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Turning Heads: Development of Vertebrate Branchiomotor Neurons

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Abstract

The cranial motor neurons innervate muscles that control eye, jaw, and facial movements of the vertebrate head and parasympathetic neurons that innervate certain glands and organs. These efferent neurons develop at characteristic locations in the brainstem, and their axons exit the neural tube in well-defined trajectories to innervate target tissues. This review is focused on a subset of cranial motor neurons called the branchiomotor neurons, which innervate muscles derived from the branchial (pharyngeal) arches. First, the organization of the branchiomotor pathways in zebrafish, chick, and mouse embryos will be compared, and the underlying axon guidance mechanisms will be addressed. Next, the molecular mechanisms that generate branchiomotor neurons and specify their identities will be discussed. Finally, the caudally directed or tangential migration of facial branchiomotor neurons will be examined. Given the advances in the characterization and analysis of vertebrate genomes, we can expect rapid progress in elucidating the cellular and molecular mechanisms underlying the development of these vital neuronal networks.

Keywords

hindbrain; motor neuron; rhombomere; axon guidance; anatomy; specification; neuronal migration; branchial arch; cranial; segmentation

INTRODUCTION

The vertebrate head contains muscles that control eye, facial, and head movements, epitomized by the ability of humans to generate subtle and moving facial expressions and to generate sounds that underlie language and speech. Although more primitive aquatic and terrestrial vertebrates lack the vocal skills of mammals and birds and the facial dexterity of mammals, all animals form homologous muscles that are essential for eye, jaw, and tongue movements associated with tracking, capturing, chewing, and swallowing food. The activities of these muscles are controlled by clusters of motor neurons, collectively called the cranial motor neurons and located in the brainstem (midbrain and hindbrain). There is keen interest in understanding the mechanisms underlying cranial motor neuron development, mainly because the anatomy and organization of these neurons within the brain provide an excellent framework to address fundamental questions of neural development.

Overview of Neuroanatomy of Cranial Motor Neurons

Cranial motor neurons can be classified into three types: somatomotor, visceromotor, and branchiomotor (Kandel et al., 2000). Somatomotor neurons are found in the midbrain and hindbrain, and innervate muscles that control eye and tongue movements. The visceromotor

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neurons (also called general visceral motor neurons) are found in the hindbrain, and innervate parasympathetic neurons that innervate tear glands, sweat glands, and the smooth muscles and glands of the pulmonary, cardiovascular, and gastrointestinal systems. The branchiomotor neurons (also called special visceral motor neurons) are located in the hindbrain, and innervate branchial (pharyngeal) arch-derived muscles that control jaw movements, facial expression, the larynx, and the pharynx. During development, the cranial motor axons exit the brainstem through specific cranial nerves (nIII to nXII; see below). While axons of all somatomotor neurons, except nIV, exit the brain ventrally from the basal plate of the neural tube, all subtypes of visceromotor and branchiomotor axons exit the brainstem dorsally from the alar plate (Lumsden and Keynes, 1989).

This review will focus on the branchiomotor neurons (BMNs), which are composed of several subtypes dedicated to specific functions. In humans, the trigeminal motor axons exit through cranial nerve V and innervate muscles of mastication and a few muscles of the palate, middle ear, and chin (Kandel et al., 2000). The facial motor axons exit through cranial nerve VII and innervate muscles of facial expression and muscles of the middle ear and upper neck. The glossopharyngeal motor axons exit through cranial nerve IX and innervate one muscle of the palate that dilates the pharynx. The vagal motor axons exit through cranial nerve X, and innervate several muscles of the larynx and pharynx. The spinal accessory motor axons exit through cranial nerve XI and innervate neck muscles (Kandel et al., 2000). The homologues of these motor neurons are found in other vertebrates and innervate different but homologous muscles (Lumsden and Keynes, 1989; Gilland and Baker, 1993; Kontges and Lumsden, 1996; Schilling and Kimmel, 1997). For example, in zebrafish, the trigeminal and facial motor neurons innervate muscles in the jaw and jaw-support structures, respectively, while the glossopharyngeal and vagal motor neurons innervate gill muscles (Hatta et al., 1990; Schilling and Kimmel, 1997; Higashijima et al., 2000).

Diseases Manifesting Cranial Motor Neuron Dysfunction

Developmental disorders affecting cranial motor neuron (CMN) organization and function are relatively rare. In cases of Pierre-Robin syndrome, patients exhibit feeding and respiratory problems, possibly due to defects in CMN development (Abadie et al., 2002). The Moebius syndrome and some forms of autism are associated with the loss of brainstem motor nuclei (Rodier et al., 1996; Bonanni and Guerrini, 1999).

In contrast to the rarity of CMN-associated developmental disorders, CMN dysfunction is frequently associated with symptoms of specific neurodegenerative diseases. In particular, amyotrophic lateral sclerosis (ALS) is characterized by a progressive loss of upper motor (corticomotor) and lower motor (brainstem and spinal motor) functions. Diminution of orofacial and tongue functions is an early manifestation of ALS (also called motor neuron disease; Urban et al., 2001). Several mouse models of ALS mimic symptoms of the human disease but carry mutations in different genes. In these mouse models, onset of disease symptoms is correlated with degeneration of various brainstem motor nuclei (LaVail et al., 1987; Chiu et al., 1995; Haenggeli and Kato, 2002). These data suggest that branchiomotor and somatomotor neuron loss may play a significant role in the etiology of ALS. CMN degeneration may also occur in Parkinson's disease (Hirsch et al., 1987).

COMPARATIVE ANATOMY OF BRACHIOMOTOR NEURON (BMN) ORGANIZATION

Recently, there has been considerable interest in the functional analysis of BMN development in zebrafish, chick, and mouse. To appreciate the common themes (and occasional differences) that these studies illustrate, it is essential that we understand the fundamental similarities in

the organization of the BMNs in these model organisms. Although focused on the BMNs, the following discussion also includes other brainstem efferent neurons such as the somatomotor neurons (SMNs) and visceromotor neurons (VMNs), because there are currently few molecular markers to distinguish between specific types of BMNs and VMNs.

The Developing Hindbrain

The anterior aspect of the developing neural tube undergoes varying degrees of expansion/swelling along the mediolateral axis, generating the earliest subdivisions of the brain into the fore-, mid-, and hindbrain. The hindbrain region is further partitioned into seven or eight compartments called rhombomeres. Several studies have demonstrated that rhombomeres represent polyclonal compartments (reviewed in Lumsden, 1990) that are distinguished from each other by limited cell movements (Fraser et al., 1990; Birgbauer and Fraser, 1994), differential cell adhesion (Guthrie and Lumsden, 1991; Wizenmann and Lumsden, 1997), differential cell repulsion (Xu et al., 1995, 1999; Cooke et al., 2001; reviewed in Xu et al., 2000; Cooke and Moens, 2002), and compartment-specific expression of homeobox genes (reviewed in Wilkinson and Krumlauf, 1990; Lumsden and Krumlauf, 1996). Each rhombomere contains a characteristic, segmentally reiterated set of neurons, including motor neurons and reticulospinal interneurons (Kimmel et al., 1985; Metcalfe et al., 1986; Lumsden and Keynes, 1989; Clarke and Lumsden, 1993; reviewed in Glover, 2001). The molecular bases of hindbrain segmentation, and specification of rhombomere identities have been reviewed recently (Moens and Prince, 2002) and will be later discussed only in the context of BMN specification. Cranial neural crest cells migrate ventrolaterally in three streams at the level of the even-numbered rhombomeres into the branchial (pharyngeal) arches to generate diverse cell types, including peripheral neurons and glia, cartilage, bone, and pigment cells (reviewed in Trainor and Krumlauf, 2000; Helms and Schneider, 2003; Halloran and Berndt, 2003).

Organization of BMNs and Other Hindbrain Efferent Neurons

There is a rich literature describing various hindbrain efferent neuron populations, especially the BMNs, because of the relative ease with which these neurons can be retrogradely labeled with fluorescent tracers from the cranial nerve roots. These data collectively provide us tremendous insights into the development and evolution of the vertebrate brainstem and its resident neurons (reviewed in Fritzsche, 1998; Glover, 2001). Here, I will focus on the organization of BMNs in zebrafish, chick, and mouse embryos (Fig. 1), because the array of genetic, genomic, molecular, and embryologic tools available in these organisms holds great promise for elucidating the mechanisms underlying the development of these neurons and their axonal projections. Figure 1 and the following text are based upon data or adapted from figures in the following studies: zebrafish (Kimmel et al., 1985; Hatta et al., 1990; Trevarrow et al., 1990; Chandrasekhar et al., 1997; Schilling and Kimmel, 1997; Higashijima et al., 2000); chick (Lumsden and Keynes, 1989; Simon and Lumsden, 1993; Gilland and Baker, 1993; Simon et al., 1994; Fritzsche, 1996; Lumsden and Krumlauf, 1996; Jacob and Guthrie, 2000); mouse (Carpenter et al., 1993; Gilland and Baker, 1993; Fritzsche, 1996; Studer et al., 1996; Schneider-Maunoury et al., 1997; Garel et al., 2000; Studer, 2001). The neuroanatomy described below is limited to observations up to specific embryonic stages (Fig. 1). Hence, changes occurring after these times have been omitted. In addition, the names of muscles innervated by the cranial motor neurons have been omitted for simplicity but are available from other sources (Burt, 1993; Kontges and Lumsden, 1996; Schilling and Kimmel, 1997).

