lesion. Many studies have shown that a conditioning lesion increases the rate of regeneration (McQuarrie, 1986; Bisby and Pollock, 1983; Bisby, 1985; Oblinger and Lasek, 1984). This regeneration is associated with expression of genes and proteins from the cell body. In general, the proteins produced during regeneration are the same as those associated with axonal growth in embryos. These substances include growth associated proteins (GAPs), tubulin, and actin.

There are two main hypotheses regarding peripheral nerve regeneration after a conditioning lesion (Sjöberg and Kanje, 1990). The first is that the local environment surrounding the growth cone controls the rate of axonal elongation. The conditioning lesion causes degeneration in the distal nerve, so that when the test lesion is made the axons should grow more rapidly along pathways vacated by the previously degenerated axons. It is thought that changes in non-neuronal cells, Schwann cells in particular, and trophic factor production are somehow responsible for neurite outgrowth (Bray et al., 1978). The outgrowth of neurites starts within 3 hours after a crush injury (Sjöberg and Kanje, 1990). Therefore, this initial early outgrowth of axons (i.e., growth cone formation) occurs without support from the cell body. This suggestion is supported by the observation that the distal segment of severed axons forms growth cones *in vitro* (Bray et al., 1978; Shaw and Bray, 1977; Wessells et al., 1978).

The second hypothesis is that outgrowth of axonal sprouts is enhanced in conditioned nerves because they are already activated when the second injury occurs. It is assumed that the nerve cell bodies have made necessary adjustments and axons have already synthesized the materials necessary for regeneration (McQuarrie, 1986; Ducker et al., 1969). It is not clear what signals between cell body and axon tip regulate the transition to regenerative growth, or whether events at the axon tip are controlled in the same way as those at the cell body. The duration

of effects caused by conditioning lesions is also not clear. One accepted model for the induction of regeneration involves the cell body invoking changes in at least one trophic factor normally derived from the target; this is retrogradely transported to the cell body through the damaged axon. However, the earliest regenerative sprouting at the axon tip can occur within a few hours of axotomy, too rapidly for the cell body to have been informed.

Investigators have determined at least two physical mechanisms that alter regeneration rate: alterations in the cell body and the environment surrounding the injured axon. Each may act in an additive fashion to produce a maximal conditioning lesion phenomenon (Bisby and Pollock, 1983). Control by the axonal environment might be exerted either by vacant channels to guiding axonal regrowth in the previously degenerated axons or perhaps through a diffusible product of degeneration that stimulates the growth of axons (Bisby and Pollock, 1983). Superimposition of the conditioning and test lesions on the sciatic nerve cause a positive reparative effect on motor neurons. When axons grew into predegenerated nerves their elongation rate was further increased above that obtained when the conditioning lesion was distal to the test lesion (Bisby, 1985). Recovery was accelerated by conditioning; the entire recovery curve shifted to earlier time intervals in conditioned nerves and the initial rate of recovery was greater. This observation was consistent with a more rapid elongation of axons along the peripheral nerve trunks and a more rapid invasion of the denervated muscles. This increased regeneration rate was associated with an earlier recovery of function.

Axons also regenerate *in vitro* much more vigorously from explants taken during the period of embryonic axonal growth, or from older explants whose axons have previously been induced to regenerate (Collins and Lee, 1982). The enhanced regenerative response following a conditioning lesion may be due to the early availability in the axon of molecules associated with regeneration.

Electromagnetic Field Stimulation

The influence of electric and electromagnetic fields on growth and differentiation of nerve tissue *in vitro* has been reported (Orgel et al., 1984; Cooper and Schliwa, 1985). The effects of applying pulsed electromagnetic fields (PEMF) to injured nerves *in vivo* in animal nerve injury models have been the subject of several investigations (Orgel et al., 1984; Aebischer et al., 1987). *In vitro*, cultures treated with PEMF showed a significant increase in neurite outgrowth relative to controls (Sisken et al., 1990).

In vivo exposure to PEMF before lesioning of rat sciatic nerve produced an increase in axonal sprouting after lesioning (Sisken et al., 1989). In studies on a nerve crush model, rats placed immediately after lesioning in a pulsed magnetic field showed a 22% increase in the rate of axonal sprouting relative to controls (Wilson and Jagadesch, 1976). This increase was found to be approximately equal to that obtained with biochemical (collagen) or hormonal agents (growth hormone) and was not dependent upon primarily invasive delivery systems. A comparable

enhancement of regeneration was obtained when the animals were exposed daily for 7 days before lesioning. This response resembles the response described for a "conditioning" lesion (McQuarrie, 1986; Bisby and Pollock, 1983). The same effect was found in one transection model of rat sciatic nerve in which gait analysis was performed 4–6 months following transection, where PEMF signals were found to significantly improve performance relative to untreated controls.

Electromagnetic stimulation has also been found to influence the synthesis of new polypeptides in a rat sciatic nerve transection model. PEMF has been shown to alter the distribution pattern of polypeptides in the injured nerve. Transection injury alone changed the pattern, indicating a "stress" response. Transection combined with PEMF reduced the stress response changes, altering the quantitative distribution of new polypeptides, which are potent promoters of embryonic neurite growth in culture (Bozyczko and Horwitz, 1986; Rogers et al., 1983).

E. Neurotransmitters

Various neurotransmitters contribute to neurite outgrowth as positive or negative influences (Lander, 1987). For example, the administration of nicotinic cholinergic blocking agents (e.g., curare) to retinal explant cultures enhances the outgrowth and elongation of ganglion cell axons (Lipton et al., 1988a,b). Activation of glutamate receptors in the hippocampal formation inhibits neurite outgrowth (Mattson et al., 1988). The vasoactive intestinal peptide (VIP) enhances axon outgrowth from spinal motor neurons (Brenneman and Eiden, 1986) and hippocampal neurons (Lipton and Kater, 1989).

F. Non-Neural Cells

Schwann Cells

The proliferation of Schwann cells during degeneration and subsequent regeneration has been well documented (Abercrombie and Johnson, 1946; Bradley and Ashbury, 1970; Pellegrino and Spencer, 1985; Ramon y Cajal, 1928a). The first wave of Schwann cell proliferation during degeneration occurs at 3–4 days post-transection followed by an axonal regrowth stage occurring at 2–3 weeks (Pelligrino et al.,1986). The role of Schwann cells and hematogenous macrophages in myelin degradation during degeneration of the rodent sciatic nerve had been examined. It was found that before the appearance of adherent macrophages, the myelin sheath fragments into ovoids, small whorls of myelin debris appear within Schwann cell cytoplasm, and the Schwann cell displays numerous lipid droplets (Stoll et al., 1989). Myelin basic protein processed by macrophages was suggested to promote the proliferation of Schwann cells during degeneration (Baichwal and DeVries, 1989). One Schwann cell mitogen has been isolated from regenerating nerves; this factor is an axonal surface proteoglycan–growth factor complex (Ratner et al., 1988). The adhesion and proliferation of Schwann cells is rapidly

and transiently induced during degeneration, and this phenomenon is maintained longer in the presence of regenerating axons (Komiyama et al., 1991).

Schwann cells also respond to nerve injuries by migrating toward the injury (Ramon y Cajal, 1928b; Ide et al., 1983). These cells are capable of binding to one another to form Schwann cell bands; upon these "bands of Bungner" neurites traverse the site and enter the distal stump void of debris (Abercrombie and Johnson, 1946). They also produce type IV collagen and incorporate it into the surrounding substrate (Carey et al., 1983). It has been demonstrated that the proliferation of Schwann cells can be initiated by contact with regenerating axons or even a fraction of axonal membranes (Salzer et al., 1980; Wood and Bunge, 1975).

N-CAM is on the surface and basal laminae of Schwann cells (Rieger et al., 1988) and appears to mediate Schwann cell adhesion (Remsen et al., 1990). It shares a common epitope with myelin-associated glycoprotein (Madison et al., 1987), suggesting that it may also be involved in myelin formation. The localization of N-CAM on Schwann cells responding to nerve injuries (Kruse et al., 1984) suggests that the molecule participates in or even regulates the PNS response to injury.

Nonmyelinated Schwann cells form slender processes that are N-CAM positive in the regenerating adult mouse sciatic nerve for 2 to 6 days during and after degeneration of axons; only a few myelinating Schwann cells express N-CAM (Landmesser et al., 1990). Growth cones and regrowing axons express N-CAM at contact sites with fibroblast-like cells on the cut ends of nerve stumps; regrowing small-diameter axons were N-CAM positive where they contacted each other or contacted Schwann cells (Landmesser et al., 1990). Schwann cells associated with degeneration myelin also expressed N-CAM (Martini and Schachner, 1988). The presence of extracellular N-CAM near an injury suggests that its secretion could be an attractant for neurites or migrating Schwann cells.

Oligodendroglial Cells

Studies of CNS glia are important because they assist us in understanding the relative lack of CNS regeneration where peripheral glia are not normally present. For example, homografts of sciatic nerves, when applied to injured spinal cords, induced significant numbers of central fibers to grow through them (Benfey and Aguayo, 1982). Transplants of Schwann cell cultures into spinal cord cavities (Kunlengel et al., 1990a,b) induced significant regeneration of motor pathways in young animals, supporting a widely held theory that these peripheral glia are the source of the most critical components of nervous system regeneration. Furthermore, the lack of these components in central glia would correlate with the central nervous system's relative inability to regenerate and restore lost functions.

Oligodendroglial cells and the myelin they produce both inhibit neurite outgrowth *in vitro* (Bandtlow et al., 1990). When antibodies to myelin membrane proteins of M_r 35,000 and 250,000 were applied to damaged spinal cords, significant sprouting of motor fibers was observed (Schnell and Schwab, 1990). There-

fore, the neutralization of oligodendrocytic factors that inhibit regrowth demonstrate the capacity of central nervous system neurons to regenerate.

Astrocytes

The contributions of these glial cells to the development of the CNS and its response to injury are complex. Astrocytes promote axon elongation during development, but this ability is absent in adults (Silver and Sidman, 1980). In adult nervous tissue, astrocytes form tumors and scars in response to many cues, including injury (Letourneau, 1985). A family of adhesion proteins that includes cytotactin (Grumet et al., 1985a,b), J1/tenascin (Chiquet-Ehrisman et al., 1986), and astrotactin (Edmonson et al., 1988) has been isolated recently that participates in adhesive mechanisms of astroycytic development and regeneration. It has been suggested that these molecules mediate regional order by establishing temporary boundaries that define functional units in developing cortical regions (Steindler et al., 1989a,b) and regenerating neuromuscular systems (Daniloff et al., 1989; Martini et al., 1990).

Fibroblasts

Fibroblasts are known to produce fibronectin, a high-molecular-weight glycoprotein of the extracellular matrix that supports nerve outgrowth (Pierce et al., 1988; Ruoslahti et al., 1981). Transforming growth factor β (TGF-β-I) promotes growth and proliferation of many cells, especially fibroblasts (Heine et al., 1987; Kimchi et al., 1988). Fibronectin and the neural cell adhesion molecule do not work in concert during the development of the neural crest; a reciprocal staining pattern was observed in basement membranes of neuroepithelial cells and the extracellular matrix (Thiery et al., 1982). Contributions by fibroblasts similar to these could conceivably occur during regeneration, as fibroblasts are numerous around injuries.

G. Other Potential Contributors to Nerve Regeneration

This section is meant to provide an overview of several factors that are currently being tested for activity in regeneration.

Glial-Derived Nexin

Also known as protease nexin I (Barker et al., 1980), glial-derived nexin (GDN) is a serine protease of M_r 41,700 (Sommer, 1987). It was identified in culture supernatants of glioma cells by its neurite outgrowth-promoting activity (Monard et al., 1973). GDN promotes neurite outgrowth *in vitro* from neuroblastoma cells (Monard et al., 1983; Gurwitz and Cunningham, 1988) and sympathetic neurons (Zurn et al., 1988). Following crushes of sciatic nerves, the amount of GDN messenger RNA and released GDN are transiently increased (Meier et al., 1989). This increase peaked 6 days after the injury; since most activity was noted 3 to 7

days after the injury, it was suggested that this molecule is likely to be involved in chromatolysis or degeneration of injured nerves (Meier et al., 1989).

Proteoglycans

Proteoglycans consist of a protein core upon which glycosaminoglycan chains are covalently linked (Hassell et al., 1986). Chondroitin sulfate (Kiang et al., 1981) is the predominant glycosaminoglycan in adult mammalian brains. Heparin sulfates are also well-documented components of brain parenchyma (Ripellino and Margolis, 1989). The observation that the substrate adhesion molecule cytotactin/tenascin binds to a chondroitin sulfate protein implicates it as a molecule that contributes to the orderly formation and reformation of the nervous system (Margolis and Margolis, 1989).

Plasminogen Activator

Glial cells secrete plasminogen activator (PA) to generate plasmin activity; this controls a cascade of proteolytic activities that degrade extracellular components, regulate plasticity, and remodel the nervous system (Soreq and Mishkin, 1981; Alvarez-Buylla and Valinsky, 1985; Kalderon, 1984; and Krystoskek and Seeds, 1984). CNS astrocytes are equipped with these capabilities for a limited developmental period (Kalderon et al., 1990).

Thy-1

The Thy-1 molecule of neurons is a glycoprotein of the neural membrane that constitutes approximately 5% of the neural cell surface (Morris, 1985; Campell et al., 1981). A receptor on astrocytes that binds to Thy-1 has been isolated (Dreyer et al., 1989). Data show that binding of neurons to astrocytes controls neurite outgrowth and regeneration. Three studies have shown that the application of Thy-1 antibodies enhances neurite outgrowth *in vitro* (Leifer et al., 1984; Lipton et al., 1988b, and Manhanthappa and Patterson, 1987). It is an endogenous inhibitory regulator of neural growth which, when down-regulated or blocked, is permissive for both growth and regeneration (Manhanthappa and Patterson, 1987).

III. CONCLUSION

More complete understanding of nerve regeneration would represent a landmark in favor of patient recovery from nerve damage. It would also establish a strong base upon which central nervous functions could be explored. It is obvious from the material presented in Table 1 that regeneration is not merely an expression of developmental events, but a complex series that includes many developmental contributions. These factors all interact to produce the ultimate effects.

The best candidates for initiators of nerve degeneration (chromatolysis) are the trophic factors β-NGF and CNTF produced in response to nerve injuries (Table 1). Although N-CAM appears to be a likely candidate for initiating nerve regeneration,

Table 1. Summary of Factors

	Degeneration				*Regeneration*				*Reinnervation*				*Remyelination*			
Factor	*I*	*P*	*T*	*Ref**	*I*	*P*	*T*	*Ref*	*I*	*P*	*T*	*Ref*	*I*	*P*	*T*	*Ref**
Adhesion molecules																
Laminin						+		23		+			+			6
Fibronectin						+		19				19				
Cytotactin						+		5		+						
N-CAM					+			3,4				3,4		+		18
Ng-CAM						+		3,4								
D-2 protein						+		15								
N-cadherin						+		24								
Trophic factors																
NGF						+		8						+		25
B-NGF	+			16	+			10								
IGF-1					+	+		9								
GAP-43						+		7		+		7		+		7
CNTF	+	+		22												
Cells																
Schwann cells			+	1	+	+		17					+		+	1,12,17
Oligodendroglia							+	2								
Astrocytes							+	15								
Fibroblasts						+		19								
GDN								14								
Proteoglycans						+		13								
Plasminogen activator						+		21								
Thy-1						+		11								

Notes: Table includes established and hypothesized contributions of each factor to the overall response to nerve injury, and nerve degeneration, regeneration, muscle reinnervation, and nerve remyelination. Abbreviations for involvement in processes: I, initiator; P, promotor; T, terminator.

*References: [1]Abercrombie and Johnson, 1946; [2]Bandtlow et al., 1990; [3]Daniloff et al., 1986a; [4]Daniloff et al., 1986b; [5]Daniloff et al., 1989; [6]Foidart et al., 1980; [7]Gispen et al., 1990; [8]Gunderson and Barrett, 1979; [9]Ishii et al., 1989; [10]Kanje et al., 1990; [11]Leifer et al., 1984; [12]Madison et al., 1987; [13]Margolis and Margolis, 1989; [14]Meier et al., 1989; [15]Nolan et al., 1987; [16]Raivich et al., 1989; [17]Ramon y Cajal, 1928b; [18]Rieger et al., 1988; [19]Ruoslahti et al., 1981; [20]Silver and Sidman, 1980; [21]Soreq and Mishkin, 1981; [22]Stockli et al., 1989; [23]Takeichi, 1988; [24]Taniuchi et al., 1986; [25]Terranova et al., 1980.

there are at least a dozen factors that may promote the process (Table 1). The extent of their participation is not yet clear.

Candidates for muscle reinnervation include adhesion molecules, fibronectin, N-CAM, NGF, and GAP-43. The best candidate for initiation of nerve remyelination is laminin, although N-CAM appears to be involved in regulation.

The differential between CNS versus the PNS regeneration is not due to the exclusive presence of Schwann cells in the periphery. However, Schwann cells are one of the most critical components of the process.

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TROPHIC ACTIONS OF GONADAL STEROIDS ON NEURONAL FUNCTIONING NORMALLY AND FOLLOWING INJURY

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Advances in Neural Science,
Volume 2, pages 131–152.

ISBN: 1-55938-625-8

ABSTRACT

Neuroendocrine research has clearly established that gonadal hormones are capable of having profound trophic effects on the nervous system. These neurotrophic actions of gonadal steroids extend to normal aspects of neuronal functioning such as cell growth, metabolism, cell survival and development, neurite outgrowth, and synaptogenesis. In the first part of this chapter, some of the neurotrophic effects of gonadal steroids on normal developing and adult brain and spinal cord neurons will be reviewed. In the latter portion, we will discuss our recent experiments in which we have exploited these neurotrophic properties of gonadal steroids in enhancing the motor neuron's response to injury.

I. EFFECTS OF GONADAL STEROIDS ON NEURONAL GROWTH

Work over the past several decades has shown that estrogen plays an important role in mediating both the behavioral and endocrine aspects of reproduction in the female rat. The arcuate nucleus (ARC) and ventromedial nucleus (VMN) of the hypothalamus, medial preoptic area (MPOA), and amygdala (AMY) have been shown to concentrate estrogen, with the ventrolateral portion of the VMN (VL-VMN) and the MPOA containing the highest percentage of hormone-concentrating neurons (Pfaff and Keiner, 1973; Morrell and Pfaff, 1978). In an ultrastructural study of the effects of estrogen on neuronal morphology, Cohen and Pfaff (1981) found increased stacking of rough endoplasmic reticulum (RER) as well as an increased number of dense-core vesicles in VL-VMN neurons in ovariectomized (OVX) rats with long-term (15-day) estrogen treatment. On average, 40.5% of hormone-treated VL-VMN neurons contained stacked RER, compared to 16.2% of cells in the control OVX group. Dense-core vesicles reached a mean number of 17.4 in hormone-treated VL-VMN neurons from estrogen-treated rats compared to 5.7 in the control OVX group. It was hypothesized that the increased stacked RER and dense-core vesicles are indicative of increased synthesis of secretory proteins, which may mediate the estrogen-dependent lordosis response in the female rat (Cohen and Pfaff, 1981).

In a later study utilizing ultrastructural and morphometric analysis, Jones et al. (1985) have also shown significant effects of estrogen on rat VL-VMN neurons. In

OVX rats, VL-VMN neurons were found to contain small ellipsoid nuclei with extensive invaginations of the nuclear envelope and clumps of heterochromatin scattered throughout the nucleoplasm. After an acute, 2-hour exposure to estrogen, nuclear shape was altered from ellipsoid to spherical with overt swelling of the nucleus, nucleolus, and the cell body. A decrease in the number of clumps of heterochromatin and an increase in the frequency of cells containing stacked RER were observed in the estrogen-treated VL-VMN cells. These morphological changes suggest rapid effects of the steroid on the transcriptional/translational activities of the neuron (Merriam, 1969; Busch and Smetana, 1970; Vic et al., 1980), as well as a growthlike action of estrogen on responsive neurons.

II. EFFECTS OF GONADAL STEROIDS ON NEURONAL METABOLISM

A. RNA Studies

As detailed above, the results of studies examining the morphological consequences of estrogen exposure on hypothalamic neurons led to the hypothesis that steroid hormones can influence cell metabolism in a trophic, anabolic manner. In a tritiated uridine autoradiographic study (Jones et al., 1986b), levels of newly synthesized RNA were determined in neurons in the ARC, POA, VMN, and AMY neurons following long-term estrogen treatment. In OVX rats chronically treated with estrogen for 15 days, the results indicate a significant increase in labeling in ARC and POA neurons (50% and 52%, respectively), whereas no effects were seen in the VMN and AMY. Significant increases in mean somal area of ARC and POA neurons (16% and 43%, respectively) were also found, whereas no effects were noted in the other regions studied. The lack of effect of the hormone on VMN neurons was unexpected, since morphological changes in rough endoplasmic reticulum stacking (Cohen and Pfaff, 1981) and nuclear structure (Cohen et al., 1984) have been seen after 15 days of estrogen treatment.

As measured by *in situ* hybridization, the effects of short-term (6 hour), intermediate (24 hour), and long-term estrogen implants on levels of ribosomal RNA were examined in VMN, ARC, and AMY neurons of OVX rats (Jones et al., 1986a). A significant increase in the mean number of grains was found in the VL-VMN and ARC nuclei, relative to control, after 6 hours (70% and 30%, respectively) and 24 hours (60% and 62%, respectively) of estrogen treatment, whereas no effect was seen following chronic 15-day exposure to the hormone. In contrast, no change in mean number of grains was seen in the DM-VMN, an area with few estrogen-concentrating cells, and AMY neurons at any of the time points examined. An interesting pattern of change between the VL-VMN and ARC was observed in this study. In the VL-VMN, levels were maximal at 6 hours, with a slight decrease at 24 hours. In the ARC, levels were low at 6 hours and then peaked at 24 hours. These results, suggesting temporal and regional specificity of action on hormone-respon-

sive areas of the brain, led to subsequent work exploring the effects of steroids on rRNA processing in individual neurons (Jones et al., 1990). In an *in situ* hybridization experiment utilizing two types of rDNA probes, one specific to precursor rRNA and another specific to stable rRNA, the estrogenic regulation of ribosomal RNA was studied in VL-VMN and ARC hypothalamic neurons. Hormone was administered for 15 minutes, 30 minutes, 2 hours, or discontinuously (2 hours on/7 hours off/2 hours on). This discontinuous mode of hormone administration was determined to be adequate estrogen priming for progesterone-facilitated lordosis behavior at 24 hours (Parsons et al., 1982). The results indicate significant increases in levels of precursor RNA only in VL-VMN neurons as early as 30 minutes, with levels doubling at 2 hours. No difference in product RNA was seen at any time point in either the VL-VMN or DM-VMN. From these studies, it has been hypothesized that estrogen acts by rapidly altering transcription of rRNA genes rather than affecting the stability of already transcribed rRNA (Jones et al., 1990).

B. Protein Studies

It has been well established that the MPOA is involved in mediating the effect of androgens on male rodent sexual behavior, and VL-VMN plays an important role in mediating the estrogen-dependent lordosis response in the female rat (Hart, 1974; Larsson, 1979; Pfaff, 1980; Rubin and Barfield, 1983). It has been suggested that the behavioral effects of the gonadal steroids are mediated through changes in protein synthesis induced by hormones in the MPOA and VMN. In a study by Scouten et al. (1985), the effect of gonadectomy on protein synthesis in the MPOA and VMN was compared to intact levels in male and female rats. Following separation by two-dimensional gel electrophoresis, silver staining, and quantification by optical densitometry, it was discovered that many proteins differed between the MPOA of intact males and estrous-induced females, but there were few differences in the VMN protein profile between these two groups. This could be explained in part by the existence of a sexually dimorphic nucleus within the POA (SDN-POA), which was part of the tissue collected from this area. Also, several reversal patterns were noticed in the MPOA and VMN. It was concluded from this study that the effects of steroids on protein regulation are gender and region specific and that future studies would be helpful in defining the relationship between individual proteins and the expression of sexual behavior in both sexes.

In a study by Rodriguez-Sierra et al. (1987), the protein profile of the ARC–median eminence complex (AM) in response to estrogen treatment was examined. During normal puberty or after a pulse of estrogen during the prepubertal period in female rats, an increase in the number of synaptic contacts can be observed in the AM coincidental to a significant increase in several proteins in this area (Clough and Rodriguez-Sierra, 1983; Rodriguez-Sierra et al., 1986). Utilizing two-dimensional gel electrophoresis and autofluorography methods, these investigators identified changes in radioactively labeled amino acid incorporation within

the first 2 days following estrogen treatment. It was concluded that estrogen has a definite role in the regulation of protein synthesis in the AM, although additional work is necessary to determine the relationship of these proteins to hormone-induced synaptogenesis.

The ability of estrogen to alter protein synthesis in the VL-VMN and POA cells was also studied by Jones et al. (1988). In contrast to the studies previously mentioned, the effect of estrogen on protein synthesis in these two regions was examined very early after hormone exposure. OVX adult female rats were treated with either acute or discontinuous estrogen treatment. Two-dimensional gel electrophoresis analysis of ^{35}S-labeled proteins in both the VMN and POA showed that either hormone paradigm is sufficient to induce or repress synthesis of several proteins. Also, in both areas of the brain, the population of proteins affected after 2 hours of hormone treatment was different from those following discontinuous hormone treatment. Earlier work by Jones et al. (1987) has shown that estrogen and progesterone can have synergistic and independent effects on identical brain regions. These studies suggest that gonadal steroids have functions unique to specific areas of the brain and that they accomplish their action through the regulation of cell-specific proteins (Jones et al., 1988).

III. EFFECTS OF GONADAL STEROIDS ON NEURONAL SURVIVAL AND DEVELOPMENT

A. Spinal Nucleus of the Bulbocavernosus

Located near the midline of the ventral horn in the fifth and sixth lumbar segments of the rat spinal cord is a population of large, densely staining multipolar motoneurons that innervate two perineal muscles important in controlling penile reflexes in male copulatory behavior, the levator ani and bulbocavernosus muscles (Breedlove and Arnold, 1981; Sachs, 1982; Hart and Melese-d'Hospital, 1983). This sexually dimorphic nucleus, the spinal nucleus of the bulbocavernosus (SNB), and the levator ani and bulbocavernosus muscles are absent or reduced in normal adult female rats. Adult male rats have approximately 200 SNB motoneurons, whereas adult female rats have about 60 SNB cells (Breedlove and Arnold, 1980). The few SNB cells that are present in the adult female rat innervate the nonsexually dimorphic anal sphincter muscle (McKenna and Nadelhaft, 1984). Adult male SNB motoneurons bind tritiated testosterone or dihydrotestosterone in greater amounts than motoneurons in the same spinal cord segment and do not bind estrogen or any of its metabolites (Breedlove and Arnold, 1980; 1981).

To explore the hypothesis that sexual dimorphism in the SNB is due to the action of androgens during development and adulthood, Breedlove and Arnold (1981) conducted a series of experiments in adult male, adult female, and genetically altered male rats with the testicular feminization mutation (*tfm*), an X-linked recessive gene defect (Lyon and Hawkes, 1970). The gonads of *tfm* rats produce

high levels of testosterone, but the genitals of these animals are feminized because of the lack of androgen receptors. Male rats with this mutation display a 85–90% reduction of androgen receptors in the anterior pituitary, hypothalamus, preoptic area, and cortex (Naess et al., 1976; Breedlove and Arnold, 1981). These investigators found no differences in SNB cell number in either normal male or female rats following adult gonadectomy or adult testosterone treatment. Furthermore, the results indicated no significant difference in cell number between intact animals, animals gonadectomized as adults, and gonadectomized animals injected daily with testosterone. Although no effect was seen in adult SNB cell number in either sex, hormone manipulation did affect adult SNB cell size. All male groups, except *tfm* rats, had larger SNB cells with larger nuclei compared to intact females. Castration of adult males resulted in a 17% decrease in somal size, whereas testosterone-treated OVX female rats showed a 22% increase in cell size (relative to respective controls). Perhaps the most interesting finding of this study was that the *tfm* rats had fewer SNB cells than their normal male littermates. In fact, the number and size of SNB cells in *tfm* rats were similar to those in female rats. Upon inspection of the perineal muscles innervated by the SNB, it was noted that these muscles were absent in adult female and *tfm* male rats. All other males had easily identifiable perineal muscles, except for the castrated males, which displayed atrophy of these muscles. These investigators concluded that sexual dimorphism in both SNB motoneurons and their target musculature is due to the organizational effect of perinatal androgen exposure. Thus, the lack of effect in the *tfm* rats suggests that the differences in cell number are due to the action of androgens and their receptors during development.

In a later study a critical developmental period, coincident with normal SNB cell death, was determined to be androgen dependent (Nordeen et al., 1985). The results of this study demonstrated two distinct phases in the development of the rat SNB. The first phase occurs between embryonic (E) days 18 and 22 and is characterized by a sharp increase in the number of SNB cells in both male and female embryos. At E18, SNB cell number is similar between the sexes. The increase in SNB cell number between E18 and E22 results in many more neurons than would be present in the adult male or female. Between E22 and postnatal day (P) 10, a marked decrease in SNB cell number occurs in both groups, but this loss is more dramatic in females. In the female, 70% of SNB motoneurons undergo a morphological sequence indicative of degeneration compared to 25% of male SNB cells. By P10, the number of SNB cells in both sexes has reached adult levels, with the SNB virtually absent in females. In contrast, females treated perinatally with testosterone throughout this critical developmental period did not statistically differ in SNB cell number when compared to normal males. It was concluded from these results that sex differences in SNB cell number were due to the influence of androgens on normally occurring cell death. Most importantly, perinatal androgen treatment can attenuate this programmed cell death. Thus, gonadal steroids appear to interact with factors that are important for motoneuron survival such as axonal growth, synapse

formation, availability of postsynaptic sites, and/or the presence of neurotrophic factors secreted by muscle (Nordeen et al., 1985).

In another study, the developmental pattern of *tfm* SNB cells was found to be similar to that of normal males, suggesting that the initial cell increase seen through E20 is androgen independent (Sengelaub et al., 1989). Like normal rats, the same two phases of development are seen in the *tfm* animal. At E18, SNB cell number does not differ between the *tfm* and normal embryos. Between E18 and 20, the motoneuron increase in the *tfm* rats was the same as that noted in normal males and, by E20, SNB cell number was significantly greater in both male groups when compared to normal females. However, this increase in *tfm* SNB cells reached a plateau, such that by E22, normal males were found to have more SNB cells than either the *tfm* or female rats. After E22, the decline in *tfm* SNB cell number mirrored that seen in females, and by P10, SNB cell number was not significantly different between *tfm* male and female pups. To explain the early masculinization of the *tfm* SNB prior to E22, the authors suggested that the small number of receptors present in these mutant male embryos is initially adequate to develop the male pattern of SNB development. It was also suggested that at this prenatal age, androgen number and/or function may be normal in *tfm* male rats, with androgen insensitivity becoming apparent only during later stages of development. Alternatively, the sex difference in SNB number may be unrelated to androgens at this early time point. However, these investigators ultimately concluded that after E20, the difference in SNB development between *tfm* and normal males is androgen dependent.

