PHYSIOLOGICAL REVIEWS
Vol. 80, No. 2, April 2000
Printed in U.S.A.

Development of Specific Connectivity Between Premotor Neurons and Motoneurons in the Brain Stem and Spinal Cord

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Glover, Joel C. Development of Specific Connectivity Between Premotor Neurons and Motoneurons in the Brain Stem and Spinal Cord. *Physiol. Rev.* 80: 615–647, 2000.—Astounding progress has been made during the past decade in understanding the general principles governing the development of the nervous system. An area of prime physiological interest that is being elucidated is how the neural circuitry that governs movement is established. The concerted application of molecular biological, anatomical, and electrophysiological techniques to this problem is yielding gratifying insight into how motoneuron, interneuron, and sensory neuron identities are determined, how these different neuron types establish specific axonal projections, and how they recognize and synapse upon each other in patterns that enable the nervous system to exercise precise control over skeletal musculature. This review is an attempt to convey to the physiologist some of the exciting discoveries that have been made, within a context that is intended to link molecular mechanism to behavioral realization. The focus is restricted to the development of monosynaptic connections onto skeletal motoneurons. Principal topics include the inductive mechanisms that pattern the placement and differentiation of motoneurons, Ia sensory afferents, and premotor interneurons; the molecular guidance mechanisms that pattern the projection of premotor axons in the brain stem and spinal cord; and the precision with which initial synaptic connections onto motoneurons are established, with emphasis on the relative roles played by cellular recognition versus electrical activity. It is hoped that this review will provide a guide to understanding both the existing literature and the advances that await this rapidly developing topic.

I. INTRODUCTION

Sherrington made no bones about the role of muscle (66): "The skeletal muscles are the motor machinery for all the life of the animal which the older physiologists were wont to call the 'life of external relation.' Of the importance of that life of external relation the moralist has written that even in man the crown of life is an action, not a thought. Should we demur to this distinction, we can still endorse the old adage that to move things is all that mankind can do, and that for such the sole executant is muscle, whether in whispering a syllable or in felling a forest."

Obviously, that man and other animals can activate ensembles of muscles in exquisitely coordinated patterns, either in smooth sequences or fractionated steps, indi-

cates a highly specific neural mechanism for selectively activating the motoneurons that drive muscle. What may be less obvious is how early the selective activation of motoneurons arises during the development of the animal. The motor precocity of many species, whose newborn possess an impressive repertoire of skills immediately upon or within hours of birth, is foreshadowed by a remarkable degree of motor coordination within the egg or womb. Even less precocious neonates, such as the human infant, exhibit an array of reflexive and willed behaviors that are clearly choreographed before birth. Indeed, embryos move, and their movements are far from haphazard. How is the neural substrate for coordinated movement constructed in the embryo?

Neuroembryologists have been interested in this question for decades. Although we are still far from a complete answer, recent research on several fronts, from the molecular to the behavioral, has illuminated many features that are likely to be classified in the future as common principles of motor development. These provide the motivation for and the subject of this review. My aim here is to give the physiologist access to a comprehensive body of literature and to convey the excitement of recent developments, without overwhelming with a plethora of details.

A. Scope of the Review

The review is intended to be digested at one relaxed sitting, perhaps along with a glass or two of wine. It is therefore necessary to restrict its content to a few manageable courses. I have selected three. The first covers the establishment of functional identity, focusing on the mechanisms that determine when and where particular neuron types arise. The second covers the pathways on which neurons may extend their axons, and which ultimately provide and restrict their access to potential synaptic targets. The third covers the actual establishment of connectivity, specifically its dependence on axon pathway choice, target selection, timing, and neural activity.

For didactic reasons, I have focused on the somatic motoneurons and a selection of premotor and sensory inputs known or presumed to have a monosynaptic relationship to the motoneurons. The somatic motoneuron has been a preferred subject of inquiry into the development of synaptic specificity for many years. There is now compelling experimental evidence in a range of species from flies to birds that motoneurons establish appropriate connections with their target muscles from the outset (18, 76, 166). In vertebrates, this is accomplished by a predetermination of the motoneurons to recognize particular pathways in the periphery, such that their axons are directed unerringly to a particular muscle (76). In the realm of synaptic specificity, these features make the somatic motoneuron the gold standard by which other neuron types may be compared. What about their inputs? Because the motoneurons are predetermined, their monosynaptic inputs must somehow recognize the appropriate motoneurons if they are to engender motor circuitry with the capacity to generate specific movements. It is therefore of great interest to elucidate whether premotor neurons are also predetermined and whether they unerringly establish appropriate connections. Polysynaptic inputs and modulatory inputs to motoneurons cannot be addressed in the same context and are not considered in this review.

It is impossible to cover results obtained from all vertebrate species in which motor development has been studied. Instead, I have selected studies that best exemplify particular phenomena, regardless of species. At the same time I try to indicate generality by selected reference to other studies (and other species) that support, oppose, or complicate the featured finding or interpretation. Among the most commonly studied species, and those which receive the most attention here, are the zebrafish (*Danio*), the African clawed toad (*Xenopus*), the bullfrog (*Rana*), the chicken (*Gallus*), the oppossum (*Monodelphis* and *Didelphis*), the mouse (*Mus*), and the rat (*Rattus*).

Anamniote vertebrates (agnathans, fish, and amphibians) contain two distinct sets of neurons: a set of primary neurons and a set of secondary neurons. The primary neurons (generated first during development) are few in number and in many cases can be individually identified and characterized by a unique pattern of synaptic connectivity. They appear to be a specific evolutionary adaptation to the larval life phase of anamniotes. The secondary neuron population, generated later, is larger and is organized more along the lines of the amniote vertebrates. In amniotes (reptiles, birds, and mammals), neurons exist predominantly as functionally identifiable populations or pools whose constituent neurons share the same basic pattern of synaptic connectivity. Thus, depending on the species and the stage of embryonic development, the review deals both with individual patterns of connectivity, that is, connections from single identifiable presynaptic neurons onto single identifiable motoneurons, as well as regional patterns of connectivity, that is, connections from populations of presynaptic neurons onto populations of motoneurons.

The events described occur during embryogenesis. A general synopsis of embryogenetic events is beyond the scope of this review. Although the accompanying figures provide a workable mental image of what is going on, readers unfamiliar with the processes of gastrulation and neurulation and with such terms as neural plate, neural tube, notochord, and neural crest, may benefit from a quick perusal of the relevant sections of an embryology text.

II. ESTABLISHMENT OF FUNCTIONAL IDENTITY

The somatic motoneurons provide a classic example of topographic organization in the nervous system. The motoneurons that innervate a given limb muscle are clustered in a coherent pool within the lateral motor column of the spinal cord. Each motoneuron pool is largely segregated from other motoneuron pools that innervate other limb muscles. Thus the somatic motoneurons of vertebrates to a large extent can be identified functionally on the basis of their positions within the spinal cord and hindbrain, because these positions are systematically related to their peripheral axon trajectories and target muscles. Of particular importance from a developmental perspective is that the axons from different motoneuron pools project along their appropriate trajectories and innervate their appropriate muscles from the outset, indicating that already before axon outgrowth begins motoneurons are in some way or another predetermined to seek out particular targets (reviewed in Ref. 76). How this predetermination might occur is a major focus of this section.

Topographic organization is also found in the central pathways of motor systems. The somatotopic organization of primary motor cortex reflects the systematic mapping of corticospinal inputs onto motoneurons, such that the spatial relationship among peripheral muscles is represented on the cortical surface (see Ref. 280). Similar though courser somatotopic relationships have been reported for the spinal projections from the red nucleus and the lateral vestibular nucleus (263). Vestibulo-ocular neurons are clustered into coherent pools, each of which innervates a specific set of extraocular motoneurons (111, 112). Within the frog spinal cord, there is evidence that interneurons controlling limb movements are organized into longitudinal columns whose differential stimulation drives the limb to distinct positions (25, 105). Even the polymorphous tongue is innervated by separate pools of protruder and retractor motoneurons, each of which receives input from specific, segregated pools of premotor interneurons (56). At the level of both the motoneurons and the premotor interneurons, the motor system of vertebrates is to a remarkable extent segregated into functionally identifiable neuron pools occupying specific positions.

How are these identities determined? How is a neuron at a specific position within the nervous system directed to innervate another neuron at another specific position, in a characteristic spatial pattern that is recognizable in all individuals of the same species, and, in some cases, across species? Before I address these questions within the arena of the vertebrate motor systems, it may be useful to describe briefly how cellular identity in general is thought to be determined during embryonic development.

A. Factors That Contribute to the Determination of Cell Identity

1. Heredity versus environment

Any cell within the embryo is subject to two main classes of factors that may influence its further differentiation. The first class consists of factors that are directly inherited from the cell's progenitors. These include patterns of DNA modification, cytoplasmic proteins that can regulate gene expression, and membrane proteins that function as receptors for extracellular molecules. The second class consists of factors that impinge on the cell from the outside environment. These include diffusible molecules that interact with cell membrane receptors or that cross the membrane into the cytoplasm, molecules that are bound to the extracellular matrix or the surfaces of other cells and that interact with cell membrane receptors, and purely physicochemical factors such as pH, pressure, and electrical potential.

An important descriptive method that is often used as a first approach to defining which factors influence a given cell is "lineage tracing." The lineage of a cell is traced by marking individual progenitor cells so that all progeny can be visualized and then identifying the sequence of progenitors that gives rise to the cell in question. Lineage tracing by itself does not distinguish between inherited and environmental factors, because the lineage obtained not only invests a cell with a line of inheritance but also places the cell and its progenitors in a specific environmental context (see Ref. 299). It does, however, provide a history of the cell that can function as the basis for experimental manipulations that test the relative roles of inherited and environmental factors. Strictly speaking, this requires the placement of single cells into novel environments to challenge the autonomy of inherited factors.

2. Classes of relevant molecules

Among the many molecules that could be at play in determining cell identity, and which necessarily fall into the categories of inherited and environmental factors, certain types have received particular attention in recent years. Those that are most germane to the material presented in this review are introduced briefly below.

A) TRANSCRIPTION FACTORS. Transcription factors are intracellular proteins that can bind to DNA, typically as protein complexes, and regulate the transcription of genes. Many are classified and named on the basis of the particular DNA-binding domain they contain. For example, the Hox proteins, among the most notorious tran-

scription factors, are so named because the first to be identified are coded by genes whose mutation (in the fruit fly *Drosophila*) causes homeotic transformations (transformations of one body structure into another), and because the written sequence coding the DNA-binding domain can be neatly circumscribed by a rectangular box. The box received the moniker "homeobox," and "Hox" is short for homeobox. Other classes of transcription factors that have achieved notoriety in the world of neuroembryology include "Pax" (paired box), "zinc-finger," "winged helix," and "bHLH" (basic helix-loop-helix) proteins, the names of which reflect either the structure of the DNA-binding domain itself (for example, zinc-finger, winged helix, and basic helix-loop-helix) or the way in which the respective written coding sequence is circumscribed on paper (for example, paired box) (26, 151, 175, 241, 261, 303, 306). Distinctions among different transcription factor classes are not always clear-cut, however, because DNA-binding domains exhibit variations and different types can coexist in the same protein (see Ref. 241).

B) SIGNALING MOLECULES. A variety of secreted and integral membrane proteins function as extracellular signals that regulate the differentiation of cells bearing appropriate receptors. Secreted proteins can diffuse away from the cell of origin and influence other cells at a distance, whereas integral membrane proteins necessitate cell contact. Autoregulation through the expression of both signal and receptor by the same cell is not an uncommon phenomenon. Families of secreted proteins that have been implicated in the regulation of cell identity in neural development include the Wnt (wingless/int), hedgehog, and transforming growth factor-β (TGF-β) protein families (39, 150, 214, 225, 257). Such proteins can either diffuse freely or become bound to extracellular matrix, or both. Another family of secreted proteins that may be involved in regulation of cell identity but which are better known for their role in preventing cell death are the neurotrophins (28, 51, 198). These include nerve growth factor (NGF), brain-derived growth factor (BDNF), and neurotrophins (NT) 3 and NT4/5. The delta and notch families of integral membrane proteins mediate local inhibitory interactions that regulate the way cells adopt specific phenotypic fates (41). A family of integral membrane proteins that are involved in guiding axon outgrowth and possibly in the regional determination of neuronal identity is the ephrins (91).

C) SIGNAL RECEPTORS. To respond to signaling proteins, a cell must bear integral membrane proteins that function as receptors. One family of such receptors that receives some attention in this review is the trk tyrosine kinases, which function as the membrane receptors for a variety of signaling proteins, including the neurotrophins, and which mediate transduction of the extracellular signal to intracellular events (16). Another family of tyrosine kinases, the Eph receptors, function as the receptors for ephrins (91).

In principle, each of these factor types can be inherited, either as extant proteins or in the form of latent mRNA. Only the signaling proteins would be included in the category of environmental factors, although some proteins may function both as signaling molecule and receptor. The relative importance of heredity and environment in the determination of cell identity thus boils down to the potential for complex interplay among these various types of molecule. For example, an inherited transcription factor could initiate the expression of a particular membrane receptor by a cell, enabling the cell to respond to signaling proteins produced by other cells in the vicinity, or, in the case of neurons, at distant sites contacted by axons. In response to these signals, the cell could express additional transcription factors, which in turn could either repress or induce the expression of yet other receptors and transcription factors. Different sets of transcription factors and receptors could be passed on to progeny if the cell divides, or could induce the expression of proteins characteristic of a differentiated cell type if the cell is postmitotic. The possible combinations of these factors are virtually limitless. That specific cell types arise at specific locations in the embryo indicates that certain combinations of factors reliably emerge by virtue of the past histories of the cells and that they systematically restrict future interactions. The challenge therefore lies in establishing whether the cell type of interest arises autonomously from a single lineage or potentially from others, determining which factors are expressed and experienced by the relevant lineage(s), and describing how these control the expression of the appropriate differentiated characters. It should come as no surprise that completing such an endeavor requires many person-years of labor.

B. What Makes a Motoneuron?

1. Birth and lineage

Motoneurons are found from mesencephalic to spinal levels of the neuraxis and are among the first neurons to be born in the nervous system (being preceded in amniote species only by a few specific classes of interneurons 9, 140, 218, 219). In anamniotes, the set of primary neurons includes motoneurons, interneurons, and sensory neurons, all of which are generated nearly synchronously (although the sensory Rohan-Beard neurons are the very first to be born, Ref. 184). By comparison of birthdates and proliferative densities in the spinal cord, it was deduced some years ago that motoneurons are born in the most ventral region of the neural tube (140), but definitive proof in the form of lineage tracers directed specifically to ventral progenitors has been obtained only recently (83).