Trigeminal (cranial nerve V) BMNs—The nV motor axons innervate several muscles, including those of mastication, derived from the mesenchyme of the first branchial arch. The motor neuron cell bodies arise in rhombomeres 2 and 3 in all three species (Fig. 1). Similar to mouse and chick, the neurons in r2 in zebrafish extend axons dorsolaterally to their exit point (Fig. 2C). The axons of BMN cell bodies in r3 initially extend dorsolaterally, then turn rostrally

to reach their exit point in r2. In all species, the anterior turn occurs where the motor axons meet the ingrowing trigeminal sensory axons, suggesting a role for the afferent projections in motor axon guidance to the exit point (Moody and Heaton, 1983a; Ma et al., 2000; see below). In addition, the cranial nerve exit points may provide chemoattractant cues (Guthrie and Lumsden, 1992; see below). The motor neuron cell bodies are located medially early in development, then migrate laterally to their final positions, and in chick, this migration requires the ingrowth of trigeminal sensory neurons (Moody and Heaton, 1983b).

Facial (cranial nerve VII) BMNs—The nVII motor axons innervate muscles derived from the second branchial arch. The motor neurons are generated in r4, with a few cells also induced in r5 in chick (Fig. 1). In mouse and zebrafish, these neurons migrate caudally from r4, adjacent and parallel to the floor plate, into r6 (mouse) or r6 and r7 (zebrafish; Fig. 2). The cell bodies appear to migrate into a leading edge protrusion, in a process called perikaryal translocation (Book and Morest, 1990). As the cell bodies migrate caudally out of r4, they leave behind an axonal process that reflects the migratory path. In addition, the motor neuron growth cones migrate dorsolaterally toward their exit point in r4 (Fig. 2D). As a result, the axonal projections of these neurons have a characteristic looping morphology or genu. In zebrafish, the motor neurons are generated in a small domain in the center of the anteroposterior span of r4. As a result, the axons composing the facial genu in zebrafish are tightly bundled (Fig. 2A). In contrast, motor neurons are generated along the entire anteroposterior span of r4 in mouse, and the axons forming the facial genu are spread out, generating a fan-shaped structure. The facial genu loops medially around the abducens (nVI) somatomotor nucleus located in r5 and r6 (Fig. 1; see below). Some chick motor neurons also undergo limited caudal migration, but they do so without forming a genu (Jacob and Guthrie, 2000). In all three species, the motor neurons migrate laterally away from the floor plate to their final positions.

Glossopharyngeal (cranial nerve IX) BMNs—These motor neurons are induced in rhombomere (r) 6 in mouse, and in r6 and r7 in chick. The cell bodies remain stationary, and their axons extend dorsolaterally toward their exit point in r6. In contrast, the zebrafish glossopharyngeal motor neurons are located in r7, and their axons form a genu, which is characteristic of caudal migration. This result, together with the observation that these BMNs are absent in the zebrafish *valentino* mutant that exhibits defective r5 and r6 (but not r7) development, suggests that the nIX neurons are born in r6 in zebrafish as well, then migrate caudally into r7 (Chandrasekhar et al., 1997). The nIX motor axons innervate a single muscle derived from the third branchial arch.

Vagal (cranial nerve X) and Accessory (cranial nerve XI) BMNs—These motor neurons are generated in r7 and the caudal hindbrain (r8; Fig. 1). In zebrafish, these neurons arise exclusively from the caudal hindbrain (Fig. 2A,B,F). In chick, the nX and nXI axons exit through a common root in r7 (Lumsden and Keynes, 1989; Gilland and Baker, 1993), while in mouse, the nX axons exit the brainstem in r7, and the nXI axons exit in r8/rostral spinal cord (Gilland and Baker, 1993). The nXI motor nucleus has not been described in zebrafish (Wullimann et al., 1996). The nX motor axons innervate muscles derived from the mesenchyme of the fourth, fifth, and sixth branchial arches.

Otic and Lateral Line Efferent (OLe) Neurons—The otic efferent axons (cranial nerve VIII; all species) and lateral line efferent axons (zebrafish) also exit in r4 in close association with the facial (nVII) BMN axons (Fig. 1). Therefore, lipophilic dye application at the r4 exit point retrogradely labels the facial BMNs and these efferent neurons. In chick, a large number of otic efferent cell bodies migrate across the floor plate in r4 (Simon and Lumsden, 1993). The mouse otic efferent neurons also migrate within r4, but in a manner similar to the facial BMNs, adding fibers at the level of the nVII genu (Fritzsche, 1996). Furthermore, the neurons

extend prominent secondary processes that cross the floor plate. In zebrafish, all efferent neurons exiting from r4 migrate into r6 and r7, and there are no molecular markers to distinguish between the facial BMNs, and the otic and lateral line efferent neurons (Chandrasekhar et al., 1997; Higashijima et al., 2000; Bingham et al., 2002). However, a subset of “facial BMNs” has prominent lateral processes that cross the floor plate (Fig. 2E; Higashijima et al., 2000; A.C., unpublished observations). Because otic efferent neurons in chick and mouse have contralateral projections, it is likely that the zebrafish efferent neurons with contralateral projections represent otic and lateral line efferent neurons (Fig. 1).

SMNs—The oculomotor (cranial nerve III), trochlear (cranial nerve IV), and abducens (cranial nerve VI) motor neurons innervate extraocular muscles in all species. The oculomotor (nIII) neurons are located in the midbrain and innervate four muscles. The trochlear motor neurons are located adjacent to the floor plate in all species, either in r1 (chick and mouse) or in the caudal midbrain/r0 (zebrafish; Moens and Prince, 2002). The nIV motor axons extend dorsolaterally away from the floor plate, and exit the neural tube dorsally to innervate an extraocular muscle located contralaterally. The abducens motor neurons are generated only in r5 (mammals) or in r5 and r6 (chick and zebrafish). The nVI motor axons exit the hindbrain ventrally through multiple rootlets, extend rostrally, and innervate one extraocular muscle. The hypoglossal (cranial nerve XII) motor neurons innervate tongue muscles in chick and mouse (Lumsden and Keynes, 1989; Gilland and Baker, 1993), but neither the neurons nor their targets have been described in zebrafish (Wullmann et al., 1996). The motor axons exit the hindbrain ventrally through rootlets located close to the floor plate.

VMNs—These motor neurons innervate parasympathetic ganglia controlling glandular secretions and smooth muscles associated with several organ systems. No VMNs have been definitively identified in zebrafish at the early stages examined (see Higashijima et al., 2000). In chick and mouse embryos, only VMNs associated with cranial nerve VII have been described. In both species, the facial VMNs arise laterally in r5, and their axons extend dorsostrally to exit with the VIIth cranial nerve. The mouse facial VMNs constitute the superior salivatory nucleus, and their axons exit the hindbrain as the intermediate nerve (Fritsch, 1996), while the chick facial VMN axons exit the hindbrain to form the palatine nerve (Jacob and Guthrie, 2000).

Overlap in BMN Subtypes—In all three species, some BMN subtypes occupy the same rhombomeres. In mouse and zebrafish, caudal migration of facial (nVII) BMNs results in the colocalization of facial and glossopharyngeal (nIX) BMNs in r6 (mouse) or r7 (zebrafish) (Fig. 1). Because facial BMNs are induced in r4, their identity, including migratory behavior, is likely to be specified in r4. Therefore, the nVII neurons are likely to maintain their specific identity even after they have migrated into caudal rhombomeres that generate other BMN subtypes. Indeed, in zebrafish, the nVII BMNs in r6 and r7 specifically express the cell adhesion marker *TAG-1* (Warren et al., 1999), while the nIX BMNs in r7 specifically express a different cell adhesion marker, *Dm-Grasp* (Kanki et al., 1994; Fashena and Westerfield, 1999), indicating that the two BMN subtypes maintain unique identities in the same rhombomere (Chandrasekhar et al., 1997). Interestingly, chick r7 generates glossopharyngeal and vagal BMNs, but it is not known how axons of the two subtypes pathfind through the same tissue to exit from different locations (Fig. 1). It is also notable that, in all three species, the facial and glossopharyngeal BMNs share rhombomeres to varying degrees with the abducens SMNs, VMNs, and otic/lateral line efferent neurons. However, the overlap may not be significant in every instance because of the characteristic, mutually exclusive locations of the neuronal cell bodies of each efferent type within the rhombomere. The role of differential cell body positions in specifying neuronal identities will be discussed later.