B. Dorsolateral Nucleus of the Spinal Cord

In addition to the SNB, another sexually dimorphic motoneuron population exists in the rodent spinal cord. The dorsolateral nucleus (DLN) is located at the same lumbar spinal cord segments as the SNB, and together the two nuclei are considered the rodent equivalent of Onuf's nucleus (Schroder, 1980; Sengelaub and Arnold, 1989). The DLN lies in the dorsal portion of the ventral horn between the SNB and the lateral motor columns, and as with the SNB, the DLN is significantly larger in the adult male than in the adult female rodent. As demonstrated by autoradiographic studies, adult DLN motoneurons concentrate androgens (Breedlove and Arnold, 1980). The DLN innervates the ischiocavernosus (IC) muscle, which is present at birth in female rodents but atrophies in the absence of androgens. However, the developmental profile of DNL cells is slightly different than the SNB. At E18 there are approximately 1200 DLN cells present in both male and female rat embryos. This is in contrast to the SNB, which does not reach maximal cell number until E22. After E20, DLN cell numbers decline sharply. Like the SNB, by P10 the adult number of cells is reached and males have significantly more DLN neurons than females. Perinatal androgen treatment of females was found to attenuate this cell loss (54% in males versus 74% in females) to typical male levels. It was hypothesized that the significant increase in SNB motoneuron number between E18 and

E22 is due to a migration of cells from the DLN followed by a period of androgen-regulated cell death in both nuclei (Sengelaub and Arnold, 1989; Nordeen et al., 1985). This hypothesis was substantiated by the finding that at E18, following HRP injection into the target muscles, there was little labeling of cells in the medially located SNB, with large numbers of cells labeled laterally across the ventral horn and in the lateral motor columns in both sexes (Sengelaub and Arnold, 1989). Despite cell migration from the DLN to the SNB, sexual dimorphism is primarily due to androgenic regulation of cell death. This cell death, beginning between E20 and E22 in the SNB and DLN, reduces cell number to adult levels by P10 (Sengelaub and Arnold, 1989).

C. Medial Preoptic Area

Located within the medial preoptic area of the rat hypothalamus is another sexually dimorphic region first identified by Gorski and co-workers in 1978 (Gorski et al., 1978). Early work determined the importance of this region in the control of masculine sexual behavior and the cyclic release of gonadotropins responsible for ovulation, and set the stage for later discoveries that true structural differences exist between the sexes (Gorski, 1966, 1971; Arnold and Gorski, 1984). The sexually dimorphic nucleus of the preoptic area (SDN-POA) was described as an area of intensely staining neurons three to seven times greater in volume in the adult male when compared to the adult female rat. Castration of newborn male rats resulted in a 50% reduction of volume of the SDN-POA at adulthood, and neonatal replacement of hormone reversed the effects of castration (Gorski et al., 1978; Jacobson et al., 1981). In a later study, the existence of this nucleus was confirmed following the discovery that the number of neurons located within this nucleus was approximately 1900 in the adult male and 640 in the adult female rat (Gorski et al., 1980). In addition to the increased number of neurons, males had significantly larger cells as well as a greater percentage of large cells within the SDN-POA. Neonatal female rats treated with a single injection of androgen showed an increase in volume of the adult SDN-POA compared to the appropriate vehicle-treated animals (Gorski et al., 1978; Jacobson et al., 1981). Early prenatal androgen treatment of female pups resulted in SDN-POA volumes equal to that of males (Dohler et al., 1982a). Consistent with the concept that testosterone aromatization to estrogen is necessary for masculinization of the brain is the discovery that perinatal estrogenic treatment of female rat pups resulted in adult SDN-POA volumes similar to that of the adult male (Gorski et al., 1981; Dohler et al., 1982b). Prior to these studies, the view was held that sex differences in behavior and reproductive function were due to subtle anatomical differences in the neuronal circuitry responsible for their manifestation, since behaviors characteristic of one sex (i.e., lordosis) could be induced in the opposite sex in the proper hormonal environment (Arnold and Gorski, 1984). With the discovery of significant gender-based differences in the central nervous system,

it is now accepted that sexual dimorphism of these structures is due to the perinatal influence of gonadal steroids.

D. Anteroventral Periventricular Nucleus of the Preoptic Area

In contrast to the effect of androgens on increasing the size of the SDN-POA described by Arnold and Gorski (1984), Ito et al. (1986) have shown that androgens *decrease* the volume of an adjacent region, the anteroventral periventricular nucleus of the preoptic area (AVPv-POA). This region is larger and more densely staining in the female compared to the male (Bleier et al., 1982) and appears to be involved in the cyclic release of gonadotropins (Terasawa et al., 1980). The results of a later study (Murakami and Arai, 1989) demonstrate that the decrease in volume of the AVPv-POA is due to a decrease in the number of cells in the nucleus. In female rats injected postnatally with testosterone, an increased rate of degenerating cells after postnatal day 7 was reported, resulting in the presence of degenerating cells when they were virtually absent in normal female pups. These investigators suggested that androgens prolong the period of cell death in the developing AVPv-POA, which then results in decreased cell numbers and the apparent sexual dimorphism of this brain region. Thus, androgens may prevent or induce cell death in the sexually dimorphic nuclei.

E. Nonsexually Dimorphic Motoneurons

Work by Hauser and Toran-Allerand (1989) with spinal cord cultures found that androgens promote the survival of nonsexually dimorphic motoneurons. In this study, ^{3}H-thymidine autoradiography was used to determine the birthdate of lumbosacral motoneurons in developing embryos. At E12, the spinal cords were dissected from the embryos (prior to the period of naturally occurring cell death) and cultured alone in the presence of mouse thigh muscle and/or treated with a mixture of testosterone and 5-dihydrotestosterone (DHT) for 1 month. Following serial sectioning of embedded explants and autoradiographic processing of slides, it was discovered that exposure to androgens resulted in a 52% increase in the number of ^{3}H-thymidine-labeled cells. Since there was increased labeling of cells regardless of the presence of the target muscle, it appears that androgens have a direct "neuronotrophic" effect on the motoneurons (Hauser and Toran-Allerand, 1989). The possibility that the androgens have a mitogenic effect on the developing motoneurons was considered unlikely since androgen administration was delayed 2 days after ^{3}H-thymidine treatment. One of the most interesting findings of this study was the increase in cell number in all areas of the spinal cord, regardless of regions that are considered acutely responsive to androgen treatment during development (the sexually dimorphic SNB and DLN). Since all the cells that were labeled at E10 are located in the same region as future adult ventral horn motoneurons that contain androgen receptors, it was hypothesized that the increased cell survival may be androgen receptor mediated.

IV. EFFECTS OF GONADAL STEROIDS ON NEURITE OUTGROWTH AND THE CYTOSKELETON

A. Neurite Outgrowth

The first study demonstrating that gonadal steroids promote neurite outgrowth was completed by Toran-Allerand in 1976. Hypothalamic and preoptic explants taken from neonatal mice showed a dramatic proliferation of neuronal processes in response to exogenous estrogen or testosterone. Such robust outgrowth was characterized by extensive arborization and the formation of dense plexuses of neuronal processes extending nearly three times as far as control explants. It was noted that the induction of neurite outgrowth was limited to a small number of cells, which suggested that the effect was specific to the steroid-sensitive cells rather than the entire neuronal explant population. One of the most interesting findings of these studies was that cell cultures treated with nutrient medium containing antibodies specific to estrogen displayed a retardation of neurite outgrowth and maturation only in those regions shown to be steroid sensitive (Toran-Allerand, 1976). The retardation of growth by antibodies against estrogen and the lack of effect by dihydrotestosterone (a nonaromatizable androgen) strengthens the hypothesis that intraneuronal aromatization of androgen to estrogen is a critical element for the male pattern of sexual differentiation (Toran-Allerand, 1984). The results of these experiments, designed to explore the cellular events responsible for sexual differentiation of the brain, argue against the simplistic view that an individual is "programmed" to follow a female pattern of development unless exposed to androgens during an early critical period. Rather, the male and female patterns of development may both require active induction by steroid, as illustrated by the dense neurite proliferation following estrogenic treatment of hypothalamic explants (Toran-Allerand, 1976).

In the canary, there exist several well-defined brain nuclei that are related to the production of song, a behavior normally expressed by the male and not the female bird. In the past, it was thought that the neural system responsible for the production of song can be influenced by gonadal steroids only during the early stages of development. Studies by Nottebohm (1980) showed that adult neonatally ovariectomized females treated with testosterone, but not estrogen or DHT, were induced to sing and that the volume of the robustus archistriatalis (RA), a forebrain nucleus for song control, was increased in these animals. Further studies by these investigators showed that testosterone increased the total dendritic length and branching of neurons in the RA compared to estrogen- and DHT-treated birds (DeVoogd and Nottebohm, 1981). Because of the extensive androgen-induced branching, it was suggested that each of the larger RA neurons was now able to receive a greater number of afferent inputs from neighboring cells. An important finding was that animals treated with estrogen or DHT showed dendritic length and branching intermediate between OVX controls and testosterone-treated females. These inves-

tigators concluded that gonadal steroids have an effect on dendritic length in adult avian brain.

In a study by Kurz et al. (1986), variations in androgen levels in the adult mammal were found to have profound yet reversible effects on the dendritic length of SNB motoneurons, suggesting an amazing structural plasticity of adult motoneurons. SNB neurons were examined in normal and castrated adult male rats, utilizing retrogradely transported horseradish peroxidase (HRP) to determine dendritic length and arborization. In addition, the cross-sectional somal area of SNB motoneurons was measured in response to hormonal manipulation. The results indicate a reduction in dendritic length and somal area in adult males that have been castrated for 6 weeks (56% and 45%, respectively). This decrease in dendritic length and cell size could be reversed to normal intact levels following 4 weeks of testosterone treatment. The trend of reduced somal area following castration was found to be consistent with earlier studies by Breedlove and Arnold (1981). These investigators discussed the possibility that HRP uptake was diminished in the castrated animals because of their androgen deficiency, resulting in artificially low dendritic lengths. However, the number of labeled SNB cells was equal between the castrated and normal intact males. Also, the maximum distance from the cell body that a dendrite was labeled was consistent across all groups examined. From this study, it was concluded that androgens are involved in the regulation of dendritic length, which may ultimately affect the neuronal circuitry of SNB motoneurons. The retraction of dendrites may result in a decrease in membrane area available for afferent synaptic input, a decrease in the number of efferent synaptic contacts, and a change in the physiology of the motoneurons due to the removal of trophic factors from neighboring cells (Kurz et al., 1986). During the lifetime of a normal male rat, androgens increase to high levels at puberty but decline as the animal ages beyond its optimal reproductive period. As demonstrated by the present study, SNB motoneuron dendritic extension and retraction are highly dependent on the animal's hormonal environment. Since androgen levels correlate with the frequency of copulatory behavior, structural changes in SNB dendritic morphology may ultimately be manifested in the display of reproductive behavior.

As discussed previously, embryonic exposure to androgens permanently alters SNB cell number, whereas androgenic manipulation during adulthood alters cell size and dendritic length. A study by Goldstein et al. (1990) examined the developmental pattern of SNB motoneuron dendritic morphology in postnatal male rats with and without the presence of androgens. This group of investigators determined that the postnatal development of SNB dendritic morphology is biphasic, with a period of extensive outgrowth followed by dendritic retraction to adult lengths. Utilizing retrogradely transported HRP to visualize SNB motoneurons, it was discovered that normal male rats undergo dendritic growth between P7 and P28. The earliest time point examined was P7, to allow for the completion of naturally occurring SNB cell death. Between P7 and P28, dendrites grow to nearly five times the length observed at the end of the first postnatal week, resulting in lengths than

are nearly twice those of normal adults. Rapid growth is then followed by dendritic retraction to adult levels by P49. At P70, no difference in dendritic length was seen compared to the 7-week-old animals. In animals that were castrated during early postnatal development (P7) or during puberty (P28), SNB dendritic length was significantly altered. Animals castrated at the end of the first postnatal week (early castrates) never display the extensive dendritic growth noted in normal animals between the first and fourth weeks of age. In fact, the dendritic morphology in these early castrates remained stunted into adulthood. Early castrated animals that were injected daily with testosterone show the normal developmental pattern of dendritic morphology. In contrast, males that were castrated coincidentally with puberty showed a gradual decline in dendritic length that resulted in significantly shorter dendrites compared to normal males at P70. These investigators concluded that while there is a period of retraction in normal animals between P28 and P49, the distribution of dendrites appears unchanged, suggesting a "pruning" of branches rather than a retraction of primary processes of the dendritic arbor. An interesting observation was that SNB dendrites display adult morphology at P49, when there is only half the adult level of circulating androgen (Resko et al., 1968; Moger, 1977). While adult levels of hormone are not attained until several weeks later, the onset of copulatory behavior controlled by the SNB system is present at P49 (Sodersten et al., 1977; Sachs and Meisel, 1979), which coincides with the development of mature SNB dendritic morphology. As discussed by these investigators, early castrates receiving testosterone injections did not show above-normal increases in dendritic lengths at P28, despite higher-than-normal hormone titers. It was suggested that the developing neurons are at a "metabolic limit" and that the presence of high levels of exogenous androgen cannot stimulate additional development of SNB dendritic arbor. It was postulated that dendritic lengths decline after P28 because of an increasing hormonal demand of SNB motoneurons. Paradoxically, this period of dendritic retraction coincides with puberty, when the levels of circulating androgens begin to rise. At P28, the hormone requirement of SNB motoneurons may increase dramatically. Despite increasing androgen titers, hormone levels may be insufficient to promote dendritic growth or stabilization of neurite length, resulting in retraction of the SNB dendritic arbor.

B. Cytoskeleton

The influence of gonadal steroids on the expression of cytoskeletal components was examined by Ferreira and Caceres (1991). Since microtubule assembly is critical to the elongation of neurites (Mitchison and Kirschner, 1988), these investigators explored the hypothesis that steroids promote neurite outgrowth by altering the expression of microtubule-associated proteins (MAPs). In medial basal hypothalamic cell cultures harvested from E17 or E19 rats, it was discovered that the addition of 17-β estradiol resulted in a significant increase in axonal and dendritic length as well as neurite branching per cell compared to untreated cultures. Utilizing

immunocytochemistry methods, a threefold induction of tau proteins that preceded and accompanied an increase in stable microtubules was noted in cells treated with estrogen. However, the induction of MAPs was selective for tau, since levels of total tubulin, MAP-1a, and MAP-2 did not change from control levels. The selective induction of tau proteins is consistent with the idea that tau is involved in microtubule stability, whereas MAP-1a and MAP-2 are involved in microtubule assembly (Drubin et al., 1988; Matus, 1988; Ferreira et al., 1989). These investigators therefore concluded that the estrogen-induced increase in tau protein results in a larger population of stable microtubules, which ultimately results in enhanced neurite outgrowth.

As presented above, emerging evidence suggests that gonadal steroids influence neurite outgrowth through the expression of cytoskeletal components. A study by McQueen et al. (1990) has shown that the lack of androgens can result in the abnormal expression of glial fibrillary acidic protein (GFAP) in hypothalamic glial cells. Utilizing *tfm* mice, these investigators have found that GFAP is abnormally expressed in this androgen-insensitive animal model. In the normal mouse, GFAP immunostaining resulted in light staining of astrocytes in the periventricular (PVH) and very few GFAP-positive cells in the dorsomedial hypothalamic nucleus (DMH) and the VMN. Ependymal tanycytes in the PVH and tanycytic processes arcing toward the median eminence through the arcuate nucleus were strongly stained. In contrast, *tfm* mice displayed an increase in staining at the ventricular border of the PVH and DMH. The most dramatic finding was the strong staining of cells in the ARC that obscured the tanycytic processes. In addition, a 10-fold increase in GFAP-positive astrocytes was noted on either side of the ventricle. The abnormal GFAP staining was confined to the above-mentioned regions and did not extend to other hypothalamic areas. These investigators concluded that astrocytes are extremely sensitive to neural dysfunction rather than indicators of extreme CNS damage.

V. EFFECTS OF GONADAL STEROIDS ON SYNAPTOGENESIS

One of the first studies to demonstrate changes in synaptic contacts on mammalian spinal motoneurons due to androgen exposure was conducted by Leedy and co-workers (Leedy et al., 1987). Ultrastructural analysis of SNB synaptic contacts in adult male rats that had been castrated for 6 weeks (no T) showed a decrease in several parameters compared to animals that had been castrated for 6 weeks but had received testosterone capsules throughout the entire period (long-term T). The mean percentage of somal motoneuron membrane apposed by synaptic terminals as well as the number of terminals per 100 mm of membrane was significantly decreased in the no T group compared to the long-term T group (approximately 27% versus 35% and 18 versus 23, respectively). An interesting finding of this study was that castrated animals that were implanted with androgen 48 hours prior to

sacrifice (short-term T) were intermediate between the no T and long-term T groups in both parameters examined. It was concluded that the amount of synaptic input to SNB cells is sensitive to circulating androgen levels in the adult male rat and that as little as 48 hours of hormone exposure in long-term castrated animals is adequate to induce synaptic changes within this population of spinal motoneurons.

In studies by Matsumoto and co-workers (1988a), androgens were shown to have a significant influence on the maintenance of synaptic inputs to SNB motoneurons in adult male rats. In an electron microscopy study, the number of synaptic afferent contacts to the SNB cell body and proximal dendrites was quantified. These contacts were classified as synaptic (apposition of an axon terminal with visible synaptic vesicles and thickening of the pre- and postsynaptic membranes), synaptoid (contact of an axon terminal with no membrane specialization), or neuron–neuron (direct apposition of cells with desmosome-like membrane specialization). Results indicate that most neural contacts in the intact animals were synaptic in nature, with very few synaptoid or neuron–neuron contacts. Significant decreases in SNB somal perimeter and frequency, size, and percentage covering of synaptic contacts on both the soma and dendrites were found after 4 weeks of castration compared to intact control animals. The frequency of synaptic contacts in the castrates was 42% of intact animals, whereas the size of the contacts was 82% of intact levels. Castration reduced the amount of membrane covered by synapses to approximately 30% of intact levels. Although no change in synaptoid contacts was noted, frequency and percentage covering of neuron–neuron contacts on the soma were decreased in the castrates. In another group of animals, 4 weeks of androgen replacement starting on the day of castration surgery resulted in neural contact profiles indistinguishable from that of intact animals. These investigators concluded that androgens are essential for the maintenance of normal SNB motoneuron synaptic architecture in the adult male rodent.

In the second of a series of experiments by this laboratory (Matsumoto et al., 1988b), the effects of androgens on SNB motoneuron gap junctions were examined. It was determined that not only do SNB motoneurons possess gap junctions between cells, but the number and diameter of these structures on each cell are particularly sensitive to hormonal manipulation. Utilizing electron microscopy and freeze fracture methods, animals that had been castrated for 4 weeks showed a decrease in the number of gap junctions per cell, the number of cells with gap junctions, and the size of these structures compared to intact animals. Animals that had been treated with testosterone for 4 weeks immediately after castration showed gap junction profiles similar to those of the intact animals. Since these tissue samples were taken from the study mentioned previously (Matsumoto et al., 1988a), membrane lengths could be estimated to determine the percentage of SNB membrane covered by gap junctions. Results indicate that intact and androgen-treated castrated rats had six times the percentage of SNB membrane covered by gap junctions compared to the castrated animals. These investigators concluded that androgens regulate the number of gap junctions between SNB motoneurons and

therefore the extent of electrical connectivity between them. This electronic coupling may then allow depolarization of the entire SNB nucleus in response to excitatory afferent input to just one area of the nucleus.

VI. ROLE OF GONADAL STEROIDS IN NEURONAL INJURY AND REPAIR

Given that many of the neuronal processes normally affected by gonadal steroids are important for neuronal regeneration, we have begun to explore the role of gonadal steroids in improving the reparative response of injured motor neurons. The model system utilized is that of the hamster facial motoneuron, a well-documented system in which to study nerve regeneration (LaVelle and LaVelle, 1984). In the adult hamster, facial motoneurons undergo a robust response to crush or cut axotomy of the facial nerve at the level of the stylomastoid foramen. The pattern of the axon reaction in these neurons appears to occur independently of the target muscle, such that the neurons will undergo a defined reactive sequence and return to a "normal" state whether or not reconnection to the facial muscles is allowed to occur. Following injury, the animal displays a characteristic unilateral facial paralysis, associated with drooping of one corner of the mouth, flattened and paralyzed vibrissae, and loss of the eyeblink reflex. Return of movement can be readily observed during a 2–3-week postoperative period. Finally, it is well established that facial neurons contain androgen receptors (Yu and McGinnis, 1986). Thus, the facial motoneuron represents an ideal system in which to study the effects of androgens on the reparative response after nerve injury.

A. Effects of Testosterone on Recovery from Facial Paralysis

Our initial studies of gonadal steroid action on injured neurons centered on determining whether subcutaneous administration of TP at the time of facial nerve crush could positively affect functional recovery from the subsequent facial paralysis (Kujawa et al., 1989). In work by Yu and collaborators on the effects of testosterone propionate (TP) on the regeneration of axotomized hypoglossal neurons (Yu, 1982a,b; Yu and Srinivasan, 1981; Yu and Yu, 1983), demonstration of HRP-labeled hypoglossal cell bodies was the criterion used to establish acceleration of reconnection to the target muscles. From this work, they hypothesized that TP increases the rate of axonal regeneration. In one of their studies (Yu and Yu, 1983), they discussed the possibility that the presence of more HRP-labeled neurons in the TP-treated animals could be due to a TP-induced increase in the uptake and transport of HRP from the terminals as opposed to an actual acceleration of the regeneration process. We therefore chose to circumvent this potential problem in data interpretation by using functional return of movement as our criterion for accelerated recovery. For these experiments, adult male hamsters received right facial nerve crush axotomies and simultaneous silastic implants of TP.

In our preliminary experiment, the amount, frequency, and mode of administration of TP employed by Yu and Srinivasan (1981) were utilized, and vibrissae function was monitored following crush axotomy. While the initial stages of return of vibrissae function, involving involuntary quivering movements, were accelerated near the end of the first week and beginning of the second week following crush axotomy of the facial nerve, return of full voluntary vibrissae movement was about the same in the TP-treated and control animals (Kujawa et al., 1989).

In the next experiment, the amount and frequency of TP administered to the animals were doubled in order to maximize the accelerative tendency of TP observed in the preliminary experiment. In addition, functional recovery from facial paralysis was further subdivided into return of eyeblink, full vibrissae movement, and complete recovery. Complete recovery included full vibrissae movement and return of the vibrissae from a posterior, flattened orientation to the normal anterior orientation observed on the unoperated left side of the animal. The greatest accelerative effect of TP on functional recovery was observed in the second week, with a 4-day difference in demonstration of the full blink reflex present between the hormone-injected and the control animals (Kujawa et al., 1989). A less dramatic, but still evident, accelerative effect of TP was also noted in return of full vibrissae movement and complete recovery.

Given that a greater hormonal effect on recovery from facial nerve paralysis was noted with the increased dosage of TP, the third experiment that was done in this study involved an alteration in the mode of administration of the hormone from daily injections to subcutaneous implantation of silastic capsules containing 100% crystalline TP. In addition, the surgical procedures were done on castrated male hamsters to remove the endogenous source of the hormone. This dual approach allowed separation of endogenous from exogenous hormone effects and the action of a continuous supply of exogenous hormone to be determined within the same experimental paradigm. A pronounced acceleration of recovery of the blink reflex, full vibrissae movement, and normal orientation of the vibrissae were observed in the TP implant group as compared to the castrated control group (Kujawa et al., 1989). As in the previous experiments, the accelerative effect of TP on recovery from facial paralysis was most obvious during the second week following crush axotomy of the facial nerve. Finally, no differences in the regenerative response of castrated control versus noncastrated control animals were found to occur. Instead, the addition of a significant amount of exogenous TP to the system may be necessary to observe any functional benefits of the hormone.

In summary, the results of this study (1) suggested that TP accelerates motor neuron axonal regeneration, (2) established that continuous exposure to high levels of exogenous hormone has the greatest effect on functional recovery from facial paralysis, (3) indicated that endogenous hormones contribute little in the way of functional benefits following nerve injury, and (4) suggested that the effects of TP on functional recovery from facial paralysis are exerted at the level of the neuron.

To test the hypothesis that TP acts at the level of the injured neuron rather than the muscle, the temporal requirements of TP exposure necessary for acceleration of recovery from facial paralysis to occur following facial nerve crush were examined in our next study (Kujawa and Jones, 1990). For each of two series of experiments, adult castrated male hamsters were subjected to crush axotomies of the facial nerve at its exit from the stylomastoid foramen. In the first experimental paradigm, one-half of the animals with facial nerve crush axotomies received subcutaneous TP capsules beginning on postoperative (PO) day 6 and continuing throughout the regeneration period, with the remainder of the animals sham-implanted. The results indicated that, without the early exposure to TP, the accelerative effects of the hormone on facial nerve regeneration were abolished (Kujawa and Jones, 1990). In the second experimental paradigm, one-half of the animals with facial nerve crush axotomies received subcutaneous TP implants immediately after crush and until PO day 7, with the remainder of the animals sham-implanted. The results indicated that an early, discontinuous dose of TP immediately after crush surgeries was sufficient to produce a full accelerative effect on the return of the semi-blink and complete recovery, and a partial accelerative effect on the blink reflex and full vibrissae movement (Kujawa and Jones, 1990).

B. Effects of Testosterone on the Regenerative Properties of Injured Motoneurons

Collectively, the results from our behavioral studies, as well as the data from other systems (Vita et al., 1983; Yu, 1982a,b; Yeagle et al., 1983), argue toward a priming effect of the hormone exerted predominantly at the neuronal level that may parallel the conditioning lesion effect reported by others (McQuarrie, 1978, 1981; Oblinger and Lasek, 1984). We therefore hypothesized from these studies that the mechanism of TP action on injured neurons involves alterations in axonal regenerative properties, that is, the rate of regeneration and/or the delay of sprouting.

This hypothesis was tested using fast axonal transport of radioactively labeled proteins (Oblinger and Lasek, 1984). As in the previously described behavioral studies, adult castrated and intact males, and intact females, were subjected to right facial nerve crush axotomy at the stylomastoid foramen. One-half of the axotomized animals in each group received subcutaneous implants of testosterone, and the remainder of the animals were sham-implanted. Systemic administration of testosterone resulted in a 26–30% increase in the rate of regeneration of the fastest growing population of axons in the male experimental groups, regardless of whether the animal was castrated or not (Kujawa et al., 1991), and a 10% increase in the rate of regeneration in the female experimental groups (Kujawa et al., 1991). This effect of testosterone on axonal outgrowth rates paralleled those found in conditioning lesion studies utilizing the rat sciatic nerve as a model system (McQuarrie, 1978, 1981; Oblinger and Lasek, 1984; Forman et al., 1980). Oblinger and Lasek (1984) found a 25% increase in axonal regeneration rates of sensory

neurons when a conditioning lesion preceded the testing lesion. Previous work by McQuarrie (1981) identified a 24% increase in the rate of regeneration of motor axons in the sciatic nerve. The conditioning paradigm involves two lesions, an initial "priming" lesion followed later by a "testing" lesion to elicit accelerated outgrowth. It has been suggested that the conditioning phenomenon can be applied to human nerve injuries where the initial insult is considered the conditioning lesion and later removal of the proximal stump neuroma becomes the testing lesion (McQuarrie, 1981). Under this treatment regimen, accelerated return of motor and sensory function might be expected to occur. Our results suggest that systemic administration of testosterone following immediate repair of the severed epineural coverings would eliminate the need for additional surgery involving a testing lesion, while capitalizing on the accelerated nerve outgrowth.

It is interesting to note that testosterone increased the rate of regeneration to a greater extent than functional recovery of facial movements (25–30% versus 10–15%). While the reasons for this are not known, there are a number of possible explanations. First, it may simply be that the behavioral testing is a less sensitive measure than the radioisotopic method for determination of axonal regeneration rates. Second, since the procedures used in this study provide information about only the fastest growing population of axons, there may be differential effects of the steroid on the bulk of more slowly growing fibers. Thus, steroids may affect initial events associated with regrowth of the faster fibers but may not have as pronounced an effect on events associated with subsequent regrowth of the slower fibers. Future electron microscopic studies examining the composition of the regenerating nerve at various distances from the crush site might provide insight into this question.

Administration of testosterone to intact females elicited an increase in the rate of axonal regeneration, albeit to a lesser extent than in intact males. In the ovary, aromatase enzymes, under normal physiological conditions, convert testosterone to estradiol (Hadley, 1988). These enzymes are present in brain tissue as well as many other peripheral organs and may play an important role in the neuronal processing of testosterone (Krey et al., 1982). Thus, the reduced effect of TP on axonal regeneration in intact females could have been due, in part, to its conversion to estradiol and subsequent inability to bind to androgen receptors within facial neurons (Naftolin et al., 1975; Naftolin and Ryan, 1975). However, no improvement was noted when the nonaromatizable form of testosterone, dihydrotestosterone (DHT), which has biological effects similar to those of TP (Whalen and Debold, 1974), was utilized in subsequent experiments. Why the female is less responsive, in terms of percentage increase in rate, than the male is unknown but may be related to the inherent differences in the regeneration rates between gender. Since the rate of facial nerve regeneration in intact females not given testosterone is within the same range as males administered testosterone, it may be that the system is only capable of achieving a preset maximal level. If so, this would imply that intact females with an inherently faster regeneration rate would reach the maximal level

with a smaller hormone-induced increase. Examination of the literature supports this idea, in that maximal regeneration rates are, with few exceptions, within ranges similar to those found in hormone-treated females in this study (McQuarrie, 1981; Oblinger and Lasek, 1984; Bisby, 1985; Griffin et al., 1976).

These data suggest that the mechanism by which gonadal steroids act in the injured nervous system is partly through the differential regulation of the regenerative properties of the injured cell, presumably via hormone receptor-mediated action at the level of the neuronal genome. If so, what genes would be predicted to be affected by TP following axon injury? As described in the first section of this review, gonadal steroids have been shown to affect neurite outgrowth, partly through regulation of cytoskeleton-associated proteins. In the conditioning lesion paradigm, alterations in the synthesis and transport of major cytoskeletal proteins such as tubulin appear to be involved in the mechanism of accelerated growth (McQuarrie, 1983). We have therefore initiated a series of experiments designed to examine the effects of gonadal steroids on cytoskeletal gene expression after facial nerve injury (Jones and Oblinger, 1994). Our preliminary results suggest that TP does differentially regulate the expression of the tubulin gene family after injury. βII tubulin mRNA levels were found to be significantly elevated 2 days after axotomy plus TP, but not after axotomy alone. By 7 days after axotomy, βII tubulin levels were elevated in both groups, but to a greater extent in the group supplemented with TP. In contrast, no steroidal effects on βIII tubulin or α-tubulin were observed.

C. Effects of Testosterone on the Nerve Cell Body Response to Injury

The evidence we have obtained thus far from the behavioral, regeneration, and tubulin studies suggests that testosterone primes the neuronal cell body in such a way as to accelerate the "switch" from a normal to a reparative state. One way to test this hypothesis is to examine the effects of gonadal steroids on the polymerase I system in an injury paradigm. As stated before, one of the earliest events that occurs in a target cell following exposure to the hormone is activation of the polymerase I system, with the result being a rapid and dramatic increase in ribosomal RNA. Following axotomy, nucleolar changes consistent with increases in rRNA levels also comprise the initial events preceding successful regeneration (Jones and LaVelle, 1986b). We therefore used *in situ* hybridization and rDNA probes to determine the effects of TP on rRNA levels following axotomy (Kinderman and Jones, 1993). The preliminary data indicate that, following axotomy, rRNA levels begin to increase within the first 24 hours after injury, peak at 2 days after axotomy, and then decline. When TP is administered in conjunction with facial nerve cut, the levels of rRNA begin to increase immediately, so that by 6 hours postinjury they already reach the maximum achieved at 2 days with injury alone. In addition, the peak increase in rRNA is approximately double that with injury alone. Thus, it appears that TP activates molecular events associated with the injury

response in accordance with the acceleration in functional recovery and regeneration rate. In conclusion, the available evidence to date suggests that many of the aspects of neuronal functioning affected by gonadal steroids under steady-state conditions are also significantly affected by steroids under stress conditions such as axon disconnection. This argues for the therapeutic usefulness of gonadal steroids in activating and/or accelerating the reparative response of neurons to injury, a concept that will be exciting to test in future clinical studies.

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ARE EPIGENETIC FACTORS INVOLVED IN THE NORMAL EXPRESSION OF NEURONAL PHENOTYPES DURING SPINAL DEVELOPMENT?

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Advances in Neural Science,
Volume 2, pages 153–167.

