A NOVEL PATTERNING OF INTERNEURONS IN HINDBRAIN REVEALS NEW PRINCIPLES OF ORGANIZATION

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The work described here focuses on a novel patterning of interneurons where stripes of excitatory and inhibitory neurotransmitter phenotypes are present in an alternating fashion from medial to lateral in the hindbrain of larval zebrafish. I investigated the hypothesis that these stripes represent an underlying template for the organization of neural circuits in hindbrain. Using photoconvertible proteins, I tracked cells of different ages within stripes identified by transgenic lines expressing fluorescent proteins exclusively in neurons with shared neurotransmitter or transcription factor expression. I found that stripes maintain an age-related organization from ventral to dorsal for old to young cells in most hindbrain regions at 4 dpf, a time when zebrafish are freely swimming. Using dye backfills and stochastic labeling of membrane-tagged fluorescent proteins, I examined the projection patterns of neurons within inhibitory stripes and found that neurons within a stripe share a common morphology and mediolateral projection pattern. This broad patterning exists in a specific circuit, the startle response circuit of the Mauthner cell, where lateral stripe neurons project laterally onto the lateral dendrite and middle stripe neurons project more medially onto ventral dendrite regions. Since an age-related patterning exists within one stripe, I looked for differences within one stripe, the medial excitatory stripe identified by expression of the alx transcription factor. Using photoconvertible proteins, stochastic expression of membrane-tagged fluorescent

proteins, whole-cell patch clamp, and calcium imaging, I found that both structural and functional patterning within a single stripe where an orderly patterning based on the age of a neuron exists for the cell body position within the stripe, the projections of these cells within the neuropil, the input resistance of these cells, and possibly, recruitment during different speeds of swimming. I also showed that there is a topological transformation from spinal cord to hindbrain both by age and by transcription factor expression. These findings indicate that a broad organization of hindbrain exists, where stripes represent a simple toolkit that is comprised of interneuron types arrayed into stripes, and, within a stripe, cells comprising a single class of interneurons are organized dorsoventrally within a stripe by age, excitability and wiring.

BIOGRAPHICAL SKETCH

Amina Kinkhabwala is the middle of five children. She grew up in Memphis, TN and followed in her older brothers' footsteps by studying physics beginning in high school. She attended Columbia University for her undergraduate degree in physics, and during that time, became fascinated by the organization of complex systems, especially the brain.

In August 2001, she joined the doctoral program for physics at Stony Brook University. In May 2003, she joined the lab of Dr. Joseph Fetcho, and in May 2004, she received her Masters degree in Physics and transferred to the doctoral program for Neurobiology and Behavior in August 2004. She has been in Dr. Fetcho's lab at Cornell during her time at Cornell.

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CHAPTER 1

INTRODUCTION

The work described here is focused on a novel patterning of interneurons where stripes of excitatory and inhibitory neurotransmitter phenotypes are present in an alternating fashion from medial to lateral in the hindbrain of larval zebrafish. I investigated the hypothesis that these stripes represent a wiring template by studying the organization of interneurons within these stripes. This study is comprised of 5 chapters. In the present chapter, I review background material concerning spinal and hindbrain organization. In chapter 2, I examine both the development and age-related patterning of stripes and show that stripes of interneurons are dorsoventrally organized by age at a time when zebrafish are freely swimming. I also show that there is a topological transformation from spinal cord to hindbrain, both by age and by transcription factor expression. In chapter 3, I show that stripes identify populations of interneurons with similar morphologies and mediolateral projections, and thus, stripes represent an orderly underlying pattern for neural circuits in hindbrain. In chapter 4, I describe both structural and functional patterning within a single stripe and show that the medial excitatory stripe maintains an orderly patterning based on the age of a neuron for the cell body position within the stripe, the projections of these cells within the neuropil, the input resistance of these cells, and possibly, recruitment during different speeds of swimming. This shows that, within a population of cells identified by similar morphology, transcription factor expression, and neurotransmitter expression, there is an orderly arrangement of input resistance and projection patterns associated with the dorsoventral position of the cell body within the stripe. In chapter 5, I summarize the results described in this work and make predictions about a possible broad organization of hindbrain, where neural circuits arise from a simple

toolkit that is comprised of interneuron types arrayed into stripes, and within a stripe, cells comprising a single class of interneurons are organized dorsoventrally by age, excitability and wiring.

The motivation for these studies within hindbrain has been guided by recent discoveries of the organization of spinal circuits involved in motor coordination across vertebrates. I will first review the key principles of spinal organization that are relevant for the present study: transcription factor organization defines functional classes of interneurons, age-related recruitment during different strengths of movement are present within a transcription factor class, and a functional topography for recruitment during different swimming speeds is present both within and across spinal classes. Although the present study concerns a novel patterning of hindbrain interneurons, much work has revealed other basic principles for hindbrain organization. In this chapter, I will review the various types of segmental organization that rostrocaudally section the hindbrain, as well as recent work that has shown patterning that is continuous across the rostrocaudal extent of hindbrain, including the serial repetition of cell types across segments and transcription factor patterning.

Functional organization of spinal cord

Transcription factor map of progenitor cells

The coordination of different muscle groups for movements requires a variety of cell types. For instance, inhibitory neurons that project contralaterally maintain left/right coordination [1] while ipsilaterally projecting inhibitory interneurons maintain appropriate timing for movements at faster speeds [2]. Thomas Jessell's lab has revealed that there is an orderly patterning early in development for the formation of these distinct cell types [3].

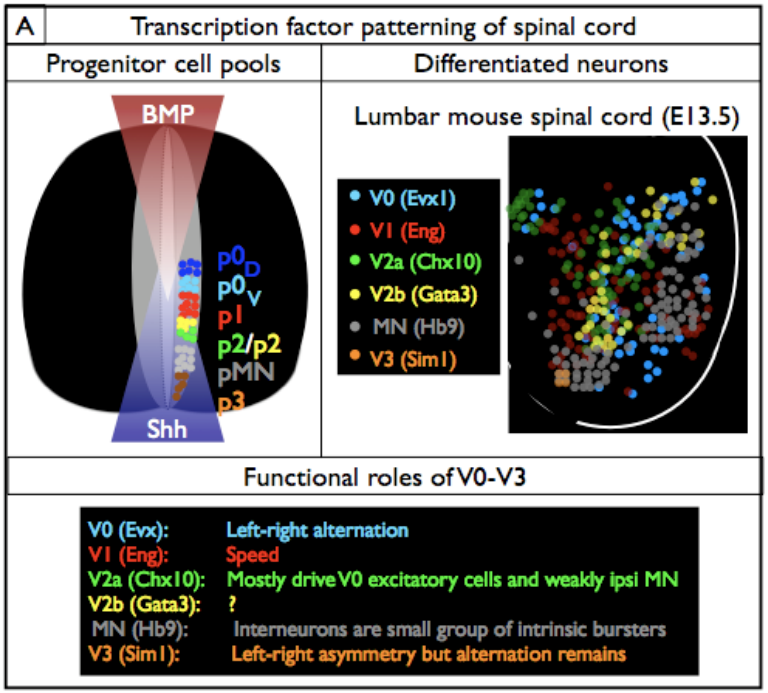
Molecular studies within spinal cord have revealed that, during development, the patterning of progenitor cell identities with unique dorsoventral positions in spinal cord is determined by opposing gradients of two morphogens. Sonic hedgehog (Shh) is ventrally secreted by the notochord and ventral spinal cord [3], and bone morphogenetic protein (BMP) is secreted by the alar plate and by dorsal spinal cord [4]. These factors set up discrete zones along the dorsoventral axis of spinal cord [3, 4]. Here I focus on those located more ventrally. Within ventral spinal cord, cross repressive interactions between proteins derived from dorsal BMP and ventral Shh gradients establish dorsoventral boundaries for progenitor zones [3]. These ventral zones were defined in spinal cord as p0, p1, p2, pMN, and p3 from dorsal to ventral and are illustrated in the left panel of figure 1.1A. Gradients of BMP and Shh are illustrated as well as the dorsoventral segregation of progenitor zones in ventral spinal cord that arises from the Shh gradient of expression. Studies have shown that this patterning depends upon the gradient of Shh signaling [3-9].

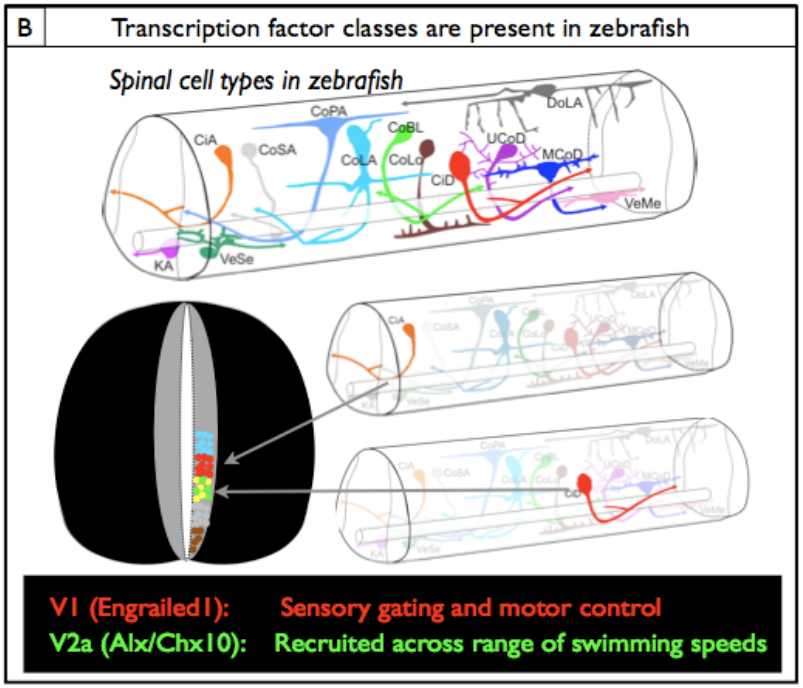
Cell types identified by transcription factors

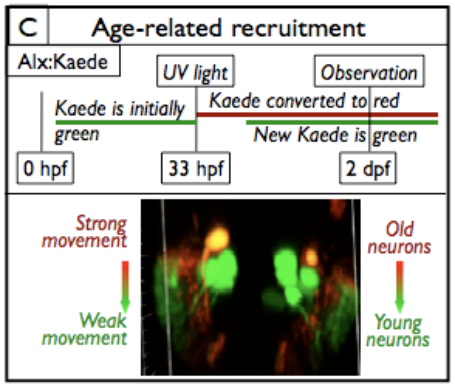
This homeodomain code present in progenitor cells persists in newly differentiated neurons in the form of a second set of transcription factors [3]. This patterning is depicted in the right panel of figure 1.1A. Neurons are color-coded based on their progenitor zone origins (from color-coded progenitor zones in the left panel). The legend summarizes the names of each cell type, and, in parentheses, the transcription factor expression that sets them apart from other spinal neurons.

Although these neurons arose from an orderly patterning of progenitor cells, neurons of different transcription factor types are intermingled in spinal cord. In figure 1.1A, the right panel depicts approximate positions of neurons of different transcription factor types in the lumbar region of an E13.5 mouse spinal cord. This

Figure 1.1 Organization of spinal neurons **(A)** Top left panel shows a summary of the patterning first described in [3]. Sonic hedgehog gradients establish an early patterning of progenitor pools located in restricted regions of the ventricular zone. These pools are depicted by different colors and named p0, p1, p2, pMN, and p3 from dorsal to ventral. The right panel shows an illustration of the positions of the neurons in the ventral horn of the lumbar mouse spinal cord that arise from different progenitor pools. These neurons express unique transcription factor patterns and are color coded accordingly. Approximate positions of each type were derived from staining at E13.5 in figure 1 of [10]. In the bottom region of panel **A**, a summary of the functional roles for these different transcription factor neuron classes is shown. Functional roles were described in the following references: V0 neurons coordinate left-right alternation [1]; V1 neurons regulate the speed of movements [2]; V2a neurons play a role in coordinating left-right locomotor activity [10]; HB9 neurons are intrinsic bursters but functional roles are not established [11-14] and V3 neurons maintain balanced rhythm for walking [15]. **(B)** Transcription factors mark interneuron classes in the zebrafish spinal cord. In the top of figure B, an illustration indicates the variety of spinal cell types present in zebrafish spinal cord, first described in [16] (illustration by D McLean). Below this illustration, two types of neurons are shown, a CiA (Circumferential ipsilateral Ascending) neuron and a CiD (Circumferential ipsilateral Descending) neuron. In [17], CiA neurons were shown to express Engrailed1 and participate in sensory gating and inhibition of ipsilateral motoneurons during swimming. In [18], CiD neurons were shown to express alx and be recruited during a range of swimming frequencies. An illustration of the progenitor regions that these cells are derived from is shown in the middle frame, and the functional roles depicted for these cells in these studies is summarized in the bottom legend of panel B. **(C)** Age-related recruitment for a transcription factor class of spinal neurons. Here, an illustration of the recruitment based on the age of a neuron described in [18] is shown. A cross section image of the spinal cord of an alx:Kaede transgenic fish, photoconverted at 33 hpf to converted old neurons to red and imaged at 2 dpf to determine positions of old (red) and young (green) neurons is shown. This study showed that the oldest alx neurons are dorsally positioned and recruited during the strongest movements, mid-dorsoventral alx neurons are recruited during intermediate strengths of movements and are slightly younger, and the ventral-most alx neurons are even younger and are recruited during the weakest strength of movements. **(D)** Functional topography for swimming in larval zebrafish is present within and across spinal neuron classes. In the left panel, an illustration of both recruitment patterns and input resistance is shown. Ventral neurons have high input resistances and are recruited during slow swimming while more dorsal neurons have lower input resistances and are recruited during faster swimming bouts. For excitatory neurons, this topography is present within a class of neurons for CiDs (alx neurons) and across two classes where MCoDs are ventral and recruited during slow swimming and CiDs recorded from in this study were more dorsal and recruited during faster swimming. On the right, a similar functional topography is shown for inhibitory interneurons but from dorsal to ventral for slow to fast swimming recruitment and low to high input resistance. Figure is adapted from [19].