Cranial Nerve Exit Points—BMNs and VMNs exit by means of common nerve roots in the dorsal neural tube, whereas SMNs exit through separate rootlets in the ventral neural tube. In chick and mouse, the BMN/VMN nerve roots are located in the alar plate of the neural tube, while the exit points in zebrafish appear to be located much more ventrally (Fig. 2C,D,F). In chick, the exit points are generated by the aggregation of late-migrating neural crest cells (Niederlander and Lumsden, 1996), which express *cadherin7* (Nakagawa and Takeichi, 1995). *Krox20*-expressing neural crest cells are found in distinct foci adjacent to r2 and r4 in mouse embryos, marking the putative nV and nVII cranial nerve exit points (Wilkinson et al., 1989; Niederlander and Lumsden, 1996). *Krox20*-expressing cells at similar locations have not been described in zebrafish (Oxtoby and Jowett, 1993) up to 30 hours postfertilization (hpf), although substantial cranial neural crest migration has occurred by 24 hpf (Schilling and Kimmel, 1994).

Variability in BMN Number Across Species—While the relative sizes of the embryos make a comparison of absolute neuronal numbers meaningless, it is noteworthy that the size of the germinative zone generating BMNs relative to the anteroposterior extent of a given rhombomere is much smaller in zebrafish compared with chick and mouse. For example, the zebrafish trigeminal (nV) motor neurons are generated in a zone that occupies approximately 25% of the anteroposterior length of r2 or r3 (Fig. 2A), whereas the chick and mouse neurons are generated along the entire anteroposterior extent of the ventricular surfaces of r2 and r3 (Fig. 1). The disparity is even more pronounced in the numbers of glossopharyngeal neurons generated in zebrafish relative to mouse and chick. The functional bases, if any, for these differences are not known.

MECHANISMS UNDERLYING BRANCHIOMOTOR NEURON DEVELOPMENT

Mutations in several zebrafish and mouse genes lead to defective development of BMNs (summarized in Tables 1 and 2, respectively). In the following sections, I review our current understanding of BMN development in zebrafish, chick, and mouse, at the levels of neuronal induction, specification, cell body migration, and axon pathfinding. Some of these topics have been reviewed recently in the context of hindbrain segmentation (Moens and Prince, 2002), and motor neuron specification and pathfinding (Briscoe and Ericson, 2001; Jacob et al., 2001; Shirasaki and Pfaff, 2002). There is also some overlap between this review and a recent review on the development of cranial nerves in mouse (Cordes, 2001).

GUIDANCE OF BRANCHIOMOTOR AXONS

There are two phases in the extension of BMN axons to their target muscles. In the first phase, the motor axons grow within the hindbrain away from the floor plate toward the appropriate exit points in the dorsal neural tube (Figs. 1, 2). In the next phase, the axons exit the neural tube, extend into the appropriate branchial (pharyngeal) arches, and stop and make synapses on their appropriate target muscles. Several axon guidance molecules and receptors are expressed in interesting patterns in BMNs and surrounding tissues in various species, consistent with roles for attractive or repulsive interactions in guiding the BMN axons to their targets. However, the instances where specific functions have been defined are limited and come primarily from mouse knockouts and from the explant studies of Guthrie and colleagues (reviewed in Jacob et al., 2001).

BMN Axon Guidance Within the Hindbrain

Chemorepulsion away from the floor plate—BMN cell bodies remain ipsilateral in all species during the period of axon guidance to target tissues. Growth cones presumably form on the surface of the neurons distal to the floor plate, extending the axons away from the midline. In the zebrafish *cyclops* mutant lacking floor plate cells, nVII BMN axons (and cell

bodies) cross the midline, suggesting the absence of a midline repellent (Chandrasekhar et al., 1997). Various families of axon guidance molecules could potentially regulate the outgrowth of BMN axons away from the floor plate and are discussed below.

Netrins: Mouse trochlear (nIV) axons are repelled by *netrin1*-expressing cells in vitro (Colamarino and Tessier-Lavigne, 1995). Furthermore, BMN axon outgrowth from rat hindbrain explants is reduced in the presence of Netrin1 (Varela-Echavarría et al., 1997), and netrin receptors *Unc5h1* and *DCC* are expressed in rat cranial motor neurons shortly after the onset of axon outgrowth (Barrett and Guthrie, 2001). In zebrafish, *netrins* are expressed in the ventral hindbrain (Lauderdale et al., 1997; Strahle et al., 1997) and genes encoding netrin receptors are expressed rather broadly in the hindbrain at the time of BMN axonogenesis (Hjorth et al., 2001; Shen et al., 2002). Together, these data suggest that Netrin-mediated signaling may play a role in the repulsion of BMN axons from the midline.

Slits: While mouse homologues of the midline repellent *slit* (Brose et al., 1999) are expressed in the ventral hindbrain (Yuan et al., 1999; Holmes and Niswander, 2001), a slit receptor (*robo*) is expressed only in the branchial arches (Yuan et al., 1999). Similarly, while zebrafish *slits* are expressed in floor plate cells at the time of BMN axonogenesis (Yeo et al., 2001), *robos* are not expressed in BMNs (Challa et al., 2001; Lee et al., 2001). These data suggest that chemorepulsion of BMN axons away from the floor plate may not be mediated by Slit-Robo interactions.

Semaphorins: Because a zebrafish homologue of the chemorepellent semaphorin plays a role in guiding BMN axons outside the hindbrain (Xiao et al., 2003; see below), semaphorins may also play a role in keeping BMN axons away from the floor plate. BMN axon outgrowth from rat hindbrain explants is reduced in the presence of Sem D (Sema3A) (Varela-Echavarría et al., 1997). Of interest, BMN axons exit normally from the hindbrain in mice defective for *Neuropilin2*, a Sema3A receptor that is expressed in BMN nuclei (Chen et al., 1997, 2000; Giger et al., 2000), and in mice defective for *Sema3A*, which is expressed in the ventral hindbrain (Taniguchi et al., 1997; Varela-Echavarría et al., 1997; Catalano et al., 1998). However, because BMN axon outgrowth within the hindbrain was not specifically examined in the mutant embryos, defects in axon outgrowth at the ventral midline, and a specific role for semaphorins in this process, cannot be ruled out.

Ephrins: In the zebrafish (vertebrate) hindbrain, ephrin–Eph repulsive interactions play essential roles in generating rhombomere boundaries (Xu et al., 1995, 1999; Cooke et al., 2001; reviewed in Xu et al., 2000; Cooke and Moens, 2002). However, neither the receptors nor the ligands are specifically expressed in the floor plate or BMNs, discounting a role for these molecules in the repulsive guidance away from the floor plate. However, Eph–ephrin interactions may play a role in BMN axon guidance in the periphery (see below).

Chemoattraction toward exit points—In addition to repulsive cues directing BMN axons away from the floor plate, chemoattractant cues may direct axon outgrowth toward the nerve exit points. When chick rhombomeres 3 and 5 are replaced after a 180-degree reversal, a majority of nV and nVII motor axons grow correctly toward their appropriate exit points located near the new rostral margins (previously caudal) of the reversed rhombomeres (Guthrie and Lumsden, 1992). These results suggest that diffusible attractive cues originating from the cranial nerve exit points may steer the BMN growth cones away from the floor plate and out of the hindbrain. Exit point tissues are derived from the neural crest (Niederlander and Lumsden, 1996) and specifically express a cell adhesion molecule cadherin7 in chick (Nakagawa and Takeichi, 1995). However, exit point chemoattractant(s) have not yet been identified.

Other factors such as axon-substrate interactions also influence BMN axon outgrowth. The trigeminal (nV) sensory afferent and motor efferent fibers are closely associated within the hindbrain and peripherally as the axons extend into the first branchial arch (Moody and Heaton, 1983a,b; Chandrasekhar et al., 1997). In zebrafish, the nV afferent axons grow into the hindbrain (Metcalf et al., 1990) several hours before the exit of nV motor axons from the hindbrain (Higashijima et al., 2000), consistent with a role for nV afferent axons in nV motor axon guidance. When chick nV afferent axons are eliminated by ablation of the trigeminal ganglion, nV motor axons fail to exit the hindbrain (Moody and Heaton, 1983a). Similarly, nV motor axons fail to exit the hindbrain in *neurogenin1* knockout mice, where the trigeminal ganglion is missing (Ma et al., 2000). These results suggest that nV sensory axons may play similar roles in all species, perhaps serving as a substrate for the outgrowth of nV motor axons.

BMN Axon Guidance in the Periphery

Upon exiting the hindbrain, BMN axons extend ventrally into their appropriate target branchial arches. This outgrowth is mediated by a combination of chemoattractive and chemorepellent cues expressed in the branchial arches and ensures that the motor axons will enter the arch mesenchyme and be directed (channeled) to the appropriate muscle targets. None of the cues identified thus far impart target specificity to the BMN axon outgrowth process.