ISBN: 1-55938-625-8

ABSTRACT

The present brief review deals with factors and developmental events that may contribute to the initiation of defined phenotypes leading to the specialization of motor and sensory neurons in the spinal cord. Is the initiation of phenotypic expression related to the rigorous chronobiology associated with the genesis of neural circuitry, as in the establishment of specific connections with peripheral and central target tissues and/or afferents? The search for developmental events that trigger differentiation in the spinal cord should contribute to the understanding of motoneurons, which in turn could provide a more complete understanding of the spinal cord.

I. INTRODUCTION

The cells of an embryo do not develop in isolation, and epigenetic factors often are responsible for the development of cellular diversity. In the nervous system, this has been confirmed through the studies of neural crest derivatives that give rise not only to the many different cell types of the peripheral nervous system but also to the melanocytes of the skin and to some mesenchymal tissues of the head (Le Douarin, 1982). These studies have pointed out the importance of the cell's microenvironment in determining the fate of neural crest cells. A microenvironment, to name a few possibilities, could be other cells, the extracellular matrix encountered during migration, hormones, afferent or efferent axonal systems, glial cells, etc. Also, through a particular lineage, a cell acquires the *competence* to respond to a signal. Therefore, lineage is also of importance, but this aspect will not be dealt with here.

The central nervous system (CNS) is characterized by an exceptional diversity of cells. Moreover, this system is also distinguished by a complexity in intercellular communications that is unmatched in the organism. When young neuroblasts leave the mitotic cycle during neurogenesis, they are poorly differentiated and they all look alike, yet they represent the source of all the different cell types of the adult CNS (Fig. 1). In the adult brain, neurons will differ from one another in their size, axonal lengths, targets, dendritic trees, numbers of their afferents, neurotransmitters or neuromodulators, and their receptors.

Motoneurons of the developing spinal cord are subjected to epigenetic influences arising from the periphery, propriospinal neurons and the supraspinal segments of the CNS. Although they share numerous characteristics, it is now obvious that motoneurons do not constitute a homogeneous population of cells.

II. PHENOTYPIC EXPRESSION OF MOTONEURONS

Based upon morphological, physiological, and molecular approaches, motoneurons have been subdivided into different classes or subpopulations. In the ventral horn, they are arranged in columns that innervate muscles with similar

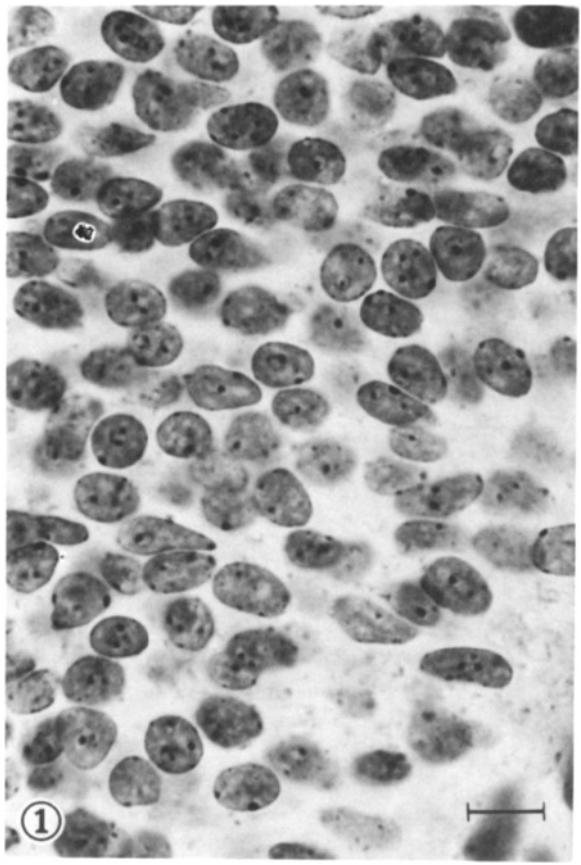

Figure 1. Transverse section of spinal cord of E12 rat embryo. This high magnification photomicrograph of the ventral horn shows a field of poorly differentiated neurons that have been generated less than 24 hours before. These all look morphologically similar. Among them, prospective motoneurons and interneurons are the recipients of a stream of information that originates from the periphery and supraspinal segments of the central nervous system. What are the developmental events that will orient their morphological and biochemical differentiation? Methacrylate section stained with toluidine blue. Scale bar, 10 μm.

functions. There are α- and γ-motoneurons. Two classes of α-motoneurons, called "tonic" or "phasic," supply muscle fibers of different types—respectively, slow or fast oxidative glycolytic muscle fibers. Motoneurons also receive various inputs from the periphery. To name a few, there are monosynaptic excitations from primary and secondary afferents originating from neuromuscular spindles, disynaptic excitations from flexor reflex afferents, and disynaptic inhibitions through Renshaw

cells or Ia inhibitory neurons. Supraspinal and propriospinal afferents are also heterogeneous and interact in a nonuniform manner with the motoneuronal pool.

Vertebrate motoneurons are also characterized by specific phenotypes. Indeed, in the neuromuscular system, among the well-determined phenotypes, it is well known that vertebrate motoneurons are cholinergic, as shown by electrophysiological data (De Feudis, 1974), and have high choline acetyltransferase (ChAT) activity (Kato and Murashima, 1985). The expression of this particular phenotype is a characteristic of all the somatic motoneurons innervating the striated skeletal muscular tissue. However, it has been shown that motoneurons also expressed various phenotypes as shown by developmental, anatomical, physiological, and immunocytochemical studies. For example, several subpopulations of developing motoneurons have been characterized by their expression of various peptides such as calcitonin gene-related peptide (CGRP), somatostatin, or vasoactive intestinal polypeptide (VIP) (Villar et al., 1989).

A. Expression of Amino Acids by Spinal Motoneurons

Among the phenotypes expressed, recent studies have surprisingly shown that γ-aminobutyric acid (GABA), the main inhibitory neurotransmitter in the nervous system of both invertebrates and vertebrates, is also expressed by the majority of vertebrate spinal motoneurons (Fig. 2) (Philippe et al., 1990). While it is well known that the motor innervation of skeletal muscles is controlled by a cholinergic

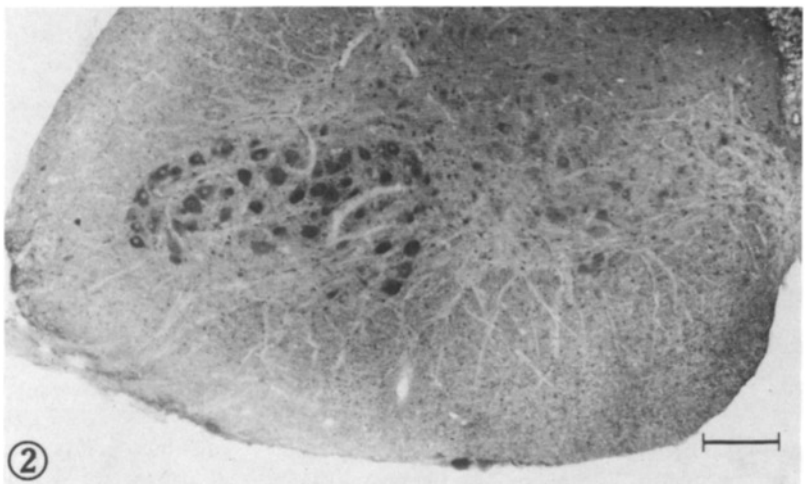

Figure 2. Transverse section of young chick spinal cord immunostained for GABA. After incubation with GABA antiserum, immunostaining is mainly observed in lamina IX and occasionally in lamina VII of the ventral horn. The large GABA-positive cell bodies correspond to motoneurons. Vibratome section. Scale bar, 165 μm.

excitatory system, motor nerve terminals express the capacity for synthesizing not only acetycholine but also GABA in human and primate neuromuscular junctions (Chan-Palay et al., 1982a,b). Hence, we verified whether these populations of large GABA multipolar neurons of the ventral horn do in fact correspond to somatic motoneurons. For that purpose, GABA immunohistochemistry was performed on chick spinal cords retrogradely labeled by fluorescent latex microspheres injected within several skeletal muscles 24 hours before sacrifice. Following this double labeling experiment, retrogradely labeled neurons also displayed a GABA-like immunoreactivity. Since only motoneurons contained fluorescent latex microspheres, it became obvious that a subpopulation of chick motoneurons does express a GABA-like immunoreactivity. Moreover, large myelinated axons located in the ventral root also exhibited clear immunostaining, suggesting that GABA is transported to the peripheral tissues, especially to skeletal muscles (Philippe et al., 1990). This hypothesis is indeed corroborated by the presence of GABA-immunoreactive typical neuromuscular junctions observed in cryostat sections of skeletal muscles of the hindlimb. The latter finding is in keeping with recent observations that a few nerve fibers stained with GABA antiserum were observed in the cranial motor nerves of chick embryo as well as in neuromuscular junctions (Von Bartheld and Rubel, 1989).

Hence, these colocalizations raise the question as to whether GABA has an exclusively inhibitory role as a neurotransmitter, or whether it may participate in the normal regulation of nerve–muscle interactions during development, as suggested by Chan-Palay et al. (1982a), or whether it is involved in the metabolism of cholinergic motoneurons. Indeed, it has been suggested that several neurotransmitters may have functions in the developing nervous system that differ from their "classical" role in synaptic transmission (Buznikov et al., 1970; Olson and Seiger, 1972; McMahon, 1974). Interestingly, neurotrophic effects of GABA have been described during development (Wolff et al., 1978; Wolff, 1981).

B. Putative Functions of GABA in the Neuromuscular System

The unexpected presence of cytoplasmic GABA-immunoreactive material in large motoneurons of the chicken raises many questions about the role of GABA in the avian and more generally in the vertebrate neuromuscular system. First, is GABA expressed only in avian motoneurons or is it also found in motoneurons of other vertebrates, particularly mammals?

Experiments performed on the spinal cord of the adult squirrel monkey (*Saimiri sciureus)* have shown that a subpopulation of motoneurons also expresses a clear GABA-like immunoreactivity in these species (Philippe et al., 1990). These results are consistent with previous detection of substantial glutamate decarboxylase (GAD) activity in almost all the cell bodies of the spinal motoneurons of rabbits and hens. Moreover, experiments performed *in vitro* on embryonic mouse and rat motoneurons have also detected the presence of intracellular GABA. However, a

study of GABA immunoreactivity in the adult rat spinal cord has shown that motoneurons are free of GABA immunoreactivity. This discrepancy between data obtained in rodents and in other species may have several explanations: (1) possible differences in methodological factors such as the composition of the fixative solution or, more likely, (2) a transient expression of this transmitter according to the species, the age, and/or the physiological activity of the animal.

In order to define a possible role for GABA in the vertebrate neuromuscular system during ontogenesis, it will be necessary to determine the source of this amino acid: Is it the product of the biosynthetic enzyme glutamic acid decarboxylase (GAD) and/or the result of a high-affinity uptake?

After incubation of slices from lumbosacral spinal cord with antibodies raised against GAD or glutamate (Fig. 3), a clear immunoreactivity was detected with both antisera in the majority of GABA-immunoreactive motoneurons. Combined pre- and postembedding immunocytochemical procedures showed colocalization of glutamate and GABA in most motoneurons, even though occasionally cell bodies immunostained either exclusively with GABA or glutamate. Since GABA, GAD, and glutamate colocalize in some chick motoneurons, it appears that the expression of GABA by these neurons is indeed due to an intracellular synthesis (Gaulin et al., 1992). These results can be corroborated by the fact that no high-affinity uptake of ^{3}H-GABA is observed. Indeed, after incubation of spinal cord slices with ^{3}H-

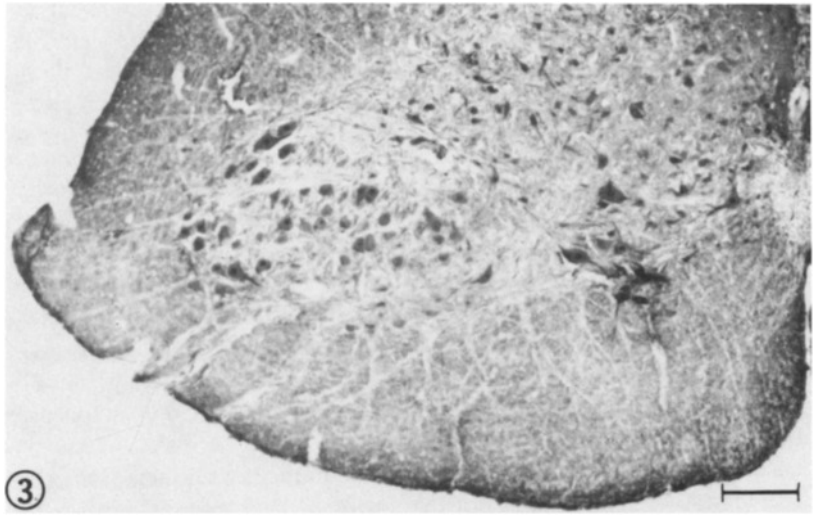

Figure 3. Transverse section of young chick spinal cord immunostained for glutamate. After incubation with glutamate antiserum, the immunoreaction is mainly in lamina IX and occasionally in lamina VII of the ventral horn. The large immunostained cell bodies correspond to motoneurons. Note that the staining pattern is similar to that for GABA. Vibratome section. Scale bar, 200 μm.

GABA, the radioisotope is not concentrated in motoneurons but mainly is found within glial cell bodies; GAD, the rate-limiting enzyme of GABA biosynthesis, is strongly expressed by chick motoneurons, as is glutamate, its immediate precursor (Gaulin et al., 1992).

It is generally accepted that a neurotransmitter is synthesized by neuronal cell bodies and released at synaptic terminals. At nerve endings, there are high-affinity uptake mechanisms that reduce the amount of free transmitter in the cleft region. Uptake by specialized membrane transport mechanisms represents the general way by which the postsynaptic actions of neurotransmitters are stopped following their release. Since the present data clearly show that motoneurons, as well as nerve endings in muscle, are devoid of high-affinity uptake of GABA and glutamate, it is suggested that these amino acids do not function as neurotransmitters in this system.

Moreover, since only a transient expression of GABA has been detected in cholinergic motor cranial nerves of the chick embryo (Von Bartheld and Rubel, 1989), it seems unlikely that GABA and glutamate function as neurotransmitters in chick cranial or spinal motoneurons. The absence of ^{3}H-GABA and ^{3}H-glutamate uptake in motoneuron perikarya, as well as the chemical heterogeneities displayed by these motoneurons, supports this assumption. This chemical heterogeneity is in agreement with results reported by Villar et al. (1989) showing that motoneuron subpopulations were significantly different according to their expression of various neuropeptides, such as CGRP, VIP, or somatostatin.

Since GABA, GAD, and glutamate immunostaining intensities are much stronger in lamina IX than in lamina VII following the numbering scheme for laminae of Martin (1979), the expression of amino acids in the majority of but not all motoneurons could be related to cellular metabolic activities. Indeed, it has been reported that in the CNS, glutamate is a key compound in several metabolic pathways within nerve cells (Hertz et al., 1983). Bicker et al. (1988) suggested that this amino acid may have multiple roles in cellular metabolism. Nevertheless, these considerations do not rule out the possibility of a role for GABA in modulating neurotransmission (Chan-Palay et al., 1982a,b). Perhaps GABA may have a secondary function in fine-tuning the actions of other neurotransmitters (Matsumoto, 1989). For example, a modulatory action of GABAergic agents on ACh release has been well documented in parasympathetic systems (Erdö and Wolff, 1990). This modulation could take place through the activation of an appropriate presynaptic receptor involved in the transmitter release process (Burnstock and Osborne, 1983).

Several hypotheses also argue in favor of a trophic role for GABA in the neuromuscular system. Indeed, in the developing neuromuscular junction, it has been suggested that GABA is involved during development in the regulation of acetylcholine receptors (Von Bartheld and Rubel, 1989), or as a neurotrophic factor in developing cranial nerves or their target tissues, in such processes as protein synthesis, neurite elongation, or synaptogenesis (Campbell et al., 1966; Wolff, et al., 1978; Wolff, 1981; Meier and Jorgensen, 1986).

III. ARE ENVIRONMENTAL FACTORS INVOLVED IN THE EXPRESSION OF NEURONAL PHENOTYPES?

The gradual establishment of a complex cellular environment in association with the spinal motoneurons is very likely to play an important role in determining their various phenotypes. Both peripheral and central interactions have been shown to be vital for motoneuron survival during development and in adults (Henderson, 1988).

A. Peripheral Environment

Motoneurons receive various types of information from the periphery through the primary sensory neurons of dorsal root ganglia (DRG), either directly by monosynaptic contacts or indirectly by polysynaptic pathways with local interneurons. Can these afferent systems and/or peripheral target tissues be involved in the elaboration of motoneuronal phenotype diversity?

There is as yet little information in the literature to answer this major question. However, the primary sensory neurons constitute an interesting example of a correlation between a defined peripheral target tissue and the expression of a particular phenotype. Furthermore, developmental studies involving DRG neurons also suggest a causal relation between the initiation of some phenotypes and the presence of specific contacts with peripheral and central target tissues.

Although originating from a common source, namely the neural crest, the primary sensory neurons are composed of two main classes: the large A and the small B neurons which, in the chick, are located respectively in the lateroventral (LV) and the mediodorsal (MD) areas of the DRG. Between these two classes of neurons, several subclasses may be further identified, for example, by their neurotransmitter or chemical content or their peripheral projections (Carr et al., 1989; Kazimierczak et al., 1986; Lawson et al., 1987; Omlin et al., 1985; Philippe et al., 1986; Rambourg et al., 1983).

Indeed, it recently has been shown that the sensory innervation of skeletal muscles and skin, at least in part, occurs by way of two subpopulations (A1 and B1) of sensory neurons expressing a calbindin immunoreactivity (Philippe and Droz, 1988, 1989). In the E10 (embryonic day 10) chick embryo, about 20% of neuroblasts are immunostained with calbindin antibodies. This percentage is fairly stable during ontogenesis as well as after hatching (Fig. 4). Furthermore, the initiation of calbindin expression within these two subpopulations of sensory neurons during development is dependent on the presence of peripheral tissues and mainly skeletal muscle tissue. Indeed, after excision of one hindlimb at E6, all of the ipsilateral DRG cells remain free of calbindin immunoreactivity after 10 days of incubation, but such is not the case when the central targets are destroyed in the spinal cord at the same age (Philippe et al., 1988). In this particular situation, it is

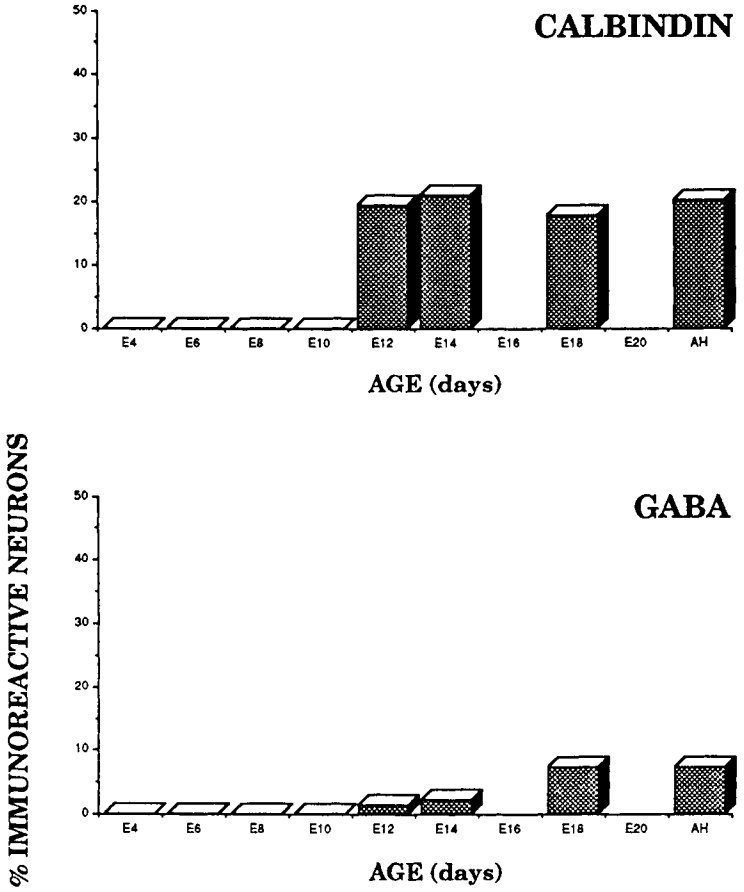

Figure 4. Percentage of immunostained primary sensory neurons in the lumbosacral chick DRG at various developmental stages. (**A**) At E11, the percentage of calbindin-immunoreactive sensory neurons is about 20% of the total, and this percentage remains fairly constant during ontogenesis and after hatching (Philippe and Droz, 1988). (**B**) In contrast, about 1.4% of sensory neurons are GABA-immunoreactive at E12, and this percentage increases progressively during ontogenesis to reach 7.2% after hatching (Roy et al., 1991). (**C**) At E5, about 5% of sensory neurons are SP-immunoreactive. This percentage increases to 95% at E8 and progressively decreases to 50% pre- and post-hatching (Duc et al., 1991). (continued)

likely that the initiation of calbindin phenotypes is triggered by the establishment of a link with the peripheral target tissues.

Among other putative neuronal phenotypes, amino acids are also expressed by defined subpopulations of primary sensory neurons. For example, when DRGs of chick embryos were immunostained with antibodies raised against GABA, no

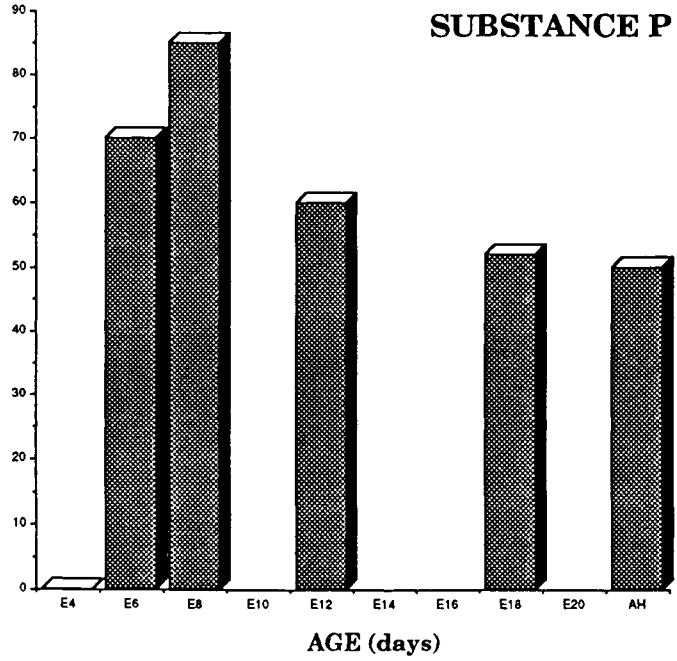

Figure 4. (continued)

reactive element was observed before E12. At this stage, at which all peripheral connections with target tissues are established, 1.4% of sensory neurons were immunostained with a light intensity in the whole perikaryon (Fig. 4B). From E18 to hatching, the percentage of immunostained cells increased to reach an average of 7.2%. From 1 to 12 days after hatching (Fig. 5), a mean of 7.3% of neuronal cell bodies exhibited a clear GABA immunostaining (Roy et al., 1991). Most of the immunostained neurons could be related to two subpopulations of sensory neurons: the large A1 and the small B1 subclasses, as defined previously by morphological criteria (Philippe and Droz, 1988).

Among the peripheral target tissues that were examined after immunostaining with antiserum raised against GABA, a few immunoreactive nerve fibers were located both in the skin and in skeletal muscles. On the other hand, there were no immunoreactive nerve fibers in the Achilles tendon or in autonomic paravertebral ganglia.

Within subcutaneous connective tissue, some nerve endings located in a tactile organ, the Herbst corpuscle, were immunostained. Within skeletal muscle, immunoreactive nerve terminals were located in neuromuscular spindles where they occurred most often around the intrafusal muscle fibers. These results strongly

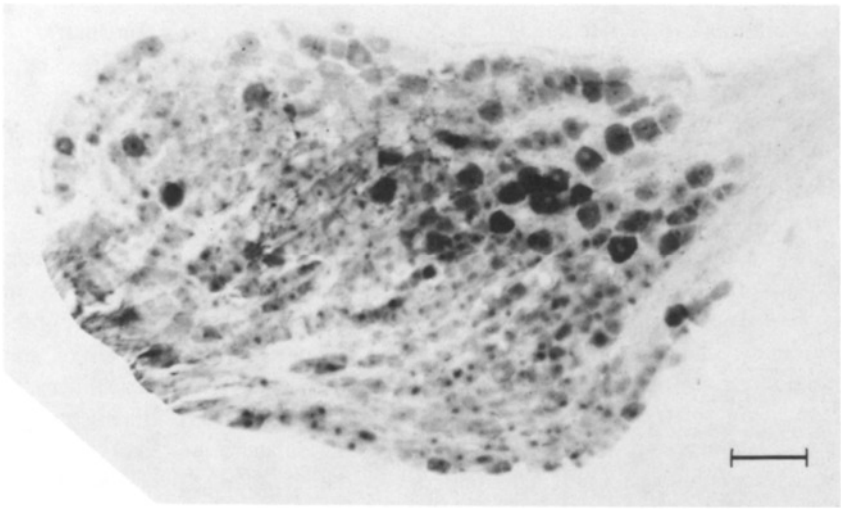

Figure 5. Expression of serotonin by a subpopulation of primary sensory neurons of chick DRG. Hatched chick DRG immunostained for serotonin. A few sensory neurons are strongly immunostained. Most of these are of large diameter. Occasional small sensory neurons are also serotonin-positive. Vibratome section. Scale bar, 100 μm.

suggest that sensory innervation of tactile receptors and skeletal muscles is, at least in part, by way of the GABA-immunoreactive neurons. These observations may indeed be corroborated by the fact that a retrograde tracer inserted within the Sartorius muscle was retrogradely transported to both the immunostained A1 and B1 neuronal cell subclasses. In the latter situation (compare with calbindin), the initiation of GABA expression is progressive; it is likely that initiation is dependent upon the establishment of connections with not only peripheral target tissues but also with central tissues. Indeed, the central connections are not yet all established at E12. Moreover, it also could be possible that GABA expression is dependent on the initiation of the progressive functional activity of the peripheral tissues.

According to these data, it appears that GABA is colocalized with calbindin and may be a putative transmitter involved in the peripheral sensory innervation of muscles. However, whereas GAD activity has been shown within the DRG, the presence of GABA within defined subpopulations of sensory neurons and their related peripheral target tissues does not guarantee that this classical inhibitory neurotransmitter is synthesized by these cell bodies. Indeed, since in the spinal cord synaptic terminals of sensory neurons have receptors for GABA, it is possible, as suggested by Barbaresi et al. (1985), that the receptor–ligand complexes might be internalized and retrogradely transported to defined sensory neurons. This may result in the observed progressive immunolabeling.

Furthermore, it is interesting that the neurons which are responsible for the sensory and motor innervation of skeletal muscles are each characterized by the presence of GABA immunoreactivity. Hence, it is possible, as suggested by Chan-Palay et al. (1982a,b), that this amino acid participates during development in the normal regulation of not only motor nerve–muscle interactions but sensory nerve–muscle interactions as well.

Among other putative transmitters that recognize neuronal subpopulations, dopamine immunoreactivity is first expressed by 0.8% of chick primary sensory neurons at E10 and reaches a percentage of 5.6% before hatching. After hatching, 8.6% of sensory neurons exhibit a clear immunostaining. According to the immunostaining of nerve terminals located in various peripheral target tissues, these immunoreactive neurons are responsible, at least in part, for the sensory innervation of the skin and paravertebral ganglia (Philippe et al., 1992). Other studies of catecholaminergic primary sensory neurons suggest that this phenotype may be related to the innervation of specific peripheral targets such as the carotid body.

A subset of sensory neurons, immunostained with serotonin antibodies, is also involved in the sensory innervation of skin and tendons (Zhou et al., 1992). Indeed, within lumbosacral dorsal root ganglion cells of the chick, serotonin immunoreactivity is first expressed in 0.4% of the whole population of sensory neurons at E13, the stage at which all peripheral target tissues could be contacted. Moreover, this is the stage at which cutaneous afferents have been suggested to invade the dorsal laminae of the spinal cord (Davies et al., 1989). The percentage of serotonin-immunoreactive cells increases progressively to 6% before hatching (Fig. 5). After hatching, the percentage was fairly stable at about 7%.

Hence, among the various phenotypes normally expressed by primary sensory neurons, a given neurotransmitter or substance can be related to specific somatic or autonomic sensory functions. The initiation of these phenotypic expressions seems to be dependent upon the establishment of connections with peripheral and/or central targets. However, this is not the case for all phenotypes. Indeed, substance P (SP), for example, is expressed by the majority of primary sensory neurons at E5–E7 (Fig. 4) (Duc et al., 1991), which are stages at which peripheral and central connections are not yet established.

Since the primary sensory neurons project their axons to the CNS, it is likely that their phenotypes are also influenced by central connections (as well as by their peripheral target tissues). It is also likely that they influence or participate, with supraspinal afferents, in the initiation and the maintenance of phenotypic expression of interneurons and motoneurons.

B. Central Environment

We have focused until now on the possible link between the establishment of a connection with a target and the appearance of a neuronal phenotype. We will now turn our attention to the afferents of the motoneurons and more particularly to the

supraspinal afferents originating from the brainstem. The adult motoneurons receive an average of 10,000 synapses from the periphery, supraspinal levels, and propriospinal neurons. Furthermore, supraspinal and propriospinal afferents are various and unequally distributed upon the motoneuronal pool. How is this precise circuitry established during development, and how does its arrival in the ventral horn influence motoneuron development?

Different studies dealing with the ontogenesis of supraspinal descending pathways show that each system arrives at the level of the spinal cord following a very precise chronology. This now has been established for several vertebrate classes like the zebrafish (Kimmel et al., 1982; Mendelson, 1986a,b), *Xenopus* (Forehand and Farel, 1982; Nordlander et al., 1985; ten Donkelaar et al., 1981); chick (Okado and Oppenheim, 1985; Shiga et al., 1991); opossum, a marsupial mammal (Cabana and Martin, 1982, 1984); and more recently for a eutherian mammal, the rat (Auclair et al., 1992). The sequence of arrival of supraspinal neurons during development in the spinal cord is surprisingly consistent across the various species studied until now. Hence, the hindbrain reticular formation, some vestibular neurons, and a midbrain nucleus associated with the medial longitudinal fasciculus (very likely representing the interstitial nucleus of the medial longitudinal fasciculus) are the first neuronal groups to reach the spinal cord in the zebrafish, *Xenopus*, chick, opossum, and the rat. This is followed by the pontine and the mesencephalic reticular formations. Some of the earliest descending supraspinal projections establish synapses directly with motoneurons in the chick (Shiga et al., 1991). In the same species (Shiga et al., 1991) and the opossum, it is the propriospinal neurons that apparently establish the first synapses with the motoneurons. These data are likely to be important when the time comes to look for developmental events or factors that regulate the morphological and biochemical differentiation of motoneurons. The sequential arrival of these different afferent axons in the spinal cord progressively introduces new cell interactions that likely lead to the orderly morphological and biochemical differentiation of motoneurons.

IV. CONCLUSIONS

The main objective of our work has been the search for factors or developmental events that contribute to the initiation of defined phenotypes which lead to the specialization of motor and sensory neurons. Since afferents as well as phenotypes develop according to a precise chronobiology, it is relevant to search for a cause-and-effect relationship. In other words, is the initiation of phenotypic expression related to the rigorous chronobiology associated with the genesis of neural circuitry, like the establishment of specific connections with peripheral and central target tissues and/or afferents?

The search for developmental events that trigger further differentiation in the spinal cord is not new, but the questions that are raised here suggest that a thorough knowledge of these events will become more and more important to attaining a

complete understanding of the motoneurons of the spinal cord. Although most of these questions cannot be answered at the moment, they nevertheless give an insight into what should be studied in the future to achieve a more complete understanding of the spinal cord.