Lineage tracing in the chicken embryo (191) has shown that progenitors that give rise to spinal motoneurons can also give rise to other neuron types, although the number of separating generations is not known. In the zebrafish, the last few divisions of a progenitor can give rise to both motoneurons and other neuron types (170). Progenitors in *Xenopus* have been found to generate only motoneurons at the last one or two divisions (130), and some progenitors in the hindbrain of the chicken embryo have been reported to give rise to only motoneurons (83, 207). As discussed in the next section, this diversity in the genealogical patterns that give rise to motoneurons is probably due to a predominant influence of environmental factors in specifying motoneuron identity.

2. Environmental factors play the major role

During gastrulation and neurulation, when the neural plate is induced and begins to form the neural tube, a mesodermal structure called the notochord forms directly beneath the midline of the nascent nervous system (Fig. 1). Within a short time, cell types characteristic of the ventral neural tube differentiate in the region overlying the notochord. These include the cells of the floor plate (the structure that eventually forms the raphe of adult brains) and the motoneurons located on either side of the floor plate. In the chicken embryo, transplantation of pieces of notochord to ectopic locations alongside the dorsal region of the neural tube leads to the differentiation of floor plate and motoneurons there, where they are normally never found (262). Conversely, removal of the notochord beneath a stretch of the neural plate, either surgically in the chicken embryo (86, 262) or mutationally in the mouse (30), prevents the differentiation of floor plate and motoneurons in that stretch of the neural tube. Instead, the ventral region contains dorsal cell types (Fig. 2). Similarly, rotation of hemisegments of hindbrain neural tube about the dorsoventral axis leads to the differentation of motoneurons in the now ventrally positioned dorsal tissue, not in the dorsally positioned ventral tissue (291). The simple interpretation is that cells in the neural tube are initially naive and can be induced by signals from the notochord to initiate specific patterns of differentiation.

The signaling that mediates the induction of ventral cell types is complex and appears to involve multiple signal pathways. The notochord induces the floor plate in a process that requires contact with the overlying neuroepithelium, and the floor plate and notochord together induce adjacent cells to differentiate into motoneurons. Notochord- and floor plate-derived signals can be dissociated. If a piece of notochord is placed close to but not in contact with the dorsal neural tube; motoneurons but not floor plate cells differentiate there (344, see also Ref. 309). A similar dissociation is revealed by genetic mutations in the zebrafish and mouse wherein motoneurons differentiate in the absence of floor plate cells (55, 106, 124, 131, 216, 304, 316). Together with the fact that implants of floor plate alone can induce ectopic motoneurons (262), these results indicate that signals from the floor plate are sufficient, but not necessary, for the induction of motoneurons.

Several observations have also questioned whether the notochord is necessary for the determination of ventral cell types. Differentiation of motoneurons and other ventral cell types has been observed in some studies despite the experimental deletion of the notochord (13, 46), and in certain zebrafish mutants that lack a notochord (106, 124). Evidently, however, the apparent noto-

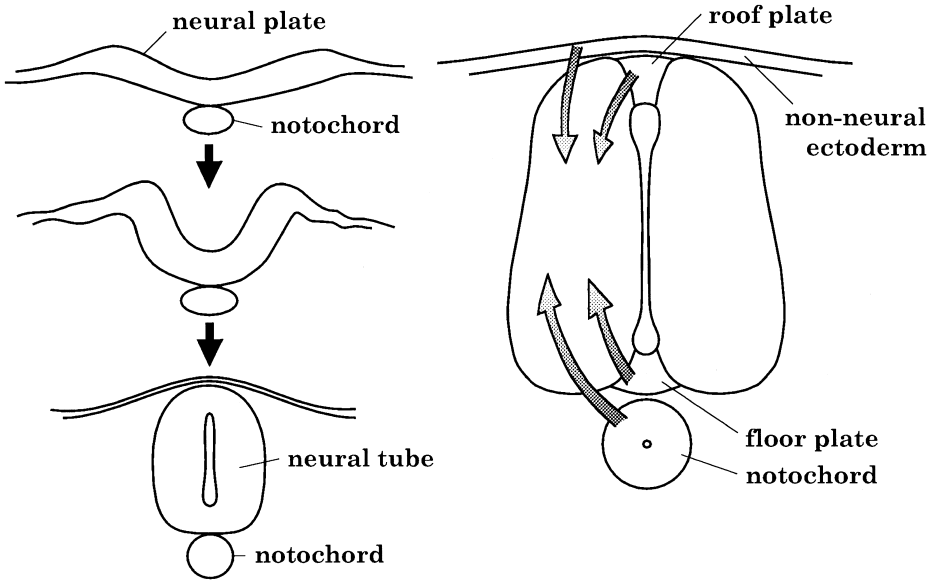

FIG. 1. The neural tube, forerunner of the central nervous system, is formed through invagination of the neural plate, which is continuous with the surface ectoderm. At both neural plate and neural tube stages, and possibly even earlier stages, signals from neighboring tissues impinge on the neuroepithelium, inducing different cell types at different dorsoventral locations. Among these are the floor plate and roof plate cells, which themselves generate signals that contribute to the inductive process.

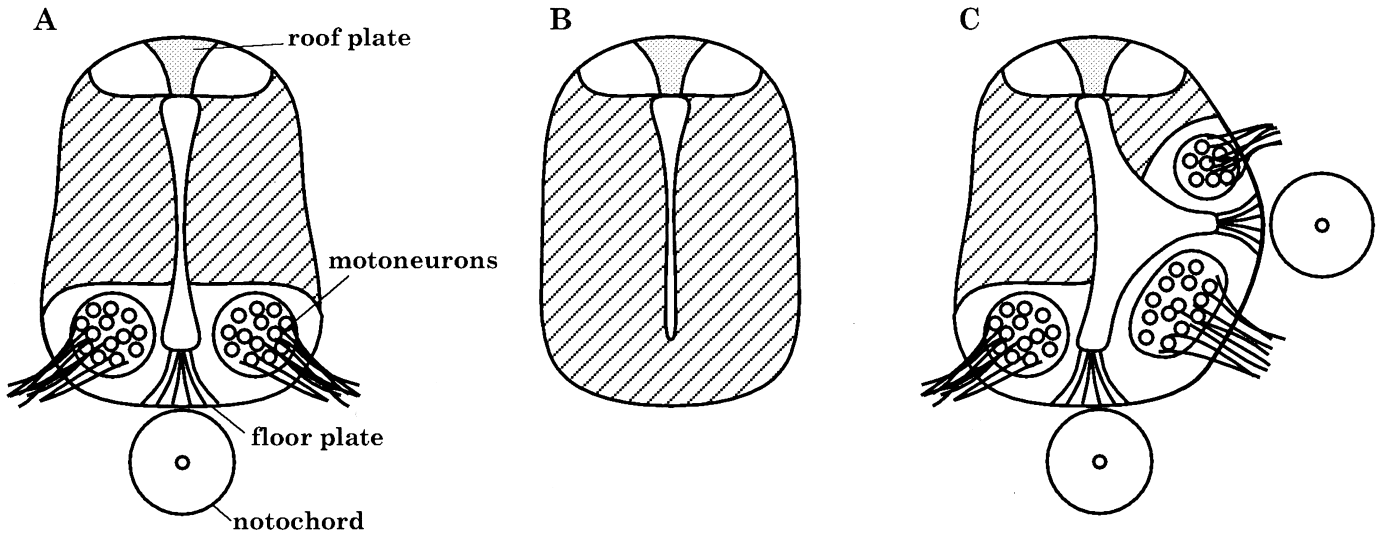

FIG. 2. The role of the notochord in induction of ventral cell types within the neuroepithelium is demonstrated by experimental manipulations. Normally, floor plate and roof plate are located at respectively ventral and dorsal poles of the neural tube, motoneurons differentiate somewhat lateral to the floor plate, and antigens expressed by interneurons, here indicated by hatching, appear dorsal to the motoneurons (*A*). Removal of the notochord before differentiation leads to expression of dorsal antigens throughout (*B*), whereas ectopic notochord tissue induces floor plate and motoneurons at dorsal locations (*C*).

chord-independent induction of motoneurons is due to signals derived from notochord precursor cells before either the notochord or the neural tube develop as overt structures (124, 344). Indeed, zebrafish primary motoneurons recently have been shown to be induced by early signals from notochord precursors, whereas secondary motoneuron induction is dependent on later signals from the notochord proper (19).

The molecular mechanisms of notochord signaling are the focus of intense research activity (58). The notochord has been shown to synthesize and secrete a specific signal protein, sonic hedgehog, that impinges on the overlying neural tissue and appears to play the principal role in inducing floor plate and motoneurons (270). Blocking the sonic hedgehog signal with specific antibodies prevents the differentiation of floor plate and motoneurons (80), and ectopic expression of sonic hedgehog leads to ectopic induction of floor plate in both anamniote and amniote embryos (67, 174, 270, 275). In contrast, genetic knockout of sonic hedgehog expression in the zebrafish only partially disrupts the differentiation of floor plate (279), indicating the presence of additional parallel signals (see also Refs. 190, 304). The inductive effect of sonic hedgehog on the neuroepithelium has been shown to have two critical periods: an early period wherein notochord precursors induce naive neural plate progenitors to produce ventral cell types and a later period wherein the ventralized progenitors are induced to produce different ventral cell types depending on the concentration of sonic hedgehog (80, 81, 276). The concentration dependence has been assayed quantitatively in vitro. Low concentrations of sonic hedgehog induce motoneurons, whereas higher concentrations induce floor plate at the expense of motoneuron differentiation (271). Given the presumed diffusion of sonic hedgehog from the notochord, this concentration dependence goes a long way toward explaining why floor plate and motoneurons arise where they do.

Recent progress has also provided glimpses into the molecular cascades that are initiated by sonic hedgehog and that result in the differentiation of ventral cell types. Sonic hedgehog signaling activates intracellular signals that are negatively regulated by protein kinase A (78, 128, 212, 319) and which in turn lead to the induction or repression of several transcription factors and extracellular matrix proteins in the ventral neural tube (33, 37, 55, 145, 193, 209, 213, 216, 274, 310). One candidate receptor for sonic hedgehog, a transmembrane protein called patched, has been identified (121, 211, 302). Patched is expressed transiently in the presumptive floor plate but disappears as the floor plate differentiates. Curiously, mutations in patched lead to expansion of the floor plate at the expense of other neural cell types, suggesting that patched has a constitutive repressive activity on the genes that sonic hedgehog normally induces locally at the midline (120). In the absence of patched, transcription of these genes is permitted throughout the neural tube. The regulatory actions in this pathway are both poorly understood and complex, including feedback and mutual inhibitory interactions among sonic hedgehog, patched, and certain target genes (55, 121, 212, 216, 273, 274, 316). The way cells respond to sonic hedgehog also appears to be

regulated by the activity of other signaling systems, such as the delta/notch pathway (10), which is known to control neural specification through local inhibitory actions (41). Moreover, there appear to be species-specific differences. This complexity notwithstanding, the end result is the establishment of sequential zones in the ventral region, each of which is characterized by the expression of a specific set of genes and the differentiation of a specific cell type. One of these zones contains the motoneurons.

3. Patterning within the motoneuron population

Once the motoneurons are determined within the ventral neural tube, what underlies their patterning into specific functional subclasses? During their differentiation, the motoneurons of amniotes migrate to different mediolateral and dorsolateral locations and segregate into distinct motor columns. Each motor column selects a characteristic axonal pathway in the periphery that connects the column to specific peripheral targets. Classically, three main columns have been described in the spinal cord, but each has functional subdivisions (reviewed in Ref. 317). The medial motor column extends the entire length of the cord, although with decreasing numbers at lumbar levels, and innervates axial musculature. Its medial subdivision innervates generally dorsal axial muscles via the dorsal ramus, whereas its lateral subdivision innervates generally ventral axial muscles via the ventral ramus. The lateral motor column is located only at brachial and lumber levels and innervates limb musculature. Its medial subdivision innervates limb muscles derived from the ventral embryonic muscle mass, whereas its lateral subdivision innervates limb muscles derived from the dorsal embryonic muscle mass. The sympathetic column is located only at thoracic and upper lumbar levels and innervates peripheral sympathetic ganglia. Within the hindbrain, somatic motoneurons and parasympathetic neurons are also organized into columns that are interrupted rostrocaudally in relation to individual cranial nerves. Parasympathetic neurons are also found within the sacral spinal cord.