Chemorepellent Mechanisms—Semaphorins play a definitive role in guiding BMN axons into the branchial arches. In zebrafish, genes encoding two secreted semaphorins, *sema3A* and *sema3D*, are expressed in the branchial arch epithelium and mesenchyme, respectively (Halloran et al., 1998, 1999), while a transmembrane semaphorin (*sema4E*) is expressed in the arch epithelium (Xiao et al., 2003). Morpholino-mediated knockdown of *sema4E* leads to defasciculation of nVII axons, suggesting that *Sema4E* normally functions to restrict nVII axon extension to the second arch mesenchyme (Xiao et al., 2003). Furthermore, when *sema4E* is ubiquitously expressed in the embryo, BMN axon outgrowth into the branchial arches is specifically eliminated, indicating that *Sema4E* may play a significant role in the guidance of all BMN axons (Xiao et al., 2003). In mouse, a *Sema3A* receptor gene, *Neuropilin2*, is expressed in BMNs (Chen et al., 2000), whereas *Sema3A* is expressed in the first and second branchial arches (Taniguchi et al., 1997). The nV and nVII cranial nerves (containing nV and nVII BMN axons, respectively) are slightly defasciculated in *Neuropilin2* knockout mice (Chen et al., 2000; Giger et al., 2000) and more severely defasciculated in *Neuropilin1* and *Sema3A* knockout mice (Kitsukawa et al., 1997; Taniguchi et al., 1997). These data suggest strongly that Semaphorin-mediated repulsive interactions channel the extension of BMN axons into the branchial arch mesenchyme but are likely not involved in guiding axons of different BMN subtypes to specific arch targets.

Chemoattractive Mechanisms—In rat brainstem explant culture, axons of specific BMN subtypes extend robustly toward branchial arch explants, suggesting strongly that arch-derived chemoattractants play a role in BMN pathfinding in the periphery (Caton et al., 2000). A major component of the chemoattractants appears to be hepatocyte growth factor (HGF), because HGF-coated beads attract BMN axons, and anti-HGF antibodies inhibit BMN axon outgrowth toward branchial arch explants or HGF-coated beads (Caton et al., 2000). *HGF* is expressed at the appropriate time in branchial arches in rat and chick, and the gene encoding the HGF receptor *Met* is expressed in most mouse BMNs (Caton et al., 2000). Nonetheless, all BMN axons exhibit normal morphologies in *HGF* and *Met* knockout mice, suggesting either that HGF-Met interactions are not needed for BMN axon guidance in mouse or that HGF functions redundantly with other branchial arch chemoattractants (Caton et al., 2000). Indeed, explant studies show that BMN axon outgrowth is also promoted by brain-derived neurotrophic factor (BDNF), ciliary-derived neurotrophic factor, and cardiotropin (Naeem et al., 2002). Moreover, no BMN defects are evident in *BDNF* knockout mice (Ernfors et al., 1994; Jones et al.,

1994), consistent with the nonredundant nature of arch-derived chemoattractants. In chick, some ephrin ligands are expressed in specific arches, whereas EphA receptor tyrosine kinases are expressed in a subset of BMNs (Kury et al., 2000), suggesting that ephrins may also function as branchial arch cues.

INDUCTION OF BRANCHIOMOTOR NEURONS

The role of the hedgehog family of secreted proteins in inducing motor neurons at all axial levels in the vertebrate neural tube has been well established in the last decade (reviewed in Tanabe and Jessell, 1996; Briscoe and Ericson, 2001). In chick explant studies, *sonic hedgehog* (*shh*)-expressing tissue such as the notochord or recombinant Shh protein are sufficient to induce motor neurons in ventral neural tube explants derived from various axial levels, and this effect is blocked by anti-Shh antibodies (Roelink et al., 1995; Tanabe et al., 1995; Ericson et al., 1996). Furthermore, motor neurons are completely absent in *Shh* knockout mouse embryos (Chiang et al., 1996; Litingtung and Chiang, 2000a). Because zebrafish express multiple *hedgehogs* in the floor plate and notochord (Krauss et al., 1993; Ekker et al., 1995; Currie and Ingham, 1996), motor neurons are decreased but not lost completely in the zebrafish *shh* mutant (Chandrasekhar et al., 1998; Schauerte et al., 1998). However, removing multiple hedgehog activities leads to near absence of all motor neurons (Beattie et al., 1997; Bingham et al., 2001).

The roles of other components of the Hh signaling pathway, such as the Patched (Ptc) and Smoothened (Smo) transmembrane proteins, and the Gli transcription factors (Ingham, 1998; Litingtung and Chiang, 2000a; Ingham and McMahon, 2001) in motor neuron induction have also been investigated. In the absence of Hh ligand, Ptc inhibits Smo function, leading to repression of Hh-responsive genes. Hh binding to Ptc relieves inhibition of Smo, resulting in the activation of Gli transcription factors and Hh-responsive gene expression. In *Ptc* knockout mouse embryos, the neural tube is ventralized due to ectopic activation of Smo function (and Hh signaling), likely resulting in the induction of ectopic motor neurons at all axial levels (Goodrich et al., 1997). Conversely, expression of a *Ptc* deletion variant that inhibits Smo function irrespective of the presence of Hh ligand leads to dorsalization of the neural tube and to the cell-autonomous repression of motor neuron induction in chick embryos (Briscoe et al., 2001). While multiple *ptc* genes are expressed in the zebrafish neural tube (Concordet et al., 1996), no functional analyses have been carried out. Mutations in zebrafish *smo* lead to a severe reduction or loss of cranial and spinal motor neurons (Chen et al., 2001; Varga et al., 2001; G. Vanderlaan and A.C., unpublished observations), underscoring the central role of this receptor in motor neuron induction. Because the mouse genome contains only one *Smo* gene, it is likely that motor neuron induction will also be severely reduced in *Smo* knockout embryos, although this has not yet been examined (Zhang et al., 2001).

The roles of the Gli transcription factors in motor neuron induction appear to be less conserved among vertebrates. Multiple *gli* genes are expressed in the developing neural tube of all vertebrates (Lee et al., 1997; Sasaki et al., 1997; Ding et al., 1998; Matise et al., 1998; Ruiz i Altaba, 1998; Karlstrom et al., 1999, 2003). Furthermore, Gli1 orthologs function only as activators, while Gli2 and Gli3 orthologs exhibit activator and repressor activities (Ruiz i Altaba, 1999; Sasaki et al., 1999). Motor neurons (presumably at all axial levels) are induced normally in mouse *Gli1*, *Gli2*, and *Gli3* single knockouts (Ding et al., 1998; Matise et al., 1998; Park et al., 2000), and in *Gli1:Gli2* and *Gli1:Gli3* double mutants (Ding et al., 1998; Park et al., 2000). These data suggest that, in mouse, Shh signaling may induce motor neurons indirectly by suppressing Gli repressor function or that Gli-independent pathways may be involved (Krishnan et al., 1997). Consistent with the former, motor neurons are induced in the ventral neural tube of *Shh:Gli3* double mutants, indicating that Shh signaling antagonizes the function of Gli3 in repressing motor neuron induction (Litingtung and Chiang, 2000b).

Furthermore, dorsoventral patterning is normal in the double-mutant neural tube, suggestive of Shh-independent patterning mechanisms (Persson et al., 2002). In sharp contrast to the mouse *Gli1* mutant phenotype, loss of *gli1* function in zebrafish (Karlstrom et al., 2003) leads to reduction or loss of cranial motor neurons but not spinal motor neurons (Chandrasekhar et al., 1999). In addition, zebrafish *gli2* function appears to be dispensable for ventral neural tube development (Karlstrom et al., 2003). That motor neuron development is blocked only in the head in zebrafish *gli1* mutants suggests either that *gli1* may function redundantly with other *glis* to generate motor neurons in the spinal cord, or that *gli1* may be required for specifying motor neuron identity in the head (Chandrasekhar et al., 1999). Of interest, expression of a dominant-negative mouse Gli3 protein that inhibits all Gli-mediated transcription blocks motor neuron specification in the chick ventral neural tube (Persson et al., 2002), indicating that Gli activator function is necessary for motor neuron induction in chick. Furthermore, overexpression of *Xenopus gli1* or *gli2* leads to the generation of ectopic motor neurons (Ruiz i Altaba, 1998). These data suggest similar functions for *gli* genes in motor neuron induction between zebrafish, chick, and frog. Therefore, the different phenotypes of *gli* mutants in mouse and zebrafish may reflect species-specific differences in *gli* expression and/or function in motor neuron progenitors or the existence of Gli-independent pathways for inducing motor neurons in mouse.

SPECIFICATION OF BRANCHIOMOTOR NEURONS

Given the distinct identities of motor neurons born in adjacent rhombomeres, it is not surprising that mutations in several mouse and fish genes that change or eliminate specific rhombomere identities lead to pronounced deficits in the development of particular BMN subtypes (Tables 1, 2). In contrast, fewer genes have been identified that are specifically expressed in BMN progenitors and that are more directly (proximally) required for their specification.