A
Transcription factor patterning of spinal cord
Progenitor cell pools
Differentiated neurons
BMP
Shh
p0D
p0V
p1
p2/p2
pMN
p3
Lumbar mouse spinal cord (E13.5)
V0 (Evx1)
V1 (Eng)
V2a (Chx10)
V2b (Gata3)
MN (Hb9)
V3 (Sim1)
Functional roles of V0-V3
V0 (Evx): Left-right alternation
V1 (Eng): Speed
V2a (Chx10): Mostly drive V0 excitatory cells and weakly ipsi MN
V2b (Gata3): ?
MN (Hb9): Interneurons are small group of intrinsic bursters
V3 (Sim1): Left-right asymmetry but alternation remains


B
Transcription factor classes are present in zebrafish
Spinal cell types in zebrafish
CiA
CoPA
CoSA
CoBL
CoLA
CoLo
CiD
UCoD
MCoD
DoLA
VeMe
KA
VeSe
V1 (Engrailed1): Sensory gating and motor control
V2a (Alx/Chx10): Recruited across range of swimming speeds


C
Age-related recruitment
Alx:Kaede
UV light
Observation
Kaede is initially green
Kaede converted to red
New Kaede is green
0 hpf
33 hpf
2 dpf
Strong movement
Weak movement
Old neurons
Young neurons

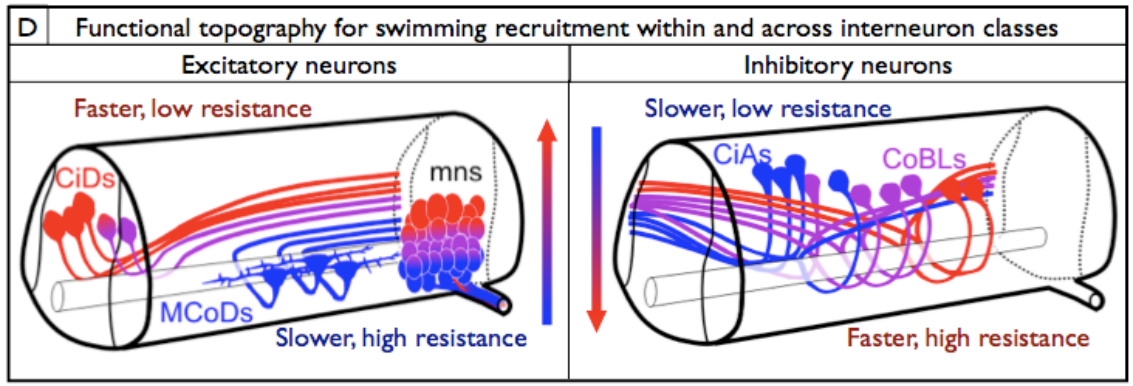

D
Functional topography for swimming recruitment within and across interneuron classes
Excitatory neurons
Inhibitory neurons
Faster, low resistance
CiDs
mns
MCoDs
Slower, high resistance
Slower, low resistance
CiAs
CoBLs
Faster, high resistance

illustration was derived from panels of figure 1 in [10] where staining of different transcription factor regions is shown. Here, panels were overlaid and positions of neurons were approximated and merged into one frame of reference. Neurons of different transcription factor types are intermingled.

This classification of neurons by transcription factor phenotypes defines both morphological and, to some extent, functional classes [2, 3, 10, 15, 20]. Morphological classes are subdivided as follows: V0 neurons express Evx1/2 transcription factors (p0-derived) [1], V1 neurons express Engrailed1 (p1-derived, [2]), V2 neurons are subdivided into V2a (Chx10 expression) [10] and V2b (Gata3 expression) and both types are derived from the p2 progenitor domain, MN express Hb9 (pMN-derived, [5]), and V3 neurons express Sim1 (p3-derived) [15]. In the following sections, we will briefly review the morphology and possible functional roles of these subtypes of neurons in mice (occasionally, for chicks as well). A summary of the functional subtypes to date is shown in figure 1.1D.

V0 interneurons coordinate left/right alternation

V0 interneurons are derived from the p0 progenitor zone, a population of progenitor cells that express Dbx1 [1, 21]. V0 neurons are subdivided into two populations; both populations are commissural interneurons but the excitatory subtype expresses Evx1/2 and the inhibitory subtype lacks Evx1/2 expression [1, 21]. A recent study used genetic knockouts to determine the role of the inhibitory type during walking in a fictive preparation of mouse spinal cord [1]. Since the Dbx1 transcription factor is involved in a regulatory program that coordinates the number of neurons of each subtype generated, a combination of Dbx1 and Evx1 knockout experiments was used to elucidate the role of the inhibitory neurons that lack Evx1 expression.

When the Dbx1 gene is knocked out, Dbx1 derived neurons are eliminated; however, an increase in dl6, a dorsal spinal interneuron class, and En1 (V1) neurons is present. The Dbx1 progenitor domain is subdivided such that dorsal progenitor cells give rise to inhibitory Dbx1 derived neurons while ventral progenitor cells give rise to Evx1 expressing excitatory neurons [1, 21]. This is illustrated in the left panel of figure 1.1A, where green and yellow cells are dorsoventrally segregated within the p0 progenitor zone. This study suggests that the elimination of inhibitory V0 neurons, derived from the dorsal p0 domain, results in an increase in dl6 neurons. Dlx6 neurons are derived from the dorsally adjacent progenitor domain and share expression of Dbx2 with Dbx1 progenitor cells. They suggested in a previous study that an absence of excitatory V0 neurons results in an increase in V1 neurons that are derived from a ventrally adjacent progenitor domain to the excitatory Dbx1 progenitor domain [21].

These mice showed an increase in cocontractions between the left and right flexor/extensor motor neurons during fictive locomotion [1]. When Evx1 is selectively targeted and Dbx1 neurons that do not express Evx1 remained (inhibitory Dbx1-derived V0 neurons), no deficits in motor coordination are observable [1]. This suggests that inhibitory V0 neurons might play a role the coordination of left-right alternation during walking.

V1 interneurons regulate the speed of the locomotor step cycle

Engrailed1 (En1) interneurons are ipsilateral ascending glycinergic neurons that innervate motor neurons in vertebrates [17, 22-24]. These neurons, known as V1 neurons in mice, can be severely reduced in number by Pax6-knockout (a transcription factor present in p1 progenitor cells) or by En1-DTA (Diphtheria Toxin A-chain protein) knock-in [2]. In Pax6-knockout mice, an increase in commissural V0 and V3

neurons are present and lumbar spinal neurons are present in normal numbers. A decrease in V2 ipsilateral excitatory neurons is also observed. However, no changes in interneuron specification are present in En1-DTA mice.

In mice, the V1 interneurons form at least two classes of interneurons known as Renshaw cells that provide feedback inhibition onto motoneurons and Ia interneurons that are thought to help coordinate ipsilateral flexor/extensor activity [23, 25-28]. In both Pax6-knockout mice and En1-DTA spinal cords, an increase in step-cycle period and motor neuron burst duration are present [2]. Motor neurons remain depolarized and fire action potentials for a long period of time in the absence of these ipsilateral inhibitory interneurons. Therefore, ipsilateral inhibitory interneurons are required to shorten burst duration to increase the speed of movement in mice. This deficit in fast locomotor behavior is also present in adult mice lacking En1 neurons. Studies were also conducted using allatostatin inactivation to avoid developmental changes that may occur with mice that develop with a substantial decrease of En1 neurons, and the results were consistent with the studies mentioned above with a twofold increase in step-cycle period [2]. Surprisingly, ipsilateral phasic activity is maintained. Therefore, V1 neurons are not required for the coordination of ipsilateral flexor/extensor phasic activity, but are required to regulate the speed of walking movements.

V2 interneurons provide drive for V0 neurons involved in left-right alternation

V2 neurons are subdivided into V2a and V2b populations. V2a neurons express the Chx10 transcription factor and are predominately glutamatergic ipsilaterally projecting, descending neurons. A recent study showed that the removal of this cell type disrupts locomotor activity [10]. This study showed that the expression of the Diphtheria Toxin A-chain (DTA) protein, selectively driven in

Chx10 neurons, selectively ablates neurons of this type. When these neurons are genetically ablated during development in Chx10:DTA mice, alternation between the opposite sides of the body is less stringently coupled while the coordination of ipsilateral flexor/extensor phases is maintained [10].

In pharmacologically induced rhythmogenesis of isolated regions of the spinal cord of Chx10:DTA mice, variability in burst amplitude and cycle period indicates that these neurons might be involved in stabilizing the locomotor rhythm. Also, a loss of alternation was observed between left and right while ipsilateral phasic activity was maintained. Backfilling experiments suggest that V2a neurons contact CINs, a subset of which were V0 neurons, and thus, could provide drive to contralaterally projecting neurons.

Brainstem stimulation in these mice in which V2a neurons were genetically ablated elicited transient and uncoordinated activity, suggesting V2a neurons are spinal mediators of brainstem activity; however Chx10 neurons are present in the brainstem as well. Sensory afferent stimulation in isolated spinal cord preparations of these mice either elicited no activity or tonic, nonrhythmic activity, suggesting that V2a neurons play a role in sensory evoked locomotor coordination.

This work suggests that ipsilateral excitatory neurons (V2a) provide excitatory inputs to contralaterally projecting V0 neurons that coordinate left-right alternation. However, in zebrafish, a group of neurons that express the alx transcription factor that is homologous to Chx10 in mice are present and will be discussed in more detail in a later section [18, 29]. Briefly, alx neurons in zebrafish are thought to play a role in the coordination of ipsilateral motor drive for faster swimming speeds by directly exciting motor neurons independent of a commissural excitatory pathway [18, 29]. However, pectoral fins are not active at these faster swimming speeds. It is possible that alx neurons drive commissural interneurons in the pectoral fin circuit in zebrafish, as do

the Chx10 neurons in mice. Conversely, a role, like the alx cells play in zebrafish, for ipsilateral coordination might be present in mice for Chx10 neurons for the coordination along the body, either for axial movements or between limbs on the same side of the body. The actual locomotor movements of Chx10:DTA mice were not described in the previous work and activity patterns shown in this study were very slow. Studies of a possible role for Chx10 neurons at faster movement speeds would allow for a more direct comparison with their roles at higher speeds in zebrafish.

Hb9 neurons

Hb9 interneurons are glutamatergic interneurons derived from the pMN progenitor pool that also generates motor neurons [14]. Genetic targeting for the silencing of Hb9 neurons without affecting other neuron types has not been possible; therefore, the functional role that these cells might play cannot be determined by targeted ablation. Previous studies have shown that this small population of interneurons may play a role in central pattern generation. These cells, to some degree, exhibit electrical coupling onto one another (however, this is not necessary to maintain synchronization within the population, suggesting that common synaptic inputs synchronize activity within the population) [12], exhibit strong postinhibitory rebound [14], maintain membrane potential oscillations during chemically-induced locomotion [14], are endogenous bursters [13], and possess a persistent sodium current that can generate pacemaker activities [30, 31]. Despite these suggestions for rhythmic capabilities of Hb9 interneurons, their functional role in maintaining specific features of rhythmic coordination is unknown at this time.

V3 interneurons are involved in the symmetrical matching of left/right burst duration

V3 neurons are glutamatergic neurons that predominately project to contralateral motor regions such as motor neurons and Ia interneurons [15]. These neurons in P0-3 mice maintain high input resistance values of 420-500 MOhm and possess spike frequencies up to 40 Hz with low spike frequency adaptation, indicating a linear input-output relationship for input current and action potentials which is an important feature for neurons involved in central pattern generation in order to maintain coordination over a variety of speeds. However, these cells are not pacemaker cells since they do not exhibit rhythmic activity in the presence of neuromodulators. Expression of the tetanus toxin light chain subunit (TeNT) has previously been shown to attenuate synaptic transmission [32, 33]. In this study, expression of TeNT was driven selectively in V3 neurons. Chronic TeNT disruption of V3 neurons causes irregularity in both L2 flexor and L5 extensor ventral roots both by increased variability in the duration of motor bursts and in the length of step cycle period in 3-6 month old mice. To determine if these changes were the result of the disruption of V3 neurons or the result of a subsequent reconfiguration of the locomotor central pattern generator, the allatostatin receptor system [2, 34] was used to quickly inactivate cells, and an increased variance in the duration of flexor bursts on either side in isolated spinal cords was observed after the addition of allatostatin, confirming the TeNT results. An asymmetry in flexor bursts on either side is also present. These neurons appear to be involved in the symmetrical matching of motor outputs on either side, but left-right alternation was consistently maintained.

Transcription factors define functional types in zebrafish spinal cord

In zebrafish, a variety of cell types in spinal cord were defined by morphological features in a previous study [16]. These neurons are depicted at the top

of figure 1.1B. Recently, studies have shown that some of these cell types express transcription factors that are homologous to those expressed in the mouse spinal cord [17, 18]. The neurons that express homologous transcription factors maintain the same neurotransmitter phenotype and gross morphology of those in mice [17, 18].