ACKNOWLEDGMENTS

The authors express their deep gratitude to Mrs. Gabrielle Audet and Mrs. Louise Bertrand for their technical assistance, and to the following graduate students: François Auclair, Marie-Claude Belanger, France Gaulin, Changman Zhou, and Nadine Leblanc. This work was supported by MRC, FRSQ, and the Association of Friedreich Ataxia of Canada.

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PLASTICITY OF DESCENDING SPINAL PATHWAYS IN DEVELOPING MAMMALS

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Advances in Neural Science,
Volume 2, pages 169–204.

ISBN: 1-55938-625-8

ABSTRACT

In this chapter we review the evidence for anatomical plasticity of descending spinal pathways in developing mammals. Corticospinal axons have been shown to grow around a lesion of the medullary pyramids in neonatal hamsters and cats and around a lesion of the spinal cord in neonatal rats and cats. Such axons use intact tissue to circumvent the lesion and often take an aberrant route to reach their destination. In neonatal hamsters and cats, most corticospinal neurons fail to survive transection of their axons when the lesion is made at medullary levels, and plasticity is thought to result primarily from late growth, that is, the arrival of late growing axons and/or growth of collaterals from axons that normally terminate rostral to the lesion. Late growth also helps account for corticospinal plasticity in neonatal rats when the lesion is made at cervical levels, but regeneration of cut axons has been documented.

In contrast to cortical axons, axons from the brainstem do not grow around a lesion of their spinal pathway in neonatal rats, cats, or hamsters. At least some of them do so in neonatal opossums, however. The North American opossum, *Didelphis virginiana*, is born in a very immature state, 12–13 days after conception, making it possible to manipulate brainstem–spinal axons at earlier stages of development than present at birth in rats, cats, and hamsters. Evidence for plasticity is particularly compelling for rubrospinal axons. Rubral axons, like cortical ones, grow around a lesion to innervate appropriate areas caudal to it, but they do so at earlier stages of development than cortical axons. Most rubrospinal neurons fail to survive axotomy during the critical period for plasticity, and the axons that circumvent the lesion are primarily late growing ones. Neuronal survival after axotomy increases with age. It appears that vestibulospinal and reticulospinal axons also grow around a lesion in neonatal opossums and that the critical period for such growth ends earlier than that for rubrospinal axons. Based on the developmental history of descending spinal pathways and the critical periods for plasticity of the ones studied to date, we have proposed that axons that reach the spinal cord first lose their potential for plasticity before axons that arrive at a later date.

In vitro evidence for growth of brainstem axons across a complete transection of the spinal cord has been reported using opossum neonates (the South American opossum, *Monodelphis domestica*) and fetal rats. Using the North American opossum, it has been shown that such growth also occurs *in vivo* and that axons from all of the brainstem areas that normally innervate the spinal cord caudal to the lesion contribute to it.

It is of interest to ask why descending spinal axons fail to grow around or through a lesion of the spinal cord at later stages of development and in the adult animal. The results of studies to date suggest that decreased growth potential, the development of myelin, astrocytic maturation, and the development of a glial response to lesioning may play a role. Further study of developmental plasticity, and the factors that limit it with age, may suggest therapeutic strategies that can facilitate axonal regeneration in the injured spinal cord of adult mammals, including humans.

I. INTRODUCTION

Damage to the sensorimotor cortex of developing mammals often results in less neurological deficit than in adults (Kennard, 1936, 1938, 1940, 1942). The basis for this phenomenon, the so-called infant lesion effect, is not always clear; but reorganization of cortical projections probably plays a role (Burgess et al., 1986; Burgess and Villablanca, 1986; Gomez-Pinilla et al., 1986; Villablanca et al., 1986, 1988; Leonard and Goldberger, 1987a,b; Villablanca and Gomez-Pinilla, 1987; Armand and Kably, 1992). Sparing of function and anatomical reorganization have also been reported after spinal cord lesions in developing mammals. When spinal cord lesions are made in neonatal kittens, for example, sparing of tactile placing occurs which has been attributed to growth of cortical axons around the lesion (e.g., Bregman and Goldberger, 1982, 1983a,b). Such growth does not take place in adult cats, nor does the behavioral recovery associated with it. Recovery of reflexes (contact placing and hopping) caudal to spinal cord hemisection also occurs in neonatal rats (Kunkel-Bagden et al., 1992). It is of interest, however, that some aspects of locomotor recovery are actually greater in rats lesioned as adults (Kunkel-Bagden et al., 1992), suggesting that recovery of function after lesions is not always greater in developing animals than in adults (see also Glassman, 1973; Nonneman and Isaacson, 1973; Passingham et al., 1978; Karte-Tillotson and Castro, 1979; Bregman and Goldberger, 1983a,b; Keifer and Kalil, 1991). It appears clear, however, that anatomical plasticity is much more robust during development than at maturity. In this chapter we explore the degree to which spinal axons, particularly those that originate at supraspinal levels, are able to grow around or through a lesion of their pathway in developing mammals and whether such plasticity results from regeneration of cut axons or simply new growth. We will also consider changes with maturity that may limit anatomical plasticity. These issues will be addressed by reviewing selected literature and by describing the results of experiments from our own laboratory.

II. DEVELOPMENTAL PLASTICITY OF DESCENDING SPINAL PATHWAYS

A. Corticospinal Plasticity

The corticospinal (pyramidal) tract is particularly large in humans (see the review by Armand, 1982), and damage to it is probably a major factor in the paralysis produced by spinal cord injury. Although corticospinal axons do not grow around a lesion in adult mammals, they are capable of doing so during development. In now classical experiments, Kalil and Reh (1979, 1982) used an orthograde tracing technique in the neonatal hamster to show that cortical axons grow around a lesion of the medullary pyramids. It will be recalled that corticospinal axons are a major component of the pyramids. When pyramidotomy was performed on one side at 2

days of age, aberrant cortical axons could be followed into the intact pyramidal tract and traced to their normal targets. When the same lesion was made between postnatal days (PD) 4 and 8, cortical axons took an abnormal route but still reached their destination. Reh and Kalil (1982) also studied corticospinal development in the hamster and found that the number of axons in the pyramidal tract showed little increase between PD3 and PD7 and that growth cones, which were present in small numbers at PD3, were rare at PD7. Since it appeared that new axons were not added to the pyramidal tract during the critical period for plasticity, they concluded that growth of cortical axons around a lesion of the pyramidal tract resulted from regeneration of cut axons.

The results of subsequent investigations supported the notion that cortical axons are able to grow around a lesion of their pathway (Bregman and Goldberger, 1982, 1983a,b; Schreyer and Jones, 1983; Tolbert and Der, 1987); but some of them suggested that such plasticity resulted from growth of late arriving axons, not regeneration of axons transected by the lesion. The experiments of Tolbert and Der (1987) addressed this issue directly in the kitten. These investigators made injections of the long-lasting fluorescent marker Fast Blue (FB) into the spinal cord between PD2 and PD5 to prelabel corticospinal neurons, and 5–8 days later they transected the pyramidal tract unilaterally. In parallel experiments they had shown that cortical axons grew around a lesion of the pyramidal tract during that time period. When the animals were sacrificed 29–79 days later, they found no evidence for FB-labeled neurons in the cerebral cortex ipsilateral to the lesion, although cortical neurons were labeled on the contralateral side. These results were interpreted to suggest that corticospinal neurons failed to survive axotomy during the critical period for plasticity and thus did not support regenerating axons. Since it had been shown in opossums (Cabana and Martin, 1984, 1985) and rats (Stanfield and O'Leary, 1985; O'Leary and Stanfield, 1986) that cortical axons form the pyramidal tract in a temporally staggered manner, Tolbert and Der suggested that the growth of cortical axons around a lesion of that tract resulted from redirection of late-growing, undamaged axons.

Subsequent studies, using a similar prelabeling technique, showed that corticospinal neurons also degenerate after pyramidotomy in hamsters if it is performed at early postnatal ages (Merline and Kalil, 1990). It was concluded, therefore, that developmental plasticity of the corticospinal tract in hamsters, as in cats (Tolbert and Der, 1987), results primarily from new growth, not regeneration of cut axons, as suggested previously. Merline and Kalil emphasized, however, that new growth could result from collateral sprouting by undamaged axons that normally terminate rostral to the lesion as well as by deflection of late-growing axons.

In the rat, corticospinal plasticity may result from regeneration of cut axons as well as late growth. In experiments performed by Bates and Stelzner (1989, 1993) FB was injected into the cervical spinal cord of neonatal rats at PD2, PD4, and PD10, and 2 days later it was aspirated. This procedure removed the FB and lesioned the corticospinal tract at the same time. Two months later another fluorescent

marker, Diamidino Yellow (DY), was injected caudal to the lesion, and after a 2-day survival the animals were sacrificed and perfused so that the brain could be fixed for frozen sectioning and viewing under a fluorescent microscope. FB-labeled neurons were observed in the motor cortex contralateral to the lesion at all ages, suggesting that many corticospinal neurons survived axotomy. In the PD2 and PD4 cases a small number of the FB-labeled neurons also contained DY, indicating that they not only survived the lesion but that they regenerated an axon, or axons, around it to incorporate DY. Cortical neurons were also labeled by DY alone. Such neurons had apparently not been axotomized but supported axons that grew around the lesion to take up DY. It should be noted that the lesions had been made at medullary levels in the hamster and cat, whereas they were made at cervical levels in the rat. It is possible, therefore, that corticospinal neurons survive cervical lesions better than lesions of the medullary pyramids and that neuronal survival, rather than species differences, accounts for the presence of regeneration in the rat.

As indicated in the introduction, corticospinal plasticity may be associated with the sparing of function seen after spinal cord lesions in developing mammals. Bregman and Goldberger (1982, 1983a,b) made partial hemisections of the spinal cord in adult cats and kittens. Adult cats never showed recovery of tactile placing caudal to the lesion, a function dependent upon the corticospinal tract, but animals operated as neonates did. Retrograde labeling studies in the neonates provided evidence for growth of cortical axons around the lesion, and ablation of the sensorimotor cortex contralateral to the lesion, the origin of the aberrant corticospinal tract, resulted in loss of the spared placing.

In rats, and presumably in other species, the critical period for corticospinal plasticity can be extended by transplants of fetal spinal cord (Bregman et al., 1989). Bregman and colleagues overhemisected the spinal cord at different stages of development and, in some cases, the lesion cavity was filled with the transplant. One to nine months later, the orthograde transport of horseradish peroxidase was employed to study corticospinal axons. In these experiments, cortical axons could be traced into the transplants well after the critical period for plasticity defined in animals without transplants. It was noted that cortical innervation of the transplant decreased with age of the recipient, although it sometimes occurred even in relatively mature animals. The authors suggested that decreased growth potential of cortical axons and changes in the environment through which they grow both contribute to loss of plasticity.

The studies reviewed above indicate that growth of corticospinal axons around a lesion is vigorous in newborn hamsters, rats, and cats and that a critical period exists for it. There is some disagreement, however, over whether plasticity results from new growth (hamster and cat) or from a combination of new growth and regeneration of cut axons (rat). This discrepancy may reflect species differences (e.g., differences in the maturation of corticospinal axons at birth in different species), differences in the age of the animal at the time of lesioning, and/or differences in the level of the lesion.

B. Brainstem–Spinal Plasticity

Although cortical axons grow around a lesion of their pathway in neonatal hamster, rats, and cats, brainstem axons do not (e.g., Prendergast and Stelzner, 1976; Bregman and Goldberger, 1982, 1983a,b). Corticospinal development continues postnatally in hamsters, rats, and cats (Donatelle, 1977; Bregman and Goldberger, 1982, 1983a,b; Reh and Kalil, 1982), when many corticospinal neurons are still programmed for axonal elongation. In contrast, brainstem axons grow into the spinal cord prenatally (Prendergast and Stelzner, 1976; Bregman and Goldberger, 1982, 1983a,b; Lakke and Marani, 1991), and by birth they may have lost their potential for plasticity. Based on such reasoning, we have hypothesized that brainstem axons, like cortical axons, will grow around a lesion of their pathway if it is made at stages of development earlier than that present at birth in hamsters, rats, and cats. That hypothesis would be difficult to test in those species, however, because of the prenatal development of brainstem–spinal connections, but it proved to be testable in the North American opossum because most brainstem–spinal development occurs postnatally in that species (Cabana and Martin, 1981, 1984; Martin et al., 1992). Opossums are marsupials whose young are born in a very immature state, 12–13 days after conception (McCrady, 1938).

Plasticity of the Rubrospinal Tract

We began our studies of brainstem–spinal plasticity by focusing on the rubrospinal tract because rubral axons grow into the spinal cord later than most axons from the brainstem (Cabana and Martin, 1981, 1984; Martin et al., 1992), and they are almost entirely crossed (see review by Xu and Martin, 1991b). The relatively late development of the rubrospinal tract made it possible to lesion rubral axons at very early stages of development, and its laterality made it possible to interpret the results of the experimental manipulations described below.

Retrograde Labeling Studies. In our initial experiments (Martin and Xu, 1988; Xu and Martin, 1989), we cut the rubrospinal tract at thoracic levels in developing and adult opossums. We knew from previous studies (Cabana and Martin, 1985) that rubral axons were present at the lesion site in all cases. After approximately 30 days, the animals were reanesthetized so that injections of FB or wheat-germ agglutinin conjugated to horseradish peroxidase (WGA-HRP) could be made several segments caudal to the lesion and ipsilateral to it. After an appropriate survival time for retrograde labeling, the animals were reanesthetized, sacrificed, and perfused so that the spinal cord and brain could be removed for sectioning and, in the cases subjected to WGA-HRP injections, processing for HRP (Mesulam, 1978).

In adult animals and older pouch-young (e.g., the EPD54 case in Fig. 1), rubral neurons were not labeled contralateral to the lesion. In younger animals, however, labeled neurons were found in the contralateral red nucleus (Fig. 1), and it was our

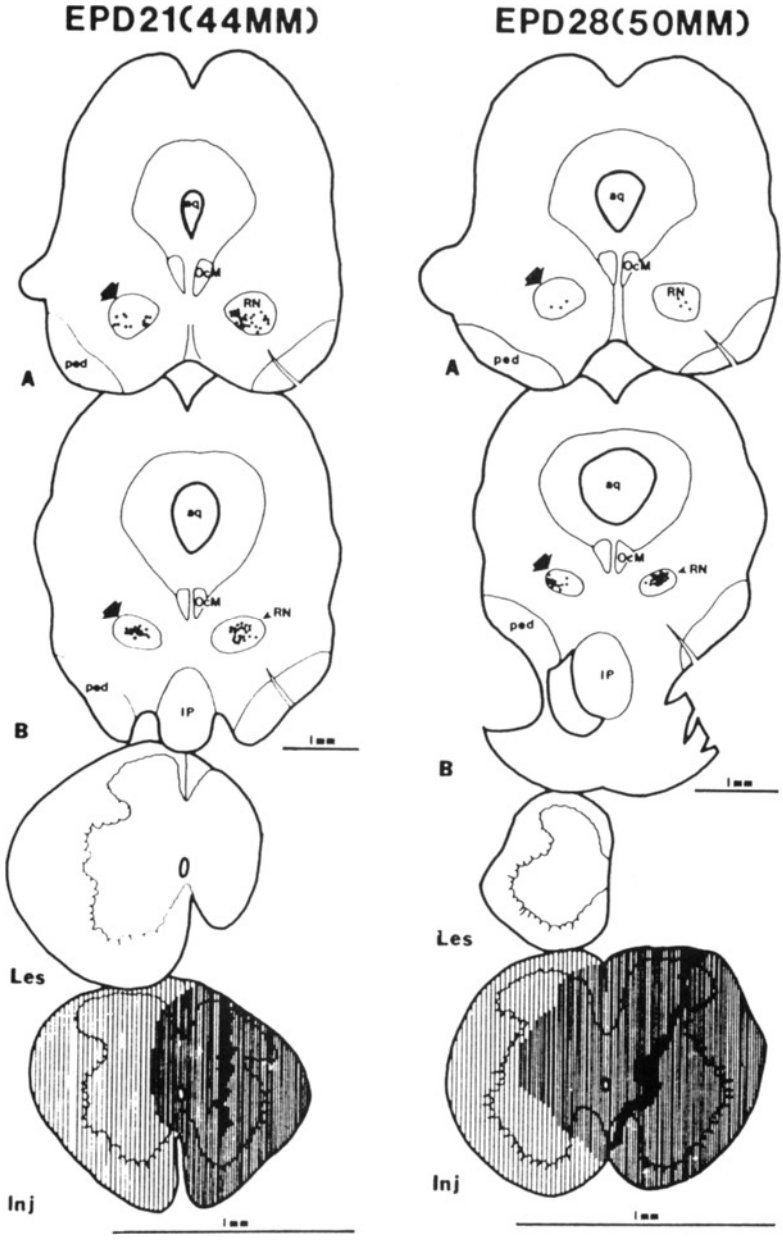

Figure 1. Plots of rubral labeling (section A, B) produced at different stages of development by spinal injections of WGA-HRP (Inj) made approximately 30 days after lesions (Les) that included the rubrospinal tract. The midbrain sections are stacked from rostral (**A**) to caudal (**B**); the red nucleus contralateral to the lesion and injection is indicated by *arrows.* (From Xu and Martin, J. Comp. Neurol. **1989**, *279.*) (continued)

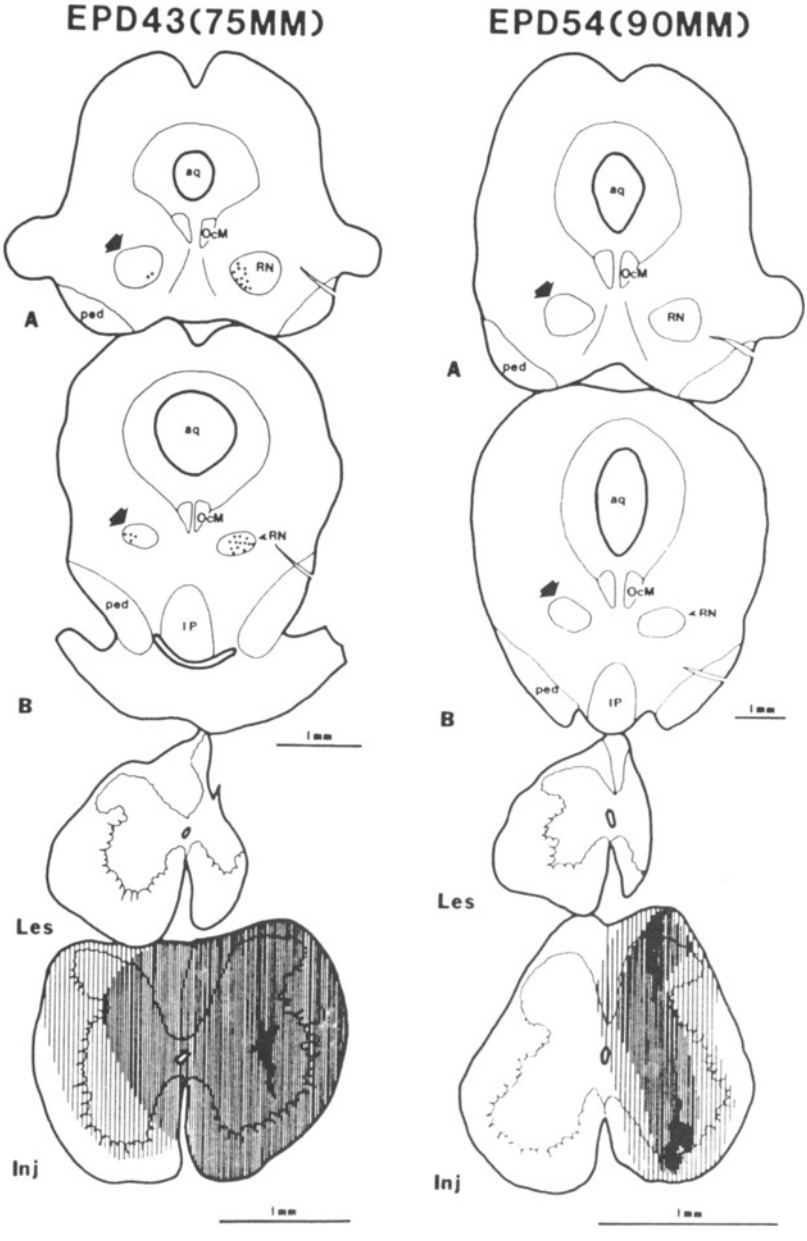

Figure 1. (continued)

interpretation that they supported axons that had grown around the lesion to incorporate the marker. Although the results from the cases plotted in Figure 1 would suggest that rubrospinal plasticity occurs as late as estimated postnatal day 43 or beyond, those obtained from more recent studies indicate that it may not extend much beyond postnatal day 30.

Orthograde Labeling Studies. To confirm the results obtained using retrograde tracing techniques, we also studied rubrospinal plasticity by using orthograde tracing methods (Martin and Xu, 1988; Xu and Martin, 1989). In those experiments, the rubrospinal tract was cut at different ages and in the adult animal, as described above; but after a 30- to 60-day survival, the red nucleus contralateral to the lesion was injected with WGA-HRP in order to orthogradely label rubrospinal axons. After the appropriate survival for orthograde labeling, the animals were sacrificed and perfused so that the removed brain and spinal cord could be processed for HRP.

At all ages, rubrospinal axons were labeled in their normal position within the dorsal part of the lateral funiculus and within laminae V–VII of the grey matter rostral to the lesion (Figs. 2A and 3). Labeling at the lesion site and caudal to it varied, however, depending on the age of the animal and the extent of the lesion. When the lesion was made in adult animals and older pouch-young, labeled axons in the white matter immediately rostral to the lesion often appeared swollen and abnormal, and labeling within the grey matter was denser than at more rostral levels (compare Figs. 2A and B). Labeled axons could not be traced caudal to the lesion in such cases (Fig. 2D). At early stages of development, however, labeled axons could be followed caudal to the lesion (Fig. 3). In the case illustrated on the left in Figure 3 and documented in Figure 4A, the spinal cord had been hemisected and rubral axons were located at the edge of the remaining part of the dorsal funiculus. Caudal to the lesion, rubral axons did not return to their normal position in the lateral funiculus, but coursed in the lateral part of the dorsal funiculus. In spite of their abnormal position, rubral axons coursed for a considerable distance caudal to the lesion and innervated areas of the grey matter appropriate to them. In cases with less extensive lesions, rubral axons grew through the remaining white or grey matter (Figs. 3 and 4B) and returned to the dorsal part of the lateral funiculus caudal to the lesion. As in the above case, they grew caudal to the lesion and innervated appropriate areas. The results of the orthograde tracing experiments thus supported those obtained by retrograde tracing methods and, together, they suggested that rubral axons are able to grow around a lesion of their spinal pathway during early development and that a critical period exists for that plasticity. As predicted, the critical period for rubrospinal plasticity ends earlier than that for corticospinal plasticity.

Prelabeling Experiments. The issue then became whether rubrospinal plasticity resulted from true regeneration (i.e., regeneration of cut axons) or growth of axons that were not damaged by the lesion. In an initial attempt to address that

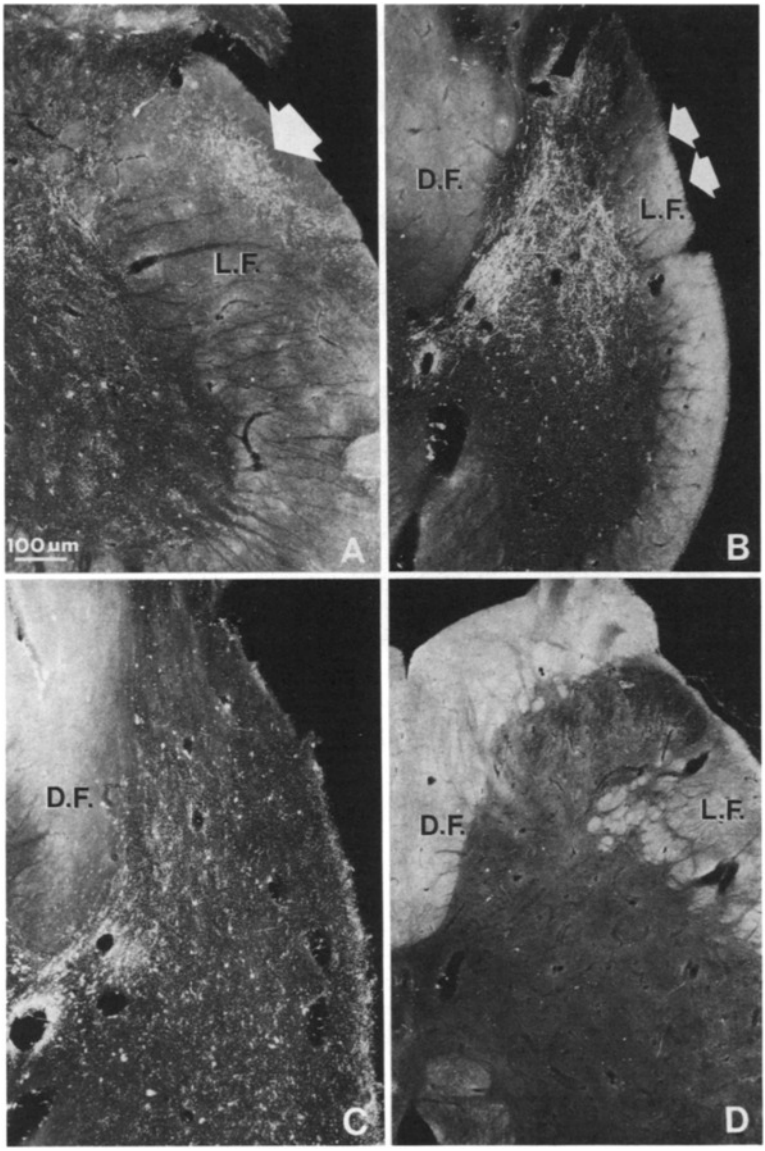

Figure 2. Dark-field photomicrographs of rubrospinal labeling in the cervical enlargement (**A**), immediately rostral to the deepest part of the lesion (**B**), at the deepest part of the lesion (**C**), and caudal to it (**D**), in an animal subjected to transection of the rubrospinal tract in the caudal thoracic cord at PD54 and an injection of WGA-HRP into the contralateral red nucleus 60 days later. Labeled axons in the lateral funiculus (L.F.) are indicated by arrows in **A** and **B**. The dorsal funiculus (D.F.) is indicated in **B–D**. The bar in **A** can be used for all figures. (Modified from Xu and Martin, J. Comp. Neurol. **1989**, *279*.)

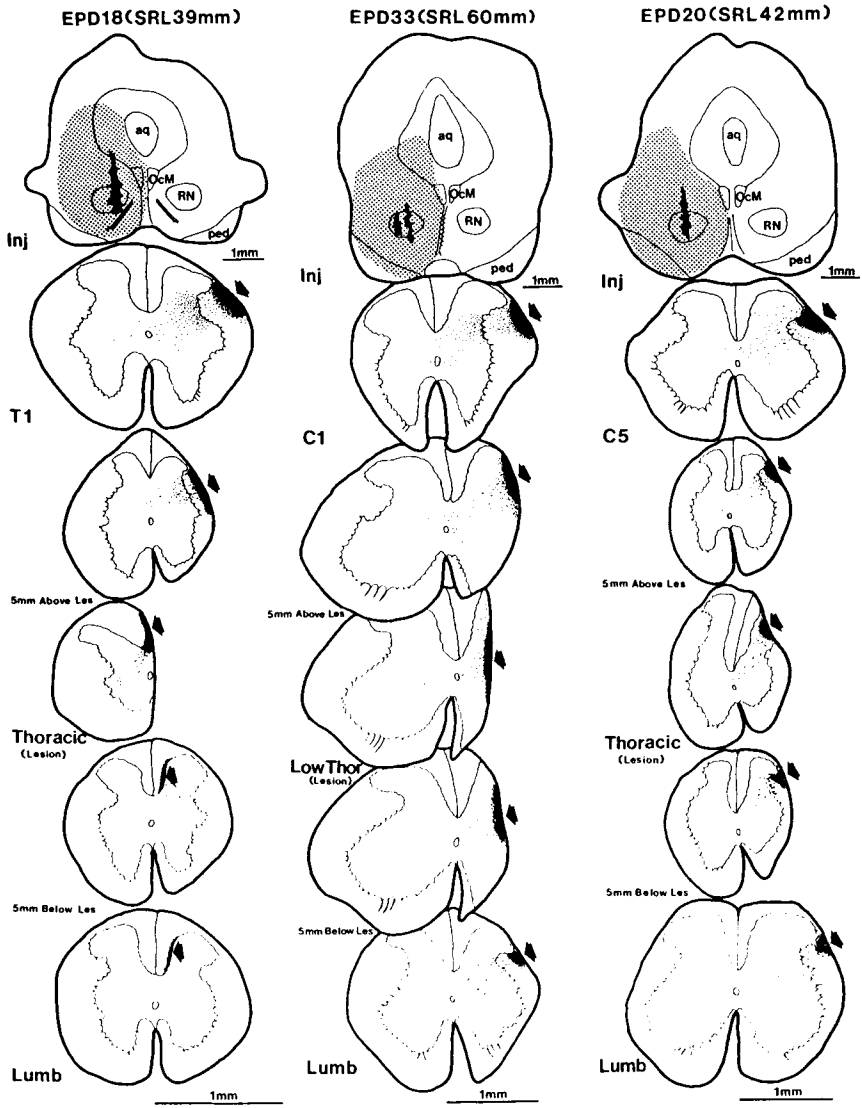

Figure 3. Plot of the rubrospinal labeling (*arrows*) present well rostral to the lesion (T1, C1, and C5), 5 mm rostral to the lesion, at the deepest part of the lesion (Thoracic or Low Thor), 5 mm caudal to the lesion, and at lumbar (Lumb) levels in three cases subjected to lesions at EPD18, EPD33, and EPD20 and injections of WGA-HRP into the contralateral red nucleus approximately 30 days later. (From Xu and Martin, J. Comp. Neurol. **1989**, *279*.)

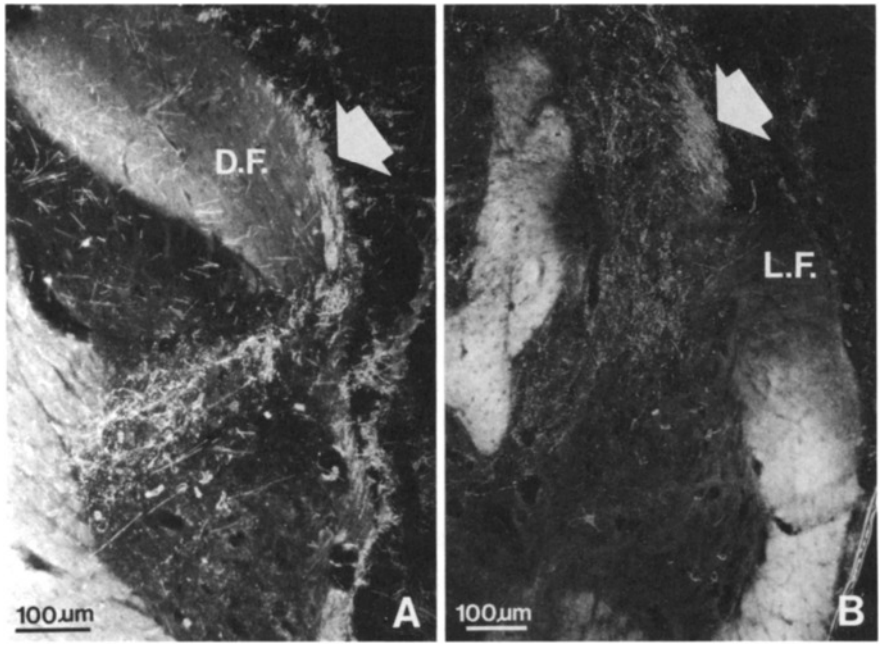

Figure 4. Dark-field photomicrographs of rubrospinal labeling (*arrows*) at the lesion site in the cases plotted on the left and right in Figure 3. (From Xu and Martin, J. Comp. Neurol. **1989**, *279*.)

question, we performed a prelabeling experiment similar to that of Tolbert and Der (1987). Rubrospinal neurons were prelabeled in adult and developing opossums by unilateral or bilateral injections of FB into the thoracic cord, and after the appropriate survival time for retrograde labeling, the rubrospinal tract was cut unilaterally, several segments rostral to the injections. After a 30-day survival, the animals were sacrificed so that sections through the red nucleus could be examined for labeled neurons. Although a few neurons were labeled in the red nucleus contralateral to the lesion during the critical period for plasticity (Fig. 5, A and C), they were not nearly as numerous as those labeled on the ipsilateral side (Fig. 5, B and D). When counts of labeled neurons were compared on the two sides, only 25% had survived (Xu and Martin, 1992). In contrast, 75% survived in the adult animal (Xu and Martin, 1990). We interpreted these results to suggest that relatively few neurons survive axotomy during the critical period for plasticity and that their numbers increase with age.