Genes Regulating Rhombomere (and BMN) Identities

The genes analyzed thus far with demonstrated roles in specifying rhombomere (and BMN) identities belong mostly to the *hox* gene families and other transcription cofactors essential for Hox-mediated gene regulation (*pbx* and *meis* genes) or *hox*-regulated target genes (*gata* genes). Other rhombomere-identity genes also encode transcription factors (*krox20* and *valentino/kreisler*) that likely function upstream of *hox* genes. The roles of these genes in establishing and maintaining rhombomere identities have been reviewed recently (Moens and Prince, 2002). I will describe below the consequences of mutations in these genes for BMN development.

Krox20—This gene, encoding a zinc finger transcription factor, is specifically expressed in rhombomeres 3 and 5 from the earliest stages of development in all vertebrates examined, suggesting that it imparts specific identities to these compartments. In mouse *Krox20* mutants, r3 and r5 appear to be deleted and the mutant hindbrain is reduced in length (Swiatek and Gridley, 1993; Schneider-Maunoury et al., 1993). The trigeminal (nV) motor neurons are reduced in number or missing. In some mutants, motor neurons found in r2 exit the hindbrain in r4, characteristic of facial (nVII) motor neurons, suggesting either that nV axons in r2 are strongly attracted to the r4 exit point (possibly due to transfection of the nV neurons into nVII neurons), or that some nVII neurons migrate anteriorly into r2 in the absence of r3 territory (Schneider-Maunoury et al., 1997). The nVI somatomotor neurons normally found in r5 (Fig. 1) are absent in mutants. Specification of the nVII, nIX, and nX neurons and axonal pathfinding is not affected in mutant embryos, consistent with the normal development of the rhombomeres (r4, r6, r7, and caudal hindbrain) within which these neuronal populations arise (Swiatek and Gridley, 1993; Schneider-Maunoury et al., 1993, 1997). However, caudal migration of nVII

motor neurons from r4 is defective due to the absence of r5 (Garel et al., 2000; see also Studer, 2001).

Valentino/Kreisler—Mutations in the zebrafish *valentino* (Moens et al., 1996) and mouse *Kreisler* genes disrupt the structure and function of a bZIP transcription factor MafB (Cordes and Barsh, 1994; Moens et al., 1998), leading to aberrant development of r5 (mouse, fish) and r6 (fish). *Valentino/mafB* is expressed in r5 and r6. In both species, the nVI somatomotor neurons are absent (Moens et al., 1996) and correlates with *mafB* expression in the rhombomeres that give rise to these neurons (Fig. 1). In *Kreisler* mutants, the nVII motor neurons are generated in r4 and undergo normal caudal migration, but the visceromotor neurons that develop in r5 (Fig. 1) are missing (McKay et al., 1997; Garel et al., 2000). In *valentino* mutants, nVII motor axons are defasciculated and the motor neurons are poorly clustered, likely due to aberrant caudal migration of the neuronal cell bodies (Chandrasekhar et al., 1997). In addition, the nIX motor neurons are missing, probably because they are normally induced in r6 and subsequently migrate into r7. Branchiomotor and somatomotor neuron populations in unaffected rhombomeres develop normally in mutant embryos (Chandrasekhar et al., 1997).

Hoxa2—This paralog group 2 gene is expressed in the hindbrain from the r1/r2 boundary caudally and is the only *hox* gene expressed in r2 (Prince and Lumsden, 1994; Prince et al., 1998; reviewed in Lumsden and Krumlauf, 1996). In mouse *Hoxa2* mutants, r2 identity is lost, whereas more caudal rhombomeres are patterned normally (Gavalas et al., 1997). Correlating with loss of r2 identity, many motor neurons in the presumptive r2 and r3 region project axons that exit the hindbrain from r4. This finding could result from the loss of nV motor neuron identity or the loss of cranial nerve V exit point in r2 due to misspecification of the r2-derived neural crest that contributes to exit point tissue (Niederlander and Lumsden, 1996; Gavalas et al., 1997). The latter possibility appears unlikely because *hoxa2* is normally not expressed in r2-derived neural crest (Prince and Lumsden, 1994; Prince et al., 1998) and, therefore, may not be involved in the formation of nV exit point tissue. Furthermore, ectopic expression of chick *hoxa2* anterior to r2 leads to the formation of trigeminal-like motor neurons in r1 (Jungbluth et al., 1999), consistent with a direct role for *Hoxa2* in specifying nV identity.

Hoxa1 and Hoxb1—These paralog group 1 genes are expressed from the r3/r4 boundary caudally, and while *Hoxa1* expression is transient, *Hoxb1* is subsequently expressed at high levels in r4 (Wilkinson et al., 1989; Prince et al., 1998). In *Hoxa1* knockout mice, r5 appears to be deleted and r4 is reduced, shortening the hindbrain by one rhombomere (Carpenter et al., 1993). Development of nV, nIX, and nX motor neurons is not affected, although the nIX and nX cell bodies are displaced anteriorly relative to wild-type embryos due to the absence of r5. Although nVII motor neurons are generated in mutants, they fail to migrate caudally (Carpenter et al., 1993; Gavalas et al., 1998). In addition, many nVII-like motor neurons are located in r3, suggestive of extensive mixing between the r3 and r4 domains in mutants.

In *Hoxb1* mutants, r4 and nVII motor neuron development are specifically affected (Goddard et al., 1996; Studer et al., 1996). Motor neurons appear to be generated normally in r4, but fail to migrate caudally into r5 and r6, indicating that they may not be properly specified. The facial motor nucleus cannot be detected postnatally in mutant mice, suggesting that the nVII neurons may degenerate due to improper specification and/or migration (Goddard et al., 1996; Studer et al., 1996). In *Hoxa1:Hoxb1* double mutants, the BMN defects are exacerbated, indicating that the two genes function synergistically to regulate rhombomere identities and the development of resident neuronal populations (Gavalas et al., 1998; Rossel and Capecchi, 1999). In accordance with the mouse data, morpholino-mediated knockdown of *hoxb1a* function in zebrafish leads to the complete and specific inhibition of nVII neuron migration into r6 and r7, in a cell-autonomous manner, with no effects on the development of the nV and nX motor neurons (McClintock et al., 2002). These observations suggest that loss of *hoxb1*

function may lead to the mis-specification of migratory nVII neurons as nonmigratory nV neurons. Consistent with this suggestion, ectopic expression of *hoxb1b* in zebrafish leads to the apparent transformation of motor neurons in the r2 region into migratory nVII-like neurons (McClintock et al., 2002; S. Bingham and A.C., unpublished observations). Furthermore, when mouse *Hoxb1* is ectopically expressed in chick r2, resident BMNs extend axons into the second branchial arch, suggesting that motor neurons in the presumptive r2 region are misspecified as nVII neurons (Bell et al., 1999).

Pbx Genes

Multiple genes encoding this class of Hox-binding transcriptional cofactors have been identified in mouse and zebrafish (Popperl et al., 2000; Waskiewicz et al., 2002). Zebrafish *pbx* genes are expressed in broad overlapping domains, and *pbx2* and *pbx4* are expressed sufficiently early to play direct roles in generating rhombomere identities (Waskiewicz et al., 2002). In *lazarus* mutants deficient in *pbx4* function (Popperl et al., 2000), r4 fails to develop normally and nVII BMNs exhibit a cell-autonomous failure to migrate caudally (Cooper et al., 2003), a phenotype that is identical to that of embryos deficient in the function of a binding partner Hoxb1a (McClintock et al., 2002; see above). Furthermore, nV motor axons frequently exit from r4 (a non-cell autonomous effect), consistent with an effect on the identities of r2 and r3 (Cooper et al., 2003; see mouse *Hoxa2* phenotype above). When all maternal and zygotic *pbx* function is removed through an elegant combination of genetics and antisense morpholino treatment, the entire hindbrain spanning r2–r7 acquires an r1 “ground state” identity, and nV, nVII (and likely nIX) BMNs are eliminated and replaced by a small number of efferent neurons with r1 identity (Waskiewicz et al., 2002). In these *pbx*-less mutants, the caudal hindbrain is relatively unaffected, and a few nX motor neurons still differentiate. Development of rhombomeres and BMNs has not yet been characterized in the mouse *pbx1* mutant (Selleri et al., 2001).

Meis genes—Multiple *meis* genes, which also encode Hox-binding partners, have been characterized in zebrafish and are expressed in dynamic patterns during hindbrain development (Vlachakis et al., 2001; Waskiewicz et al., 2001; Zerucha and Prince, 2001; Choe et al., 2002). When Meis function is disrupted by sequestering the proteins in the cytoplasm with a Pbx4 fragment (see above), the development of r3, r4, and to some extent, r5 is severely affected (Choe et al., 2002). Consequently, the nV neurons in r3 are lost, and the nVII neurons are variably affected, but the defects need to be further characterized. The role of *meis* genes in hindbrain (and BMN) development has not been studied in mouse.