Two examples are shown in figure 1.1B. The top illustration depicts a CiA (Circumferential ipsilateral Ascending) neuron. This neuron expresses the Engrailed1 transcription factor that is homologous to the mouse Engrailed transcription factor [17]. These neurons arise from the p1 domain as illustrated and are ipsilateral inhibitory neurons [17]. Below the CiA neuron in figure 1.1B, the morphology of a CiD (circumferential ipsilateral descending) neuron is shown. This cell type expresses the alx transcription factor that is homologous to Chx10, both of which define a class of ipsilateral excitatory interneurons [10, 18]. Alx neurons are derived from the p2 progenitor domain as illustrated [18]. The functional roles of these neurons have been described as well and will be reviewed in the next two sections and are summarized at the bottom of figure 1.1B.

V1 neurons

In both chicks [25] and mice [2, 26], the post mitotic pool of neurons expressing Engrailed-1 has been shown to be functionally heterogeneous. In the zebrafish, the Engrailed-1 population is a homogeneous population defining all of the ipsilateral inhibition in both fish and frogs [17, 22]. V1 neurons in zebrafish spinal cord, also called CiAs (circumferential ipsilateral ascending), provide inhibition to both sensory and motor pathways. These neurons possess at least two functional roles but represent one distinct population of neurons with extensive axonal arbors innervating a range of dorsoventral regions in spinal cord

CiA neurons have been shown to play a role in gating the flow of sensory information during swimming [17, 35]. CiAs inhibit CoPA (commissural primary ascending) interneurons, suggesting they play a role similar to that of a spinal neuron in Xenopus [36-38] that is responsible for gating the flow of information through a sensory reflex pathway involving Rohon-Beard (RB) neurons. CoPA neurons receive inputs from primary sensory afferents of Rohon-Beard neurons [17, 35]. When an animal bends to one side sensory driven motor output on the opposite side is inhibited. The rhythmic inhibition of CoPAs by CiAs during swimming suggests that CiAs are involved in gating sensory information. These neurons are also present in Xenopus and express Engrailed1 [22].

CiAs also inhibit motor neurons directly on the ipsilateral side, possibly serving the same function in zebrafish as described earlier in mice [2] to enable an orderly timing of rhythmic coordination during fast movements. These neurons also inhibit ipsilateral excitatory interneurons involved in motor control. It is possible that, later in life, these neurons in fish and frogs form diverse functional classes of interneurons that match the heterogeneity seen in mice.

V2a neurons

In zebrafish, V2a neurons have been identified to be CiDs (Circumferential ipsilateral Descending) [18]. These neurons express the alx transcription factor that is homologous to Chx10 in mice [39]. They also express Nkx6.1, the transcription identifying precursors cells in the p2 region in mice [3, 40] as well as in postmitotic neurons derived from this region [40]. Alx neurons are rhythmically active during swimming and a subset is active during escape responses. These neurons form monosynaptic connections with motoneurons, and play a central role in rhythm

generation in zebrafish. A similar cell type is also present in xenopus and is implicated in both swimming and struggling [41].

Dorsoventral age-related organization within a transcription factor class is related to the pattern of recruitment during behavior

Within the alx transcription factor class of neurons in zebrafish, there are functional differences associated with the age of a neuron. The oldest alx neurons are involved in stronger movements such as escape and fast swimming while younger neurons are recruited at slower swimming speeds [18]. The oldest alx neurons are also positioned dorsal with respect to younger alx neurons. In the bottom panel of figure 1.1C, a cross section within the spinal cord of a transgenic line expressing Kaede, a photoconvertible protein, in alx neurons is shown. In the top panel, an illustration of a photoconversion experiment is shown. Alx:Kaede transgenic fish were exposed to UV light at 33 hpf to convert Kaede protein from green to red and were imaged at 2 dpf. Neurons expressing Kaede at 33 hpf had red expression while later differentiating neurons lacking Kaede expression at 33 hpf were exclusively green. The oldest neurons were dorsal (top) relative to younger neurons (shown to right of image). In this study, the ventral to dorsal axis of old to young neurons reflects a recruitment pattern for strong to weak movements. Arrows on either side of the image in figure 1.1C illustrate that the patterning from young to old and for recruitment from slow to fast movements are both present from ventral to dorsal.

Functional topography for swimming within and across cell types defined by transcription factors in spinal cord

The functional organization of the alx neurons in zebrafish extends broadly to other cell types. Recent studies of zebrafish showed that several neuronal types are

recruited in dorsoventral patterns in a manner correlated with their input resistance [19]. This patterning is present in motor neurons as well as excitatory and inhibitory interneurons involved in coordinating swimming behavior and is present continuously across cells of different morphological types. Within the population of interneurons studied, the pattern could not be directly explained by the soma size of a given neuron. Within a neurotransmitter phenotype, this pattern is present and progressed across cells of different transcription factor phenotypes. For instance, neurons expressing the alx transcription factor, CiDs in zebrafish, are ipsilateral descending glutamatergic interneurons recruited during most swimming speeds, but the dorsal (low resistance) alx neurons are recruited only during the fastest movements [18, 19]. However, in more ventral regions in spinal cord there is a cell type not expressing alx (potentially expressing Evx2) that possesses a large cell body but a very low input resistance and is recruited during slow swimming. When these recruitment patterns are overlaid, they form one trend that follows the functional topography also seen in motor neurons. Inhibitory cells of two types are also recruited within an opposing functional topography of dorsal to ventral recruitment for slow to fast swimming that matches their input resistance.

Figure 1.1D shows an illustration adapted from the study described above [19]. On the left, both motoneurons and excitatory neurons are recruited from ventral to dorsal for slow to fast swimming. On the right, inhibitory neurons are recruited in an opposing fashion. For both excitatory and inhibitory neurons, the recruitment topography is correlated in the same direction with input resistance: low input resistance neurons are only recruited during the fastest movements while higher input resistance neurons are recruited during slow swimming. This patterning defines a broad organization of spinal interneurons since it extends across multiple cell types.

Shared features of spinal cord and hindbrain organization

In spinal cord, we described earlier how the transcription factor organization for ventral classes arises from early gradients of Shh and BMP [3, 4]. Shh is present throughout ventral regions of hindbrain as well [42]. Since the hindbrain has many similarities to spinal cord [43], it is possible that the hindbrain is a rostral specialization of spinal cord. We hypothesized that patterns described above for spinal cord might be present within the hindbrain and that the neurotransmitter stripes might represent an orderly patterning that is related to the spinal organization of neural circuits.

Although similarities exist between the spinal cord and hindbrain, many features of hindbrain organization and development are distinct from those of spinal cord. In the following sections, basic features of hindbrain organization that distinguish it from spinal cord, namely, segmental and nuclear organization, will be reviewed. Segmental organization in the hindbrain defines unique identities for each segment; however, previous studies have also shown that similarities exist between hindbrain segments. I will review these similarities in the final section of this chapter.

Segmental organization of hindbrain

Features of hindbrain patterning are present even in amphioxus [44-50], an ancestor of the vertebrate lineage, suggesting they were present even in the earliest vertebrates. Amphioxus expresses the Islet transcription factor in a serially repeated manner within motoneurons [45]. Amphioxus also exhibits a segmental hox patterning [47]. However, physical segmental bulges delineating segments in hindbrain only begin to appear in lamprey, as does the neural crest [47]. In lamprey, reticulospinal neurons and cranial nerve roots are positioned in specific rhombomere segments; however, it is argued that the interdependence of the hox code and cranial

nerve identities along hindbrain does not arise until the time of gnathostomes [47]. Within vertebrates, several of these features of hindbrain segment patterning are tightly conserved and will be discussed here.

The number of rostrocaudally arrayed segments (rhombomeres) present in the hindbrain of vertebrates is either seven or eight (r1-8). Segmentation is present at the anatomical, morphological, and molecular levels (reviewed in [51]). The patterning is initiated by a signaling center comprised of krox-20 expression in rhombomeres 3 and 5 across vertebrates [51, 52]. Hindbrain segments define specific regions for body patterning as well. Neural crest derived from rhombomeric regions migrates to specific pharyngeal arches to form bone, cartilage, and connective tissue [53]. In zebrafish, for example, neural crest cells from r1-r3 migrate to the first pharyngeal arch to form jaw structures [54]. These structures are innervated by the Vth (trigeminal) cranial branchiomotor nerve, whose cell bodies reside in r1-r3 [55]. The same sort of patterning is observed for the second and third pharyngeal arches.

Anatomically, segmentation is visible in many vertebrate species as dimples at segment boundaries and bulges at segment centers. This is transiently visible at the 18 somite stage (18-20 hpf) in zebrafish [56]. The r4 territory is defined first with the r3/4 boundary established, then r4/5, r1/2, r2/3, and r6/7 boundaries are present (reviewed in [51]). The r7/8 boundary is not present by anatomical or molecular approaches, suggesting that the neuronal organization of posterior hindbrain may differ from more rostral hindbrain regions in zebrafish [57] and in chicks [58]. R1 is also distinctly different and forms an intimate tie with cerebellar regions [59-61].

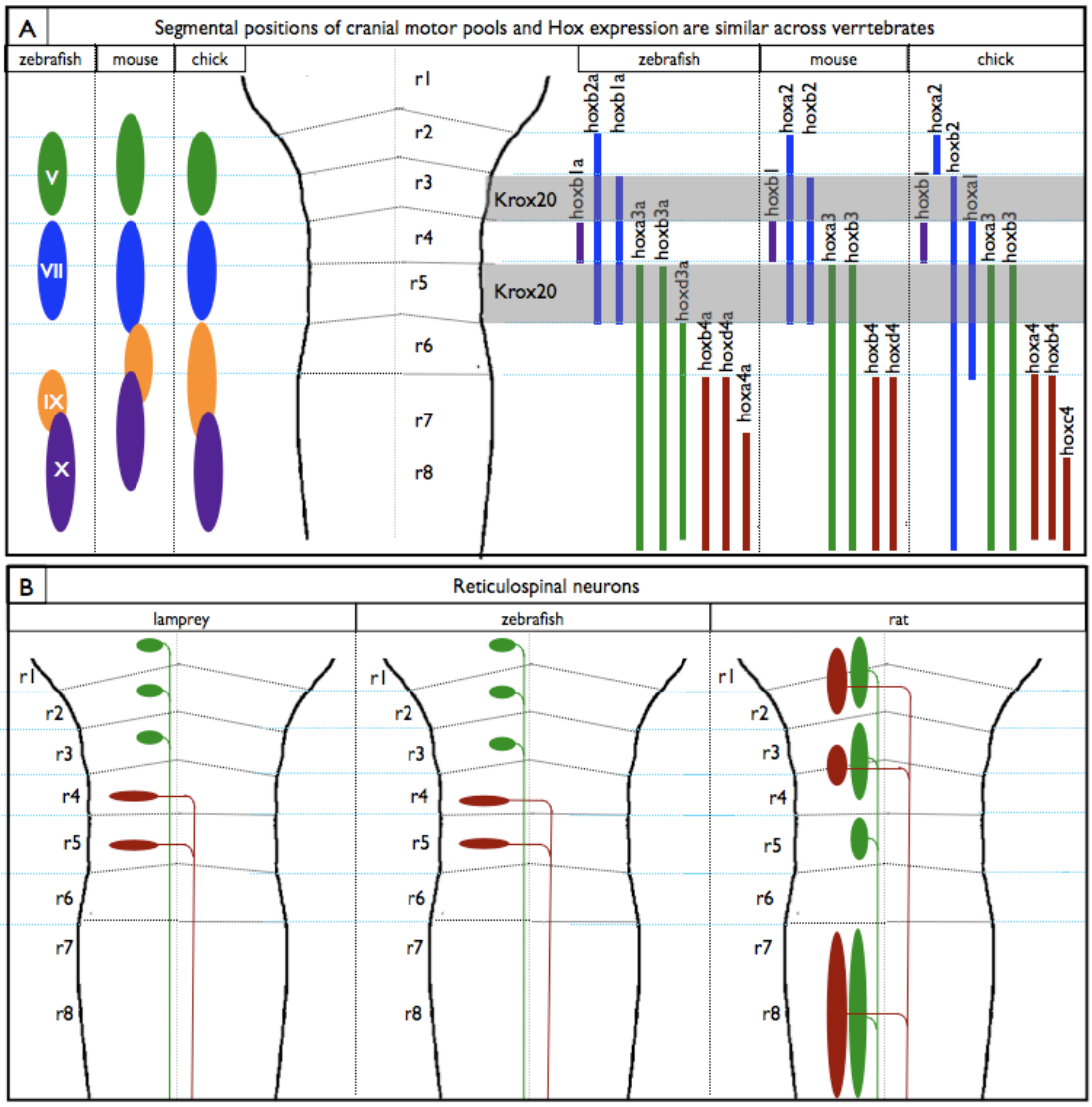
Another general organizational principle is the iteration of even-odd rhombomeres. For instance, in zebrafish, reticulospinal neurons are laterally positioned in even-numbered rhombomeres and more medially in odd rhombomeres [62, 63]. In both chick and zebrafish, branchiomotor nerves exit hindbrain from even-

numbered rhombomeres [55, 64], neural differentiation is delayed in odd-numbered rhombomeres [51], cranial neural crest cells migrate from even-numbered rhombomeres [53, 54], and even and odd rhombomeres possess cells with different attractive/repulsive components and can sort into even and odd pools when intermixed [65-68].

Molecular patterns also are segmental in nature; molecular cues for segmental identity are most strikingly observed by the distinct expression of several homeotic genes across hindbrain. Early expression of krox-20 and other genes are involved in initiating early segment patterning [51], and later segmental expression of hox genes may play a role in establishing both unique and segmentally repeated cell types. The similar expression of krox-20 in rhombomeres 3 and 5 in zebrafish [52], chick [69], and mice [70] as well as hox gene expression confined to specific rhombomere segments [51, 69, 71] is illustrated on the right in figure 1.2A.