It is of interest to ask why most rubrospinal neurons failed to survive axotomy during the critical period for plasticity and yet most of them survived in the adult animal. One possibility is that axotomized neurons were deprived of trophic factors from their targets during development (see Sofroniew et al., 1990, for a review)

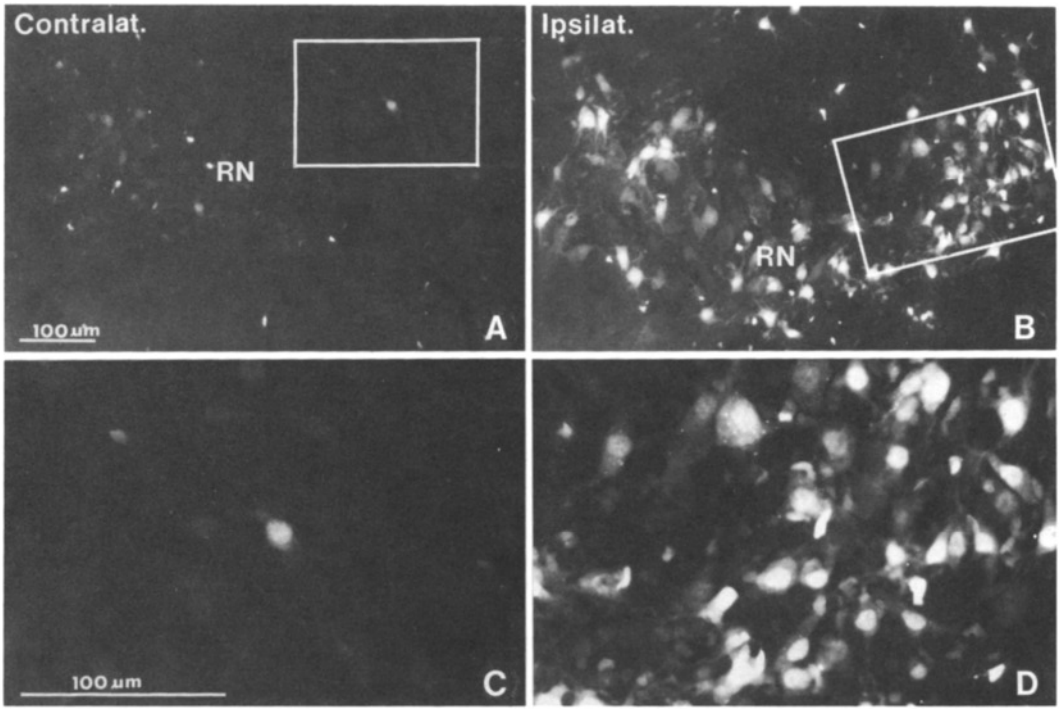

Figure 5. Photomicrographs of labeled neurons in the red nucleus contralateral (Contralat.) and ipsilateral (Ipsilat.) to a caudal thoracic injection of FB made at PD26. Three days later, a lesion of the rubrospinal tract was made four segments rostral to the injection. The animal was sacrificed 30 days later. Although the injection *was large enough to label neurons in the ipsilateral red nucleus (**B** and **D**), only a* few rubral neurons were labeled on the contralateral side (**A** and **C**). In cases with comparable injections, but no lesion, the number of labeled neurons in the contralateral red nucleus far exceeded that in the ipsilateral one. The area within the rectangles in **A** and B are shown in **C** and **D**, respectively. The bar in **A** can be used for **B** and that in **C** can be used for **D**. (From Xu and Martin, J. Comp. Neurol. **1989**, *279*.)

and that those factors became less important with age. In that regard, it is interesting that neurotrophins, specifically BDNF and NT-3, rescue axotomized rubrospinal neurons in postnatal day 3 rats (Diener-Ostfield et al., 1993) and that BDNF prevents atrophy of rubrospinal neurons after axotomy in adult rats (Tetzlaff et al., 1993). It is also possible that the immature neurons of the red nucleus were committed to axonal elongation and had not yet established synapses (Merline and Kalil, 1990) or sustaining collaterals rostral to the lesion (Frye and Cowan, 1972). The development of synapses and collaterals may, of course, be related to incorporation of available trophic factors. In addition, a simple distance effect may have been operative, since axotomy was performed closer to the cell body in developing animals than in adults. Whatever the explanation, we interpreted our results to suggest that relatively few neurons survived axotomy during the critical period for plasticity and that plasticity resulted primarily from new growth, that is, deflection of late growing axons and/or the caudal growth of collaterals from undamaged axons that normally terminate rostral to the lesion. That interpretation is consistent with the relatively protracted development of the rubrospinal tract (Cabana and Martin, 1986).

Double Labeling Experiments. To obtain more direct evidence for new growth in rubrospinal plasticity and to determine if regeneration of cut axons also occurs, we performed double labeling experiments similar to those used by Bates and Stelzner (1989, 1993). FB was injected unilaterally or bilaterally into caudal thoracic or rostral lumbar levels of the spinal cord during the critical period for plasticity (Fig. 6, FB Injection). After approximately 4 days, the pups were reanesthetized and subjected to a lesion of the rubrospinal tract four to five segments rostral to the injection(s) (Fig. 6, Lesion). During the same surgery, the injected bolus of FB was removed by gentle suction (see documentation in Fig. 7D) so that it would not be available to late-growing axons. The animals were allowed to survive for about 30 days before the second marker, DY, was injected between the site of the FB injection and the lesion (Fig. 6, DY Injection, and Fig. 7C). The intent of the second injection was to label any rubral neurons whose axons had grown around the lesion during the 30-day survival. The pups were maintained for about 5 days before sacrifice, and sections through the red nucleus were searched with a fluorescence microscope for neurons labeled by FB, DY, or both markers (Fig. 6, Observation). Only a few neurons in the red nucleus contralateral to the lesion were labeled by FB alone, supporting the results of the prelabeling studies described above. In contrast, many neurons were labeled by DY alone (Fig. 7A, solid arrows), indicating that their axons were not present in the caudal cord when FB was injected, but that they had grown around the lesion during the 30-day survival to incorporate DY. A few double-labeled neurons were also found (Fig. 7A, open arrow). The presence of FB in such cells indicated that they survived axotomy, and the presence of DY suggested that they supported an axon (or axons) that grew around the lesion to at least the level of the second injection. The results from one case are plotted in

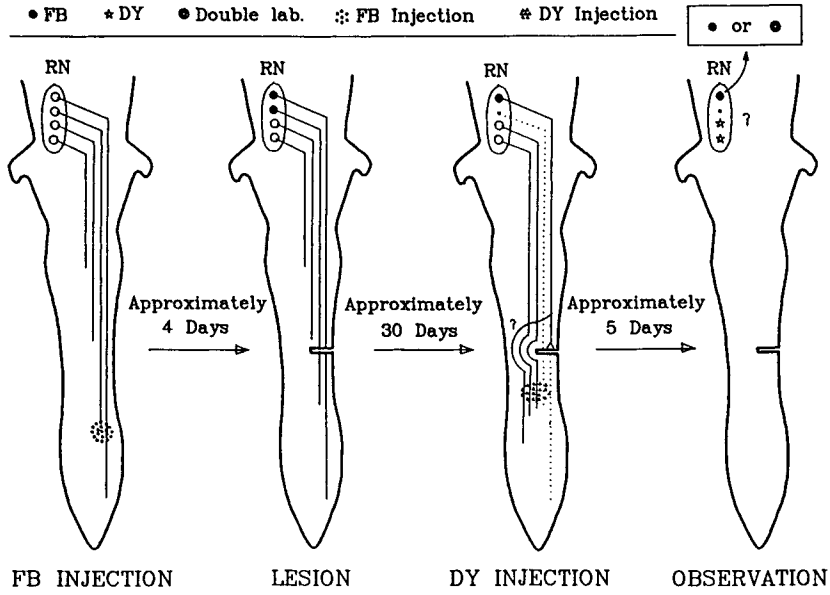

Figure 6. Schematic drawing of the experimental paradigm employed for the double-labeling study. Fast Blue (FB) injections were made into the caudal thoracic or rostral lumbar cord at different ages (FB injection). Approximately 4 days later, after rubrospinal neurons had been labeled by FB, a lesion of their axons was made several segments rostral to the injection (Lesion). Approximately 30 days later, a second marker, Diamidino Yellow (DY), was injected into the cord between the FB injection and the lesion (DY Injection). Five days later, the animals were sacrificed and the red nucleus (RN) was examined for neurons labeled by one or both markers (Observation). FB was removed by suction when the lesion was made so that it would not be available to late-growing axons during the 30 day survival. (Modified from Xu and Martin, *J. Comp. Neurol.* **1991**, 313.)

Figure 8. Our interpretation of the above results was that rubrospinal plasticity results primarily from growth of late-arriving axons, as suggested for corticospinal plasticity in hamsters (Merline and Kalil, 1990) and cats (Tolbert and Der, 1987), but that regeneration of cut axons also occurs. It will be recalled that regeneration also contributes to corticospinal plasticity in the rat (Bates and Stelzner, 1989, 1993).

The relatively minor role for true regeneration in rubrospinal plasticity may simply reflect the failure of most rubrospinal neurons to survive axotomy during the critical period. This hypothesis is supported by the results of transplant studies. When fetal spinal cord was placed into the lesion cavity of spinal cords hemisected at midthoracic levels in neonatal rats, rubrospinal neurons survived axotomy (Bregman and Reier, 1986), and many of them supported axons that regenerated into the transplant (Bregman and Bernstein-Goral, 1991; Bernstein-Goral and Bregman, 1993). It is possible that the transplants supplied trophic factors that resereved oxotomized rubrospinal neurons, increasing the number of axons available

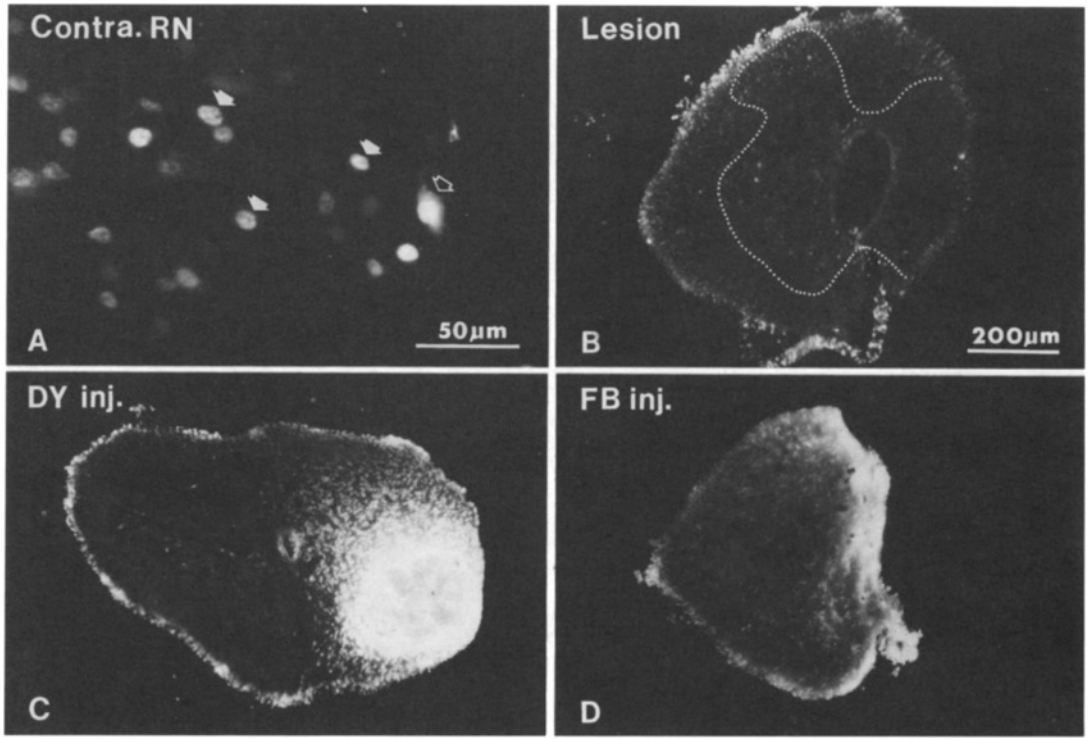

Figure 7. Fluorescence photomicrographs of rubrospinal neurons labeled by Fast Blue (FB) alone, by Diamidino Yellow (DY) alone (**A**, *solid arrows*), and by both markers (*open arrow*) contralateral to the lesion from a case subjected to unilateral injection of DY (**C**) and FB. The lesion was made at PD22. At the time of the lesion, residual FB was removed so that it was not present at the injection site (**D**). The bar in **B** can be used for **C** and **D**. The grey matter is outlined in **B**. (From Xu and Martin, J. Comp. Neurol. **1991**, *313*.)

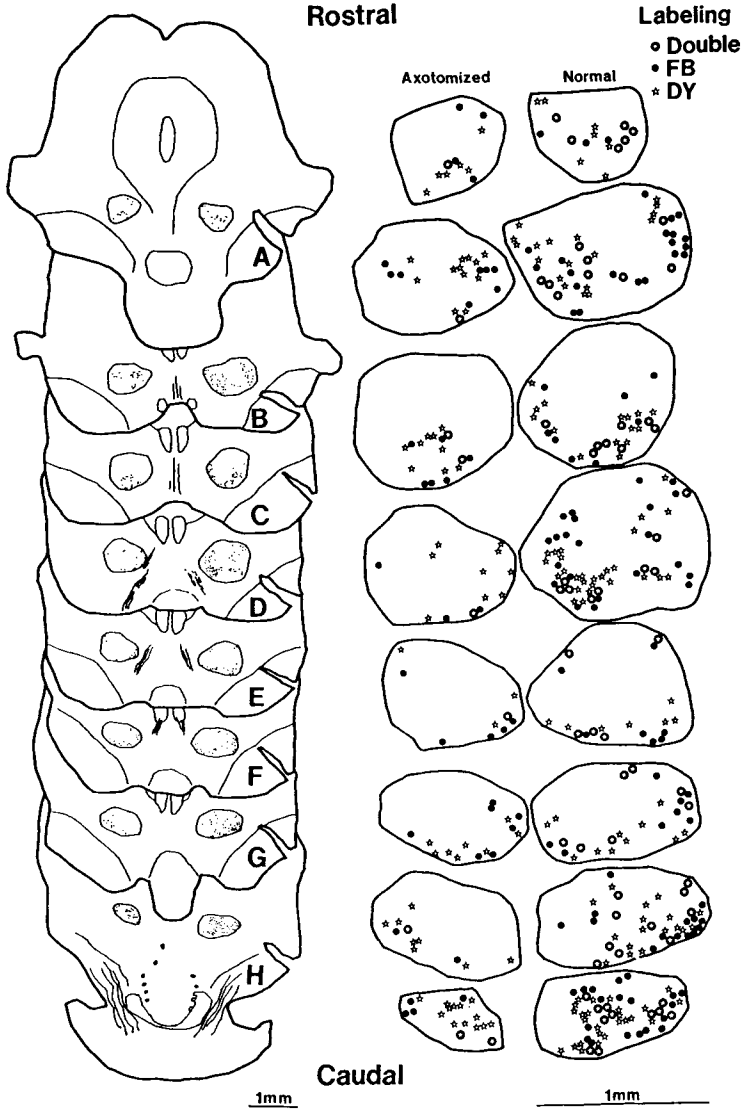

Figure 8. Plot of Fast Blue (FB) (*dots*), Diamidino Yellow (DY) (*stars*), and double-labeled (*stars on dots*) neurons from a case subjected to bilateral injections of FB and DY and a unilateral lesion at PD22. The levels of the red nucleus plotted were drawn on the left. Note that the number of labeled neurons on the side contralateral to the lesion was less than on the ipsilateral side. In the contralateral red nucleus, most of the labeled neurons were labeled by DY alone. (Modified from Xu and Martin, *J. Comp. Neurol.* **1991**, 313.)

for regeneration. That hypothesis is supported by evidence that neurotrophins, specifically BDNF and NT-3, rescue axotomized rubrospinal neurons in postnatal day 3 rats (Diener-Ostfield et al., 1993).

Plasticity of Selected Vestibulospinal and Reticulospinal Axons

The results described above indicate that rubral axons, like cortical axons, are able to grow around a lesion of their spinal pathway and that a critical period exists for that plasticity. It became our working hypothesis that all supraspinal axons are able to grow around a lesion at some stage of development, although differences in critical periods may exist (Xu and Martin, 1991a). Based on comparison of the critical periods for rubrospinal and corticospinal plasticity, it seemed reasonable to suggest that axons which grow into the spinal cord early, lose their potential for plasticity before axons that reach it at a later date. To test that hypothesis further, we initiated studies designed to determine whether axons from the medial pontine reticular formation and the dorsal part of the lateral vestibular nucleus are able to grow around a lesion of their spinal pathway and, if so, to determine the critical period(s) for their plasticity. We know from previous studies (Cabana and Martin, 1981, 1984; Martin et al., 1992) that reticular and vestibular axons grow into the spinal cord before axons from the red nucleus, so our hypothesis would predict that the critical periods for reticulospinal and vestibulospinal plasticity end earlier than that for rubrospinal axons.

In order to study reticulospinal and vestibulospinal plasticity, we hemisected the thoracic cord at PD20, well within the critical period for rubrospinal plasticity, and approximately 30 days later made bilateral injections of FB several segments caudal to the lesion. After an appropriate time for FB labeling, the animals were sacrificed and frozen sections of the brainstem were examined for labeled neurons. As expected, rubral neurons were labeled contralateral to the lesion (Fig. 9, A and B), indicating that their axons had grown around it to incorporate the marker. In contrast, neurons were not labeled in the medial part of the pontine reticular formation or in the dorsal part of the lateral vestibular nucleus ipsilateral to the lesion (Figs. 9D–F and 10, B and D), although they were labeled on the contralateral side (Figs. 9D–F and 10, A and C). Spinal projections from both areas are exclusively ipsilateral (Martin et al., 1992). When the same experiment was performed at PD12, however, a few neurons were labeled in the dorsal part of the lateral vestibular nucleus ipsilateral to the lesion (Fig. 11B), and when it was performed at PD5, some were also labeled in the medial part of the pontine reticular formation on the same side (Fig. 11D). We have interpreted these results to suggest that vestibular and reticular axons, like rubral axons, are able to grow around a lesion, but that the critical period for their plasticity ends earlier than that for rubrospinal axons. In some of the PD5 cases referred to above, there was evidence for regeneration of spinal cord tissue at the lesion site and growth of reticular and vestibular axons through the lesion, not around it.

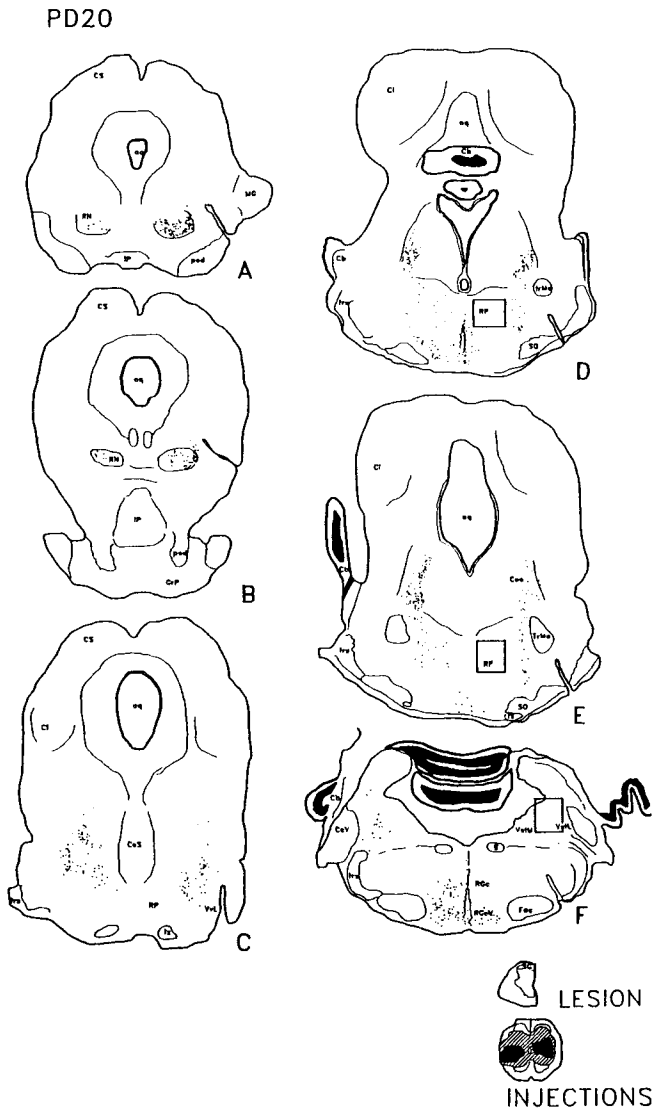

Figure 9. Plot of labeled neurons (*dots*) in an opossum subjected to hemisection of the spinal cord on postnatal day (PD) 20 (Lesion) and bilateral injections of Fast Blue (Injections) caudal to the lesion 30 days later. The sections are arranged from rostral (**A**) to caudal (**F**), and the regions of the pontine reticular formation and lateral vestibular nucleus referred to in the text are indicated by the rectangles in sections **D–F**.

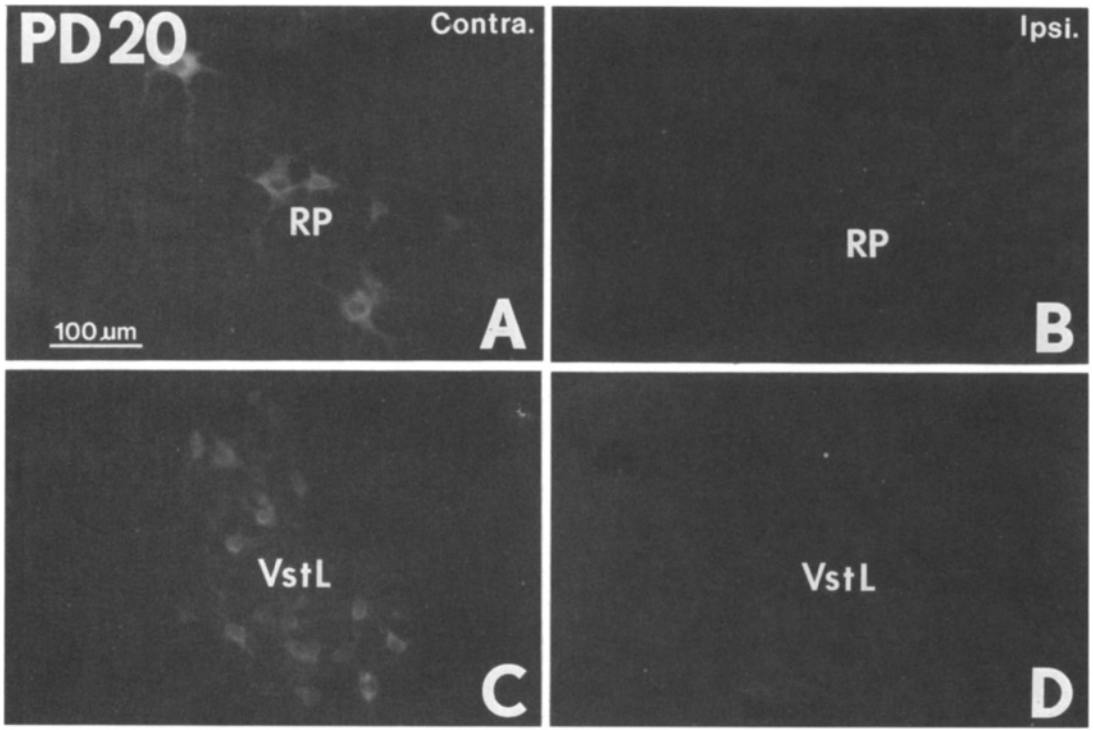

Figure 10. Photomicrographs of the medial pontine reticular formation (RP) and dorsal part of the lateral vestibular nucleus (VstL) from a case subjected to bilateral injections of Fast Blue (FB) into the rostral lumbar cord 30 days after hemisection of the thoracic cord at postnatal day (PD) 20. Neurons were not labeled ipsilateral (Ipsi) to the lesion (**B** and **D**) in either area, although they were labeled on the contralateral (Contra) side (**A** and **C**).

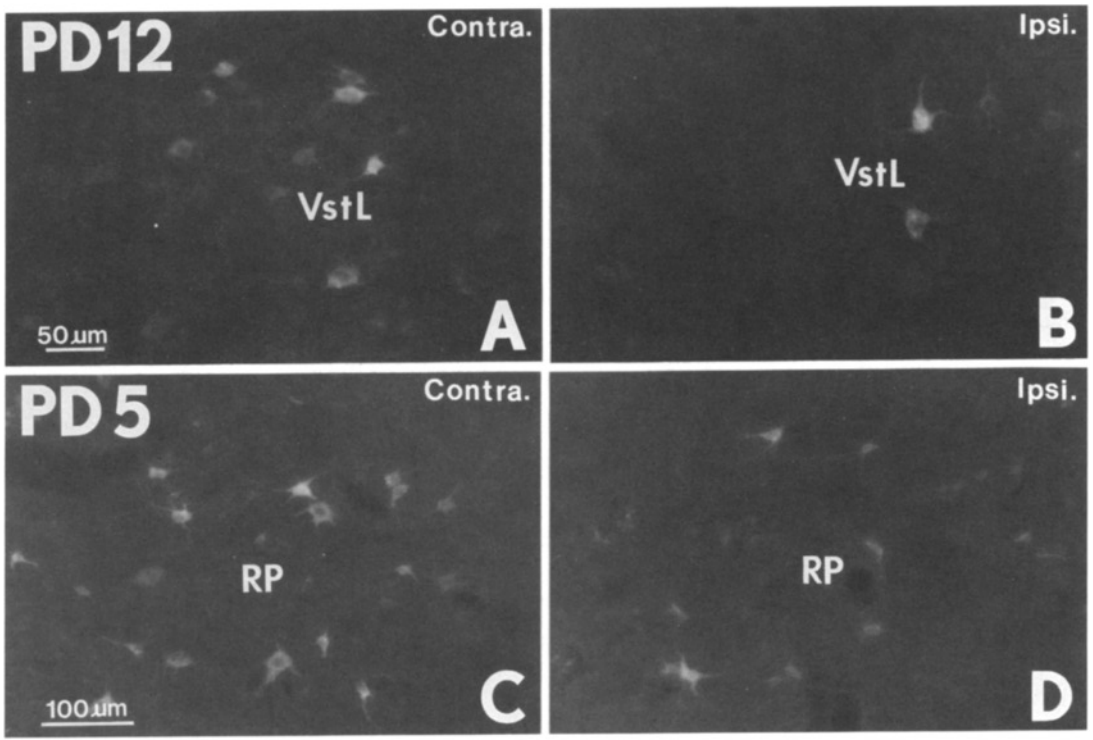

Figure 11. Photomicrographs of the labeling produced in the dorsal part of the lateral vestibular nucleus (VstL) and the medial pontine reticular formation (RP) by bilateral injections of Fast Blue (FB) into the rostral lumbar cord 30 days after hemisection of the thoracic cord at postnatal days (PD) 12 and 5. At PD12, neurons were labeled in the VstL ipsilateral (Ipsi) to the lesion (**B**) and at PD5 they were also labeled in the RP on the same side (**D**). Labeling on the side ipsilateral to the lesion (**B** and **D**) was never as great as that on the contralateral (Contra) side (**A** and **C**).

Evidence for Growth of Brainstem Axons Through a Complete Transection of the Spinal Cord

In most of the experiments described above, growth of supraspinal axons occurred around the lesion, not through it. Evidence for growth of brainstem axons through regenerated cord was present in some cases, however, and it has been shown using neonatal opossums (the South American opossum, *Monodelphis domestica*) and fetal rats (Saunders et al., 1992; Treherne et al., 1992; Woodward et al., 1993) that at least some brainstem axons can grow through a crush lesion of the cord *in vitro*. A critical period has been established for such growth in *Monodelphis* (Varga et al., 1993), and locomotor activity has been reported to be relatively normal in weaned pups whose spinal cords had been crushed at PD5–7 (Saunders et al., 1994). Based on such results, we predicted that comparable growth would occur *in vivo* in the North American opossum.

In order to test our prediction, we transected the midthoracic cord of anesthetized pups at PD5 and asked whether supraspinal axons would grow through the lesion (Martin et al., 1994). In some cases, the pups were sacrificed shortly after surgery so that sagittal sections of the spinal cord could be cut and stained for Nissl substance to document that our lesioning method resulted in a complete transection. The remaining pups were returned to the vivarium with their mother for about 30 days to allow for growth through the lesion. After the appropriate survival period, the operates were reanesthetized and reoperated so that bilateral injections of FB could be made several segments caudal to the lesion. After a survival of 5–7 days for retrograde transport of the injected marker, the pups were sacrificed by an overdose of anesthetic and perfused so their brain and spinal cord could be removed and sectioned for examination with a fluorescent microscope. When the spinal cord was examined prior to sectioning, evidence for regeneration was clearly present at the lesion site. In fact, the lesioned segment could only be identified because it was somewhat thinner than normal. Sections through the regenerated cord were examined to be sure that FB had not spread to the lesion site, and after that was established, the coverslips were removed so that the sections could be stained for Nissl substance. Examination of the Nissl-stained material revealed that recognizable spinal cord tissue was present at the lesion site, although it was somewhat disorganized. In all cases, neurons were labeled in each of the brainstem areas labeled by comparable injections in age-matched, unlesioned controls. Cortical axons do not grow caudal to midthoracic levels in the opossum (Cabana and Martin, 1985), so cortical labeling would not be expected.

In additional experiments, we transected the spinal cord at PD12 and made the FB injections 30 days later, as described above. When the pups were sacrificed 5–7 days later, the spinal cord was very thin at the lesion site and, histologically, few neurons were found. Labeled neurons were present at brainstem levels, however, although they were much fewer in number than in the PD5 cases and they were not found in all of the areas labeled in age-matched controls. Rubral neurons were

labeled in all cases, but few neurons were labeled in either the pontine reticular or lateral vestibular nucleus. We have interpreted these results to suggest that the spinal cord regenerates after complete transection if the lesion is made early enough in development and that brainstem axons grow through the lesion in large numbers. The end of the critical period for such growth has not yet been established, but it is apparently sometime after PD12. Based on results in developing chicks (Hasan et al., 1993), it is reasonable to predict that the regeneration of cut axons contributes to the growth just described.