Recent studies have shown that these motoneuron subclasses can be distinguished by their patterns of gene expression (Fig. 3). During their differentiation, vertebrate neurons express a number of Hox genes belonging to the LIM family.[1] Some LIM genes are expressed specifically by motoneurons, whereas several other LIM genes are expressed both by motoneurons and other neurons (200, 317). Strikingly, particular combinations of LIM gene

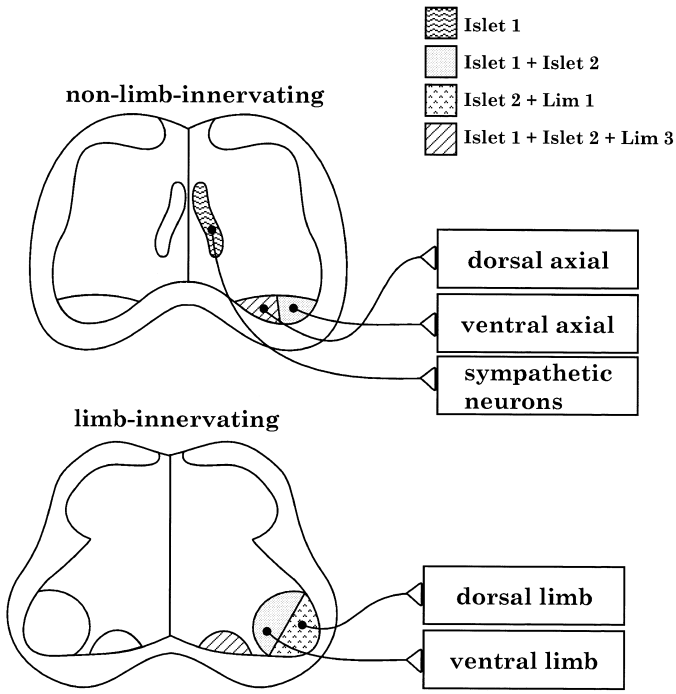

FIG. 3. The combinatorial expression pattern of LIM homeobox genes is correlated with different classes of spinal motoneurons in the chicken embryo. In non-limb-innervating segments, sympathetic preganglionic motoneurons and medial and lateral components of the medial motor column are differentiated by LIM gene expression; in limb-innervating segments, the medial motor column and medial and lateral components of the lateral motor column are differentiated. [Adapted from Tsuchida et al. (317).]

expression sort motoneurons according to their identities, both in amniotes and anamniotes (11, 308, 317, 324). In the chicken embryo, each motor column subdivision is characterized by a specific pattern of LIM gene expression. Thus the pattern of LIM gene expression is correlated with both the spinal position and peripheral axon trajectory of a motoneuron. However, the expression pattern is best correlated with peripheral axon trajectory, a fact that is demonstrated by the rhomboideus motoneuron pool, whose peripheral axon trajectory is atypical for its columnar location. In this case, the pattern of LIM gene expression is appropriate for the peripheral axon trajectory, not the columnar location (317). Because the LIM genes are expressed before axon outgrowth, the pattern of expression in fact predicts the peripheral pathways taken by the different motoneuron columns. The implication is that the LIM transcription factors control the expression of as yet unidentified membrane receptors that enable the motoneuron axons to discriminate among the available peripheral pathways. Each specific combination of LIM transcription factors evidently dictates the expression of a particular set of membrane receptors that then steers an axon onto a particular pathway and thus to a particular target region. Recent studies in *Drosophila*, in which misexpression of specific LIM genes leads to pre-

[1] The LIM family is named after the genes *Lin-11*, *Islet-1*, and *Mec-3*, the first genes of this class to be characterized. *Lin-11* and *Mec-3* regulate cell fate in the nematode *C elegans*; mutation affects lineage and mechanosensory neuron phenotypes, respectively (100, 329). *Islet-1* expression was first discovered in the pancreatic islet cells of the adult rat (159).

dictable changes in axon pathfinding, provide direct support for the idea that the LIM combinatorial code instructs motoneurons to follow specific peripheral paths (315). Similar misexpression of specific LIM genes in mice switches the point at which motoneuron axons exit the neural tube between a ventral site (characteristic of most somatic and visceral motoneurons) and a dorsal site (characteristic of trigeminal, trochlear, and spinal accessory motoneurons), indicating a similar instructive role in guiding initial axon trajectories within the central nervous system (284).

The restriction of the different motor columns to particular rostrocaudal levels does not appear to arise from rostrocaudal differences in notochord signals (311), but rather through positionally restricted signals from the paraxial mesoderm, from which arise the somites on either side of the neural tube (77). Thus notochord signals establish the generic motoneuron phenotype, whereas paraxial mesoderm signals establish the rostrocaudal subdivisions as defined by LIM and other gene expression patterns.

Motoneurons are further subdivided into motoneuron pools, each of which projects along a more specific pathway to innervate a particular muscle. LIM genes appear to control this differentiation as well, at least in anamniotes (11). In the zebrafish, each of the three identified primary motoneurons per spinal hemisegment has a characteristic rostrocaudal position and projects along a specific peripheral pathway to innervate a specific block of axial musculature (75) (Fig. 4). Each projects along its specific pathway in the absence of the others, demonstrating an independent ability to recognize and discriminate among the pathways (75). The combinatorial expression pattern of the LIM genes assayed to date in zebrafish distinguishes one of the three motoneurons from the others; it is plausible that assaying the expression of additional genes will distinguish all three uniquely. The motoneuron type-specific pattern of LIM gene expression evolves gradually from a less specific pattern, but the final pattern is present before axon outgrowth, again implicating the LIM genes in the determination of peripheral axon pathfinding.

Of particular importance is the finding that LIM gene expression is determined in the zebrafish motoneurons according to their micropositions within the spinal segment (11). None of the three motoneurons is commited to a particular identity at an early stage but becomes so by virtue of a highly resolved system of positional information within the neural tube (Fig. 4). If transplanted to a different rostrocaudal location more

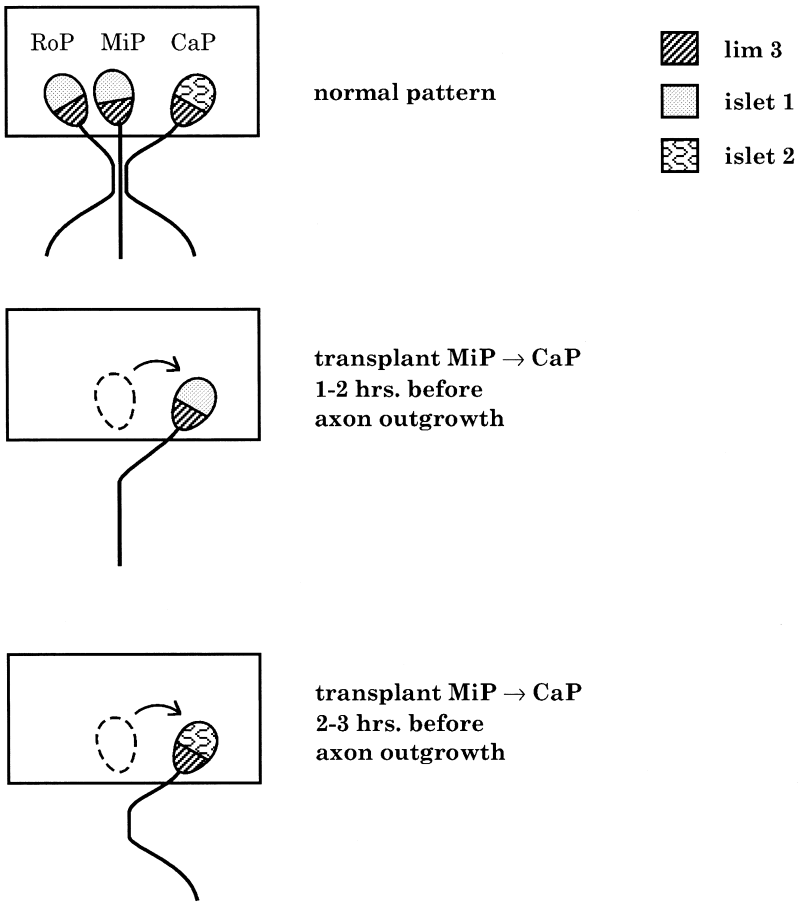

FIG. 4. The combinatorial expression pattern of LIM homeobox genes is correlated with individual motoneuron identity in the zebrafish embryo. Rostral, middle, and caudal primary motoneurons (RoP, MiP, and CaP, respectively) each have stereotyped locations, peripheral axon trajectories, and muscle targets. Both motoneuron identity and the corresponding pattern of LIM gene expression are determined during a specific window of time as a function of cell position.

than 2 h before axon outgrowth begins, a motoneuron differentiates according to its new location. If transplanted to a different rostrocaudal location less than 1 h before axon outgrowth begins, it differentiates according to its original location. Clearly, there are different inductive signals at the different locations that impose particular identities onto the motoneurons. Similar results have been obtained in the chicken embryo, where sets of motoneuron pools transplanted to ectopic segmental locations differentiate either according to their new or original locations depending on how early the transplantation is performed (185, 217). In the zebrafish, transplantation before commitment also initiates a new pattern of LIM gene expression appropriate for the new location, whereas transplantation after commitment does not alter the LIM pattern already expressed (11). Thus the pattern of LIM gene expression is determined by the microenvironment and in turn predicts the differentiation of specific motoneuron identity. Recently, retinoic acid has been implicated as a potential local cue within the spinal microenvironment (92, 297, 298).

In amniotes, several other genes have been shown to be expressed specifically by subsets of motoneurons, in patterns different from the LIM gene-associated subdivision. For example, a transcription factor of the winged helix family is expressed in a subset of thoracic and lumbar motoneuron pools (61); specific members of the ETS family of transcription factors are expressed in restricted subsets of brachial and lumbar motoneuron pools (206), and two specific Eph receptors are expressed, respectively, in limb-innervating motoneurons (311) and in a subdivision of the medial motor column and a subset of axial muscles (168). Combinatorial expression of such genes may provide the final determinants of motoneuron identity in amniotes; indeed, the expression of specific combinations of ETS and LIM genes defines individual motoneuron pools in the lumbar spinal cord (206). Of particular interest is the dependence of ETS gene expression on signals from the limb, as the motoneuron axons grow into the periphery (206). This means that motoneuron determination may include a phase of peripheral specification, a phenomenon that will be dealt with in more detail in section II*D3*. The restricted expression of Eph receptors by subsets of motoneurons and muscles is also especially intriguing, because Eph receptors are known to be involved in guiding axon outgrowth (see sect. III*B*) and could provide the mechanistic link between positional determination of motoneuron pools and their directed axonal growth to the appropriate target muscles.

Following their determination, most motoneurons remain near their ventromedial origins in the neural tube (although thickening of the ventral neuroepithelium in the hindbrain displaces some motoneuron pools to relatively dorsal locations). Within the ventral territory, the bulk of the motoneurons migrate different distances mediolaterally as they sort into columns. Some motoneurons, however, move to entirely different neighborhoods. For example, the trigeminal motoneurons in the hindbrain and the sympathetic preganglionic neurons in the spinal cord migrate actively from their ventral origins to more dorsal positions (236, 260, 264), and the motoneurons that innervate the superior rectus muscle of the eye migrate across the midline to take up residence on the opposite side (239, 266). These peregrinations are highly specific, determine the definitive positions of the motoneurons in question, and therefore could influence their accessibility to synaptic inputs. They also occur after the motoneurons have extended axons, and hence after the onset of LIM gene expression. LIM and other gene expression may therefore play a role in determining patterns of motoneuron migration as well as patterns of motoneuron axon outgrowth.

C. What Makes a Premotor Interneuron?

1. Birth and lineage

Premotor interneurons are found not only in close proximity to their target motoneurons but also at more far-flung locations, the latter exemplified by the descending premotor projections from primary motor cortex and brain stem to the spinal cord. Generation times are diverse, reflecting the different times of maturation of the brain regions in which the premotor interneurons reside. Most studies of interneuron generation have not discriminated between premotor interneurons and other types of interneurons. In both the brain stem and the spinal cord of amniotes, some interneurons, including reticular neurons in the brain stem and dorsally located interneurons in the spinal cord, are born before or at the same time as the motoneurons (9, 72, 87, 140, 218, 219). The early generated reticular neurons might include premotor interneurons (though definitive information is lacking on this point), whereas the early generated spinal interneurons are probably sensory (72, 87). Most interneurons in the brain stem and spinal cord of amniotes, and thus most premotor interneurons, are born after the generation of motoneurons (5–9, 218, 219). In the anamniote *Xenopus*, there is a general tendency for primary neurons to be generated in a dorsal to ventral sequence. The dorsally located sensory Rohon-Beard neurons are among the first born, and other neurons are born later, but the precise temporal relationship between interneuron and motoneuron generation has not been described (130).

As noted above, lineage tracing in the chicken (191) has shown that progenitors that give rise to spinal motoneurons can also give rise to other neuron types, including interneurons. In the spinal cord of *Xenopus*, some progenitors give rise to only a specific type of interneuron (commissural, ascending, descending, local) during their

last one or two divisions, whereas others give rise to different types of interneurons and glial cells during their last one or two divisions (130). The functional identity of the interneuron types was not determined, although some of the descendent commissural interneurons almost certainly include premotor interneurons (50). Finally, in the hindbrain of the chicken embryo, progenitors have been reported to give rise to only interneurons of a specific type, such as vestibulospinal interneurons (207). No consistent pattern emerges from interneuron genealogy. On the other hand, a number of studies provide strong indications that environmental factors play the major role in determining the identity of premotor interneurons.

2. Potential environmental determinants

The first indication of a role for environmental factors is that the location of different interneuron classes within the neuroepithelium is in some cases systematically related to axon trajectories, just as is the case for motoneurons. Commissural versus ipsilaterally projecting interneurons in the spinal cord and brain stem are typically segregated in the transverse plane to varying degrees (47, 68, 69, 108, 109, 115, 129, 182, 232, 268, 287, 290, 342). Projection interneurons in the hindbrain with ascending versus descending axons are segregated both in the transverse plane and rostrocaudally (107–112). In anamniotes, identified premotor interneurons with stereotypic positions in the neuroepithelium have specific axon trajectories (129, 182, 232).

The general correlation between interneuron position and axon trajectory can be extended in some cases to more specific relationships between identified premotor interneuron types and their motoneuron targets. Within the vestibular nuclei of the chicken embryo, for example, vestibulospinal and vestibulo-ocular neurons are organized into coherent clusters with different axonal trajectories and motoneuron targets (110–112). The vestibulospinal and vestibulo-ocular interneurons are segregated from each other along the rostrocaudal axis (259). Within each of these populations, different pools are segregated according to their axon trajectories, and, in the case of the vestibulo-ocular premotor interneuron pools, according to the extraocular motoneuron pools they innervate (112). Premotor interneurons that innervate specific subclasses of trigeminal and hypoglossal motoneurons in the rat and duck are also segregated to varying degrees (29, 56, 201).

There is also a striking correlation between gene expression patterns and interneuron position. A growing number of transcription factors and other genes are being described that are expressed in different domains in the transverse plane of the neural tube (308). The expression of different Pax genes, for example, defines different dorsoventral domains within the ventricular zone of the spinal cord and hindbrain (122, 163), as does the dynamic expression of certain Hox genes (167). Repression of Pax6 expression is in fact an essential step in the induction of ventral progenitors by sonic hedgehog (81). In the hindbrain, different pools of vestibulospinal, vestibulo-ocular, and reticulospinal neurons lie in different rostrocaudal and mediolateral domains that are characterized by different patterns of Hox and Pax gene expression (14, 53, 107, 109, 162). Some of the LIM genes that motoneurons express, as well as other genes not expressed by motoneurons, are expressed differentially in interneurons that derive from different dorsoventral levels (38, 81, 83, 86, 136, 194, 204, 216) (Fig. 5). Several studies present evidence that the expression of specific transcription factor combinations by interneurons or their precursors is correlated with axon trajectory or other phenotypic characters (109, 136, 194, 233).

The second indication that environmental factors are pivotal in determining premotor interneuron identity comes from the same kinds of environmental manipulations that were discussed in connection with motoneuron determination, for these also alter the determination of cells that normally differentiate into interneurons. For example, preliminary studies in which specific interneuron types in the zebrafish spinal cord have been transplanted to ectopic positions have shown that, as is the case for motoneurons, commitment to a specific axon trajectory occurs shortly before axon outgrowth begins (75). Thus it appears that at least some interneuronal attributes are determined by position-specific signals within the microenvironment of the neural tube. What are these signals and from whence do they arise?