Rhombomere boundaries are physical entities that display transcription factor expression distinct from rhombomere centers and may largely contain glial cells [72, 73]. They are regions of enlarged extracellular space that form conduits for circumferentially projecting axons in hindbrain [73]. These boundaries are suggested to play a role in axon guidance and cell migration. This type of boundary is crucial in the patterning of the drosophila imaginal disk where cells of the dorsal and ventral regions interact to induce vestigial gene expression in the boundary region [74]. Rhombomere boundaries have been shown to be distinct regions in hindbrain [75] that form glial ‘curtains’ in both zebrafish and chicks [76, 77]. These boundary properties may play a role in cell lineage restriction to segments of origin [78, 79].

Figure 1.2 Segmental patterning of hindbrain is conserved across vertebrates. **(A)** Positions of cranial motor pools and Hox expression in vertebrates. Positions of cranial motor pools V,VII,IX, and X are illustrated on the left for zebrafish, mouse, and chick, reviewed in [80].Despite slight differences (see positions of IX and X motor pools), the segmental positions of these motor pools are strongly conserved for these three vertebrates shown. On the right, Hox genes are patterned in a segmental fashion that is conserved across vertebrates (zebrafish: [71], comparison between zebrafish and mouse Hox expression reviewed in [51], and chick expression reviewed in [69]). Also shown on the right is the conserved patterning of Krox20 in rhombomeres 3 and 5 in the hindbrain of vertebrates. Krox20 has been shown to be a key regulator of rhombomere specific gene expression [81, 82]. Krox20 expression is localized to rhombomeres 3 and 5 for all vertebrates that have been examined (zebrafish: [52], xenopus and chick: [69], and mouse: [70]). **(B)** Positions of reticulospinal neurons across vertebrates. Medial reticulospinal neurons project ipsilaterally while more lateral reticulospinal neurons project contralaterally [80, 83].


A
Segmental positions of cranial motor pools and Hox expression are similar across verrtebrates
zebrafish
mouse
chick
V
VII
IX
X
r1
r2
r3
r4
r5
r6
r7
r8
Krox20
Krox20
hoxb2a
hoxb1a
hoxb1a
hoxa3a
hoxb3a
hoxd3a
hoxb4a
hoxd4a
hoxa4a
hoxa2
hoxb2
hoxb1
hoxa3
hoxb3
hoxb4
hoxd4
hoxa2
hoxb2
hoxb1
hoxa1
hoxa3
hoxb3
hoxa4
hoxb4
hoxc4
B
Reticulospinal neurons
lamprey
zebrafish
rat

Nuclear organization of hindbrain

Cell types are present in tightly conserved rhombomere regions across vertebrates. The hindbrain contains 8 of the 12 pairs of cranial nerves (V through VII) responsible for sensory and motor components of taste, hearing, balance, mastication, facial expressions, some eye movements, and in terrestrial vertebrates, the secretion of tears and saliva [84]. Hindbrain nerves also innervate pharyngeal arches that are also segmentally arrayed [51]. Along with cranial motor pools, the reticulospinal interneurons in the zebrafish maintain an orderly segmental patterning in hindbrain [57, 62]. Other cell types have also been shown to conform to this segmental patterning [85, 86]. Although physical segmentation is only transiently present, the early segmentation leaves an imprint on the neural patterning of the hindbrain that persists into the adult stages.

Nuclei are confined to specific segmental regions

Studies involving grafts of specific rhombomere segments from quail hindbrain homotopically into chick embryos have revealed that hindbrain nuclei are localized to specific rhombomere regions [87]. For instance, nucleus angularis is localized to r3-6, nucleus laminaris to r5-6, nucleus magnocellularis to r6-7, nucleus ovilaris superior to r5, and nuclei of the lateral lemniscus arise within r1-3 [87].

Although rhombomeres 7 and 8 are not divided by a physical boundary, chick/quail grafts within this region show that nuclei are localized to specific regions that have been named pseudorhombomeres 7-11 [88]. Some regions, such as vestibular, trigeminal columns, the reticular formation, and pontine nuclei did however selectively migrate into host hindbrains, indicating that they did not remain confined to their pseudorhombomeres of origin.

Nuclear organization is conserved across vertebrates

The nuclear organization for sensorimotor processing centers in hindbrain is a conserved feature across vertebrates. For example, the gravity sensing system is comprised of peripheral sensors of changes in gravity in the form of utricular and saccular hair cells [89]. The fundamental pathways for gravity sensing are conserved from birds through mammals [89]. The nucleus vestibularis tangentialis is comprised of a group of secondary vestibular neurons positioned in lateral regions of hindbrain along the vestibular nerve [90]. This nucleus has been observed in the hindbrain of reptiles, bird, and fish [89, 91].

Another example is the vocal acoustic network. In batrachoidid fish, rhythmically firing cells within caudal hindbrain control the timing of contractions of vocal muscles attached to the swim bladder that are responsible for producing calls [92]. A recent study has shown that the location within caudal hindbrain of this vocal-acoustic circuit is present in fish and all major lineages of vocal tetrapods [92].

Hox genes and nuclear organization

A marker of rhombomere identity described earlier in this section is the expression of transcription factors in a rhombomere-specific pattern [51, 69, 71]. On the right of figure 1.2A, a summary of hox expression in zebrafish, chick, and mouse shows the similarities in different species [51, 69, 71]. On the left in this figure, positions of cranial motor pools are shown to be localized to specific rhombomere regions [80]. Since nuclei arise from specific segments, the question arises of whether the formation of hindbrain nuclei depends on hox gene expression. In a recent study, boundaries of hox gene expression in pseudorhombomeres 7-11 was shown to overlap with boundaries of nuclei [93]. In this study, they show that spaced steps of hox gene

anterior boundaries are correlated with the rostrocaudal positions of different sensory and motor columns as well as the reticular formation [93].

To show that hox genes play a role in nuclei formation, one study used the chick/quail grafting experiment described above but with heterotypic instead of homotypic grafting. They showed that when rostral regions of quail brains at the 5-somite stage (before hindbrain boundaries are visible) are transplanted to caudal regions of chick embryo hindbrains, the cells that arise have changed to correlate with their new, more caudal position [94]. More strikingly, studies have shown that overexpression of hox genes can transform rhombomere 2 into a rhombomere 4 identity, with duplicates of the Mauthner cells, normally present in rhombomere 4, also present within rhombomere 2 [95]. When normal, rhombomere 4 positioned Mauthner cells were lesioned, the rhombomere 2 Mauthner neurons were sufficient to produce an appropriate startle performance [96]. Therefore, hox genes can initiate a program for the formation of a unique identity of a rhombomere segment for the production of cell types and their functional projections.

Transcription factors identify subpopulations within a nucleus

The function that specific nuclei perform can require more than one cell type. For example, the tangential nucleus is comprised of principal cells (80% of neurons), elongate cells (20%), and giant cells (<1%) that differ in their morphological features and numbers of cells within the nucleus [90]. The two major cell types also differ in their active membrane properties and response to vestibular nerve stimulation, suggesting distinct functional roles for different cell types within this nucleus [97].

Since nuclei can be heterogeneously populated, previous studies have investigated how cell types for specific nuclei are derived. Although hox genes are present across entire nuclear regions of hindbrain, recent studies have shown that

subpopulations of neurons within a nucleus express different types of transcription factors. For example, Tlx-3 is present in distinct subcomponents of trigeminal and vestibular nuclei [98]; Math5 is expressed in subpopulations within the ventral cochlear nucleus [99]; and Olig3 expressing neurons contribute to the nucleus of the solitary tract and to precerebellar nuclei [100]. Therefore, transcription factor expression reveals that subpopulations of neurons within nuclei, and, in some cases, shared across nuclei, are present, suggesting that subtypes of neurons can be shared across functionally distinct nuclei. This indicates a two-tier program of specialization. First, rhombomeres express unique patterns of hox genes, and second, cells within (and across) rhombomeres express transcription factors to define different cell types within a rhombomere where hox expression is uniform.

Development of hindbrain nuclei

There is considerable work that explores the patterns of differentiation of neurons in hindbrain rhombomeres. Here I review some of this work that bears upon my studies of the differentiation of neurons within neurotransmitter stripes in hindbrain. Since nuclei are also localized to specific local regions within rhombomeres, one important question is the origin of the cells that contribute to different nuclei. This has been tracked by using quail/chick grafts where homotypic grafts of quail hindbrain regions of different dorsoventral positions into chick embryos revealed the origins of nuclei from alar and basal plates [101]. Motor nuclei were derived from the basal plate, sensory nuclei from the alar plate, and the reticular formation arose from both alar and basal plate regions. The timing of migrations in different directions to form nuclei was investigated and a temporal order for different movements was found. From E5-8, dorsoventral and ventrodorsal movements are present with dorsoventral streams more prominent; from E8-9, the marginal stream

was found to cross the midline; and beyond E12, longitudinal migrations in ventral regions both rostrocaudal and caudorostrally were seen. Fibers in the marginal zones were evident before the onset of cell body migration, indicating that, to some degree, connections are established before migrations to form nuclei begin.

In the vestibular system, the tangential nucleus in chicks is the primary vestibular nucleus whose two main neuron types both migrate and begin to differentiate between 5 and 8 days [102]. The earliest synapses were received at 5 days on primitive processes and subsequently on their cell bodies by longitudinal fibers of unknown origin. However, primary vestibular afferents did not form identified synapses on the developing tangential neurons until 7.5 days.

In the avian auditory system, localization of sound in space begins with the bilateral innervation of nucleus laminaris (NL) by nucleus magnocellularis (NM) neurons. In one study, the developmental timing of different events for the organization of this auditory system was studied [103]. In the auditory system, NM neurons are present at E6, and the auditory anlage is organized into two nuclei, NM and NL in a rostral to caudal fashion between E6-8. NM neurons were present throughout the extent of the anlage at E6. NL was present in the ventral anlage. Ipsilateral NM fibers contacted the developing NL at E8, well after the NM collaterals had projected contralaterally. Ipsilateral connections between NM and NL neurons coincided with the arrival of VIIIth nerve fibers in NM. Therefore, extensive pathfinding and morphological rearrangement of central auditory nuclei occurred well before the arrival of cochlear afferents.

Age-related patterning

By 2 dpf, the majority of hindbrain neurons in zebrafish have differentiated [76]. There is a progressive filling in where neurons migrate ventrally away from the

ventricular zone, and, between 1 and 2 dpf, a ventral to dorsal expansion in the region of differentiated neurons occurs [76]. However, the age-related organization of this patterning has not been addressed; it has been assumed that the oldest neurons are ventral and those more dorsal are younger, which would be the case if dramatic migrations, such as those for patterning the cortex into layers [84], are not present in hindbrain. In chapter 2, we address the age-related patterning by using photoconvertible proteins in transgenic zebrafish to track the positions of neurons of different ages within neurotransmitter stripes in embryos and larval zebrafish. Below, we summarize prior results showing age differences within hindbrain; however, no study has shown age-related patterning within one neuron class as was shown for alx neurons in zebrafish spinal cord [18].

In the avian cerebellar rhombic lip, early born cells migrate to rhombomere 1 while later born cells migrate ventrally but cease migration at the lateral edge of the cerebellum [104]. Therefore, in this case, early and late born cells form two different types of neurons. Early born neurons are present in rhombomere 1, and late born neurons populate the cerebellum

Within the inferior olive of the rat, the cytoarchitecture was linked to an age-related organization in a previous study [105]. In the inferior olive complex, ION (inferior olivary nucleus) neurons born at the same time are arrayed in clusters within the adult structure. Interestingly, the mediolateral age-related patterning is present and reversed between rostral and caudal regions. This arises from the lack of lateral to medial migration in the MAOc (the caudal region of the medial accessory olive).

The vestibular nucleus of rat is subdivided into LVe (lateral vestibular nucleus), IVe (inferior vestibular nucleus), and MVe (medial vestibular nucleus) where each subnucleus maintains different projections [106]. In one study, these subnuclei were found to form in a stepwise manner [106]. At E12, most neurons in

LVe were generated; IVe neurons were equally generated on E12 and E13; and MVe neurons across E12-14 with a peak at E13.

In summary, neurons of different ages can populate different brain regions as was shown for rhombomere 1 and the cerebellum [104]. Within nuclei, neurons of different ages can be mediolaterally segregated, as described within the inferior olive of rats [105]. In nuclei subdivided based on projection patterns, subdivisions can arise in a stepwise manner during development as was shown for the vestibular nucleus of rats [106]. Also, indications that circuits within nuclei might wire before peripheral sensory nerves project to these regions suggests that interneuron wiring might occur before primary sensory information is received, as was shown in the avian auditory systems [103].