III. FACTORS THAT MAY CONTRIBUTE TO LOSS OF DEVELOPMENTAL PLASTICITY

Failure of descending spinal axons to grow around or through a lesion after specific critical (permissive) periods of development could be related to reduced ability to generate and/or sustain axonal elongation and/or changes in the environment through which axonal elongation occurs. The significance of the environment is emphasized by the finding that severed axons of the central nervous system can regenerate into peripheral nerve grafts even in adult animals (Cajal, 1928; Richardson et al., 1980; David and Aguayo, 1981; Benfey and Aguayo, 1982). Schwann cells of peripheral nerve grafts supply a suitable environment for axonal elongation (Schwab and Thoenen, 1985; Carbonetto et al., 1987; Savio and Schwab, 1989) as well as trophic factors: NGF (Heumann et al., 1987), brain-derived neurotrophic factor (Acheson et al., 1991), and, possibly, epidermal growth factor (Toma et al., 1992). In the studies described below, we have begun to address loss of growth potential and changes in the environment traversed by rerouted axons as factors that may limit developmental plasticity.

A. Synthesis of Growth-Associated Proteins

If diminished ability to initiate and/or sustain axonal growth is the major factor in loss of developmental plasticity, it might be predicted that the end of the critical period for plasticity would correlate with a marked decrease in growth-associated proteins (Skene and Willard, 1981; Kalil and Skene, 1986; Meiri et al., 1986; Moya et al., 1989; Skene, 1989). To address that issue, we studied the development of one such protein, GAP-43, using an antibody (9-1E12) that recognizes all of its known epitopes (Goslin et al., 1988, 1990).

As expected, GAP-43-like immunoreactivity (GAP-43-LI) was abundant in the dorsal part of the lateral funiculus, the location of rubrospinal axons (Martin and Dom, 1970), and the ventral funiculus, the location of lateral vestibulospinal and pontine reticulospinal axons (Martin et al., 1975, 1979), during the critical periods for growth of rubrospinal, vestibulospinal, and reticulospinal plasticity around or through a lesion. Immunoreactivity was still present in both areas, however, well after such growth no longer occurs (Fig. 12, A and B). GAP-43-LI decreased

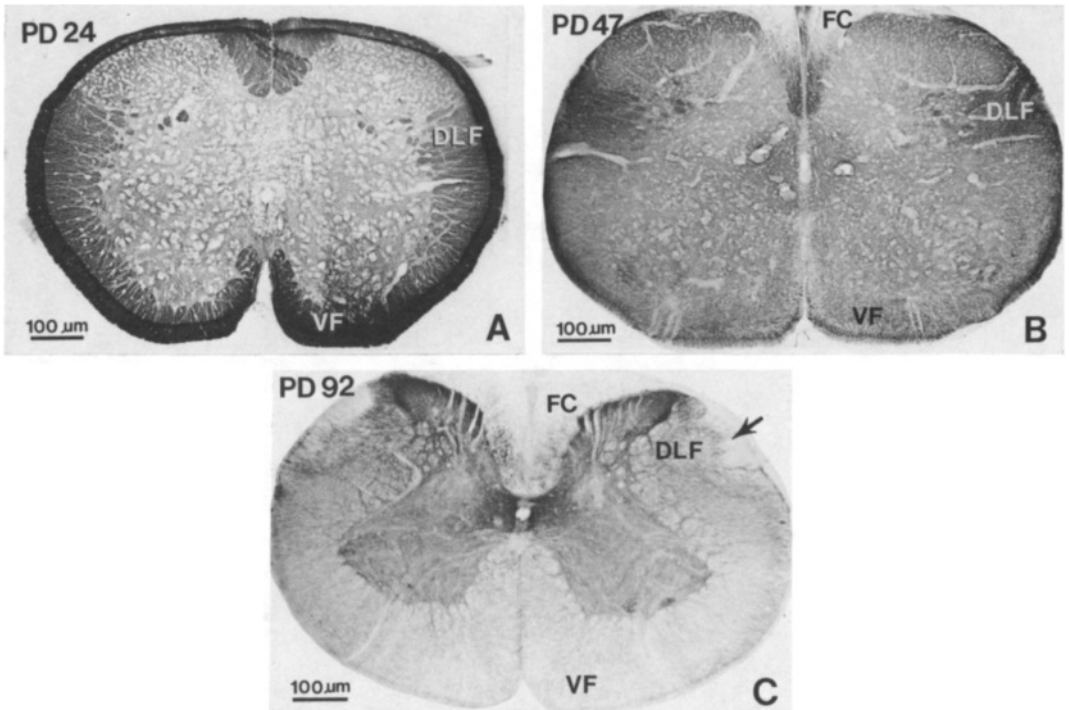

Figure 12. Photomicrographs of GAP-43-like immunoreactivity in the cervical spinal cord of opossums sacrificed at postnatal days (PD) 24, 47, and 92. The dorsal part of the lateral funiculus (DLF), the ventral funiculus (VF), and the fasciculus cuneatus (FC) are indicated. The *arrow* points to the dorsal spinocerebellar tract.

markedly in the ventral funiculus with age (compare Fig. 12, A–C), but it remained relatively high in the dorsal part of the lateral funiculus (Fig. 11C), even in the adult animal. The only nonimmunostained area of the lateral funiculus at later stages of development (Fig. 12C, arrow) and in the adult animal was the dorsal spino-cerebellar tract (Hazlett and Martin, 1971). Evidence for the presence of GAP-43 after the end of the critical period for plasticity has also been reported for corticospinal axons in the rat (Stelzner and Strauss, 1991). One interpretation of these data is that supraspinal neurons continue to synthesize and transport GAP-43 after the critical periods for plasticity and that they still have some potential for regeneration. It is possible, of course, that the presence of GAP-43 reflects synaptogenesis and/or synaptic remodeling and not the ability to support axonal elongation. There is evidence that GAP-43 expression in neurons is negatively regulated by a factor or factors transported from the terminal fields of its axons (Benowitz et al., 1990), so it is conceivable that GAP-43 synthesis and transport do not diminish until synaptic terminals are established. In any case, these observations suggest that diminished growth potential with age is not the only factor in loss of developmental plasticity and that changes in the environment traversed by regenerating and late-growing axons also contribute.

B. Myelin Formation

Schwab and Caroni (1988) have demonstrated that myelin-forming oligodendrocytes and CNS myelin are nonpermissive substrates for neurite growth. Two proteins were isolated from CNS myelin, and when they were neutralized by an antibody specific to them, the inhibitory function of myelin on axonal elongation was diminished (Caroni and Schwab, 1988a,b; Schnell and Schwab, 1990; Kapfhammer et al., 1992). It has also been shown that corticospinal axons exhibit some degree of regeneration if myelin formation is suppressed by x-irradiation (Savio and Schwab, 1990; Schwab and Schnell, 1991). Since myelin of the central nervous system inhibits axonal growth and regeneration, it is possible that some aspect of myelin formation contributes to the end of the critical period for descending spinal plasticity. The proteins in myelin that inhibit axonal growth are present early in development, even before compact myelin can be identified (Caroni and Schwab, 1989). Since myelin basic protein and galactocerebrosides are present at very early stages of myelin formation, it is possible that their appearance coincides with that of the above and/or other inhibitory proteins. We have, therefore, used immunohistochemistry to study the development of myelin basic protein and galactocerebroside-like immunoreactivity in the opossum's spinal cord and asked whether its appearance in areas traversed by normal and rerouted axons of the red nucleus correlates temporally with the end of the critical period for rubrospinal plasticity (Ghooray and Martin, 1993a).

Myelin basic protein (MBP)-like and galactocerebroside (GalC)-like immunoreactivity (LI) appeared in the dorsal part of the lateral funiculus, the area that contains

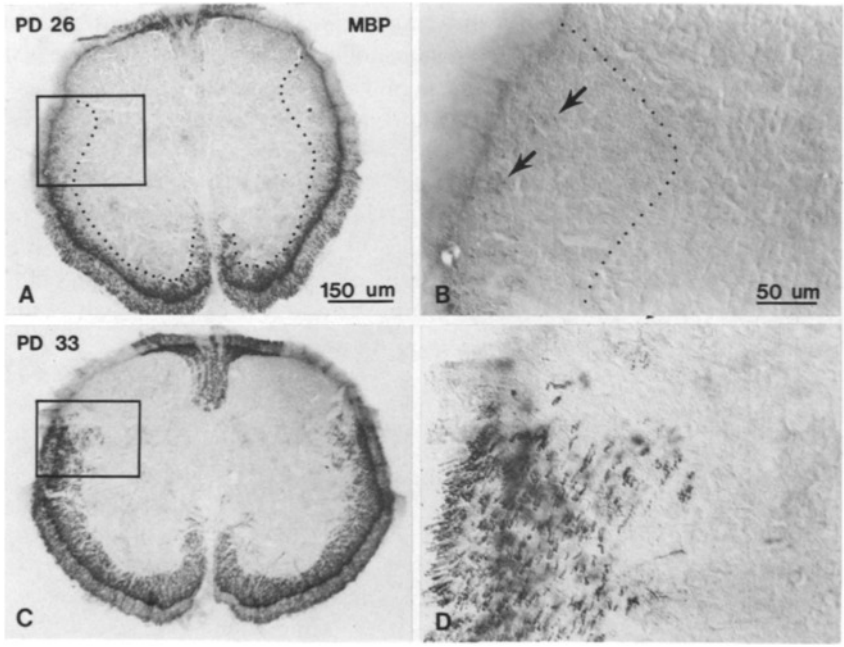

Figure 13. Photomicrograph of sections through the thoracic cord immunostained for myelin basic protein (MBP) at postnatal days (PD) 26 (**A**) and 33 (**C**). The rectangles in **A** and **C** outline the areas shown at higher magnification in **B** and **D**, respectively. The *arrows* in **B** point to a few scattered, lightly immunostained structures. The size indicators in **A** and **B** can be used for **C** and **D**, respectively.

rubrospinal axons, around the end of the critical period for growth of rubral axons around a lesion, and in the dorsal horn, an area traversed by rerouted axons in the plasticity experiments, shortly thereafter. The end of the critical period usually occurs between PD26 and PD30. Figure 13, A and B, documents the initial appearance of MBP-LI in the dorsal part of the lateral funiculus at PD26, and Figure 13, C and D, shows its development by PD33. Although there is not an exact correlation between the end of the critical period and the expression of MBP and GalC, it takes time for rubral axons to grow around the lesion, and that time period is unknown. Opossums have a protracted development, and it is possible that individual variation in the appearance of myelin and/or the end of the critical period for plasticity contribute to the apparent temporal disparity. In any case, our results are consistent with the hypothesis that proteins in myelin that inhibit axonal growth contribute to the end of the critical period for growth of rubrospinal axons around a lesion of their pathway.

MBP-LI was present in the ventral funiculus, the location of vestibulospinal and reticulospinal axons, well before it was found in the dorsal part of the lateral

funiculus, the location of the rubrospinal tract (Fig. 13A), so it is possible that the development of myelin also contributes to loss of vestibulospinal and reticulospinal plasticity. The corticospinal tract is the last descending spinal pathway to show MBP-LI, and axons within it are able to grow around a lesion well after brainstem axons no longer do so. Since the end of the critical period for growth of brainstem axons across a transection of the spinal cord has not yet been identified in *Didelphis*, we do not know whether it correlates temporally with myelin formation. It is of interest, however, that myelin-associated growth inhibitors are present in the spinal cord of the South American opossum, *Monodelphis domestica*, by the end of the critical period for growth of axons across a crush of the spinal cord *in vitro* (Schwab et al., 1993).

C. The Development of Radial Glia and Astrocytes and their Response to Lesioning

Normal Development of Radial Glia and Astrocytes

We studied radial glia and astrocytic development immunohistochemically using antibodies against vimentin and glial fibrillary acidic protein (Ghooray and Martin, 1993b). Vimentin (Vim) is present in radial glia and immature astrocytes (Pixely and de Vellis, 1984; Voigt, 1989; Yanes et al., 1990; Tohyama et al., 1991), whereas glial fibrillary acidic protein (GFAP) is found within radial glia and mature astrocytes (Eng et al., 1971; Bignami and Dahl, 1976; Levitt and Rakic, 1980). Vim tends to disappear with maturity and is replaced by GFAP (Pixely and de Vellis, 1984; Raff et al., 1984). Our developmental results showed that: (1) vimentin-like immunoreactivity (Vim-LI) is present in radial glia of the opossum's spinal cord at birth and it does not disappear completely with maturity; (2) GFAP-like immunoreactivity (GFAP-LI) is present in radial glia of the ventral cervical cord at birth, and its subsequent expression with age follows ventral to dorsal and rostral to caudal gradients; and (3) the transition from radial glia to relatively mature-appearing astrocytes also follows ventral-to-dorsal and rostral-to-caudal gradients.

The transition from immature radial glia to mature-appearing astrocytes was particularly interesting. Figure 14, A and C, documents the presence of radial glia in the lateral funiculus at PD21, that is, during the critical period for growth of rubrospinal axons around a lesion, and Figure 14, B and D, illustrates the presence of mature-appearing astrocytes in the same area by PD30, around the end of the critical period. The transition from radial glia to mature-appearing astrocytes occurred earlier in the ventral funiculus (Fig. 14, E and F), the location of vestibulospinal and reticulospinal axons. Immature glia have been shown to support growing axons (Silver et al., 1982; Silver and Ogawa, 1983), and when they are transplanted into the mature brain, they apparently elicit axonal growth (Smith et al., 1986). Immature glia may support growing and regenerating axons by mechanical guidance (Singer et al., 1979), the production of appropriate extracellular matrix molecules (e.g., laminin, fibronectin and heparin sulfate proteinglycans, Carbon-

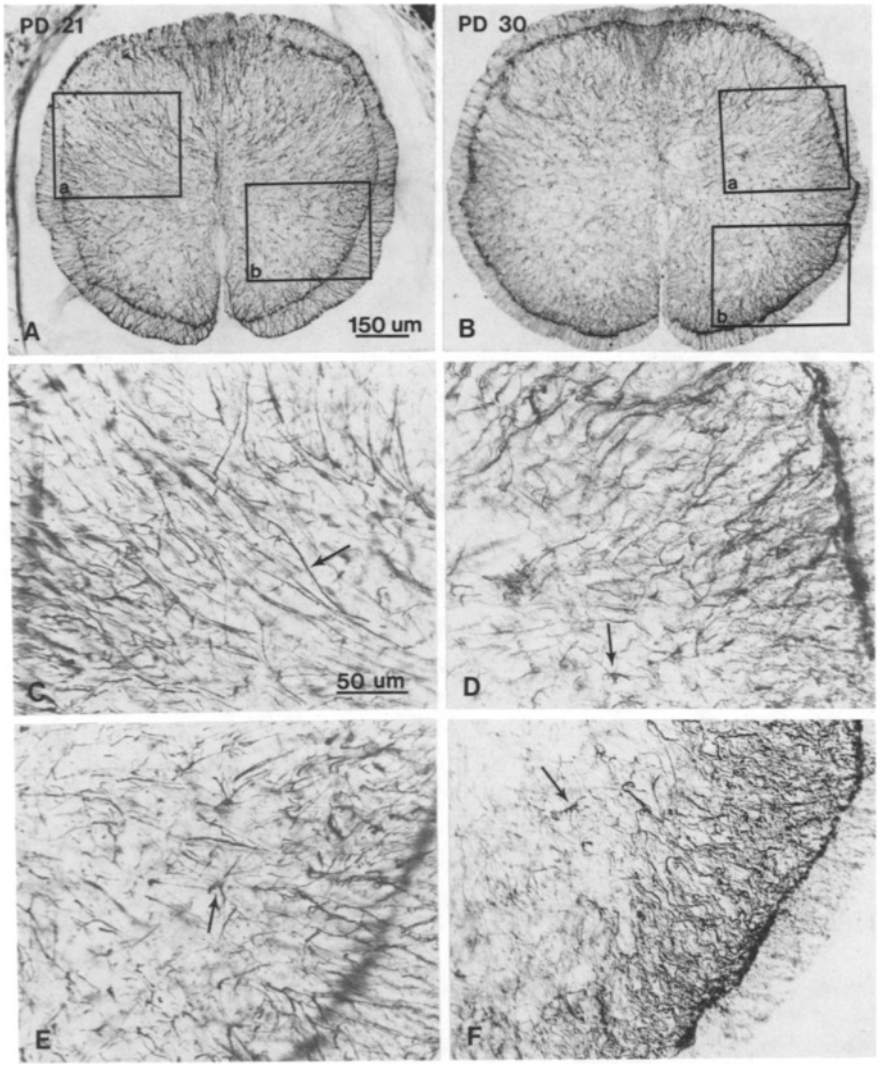

Figure 14. Photomicrographs of sections through the thoracic cord immunostained for glial fibrillary acid protein at postnatal days (PD) 21 (**A**, **C**, and **E**) and 30 (**B**, **D**, and **F**). The area outlined in rectangles **a** and **b** in **A** are shown in **C** and **E** and the areas within **a** and **b** in **C** are shown in **D** and **F**. The *arrow* in **C** indicates the process of a radial glial cell, whereas those in **D–E** point to mature-appearing astrocytes. The size indicator in **A** can be used for **B** and that in **C** can be used for **D–F**. (Modified from Ghooray and Martin, *Glia* **1993b**, 9.)

etto et al. 1983, 1987), or the production of tropic and/or trophic factors (Bregman and Reier, 1986; Haun and Cunningham, 1987; Repka and Cunningham, 1987; Bregman, 1988; Bregman et al., 1989). Loss of one or more of these functions might contribute to the end of the critical period for plasticity of descending spinal pathways.

The Development of an Astrocytic Response to Lesioning

It has been suggested that the development of a glial response to injury inhibits growing or regenerating axons (e.g., Reier et al., 1983). It is possible, therefore, that loss of developmental plasticity in descending spinal pathways results from the development of a glial response to lesioning. This issue was addressed in two series of experiments (Ghooray and Martin, 1993c). In one series, the spinal cord of anesthetized pups was hemisected at midthoracic levels, and the animals were allowed to survive for 2 weeks before being anesthetized again, sacrificed, and perfused for GFAP immunohistochemistry. Two weeks after hemisecting the thoracic cord at PD21, prior to the end of the critical period for growth of rubrospinal axons around a lesion, a relatively mild astrocytic response was observed in the "white" matter. When the lesion was made at PD26, near the end of the critical period (PD26–30), hypertrophied astrocytes were present at the grey/white matter junction and cystic cavitations were observed.

A second series of animals was allowed to survive for 4 weeks after lesioning, the survival time used in the plasticity experiments. In such cases a second operation was performed 4–5 days before sacrifice so that FB could be injected bilaterally two or three segments caudal to the lesion. The intent of the FB injection was to label rubral neurons whose axons had grown around the lesion so that the development of a glial scar and the presence of rubrospinal plasticity could be assessed in the same animal. In such cases, a glial response was seen earlier than in the ones that survived for only 2 weeks. When the lesion was made at PD15, well within the critical period for rubrospinal plasticity, evidence for a glial response was limited to the ventral funiculus and the ventral part of the lateral funiculus. As expected, rubral neurons were labeled contralateral to the lesion, suggesting that they supported axons that had grown around it. When the lesion was made at PD21 (Fig. 15A), still within the critical period for plasticity, the glial response extended into that part of the lateral funiculus occupied by rubral axons (Fig. 15, B–D). In spite of the glial response, rubral neurons were still labeled contralateral to the lesion (Fig. 15E), although they were not as numerous as those labeled ipsilaterally (Fig. 15F). When the lesion was made at PD26, near the end of the critical period, cavitation was present at the lesion site (Fig. 16A), and astrocytes were hypertrophied at the grey/white matter junction (Fig. 16, B–D). In such cases, rubral neurons were not labeled contralateral to the lesion (Fig. 16E), although they were labeled on the ipsilateral side (Fig. 16F). An astrocytic response was not observed in the grey matter of the dorsal horn, an area used by rubral axons to grow around a lesion of their pathway, until well after the end of the critical period.

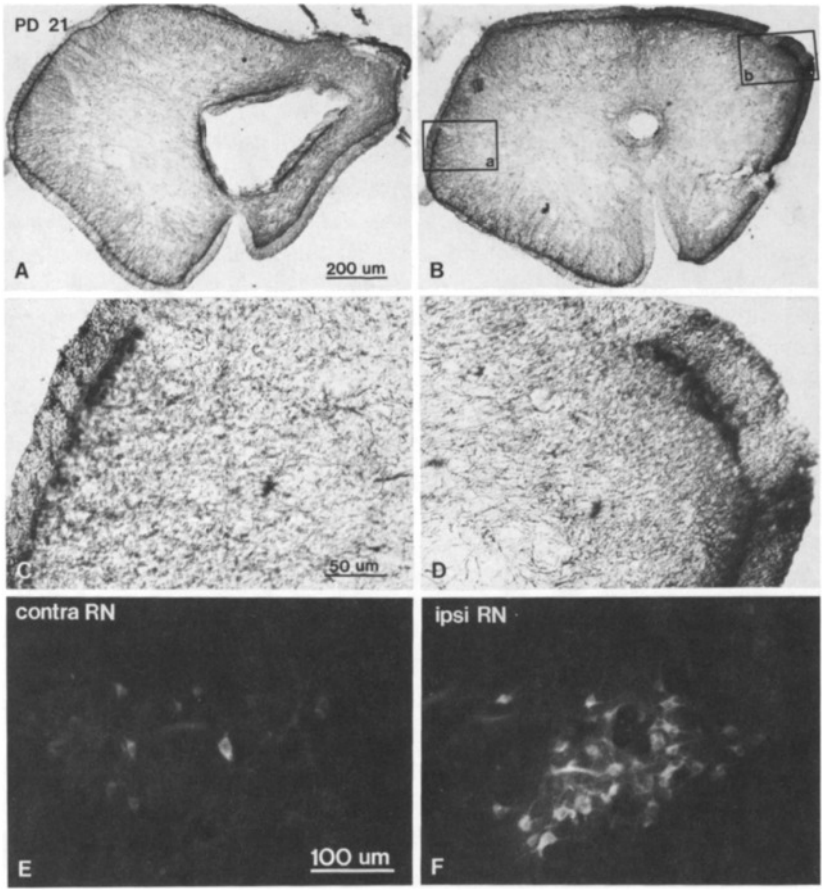

Figure 15. Photomicrographs of sections immunostained for glial fibrillary acid protein through the lesion (**A**) and rostral to it (**B**) from an animal subjected to hemisection of the thoracic cord (right side of **A**) at postnatal day (PD) 21 and sacrificed 30 days later. The areas outlined in rectangles **a** and **b** of **B** are shown in **C** and **D**, respectively. Neurons labeled by bilateral injections of Fast Blue caudal to the lesion prior to sacrifice are shown in fluorescent photomicrographs (excitation wavelength = 360 nm) from the contralateral red nucleus (contra RN) in **E** and the ipsilateral red nucleus (ipsi RN) in **F**. The size indicator in **A** can be used for **B**, that in **C** can be used for **D**, and that in **E** can be used for **F**. Note that neurons are labeled in the red nucleus contralateral to the lesion (**E**), even though a glial reaction is present at the lesion (**A**) and rostral to it (**B** and **D**).(Modified from Ghooray and Martin, *Glia* **1993c**, 9.)

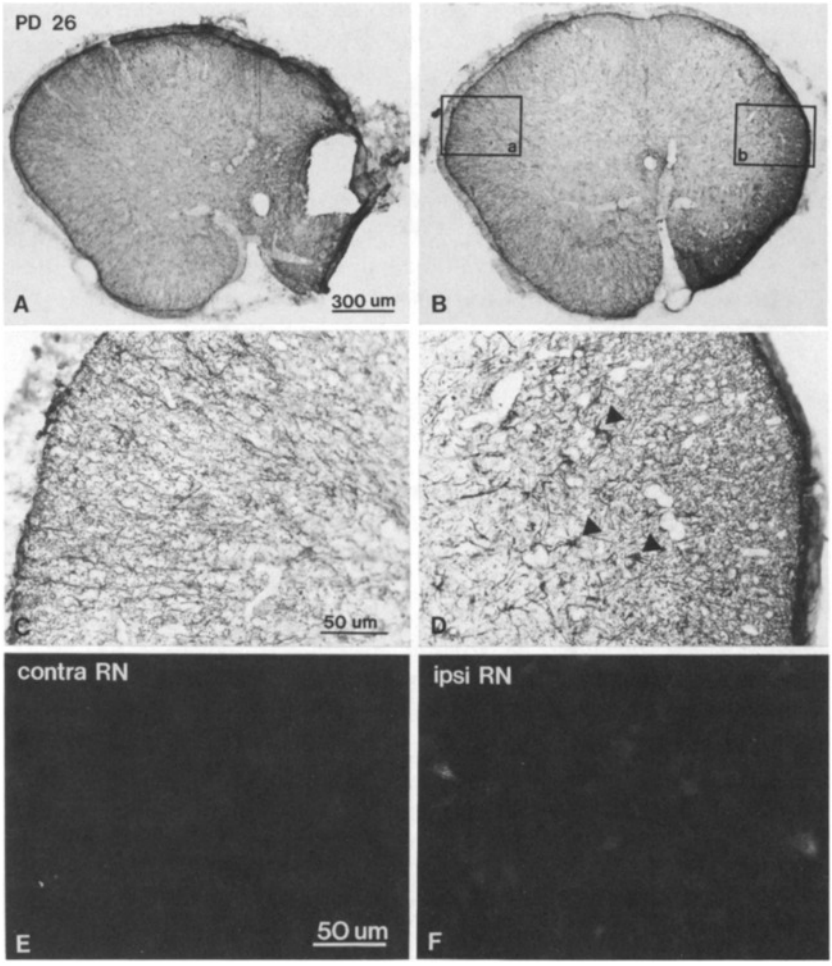

Figure 16. Photomicrographs of sections immunostained for glial fibrillary acidic protein through the lesion (**A**) and rostral to it (**B**) from an animal subjected to hemisection of the thoracic cord at postnatal day (PD) 26 and sacrificed 30 days later. The areas outlined in rectangles **a** and **b** of **B** are shown in **C** and **D**, respectively. Note the presence of a glial reaction at the lesion site (**A**) and rostral to it (**B** and **D**), which includes cysts (**A**) and hypertrophied astrocytes (*arrowheads* in **D**). Fluorescence photomicrographs (excitation wavelength = 360 nm) from the same case (**E** and **F**) show that neurons were not labeled in the red nucleus contralateral to the lesion (contra RN) by bilateral injections of Fast Blue caudal to it, although they were labeled on the ipsilateral side (ipsi RN). The size indicator in **A** can be used for **B**, that in **C** can be used for **D**, and that in **E** can be used for **F**. (Modified from Ghooray and Martin, *Glia* **1993c**, 9.)

Although failure of rubrospinal axons to grow around a lesion did not correlate temporally with the initial appearance of a glial response to lesioning, it did correlate with the development of hypertrophied astrocytes at the grey/white matter junction and the appearance of cystic cavities. The development of cystic cavities suggests that macrophages had invaded the lesion and that the cavity left after phagocytosis had been walled off by astrocytic processes. The effect of this process and the products produced by it on axonal elongation may be important. For example, chondroitin sulfate proteoglycan (SC-PG) is up-regulated in areas of glial scarring in adult mammals, and CS-PG inhibits growing axons (e.g., Mckeon et al., 1991; Pindzola et al., 1993). It may be, therefore, that the ability to produce CS-PG after lesioning is a factor in loss of developmental plasticity.

IV. SUMMARY AND CONCLUSIONS

The results of the experiments reviewed herein show that corticospinal, rubrospinal, vestibulospinal, and reticulospinal axons grow around a lesion of their pathway in developing mammals, although they do not do so in adults. In fact, brainstem axons will grow through a spinal lesion, including a complete transection, if the lesion is made early enough during development. New growth (i.e., growth of late arriving axons that are deflected by the lesion, and/or caudal growth of collaterals from axons that normally terminate rostral to the lesion) appears to be the major contributor to growth around a lesion. Regeneration of cut axons has also been documented, however, and the degree to which it occurs may depend upon the number of neurons that survive axotomy. The number of surviving neurons increases with age, although they no longer regenerate a process around the lesion. The relative contributions of new growth and regeneration to growth through a lesion have not yet been established in mammals.

Our results suggest that descending spinal axons lose their potential for plasticity according to different timetables. Based on comparison of the developmental history of descending spinal pathways with the critical periods for plasticity of those studied to date, we have proposed that axons that grow into the spinal cord first lose their potential for plasticity before axons that arrive at a later date.

If loss of plasticity in different pathways occurs according to the sequence of their growth into the spinal cord, it seems reasonable to suggest that the end of the critical period for plasticity correlates with diminished growth potential with age. It is apparently not that simple, however. Axons are still added to the rubrospinal tract after the end of the critical period for rubrospinal plasticity (Xu and Martin, 1991a), and the results of GAP-43 studies suggest that the rubral axons may still be capable of growth. Although diminished growth potential with age probably contributes to loss of developmental plasticity, it seems likely that changes in the environment encountered by growing axons are also factors. Such changes include the development of myelin and astrocytes and the appearance of a glial response to injury.

In the opossum there is a loose temporal correlation between the development of myelin and loss of descending spinal plasticity, suggesting that myelin proteins that impede axonal growth contribute to the end of plasticity. Direct evidence for a cause-and-effect relationship between myelin formation and loss of developmental plasticity is available in the chick (Keirstead et al., 1992).

Rubral axons are able to grow around a lesion in the face of an immature glial response, but they do not do so when reactive astrocytes can be identified at the grey/white matter interface and cystic cavities are present at the lesion site. A correlation between the development of cystic cavitation and the end of the critical period for corticospinal plasticity has been reported for the rat (Firkins et al., 1993).

Recovery of function and restoration of supraspinal connections also takes place after spinal lesions in developing amphibians (Forehand and Farel, 1982; Beattie et al., 1990) and chicks (Hasan et al., 1990, 1993; Shimizu et al., 1990), and in both groups, regeneration of cut axons occurs. In developing amphibians and chicks, as in developing opossums (Saunders et al., 1992; Treherne et al., 1992; Woodward et al., 1993; and experiments described herein), brainstem axons are able to grow across a complete transection of the spinal cord.

One interpretation of the results reported to date is that the mammalian spinal cord regenerates after lesions at early stages of development and that brainstem axons grow through the lesion. When regeneration of spinal cord tissue no longer occurs, brainstem axons and (depending upon the species and level of the lesion) axons from the cerebral cortex grow around the lesion if they have the necessary potential for growth and an environment that permits it.

Although descending spinal axons are able to grow around and/or through a lesion in developing amphibians, birds, and mammals, there is little evidence for comparable growth of ascending spinal axons. In neonatal rats, dorsal root axons do not grow around a lesion of the fasciculus gracilis at rostral cervical levels (Lahr and Stelzner, 1990), although cortical axons, which also occupy the dorsal columns, do so readily (Bernstein and Stelzner, 1983; Schreyer and Jones, 1983; Bates et al., 1988). Dorsal root axons may be relatively mature in newborn rats, however, since they have already reached the nucleus gracilis (Lahr and Stelzner, 1990). In contrast, cortical axons have just begun to enter the cord (Donatell, 1977; Schreyer and Jones, 1983). Even in developing amphibians, where plasticity might be expected, ascending axons fail to grow across a spinal transection, although descending axons do so readily (Forehand and Farel, 1982; Beattie et al., 1990). We have obtained evidence, however, that axons within the fasciculus gracilis (unpublished results) and dorsal spinocerebellar tract (Terman et al., 1994) will grow through a lesion of their spinal pathway at early stages of development in opossums, suggesting that they have at least some potential for plasticity.

Studies such as those described herein have potential importance for spinal cord injury research. As more is learned about developmental plasticity and the factors that limit it with age, it may be possible to design therapeutic strategies that facilitate axonal regeneration in the injured spinal cord of adult mammals, including humans.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the technical, typing, plotting, and proofing help of Ms. Mary Ann Jarrell and the photographic assistance of Mr. Karl Rubin. We are also grateful to Drs. Barbara Bregman and Dennis Stelzner, who offered comments and criticisms on early drafts. Experimental work by the authors was supported by USPHS grants NS-25095 and NS-10165.

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DEVELOPMENT OF THE MAMMALIAN AUDITORY HINDBRAIN

Frank H. Willard

Dedication. This review is dedicated to the memory of Gopal D. Das. I wish to thank Dr. Das for sharing with me his profound love of brainstem organization and beginning my interests in the subject as a first-year graduate student. I would also like to thank him for suggesting this review and for his encouragement and understanding.