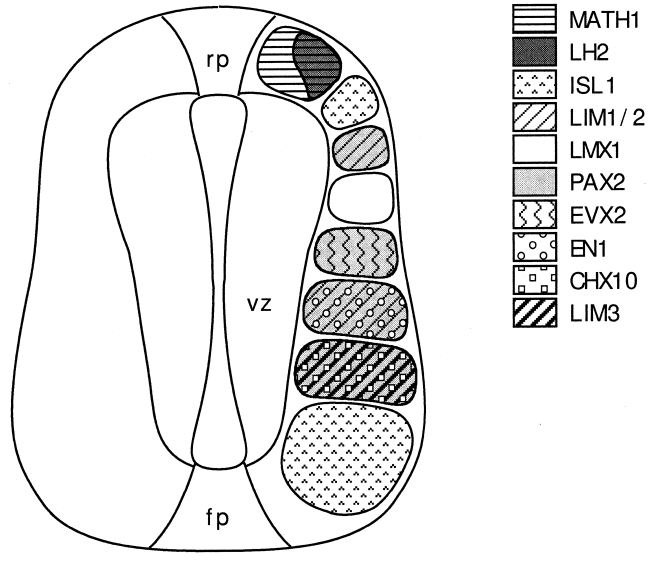

FIG. 5. Spatial patterns of gene expression arising from bipolar inductive signaling define different classes of interneurons in the neural tube. In most cases, it is unclear how these classes relate to functional phenotypes. rp, Roof plate; fp, floor plate; vz, ventricular zone (where neurons are generated).

We recall that when the notochord is removed from beneath a stretch of the spinal neural tube, the ventral region of the neural tube expresses dorsal cell types (345). This shows that an absence of inductive factors from notochord leads to a dorsalization of neuroepithelium but does not resolve whether dorsal character is a default state or is also actively determined by inductive factors. That active determination of dorsal character occurs is shown by the inductive effects exerted by the roof plate and surface (nonneural) ectoderm, both located dorsally. These tissues have been shown to secrete diffusible proteins of the TGF-β family, most notably bone morphogenetic protein (BMP) 4 and BMP7, that inhibit the induction of ventral cell types and promote the induction of dorsal cell types (12, 17, 205, 240, 288).[2] If both notochord and nonneural ectoderm are placed adjacent to naive neuroepithelium, a competitive interaction occurs in which proximity dictates the outcome; the cells immediately adjacent to the notochord differentiate ventral phenotypes, whereas a few cell diameters away expression of dorsal phenotypes is induced (54, 235).

The molecular mechanism of dorsal signaling is, if anything, more complicated than that of ventral signaling. Bone morphogenetic proteins bind to receptor complexes with serine-threonine kinase activity and lead to the phosphorylation of a number of transcription factors. An important feature is that extracellular BMP binding proteins regulate the diffusion and activity of BMP along the dorsoventral axis (225). The antagonism between ventral and dorsal signals is underscored by the expression by the notochord of secreted proteins that antagonize the action of BMP (221). The way in which ventral and dorsal signals interact is still poorly understood. Nevertheless, it is quite clear that they establish a bipolar, interactive signaling system that specifies transcription factor expression and, by extension, neuronal phenotype, as a function of position along the dorsoventral axis (Fig. 5; Refs. 225, 308).

3. Patterning within the premotor interneuron population: anatomical versus functional topography

It is important to stress that the coupling of functional identity and position is generally less prominent for interneurons than is the case for motoneurons, whose clustering into pools bears a strict relationship to target muscles. Many interneurons innervate multiple motoneuron pools, and any given motoneuron pool receives input from diverse interneuronal sources. Convergence and divergence are indeed prerequisites for the recruitment of different sets of motoneurons to different tasks at different times. Functional topography certainly exists in this interneuronal network. For example, microstimulation at different sites in the medullary reticular formation in cat activates sets of muscles in a roughly topographic order (62). Cervical interneurons active during locomotion versus limb-reaching in cats are partially segregated both longitudinally and transversely, despite innervating the same motoneurons (2, 3). The motor effects of spinal interneuron stimulation, in terms of the limb positions that are generated, map to different positions in the intermediate zone of the frog spinal cord (25, 105, 237). Although such observations suggest specific functional roles for individual interneurons, the extent to which they reflect an anatomical grouping into interneuron pools is far from clear. Anatomical topography has obvious advantages for coupling neurons in specific patterns during development, but the actual topography of interneurons is most likely a compromise between developmental and functional facility.

Given the complexity of interneuronal organization, it is not surprising that the analysis of interneuron patterning lags behind that of motoneuron patterning. It is not yet known how specific premotor interneurons acquire their identities, in part because specific premotor interneuron types have not been well characterized. The situation may be more complicated than a simple system of bipolar gradients, because not all interneuronal phenotypes are distributed in orderly, stacked domains along the dorsoventral axis. For example, one specific class of paragriseal commissural interneurons is generated ventrally and takes up residence lateral to the motoneurons (72), and some premotor interneurons even acquire definitive positions that are intermingled with those of motoneurons (for example, the intrinsic interneurons of the abducens nucleus). Thus relative position along the dorsoventral axis is probably insufficient to explain all features of the pattern. Analysis at the level of individual premotor interneuron subclasses is required, especially along the lines of transplantation experiments to challenge individual neurons with novel microenvironments.

D. What Makes a Ia Muscle Spindle Afferent?

1. Origin and position

The Ia muscle spindle afferents (hereafter referred to as "Ia afferents") represent the major source of monosynaptic sensory input to motoneurons. They originate from sensory neurons in the dorsal root ganglion (DRG). Developmentally, most of the neurons in the DRG derive from the migratory neural crest, and their early nomadic phase brings them into contact with a different set of environmental signals than are experienced by their brethren in the neural tube (34, 35, 79, 192). Recently, the neural tube proper has been shown to give rise to a

[2] BMP stands for bone morphogenetic protein. These signaling proteins were first described in the context of hematopoesis in the bone marrow.

secondary contribution of cells that emigrate out the dorsal roots and into the already formed DRG; some of these differentiate into sensory neurons (285). From these two sources, then, originate the sensory neurons that will innervate muscle, skin, and other organs peripherally, and motoneurons and interneurons centrally.

Within the DRG, the various subpopulations of cutaneous and muscle sensory neurons are rather haphazardly distributed, so there is no immediately obvious systematic relationship between the position of a sensory neuron in the ganglion and its peripheral or central targets. There is another distinction, however, that is related to position in the ganglion, at least in the chicken embryo. Sensory afferents in most vertebrates are typically divided into large- and small-diameter classes, and these project respectively in the dorsal funiculus and in Lissauer's tract (reviewed in Ref. 36). The Ia afferents belong to the large-diameter class and project in the dorsal funiculus, also at embryonic stages (70, 195, 230, 293, 294, 322). In the chicken embryo, the dorsal funiculus and Lissauer's tract afferents derive, respectively, from the ventrolateral (VL) and dorsomedial (DM) populations of sensory neurons in the DRG (73). Thus, although muscle afferents in general can derive from both the VL and the DM populations (141), Ia afferents are a subset of the VL population (this implies that muscle afferents derived from the DM population are of other types, such as type II and III afferents).

The VL neurons are born on average 1–2 days earlier than the DM neurons and attain initially larger soma sizes; the two populations are then easy to discriminate both by size and by position (125, 126; see also Ref. 228). Cell lineage analysis has shown that some neural crest progenitors give rise to both VL and DM sensory neurons, whereas some give rise to only VL neurons (96). Although sensory afferents were not categorized functionally in this study, it seems likely, given the arguments above, that lineage patterns giving rise to Ia afferents are variable, as we have seen for motoneurons and interneurons.

2. Influence of neurotrophins

Another approach to addressing what determines the identity of Ia afferents has been to examine the role of different neurotrophins. All sensory neurons require neurotrophins for survival; without them they die by apoptosis (reviewed in Refs. 28, 295). Indeed, neurotrophin levels normally are limiting, leading to the well-known naturally occurring cell death of sensory neurons during a specific period of their development (125, 126).

Beyond their role as survival factors, neurotrophins have been implicated in the differentiation of sensory afferents to specific functional subtypes (51, 198). Could the Ia afferent phenotype be related to a specific neurotrophin? Several lines of evidence indicate that the neurotrophin NT3 has a selective role in supporting the survival and differentiation of Ia afferents, at least at certain stages of their maturation. First of all, most muscle afferents are dependent on the neurotrophin NT3, but not on other neurotrophins (139, 144, 244). Indeed, NT3 is sufficient to maintain Ia afferents even in the absence of muscle and thus of any other potential target-derived factors (245). Second, the Ia afferents appear to selectively express the NT3 receptor tyrosine kinase trkC during the period of naturally occuring sensory neuron death, and application of function-blocking NT3 antibodies to chicken embryos during the same period selectively depletes Ia afferents (244). Moreover, deletion of the catalytic form of trkC in transgenic mice leads to a highly selective loss of Ia afferents (172).

Because the catalytic form of trkC transduces NT3 binding to intracellular cascades that can regulate gene expression, it has been proposed that exposure to NT3 might induce the Ia afferent phenotype among uncommitted sensory neurons (245). Because not just Ia afferents, but also other types of sensory neurons, express trkC and require NT3 for their survival during stages before target innervation (1, 82, 84, 197, 203, 313), specification of a potential Ia afferent phenotype by NT3 exposure necessarily would have to involve heterogeneity in the NT3 response or occur at a later stage.

In fact, Ia afferent sensory neurons do exhibit a second, later phase of trkC expression and NT3 dependence, during which they evidently are specifically dependent on muscle-derived NT3 for their survival. Their wholesale loss in limb-innervating segments in chicken embryos with extirpated limb buds, together with their survival in transgenic mice with NT3 expression restricted to muscle, indicates that sources of NT3 other than muscle are insignificant (245, 341). One possibility, therefore, is that only late exposure to NT3 via axonal contact with muscle permits the differentiation of the complete Ia phenotype, namely, muscle spindle innervation peripherally followed by monosynaptic innervation of motoneurons centrally.

An intriguing link between NT3 and the central side of the Ia afferent phenotype is the selective immunity of NT3-dependent sensory neuron axons to the repulsive effects of a diffusible protein present in the ventral region of the developing spinal cord (104, 231, 267, 286, 340). This protein, called collapsin-1 or semaphorin III/D in different species, inhibits the growth of specific types of axons in vitro, including those of sensory neurons that require other neurotrophins than NT3 for their survival and differentiation (208, 231, 286). The inhibitory effect of collapsin-1 is mediated by a receptor called neuropilin-1, which evidently is not expressed by trkC+ axons (134, 164, 173, 307). The expression of collapsin-1 is complex, however, in that it occurs in both dorsal and ventral regions of the spinal cord initially, although eventually

becoming most prominent in the ventral region (286). Thus only trkC+ axons are able to approach ventral targets; all others must resign themselves to innervating targets in the dorsal horn. Are Ia afferents the only trkC+ sensory neurons at this stage? No, a few cutaneous sensory neurons also express trkC late (222, 244), and indeed, a small proportion of cutaneous afferents establish ventral terminals, in the medial part of the intermediate zone (73). Thus neither trkC expression nor collapsin-1 insensitivity is uniquely specific to Ia afferents. Nevertheless, these features narrow the field and might contribute, in concert with other factors, to specifying sensory neurons as Ia afferents. It will be especially interesting to discover what regulates these features during the specification of the sensory neuron lineage from neural crest.

The role of collapsin-1/semaphorin III/D in mediating selective sensory afferent ingrowth has been questioned recently by gene knockout experiments. If collapsin-1/semaphorin III/D is responsible for restricting non-trkC+ afferents to the dorsal horn, then its elimination by gene knockout should allow massive ingrowth into the ventral horn. Two such knockouts have been generated. In one, sensory afferent projections were indistinguishable from normal, and in the other, there was a restricted ingrowth of non-trkC+ afferents along the ventral midline, hardly a wholesale invasion of the ventral horn (20, 312). One possible explanation is that other collapsins/semaphorins can repulse these afferents and that repulsion in vivo requires their concerted action. In this regard, it will be important to generate knockouts of neuropilin-1, which can also bind other collapsins/semaphorins (102).

Of course, none of this explains why the Ia afferents project to muscle in the first place. As it turns out, muscle afferents, but not cutaneous afferents, closely follow motoneuron axons as they grow into the periphery (142, 186). How this specific axon-axon attraction arises is still unknown, but it suggests a predetermination of the peripheral side of the Ia afferent phenotype, namely, peripheral axon growth to muscle.

3. Peripheral specification

Once sensory neurons are determined to be Ia afferents, they are also distinguished by their patterns of connections onto motoneurons. The functional basis of the monosynaptic stretch reflex indeed depends on the selective innervation of a motoneuron pool by the Ia afferents supplying the target muscle of that pool. Ia afferents selectively innervate appropriate motoneurons even when inappropriate motoneurons are in close proximity or made more available by removal of their own Ia afferent input (74, 98, 202). How do different sets of Ia afferents become determined to selectively innervate particular muscles and the corresponding, homonymous motoneurons?

Despite the fact that Ia afferents normally project along the appropriate sets of peripheral trajectories and innervate the appropriate sets of muscles from the outset (141), Ia afferents that are forced to innervate an inappropriate muscle target seem not to make any attempt to compensate peripherally (97, 143), a feature they also share with motoneurons (188, 336, 337). Once the afferents innervate a particular muscle, however, this subsequently dictates the selection of target motoneurons in the spinal cord, a process called peripheral specification (reviewed in Ref. 97). The capacity for different types of sensory afferents to be peripherally specified and the degree to which they can be specified is not yet completely defined. The case is strongest for Ia afferents, which, when caused to innervate a different muscle than usual, alter their central target selection accordingly, although it is not yet clear whether they absolutely shun the motoneurons they would normally have innervated (332). The implication is that the central connections of Ia afferents are always established in a pattern that maintains stretch reflex specificity (Fig. 6).