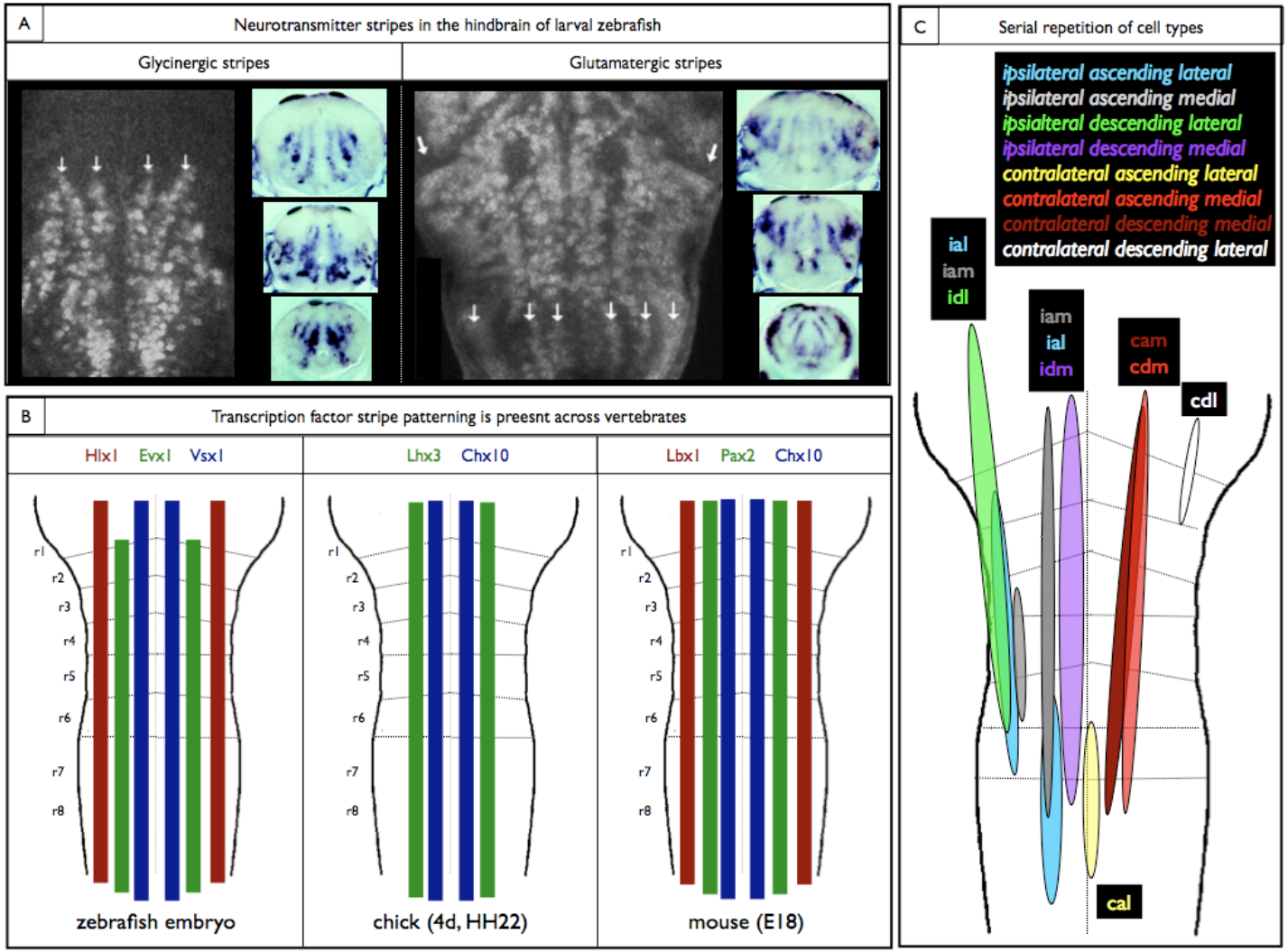
Despite these age-related patterning studies revealing hints of an age-related order, no hindbrain study has revealed an age-related difference in neurons of the same type as was shown in spinal cord for neurons expressing the alx transcription factor [18]. Since this spinal age-related pattern revealed functional differences in recruitment for neurons of the same type, understanding the age-related patterning of cells of the same type in hindbrain might reveal new principles of age-related functional organization in hindbrain.

Indications of a stripe-like patterning of hindbrain

Neurotransmitter stripes

In a previous study from the Fetcho lab, stripes of neurons by neurotransmitter phenotype were shown to exist in the hindbrain of larval zebrafish [107]. In situ hybridizations using genes specific to excitatory (VGlut2.1) and inhibitory neurons (GlyT2) were performed and examples from this study are shown in figure 1.3A. The left panel shows patterning of glycinergic neurons in hindbrain, and the right panel

Figure 1.3 Stripe patterning by neurotransmitter and transcription factor types is present rostrocaudally across hindbrain. **(A)** Neurotransmitter stripes in hindbrain. In situ hybridizations adapted from [107]. On the left, fluorescent in situ hybridizations using the GlyT2 in situ probe described in the paper are shown. In dorsal view, multiple stripes are present and indicated by white arrows. To the right in this panel, 3 cross sections at different rostrocaudal positions indicate more clearly a stripe patterning that is present for glycinergic neurons across hindbrain. In the right panel, a similar stripe patterning is shown for glutamatergic neurons. Again, fluorescent in situ hybridization was performed to label neurons of a single neurotransmitter type; here, the VGlut2 in situ probe marks glutamatergic neurons that also form stripes indicated by white arrows in dorsal view and more clearly distinguished in cross sections shown on the right. **(B)** Transcription factor stripes are present rostrocaudally across hindbrains of multiple vertebrates. Transcription factor expression is illustrated for multiple types in zebrafish, chick, and mice. Data for each was observed in the following references: Hlx1 in 30 hpf zebrafish from [108]; Evx1 in 48 hpf zebrafish embryos from [109]; Vsx1 in 35 hpf zebrafish from [110]; Lhx3 and Chx10 expression in HH22 (4 day old) chicks from [111]; and Lbx1, Pax2, and Chx10 expression in E18 mice from [112]. (C) Serial repetition of cell types across rhombomeres in chick hindbrain. In dorsal view, an illustration of regions of both ipsilaterally (left side) and contralaterally (right side) projecting neurons is shown. For each cell type, a column of neurons was found by backfilling experiments; columns for each type are present in a continuous region across at least 2 rhombomeres, and, in some cases, rostrocaudally throughout hindbrain. This illustration is an adaptation of the summary diagram (figure 7) of the study that reported this patterning: [58].


A
Neurotransmitter stripes in the hindbrain of larval zebrafish
Glycinergic stripes
Glutamatergic stripes
B
Transcription factor stripe patterning is preesnt across vertebrates
Hlx1 Evx1 Vsx1
Lhx3 Chx10
Lbx1 Pax2 Chx10
r1
r2
r3
r4
r5
r6
r7
r8
zebrafish embryo
chick (4d, HH22)
mouse (E18)
C
Serial repetition of cell types
ipsilateral ascending lateral
ipsilateral ascending medial
ipsialteral descending lateral
ipsilateral descending medial
contralateral ascending lateral
contralateral ascending medial
contralateral descending medial
contralateral descending lateral
ial
iam
idl
iam
ial
idm
cam
cdm
cdl
cal

shows the patterning of glutamatergic neurons. In each panel, the image to the left is a dorsal view of hindbrain regions and white arrows indicate stripes of neurons that are mediolaterally segregated. On the right, cross sections are shown from rostral (top) to caudal (bottom). In each cross section, neurotransmitter stripes are clearly present and are mediolaterally segregated. The work described here focuses on this stripe patterning and how relates to the organization of interneurons in hindbrain.

Transcription factor expression

Many studies have shown that transcription factors present in spinal cord are present in the hindbrain as well [99, 100, 108-134]. Some examples of this stripe patterning for zebrafish, chick, and mice are shown in figure 1.3B. In each case, a mediolateral segregation of transcription factors is present, and transcription factor expression extends rostrocaudally throughout hindbrain. In zebrafish, Vsx1 is present in a medial band in hindbrain [110], while Evx1 is present in a slightly more lateral hindbrain region [109], and Hlx1 is laterally located rostrocaudally throughout hindbrain [108]. The middle panel shows that, in chicks, Chx10 expression forms a rostrocaudal band medially and Lhx3 forms a band as well [111]. In mice, three transcription factors are present at mediolaterally-segregated positions, and a recent study showed that reticulospinal neurons that project to different mediolateral regions express different transcription factors of these three types [112]. This patterning has been hinted at previously where medial reticulospinal neurons project ipsilaterally and more lateral reticulospinal neurons project contralaterally for reticulospinal neurons across multiple rhombomere segments [80, 83] and is shown in figure 1.2B.

Segmental repetition of cell types

A previous study addressed the possibility that all neurons in hindbrain might form an orderly wiring pattern. By performing backfills in specific chick hindbrain regions and tracking backfilled cells that project to that region, rostrocaudally extending bands of cells that were mediolaterally segregated were found to exist and share projection patterns. This study suggested, before the transcription factor organization of spinal cord and hindbrain were established, that interneurons in hindbrain might be serially repeated across segments to establish a wiring template for hindbrain neural circuits [58]. This idea has some parallels with previous evidence for orderly patterns of neurons in zebrafish [62]. Figure 1.3C shows a merged view of the patterning in the chick hindbrain, where continuous columns progressing across multiple rhombomeres that identify cells with similar projections are shown. In the following chapters, we show that this is clearly the case in zebrafish, and that neurotransmitter stripes identify neurons with particular projection patterns. Since stripes also extend dorsoventrally, we can expand upon this previous hypothesis and investigate the organization within a stripe.

Summary

In this chapter, we have reviewed studies detailing the organization of spinal circuits involved in the coordination of motor behavior as well as studies describing the organization of hindbrain, both revealing segmental differences in hindbrain as well as continuous patterns such as transcription factor expression and the serial repetition of cell types across multiple segments. In the following chapters, we will show how the organization of hindbrain stripes represents a topological transformation of spinal organization. The main features of hindbrain stripes will be shown to exist across multiple rhombomere segments, indicating a conserved role of stripe

organization across segments, despite the differences within segments described here. In chapter 2, I will show that stripes maintain an orderly patterning from ventral to dorsal for old to young neurons, indicating an age-related patterning within stripes. In chapter 3, I will show that glycinergic stripes define regions of glycinergic neurons that have similar morphology and mediolateral patterns of projections. In chapter 4, I show that, within a single stripe, along with the age-related organization of cell bodies, there is an age-related organization of input resistance and wiring that might indicate a functional topography dorsoventrally along the stripe for recruitment during swimming.

CHAPTER 2

AN ORDERLY ARRANGEMENT OF NEURONS IN HINDBRAIN BY TRANSMITTER, TRANSCRIPTION FACTOR, AND AGE

Abstract

Recent studies of the spinal cord have revealed some simple patterns of organization based on both transcription factor expression and the age of a neuron. Spinal neurons arise from progenitor pools marked by dorsoventrally organized bands of transcription factor expression that direct the differentiation of specialized cell types from different regions of spinal cord [3]. In zebrafish, neurons arising from these regions at different times have different electrical properties [19] and participate in different speeds of locomotion [18, 19, 29, 135]. While there has been much progress in revealing core patterning in spinal cord, less is known about overall neuronal patterning in hindbrain except for the striking segmental organization along its rostrocaudal axis. Our prior work revealed that there are stripes of neurons with homogeneous transmitter phenotype that extend rostrocaudally through hindbrain and alternate from medial to lateral in the expression of inhibitory versus excitatory transmitter markers [107]. We show here that the transmitter stripes align with transcription factor bands, similar to those in spinal cord, but with a different orientation as a result of a topological transformation from spinal cord to hindbrain. We investigated whether an age-related patterning exists in these stripes of neurons arrayed both by transcription factor and neurotransmitter expression patterns, and whether it persists into larval stages when fish are freely swimming, and thus, when hindbrain networks have been wired. The quick developmental time of zebrafish allowed us to track neurons of different ages in vivo using transgenic lines expressing

either photoconvertible or fluorescent proteins under promoters for genes expressed in neurons sharing neurotransmitter or transcription factor phenotype. We found that an orderly patterning by age persists within hindbrain stripes throughout much of hindbrain, with the oldest neurons located ventrally, and increasingly younger ones stacked above them along the axis of the stripe. This age-related patterning might have significance for a wide variety of hindbrain neural circuits since it expands rostrocaudally through hindbrain and is present at an age in which zebrafish are freely swimming. If there are age-related functional properties in hindbrain similar to those in spinal cord, these stripes might reflect a simple functional patterning in hindbrain that underlies larval behaviors. The stripe in which a neuron belongs may define its transmitter phenotype and projection pattern and thus its wiring pattern in a network (see chapter 3). The position of a neuron within a stripe may determine its electrical properties and thus, for example, its contribution to different speeds and strengths of movements, with older, ventral neurons participating in faster, stronger movements or higher threshold functions than younger ones (see chapter 4).

Introduction

Two patterns of organization have recently been described for interneurons in spinal cord. During development, progenitor cells, as defined by patterns of expression of transcription factors, are arrayed in an orderly manner where progenitor cells expressing particular transcription factor types are positioned at specific dorsoventral positions [3]. These progenitor cells have to been shown to generate neurons that express distinct transcription factors [3] and form functional classes [1, 2, 10, 15, 22-26, 40, 135-138]. For instance, Nkx6.1 progenitor cells produce two types of cells termed V2 neurons [40, 137, 139]. V2a neurons, one of these two types, express the Chx10 transcription factor and are involved in motor coordination [10].

When these cells are removed in mice, motor coordination is disrupted [10]. Alx, a transcription factor homologous to Chx10, is present in a population of interneurons in zebrafish with a similar morphology to the Chx10 expressing neurons in mice. These cells are active during either swimming or escape maneuvers [18, 19, 29, 135], suggesting transcription factors define cells with similar structure and with similar functional roles across species.

Another pattern of organization within a transcription factor class is a correlation between the age of a neuron and its participation in different strengths of movements. In zebrafish, the Alx transcription factor neurons described above are recruited from ventral to dorsal as swimming frequency increases [18, 19, 29, 88]. Because these neurons are arranged by age, with young ones ventral and old ones dorsal [18], their recruitment follows both a position [18, 19] and an age-dependent order [18].

The presence of these simple principles that define the functional organization of interneurons by age and transcription factor expression in spinal cord raises the question of whether there might be similar patterns of organization in the brain. We have previously described a patterning of interneurons in hindbrain of stripes identified by neurotransmitter phenotype [107]. These stripes alternate between excitatory and inhibitory neurotransmitter phenotype from medial to lateral. One possibility is that these stripes reflect an orderly organization of cell types like those defined by transcription factors in spinal cord.