Advances in Neural Science,
Volume 2, pages 205–234.

ISBN: 1-55938-625-8

ABSTRACT

The normal development of language and communicative skills is contingent on normal hearing; it is therefore quite important to understand the events occurring during the ontogeny of the auditory system. Especially critical to our understanding of this process is determining which features of the auditory system are established prior to the onset of hearing and which are developed later, when the system is exposed to stimulus-driven activity. Based on this information, it will be possible to analyze the influence of early environmental activity on auditory system development and determine whether critical periods, dependent on specific acoustic inputs, are present.

As a background, this review begins by examining the cytology and connectivity of the adult mammalian auditory hindbrain, followed by a consideration of the events occurring in the normal development of auditory brainstem connections. It is concluded that major projections between the auditory nerve, cochlear nuclei, superior olives, and inferior colliculi are established early and that their adult form of laterality and topography is also present early in development. Transient expression of aberrant laterality in these connections is not seen between the auditory nerve and cochlear nuclei, or between the primary nucleus and the inferior colliculi.

Despite the early commitment of connections in the hindbrain auditory system, numerous structural features are undergoing development or remodeling during the onset of hearing. In the cochlea, significant changes are occurring in the structure of the basilar membrane and associated support cells. In the central pathways, Golgi Type II cells, or interneurons, are intercalating into the circuitry of the cochlear nucleus, synaptic connections at several levels experience remodeling, and myelination of major fiber tracts is occurring. Not only could these features alter auditory development, they may also be influenced by the acoustic environment.

The review concludes by examining data from studies that manipulated input to the auditory system. It is demonstrated that alteration of the acoustic input, such as occurs in cochlear ablation or meatal ligation, can influence nuclear size, cell number, connectivity, and cell physiology in the hindbrain auditory system. These events are expressed directly, at the level of the cochlear nucleus, as well as transneuronally in the superior olive or inferior colliculus. There are critical periods in auditory system development during which the manipulation has its greatest affect and after which its influence recedes. It is also demonstrated that some of the alterations in connectivity and cellular physiology are due to unbalancing the system, since bilaterally symmetric manipulation does not have the effect experienced with unilateral manipulation. Finally, it is pointed out that subtle manipulation, such as repetitive nonvarying stimuli, can influence the fine features of auditory system development, resulting in aberrant cellular physiology.

Thus a balanced and varied acoustic input appears to be a requisite for the normal functioning of the hindbrain auditory system. The sensitivity of the nascent system to external manipulation has profound implications for the plight of children suffering from early monaural and binaural hearing deficits and may well have an impact on the development of learning disabilities. These observations underline the necessity for further studies of the nature and timing of critical developmental periods in the ontogeny of the auditory system.

I. INTRODUCTION

The normal function of the auditory system is necessary for the appropriate acquisition of language skills in children. Consequently, developmental defects in the processing of acoustic information represent an important cause of childhood learning disabilities (Duane, 1977; Beadle, 1981; Willeford and Burleigh, 1985). A critical feature of normal auditory function is the systematic transduction of the audible frequency spectrum into a neural code using the structural properties of the cochlea (reviewed in Pickles, 1988) and the subsequent maintenance of this code by spatial segregation of neurons and axons into topographical arrays in the auditory brainstem nuclei (Guinan et al., 1972; Morest, 1973; Clopton et al., 1974; Scheibel and Scheibel, 1974; FitzPatrick, 1976; Arnesen and Osen, 1978; Morest and Oliver, 1984). An understanding of the developmental processes influencing the formation of topographic relationships in the auditory pathways and the response of these connections to external perturbations are essential steps toward understanding the congenital and environmentally induced defects affecting auditory system function and communication skills.

This first section of this article briefly reviews the structure and connectivity of the auditory peripheral receptor and the hindbrain auditory nuclei, with particular emphasis on the topographic representation of the end organ in the central pathways of the brainstem. The article summarizes evidence that the topographic order of projections in the auditory system develops early in the ontogeny of the organism, at a time prior to the onset of stimulus-driven activity. It also considers evidence that events occurring after the establishment of topographic order are influenced by environmentally derived, acoustic stimuli and that these events can be altered in their course by manipulating the auditory input.

II. THE MAMMALIAN ORGAN OF CORTI

The mammalian cochlea is a narrow, coiled tubule embedded in the petrous portion of the temporal bone (reviewed in Bast and Anson, 1949; Anson and Donaldson, 1981). This cul-de-sac is lined with several membranes, the innermost of which is the scala media, housing a closed sac of fluid, the endolymph (Dallos, 1984). Along one wall of the scala media lies a delicate structure, the organ of Corti. The base of this organ is mounted on a flexible partition, the basilar membrane, and its apex contacts the tectorial membrane. The basilar membrane is attached on either border to the bony cochlea, whereas the tectorial membrane is anchored to a bony structure only along one of its borders. A differential movement occurs between these membranous structures in response to sound pressure waves in the cochlear fluids. Positioned between these two membranes, the organ of Corti is capable of detecting their slight displacement in response to acoustic stimuli. As a result of a progressive shift in physical properties along the basilar membrane, the location of its maximal displacement to sound is related to the frequencies contained within the stimulus

(reviewed in Pickles, 1988). Low-frequency stimuli maximally displace the apex of the basilar membrane, and high-frequency stimuli maximally displace the base of the membrane.

The organ of Corti, which extends the entire length of the basilar membrane, contains the energy transducer for the auditory system. The transducer converts the mechanical displacement of the membrane into an electrochemical, sensory neural code (Davis, 1965). The stimulus coding is accomplished by several rows of hair cells, which contact the peripheral processes of the auditory nerve fibers. Each hair cell contains a polarized tuft of stereocilia on its apical surface. At the molecular level, the stimulus transducer mechanism involves mechanically operated ion channels located on the tips of the stereocilia, which respond to subtle displacements of the stereocilia along one axis of the hair cell (Pickles and Corey, 1992).

The hair cells are divided into two groups differing in their position on the basilar membrane, physical shape, and pattern of innervation (reviewed in Schuknecht, 1974). The inner hair cells are vase shaped (Smith, 1978; 1981) and form one continuous row located internal to the tunnel of Corti and closest to the modiolus at the center of the cochlea. Outer hair cells are columnar in shape (Smith, 1978; 1981) and arranged in three parallel tiers, all of which are located external to the tunnel, thus closest to the stria vascularis. Both types of hair cells are innervated at their base by the peripheral processes of spiral ganglion cells and efferent terminals of olivocochlear neurons; however, the terminal size and position on the cell base differ markedly between inner and outer hair cells (Spoendlin, 1969; Liberman, 1980; Liberman et al., 1990).

The primary afferent processes contacting the hair cells represent the dendrites of neurons located in the spiral ganglion of the modiolus. The spiral ganglion cells are classified into two categories based on their morphology (Spoendlin, 1975). Approximately 95% of the ganglion cells have large, lightly staining cell bodies surrounded by myelin sheaths; these represent the Type I cells. The remaining 5% have small, darkly staining cell bodies with little or no myelin; these form the Type II cells. Corresponding to the two types of ganglion cells are two types of primary afferent processes (Kiang et al., 1982). Radial fibers, the largest and most plentiful, are the peripheral processes on Type I ganglion cells; they pass directly outward from the ganglion to reach the inner hair cells. Spiral fibers are the peripheral processes of Type II ganglion cells; they pass outward across the tunnel of Corti, then turn and spiral basally along the organ of Corti for approximately 0.6 mm (Spoendlin, 1978).

The duality of the ganglion cell population is reflected in the differential and complicated innervation of the inner and outer hair cells. Although some species variation exists in the exact numbers, each inner hair cell receives numerous (approximately 20) large afferent terminals derived from the radial fibers, whereas the outer hair cells each receive fewer (approximately 10) afferent terminals that arise from the spiral fibers (Schuknecht, 1974; Spoendlin, 1975; Liberman et al., 1990). The absolute number of afferent terminals contacting an inner hair cell

decreases progressively as a function of distance along the base-to-apex axis of the cochlea; conversely, the number of afferent terminals contacting outer hair cells increases from base to apex (Liberman et al., 1990). There also exists a differential positioning of terminals on the hair cell base. On the inner hair cells, the largest of the afferent terminals are located on the strial side,[1] and the smaller of the afferent terminals are positioned on the modiolar face of the cell (Liberman, 1980). Conversely, within a given row of the outer hair cells, the small afferent terminals are confined to the strial side, the modiolar face of the cell being occupied by large efferent terminals (Liberman et al., 1990). Thus, the organ of Corti exhibits a base-to-apex, as well as a modular-to-strial set of gradients in hair cell innervation characteristics.

The central processes of the spiral ganglion cells form the auditory nerve, which passes out of the modiolus and through the internal auditory meatus, to eventually innervate the ipsilateral cochlear nuclei of the brainstem. The organization of axons in the auditory nerve reflects the position of their peripheral processes along the organ of Corti (Lorente de No, 1933; Lewy and Kobrak, 1936). This pattern is most clearly demonstrated in the cat, where the center of the auditory nerve contains fibers arising from the apex of the cochlea and the outer wraps of the nerve contain fibers derived from progressively more basal regions (Arnesen and Osen, 1978). Through this orderly array of projections, a topographic map of the basilar membrane representing the base-to-apex and modular-to-strial axes is transferred to the cochlear nuclei of the brainstem (Leake and Snyder, 1989). This map represents the entire audible frequency spectrum of the species, and as such, is carried through the various auditory nuclei of the brainstem and thalamus, eventually obtaining multiple representations in the temporal cortex (Aitkin et al., 1984; Webster and Garey, 1990). At all levels of the central nervous system the fidelity of the map is preserved through restricted geometric properties of axonal and dendritic domains (see below).

III. THE MAMMALIAN AUDITORY HINDBRAIN

The mammalian cochlear nucleus is partitioned into three major regions based on its cytology, fibroarchitecture, and efferent connections. These divisions are the anteroventral cochlear nucleus (AVCN), the posteroventral cochlear nucleus (PVCN), and the dorsal cochlear nucleus (DCN) (Osen, 1969a,b; Brawer et al., 1974; Willard and Ryugo, 1983; Morest et al., 1990). The auditory nerve enters the cochlear nucleus from its ventral surface and bifurcates forming ascending and descending branches (Lorente de No, 1933; 1981; Osen, 1970; Kane, 1978). Through these branches, the central processes of the auditory nerve are distributed to all three divisions of the cochlear nucleus (Powell and Cowan, 1962; Osen, 1970). A general pattern for distribution of this nerve in mammals can be described. The ascending branches pass anteriorly into the AVCN, and the descending

branches curve posteriorly to course through PVCN. The terminal portion of the posterior branches arch superiorly to enter the DCN.

The cytology of the mammalian cochlear nucleus is well documented in numerous species (Osen, 1969a; Brawer et al., 1974; Moore and Osen, 1979; Disterhoft et al., 1980; Martin, 1981; Perry and Webster, 1981; Zook and Casseday, 1982a; Webster and Trune, 1982; Willard and Ryugo, 1983; Willard and Martin, 1983; Adams, 1986; Morest et al., 1990). Although some variation occurs, in general, cell morphology and cytoarchitecture have remarkable similarities between widely divergent species. At least eight major cell types are identified based on their dendroarchitecture, cytology, and connections: spherical bushy cells, globular bushy cells, stellate cells, pyramidal (principal) cells, large multipolar (giant) cells, octopus cells, cartwheel cells, and granule cells. The stellate cells represent a heterogeneous composite of various sized cells found throughout all three divisions of the cochlear nucleus. Although many of these cell types are present in more than one division of the cochlear nucleus, each division has a unique combination (Brawer et al., 1974).

Typically the AVCN features prominent spherical bushy cells anteriorly and globular bushy cells posteriorly, with a mixture of variously sized stellate cells distributed throughout (Cant and Morest, 1979a,b; Tolbert et al., 1982; Tolbert and Morest, 1982). Scattered giant cells are seen in the dorsal portions of this division. The ascending branches of the auditory nerve form large calycine endings, which surround the spherical cells in AVCN (Held, 1893; Ramon y Cajal, 1909; Brawer and Morest, 1975; Ryugo and Fekete, 1982).

The cytoarchitecture of PVCN is dominated by a central region containing octopus cells surrounded by several smaller areas containing numerous stellate cells (Harrison and Irving, 1966; Kane, 1973). The posterior branches of the auditory nerve are tightly fasciculated as they pass through this division of the cochlear nucleus.

The DCN has a cortical structure composed of several layers of cells and fibers (Ramon y Cajal, 1909; Fuse, 1913; Kane, 1974; Osen and Mugnaini, 1981; Ryugo and Willard, 1985; Schweitzer and Cant, 1985; Blackstad et al., 1984; Willard et al., 1984). The pyramidal or fusiform cells are the principal cell type of this nucleus. Their cell bodies, which are surrounded by granule cells, are arranged in a sheet representing layer II. The apical dendrites of pyramidal cells extend superiorly into layer I, or the molecular layer, where they are surrounded by cartwheel and small stellate neurons; and their basal dendrites descend into layer III, where they are surrounded by stellate and giant cells. Axons of the descending branch enter the inferior surface of the DCN and pass vertically through layers III and II, but stop short of layer I (Cohen et al., 1972; Willard et al., 1984).

Three major efferent pathways connect the cochlear nucleus to the hindbrain auditory nuclei: the ventral acoustic stria (trapezoid body), the intermediate acoustic stria, and the dorsal acoustic stria (reviewed in Tsuchitani, 1978; Aitkin et al., 1984; Helfert et al., 1991). Although the nuances of these connections are beyond

the scope of this chapter, certain patterns provide a background to the developmental studies. The spherical bushy cells and stellate cells of anterior AVCN project axons through the ventral acoustic stria to reach the medial superior olivary nucleus bilaterally (Jois et al., 1990; Casseday et al., 1992). The globular bushy cells of posterior AVCN send their axons through the ventral acoustic stria to reach the contralateral medial nucleus of the trapezoid body (Friauf and Ostwald, 1988; Smith et al., 1991; Kuwabara et al., 1991; Casseday et al., 1992). The axons of octopus cells in PVCN pass through the posterior portion of the intermediate acoustic stria to reach the ipsilateral periolivary nuclei and the contralateral ventral nucleus of the lateral lemniscus (Warr, 1969, 1982). Finally, the pyramidal and giant cells of the DCN project through the dorsal acoustic stria to reach the contralateral inferior colliculus (Adams, 1979; Ryugo et al., 1981; Willard and Ryugo, 1983; Ryugo and Willard, 1985).

The superior olive lies in the ventral portion of the brainstem at the pontomedullary junction. This complex consists of three well-defined nuclei, the lateral (LSO) and medial (MSO) superior olive and the medial nucleus of the trapezoid body (MNTB), and is surrounded by several periolivary nuclei (Ramon y Cajal, 1909; Lorente de No, 1947; Scheibel and Scheibel, 1974). The three main nuclei are present in most species examined to date, but considerable variation is seen in their sizes and in the appearance and nomenclature of the periolivary nuclei (Irving and Harrison, 1967; Moore and Moore, 1971; Ollo and Schwartz, 1979; Adams, 1983).

The cochlear nucleus innervates the superior olivary nuclei in a complex and only partially understood pattern (reviewed in Tsuchitani, 1978; Brugge and Geisler, 1978; Aitkin et al., 1984). The MSO is a diagonal band of cells that have a bipolar and tripolar shape, usually featuring at least two horizontally oriented dendrites (Ollo and Schwartz, 1979; Willard and Ryugo, 1983); the laterally projecting dendrites receive terminals from spherical bushy cells and stellate cells in ipsilateral AVCN, and the medially projecting dendrites receive terminals from spherical bushy cells in the contralateral AVCN (Warr, 1966; Jois et al., 1990; Casseday et al., 1992). The LSO exhibits considerable species variation in its shape, being a solid bar of cells in marsupials (Willard and Martin, 1983) and an elaborate curvilinear sheet of cells in the cat (Scheibel and Scheibel, 1974). This nucleus contains several classes of cells (Helfert and Schwartz, 1986). The predominant type, the planar-shaped principal cells, have dendrites projecting in two opposing directions (Cant, 1984). The laterally projecting dendrites receive terminals from the spherical bushy cells and stellate cells in the ipsilateral cochlear nucleus (Jois et al., 1990; Casseday et al., 1992), and the medially projecting dendrites receive terminals from the ipsilateral medial nucleus of the trapezoid body (Spangler et al., 1985; Zook and DiCaprio, 1988; Kuwabara et al., 1991). The MNTB neurons form a cluster of cells embedded in the fibers of the trapezoid body. The principal neurons of MNTB have prominent spherical somata (Morest, 1968), which receive large end bulbs. These distinctive endings arise from axons of the globular bushy cells in the contralateral cochlear nucleus (Friauf and Ostwald, 1988; Smith et al., 1991;

Kuwabara et al., 1991). As previously mentioned, the axons of MNTB neurons innervate the medially directed dendrites of cells in the LSO of the same side. This provides LSO with an indirect input from the contralateral cochlear nucleus. Thus, neurons in both MSO and LSO receive bilateral innervation arising in the opposing cochlear nuclei and are capable of responding to binaural stimuli. The convergent mapping of this binaural information is critical to its processing.

Like the auditory nerve and cochlear nuclei, the nuclei of the superior olive contain an orderly array of elements. The neurons in the medial and lateral nuclei of the superior olive are organized in a tonotopic fashion, reflecting a map of the frequency spectrum along the basilar membrane (Guinan et al., 1972). The connections between the AVCN and superior olivary complex (Cant and Casseday, 1986; Zook and Leake, 1989) and the projections between the MNTB and LSO (Spangler et al., 1985) are arranged in a topographic order. The precise alignment of these multiple topographic arrays within the superior olivary nuclei is a necessary element for the comparison of binaural stimuli (Sanes and Rubel, 1988).

Ascending projections from all of the pontomedullary auditory nuclei converge on the inferior colliculus (IC) of the midbrain (Woollard and Harpman, 1940; Moore and Goldberg, 1963; Carey and Webster, 1971; Osen, 1972; Beyerl, 1978; Adams, 1979; Kudo and Nimi, 1980; Ryugo et al., 1981; Aitkin and Kenyon, 1981; Brunso-Bechtold et al., 1981; Schweitzer, 1981; Zook and Casseday, 1982b, 1987; Willard and Martin, 1983; Willard and Ryugo, 1983; Hashikawa and Kawamura, 1983; Nordeen et al., 1983; Coleman and Clerici, 1987; Moore, 1988; Kudo et al., 1990; Oliver and Shneiderman, 1991). A specific laterality dominates the organization of these midbrain projections. In general, ascending fibers arise contralaterally in the cochlear nucleus, ipsilaterally in the ventral nucleus of the lateral lemniscus, and bilaterally in the superior olivary nuclei and dorsal nucleus of the lateral lemniscus. The degree of bilaterality in the midbrain projections from the nuclei of the superior olive varies between species. The cat (Adams, 1979), bat (Zook and Casseday, 1987), mouse (Willard and Ryugo, 1983), and rat (Beyerl, 1978) have a predominantly ipsilateral projection from MSO and a contralateral projection from LSO; whereas the opossum (Willard and Martin, 1984) and mole (Kudo et al., 1990) have a strong bilateral projection from the MSO to the midbrain.

The IC consists of a prominent central nucleus containing a laminar arrangement of cells and fibers; this nucleus is covered by a cortical structure on its dorsal and lateral borders (Morest, 1964; Genieic and Morest, 1971; Rockel and Jones, 1973a; Zook and Casseday, 1982a; Willard and Ryugo, 1983; Willard and Martin, 1983; Morest and Oliver, 1984; Oliver and Morest, 1984; Faye-Lund and Osen, 1985; Meininger et al., 1986; Oliver and Shneiderman, 1991). The orderly projection of hindbrain auditory nuclei into the IC creates a topographic representation of the basilar membrane in the fibrodendritic laminae of the central nucleus (Massopust and Ordy, 1962; Rose et al., 1963; Aitkin et al., 1972, 1975; Clopton and Winfield, 1973; Merzenich and Reid, 1974; FitzPatrick, 1976; Roth et al., 1978; Adams, 1979; Semple and Aitkin, 1979; Ryugo et al., 1981; Webster et al., 1984; Shneiderman

and Henkel, 1987; Oliver and Shneiderman, 1991). Based on the convergence of this complex array of afferent projections from the pontomedullary auditory nuclei, the inferior colliculus is thought to assemble an auditory map of space, each colliculus representing the contralateral hemisphere of acoustic space for use in reflex auditory functions (reviewed in Aitkin et al., 1984; Caird, 1991).

Thus, at all levels of the central auditory pathways, the topographic relationships representing position along the basilar membrane serve to maintain the critical features for processing auditory information. When do these maps develop in the auditory system, how do they develop, and what are the factors that influence their maturation? These questions represent the major issues addressed in the remainder of this review.

II. EARLY DEVELOPMENT OF AUDITORY HINDBRAIN CONNECTIVITY

As the neural tube forms, a small placode of cells develops lateral to the rhombencephalon (reviewed in Streeter, 1906; Bast and Anson, 1949; Willard, 1990). The placode invaginates to form the otic vesicle. The ventral surface of this vesicle gives rise to the cochlear duct. This duct grows in a spiral, creating a series of coils. The coils surround the center of the cochlea, called the *modiolus*, which is occupied by spiral ganglion cells. The number of coils per cochlea shows considerable variation between species; the guinea pig has 4.5 turns (Thorn et al., 1979), the human has 2.75 turns (Bast and Anson, 1949), and the mouse (Lim and Anniko, 1985) and the marsupial (*Monodelphis*) (Willard and Munger, manuscript in preparation) have 1.75 turns.

Growth of the spirals and growth of volume in the cochlea are two separate events (Willard, 1992). The South American (Brazilian) opossum, *Monodelphis domestica*, a species that does not respond to sound until 34 days after birth, is born with a cochlear duct with three-fourths of a turn (Willard and Munger, 1988). The duct reaches the adult number of turns (1.75) around postnatal day 8 (PD8). During this first phase of growth the absolute volume of the duct remains relatively constant. Between PD8 and PD20, the duct passes through its second phase of growth, in which it dramatically increases in size. During this phase, the volumetric growth curve for the cochlea duct is sigmoidal; halfway along its steepest phase (PD16), osteogenesis begins in the walls of the duct and future growth of the cochlea in *Monodelphis* is then curtailed (Willard, 1992).

Maturation of the cochlear epithelium occurs in a sequence of stages that have been examined in numerous species (Streeter, 1906; Van der Stricht, 1918; Wada, 1923; Anson, 1934; Larsell et al., 1935, 1944; Bast and Anson, 1949; Altmann, 1950; Shute, 1951; Belanger, 1956; O'Rahilly, 1963; Bredberg, 1967; Nakai and Hilding, 1968; Pujol and Marty, 1970; Sher, 1971; Hinojosa, 1977; Anson and Donaldson, 1981; Pujol, 1985; Burda, 1985; Willard and Munger, 1988). The first indication of sensory receptor development is the formation of the organ of Kolliker

along the sensory side of the duct. Kolliker's organ differentiates into greater and lesser epithelial ridges, with the greater epithelial ridge positioned closest to the modiolus of the cochlea (Wada, 1923; Hinojosa, 1977; Willard and Munger, manuscript in preparation). Development of the inner hair cells occurs from the greater ridge, whereas the outer hair cells arise from the lesser ridge (Wada, 1923; Lim and Anniko, 1985; Willard and Munger, manuscript in preparation). The primary afferent processes of spiral ganglion cells grow through the cochlear epithelium to contact the bases of the inner hair cells. Subsequently, they extend under the nascent tunnel of Corti, to reach the outer hair cells.

Each of the maturation stages of the cochlea sweeps along the length of the basilar membrane forming sequential waves of differentiation from base to apex (reviewed in Rubel, 1978; Willard and Munger, manuscript in preparation). Growth is initiated by a bud of mitotic cells at the apex of the developing cochlea (Willard, 1992). This initial wave of cell genesis is followed by a base-to-apex wave of cell growth to form the organ of Kolliker, a wave of differentiation of the inner then outer hair cells and, subsequently, a wave of innervation. These two concepts, the sequence of maturational stages and their base-to-apex movement along the basilar membrane, represent principles of cochlear development characteristic of all mammals examined to date (Rubel, 1978; Willard and Munger, manuscript in preparation).

The lateral wall of the otocyst gives rise to the spiral ganglion cells. Initially, a funnel-shaped mass of cells leaves the duct wall; however, the distal tail of the funnel retains its connection with the duct (Carney and Silver, 1983). As these cells migrate out of the growing duct, they accumulate in the center of its spiral, forming the modiolus. Even in the earliest stages of otocyst development, cytoplasmic processes are seen connecting the otocyst and the migrating ganglion cells (Carney and Silver, 1983; Willard and Munger, 1988). This suggests that ganglion cells migrate by a form of perikaryal translocation (as described in the central nervous system by Morest, 1969a), with each ganglion cell maintaining contact with the otocyst by its peripheral processes.

During the early stages of development in the cochlea, related events are occurring in the brainstem. At the time of ingrowth of the auditory nerve axons and their maturation, many neurons destined for the presumptive cochlear nucleus (and thus called *precochlear neurons*) are still in the process of migration. The origin for some of these precochlear cells appears to be a cytogenetic zone along the sulcus limitans at the pontomedullary junction (Willard and Martin, 1986b). As the axons of these precochlear cells begin growing toward the superior olive and midbrain, their cell bodies migrate toward the cochlear nucleus over the presumptive dorsal acoustic stria. An inflection point (or right-angle bend) at the departure site for the growing process and cell body is left in the axons of the adult dorsal acoustic stria (Willard et al., 1983). At maturity, the precochlear neurons originating from this cytogenetic zone represent the largest cells in the cochlear nucleus: the pyramidal and giant cells of DCN and the large multipolar neurons of the VCN (Willard and Martin, 1986b).

In all mammals examined (including marsupials), the auditory nerve has grown into the brainstem by birth but is not completely distributed to the three divisions of the cochlear nucleus (Schweitzer and Cant, 1984; Willard, 1990). In the opossum, the entrance of auditory axons into the brainstem coincides with the lateral migration of precochlear neurons into the nucleus (Willard and Martin, 1986b; Willard, 1990). Some of the auditory nerve fibers reach the ependymal surface of the medulla before the precochlear cells occupy the locus for the presumptive DCN. This suggests that some auditory nerve fibers are in a position awaiting the arrival of neurons destined for the cochlear nucleus. It is possible that the auditory nerve fibers offer cues used by the migrating precochlear neurons to determine the final position of their cell bodies. However, in hamsters, the auditory nerve fibers enter DCN postnatally, after many of its neurons are already present (Schweitzer and Cant, 1984); thus species variation in this mechanism is possible.

The adult morphology of auditory nerve axons is established at an early point in development, even before the DCN has reached its mature dimensions. This has been demonstrated using HRP to label the auditory nerve in an age-graded series of animals (Schweitzer and Cecil, 1992). Following an initial growth in length as the auditory nerve axons invaded the nucleus (PD0–9), there is little change in their morphology in prehearing (PD10–16) or posthearing (after PD16) animals. However, during this time (PD10–30), the volume of the DCN doubles in size; consequently, the area influenced by each auditory nerve axon diminishes. These data suggest that auditory nerve axons quickly assume adult-like distribution in the nucleus and maintain this organization as the nucleus continues to mature.

Although the general distribution of auditory nerve fibers changes little, its synaptic features undergo remodeling during maturation of both the central and peripheral processes. In the periphery, the inner hair cells are initially contacted by both afferent and efferent endings (reviewed in Pujol, 1985). Gradually, the ratio between these endings shifts to favor afferent contacts, with the inner hair cells and the efferent contacts relocated to the afferent terminals. Conversely, the initial synaptic contacts are made by afferent terminals on outer hair cells. Subsequently, these afferent terminals are removed from the outer hair cells and replaced by large efferent terminals. All of this remodeling occurs in the time preceding and during the onset of stimulus-driven activity in the cochlea. The central process of the spiral ganglion cells is also undergoing a progressive change in morphology subsequent to their initial contacts with their target cells. Beginning as large spoon-shaped endings, the end bulbs in AVCN undergo a sequence of structural alterations to form long, thin processes characteristic of the adult bulb (Ryugo and Fekete, 1982); these events occur in the first 20 days of postnatal life. The large spoon shape of the end bulb early in development restricts the number of primary and nonprimary afferent fibers contacting a single spherical cell in the cochlear nucleus. Consequently, as the shape matures, additional room becomes available for other inputs on the cell. This protracted period of morphological development in the cochlear nucleus

coincides with the late developing phase-locking properties of neurons in the cochlear nucleus, suggesting a correlation in structure and function (Kitzes, 1990).

The ascending projections of the auditory hindbrain are also established early in the development of the organism. The sequence of development for the ascending projections to the midbrain has been examined in the opossum (Willard and Martin, 1987) and the rat (Kandler and Friauf, 1991). Using the retrograde transport of HRP, the presence of ascending midbrain connections can be detected in the opossum at PD5 (approximately equivalent to a gestational day 12–14 in the mouse or rat). Using the transport of fluorescent dyes, Friauf and Kandler (1990) labeled projections to the rat midbrain from the auditory hindbrain nuclei at birth. In both species, the ascending projections develop in parallel rather than in series. The superior olivary nuclei of the opossum project to the midbrain at a stage slightly preceding those of the ventral cochlear nucleus, which project to the midbrain a few days later (Willard and Martin, 1987). A striking feature of these studies is that the laterality of these early projections reflects the laterality of projections in the adult animal. No transient projections are observed in the development of ascending relationships between the hindbrain auditory nuclei and the inferior colliculus (Willard and Martin, 1987; Kandler and Friauf, 1991). Based on these observations, deafferentation studies that have unmasked abnormal projections (Nordeen et al., 1983; Moore and Kitzes, 1985; Moore and Kowalchuk, 1988; Kil et al., 1989) most likely are *not* the result of preserving transient connections that otherwise would have been lost.

V. PATTERN FORMATION IN THE AUDITORY PROJECTIONS OF THE HINDBRAIN

Adult auditory nerve fibers form a pattern of narrow fascicles across the cochlear nucleus in a topographic representation of the basilar membrane (Leake and Snyder, 1989). This arrangement of the auditory nerve in the cochlear nucleus is detectable very early in the development of the opossum (Willard, 1991). HRP has been placed in the cochlea of *Monodelphis* to label the central processes of the auditory nerve in an age-graded series of animals. In the youngest animals examined (PD6), auditory nerve axons are grouped in fascicles across the cochlear nucleus. At PD8, individual narrow bands of labeled fibers are present across the presumptive cochlear nucleus. When multiple turns of the cochlea are involved in the placement of HRP, multiple bands of labeled fibers are observed in the nucleus, each band separated by an interband interval. These bands are interpreted as the projections from portions of specific turns in the cochlea, and the interband interval represents the unlabeled portion of the turn between the injection sites. Very little scatter of HRP granules is seen along the sides of the bands, suggesting that the restricted axonal projections underlying the narrow isofrequency contours of the adult nucleus are established early in development (Willard, 1991). This orderly projec-

tion to the cochlear nucleus on PD8 occurs at a time well before the first indication of a functional auditory system at PD34.

The early development of spatial order in the rat auditory nerve projections into the cochlear nucleus has also been described (Angulo et al., 1990). On embryonic day 14, fibers from the basal turn of the cochlea bifurcate in the dorsomedial portion of AVCN, and those from the apical turn grow into the more lateral portions of the nucleus. The development of this topographic order also long precedes the onset of function in the rat, which occurs around postnatal days 8–10 (Crowley and Hepp-Raymond, 1966). The precision present in the auditory nerve projections as demonstrated in the opossum and rat are in agreement with the observation that the morphology of auditory nerve axons is achieved early in development, with little change in their distribution occurring as the nucleus matures (Schweitzer and Cecil, 1992).