In an extremely exciting recent development, it has been shown that motoneurons and Ia afferents that innervate a given muscle express the same combination of ETS genes and that ETS gene expression in both neuron types is dependent on signals from the limb

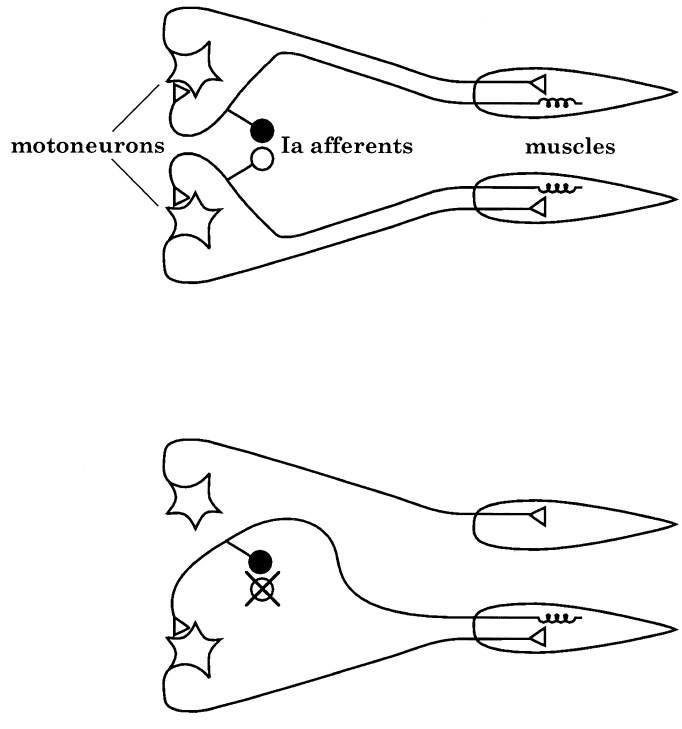

FIG. 6. A hypothetical experiment (combining features of several actual experiments) illustrating the principle of peripheral specification. Depending on which peripheral muscle they innervate, Ia afferents synapse on the homonymous motoneurons, thus ensuring appropriate reflex specificity. [Adapted from Frank and Wenner (97).]

(206). This matching of gene expression and target selection suggests that peripheral specification has the potential to establish common molecular surface markers in the two neuronal elements of the monosynaptic stretch reflex arc. The concordance between ETS gene expression by sensory neurons and motoneurons is very high for certain muscles but lower for others. This may simply mean that not all the relevant ETS genes have been identified. However, it might also reflect the actual degree of functional specificity in the stretch reflex: after all, Ia afferents are known to synapse not only on homonymous motoneurons but also on synergists and to some extent antagonists (see sect. IV*A*).

The mechanism by which Ia afferents are peripherally specified is unknown but must involve a retrograde signal from the peripheral axon that tells the neuron which muscle it has innervated. Transplantation of DRG to ectopic locations at different times in frog tadpoles has shown that peripheral specification does not occur after a certain stage of development, indicating a critical period for the process (223). Indeed, the induction by limb-derived signals of coordinated ETS gene expression in Ia afferents and motoneurons of the chicken embryo is similarly stage dependent (206).

Thus Ia afferents may be predetermined, like motoneurons, to innervate muscle, although it is still unclear how this might occur. In contrast, they are not predetermined to innervate specific central targets; rather, they appear to depend on signals from the muscle targets they innervate peripherally for instructions as to which motoneurons they should innervate centrally.

III. AXON PATHWAYS IN THE BRAIN STEM AND SPINAL CORD DURING EMBRYOGENESIS

Given that premotor interneuron and Ia afferent identities become determined at some point, how do their axons reach the motoneurons? Here I give a brief overview of the pathways on which these neurons project their axons and the mechanisms that contribute to pathway formation and recognition.

A. Early Axons Establish a Scaffold of Tracts

In the central nervous sytem, the first neurons to differentiate begin to extend their axons before the neural tube has undergone most of the morphometric change that leads to the mature structure of the brain and spinal cord. At these early stages, the neural tube is still a relatively thin epithelium with the rough topology of a cylinder sealed at both ends, albeit with a few characteristic bulges and furrows here and there. Remarkably, although distances are short and much of the epithelial surface is uncontoured, the first axons extend along very sterotyped paths, avoiding much of the available terrain. The pattern of these first "pioneer axons," as they have come to be known, in fact establishes a scaffold of axon tracts (Fig. 7, *A* and *B*) that is utilized by later axons and that with time becomes more complex as the number of axons increases (44, 64, 169). This is not to say that axons do not grow in the territory between the main staves and rungs of the scaffold, but the early axons clearly focus on the scaffold pattern. Although many of the major fiber tracts of the adult brain can be related to the tracts of this early scaffold, further development increases the complexity by adding new tracts and tract intersections.

The similarity of the early scaffold pattern in different anamniote and amniote species is compelling evidence for an evolutionary conservation of a basic plan for axon guidance in the vertebrate neuroepithelium. To be fair, there are differences. For example, some tracts, like the dorsal tract of the mesencephalic nucleus of the trigeminal nerve, are found in amniotes but not anamniotes (63), almost certainly reflecting the appearance of new neuron populations in the amniote radiation. Some of the early trajectories are less sharp in amniotes than in anamniotes, almost certainly related to the larger number of axons in amniotes. Nevertheless, one can almost imagine a zebrafish axon blithely and unerringly navigating through the neuroepithelium of an early chicken or mouse embryo.

With respect to premotor interneurons, the most important tracts in the early scaffold are the medial longitudinal fascicle (mlf) and lateral longitudinal fascicle (llf), because these serve to channel axons along the length of the neuraxis where motoneurons reside. In anamniotes (169, 243, 321), the mlf contains reticulospinal and other axon types and extends from the mesencephalon through the spinal cord, coursing just lateral to the floor plate. At early stages, the llf contains the axons of trigeminal sensory afferents in the hindbrain and of the sensory Rohon-Beard neurons in the spinal cord. Vestibulospinal and sensory axons from DRG appear later. Within the ventral white matter of the spinal cord, the axons of different types of spinal interneurons course at specific dorsoventral locations (183, 242) (Fig. 7*C*).

In amniotes, the situation is similar at early stages but becomes more complex with time (Fig. 7, *D* and *E*). Both the mlf and the llf contain axons of several types at early stages. The mlf courses just lateral to the floor plate and contains reticulospinal, medial vestibulospinal, and vestibulo-ocular axons, whereas the llf contains lateral vestibulospinal, tectobulbar, spinal trigeminal, spinocerebellar, and perhaps other axons (44, 47, 115, 287). Some axons, including some reticulospinal axons and the tectospinal axons, course longitudinally in the zone between these two tracts (115, 287). There is an initial segregation of different axon types within the llf (47) and an eventual

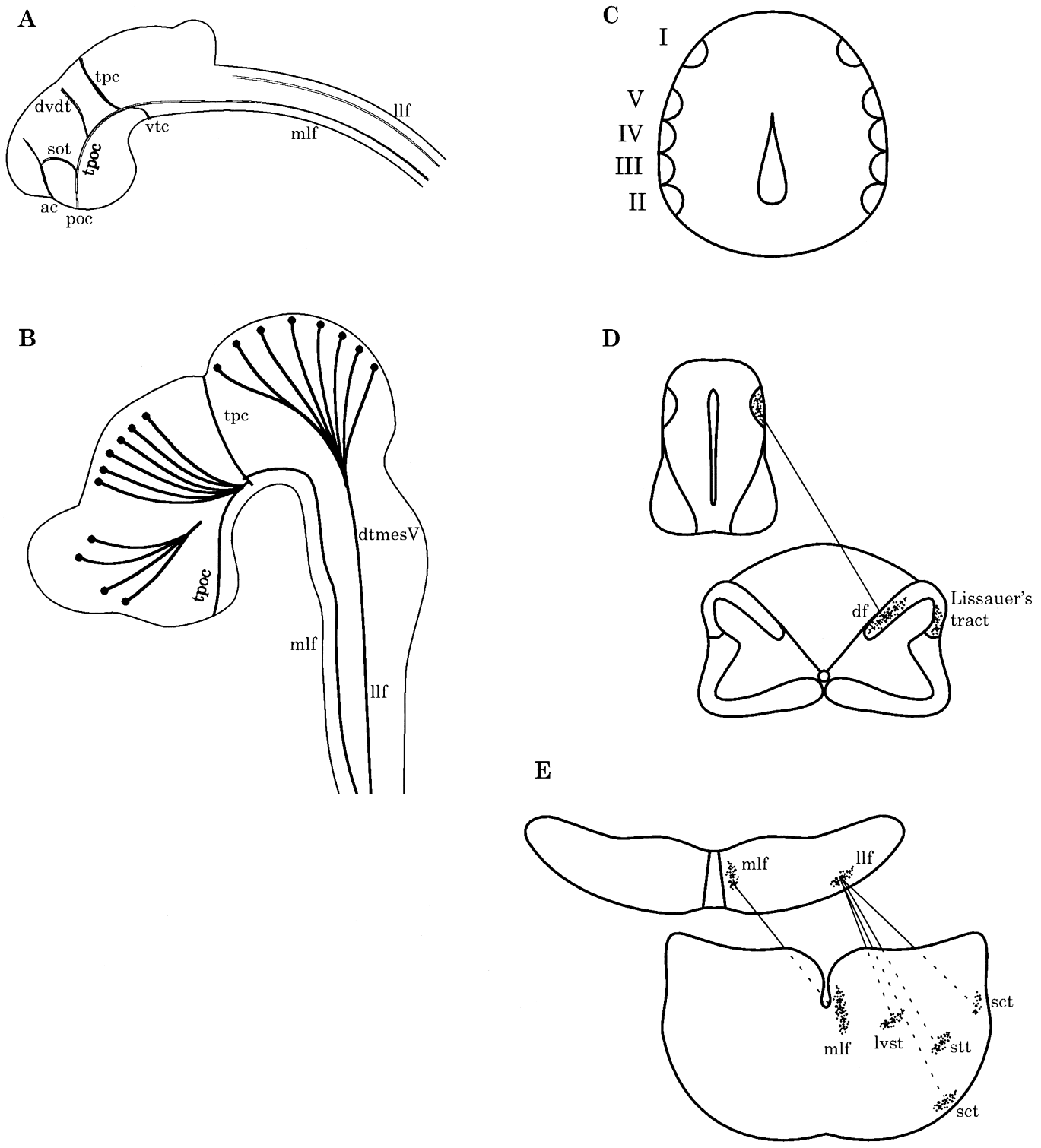

FIG. 7. Initial axon outgrowth establishes an early scaffold of axon tracts that increases in complexity with further development. *A*: early scaffold in zebrafish. [Adapted from Kimmel (169).] *B*: early scaffold in chicken. [Adapted from Chedotal et al. (44).] *C*: discrete pathways taken by axons of sensory neurons and spinal interneurons at early stages in an anamniote. Roman numerals indicate the temporal order in which the pathways are utilized, with I indicating the pathway taken by sensory axons and II—V indicating pathways taken by interneurons. [Adapted from Nordlander (242).] *D*: sensory and interneuron tract development in an amniote is illustrated in the spinal cord. [Adapted from Eide and Glover (71).] *E*: sensory and interneuron tract development in an amniote is illustrated in the hindbrain. [Adapted from Glover and Petursdottir (115).] In *D* and *E*, note the transformation of early scaffold elements to mature fiber tracts. ac, Anterior commissure; poc, postoptic commissure; sot, supraoptic tract; tpoc, tract of the postoptic commissure; dvdt, dorsoventral diencephalic tract; tpc, tract of the posterior commissure; vtc, ventral tegmental commissure; mlf, medial longitudinal fascicle; llf, lateral longitudinal fascicle; dtmesV, dorsal tract of the mesencephalic trigeminal nucleus; lvst, lateral vestibulospinal tract; stt, spinal trigeminal tract; sct, spinocerebellar tract.

separation of these types into distinct tracts as the hindbrain expands during subsequent development (115). In contrast, the mlf remains much more coherent over time. As they progress into the spinal cord, the motor components of the llf approach the mlf, and they both course at ventromedial locations, whereas the sensory components of the llf sort out to more dorsolateral locations. The dorsal columns, containing the longitudinal axons of sensory neurons, extend through the length of the spinal cord up to the lower hindbrain where the dorsal column nuclei reside. At early stages, sensory axons course in a single tract, His' bundle, whereas at later stages they course in two tracts, the dorsal funiculus and Lissauer's tract (71).

The early scaffold has been visualized by staining the growing axons themselves and is therefore only an indirect indication of the presence of cues in the neuroepithelium that guide the axons onto specific trajectories. Several studies have noted that domains of gene expression in the neuroepithelium seem to demarcate some of the elements of the scaffold, and manipulation of some of these domains perturbs the scaffold (reviewed in Ref. 338). The implication is that cell surface properties or other molecular features that guide axons are under the control of such genes and thus arise at specific locations in the neuroepithelium (338, 339).

B. Axon Guidance Cues

What guides the early axons onto the scaffold pattern and into specific tracts? At present there is little concrete information on this point. A full discussion of potential mechanisms is beyond the scope of this review, but before beginning the next section, it is probably helpful to describe some of the factors that might be at play. These can be classified in different ways; here I distinguish five factors that may be involved in axon guidance.

1. Initial polarity

Most neurons have a single axon, which issues from a single point on the cell surface. Given that all neurons (at least in the central nervous system) begin their life as an epithelial cell, the original basal-apical polarity of the neuron may be involved in the budding of the axon and its initial trajectory. Indeed, many axons are simply the extensions of the basal or apical processes of the newborn neurons (see Ref. 148 for discussion). The maintenance of original epithelial polarity as a neuron migrates and takes up its definitive position could send its axon off on a ballistic trajectory that would clearly influence which guidance cues the axon contacts subsequently.

2. Contact guidance by neuroepithelial cells or extracellular matrix

Axons often exhibit a proclivity for growing on particular substrates, both cellular and acellular. The attraction is generally accepted to be mediated by molecules for which the axon growth cones bear receptors (reviewed in Refs. 57, 146, 278). Selective growth of axons on neuroepithelial cells and on other axons has been documented in the central nervous system of both invertebrates and vertebrates, and a number of membrane and extracellular matrix proteins have been identified that may, in combination, contribute to such behavior (reviewed in Refs. 118, 314).

3. Chemoattraction

Axons can also be attracted by diffusible molecules emanating from specific structures. For example, commissural axons are attracted by diffusible molecules from the floor plate (49, 314), and cortical axon collaterals are attracted by diffusible molecules from the pontine nuclei (135). Such diffusible molecules selectively attract specific neuron types, indicating the existence of specific receptors that are differentially expressed by neurons.