The hindbrain is a rostral continuation of spinal cord and developmental studies have revealed links between them. Ventral sonic hedgehog (Shh) and dorsal bone morphogenetic protein (BMP) gradients are present early in development across both regions [42, 140]. Within spinal cord, these morphogens are responsible for the early transcription factor patterning of progenitor cells [42, 141]. The induction of

spinal cord patterning requires the expression of Cdx; loss of Cdx allows for the expansion of hindbrain regions into spinal cord and overexpression of Cdx4 disrupts the unique rhombomeric program of hindbrain development, supporting a link between spinal cord and hindbrain [142]. Many studies have indicated that bands of transcription factor expression are present in hindbrain [99, 100, 108-134]. This raises the question of whether neurotransmitter stripes corresponded to transcription factor expression bands and how these stripes develop in hindbrain relative to spinal cord.

There are hints that the age-related order and functional organization described for spinal cord might also exist in the hindbrain. The fastest network in hindbrain develops first. The earliest born neuron is the Mauthner cell, a neuron that, with a single action potential, can initiate an escape response that involves a coiling of the entire body away from a strong, and possibly threatening, sensory stimulus [143]. Some previous studies have investigated whether an age-related patterning might exist within specific hindbrain nuclei [103, 105, 106]. Until recently, however, both tracking cells of different ages in vivo as well as in vivo visualization of neurons of one specific type has not been possible; therefore, it remains unknown whether an age-related organization of cell types within hindbrain circuits might exist. Such a patterning might reflect functional divisions within a cell class defined by transcription factor expression.

The hindbrain is much larger than spinal cord and there could be considerable migration of neurons that might obscure any age-related patterning. We suspected, however, that an age-related patterning might still exist in the hindbrain of larval zebrafish since another striking organization, the stripe patterning by neurotransmitter phenotype, persists into larval stages. The rapid developmental time of zebrafish and transgenic lines expressing color change proteins allowed us to ask whether there is an

age-related order of neurons that share neurotransmitter or transcription factor expression.

We found that the transmitter stripes in hindbrain are marked by transcription factors that are some of the same ones that define neuronal classes in spinal cord [1, 10, 18, 21, 137]. The topological organization of these stripes is altered from spinal cord to the brain, but major features of the patterning are shared. The neurons within hindbrain stripes in most regions are arranged by age, with the oldest ventral and younger ones dorsal. This age related order has obvious parallels with that of spinal cord. Importantly, the stripe patterning in hindbrain is evident even after the fish is swimming suggesting that neural circuits are constructed at a time when the cells are ordered by type and age. This suggests that there may be a simple, underlying structural and functional template shared by hindbrain and spinal cord, upon which more specialized circuits are constructed.

Results

Overall age-related patterning in hindbrain compared to spinal cord

Previous work showed that there is an age related patterning in spinal cord in which the oldest spinal neurons are centrally and laterally located in cross section, with younger neurons stacked above and below them (D McLean and JR Fetcho, unpublished). We first compared the patterning of neurons in hindbrain to the spinal organization by using a transgenic line expressing a photoconvertible protein in all neurons under control of a general neuronal promoter, Huc (Huc:Kaede, [144]). We exposed these transgenic fish to UV light at 24 hours post fertilization (hpf) to photoconvert the neurons that had differentiated by then from green to red. Imaging the fish at 4 days post fertilization (dpf) revealed the positions of the oldest, red neurons in comparison to neurons that started expressing Kaede protein after 24 hpf,

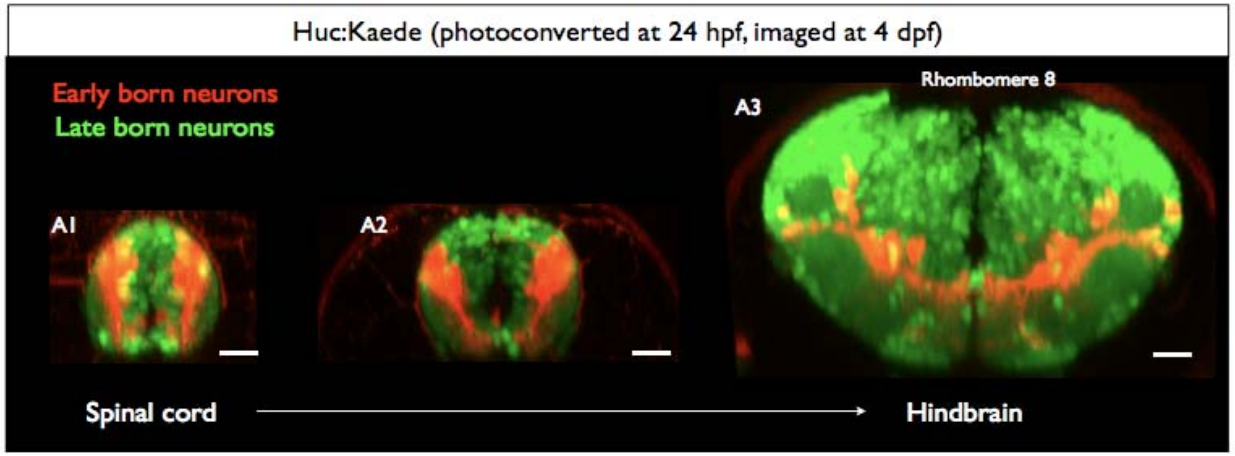
which were green only (figure 2.1A). In the spinal cord, the oldest neurons were both laterally and centrally positioned, just ventral to the dorsoventral middle of cord (figure 2.1A1). In the hindbrain, old neurons were mid-ventrally positioned in the middle region on either side of the hindbrain (figure 2.1A3). In sections from spinal cord to hindbrain, the width of the cross section increased into hindbrain with the relative positions of old and younger cells in the two regions appearing as if the spinal cord was split open at the midline and spread apart with dorsal spinal cord regions now present more laterally in hindbrain regions (figure 2.1A1-3).

The experiments described above allowed for the marking of neurons that differentiate before and after a certain time. This showed that the oldest were more ventral with younger ones above in hindbrain, suggesting that they are ordered by age from ventral to dorsal. To examine this more closely, we wanted to compare neurons that were present at a particular time with those added shortly after, rather than with all later added neurons.

To accomplish this, we photoconverted fish at either 24 hpf or 4 hours later, at 28 hpf, and imaged both converted fish at 2 dpf. In cross sections of hindbrain (figure 2.2B2-3) there were more ventrally positioned red cells at 28 hpf than at 24 with the 28 hpf neurons occupying a broader dorsoventral and mediolateral extent. In the hindbrain, the older neurons in both cases were ventral and in the middle of the hindbrain, with younger neurons on either side and above them (figure 2.2B2-3). The slightly younger neurons present by 28 hpf occupied a broader mediolateral and dorsoventral extent than at 24 hpf.

To reveal the location of neurons likely added between 24 and 28 hpf, we performed image registration to align the images of the fish converted at the two different times, using the green expression in all neurons of both fish for alignment. In

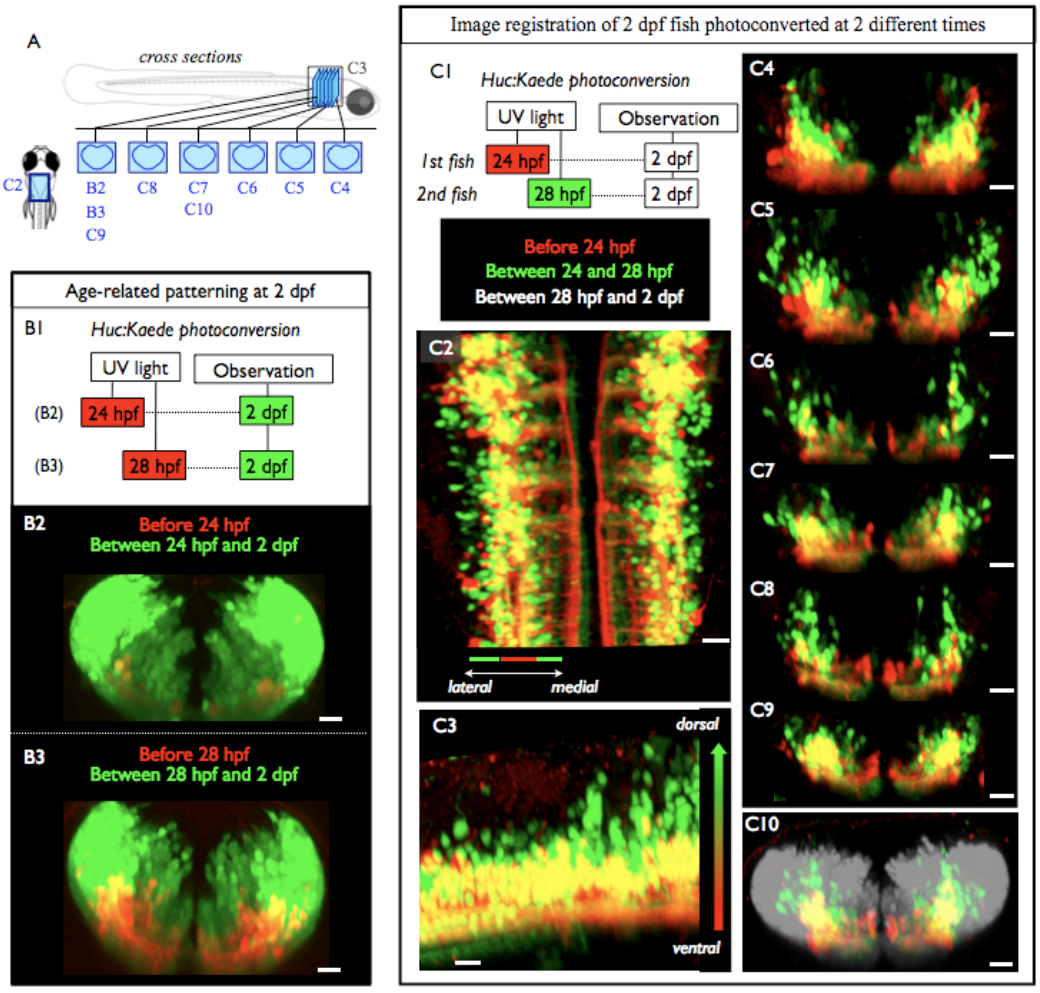
Figure 2.1 Patterning between hindbrain and spinal cord by age. Huc:Kaede transgenic fish were photoconverted at 24 hpf and imaged at 4 dpf. **(A)** Cross sections from spinal cord to hindbrain show an orderly patterning of interneurons by age. In the spinal cord (**A1**), old neurons are laterally and mid-dorsally positioned and younger neurons are more medially positioned as well as dorsal and ventral to the oldest ones. In the hindbrain (**A3**), the oldest neurons are ventrally positioned and younger neurons fill in more dorsally, as well as lateral and medial to the oldest ones. Age-related patterning is continuous from spinal cord to hindbrain. The widening in hindbrain relative to spinal cord as well as the opening of the neural tube forms a patterning in hindbrain where the spinal pattern is split open dorsally and the mediolateral segregation by age in spinal cord is transformed into a dorsoventral segregation by age within hindbrain. Scale bars = 20 um.


Huc:Kaede (photoconverted at 24 hpf, imaged at 4 dpf)
Early born neurons
Late born neurons
A1
A2
A3
Rhombomere 8
Spinal cord
Hindbrain

the aligned image we colored the red neurons from the 24 hpf photoconversion red and the red neurons from the 28 hpf conversion green (legend in figure 2.2C1). This approach shows in horizontal projections that the neurons present by 28 hpf extend both laterally and medially somewhat beyond the boundaries of those already present at 24 hpf (figure 2.2C2). This suggests that some of the cells added in those 4 hours might be added on either side of those present earlier. In a lateral view, many neurons present by 28 hpf extend above those at 24 indicating that many new cells might also be added above those present 4 hours earlier (figure 2.2C3). This expansion of the neurons from ventral to dorsal for neurons appearing between 24 and 28 hpf at 2 dpf is also evident in cross sections (figure 2.2C4-9), and suggests that neurons are added immediately above those differentiating just before them.

In cross sections across much of hindbrain (figure 2.2C4-9), slightly younger neurons are consistently either more dorsal, lateral, or medial than the oldest cluster of neurons. This patterning of neurons is consistent with the orderly addition of neurons by age, with new ones added adjacent to older ones, and the major addition occurring from ventral (old) to dorsal (young) in hindbrain. In figure 2.2C10, neurons present within each time frame are shown in three different colors in a cross section from a mid-rostrocaudal hindbrain region (red: present by 24 hpf; green: present by 28 hpf; white: present at the time of imaging, 2 dpf, see legend in figure 2.2C1). The youngest (white) neurons fill into hindbrain regions in a similar manner to those present early (green relative to red), with the youngest (white) neurons extending dorsally above the older (red and green) ones. This indicates that all neurons are patterned in an orderly manner by age at 2 dpf.