Gata2 and Gata3—These transcription factor genes are expressed in the ventral hindbrain (Nardelli et al., 1999; Bingham et al., 2002), and within this domain, mouse *Hoxb1* activates *Gata2* expression, which in turn activates *Gata3* expression (Nardelli et al., 1999; Pata et al., 1999). The mouse genes are expressed in the otic efferent neurons but not in the nV or nVII BMNs. In *Gata3* knockout mice, contralateral projections of otic efferents are severely reduced, while caudal migration of nVII motor neurons is unaffected (Pata et al., 1999; Karis et al., 2001). However, in *Gata2* knockout mice, nV and nVII neurons are reduced in number and nVII neurons fail to migrate caudally (Nardelli et al., 1999). Of interest, expression of *Nkx6.1*, which delineates a ventral neural progenitor domain containing BMNs (Vallstedt et al., 2001; Muller et al., 2003; see below), is down-regulated in *Gata2* mutants, raising the possibility that BMNs may not develop normally (Nardelli et al., 1999). Consistent with this suggestion, migration of nV and nVII neurons is severely affected in *Nkx6.1* mutants (Muller et al., 2003; Pattyn et al., 2003; see below).

Genes Regulating BMN Progenitor Identity

In addition to the above genes that regulate BMN identities indirectly, a large number of genes have been identified that are expressed in the ventral neural tube and regulate motor neuron and interneuron identities more directly. Extensive functional analyses of these genes in chick and mouse have led to a three-step model of ventral cell type specification by Shh signaling (Briscoe and Ericson, 2001). In this model, different levels of the Shh gradient regulate the expression of genes encoding two classes of transcription factors: Class I factors (Nkx2.2, Nkx2.9, Nkx6.1, Nkx6.2) are induced by Shh, and Class II factors (Pax3, Pax6, Dbx1, Dbx2, Irx3) are repressed. Cross-repressive interactions between Class I and Class II genes generate different domains of transcription factor expression in the ventral neural tube, leading to the induction of cell-type specific transcription factors such as *Islet1*, *Mnr2*, *Hb9*, *Phox2a*, and *Phox2b* in motor neuron progenitors (Ericson et al., 1992; Pattyn et al., 1997; Tanabe et al., 1998; Briscoe et al., 2000; for reviews, see Jacob et al., 2001; Shirasaki and Pfaff, 2002). Many of these genes are expressed in domains or progenitors that generate more dorsal cell types (*Pax3*, *Dbx1*, *Dbx2*, *Irx3*) or somatomotor neurons (*Mnr2*, *Hb9*, *Pax6*, *Nkx6.1*, *Phox2a*, *Phox2b*). *Islet1* is expressed in motor neurons in all species (Ericson et al., 1992; Appel et al., 1995; Varela-Echavarria et al., 1996) and is essential for motor neuron differentiation (Pfaff et al., 1996). *Nkx2.2*, *Nkx6.1*, *Nkx6.2*, *Phox2a*, and *Phox2b* are expressed in branchiomotor and visceromotor neuron precursors, and interestingly, only knockout of *Pax6*, *Nkx6.1*, and *Phox2b* generate a BMN phenotype, which is discussed below. Such functional analyses will eventually decipher the transcription factor code that completely specifies the identities of hindbrain motor neuron progenitors (Briscoe and Ericson, 2001; Takahashi and Osumi, 2002; Pattyn et al., 2003), preceding the expression of SMN-specific (Varela-Echavarria et al., 1996), VMN-specific (Pattyn et al., 2003), and BMN-specific transcription factors.

Pax6 and Nkx2.2—*Pax6* is expressed in the progenitors of the nVI and nXII somatomotor neurons (Ericson et al., 1997; Osumi et al., 1997), and *Pax6* function is required for the proper specification of nVI and nXII progenitor domains (Takahashi and Osumi, 2002). In mouse *Pax6* mutants, these somatomotor neurons are missing but there is a dorsal expansion of the *Nkx2.2* domain (containing BMN progenitors) and a corresponding increase in the number of nVII and nX BMNs (Ericson et al., 1997; Osumi et al., 1997). Interestingly, in mouse *Nkx2.2* mutants, the *Pax6* expression domain does not expand ventrally and there is no effect on the formation of various BMN subtypes (Briscoe et al., 1999). Clearly, *Nkx2.2* expression is sufficient to generate BMN progenitors in ectopic locations (as in *Pax6* mutants), but its function is not necessary within its normal ventral domain, likely due to redundancy with *Nkx2.9* (Briscoe et al., 1999).

Nkx6.1 and Nkx6.2—Mouse *Nkx6.1* is expressed in all branchiomotor and visceromotor neurons, and *Nkx6.2* is expressed in the nV and nVII BMNs (Muller et al., 2003; Pattyn et al., 2003). It must be noted that the VMNs described by Pattyn et al. (2003) include the VMNs and BMNs as defined in this review. In *Nkx6.1* mutants, all BMNs appear to differentiate normally, but the migration of nV and nVII motor neurons is greatly reduced (Muller et al., 2003; Pattyn et al., 2003; see below). In addition, the nV, nIX, and nX motor axons exhibit varying degrees of pathfinding defects in *Nkx6.1* single and *Nkx6.1:Nkx6.2* double mutants (Muller et al., 2003; Pattyn et al., 2003). *Nkx6.2* knockout mice are viable and exhibit no motor neuron defects, possibly due to complete compensation by *Nkx6.1* (Pattyn et al., 2003; M. Sander, personal communication). The inability of *Nkx6.2* to rescue motor neuron defects in *Nkx6.1* mutants may reflect temporal differences in onset of expression of the two genes.

Phox2a and Phox2b—Both homeodomain genes are expressed in somatic, visceral, and branchial motor neuron progenitors in the mouse hindbrain (Pattyn et al., 1997). However, *Phox2a* expression precedes *Phox2b* expression in the nIII and nIV somatomotor neurons,

while *Phox2b* expression precedes *Phox2a* expression in all BMN progenitors. Hence, only the nIII and nIV neurons are missing in *Phox2a* knockout mice (Pattyn et al., 1997), while all BMN progenitors fail to differentiate into motor neurons in *Phox2b* knockout mice (Pattyn et al., 2000). In zebrafish *phox2a* mutants, nIII and nIV motor neurons are lost, as in mutant mice (Guo et al., 1999). However, the roles of zebrafish *phox2a* and *phox2b* genes in BMN development have not been examined.

TANGENTIAL MIGRATION OF BRANCHIOMOTOR NEURONS

In vertebrates, all newborn neurons migrate away from the germinal (ventricular) zone to populate characteristic locations within the developing neural tube (Hatten, 1999). These migrations mostly involve movement along the radial axis (Hatten, 2002; Nadarajah and Parnavelas, 2002). However, a large number of neurons also migrate orthogonal to the radial glial fibers either along the rostrocaudal or mediolateral axes. These nonradial or tangential migrations have been increasingly recognized to play essential roles in the proper positioning of neurons in the cortex and other brain regions (Marin and Rubenstein, 2001).

Environmental Cues Regulating BMN Migration

Branchiomotor neurons of all species undergo tangential migrations along the mediolateral axis such that specific BMN subtypes occupy distinct dorsolateral locations (Simon et al., 1994; Chandrasekhar et al., 1997; Higashijima et al., 2000; Garel et al., 2000). In addition, the facial (nVII) BMNs of several vertebrates, including mouse and zebrafish but not chick, migrate tangentially along the rostrocaudal axis from their birth place in rhombomere 4 into more caudal rhombomeres as far as r7 (Gilland and Baker, 1993; Goddard et al., 1996; Studer et al., 1996; Chandrasekhar et al., 1997; Higashijima et al., 2000). Interestingly, even chick nVII neurons are capable of tangential migration into caudal rhombomeres. When a chick r4 is homotopically transplanted in the place of a mouse r4, the chick nVII neurons migrate caudally into the host (mouse) r5 and r6 (Studer, 2001), demonstrating that chick nVII neurons have the ability to respond to signals in the mouse environment and undergo caudal migration. Conversely, nVII neurons generated in a mouse r4 transplanted into the chick hindbrain do not migrate caudally, demonstrating that environmental cues necessary for initiation, and perhaps maintenance, of caudal migration are absent in the chick hindbrain (Studer, 2001).