4. Chemorepulsion

Axons can also be repulsed, both by diffusible molecules and by substrate-bound molecules. We have already made the acquaintenance of one such molecule, collapsin1/semaphorin III, in our discussion of the determination of Ia afferent identity. Repulsion typically involves a structural collapse of the axon growth cone, preventing further advance in the direction of the repellent molecule. Chemorepulsion was first discovered in the interactions between axons of different neuron types in vitro (158) but has since been observed on contact of axons with glial cells and potential target cells (reviewed in Ref. 60) and in reaction to the same molecules that function as diffusible chemoattractants (48). This suggests that the same substance may selectively attract some axons while selectively repelling other axons, a phenomenon that also presupposes the differential expression of receptors by neurons.

5. Axon-axon interactions

Because in vertebrates, and especially amniotes, most neuron types exist in multiples, each specific projection typically contains many axons, even at the "pioneering" stage. Thus axons have the opportunity to interact with each other at the same time they are reacting to other guidance cues (57). The degree of axon fasciculation and of axon diversity within a fascicle will depend on

whether interactions are homophilic, heterophilic, homophobic, or heterophobic.

C. Axon Outgrowth by Spinal Interneurons Illustrates How Multiple Guidance Cues Establish Axon Trajectory

The way in which the various cues described above interact to guide axons on characteristic trajectories can be illustrated by the behavior of spinal interneurons (Fig. 8), whose axon pathfinding has been studied in both anamniotes and amniotes (reviewed in Ref. 118). In these studies, several general classes of interneurons have been distinguished on the basis of location and pathfinding behavior. Dorsally located interneurons project circumferentially toward the midline, which they either cross (commissural) or not (noncommissural). Ventrally located interneurons generally project longitudinally without crossing the midline, although some specific populations of ventral interneurons are commissural (72). Each class probably contains premotor interneurons, but in the vast majority of developmental studies, this distinction has not been addressed.

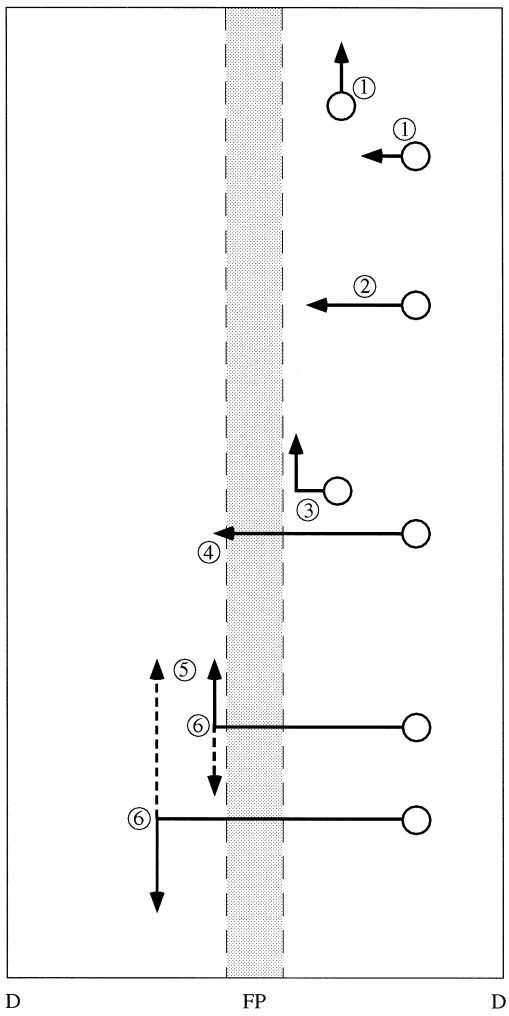

FIG. 8. Sequential mechanisms are involved in guiding the axons of spinal interneurons along appropriate trajectories. A segment of spinal cord split along the dorsal midline (D) and opened up like a book is viewed from the ventral surface, with the ventral midline floor plate (FP) shaded. Axon trajectory is established through *1*) initial polarity of axon outgrowth; *2*) circumferential guidance cues; *3*) interactions with the floor plate, via diffusible factors and contact; *4*) selection of longitudinal course; *5*) selection of dorsoventral altitude for longitudinal extension; and *6*) selection of longitudinal direction.

Circumferentially projecting interneurons are distributed along the length of the spinal cord and project on the circumferential trajectory independently of the others. There is no fasciculation and no evidence that axons guide each other around the circumference of the spinal cord. This is often interpreted to indicate a longitudinally distributed contact guidance cue that directs axons ventrally (49, 180, 343). Support for contact guidance comes from the existence of growth-promoting membrane and extracellular matrix proteins along the circumferential path and the expression by circumferential axons of potentially appropriate receptors (59, 103, 326, 343). An additional mechanism that may contribute to initiating the circumferential trajectory is the release of a diffusible chemorepellant by the roof plate (15).

As the circumferential axons approach the midline, they come under the influence of diffusible proteins secreted by the floor plate. One of these, netrin-1, is believed to exert a selective chemoattraction on the commissural interneurons, leading them to the floor plate in preparation for their midline crossing (reviewed in Refs. 49, 314, but see Ref. 210). The chemoattractive influence is not necessary for the initial circumferential trajectory, which is established even in the absence of the floor plate. The function of netrin-1 may therefore be to coax specifically the commissural axons into the floor plate while leaving the circumferentially projecting noncommissural axons unaffected. This would imply the differential expression of netrin receptors by commissural and noncommissural interneurons. Alternatively, netrin receptors might be expressed by both classes but transduce different responses. For example, the floor plate has been shown to repulse the axons of motoneurons, and in some cases, this repulsion is mediated by netrin-1 (325).

Despite the specific attraction exerted by the floor plate on commissural axons, the presence of the floor plate is not necessary for midline crossing by commissural axons. If the floor plate is ablated, either by laser microsurgery or genetic mutation, a substantial number of commissural axons (approaching 75% in zebrafish) cross the midline (123). Is there another source of midline chemoattractants? The answer appears to be yes, namely, the notochord. The notochord also expresses netrin-1 in amniotes (165, 283), and its ablation in zebrafish decreases the frequency of crossing by commissural axons;

indeed, the effect of notochord and floor plate ablation is additive (30, 123), yet, even when both are ablated, ~50% of commissural axons cross the midline. Similarly, transgenic mice with deletions of the netrin-1 gene or of the gene for its receptor exhibit substantial, but not complete, deficits in midline crossing by commissural interneurons (85, 282). Taken together, these findings are perhaps an indication that the initial circumferential trajectory is sufficiently ballistic to ensure a reasonably high rate of midline crossing.

The ability to enter the floor plate upon reaching it is dependent on interactions between specific membrane proteins on the commissural axons and on the floor plate cells. Two such proteins, axonin-1, present on commissural axon growth cones, and NrCAM, present on floor plate cells, bind heterophilically, and blocking either with antibodies inhibits the commissural axons from entering the floor plate (300, 301). The effect occurs as the axons contact the floor plate, indicating the presence of a floor plate-associated repellent that is overridden by axonin-1/NrCAM binding. Selective axonin-1 expression could therefore explain why commissural axons enter, whereas noncommissural axons turn to course alongside the floor plate. There are therefore at least two potential mechanisms for specifying commissural versus noncommissural axons: differential expression of netrin-1 (or other chemoattractant) receptors and differential expression of axonin-1 (or other membrane proteins) that regulate contact-dependent repulsion.

Given their initial attraction, how do the commissural axons escape from the floor plate as they cross to the opposite side? Somehow they must cease being attracted by netrin-1 so they can interact with guidance cues lateral to the floor plate. This has been demonstrated for commissural axons originating from the cerebellar plate, which cease responding to netrin-1 or to ectopic floor plate tissue if and only if they first cross the floor plate (289). How this occurs is still unknown, but it could involve a loss of netrin receptors, a decrement or reversal in the response induced by netrin receptor activation, or the initiation or augmentation of the response to floor plate-associated repellents.

Another difference between commissural and noncommissural interneuron classes is their response to longitudinal pathways. The ipsilateral longitudinal pathways are ignored by the commissural axons as they approach the midline yet avidly selected by noncommissural axons. After crossing the midline, however, the commissural axons select the same longitudinal pathways on the other side. As in floor plate escape, the sudden interest in longitudinal pathways suggests a change in the expression of membrane receptors upon crossing the midline. Evidence for this sort of change comes from studies of the expression of adhesion molecules by commissural axons. Upon leaving the floor plate, commissural axons in amniotes switch their expression of certain adhesion molecules that are known to play a role in axon growth and fasciculation (59, 343). In addition, the floor plate cells have been shown to transfer proteins onto commissural axons (40). How these particular molecules are involved in crossing and turning by commissural axons is not known, but their regulation suggests that interactions with the floor plate alter the receptive properties of commissural axons in preparation for subsequent changes in trajectory.

The longitudinal axons of both commissural and noncommissural interneurons acquire specific dorsoventral levels in the white matter, a feature that is especially prominent among the early longitudinal axons of anamniotes (183, 242; see also Ref. 72) (Fig. 7*C*). The mechanism underlying the choice of longitudinal trajectory is poorly understood. It may involve contact guidance on neuroepithelial cells or on other longitudinal axons already present in the white matter (24, 178, 179). There are several indications that the longitudinal trajectories are also dependent on the floor plate. If the floor plate is absent, axons may fail to turn after crossing the midline, may choose the wrong longitudinal trajectory, or may choose the right longitudinal trajectory but grow in the wrong direction (reviewed in Ref. 49). Nevertheless, many axons do the right thing (123), an indication that perhaps redundant guidance cues exist for the longitudinal pathways (179). The defects could represent the loss of anything from highly specific guidance cues to more general guidance cues. For example, commissural axons might require an interaction with the floor plate to initiate the expression of specific receptors, and hence become confused during subsequent longitudinal growth in the absence of such an interaction. Alternatively, axons might be programmed to seek specific concentration isolines of netrin-1 or other diffusible substances emanating from the floor plate, and hence fail to adopt characteristic dorsoventral altitudes in the absence of the floor plate. These possibilities remain untested.

Axons on longitudinal trajectories often fasciculate, in many cases by type, in contrast to the individualism exhibited while growing circumferentially. Several types of membrane proteins have been implicated in the fasciculation of axons (reviewed in Ref. 57), but the mechanism that patterns the fasciculation of spinal interneuron axons is poorly understood. Fasciculation probably has several roles. In the development of connections, it may serve to restrict access to particular targets as well as to ensure a concerted and ordered approach of axon populations to target regions.

In summary, spinal interneurons establish initial axon trajectories that are either circumferential or longitudinal. The choice of initial trajectory is roughly correlated with the dorsoventral position of the cell soma and could, in principle, be predetermined on that basis. As

they extend, the axons interact with matrix molecules and diffusible molecules, and, in the case of commissural axons, directly with the floor plate. They make characteristic turns, choose specific longitudinal trajectories, and fasciculate selectively. The guidance of spinal interneuron axons illustrates what appears to be a common principle in the development of axon projections (117, 118), namely, the road to a target is a hierarchical sequence of interactions, each of which may determine the conditions of the next.

IV. DEVELOPMENT OF CONNECTIVITY

The development of synaptic connections from premotor neurons onto motoneurons can be separated into two main phases. The first involves the growth of axons to the region where motoneuron targets reside and has been discussed in general terms in section III. The second involves the selective innervation of the appropriate motoneurons within the population of available motoneurons. In only a few cases have both of these phases been studied. The best-studied case is that of the Ia afferents, which is discussed first in this section. Examples of selected types of long projection premotor interneurons follow. Finally, the question of connections from local premotor interneurons onto motoneurons is discussed, mostly in the context of what can be deduced from the development of motor activity patterns.

A. Development of Connections From Ia Afferents

Ia afferent axons extend from the DRG toward the periphery and toward the spinal cord at about the same time. The central axons penetrate the dorsal margin of the cord and immediately bifurcate to extend longitudinally in an axon bundle ("His' bundle") that eventually becomes incorporated in the dorsal funiculus. In the chicken embryo, the ingrowth of Ia afferents occurs simultaneously with the ingrowth of other large-diameter afferent classes, such as cutaneous afferents, derived from the early generated VL sensory neuron population (71, 73, 230). Later arriving afferents from the DM sensory neuron population (which includes non-Ia muscle afferents) enter the spinal cord at more lateral positions and course in Lissauer's tract (71, 73; see also Ref. 234). Both timing and axon guidance could be at play in positioning Ia afferent axons in the medially located dorsal funiculus (73).

In both anamniotes (93, 322) and amniotes (52, 71, 195, 292), the Ia afferents extend substantial distances longitudinally in the dorsal funiculus before they begin to sprout collaterals into the gray matter. The delay may be because of changes in the afferents or changes in the gray matter. Afferents might be programmed to focus their metabolic resources first on longitudinal growth and then on collateral growth. Alternatively, collateral sprouting might be dependent on the appearance of chemoattractants or the disappearance of chemorepellents in the gray matter. Experiments in culture on the temporal development of collapsin1/semaphorinD-induced repulsion suggest that the disappearance of this chemorepellant from the dorsal horn permits the initial sprouting of collaterals (267, 286). Delayed sprouting of collaterals is observed in other axon systems and in at least one case appears to depend on the appearance of selective chemoattractants (252). The neurotrophin NT3, to which Ia afferent collaterals are sensitive, is expressed by motoneurons in the rat and has been proposed as a potential chemoattractant for the Ia afferents (346).

Once the Ia afferent collaterals sprout, they extend uninterrupted into intermediate and ventral regions of the gray matter. The depth of the collaterals varies among different species, but in all species brings the collaterals into close proximity to motoneuron dendrites and somata (73, 138, 147, 292, 296, 322). The ventral excursion distinguishes the collaterals of Ia afferents from the collaterals of cutaneous afferents and of the later developing muscle afferents. Neither of the latter (except for a few cutaneous afferents) penetrates beyond the dorsal horn, despite, in the case of the early developing cutaneous afferents, sprouting concomitantly with the Ia afferent collaterals (70, 73, 230, 322). As discussed earlier, in amniotes the ability of afferents to grow into the ventral reaches of the gray matter is correlated with their relative immunity to the chemorepellant effects of collapsin1/semaphorinD and perhaps other molecules, an immunity which in turn seems to be associated with trkC expression and NT3 dependency (231, 245, 286, 340). Because most if not all Ia afferent collaterals express trkC as they approach the ventral gray matter (245), they slip unhindered through the gauntlet of chemorepellant.

Thus Ia afferents appear to be differentiated from at least some other types of afferents at the time collateral sprouting begins. Moreover, the Ia afferents that innervate medial and lateral motoneuron pools, respectively, follow different ventrad trajectories in the rat embryo, suggesting differentiation within the Ia afferent population at early stages as well (296).