Figure 2.2 Positions of early born neurons of slightly different age at 2 dpf in hindbrain. **(A)** Orientation of images in this figure. **(B)** Cross sections of hindbrain for two Huc:Kaede 2 dpf fish photoconverted at different times on the previous day. The legend in **B1** illustrates the timing of the experiments in **B**. In **B2**, Huc:Kaede fish were photoconverted at 24 hpf and imaged at 2 dpf, and in **B3**, Huc:Kaede fish were photoconverted 4 hours later at 28 hpf and imaged at 2 dpf as well. In both cases, old (red) neurons are ventrally positioned and centered on either side of hindbrain. In **B3**, slightly more old (red) neurons are present in the same ventral position. **(C)** Image registration of Huc:Kaede fish at 2 dpf photoconverted at 2 different times. Image registration was performed using the Huc:Kaede green expression for two fish at 2 dpf. After alignment, the oldest neurons (by 24 hpf) are colored red and the slightly younger neurons (by 28 hpf) are colored green, and all cell present by 2 dpf, the time of imaging, are shown in white (only in **C10**) as indicated in **C1**. A dorsal view (**C2**) indicates that, rostrocaudally across hindbrain, green (younger) neurons are both more medial and more lateral to the rostrocaudal band of red (early born) neurons (**C2**, red/green bars below image indicate mediolateral regions of red and green cells). In lateral view **(C3)**, green (younger) cells are only seen more dorsal to red (older cells) at 2 dpf. Red/green bars shown to the right of the image indicate dorsoventral regions of red and green cells. **(C4-C9)** Cross sections originating from the same image registration already shown in dorsal and lateral projections in **C2** and **C3** respectively. In each cross section, slightly younger (green) neurons are consistently dorsal to red (older neurons). Here it is clear that even among closely timed neuronal differentiations, an age-related patterning of cell bodies is maintained at 2 dpf. This patterning is present for neurons of all three time periods as shown in **C10**. Scale bars = 20 um.

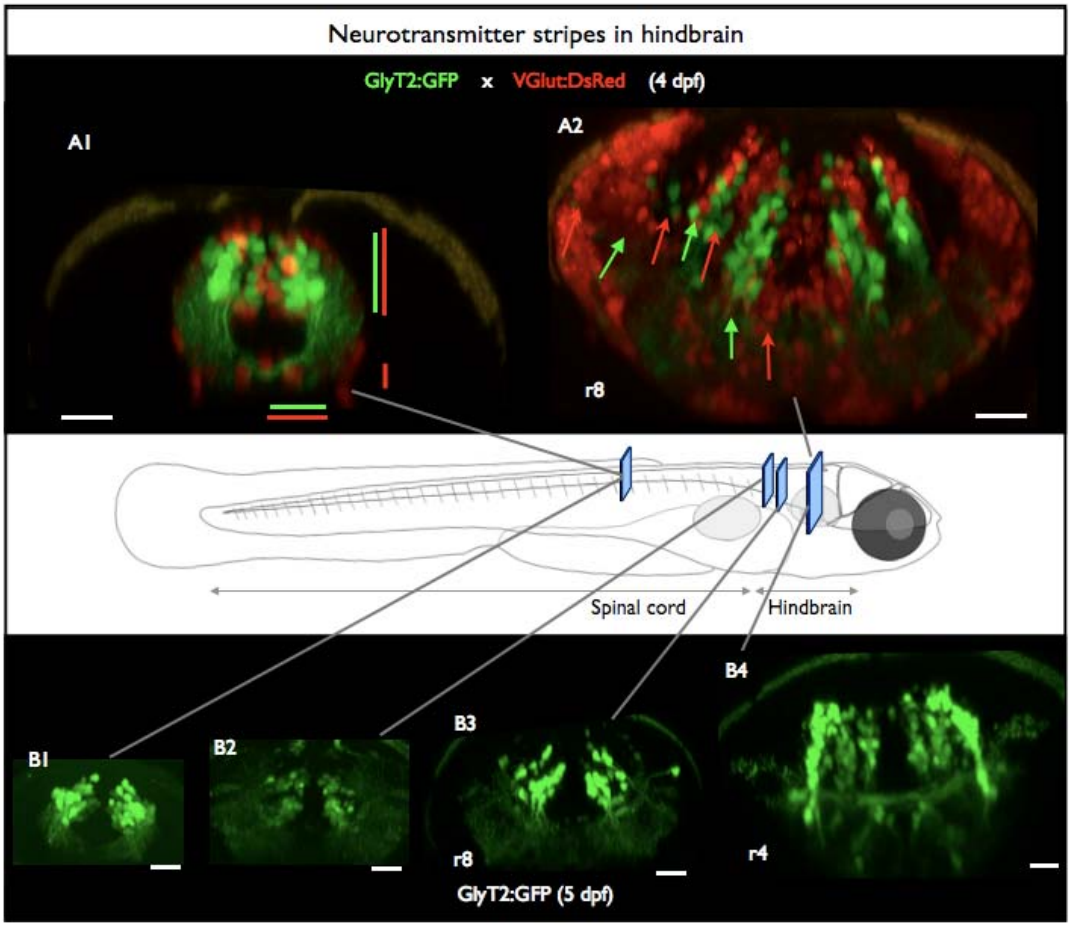

A
cross sections
C3
C2
B2
B3
C9
C8
C7
C10
C6
C5
C4
Age-related patterning at 2 dpf
B1
Huc:Kaede photoconversion
UV light
Observation
(B2)
24 hpf
2 dpf
(B3)
28 hpf
2 dpf
B2
Before 24 hpf
Between 24 hpf and 2 dpf
B3
Before 28 hpf
Between 28 hpf and 2 dpf
Image registration of 2 dpf fish photoconverted at 2 different times
C1
Huc:Kaede photoconversion
UV light
Observation
1st fish
24 hpf
2 dpf
2nd fish
28 hpf
2 dpf
Before 24 hpf
Between 24 and 28 hpf
Between 28 hpf and 2 dpf
C2
lateral
medial
C3
dorsal
ventral
C4
C5
C6
C7
C8
C9
C10

Organization by neurotransmitter type in hindbrain versus spinal cord

One of the striking features of the organization of hindbrain is the interleaved stripes of neurons that alternate between excitatory and inhibitory neurotransmitter phenotype from medial to lateral. These are evident in a cross section of the hindbrain within rhombomere 8 of a 4 dpf transgenic fish with the glycine transporter 2 promoter driving GFP and the vesicular glutamate promoter driving red fluorescent protein shown in figure 2.3A2. In chapter 3, we discuss the significance of these neurotransmitter stripes by showing that stripes of glycinergic neurons identify distinct morphological classes of inhibitory interneurons that also project to unique mediolateral positions. Since an age-related organization has been shown for spinal neurons [18], here we compare the relationship between transmitter stripes in hindbrain with that of spinal cord before looking at the age-related organization within these transmitter stripes.

Cross sections of spinal cord in the dual color Glyt2/VGlut transgenic line show that there is more intermingling of neurons with different transmitter phenotypes in spinal cord (figure 2.3A1) compared to hindbrain (figure 2.3A2). To explore the transition from the spinal cord pattern to the stripe patterning in hindbrain, GlyT2:GFP 5 days postfertilization (dpf) transgenic fish were imaged along the rostrocaudal axis of the animal. Cross sections from confocal images at different locations are shown in figure 2.3B. In the spinal cord, glycinergic interneurons are distributed uniformly, without any obvious clustering into stripes at 5 days (figure 2.3B1). More rostrally, gaps begin to appear between clusters of glycinergic interneurons that form roughly horizontal stripes segregated dorsoventrally (figure 2.3B2). Further rostrally, within hindbrain, these stripes are rotated so that they are oriented at an angle (figure 2.3B3, rhombomere 8). In rostral hindbrain segments, stripes are vertically oriented and segregated mediolaterally (figure 2.3B4, rhombomere 4). Therefore, a transformation

Figure 2.3 Patterning between hindbrain and spinal cord by neurotransmitter phenotype. **(A)** Patterning of interneurons by neurotransmitter phenotypes. Cross sections of dual expressing VGlut:DsRed and GlyT2:GFP transgenic fish at 4 dpf in spinal cord (**A1**) and hindbrain (**A2**). In the spinal cord, excitatory (VGlut:DsRed) and inhibitory (GlyT2:GFP) interneurons are intermingled (**A1**). Vertical red/green bars indicate dorsoventral regions of glutamatergic/glycinergic neurons, and horizontal red/green bars indicate mediolateral regions of glutamatergic/glycinergic neurons on the right side of the cross section shown in **A1**. In both cases, glutamatergic and glycinergic neurons are present across similar regions of spinal cord. In the hindbrain, however, stripes of neurons are present that alternate between excitatory (red) and inhibitory (green) neurotransmitter phenotypes from medial to lateral (**A2**). Red/green arrows indicate ventral regions of glutamatergic and glycinergic stripes respectively. **(B)** Glycinergic neurons in cross sections from spinal cord to hindbrain. In the spinal cord, glycinergic interneurons show no indications of stripe patterning (**B1**). More rostral, stripe patterning is present in a dorsoventral pattern (**B2**). Within hindbrain, stripe patterning is no longer dorsoventrally oriented (**B3**), and, more rostrally, stripes are segregated into different mediolateral positions (**B4**). Scale bars = 20 um.


Neurotransmitter stripes in hindbrain
GlyT2:GFP x VGlut:DsRed (4 dpf)
A1
A2
r8
Spinal cord
Hindbrain
B1
B2
B3
B4
r8
r4
GlyT2:GFP (5 dpf)

occurs from spinal cord to hindbrain, where clusters of dorsoventrally segregated neurons appear and, more rostrally, are rotated until they are dorsoventrally aligned and segregated mediolaterally (figure 2.3B1-4).

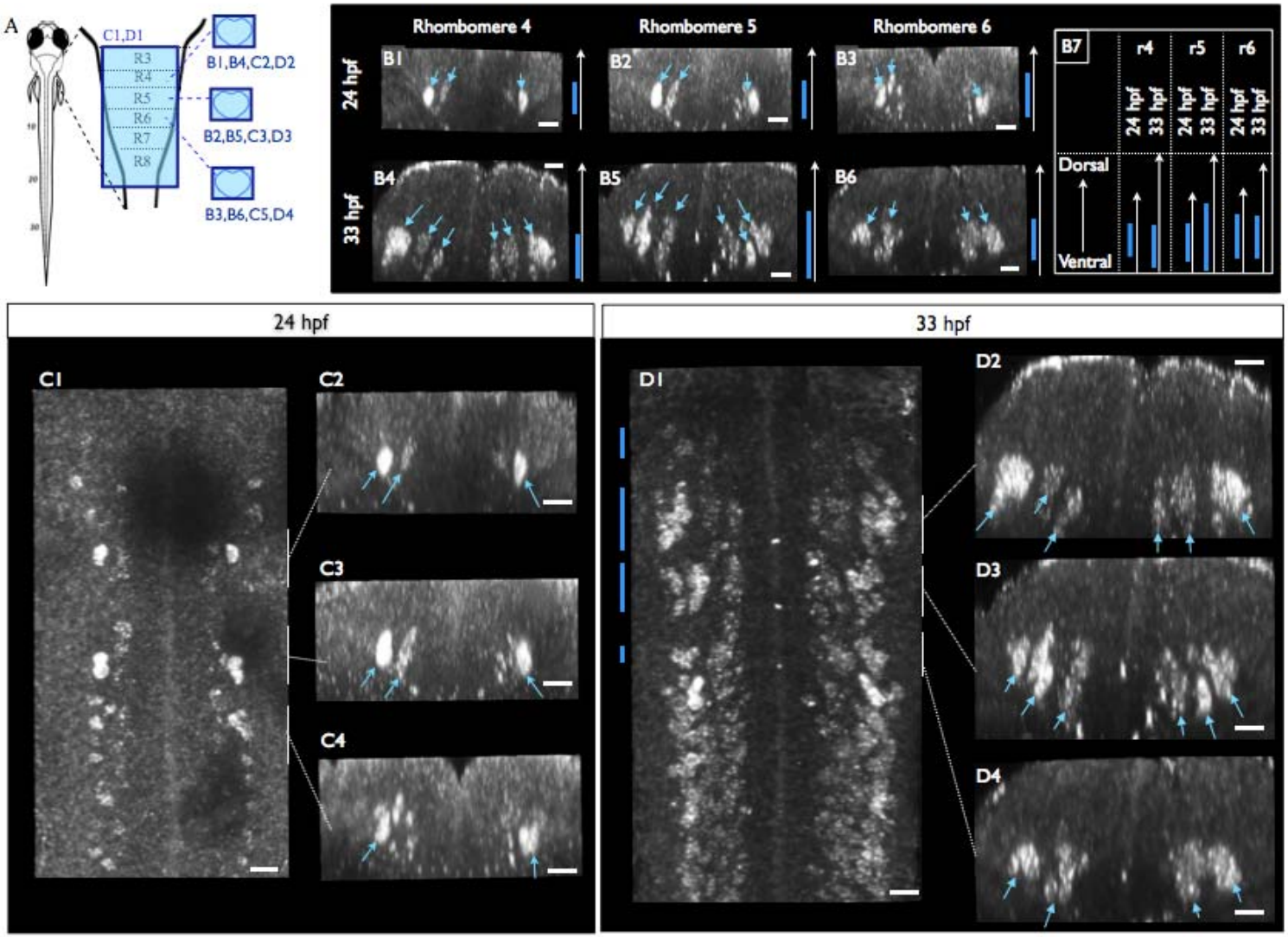
Development and age-related organization of neurotransmitter stripes

Evidence from spinal cord shows a relationship between the age of neurons of particular classes and their functional roles in different speeds of locomotion ([18], D McLean and JR Fetcho; unpublished). This, along with the striking organization into transmitter stripes in hindbrain, led us to ask how the transmitter stripes developed and whether there was any age related patterning within the stripes.

To examine when the stripes arose developmentally, we performed fluorescent in situ hybridization on zebrafish embryos at different time points using a probe that marks glycinergic interneurons (GlyT2, [107]; figure 2.4). At 24 hpf, glycinergic interneurons populated ventral regions of rhombomere centers (figure 2.4B1-3, arrows indicate dorsal tips of stripes in rhombomeres 4-6, N=6). The few labeled neurons were not clustered together; instead, spaces were present between them (arrows in figure 2.4B1-3 indicate single glycine neuron positions. Note the gaps between cells in rhombomeres 4 and 5). By 33 hpf, more glycinergic neurons are present in cross section view, and a stripelike patterning is prominent, with all three stripes present and mediolaterally segregated by gaps where glycinergic neurons are absent (figure 2.4B4-6, arrows indicate dorsal tips of stripes in rhombomeres 4-6, N=3). All glycinergic neurons present at these early times are ventrally positioned (figure 2.4B7).