Additional evidence of a role for environmental cues in mediating nVII tangential migration is provided by studies of the zebrafish *trilobite* (*tri*) mutant (Solnica-Krezel et al., 1996; van Eeden et al., 1996). In *tri* mutant embryos, nVII neurons are generated in normal numbers but fail to undergo tangential migration into rhombomeres 5–7 (Bingham et al., 2002). Consequently, nVII neurons remain in r4 but still undergo tangential migration into dorsolateral locations. Genetic mosaic analysis shows that wild-type nVII neurons are unable to migrate caudally in a mutant environment, and conversely, *tri* mutant motor neurons frequently, but not always, migrate in a wild-type environment, demonstrating a strong non-cell autonomous role for Trilobite in tangential migration of nVII neurons (Jessen et al., 2002). *Trilobite* encodes a transmembrane protein Strabismus (Jessen et al., 2002; Park and Moon, 2002), which is a key component of the noncanonical Wingless/Wnt signaling pathway that mediates planar cell polarity (PCP) in *Drosophila* and gastrulation-associated cell movements in vertebrates (reviewed in Tada et al., 2002; Tree et al., 2002; Veeman et al., 2003). High-resolution analysis of migrating motor neurons suggests that *tri* mutant motor neurons have lost the ability either to generate protusions in a polarized manner or to stably maintain polar protusions (Jessen et al., 2002). Because *trilobite* is expressed ubiquitously in the hindbrain during the period of nVII neuron migration, the challenge will be to unravel how the expression of a transmembrane protein on surrounding cells can affect the ability of migrating motor neurons to move in a directed manner. Of interest, loss-of-function or dominant-negative mutations in other components of the noncanonical Wnt/PCP signaling pathway (such as Wnt5a, Wnt11,

Glypican/Knypek, and Disheveled) that lead to defective gastrulation-associated cell movements do not cause defects in caudal migration of nVII neurons. It is tempting to speculate that Strabismus participates in another, as yet unidentified, signaling pathway to mediate tangential migration of nVII neurons along the rostrocaudal axis.

A zebrafish homologue of *prickle*, which encodes a LIM-domain protein component of the Wnt/PCP pathway, is expressed in r4 and r5 in the vicinity of, but not in, migratory nVII neurons (Carreira-Barbosa et al., 2003). Furthermore, morpholino-mediated knockdown of zebrafish *prickle* leads to defects in gastrulation-associated cell movements and tangential migration of nVII neurons (Carreira-Barbosa et al., 2003), as demonstrated previously for *trilobite*. These results suggest that Trilobite and Prickle function in the same pathway and may modulate environmental cues necessary for initiating and/or maintaining nVII neuron migration.

Cell-Intrinsic Mechanisms in BMN Migration

In contrast to the well-documented roles for environmental cues in nVII neuron migration described above (Studer, 2001; Jessen et al., 2002; Carreira-Barbosa et al., 2003), much less is known about mechanisms that act within nVII neurons to mediate tangential migration. In mouse, genes encoding various axon guidance and cell adhesion molecules are expressed in nVII motor neurons during specific migratory phases (Garel et al., 2000; Muller et al., 2003). *TAG-1* is expressed in nVII neurons in r4 and r5, whereas *Ret* and *Unc5h3* are expressed by nVII neurons migrating in r5 and r6. Furthermore, *cadherin8* and *neogenin* are expressed during dorsolateral migration of nVII neurons in r6 (Garel et al., 2000; Muller et al., 2003). Mouse *Ebf1* and *Nkx6.1* are expressed in nVII neurons throughout their migratory pathway, and regulate the expression of genes such as *Ret* and *Unc5h3* that appear to be involved in nVII neuron migration (Garel et al., 2000; Muller et al., 2003). In *Ebf1* knockout embryos, nVII neurons prematurely migrate dorsolaterally and fail to undergo complete caudal migration. These defects correlate with the abnormal expression of *TAG-1*, *Ret*, and *cadherin8* in mutant embryos (Garel et al., 2000). In *Nkx6.1* mutants, nVII neurons fail to migrate out of r4 and abnormally express *Unc5h3* and *Ret* (Muller et al., 2003). These data suggest that *Ebf1* and *Nkx6.1* regulate the expression of genes that modulate the cell surface properties of nVII neurons during different phases of migration. In *Ebf1* and *Nkx6.1* mutants, genes mediating specific migratory responses may be expressed precociously, leading to aberrant migratory behavior (Garel et al., 2000; Muller et al., 2003). The incomplete expressivity of the *Ebf1* mutant migration phenotype suggests that *Ebf1* may function redundantly with other genes in mediating nVII neuron migration. However, *Ebf1* and *Nkx6.1* appear to function in parallel, because *Nkx6.1* expression is unaffected in *Ebf1* mutants and vice versa (Muller et al., 2003).

FUTURE DIRECTIONS

Studies in several systems, primarily mouse, chick, and zebrafish, are beginning to provide a detailed perspective into the mechanisms underlying various aspects of BMN development. Nevertheless, many questions remain to be answered: What is the transcription factor code that uniquely identifies and specifies BMNs, apart from visceromotor and other efferent neurons? Are there markers that differentiate between BMNs of different subtypes, and between BMNs of the same subtype located in different (adjacent) rhombomeres? When are BMNs born, and do birth dates vary between BMN subtypes? Do different BMN subtypes require different concentrations of hedgehog signals for induction? What is the relationship between the mechanisms specifying rhombomere and BMN identities? What enables otic efferent neurons to project contralaterally, but prevents colocalized facial BMNs from doing so? Are there arch-specific guidance cues that ensure that BMN subtypes extend into their appropriate target arches? What are the mechanisms that initiate tangential (caudal) migration of facial BMNs, specify the direction of movement, and terminate migration? Does electrical activity regulate the processes of BMN migration and axon guidance?

Complementary strategies involving gain- and loss-of-function approaches and genetic and embryologic manipulations will provide answers to these questions. Genes potentially involved in BMN differentiation, migration, and axon guidance can be identified by microarray analysis or subtractive hybridization of cDNAs cloned from purified BMN subtypes, as demonstrated for other cell types (Dulac and Axel, 1995; Tietjen et al., 2003). This effort will be facilitated through use of extensive genomic sequence and expression data, which continue to accumulate and improve as current mouse, fish, and chick genome projects near completion. A complementary approach is the mutagenesis-based identification of genes that perturb BMN development in mouse and fish (Haffter et al., 1996; Leighton et al., 2001; Stryke et al., 2003). One such screen using a zebrafish BMN-expressed *GFP* line (Fig. 2A; Higashijima et al., 2000) is under way and has identified BMN-specific mutations (Table 1; H. Okamoto, personal communication). A systematic genomic scale screen using antisense morpholino oligonucleotides to knock down gene function (Nasevicius and Ekker, 2000; Chen and Ecker, 2003) may also identify genes involved in zebrafish BMN development (S. Ekker and A.C., unpublished data). While the technology for functional analysis of gene function is quite sophisticated in mouse, the state-of-the-art is improving rapidly in chick and fish. Gain-of-function analyses using electroporation and retroviral infection are well developed in chick (Swartz et al., 2001). Knockdown of gene function, using electroporated antisense morpholinos also appears feasible in chick (Kos et al., 2003). In zebrafish, genetic and chemical methods for manipulating gene expression in space and time are available (Scheer and Campos-Ortega, 1999; Halloran et al., 2000; Nasevicius and Ekker, 2000; Ando et al., 2001). Lastly, methods for measuring the electrical activity of BMNs during development are available (Fortin et al., 1995; Abadie et al., 2000; Gust et al., 2003). Given this rich repertoire of experimental tools, the prospects are very bright for deciphering the detailed molecular and genetic pathways underlying the development of branchiomotor neurons. Future challenges will include identification and ontogeny of higher-order neurons that modulate the electrical activity of BMNs, and elucidation of the mechanisms that build these neuronal circuits, which enable branchiomotor neurons to control diverse movements of the vertebrate head.

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Fig. 1.

Organization of cranial motor neurons in zebrafish, chick, and mouse. Embryo ages: zebrafish, 48 hours postfertilization (hpf); chicken, stage 25; mouse, 13 days post coitum (dpc). The hindbrains are depicted in open-book configuration (anterior to the top), with the dorsal most neural tube at the lateral margins of the diagrams. The series of bulges and the broken grey lines partition the hindbrain into rhombomeres from r0/r1 to r8. The hindbrain region anterior to r2 is defined as r0/r1, because it is composed of two distinct domains, r0 or the isthmic rhombomere anteriorly, and r1 posteriorly, which can be distinguished using molecular markers in zebrafish (Moens and Prince, 2002). Rhombomere 8 is longer than the other rhombomeres, and is also referred to as the caudal hindbrain (chb). The branchiomotor neurons (BMNs) and axons are shown in red, the somatomotor neurons (SMNs) in green, the otic and lateral line efferent (VIII, OLe) neurons in blue, and the visceromotor neurons (VMNs) in yellow (see text for details). For clarity, motor neuron populations are depicted on only one side of the neural tube. In the chick and mouse neural tubes, the alar plate (AP) and basal plate (BP) are clearly demarcated by the sulcus limitans. The zebrafish neural tube forms through different morphogenetic movements compared with chick and mouse, and the distinction between AP and BP in the hindbrain has not been described. Therefore, the AP and BP assignments are tentative in the fish neural tube. FP marks the floor plate region. The nerve exit points of the BMN, VMN, and otic efferent axons are shown as grey circles and are located dorsally in the alar plate of the chick and mouse neural tubes. In contrast, the exit points appear to be more ventral in zebrafish, and their depicted locations relative to the AP–BP boundary are tentative. The exit points of the SMN axons are located ventrally within the BP, close to the FP, in all species. The uncertainty in the location of the nerve XI exit point in the mouse embryo is indicated by a question mark. OV, otic vesicle.

Fig. 2.