The central nucleus of the cat inferior colliculus contains a tonotopic distribution of units at the ontogeny of hearing, as detected in physiological studies (Aitkin and Moore, 1975). This observation suggests that ascending projections to the midbrain have formed in their adult-like pattern by this time in development. Anatomical studies have also demonstrated the presence of topographic order in the ascending projections to the midbrain at an early stage in development in at least two species. Narrow sheets of neurons in cochlear nucleus projecting to restricted fibrodendritic laminae in the central nucleus are present in opossums on PD25–33 (Willard and Martin, 1987).[2] The early development of topographic connections to the midbrain has also been noted in the rat (Friauf et al., 1991). The oncogene c-*FOS* can be used as a marker of neuronal activity in young (PD14) animals that have been exposed to narrow-band frequency stimulation. This technique reveals a topographic series of isofrequency bands in place in the auditory pathways as they first begin to function[3]. The fact that these events have occurred prior to or during the onset of hearing suggests that the mapping functions of the brainstem auditory pathways are initially established through mechanico-spatial properties of growth rather than by acoustically driven cues.

A topographic order in the representation of characteristic frequencies is also present in the superior olive at the onset of auditory function (Sanes and Rubel, 1988). Neurons in the LSO are excited by stimuli in the ipsilateral cochlea and inhibited by stimuli in the contralateral cochlea. These binaural response properties are accomplished by the arrangement of projections from the contralateral cochlear nucleus through the MNTB (see previous description). The registration of these two maps (excitation and inhibition) in the adult opens the possibility of examining their relationships during development. Sanes and Rubel (1988) reported that these two cochleotopic maps are present at the onset of auditory function and that their maturation occurs simultaneously in the gerbil. The characteristic frequencies of excitation and inhibition for a given neuron are highly correlated in the adult gerbil (0.99). Although the two maps in the younger animals are not as precisely correlated as the adult, it is still remarkably close (0.69, as cited in Kitzes, 1990). This level

of correlation in the alignment of two maps arising from opposite sides of the brainstem requires rather precise registration of axonal projections, even at the onset of function (reviewed in Kitzes, 1990).

Thus, the development of topographic connections in the auditory brainstem occurs early in ontogeny, preceding the onset of stimulus-driven function. The early structural development of auditory nerve axons (Schweitzer and Cecil, 1992) and the early presence of their ordered distribution (Willard, 1991) strongly support this contention. Although the order in these projections seems well established early in ontogeny, maturation of other factors, as evidenced in the changing physiological properties, has been reported during the onset of hearing. Narrowing of the frequency ranges over which auditory neurons respond (sharpening of tuning curves), the expansion of the range of stimulus intensity over which neurons will respond (increasing dynamic range), and maturation of the ability to follow the phase of the stimulus (phase locking) have all been observed in the cochlear nucleus, superior olive, and inferior colliculus at various stages of development (reviewed by Brugge, 1983; Kitzes, 1990). Not all of these events can be accounted for by an extended maturation period for the cochlea. For example, neurons activated by approximately the same region of the basilar membrane, based on their characteristic frequencies, have a considerable degree of variation in the maturation of other electrophysiological properties such as their response areas and tuning curve shapes (Sanes and Rubel, 1988). This inconsistency between neurons related to similar points on the basilar membrane would not be expected if cochlear maturation is the sole rate-limiting factor in the development of functional properties in the central system. Thus, structural changes in the central auditory circuits, as well as those occurring in the cochlea, can potentially influence the maturation of the electrophysiological properties in the central auditory system. The structural changes occurring in the maturing auditory system and the response of this system to manipulation of input are the subjects of the remainder of this review.

VI. CYTOLOGICAL DIFFERENTIATION ACCOMPANYING THE ONSET OF STIMULUS-DRIVEN FUNCTION IN THE AUDITORY SYSTEM

Numerous factors involving the maturation of the cochlea are described as "rate limiting" in the development of hearing. Larsell et al. (1944) raise the possibility that support cell maturation is crucial for the onset of hearing. Pujol and Hilding (1973) suggest that the onset of hearing is related to the opening of the tunnel of Corti, since in the cat, rat, and mouse these events are closely related in time. Pujol et al. (1978) also suggest that the separate time course for innervation of inner and outer hair cells influences the progressive refinement of hearing functions in cats. Specifically, the maturation of outer hair cell innervation has been identified as influencing the onset of hearing (Pujol, 1985). The connections of the hair cells with the tectorial membrane (Kraus and Aulbach-Kraus, 1981), the internal struc-

ture of the hair cell and its membrane properties, innervation of the hair cells, and development and myelination of ganglion cells and their processes are also postulated as "rate limiting" factors in the inception of hearing (Romand et al., 1987; Brugge, 1988). Although the organ of Corti is well developed and its tunnel is open before the onset of hearing in opossums, significant structural changes in the relationship between the inner hair cells and their immediate support cells, the phalangeal cells, as well as structural changes in the basilar membrane and its tympanic epithelium, continue to occur during this time (Willard and Munger, manuscript in preparation). The cells investing the base of the inner hair cell during the period PD30–35 undergo a compression in shape as the cells of Claudius expand greatly in size along the basilar membrane of the opossum cochlea. Thus, differentiation of support cells and innervation patterns in the organ of Corti, along with changes in the physical properties of the basilar membrane, accompany the onset of hearing in most species and could influence the timing and electrophysiological properties of this event.

Along with structural changes in the cochlea, maturational events are occurring in the central nervous system during the early development of auditory functions. Differentiation of neurons in all regions of the auditory hindbrain begins long before and continues through the onset of stimulus-driven behavior in mammals. However, neuronal maturation is a complex process with differing populations of neurons developing along separate timetables. In general, Golgi Type I neurons achieve some of their adult characteristics in dendroarchitecture and axonal distribution by the onset of auditory function (Morest, 1969a,b; Schweitzer and Cant, 1985). Where they have been examined, neuronal processes at least arrive at their adult targets prior to the onset of stimulus-driven activity in the auditory system (Morest, 1969a,b; Willard and Martin, 1986b). In the DCN of the opossum, the large cells that project to the midbrain achieve their adult-like, vertical orientation by the onset of hearing; whereas the small neurons, stellate and cartwheel neurons, are still developing at this time (Willard and Martin, 1986a).

Despite their early development, Golgi Type I cells exhibit subtle changes during the period surrounding the onset of stimulus-driven function. The apical dendritic tree of pyramidal cells in the hamster DCN continues to develop its planar shape as much as 25 days after birth (Schweitzer and Cant, 1985)[4]. A classic description of the elaborate axodendritic interactions that occur during development of the medial nucleus of the trapezoid body of the cat is provided by Morest (1969a). During the 30 days between birth and onset of hearing in the ferret, the principal neurons of the medial superior olivary nucleus develop a planar-shape dendritic arbor and align in a stacked array (Henkel and Brunso-Bechtold, 1990). This period also features the transient appearance of appendages on the dendrites and somata of MSO neurons and the growth of tertiary dendritic tufts at about the onset of hearing (PD–32). Remodeling of projections from the cochlear nucleus to the contralateral MNTB is seen in the early development of the gerbil and mouse (Kuwabara et al., 1991). The axons from globular bushy cells in the cochlear

nucleus contain two calycine endings in the contralateral MNTB of these young animals. These calycine endings reach separate but closely related neurons in MNTB; as the animals increase in age the relationship between calycine pairs becomes closer, and eventually one terminal is eliminated, since double calycine endings are a rarity in the adult animal (Kuwabara et al., 1991). Remodeling of projections from MNTB cells on their postsynaptic targets in the ipsilateral LSO of the gerbil has also been reported (Sanes and Siverls, 1991). A significant reduction in the area of influence for these axons and a reduction in the number of synaptic boutons carried by individual MNTB axons in LSO occurs during the third week of life[5]. Finally, the shape, orientation, and structure of the dendritic tree of neurons in the central nucleus of the rat inferior colliculus undergo extensive changes during the first 20 days of life (Dardennes et al., 1984). Although Golgi Type I cells have achieved their adult positions and their projections are well ordered, it is possible that the subtle remodeling of synaptic arrangements between these cells and their target neurons accounts for some of the evolving features in the electrophysiological maturation of central auditory neurons, especially around the onset of hearing. Other sources for this maturation process may be found in the Golgi Type II cells.

The genesis and differentiation of Golgi Type II neurons (local circuit type of neuron) generally are late events (Morest, 1969b; Altman and Bayer, 1980) and are still occurring with the onset of auditory functions (Willard and Martin, 1986a). In the dorsal cochlear nucleus, the Golgi Type II neurons differentiate from the neuroepithelium of the rhombic lip just prior to and during the onset of acoustically driven behavior. Formation of their dendroarchitecture and development of their dendritic spines is still occurring as the animal begins to respond to acoustic stimuli (Willard and Martin, 1986a; Willard and Martin, manuscript in preparation). These features of neuronal differentiation for both Golgi Type I and II cells, occurring around the time of the onset of hearing, suggest that they could be responsive to stimulus-related modifications.

One of the last steps in the development of the nervous system is the deposition of myelin sheaths around the processes and cell bodies of neurons. With the completion of this process, axonal transmission reaches its maximal conduction velocity representing the mature state. Most studies of myelination and auditory system development have used human subjects (reviewed by Willard, 1990). Summary graphs of myelination in the human brain are presented by Lemire et al. (1975), as calculated from the data of Yakovlev and LeCours (1967). It appears that the onset of myelination and the time course for completion are in synchrony with the ontogeny and maturation of hearing in the human brain, as monitored by acoustic reflexes (Birnholz and Benacerraf, 1983) and auditory evoked potentials (Zimmerman et al., 1987).

Thus, several key events are occurring in the mammalian auditory pathways at the time of the onset of hearing. Physical changes occur in the structure of the basilar membrane and the cells supporting the sensory transducers; these changes most

likely influence the maturation of many functional parameters seen in the auditory nerve, cochlear nucleus, and other portions of the auditory system (reviewed in Brugge, 1983; Walsh and McGee, 1986). Although the major central auditory projections are in place and their topographic order established, many subtle details of connectivity and myelination in the auditory system, including the intercalation of Golgi Type II neurons into the central pathways, have yet to be completed at the onset of hearing in most mammals. These changes in the synaptic arrangement of local circuit neurons, as well as myelination, could also be important sources of environmentally induced alteration of function in the auditory system (reviewed in Willard, 1990).

Thus, continued maturation of structures at the level of the cochlea and within the central auditory neural circuits is occurring at the time of hearing onset in mammals. The response of these processes to manipulation of the auditory input represents a form of neural plasticity. How much plasticity is present in the auditory pathways and the time at which it can be expressed are significant issues in developmental auditory neurobiology.

VII. MANIPULATION STUDIES

The response of the auditory brainstem to various forms of manipulated primary input has received much interest in the past decade. At the end of the 1970s, very little was known regarding the plasticity of the auditory system; however, the situation has altered with numerous publications in the 1980s. Much of the work involving the manipulation of auditory input in development has used the chick as a model species and has been reviewed recently by Rubel and Parks (1988). The current review focuses on mammalian studies, summarizing the general principles that govern the response of the auditory system to manipulation. The changes in the cochlear nucleus subsequent to manipulation of the cochlea or auditory nerve will also be examined, followed by a review of alterations that occur at more rostral levels in the auditory system consequent to manipulation of the primary input.

The series of morphological and physiological events that occur in the development of the mammalian auditory system are reasonably consistent from species to species (Pujol and Uziel, 1988). Most importantly, when interspecies variation does occur, it is the absolute value of the time between these events that differs and not the order of events in the sequence. Studies on auditory deafferentation or deprivation seek to determine the plasticity of these developmental events. Such studies are dependent on at least three factors: (1) the point in the developmental sequence at the time of manipulation, (2) the type and extent of manipulation performed, and (3) the postmanipulative survival time. The first and third factors also have to be considered with respect to a fourth variable, that is, the time of hearing onset for the species in question. Since these variables have not been treated consistently in all studies, it is often difficult to compare results. As a caveat, it should be kept in mind that the point within this progression at which birth occurs is not consistent

between species (Pujol and Uziel, 1988), and consequently, the birth date is not a good reference for interspecies comparison of structural or physiological events in auditory system development.

Manipulation of auditory input to the brainstem leads to a reduction in the volume of the cochlear nucleus ipsilateral to the manipulated cochlea. Cochlear removal in the mouse on PD6 produces a loss in volume of the ipsilateral cochlear nucleus; the greatest loss is seen in the deep layer of the DCN and in the AVCN (Trune, 1982a). Sound deprivation by ligature of the external auditory meatus on PD10 in the rat also produces a significant reduction in cochlear nucleus volume; the effect decreases when deprivation occurs at later ages (Coleman et al., 1982). Unlike that reported in the mouse, the effect of manipulating auditory input is greater in the VCN than the DCN of the rat. Cochlear lesions on PD12 through PD24 of the ferret also result in reduction of volume in the ipsilateral cochlear nucleus, and the effect of the lesion is greater in the VCN. The volume reduction correlates inversely with the number of surviving ganglion cells (Moore and Kowalchuk, 1988). The reduction in volume goes beyond a simple loss in mass due to atrophy of the central processes of the auditory nerve, since it also occurs, albeit to a lesser degree, with deprivation studies (Coleman et al., 1982). Therefore, the volume of the cochlear nucleus appears to be sensitive to both the presence of the primary afferent fibers and to their stimulus-driven activity.

Manipulation of auditory nerve input also leads to a reduction in the average size and number of specific neurons in the ipsilateral cochlear nucleus. Ablation of the cochlea or deprivation of sound in neonatal (PD3) mice results in a decrease in the long axis of globular cell somata in the ipsilateral AVCN and principal cell somata in the contralateral MNTB by 45 days of development (Webster and Webster, 1977). Other cells in the cochlear nucleus size did not appear to be affected by this manipulation. Removal of the external auditory meatus in mice on PND-3 results in a decrement of soma size in the spherical and globular cells of AVCN by 45 days of development (Trune and Morgan, 1988a). This loss involves diminished cytoplasm in the neuron, yet individual neurons retain their normal nuclear size. However, in an earlier study, the cochlea was removed from mice at PD6, and despite a 34% reduction in the average number of cells in the cochlear nucleus by 45 days of development, there is no detectable reduction in size of neurons in the globular and small spherical cell region (Trune, 1982a). One source of discrepancy in these studies may be the differing ages of the mice at the time of manipulation, the fact that regional and cytological distinctions in the AVCN of the mouse are subtle, and the possible existence of a gradation in cell size between large and small spherical cells (Willard and Ryugo, 1983). Sound deprivation by ligature of the external auditory meatus in rats initiated on PD10 reduces significantly the size of large spherical cells in the cochlear nucleus ipsilateral to the manipulated input after 70 days of survival (Coleman and O'Connor, 1979; Coleman, 1981). Removal of the cochlea in ferrets on PD5 produces a 50% reduction in the number of large neurons in the AVCN by 90 days of survival (Moore, 1990). Similarly, removal of

the gerbil cochlea on PD7 results in a 58% reduction in neuron number in the ipsilateral cochlear nucleus by 9 weeks (63 days) after the surgery (Hashisaki and Rubel, 1989). In the first 2 days after manipulation, cell soma size is reduced by 35%; this progresses to a 58% reduction in size by 2 weeks. These results have been expanded to demonstrate that simply blocking neural activity is sufficient to induce a decrement in soma size. Exposure of the cochlea in gerbils at 4–6 weeks of age to tetrodotoxin, a nerve conduction blocker, results in a 21% decrease in soma size for the large spherical cells in the AVCN (Pasic and Rubel, 1989). A two-stage process emerges when these data are considered along with data demonstrating a loss in volume of the cochlear nucleus consequent to ipsilateral denervation of the auditory input. First, immediately after the insult, a loss of volume in the cochlear nucleus occurs presumably due to the functional deprivation of input; this volume reduction involves a loss in cell size. Second, in denervation studies, this initial loss is followed by an additional volume reduction occurring over a longer time course. The volume loss in the second phase includes the atrophy and loss of the central processes of the auditory nerve.

Altered dendritic morphology, synaptic arrangement, and cellular function of the neurons in the cochlear nucleus is seen following manipulation of ipsilateral auditory nerve input. Removal of the cochlea in mice on PD6 results in a decrease in the cross-sectional area of the dendritic domain of the AVCN bushy cells and a decrease in the length of dendritic domains for the AVCN stellate cells (Trune, 1982b). Surprisingly, no alteration in the length of the large pyramidal neurons in DCN is detected, yet the size of the deep layer in DCN diminishes considerably (Trune, 1982a). Removal of the external auditory meatus in mice on PD3 results in several alterations in cellular structure and function in the ipsilateral cochlear nucleus. The cytoplasm of the large spherical cells of the AVCN exhibits a reduction in mitochondrial number and a dispersion of rough endoplasmic reticulum (Trune and Morgan, 1988b). Concomitant with these changes, the synthesis of protein is diminished in the cochlear nucleus ipsilateral to the manipulated cochlea (Trune and Kiessling, 1988). Finally, a decrease in primary afferent terminals on AVCN spherical cells is reported, with the extra space being occupied by terminals of presumed nonauditory nerve origin (Trune and Morgan, 1988b). These findings are provocative; however, additional studies are necessary to ascertain the structural and biochemical events underlying the alterations in nuclear and neuronal volume changes.

The impact of auditory nerve deprivation also has an age-related component. Denervation has its most profound effects when it is performed in younger animals; however, changes, albeit less dramatic, can be seen when the procedure is performed in adults. A critical period for influencing cochlear nucleus volume is seen for denervation in the ferret (Moore, 1990) and the gerbil (Hashisaki and Rubel, 1989). Removal of the ferret cochlea on or before PD5 results in a 50% loss of neurons from the cochlear nucleus, whereas removal after this date has a minimal effect on cell number (Moore, 1990). Removal of the gerbil cochlea in animals on

PD7 results in a 58% loss in neuronal size over a 9-week period; however, similar manipulations at 8 and 20 weeks of age did not result in a large reduction in neuronal size (Hashisaki and Rubel, 1989). Both of these studies support the concept of a developmental window that represents a critical period for auditory nerve influence on cochlear nucleus ontogeny. After this time, the window closes and subsequent manipulation of the primary afferent fibers does not have as significant an effect on the growth of central auditory neurons.

Denervation of the auditory brainstem can result in transneuronal changes in the morphology of neurons in the superior olivary nuclei. Dendritic morphology of the MSO is altered by early postnatal deprivation. Normally the horizontal dendrites of these cells receive a binaural input, each dendrite being exposed to input from the cochlear nucleus toward which it is pointed (see previous discussion). Loss of primary input to one cochlear nucleus results in a shift in cell population in the MSO such that dendrites pointed toward the manipulated side are shorter (Feng and Rogowski, 1980). Adult deprivation also has transneuronal effects on the superior olivary complex, particularly the MNTB. Following cochlear ablation in adult cats, gliosis is seen only in the vicinity of the central distribution of the auditory nerve; however, cell shrinkage is present in the AVCN, LSO and contralateral MNTB (Powell and Erulkar, 1962). Fifteen days after cochlear ablation, the calycine endings in the contralateral MNTB showed neurofilamentous hyperplasia, with diminished numbers of mitochondria and synaptic vesicles (Jean-Baptiste and Morest, 1975). After 30 days the principal neurons receiving these calycine endings eventually shrink, dropping to 70% of their original size by 7 months post-lesion. The calycine endings in MNTB arise from axons of the globular bushy cells in the AVCN; these cells are only deafferentiated and not physically damaged in the cochlear ablations. Studies illustrating transneuronal atrophy as well as those reporting a loss in cell volume in the cochlear nucleus consequent to meatal ligature (Coleman et al., 1982) or primary afferent conduction blockade (Pasic and Rubel, 1989) support the concept that stimulus-driven neural activity is critical to the maintenance of cellular morphology in the auditory brainstem.

Morphological changes also occur at the level of the midbrain following deafferentation of the auditory brainstem. Ascending projections from the cochlear nucleus are altered by deprivation of auditory input. Removal of the right cochlea on PD6 in mice[6] reduces the number of fusiform and globular cells in the right cochlear nucleus projecting to the left (contralateral) inferior colliculus when examined 45 days later. However, in this study, additional projections to the left inferior colliculus from the left cochlear nucleus (nonmanipulated side) are not seen (Trune, 1983). Thus, an ipsilateral cochlear–midbrain projection did not appear to be enhanced; this negative finding could be a product of the age of the animal at the time of manipulation, as later studies have demonstrated. Ablation of the left cochlea in neonatal (approximately PD1) gerbils[7] did result in an increase in the number of cochlear nucleus neurons on the right, projecting to the right (ipsilateral) inferior colliculus when examined 4–12 months postlesion (Nordeen et al., 1983).

This compensatory increase in ipsilateral midbrain projections from the nonlesioned side is not seen when lesions are done in adult animals. The modification of ipsilateral midbrain projections in the gerbil has been verified by examining the axonal fields in the midbrain after cochlear ablations in animals on PD2 (Moore and Kitzes, 1985). Normally the contralateral midbrain projection has decidedly the heaviest distribution of axons from the cochlear nucleus; following cochlear ablation, this shifts to a more bilaterally balanced distribution for the projections of the cochlear nucleus from the nonlesioned side. A similar event has been reported for the ferret[8]; cochlear ablation on the right side on PD14 to PD24 results in an increased number of neurons projecting from the left cochlear nucleus to the left (ipsilateral) inferior colliculus (Moore and Kowalchuk, 1988). This abnormal projection is not present in animals having a short (>30 days) postlesion survival time. The expression of ipsilateral projections from the cochlear nucleus to the midbrain has a strong age-dependent nature; it is present in ferrets lesioned between postnatal days 14 and 24, but it is not seen in ferrets lesioned on PD90. Interestingly, bilateral removal of the ferret cochlea on PD21 did not affect the absolute number or laterality of cochlear nucleus projections to the midbrain (Moore, 1990). These studies suggest that the midbrain projections from the cochlear nucleus are sensitive to input from the cochlea and that a bilateral balance, based in auditory input, is maintained between these two projections during a critical phase of development. Removal of one cochlea during the appropriate stage upsets this balance, whereas bilateral and approximately equal manipulations can maintain the balance in the projection. The source of these enhanced projections is most likely sprouting, since investigations into the early development of the ascending projections to the midbrain have not found transient bilateral projections (Willard and Martin, 1987; Friauf and Kandler, 1990). In addition, the expression of the aberrant projections requires a postlesion survival time in excess of 30 days for ferrets, suggesting that a growth process is under way (Moore and Kowalchuk, 1988). A postlesion survival time of 6–14 days is necessary for the expression of aberrant connections in gerbils following ablation of the cochlea (Kil et al., 1989). Having considered the existence of specific critical periods for the expression of plasticity in the cochlear-midbrain projection, it is evident that the older age of the animal at the time of manipulation (with respect to the onset of hearing) and the shortened postlesion survival period could explain the lack of a projection to the ipsilateral IC in the previously mentioned study using mice (Trune, 1983).

The manipulation studies of the auditory brainstem demonstrate a reduction in nuclear volume, cell number, and size, and an altered connectivity resulting from a decrease in stimulus-driven input to the system. Just as it was possible to dissociate the reduction in the volume of the cochlear nuclear from the reduction in cellular volume (Hashisaki and Rubel, 1989; Pasic and Rubel, 1989), it is also possible to isolate the plasticity in connections from the reduction in nuclear volume. Plugging the right ears of ferrets at 24–34 days of age produces a conductive hearing loss in the right ear featuring elevated hearing thresholds demonstrable following unplug-

ging of the ears 3–15 months later. On histological examination 24–60 hours following unplugging of the ears, the overall volume of cochlear nucleus and the numbers of neurons present in the AVCN did not appear to change when compared to the unmanipulated side; however, the numbers of cells in the left cochlear nucleus cells with projections to the left (ipsilateral) midbrain are significantly increased (Moore et al., 1989). In essence, an alteration in connectivity occurred without a concomitant alteration in cell size or cell density. Thus, the mechanism fostering plasticity in the midbrain projections of cochlear nucleus cells is not the same mechanism as that producing a reduction in cell number and cell size in the cochlear nucleus in response to diminished auditory input.

The transneuronal changes following ablation of one cochlea alter the physiological characteristics of auditory processing in the nuclei rostral to the cochlear nucleus. In general, units in the inferior colliculus contralateral to the manipulation become more responsive to stimuli presented to their ipsilateral (nonmanipulated) cochlea. This is demonstrated by decreased sensitivity thresholds and wide dynamic ranges in response to stimuli presented to the remaining cochlea. Ablation of the gerbil left cochlea on PD2 significantly altered the physiological properties of units in the right inferior colliculus when the right cochlea was stimulated (Kitzes, 1984; Kitzes and Semple, 1985). Units in the right inferior colliculus demonstrate an increase in excitatory response to ipsilateral (monaural) stimuli. Thus, their response properties more closely resembled those achieved when the contralateral cochlea is stimulated in a normal gerbil. The adaptability of neurons in the IC may arise from the increased number of ipsilateral midbrain projecting neurons found in the cochlear nucleus opposite the ablation (Nordeen et al., 1983; Moore and Kitzes, 1985).

Monaural deprivation also alters binaural processing in the IC. One form of binaural processing in the IC features facilitation of neuronal activity by stimuli presented to the contralateral ear and suppression when presented to the ipsilateral ear. Ligature of the external auditory meatus in rats on PD10 results in an increased suppression of activity in the IC ipsilateral to the unoperated ear (Silverman and Clopton, 1977). This result was interrupted as a loss of facilitation from the manipulated contralateral cochlea. While this may seem contradictory to the data from Kitzes (1984) demonstrating an increase in excitation in the ipsilateral IC, comparisons are difficult since one group used monaural stimuli and cochlea ablation (Kitzes, 1990) and the other used binaural stimuli and meatal ligation (Silverman and Clopton, 1977), as discussed in Kitzes (1990). However, an additional and more salient difference exists between the paradigms used in these two studies; the gerbils had been manipulated at PD2 (10–14 days prior to the onset of hearing), at a time when they are known to be capable of growing new connections from the cochlear nucleus on the unoperated side to the ipsilateral IC (Nordeen et al., 1983). Conversely, the rat pups had been manipulated at a later date in the relative development of the auditory system (PD10, 1–2 days prior to the onset of hearing) and at a time when manipulations in mice (which have a somewhat

similar relative time frame for auditory development) do not produce aberrant ipsilateral midbrain projections (Trune, 1983).

Bilateral manipulations of the cochlea did not have the effect that unilateral manipulation exhibited (Clopton and Silverman, 1977). Bilateral ligations of the external auditory meatus did not alter the balance of facilitation and suppression present in midbrain neurons. These data resemble observations made with regard to the plasticity of midbrain-projecting neurons in the cochlear nucleus. Bilateral removal of the cochleas from ferrets did not alter the numbers or laterality of midbrain-projecting neurons in the cochlear nucleus (Moore, 1990). These results strongly suggest that a competition is established in the laterality of connections in the auditory hindbrain and that the ratio of ipsilateral to contralateral input to the midbrain is balanced by activity in the auditory nerve. Unilateral manipulations distort this balance; whereas, bilaterally symmetric manipulations do not disturb the major ascending projections.

Not just any bilateral input will produce normal development in the auditory midbrain; abnormal acoustic stimuli presented to animals with unmanipulated ears can alter the subtle details of neuronal organization in the IC. Rearing mice with binaural hearing in the presence of repetitive clicks results in a broadening of the tuning characteristics of midbrain neurons but does not alter their spontaneous firing rates or response latencies (Sanes and Constantine-Paton, 1985). Although the clicks contained a wide range of frequencies normally heard by a mouse, their repetitive nature constitutes an abnormal timing sequence. The widening of the tuning curves in response to this stimulus is hypothesized to result from a retention of inappropriate excitatory synaptic connections. Despite the loss in precision of the tuning curves, characteristic frequencies for neurons could be determined and a normal tonotopic distribution of cells is intact (Sanes and Constantine-Paton, 1985). Thus, bilateral stimuli can maintain an appropriate balance of input to the midbrain, but it appears that some normal variation in the input is needed to obtain the subtle details of the normal synaptic arrangement.

In general, the results from the studies cited in this section demonstrate the importance of a balanced and varied acoustic environment to the development of the auditory system and suggests that critical time periods exist for the formation of synaptic connections. Studies are need to examine the extent to which this system can recover from exposure to manipulated input.

VIII. SUMMARY

This review has examined the organization and development of the auditory brainstem in mammals. It has emphasized the specificity in laterality of connections in these pathways along with the precision in topographic representation of the basilar membrane seen at multiple levels of the system. It has also considered the system's normal development and has summarized the response of the auditory brainstem to peripheral manipulation during maturation.

The fundamental organization of the auditory system and its topography are established early in development, well before the onset of stimulus-driven activity. The end-organ experiences early growth and development to accommodate the ossification of the petrous portion of the temporal bone. The auditory nerve quickly grows into the brainstem and establishes its adult-like morphology and adult-like spatial order. The ascending pathways from the cochlear nucleus to the midbrain develop their mature laterality seemingly without forming transient projections to contralateral structures. Within the nuclei of the auditory brainstem, the dendroarchitecture of the Golgi Type I (projection) neurons is evident prior to the onset of stimulus-driven activity.

Despite this early commitment in the structure of the auditory pathways, numerous events are still occurring as the system becomes receptive to acoustic stimuli. At the level of the end organ, the support structures of the organ of Corti are still maturing as the organ begins responding to external stimuli. Within the cochlear nucleus, differentiation of Golgi Type II neurons is still ongoing. The establishment of synaptic morphology between central auditory neurons is occurring, especially as these local neurons circuits integrate into the projection system. Myelination of central axons continues long after the system has become receptive to external stimuli. All of these processes could play a role in the response of the auditory system to peripheral insult around the time of the onset of hearing.

Manipulation of unilateral input to the auditory system can profoundly influence structure and function. At the level of the primary afferent fibers, these effects include the volume of the cochlear nucleus, the size and number of neurons within the nucleus, and their projections to the inferior colliculus. Transneuronal effects extend to the superior olivary nuclei and inferior colliculus, influencing cytology and physiology. Manipulation of bilateral input to the auditory system also has an influence on the development of central neural circuits. An age-related factor is present in the plasticity of the auditory system, and a critical period exists beyond which manipulations are not as effective.

Multiple mechanisms are involved in the response of the auditory system to stimulus manipulation. These mechanisms can be dissociated into separate events, each responding to a different degree with various manipulations. The picture emerges of a very complex system with multiple cytological processes operating contiguously during development. Internuclear projections and their ordered spatial relationships are established without stimulus-driven activity, most likely based on cues inherent in existing brainstem components. A balance in connectivity in the auditory pathways arises by the onset of hearing that maintains the established internuclear projections, but this could also play a role in the later development of local circuit or intranuclear connections. Refinement in the physiological properties of central auditory neurons is a product of a progressively maturing input from the cochlea, as well as developmental events in the central circuits.

The sensitivity of the auditory system to external manipulation, discussed in this review, has profound implications for the plight of children suffering from early

monaural and binaural hearing deficits and may well have an impact on the development of learning disabilities. Unfortunately, not much is known about the postlesion recovery of structural and functional integrity in the auditory system. However, during the past decade the groundwork has been laid for formulating basic research into this area.

NOTES

1. The "strial side" refers to the side containing the stria vascularis; this side of the organ of Corti is furthest from the center of the cochlea, the modiolus.
2. The onset of function in these animals has been estimated at PD50 (Larsell et al., 1944).
3. The onset of stimulus-driven auditory activity is between PD11 in the rat (Carlier et al., 1979; Uziel et al., 1981).
4. Initiation of hearing in the hamster occurs around 16 days after birth (Schweitzer, 1987).
5. Hearing begins between postnatal days 14 and 16 in the gerbil (Kitzes, 1984).
6. Mice begin hearing at 10 to 12 days postnatal.
7. Gerbils begin hearing 14–16 days postnatal (Kitzes, 1984).
8. Ferrets begin hearing at 32 days postnatal.

ACKNOWLEDGMENTS

I would like to thank Drs. Laura Schweitzer, Linda Ross, and John Zook for their critical and very helpful reading of the unfinished manuscript. I would also like to thank Dr. David Moore for helpful discussions of specific issues raised in this review.

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