During the delay before collaterals start sprouting, the peripheral axons of Ia and other afferents have reached the periphery and have begun to terminate in different target tissues. Peripheral specification might therefore be sufficient to explain the selective behavior of the central collaterals during their initial ingrowth; depending on which peripheral target is contacted, specific sets of receptors could be expressed by the central axons that dictate their sensitivity to different attractive and repellant factors.

As the Ia collaterals reach the ventral horn, they begin to make functional synapses on the motoneurons.

These can be assayed electrophysiologically, providing another means of documenting specificity. Electrophysiological studies of the development of connections have focused on brachial Ia afferents in the bullfrog tadpole (99); on lumbar Ia afferents in the chicken (74, 195, 196), mouse (224), and rat embryos (177, 277, 281); and on brachial and lumbar Ia afferents in the wallaby pouch young (138).

In the chicken embryo, stimulation of muscle nerves or dorsal roots first produces monosynaptic excitatory synaptic potentials in motoneurons at about the same time the central collaterals of Ia afferents begin to reach motoneuron dendrites (195). At somewhat later stages, when Ia collaterals have reached the lateral motor column in large numbers, the monosynaptic connections are stronger. At this time, tests of the specificity of connections show that Ia afferents from a given muscle make strong monosynaptic connections onto homonymous motoneurons, but also weaker monosynaptic connections onto certain synergistic and antagonistic motoneurons (196). This pattern is maintained until hatching, indicating that little if any qualitative synaptic rearrangement occurs and suggesting that the initial pattern of connections is largely appropriate (see also Ref. 74). Support for this notion comes from a comparison with connectivity patterns in the adult cat: in the cases of weaker monosynaptic connections onto antagonist motoneurons in the chicken embryo, the same situation is found in the homologous antagonists in cat (see Ref. 196).

Similar results have been obtained in the bullfrog tadpole, where the appropriate specificity of Ia afferent connections onto several forelimb motoneuron pools is apparent from the earliest stage synaptic potentials can be recorded (99), that is, at about the time the Ia afferents first make anatomical contact with motoneuron dendrites (93, 147). In the mouse embryo, appropriate specificity is evident shortly before birth (224) but has not been assessed at earliest anatomical contact (~2 days earlier). In contrast, in the rat embryo, the proportion of inputs from antagonistic Ia afferents has been reported to be substantially higher initially, with a fall to adult levels during the first postnatal week (281). Similarly, stimulation of a single dorsal root in the rat activates motoneurons over a wider segmental range at early than late stages, suggesting a less specific initial pattern of connections that is gradually sharpened (277). In these studies, however, there is some uncertainty regarding whether the recorded synaptic actions were monosynaptic or polysynaptic (see Ref. 224 for discussion on this point). Polysynaptic inputs to motoneurons in the bullfrog tadpole, for example, have been shown to decrease in strength with development, in contrast to monosynaptic Ia inputs (99). It would appear, despite the reported difference between rat and other species, that in all species so far examined Ia afferents synapse predominantly on homonymous motoneurons from an early stage, and in at least some species of both anamniotes and amniotes Ia afferent inputs exhibit appropriate specificity from the time they are first established.

Because appropriate and inappropriate motoneuron targets can exist in close proximity and their dendrites overlap substantially (202), individual Ia afferents, and very likely individual collaterals, must discriminate among several different potential targets. Evidently they select the right ones from the outset. This sort of behavior immediately brings to mind a molecular recognition system similar to that involved in axon guidance. However, as suggested by the reported improvement of specificity in the rat, an alternative possibility exists. Ia afferent connections could initially be less specific, if rapid weakening or removal of the inappropriate connections precluded their detection by electrophysiological methods. This would require that inappropriate connections be recognized for selective removal. One way the embryo could accomplish this is through the sort of activity-dependent remodeling of synaptic connections that characterizes other sensory relays such as cutaneous primary afferent projections (199, 227) and the visual system (reviewed in Ref. 119). The basic idea for the Ia afferent system is that activity in a given motoneuron pool contracts a given muscle and thereby affects the activity of homonymous and heteronymous Ia afferents differentially, such that a millisecond-for-millisecond comparison of a motoneuron's own activity with the activity of the various Ia afferents impinging on it would allow appropriate and inappropriate connections to be distinguished and the latter subsequently removed. Indeed, in the rat spinal cord, the *N*-methyl-D-aspartate (NMDA) class of glutamate receptors, which is a molecular substrate for activity-dependent remodeling in other sensory systems, is transiently expressed in the ventral horn at the stages when the specificity of Ia connections increases (156). The potential role of activity as a regulator of specificity has been experimentally tested in different ways in the bullfrog (94) and the chicken embryo (229), as discussed below.

In the bullfrog, two types of experimental manipulation have been performed to disrupt normal patterns of activity. In the first type, muscle tendons are either cut, such that muscle contractions do not generate stretch, or their insertions are transplanted onto the opposite surface of the bone such that an extensor is transformed into a flexor (95). Both manipulations disrupt the normal pattern of afferent activity throughout the period when Ia afferent connections are formed. Despite this, Ia afferent connections develop with the appropriate specificity (95).

In the second type of manipulation, a brachial ventral root is resected shortly before Ia afferent connections are made onto motoneurons. The motoneuron axons regenerate, but in most cases reinnervate limb muscles nonspecifically (94). The normal pattern of limb movement is seriously compromised, and motoneuron and Ia afferent

activity are discoordinated. This sets up a situation in which specificity can be tested, but unfortunately also randomizes the central distribution of motoneurons innervating a given muscle. Because different motoneuron pools are not strictly segregated to begin with, this would seem to preclude the identification of original motoneuron identity that is necessary to evaluate whether the formation of specific Ia afferent connections depends on molecular recognition as opposed to activity. Fortunately, however, a feature of the normal innervation pattern exists that allows these alternatives to be discriminated, namely, Ia afferents serving different triceps muscle heads innervate all triceps motoneurons strongly, a functionally appropriate situation since the different heads of the triceps muscle are synergists. In other words, even if a triceps motoneuron cannot be identified directly as such in a normal animal, the presence of correlated strong Ia afferent inputs from the different triceps muscle heads is a virtual sure bet that the motoneuron is a triceps motoneuron. If normal patterns of activity were responsible for generating this specificity, then the tight correlation of Ia afferent inputs from different triceps nerve heads should disappear after nonspecific reinnervation of muscles by triceps motoneurons. It does not. Accordingly, Ia afferents serving different heads of the triceps muscle must all be able to recognize and select the triceps motoneuron targets even in the absence of coordinated activity (94).

In the chicken embryo, the potential role of activity has been tested more directly. Virtually all muscle contractions were blocked by chronic application of *d*-tubocurarine during the period when Ia afferent connections are made (229). In addition, *d*-tubocurarine reduces motoneuron activity itself, specifically blocking the normal correlated bursting patterns of particular motoneuron pools (189). Despite this disruption of activity, Ia afferents established the normal pattern of synaptic connectivity. Moreover, because *d*-tubocurarine also inhibits naturally occurring motoneuron death, this experiment also rules out the possibility that inappropriate connections made by Ia afferents are eliminated through the selective death of inappropriately innervated motoneurons.

Appropriately specific connections from Ia afferents onto lumbar motoneurons are also formed in the chicken embryo in the absence of inputs descending from regions rostral to the thoracic spinal cord (255). These inputs play an important role in modulating sensory afferent inputs in the adult spinal cord; their lack of influence on the development of specificity emphasizes the relatively strong autonomy of Ia afferents in correctly recognizing motoneuron targets.

To summarize, during their development, Ia afferents establish connections onto motoneurons through a series of highly specific behaviors (Fig. 9*A*). First, they project their axons selectively in the dorsal funiculus. This is probably determined by a combination of timing and selective recognition of longitudinal guidance cues. Second, they contact peripheral target muscles, which invests them with different functional identities that must be matched to specific targets centrally. This is related to the specific expression of ETS genes (206). Third, they sprout collaterals into the ventral gray matter, a feat that requires immunity to ventral chemorepellents. Fourth, upon reaching motoneuron dendrites, they selectively innervate the functionally appropriate dendrites through what appears to be a process of specific recognition, although in the rat some inappropriate connections may be made and later removed. At the time they are innervated, the motoneurons have already extended axons along characteristic pathways in the periphery and innervated specific muscles, and therefore already have distinct identities. In addition, as mentioned previously, peripheral signals induce the expression of ETS genes in the motoneurons. Accordingly, it is likely that the motoneurons can be discriminated as postsynaptic targets by virtue of specific molecular cues on their surfaces. This could potentially occur through a homophilic molecular interaction mediated by the coordinated expression of ETS genes in the motoneurons and the homonymous sensory neurons (206).

B. Development of Connections From Long-Projecting Premotor Interneurons

1. Reticulospinal projections

The reticulospinal projection is a phylogenetically ancient component of the vertebrate motor system. It represents the largest source of descending input to the spinal cord in anamniotes, where the number of reticulospinal neurons, especially the subset of primary reticulospinal neurons, is small enough that many have been individually identified on the basis of position and axon trajectory. Their presence, distribution, morphology, and function vary somewhat among anamniote species. The two best known primary reticulospinal neurons are the commissural Mauthner neuron, first discovered in fish, and the ipsilaterally projecting Müller neurons, first discovered in lamprey (see Ref. 272). Some of the salient features of Mauthner and Müller neurons in a "generic" anamniote, ignoring species differences, follow. The Mauthner neuron axon decussates at the level of the soma and descends in the medial longitudinal fascicle. It makes monosynaptic excitatory connections onto motoneurons and commissural interneurons on the contralateral side of the spinal cord and mediates the rapid early phase of reflex escape behavior in teleost fish (reviewed in Refs. 65, 89, 90; see also Ref. 272). Synapses onto motoneurons are chemical, whereas synapses onto commissural interneurons are electrical. The Müller neurons have ipsilat-

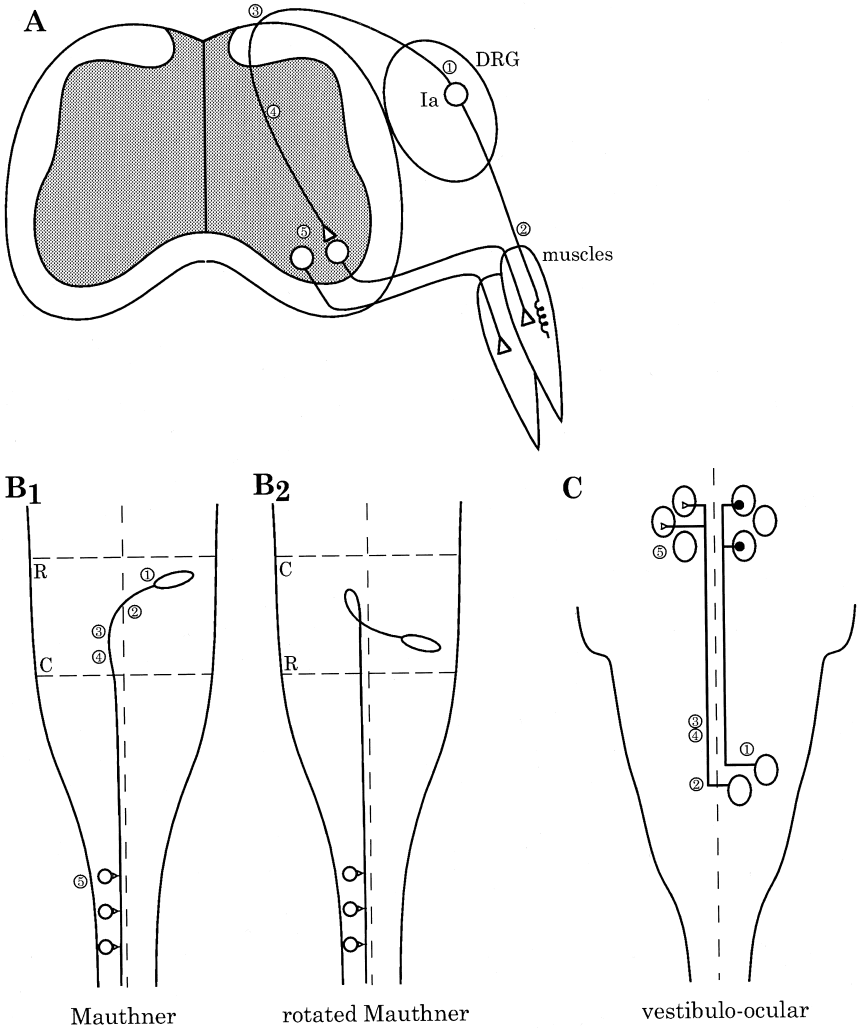

FIG. 9. Connections from premotor neurons to motoneurons are established through a series of highly specific behaviors. *A*: Ia afferents extend axons centrally (*1*) and peripherally at about the same time. The peripheral axon contacts a specific muscle (*2*), whereas the central axon bifurcates in the dorsal funiculus (*3*). After a delay, collaterals sprout into the gray matter (*4*) and reach the ventral region by virtue of immunity to ventral chemorepellents. Upon reaching motoneurons, synapses are selectively made predominantly onto the motoneurons innervating the appropriate muscle. *B1*: reticulospinal Mauthner neuron extends an axon obliquely caudad (*1*), crosses the midline floor plate (*2*), selects a trajectory in the medial longitudinal fascicle (*3*) in the caudal direction (*4*), and makes monosynaptic excitatory connections onto motoneurons (*5*). *B2*: the behavior of the axon of a Mauthner neuron in a rotated segment of hindbrain illustrates the course correction made after midline crossing. *C*: vestibulo-ocular neurons project axons toward the midline (*1*), in contrast to nearby vestibulospinal neurons that project caudally. At the midline, axons from different pools of vestibulo-ocular neurons react differently (*2*), one pool crossing the midline and the other not. Both sets of axons select a trajectory in the medial longitudinal fascicle (*3*) in a rostral direction (*4*), in contrast to the axons of nearby reticulospinal neurons, which project caudally in the same fascicle. Once they reach the region of the extraocular motoneurons, each set of vestibulo-ocular axons terminates on a characteristic set of motoneuron pools, with either excitatory (triangles) or inhibitory (solid circles) connections.

eral axons that descend in the medial longitudinal fascicle and make monosynaptic excitatory connections onto motoneurons and interneurons on the same side of the spinal cord. Synapses onto motoneurons can be chemical and electrical (272). Most of the primary reticulospinal neurons, including the Mauthner and Müller neurons, are phasically active during locomotion and other spinal motor activity (160, 161).