Location of the cranial nerve exit points in the zebrafish hindbrain. **A,B:** Dorsal views of the hindbrain, with anterior to the top, in 36 hours postfertilization (hpf) transgenic embryos expressing green fluorescent protein (GFP) in the trigeminal motor (nV), facial motor (nVII), otic and lateral line efferent (OLe), and vagal motor (nX) neurons. GFP was detected by confocal imaging (A), and by labeling with an anti-GFP antibody (B). The various motor and efferent neuron populations are found at characteristic locations in rhombomeres 2–7 (r2–r7), and the caudal hindbrain (chb) (see text for details). The broken white lines in B indicate the plane of the cross-sections depicted in C–F. oto, otic vesicle. **C–F:** Cross-sections of the hindbrain in GFP antibody-labeled embryos at the approximate axial levels indicated in B. Dorsal is to the top in all panels. nc, notochord. **C:** The nV branchiomotor neuron cell bodies in r2 are located medially, close to the floor plate, and the exit point for their axons is located dorsolaterally (arrowhead). The asterisk indicates the nVII motor axons extending into the second branchial arch. **D:** Many nVII/OLe cell bodies are still found in r4 in this embryo, immediately adjacent to the floor plate. The axons are tightly fasciculated and exit the hindbrain dorsolaterally (arrowhead). **E:** In r7, GFP-labeled processes of nVII/OLe neurons (arrowhead) cross the midline ventral to the floor plate cells. Based on their similarity to the projections of contralateral vestibuloacoustic neurons in chick and mouse, these processes are tentatively assigned to zebrafish OLe neurons (see text for details). **F:** The nX motor neurons are located more dorsolaterally compared with the nV and nVII neurons, and their axons exit from the

neural tube (arrowhead) where the cell bodies are located. Scale bar in F = 40 μm in A,B, 30 μm in C–F.

TABLE 1

Zebrafish Mutations Assayed for Defects in BMN Development^a

Genetic locus ^b	Encoded protein ^c	Mutant BMN phenotype
Mutants identified on the basis of mid-line defects		
<i>chameleon</i>	Unknown	Loss/reduction of specific subtypes ^d
<i>cyclops</i>	Nodal-related (Ndr2) ligand	Severe loss of neurons, axon pathfinding errors ^e
<i>floating head</i>	Homeodomain Transcription factor	No defects ^d
<i>iguana</i>	Unknown	Reduction of specific subtypes ^d
<i>one-eyed pinhead</i>	EGF-related ligand	No defects ^d
<i>schmalspur</i>	Foxh1 winged-helix transcription factor	Large decrease in neuron number ^d
<i>slow-muscle omitted</i>	Smoothed transmembrane protein	Total absence of all BMNs ^d
<i>sonic you</i>	Sonic hedgehog ligand	Loss/reduction of specific subtypes ^e
<i>you-too</i>	Gli2 zinc finger transcription factor	Loss/reduction of specific subtypes ^d
Mutants with defects in midbrain or hindbrain morphology		
<i>acerebellar</i>	FGF8 ligand	No defects ^d
<i>bashful</i>	Laminin α 1	Disorganized clusters ^d
<i>eisspalte</i>	Unknown	Disorganized clusters, reduction in neuron number ^d
<i>lazarus</i>	Pbx4 transcription cofactor	nV axon guidance errors; Loss of facial BMN migration ^e
<i>mind bomb</i>	E3 ubiquitin ligase	Reduction in neuron number, clusters disorganized and fused across midline ^e
<i>no isthmus</i>	Pax2.1 homeodomain transcription factor	No defects ^d
<i>otter</i>	Unknown	No defects ^d
<i>sleepy</i>	Laminin γ 1	Disorganized clusters ^d
<i>valentino</i>	MafB b-ZIP transcription factor	Disorganized nVII BMN clusters, nIX neurons absent, and defasciculation of nVII axons ^e
Mutants with defective retinotectal pathfinding		
<i>astray</i>	Robo2 transmembrane protein	No defects ^d
<i>belladonna</i>	Unknown	No defects ^d
<i>detour</i>	Glil zinc finger transcription factor	Total absence of all BMNs ^e
Mutants with defects in branchial arch development		
<i>dackel</i>	Unknown	No obvious defects ^d
<i>lockjaw</i>	Unknown	No obvious defects ^d
<i>pinscher</i>	Unknown	No obvious defects ^d
<i>schmerle</i>	Unknown	No obvious defects ^d
<i>sturgeon</i>	Unknown	No obvious defects ^d
<i>sucker</i>	Endothelin-1 ligand	No obvious defects ^d
<i>van gogh</i>	Unknown	No obvious defects ^d
Mutants exhibiting defects in gastrulation-associated cell movements		
<i>knypek</i>	Glypican 4/6 transmembrane protein	No defects ^e
<i>pipetail</i>	Wnt5a ligand	No defects ^e
<i>silberblick</i>	Wnt11 ligand	No defects ^e
<i>trilobite</i>	Strabismus transmembrane protein	Total loss of facial BMN migration, no axon pathfinding defects ^e
Mutants with somite defects		
<i>after eight</i>	DeltaD transmembrane protein	No defects ^d
<i>deadly seven</i>	Notch1 transmembrane protein	No defects in cell number or nVII axon pathfinding ^d
<i>fused somites</i>	T-box transcription factor	No defects ^d
<i>no tail</i>	T-box transcription factor	No defects ^d
Other mutants		
<i>landlocked</i> (GFP screen)	Unknown	Total loss of facial BMN migration ^e

^a BMN, branchiomotor neuron; GFP, green fluorescent protein.

^b Mutants characterized in following studies. *cyclops*: Hatta et al., 1991; *slow-muscle omitted*: Chen et al., 2001, Varga et al., 2001; *lazarus*: Popperl et al., 2000; *valentino*: Moens et al., 1996; *deadly seven*: Gray et al., 2001; *no tail*: Halpern et al., 1993; remaining mutants: Haffter et al., 1996, and references therein.

^c The citations of studies describing the molecular characterization of various mutant loci have been omitted for space considerations. Some of the studies have been cited in the text.

^d A. Chandrasekhar and colleagues, unpublished data. For most of the mutants, the BMN phenotype was assayed by islet antibody labeling at 36–48 hours postfertilization, and there were no noticeable defects in the size, organization, and distribution of the BMN clusters in various hindbrain rhombomeres. None of the mutants have been assayed for defects in BMN axon pathfinding.

^e *cyclops*: Chandrasekhar et al., 1997; *sonic you*: Chandrasekhar et al., 1998; *mind bomb*: Bingham et al., 2003; *lazarus*: Cooper et al., 2003; *valentino*: Chandrasekhar et al., 1997; *detour*: Chandrasekhar et al., 1999; *knypek*, *trilobite*: Bingham et al., 2002; *pipetail*, *silberblick*: Jessen et al., 2002; *landlocked*: H. Okamoto, personal communication.

TABLE 2

Mouse Genes With Demonstrated Roles in BMN Development^a

Gene	Encoded protein	Loss-of-function BMN phenotype ^b
<i>Shh</i>	Sonic hedgehog ligand	Loss of all motor neurons
<i>Islet1</i>	LIM and homeodomain transcription factor	Loss of all motor neurons
<i>Phox2b</i>	Homeodomain transcription factor	Loss of all branchial and visceral motor neurons
<i>Pax6</i>	Homeodomain transcription factor	Increase in nVII and nX motor neurons
<i>Sema3A</i>	Secreted chemorepellant	Defasciculation of BMN axons
<i>Neuropilin1</i>	Semaphorin receptor	Defasciculation of BMN axons
<i>Neuropilin2</i>	Semaphorin receptor	Defasciculation of nV and nVII axons
<i>Ebf1</i>	Transcription factor	Defective caudal migration of nVII neurons
<i>Krox20</i>	Zinc finger transcription factor	Decrease in nV neurons; defective caudal migration of nVII neurons
<i>Kreisler</i>	bZIP transcription factor	Loss of nVII VMNs; BMNs not affected
<i>Hoxa2</i>	Homeodomain transcription factor	Loss of nV neurons; transfecting into nVII neurons
<i>Hoxa1</i>	Homeodomain transcription factor	nVII-like neurons in rhombomere 3; loss of caudal migration of nVII neurons
<i>Hoxb1</i>	Homeodomain transcription factor	Decrease in nVII neurons; defective specification/migration of nVII neurons
<i>GATA2</i>	Zinc finger transcription factor	Decrease in nV and nVII neurons; loss of caudal migration of nVII neurons
<i>Nkx6.1</i>	Homeodomain transcription factor	Loss of caudal migration of nVII neurons; reduced dorsolateral migration of nV neurons
<i>Ngn1</i>	bHLH transcription factor	Defective nV axon pathfinding

^aSee Cordes (2001) for a more comprehensive list of genes required for the development of cranial nerves and sensory ganglia in mouse. BMN, branchiomotor neuron; bHLH, basic helix-loop-helix; n, cranial nerve; VMN, visceromotor neuron.

^bThe studies describing the defective hindbrain neuronal phenotypes are cited in the text.