In amniotes, the reticulospinal population is greatly expanded relative to anamniotes. Nevertheless, individual reticulospinal pools can be identified on the basis of position and axon trajectory (chicken, J. Glover, G. Petursdottir, A. Asgeirsdottir, and H. Bjørgvinsson, unpublished observations; mouse and rat, Ref. 14). The connectivity patterns of amniote reticulospinal neurons are less well described, but some make monosynaptic connections onto motoneurons (4, 258).

The early development of reticulospinal axon trajectories has been studied in both anamniotes and amniotes (reviewed in Ref. 115). In both, the reticulospinal neurons are the first to project to the spinal cord, in line with their early generation times. The majority of reticulospinal axons project longitudinally in the mlf, choosing either an ipsilateral or a contralateral trajectory. Commissural reticulospinal axons typically cross the midline at the same level as the parent soma. As was the case for spinal commissural interneurons, the commissural reticulospinal neurons ignore ipsilateral longitudinal pathways but select the same pathways on the opposite side.

Reticulospinal axons that project ipsilaterally versus contralaterally do so appropriately from the outset (14, 115, 226, 243, 321), much like spinal interneurons (49, 314). The choice to cross or not to cross is unrelated to time of birth or timing of axon outgrowth, and the same choices are made over protracted time periods as new reticulospinal neurons differentiate (14, 115, 226). This means that the axons follow guidance cues that are not temporally restricted. Axon trajectory is related to soma position, although the ultimate positions of some reticulospinal neurons are assumed by secondary migration after axon outgrowth and may not reflect a positional determination of axon trajectory (14, 226).

Evidence that reticulospinal axon trajectory is established sequentially by different guidance cues (Fig. 9*B*) comes from the behavior of Mauthner neuron axons after transplantation of rostrocaudally rotated portions of the hindbrain (137). Initally, the rotated Mauthner neurons project obliquely rostrad instead of caudad as they normally do, suggesting an initial ballistic trajectory defined by intrinsic polarity. They approach and cross the midline, suggesting an interaction with diffusible substances from the floor plate. On reaching the other side, most then make a course correction and project caudally in the medial longitudinal fascicle. This course correction is interesting because it shows that the cue for longitudinal growth has a directionality that is independent of the orientation of the tissue in which the axons grow. This means either that the directionality is respecified in the rotated piece of hindbrain or that the cues for longitudinal growth originate from outside the rotated piece, for example, by ingrowth of other longitudinal axons.

In *Xenopus*, the first reticulospinal axons make synaptic contacts with primary motoneurons as they grow through the spinal cord (323). At this time, the motoneurons have already extended axons into the periphery and innervated muscle. Beyond this, no analysis has been made in any species of the initial specificity with which different types of reticulospinal neurons make synaptic contacts in the spinal cord. One might ask, given the distributed effects of many of the reticulospinal neurons, whether much specificity exists. For example, the Mauthner neuron makes excitatory connections with many motoneurons and interneurons along the length of the spinal cord. Nevertheless, it must select motoneurons and the correct inhibitory commissural interneurons among other potential targets, and it must make the appropriate types of synapses, namely, chemical onto the motoneurons and electrical onto the interneurons.

Despite the lack of any direct analysis of synaptic specificity, behavioral studies have shown that coordinated swimming movements and phase-locked impulse activity in the reticulospinal neurons appear within a few hours of the arrival of reticulospinal axons in the spinal cord (323). This does not prove initially appropriate connections but places a definite and short window on the time within which any potential mistakes must be corrected. As is discussed in section IV*C*, coordinated patterns of electrical activity are unlikely to play a role in establishing the correct pattern of connections.

2. Vestibulo-ocular projections

The vestibulo-ocular system is also phylogenetically ancient and is organized on similar principles throughout the vertebrate radiation, although with interesting variants, especially in cyclostomes (101). In general, the projection from vestibulo-ocular interneurons to extraocular motoneurons exhibits a highly stereotyped pattern of crossed excitatory and ipsilateral inhibitory connections that couples the activation of specific semicircular canals to the activation of specific extraocular muscles. This stereotypy ensures that conjugate eye movements compensate for head movements such that gaze can be fixed even when the head is not.

The development of the vestibulo-ocular projections to the mesencephalic oculomotor nuclei has been studied in the chicken embryo (Fig. 9*C*). They originate from discrete pools of vestibular neurons that lie in characteristic domains within the brain stem (53, 110, 112). Some vestibulo-ocular pools project obliquely in the brachium conjunctivum, whereas some project directly medially toward the midline and then rostrally in the medial longitudinal fascicle, distinguishing them from nearby reticulospinal neurons that project caudally in the same fascicle. Each vestibulo-ocular pool projects either ipsilaterally or contralaterally and contacts specific sets of extraocular motoneuron pools. Evidently, those projecting ipsilaterally make inhibitory connections, whereas those projecting contralaterally make excitatory connections (reviewed in Ref. 112).

The choice to cross or not to cross the midline and the choice to take a rostrad trajectory in the medial longitudinal fascicle appear to be made at the outset, because the relationship between spatial domains and axon trajectories is evident at early stages of axon outgrowth (107, 111, 115). For example, a pool of vestibulo-ocular neurons that projects ipsilaterally in the medial longitudinal fascicle does so despite lying in close proximity to the lateral longitudinal fascicle, in which a neighboring vestibulospinal neuron pool projects. By analogy to the behavior of reticulospinal neurons and spinal interneurons, it seems likely that the vestibulo-ocular neurons also follow a series of guidance cues that establishes the appropriate trajectory during initial outgrowth.

The vestibulo-ocular axons reach the level of the extraocular motoneuron pools after the motoneurons have extended axons into the periphery. Collaterals do not appear until a couple of days later, echoing the delayed sprouting of collaterals exhibited by Ia afferents. As the collaterals sprout, they grow selectively toward specific regions within the extraocular motoneuron population, corresponding to the locations of the appropriate motoneuron pools (112, 116). The nearby parasympathetic motoneurons of the Edinger-Westphal nucleus are almost never contacted by vestibulo-ocular collaterals, despite being well within their reach. This selective collateral outgrowth suggests the formation of initially appropriate synaptic connections, an issue that can be addressed more directly in the future using electrophysiological recordings. Anatomically, as soon as the collaterals establish substantial numbers of terminal branches,

these are largely restricted to the appropriate motoneuron pools (112, 149).

An additional example of selective collateral outgrowth by premotor projection interneurons is found in the development of corticospinal projections. These have quite a different behavior compared with their brain stem cousins when it comes to axon outgrowth, because cortical neurons in general have a predilection for extending axons along inappropriate trajectories, anomalies that are later eliminated by wholesale retraction (reviewed in Ref. 252). When it comes to collateral sprouting, however, corticospinal axons exhibit a selective behavior similar to that of the vestibulo-ocular neurons. Collaterals of axons originating from forelimb and hindlimb regions of the sensorimotor cortex sprout selectively into forelimb- and hindlimb-innervating regions of the spinal cord, respectively (176).

To summarize, premotor projection interneurons, like Ia afferents, establish connections onto motoneurons through a series of highly specific behaviors (Fig. 9). They extend axons along the appropriate trajectories from the outset (except for cortical axons). Upon reaching the region containing target motoneurons, they may either make synaptic connections rapidly or after substantial delay. In both cases, indirect evidence suggests that the initial pattern of connections is appropriately specific. The premotor axons, like the Ia afferents, reach their motoneuron targets after the motoneuron axons have extended into the periphery. The motoneurons already have distinct identities at this point, and it is plausible that they can be discriminated by virtue of molecular cues on their surfaces.

C. Development of Connections From Local Premotor Interneurons

Studying the development of connections from local premotor interneurons onto motoneurons is hampered by their close proximity and by the multiple functions individual local interneurons may serve (see Ref. 220). Indeed, this has long hindered the description of local premotor interneuron development in amniotes. In the spinal cord of anamniotes, where fewer neurons make for an easier attack, it is quite clear that identifiable local premotor interneurons extend their axons along specific trajectories from the outset (181, 269), and behavioral and electrophysiological studies have shown that reflex and locomotory behaviors are composed of coordinated motor elements not long after interneurons reach potential motoneuron targets (27, 152, 320).

Despite the lack of direct evidence, several types of indirect evidence suggest that appropriately specific connections are made by local premotor interneurons onto motoneurons at an early stage of development in both anamniotes and amniotes.

First, behavioral analysis and electromyogram (EMG) recordings show that movements and patterns of muscle activation are coordinated shortly after their first appearance (reviewed in Refs. 21, 22, 265). In the chicken embryo, for example, flexor and extensor activity alternates at individual joints shortly after the muscles become active (23). At later stages, when embryonic motility is exuberant, what appear to be jerky and uncoordinated movements actually arise from variable patterns of coordinated interjoint movements (328). The EMG recordings and recordings from motoneurons also reveal substantial organization in activity patterns (31, 187, 248–251, 256). Refinement does occur (reviewed in Ref. 21) but evidently operates on an initially organized set of connections. In general, the local and propriospinal interneuronal projections that presumably underlie interjoint and interlimb coordination appear anatomically well before limbs become motile (42, 43, 254, 342). The functional development of these connections remains a relatively uncharted field.

Second, coordinated patterns of activity can develop *1*) before the ingrowth of Ia afferent collaterals into the cord (23), *2*) in the absence of connections from Ia afferents and from more rostral regions of the spinal cord and brain stem (32, 127, 253, 330), and *3*) in the absence of normal sensory feedback (88, 188, 327, 331; see also Refs. 157, 113), although subtle effects of sensory disruption may be obtained (334). Sensory and descending inputs have an overriding influence on the selection and coordination of motor patterns at later stages (31), presumably by gating impulse flow through the interneurons. Despite their "subservient" position in this hierarchy, the local interneurons evidently have the autonomy to synapse selectively on motoneurons and activate them in coordinated patterns in the absence of sensory and descending influences.

Third, patterns of motor activity that are characteristic for different regions of the spinal cord develop autonomously after transplantation. For example, brachial segments transplanted to lumbar levels before neuronal differentiation in the chicken embryo eventually innervate the hindlimbs but drive them in synchronous movements characteristic of the wings, whereas lumbar segments transplanted to brachial levels eventually innervate the wings but drive them in alternating movements characteristic of the legs (238). Other studies confirm that regardless of which muscles they innervate, motoneurons receive connections from interneurons that are functionally appropriate for the spinal segments in which they reside (188, 246, 327). This means that information intrinsic to different regions of the spinal cord is responsible for establishing connectivity between premotor interneurons and motoneurons. The pattern of connectivity is not dependent on retrograde signals from the muscles that inform central circuits of what they should be doing to

create sensible output. It is tempting to conclude that discriminable molecular markers of motoneuron identity provide the intrinsic information.

Curiously, the central connections that develop appropriately and are initially active despite being mismatched to the periphery become inactive at later stages of development. This evidently occurs through a fall in the net synaptic activation supplied by spinal interneurons to the motoneurons (171, 246, 247, 305). The reason for this is still unknown, but the phenomenon implies a central mechanism that can recognize and suppress motor activity that is not appropriate for the peripheral targets innervated.

Fourth, normal motor patterns develop in embryos subjected to various forms of pharmacological blockade of activity (132, 133, 189, 318; see also Ref. 333). The elimination of potentially inappropriate connections through activity-dependent processes (see also sect. IV*A*) thus does not seem to be a prominent mechanism in the establishment of appropriate motor connections (in contrast to the pivotal role of activity in many sensory systems; reviewed in Ref. 119). In none of these studies, however, has neural activity during the blockade been assessed rigorously. Moreover, blocking activity may have subtle effects on motor connectivity that are not revealed by the limited behavioral and electrophysiological tests that have been utilized. For example, activity affects the morphological, electrophysiological, and molecular development of motoneurons, all of which could influence synaptic connectivity (153–155).

These arguments for early specificity are not meant to imply that connections from interneurons to motoneurons are laid down in immutable stone. Changes do occur. Motor patterns are modified during normal development (21, 22, 256), presumably in part through the modulation of existing connections by later-developing sensory and descending inputs (32, 253, 320). Although motor refinement need not reflect the presence of initially inappropriate connections, there is still too little information available to dismiss elimination or reorganization of connections from premotor interneurons in the establishment of the mature connectivity pattern.

V. SUMMARY

It is perhaps premature to draw global conclusions about the development of specific connectivity in motor systems, when so few elements of motor systems have been studied comprehensively. Nevertheless, on the basis of the information presently at hand, the following statements illustrate what seem to be the emerging general principles.

1) Motoneuron identity is determined, evidently in a stepwise fashion, by environmental factors acting on the cell body before axon outgrowth and during axon growth into the limb. The final step emerges from a highly resolved system of positional information in which the micropositions of the motoneuron soma in the neural tube and, potentially, of its peripheral axon in the limb, are decisive for its identity. The sequelae of motoneuron determination are likely to include the expression of specific surface molecules that guide motoneuron axons onto appropriate peripheral pathways and that engender motoneurons with discriminable labels that can be used by potential premotor neurons.

2) Some aspects of premotor interneuron identity, for example, axon trajectory and target selection, are likely to be determined in much the same way as motoneuron identity. Central target selection by Ia afferents, in contrast, is determined by a process of peripheral specification, following axon outgrowth and contact of peripheral targets.

3) Both premotor interneurons and Ia afferents establish connections with motoneurons through a sequence of highly specific behaviors. The sequence begins with initially appropriate axon outgrowth along specific trajectories. This is controlled by multiple guidance cues that steer axons through a series of choice points, gradually restricting their access to potential targets.[3] Upon reaching the appropriate target region, at least some premotor axons select the appropriate motoneurons with little error, giving rise to initially appropriate connections and coordinated activity. Presumably this selection occurs through the recognition of surface labels on motoneurons.

4) Activity does not play a major role in the establishment of specific connections from Ia afferents onto motoneurons, nor evidently in the establishment of connections from premotor interneurons that underly basic elements of motor coordination. This implies that activity-dependent removal of potentially inappropriate connections, a hallmark of the development of many sensory systems, is less prominent in the development of motor connectivity.

With this paper I honor the memory of Dr. Walter Heiligenberg, teacher and friend, who died in a tragic air accident in 1994.

I thank Anne Bekoff, Judith Eisen, Eric Frank, Kristian Gundersen, Jan Jansen, and Bjarne Waaler for critical reading of the manuscript and Håvard Tönnesen for assistance in making figures.

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[3] Corticospinal neurons represent a premotor interneuron population that appears to be less specific in its outgrowth behavior, a feature that seems to be characteristic for cortical efferents in general (see Ref. 176).

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