Along with the extension of columns of neurons along the dorsoventral axis to form stripes, a rostrocaudal development simultaneously occurs (figure 2.4C-D). At 24 hpf, few glycine cells are present at different rostrocaudal positions with gaps in

Figure 2.4 Earliest differentiating glycinergic neurons are positioned ventrally and sorted into stripes initially. **(A)** Orientation of images in this figure. **(B-D)** In-situ hybridization using GlyT2 probe to mark glycinergic neurons. Reconstructed cross sections or dorsal projections of confocal images of fluorescent in situs are shown. **(B1-3,C1-4)** In situ hybridization staining for glycinergic neurons at 24 hpf (N=6). **(B4-6,D1-4)** In situ hybridization staining for glycinergic neurons at 33 hpf (N=3). **(B)** Cross sections of in situ staining are shown for rhombomere 4 (**B1**,**B4**), rhombomere 5 (**B2**,**B5**), and rhombomere 6 (**B3**,**B6**). Very few glycinergic neurons are present in these segments in the hindbrain at 24 hpf (**B1-3**). However, neurons are already sorted into stripes at this early time point with blue arrows indicating positions of single neurons. Spaces are evident between these individual, early born neurons (see **B1** for rhombomere 4 and **B2** for rhombomere 5 for clear examples of this). **(B4-6)** At 33 hpf, more neurons are present in both r4 and r5, and, in both rhombomeres, neurons remain sorted into stripes. Blue arrows indicate stripe positions. In rhombomere 6, new stripes are apparent at 33 hpf (**B6**). **(B7)** Ventral positions of glycinergic neurons within each image. **(C)** In situ hybridization to mark glycinergic neurons at 24 hpf (N=6). Few glycinergic neurons are present at this stage. In **C1**, a dorsal projection of a confocal image reveals that spaces are present rostrocaudally between glycinergic neurons. Glycinergic neurons are segmentally positioned early with gaps rostrocaudally in-between. **(C2-C4)** Reconstructed cross sections of confocal image indicate neurons are positioned ventrally and are sorted into stripes at the earliest stages. (**D**) In situ hybridization at a later time point (33 hpf) indicates the patterning of glycinergic cells in the hindbrain when more cells are present (N=3). In **D1**, a dorsal view indicates that stripes are filling into regions within each segment rostrocaudally but small gaps are still present in-between. While glycinergic neurons are filling in rostrocaudally, dorsoventral regions are simultaneously populated. In **D2-4**, cross sections show that more cells are present within rhombomeres than at 24 hpf (**C2-C4**), but all neurons are still ventrally positioned and are still sorted into stripes with three stripes now present on each side of hindbrain. Scale bars = 20 um.


A
C1,D1
R3
R4
R5
R6
R7
R8
B1,B4,C2,D2
B2,B5,C3,D3
B3,B6,C5,D4
Rhombomere 4
Rhombomere 5
Rhombomere 6
24 hpf
33 hpf
B1
B2
B3
B4
B5
B6
B7
r4
r5
r6
24 hpf
33 hpf
Dorsal
Ventral
24 hpf
33 hpf
C1
C2
C3
C4
D1
D2
D3
D4

between where glycine cells are absent (figure 2.4C1, N=6). By 33 hpf, as with the dorsoventral patterning, there is a progressive filling in of rostrocaudal regions to form rostrocaudal strings of cells (figure 2.4D1, left: blue bars indicate regions where glycine cells are present, compare these to the regions of expression at 24 hpf shown in figure 2.4D1). This is evident in figure 2.4D1, where the cells form three clear bands that extend rostrocaudally and correspond to the three glycine stripes. In cross sections, it is clear that both the rostrocaudal [figure 2.4C1 (24 hpf) and 2.4D1(33hpf)] and dorsoventral [figure 2.4C2-4 (24 hpf) and 2.4D2-4 (33 hpf)] filling in of stripes occurs simultaneously. In sum, the segregation between different glycine stripes is present in dorsal (figure 2.4D1) and cross section (figure 2.4D2-6) views at 33 hpf and there are hints of this segregation even when very few cells are present within a single rhombomere as observed at 24 hpf (figure 2.4C1-4).

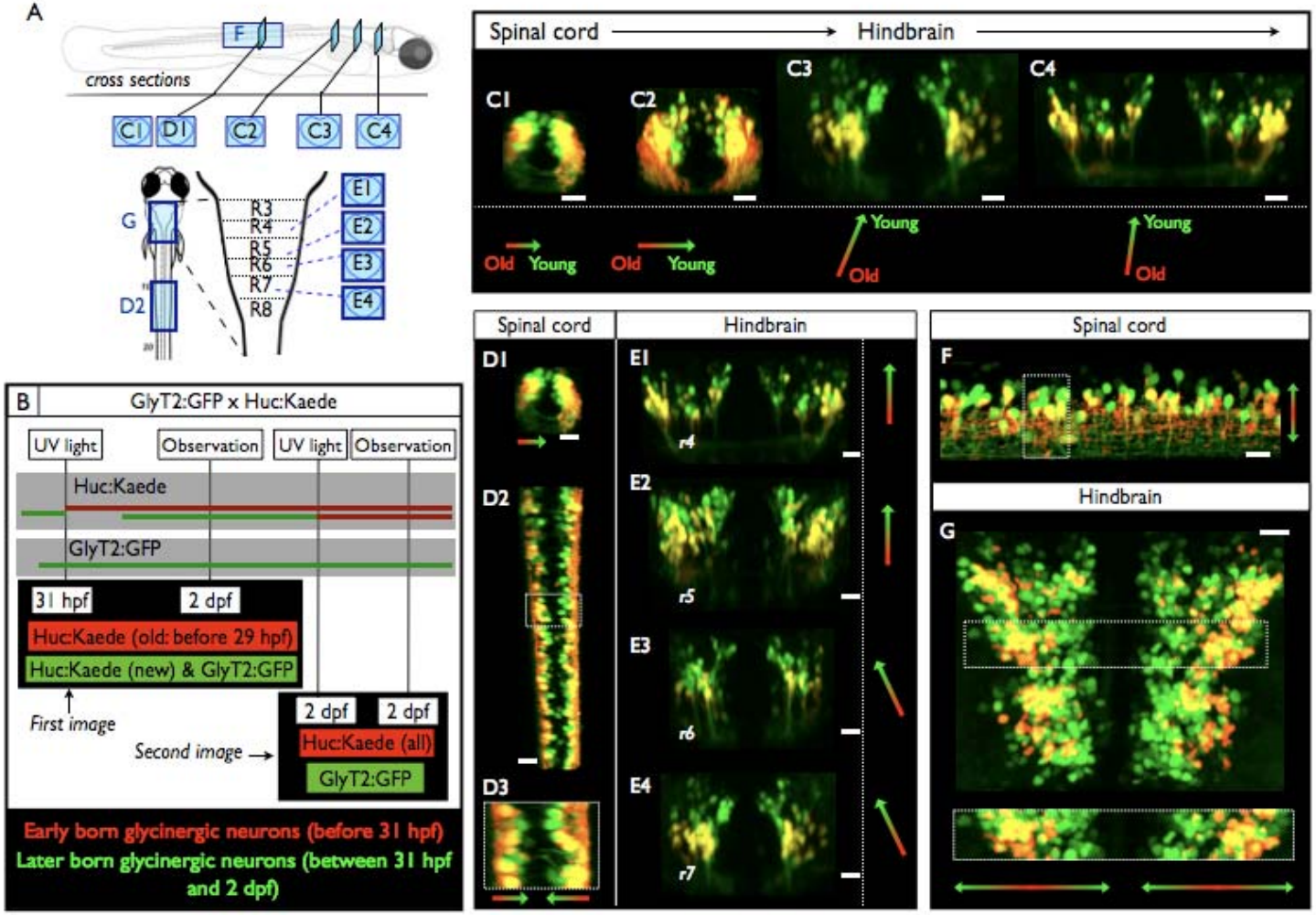
Glycinergic interneurons are present in stripes early during development and form longer continuous stripes extending rostrocaudally and dorsoventrally as more glycinergic neurons differentiate. However, in situ hybridization does not indicate the relative ages of neurons at different dorsoventral positions within these stripes. To examine this, we crossed Huc:Kaede transgenic fish with GlyT2:GFP transgenic fish so that glycinergic cells of different ages can be distinguished. Using a series of two photoconversions, one during development to photoconvert the oldest cells, and the second to photoconvert all Huc:Kaede cells to red to reveal the green overall expression pattern of GlyT2:GFP. The idea is that we can see where the oldest cells are because they were converted to red after an early photoconversion. We collected images to determine the positions of these oldest cells. We then convert all Kaede expression to red (without this second photoconversion, we could not determine whether green cells were glycinergic neurons or young neurons with green Kaede expression). After converting all of the Kaede neurons, any remaining green ones are

glycinergic cells. We can then align the images of the old (red in the first image) cells with the images of the glycinergic cells (green in the second image) and determine where the oldest glycinergic cells are positioned by finding the location of cells that are both red and green.

In figure 2.5B, an illustration of this experiment is shown. At the top, the two photoconversions of the Huc:Kaede expression are shown with red and green lines indicating the color changes at both photoconversion times, and below this, the unchanged green expression of the GlyT2:GFP expression is indicated. In the middle of the legend, the two successive confocal images acquired during the same imaging session are shown, with expression in the green and red channels from the Huc:Kaede and GlyT2:GFP transgenic lines. Image registration was performed using the green channel from both images to align all channels into one frame of reference. Subsequently, colocalization of the red channel in the first image with the green channel in the second image revealed the oldest glycinergic neurons. The green channel in the second image identifies the glycinergic neurons. These two channels are shown in figures 2.5C-G, with the red channel indicating glycinergic neurons present by 31 hpf and the green channel indicating positions of all glycinergic interneurons (see bottom of figure 2.5B for legend).

We found that glycinergic interneurons maintain an age-related patterning in both spinal cord and hindbrain that, as we might expect, matches the general patterning in the previous Huc:Kaede experiments (figure 2.1). In the spinal cord, the glycinergic neurons are in the upper half of cord, with the oldest being the most lateral in the population and younger ones medial (figure 2.5C1, N=3). In the hindbrain, the oldest glycinergic neurons occupy the most ventral positions, with many younger cells above them (figure 2.5C3-4, N=2). The age-related patterning maps onto hindbrain stripes, with ventral neurons within a stripe containing older neurons than more dorsal

Figure 2.5 Patterning in the spinal cord and hindbrain by age and neurotransmitter phenotype. **(A)** Orientation of images in this figure. **(B)** Legend for the timing of experiments in this figure. GlyT2:GFP x Huc:Kaede fish were photoconverted at 31 hpf and imaged at 2 dpf. A second photoconversion was performed to photoconvert all Huc:Kaede to red and a second confocal image was collected. In this second image, all green cells are glycinergic interneurons. In the top of **B,** red/green expression of Kaede is shown from early times to 2 dpf, indicating the conversions of green to red expression twice, once to reveal early born neurons (red in the first image) and a second time to reveal all glycinergic neurons (green in the second image). In the middle of B, red/green channels of both images are shown, indicating red/green expression of Kaede and green expression of GlyT2:GFP within both confocal stacks. Image registration of the green channels of both images was used to align the stacks, and subsequently, colocalization of the early born neurons (red in the first image) with the glycinergic interneurons (green in the second image) revealed the earliest born glycinergic interneurons. The green channel from the second image was used to compare the positions of the oldest glycinergic neurons to those of all glycinergic neurons. At the bottom, red/green expression for images in **C**-**G** obtained from the method described above is shown. Red expression indicates neurons with Huc:Kaede expression by 31 hpf, while exclusively green expression indicates neurons with Huc:Kaede expression appearing between 31 hpf and 2 dpf, the time of imaging. **(C)** Age-related patterning of glycinergic neurons is continuous from spinal cord to hindbrain. In **C1**, a cross section of spinal cord indicates that the oldest glycinergic neurons are lateral while younger ones are more medial. In the hindbrain, shown in cross section in **C4**, glycinergic neurons are sorted into stripes and the oldest neurons (red) are ventrally positioned within stripes with younger (green) ones filling in more dorsally. From spinal cord to hindbrain (**C1**-**4**), a transformation of the age-related patterning occurs, where the mediolateral segregation within spinal cord is continuously changed into a dorsoventral age-related segregation within hindbrain, which is also sorted into stripes. Below the images in **C1**-**4**, red/green arrows show the orientation of age-related segregation and illustrate the continuous transformation of the age-related patterning of glycinergic neurons from spinal cord to hindbrain that matches that shown in figure 1 for all neurons. **(D-E)** Topological transformation of age-related patterning between spinal cord (**D**) and hindbrain (**E**). In **D1**-**3**, the mediolateral segregation by age of spinal glycinergic neurons is shown in cross section (**D1**) and in dorsal view (**D2**, magnified region in **D3**). Below **D1**,**D3**, red/green arrows indicate the direction of segregation. In **E1**-**4**, cross sections within hindbrain from rhombomere 4-7 are shown, and, to the right, red/green arrows indicate the direction of age-related segregation, rotated from that in spinal cord (**D1**-**3**). In **F**, a lateral view of spinal cord shows that the oldest neurons (red) are mid-dorsoventral with younger green cells ventral and dorsal to the oldest cells (red/green bars to right of image indicate dorsoventral regions of red/green cells within the white boxed region). In **G**, a similar middle patterning of oldest cells with younger cells on either side is seen in the hindbrain; however, this time the patterning is mediolaterally oriented. Scale bars = 20 um.

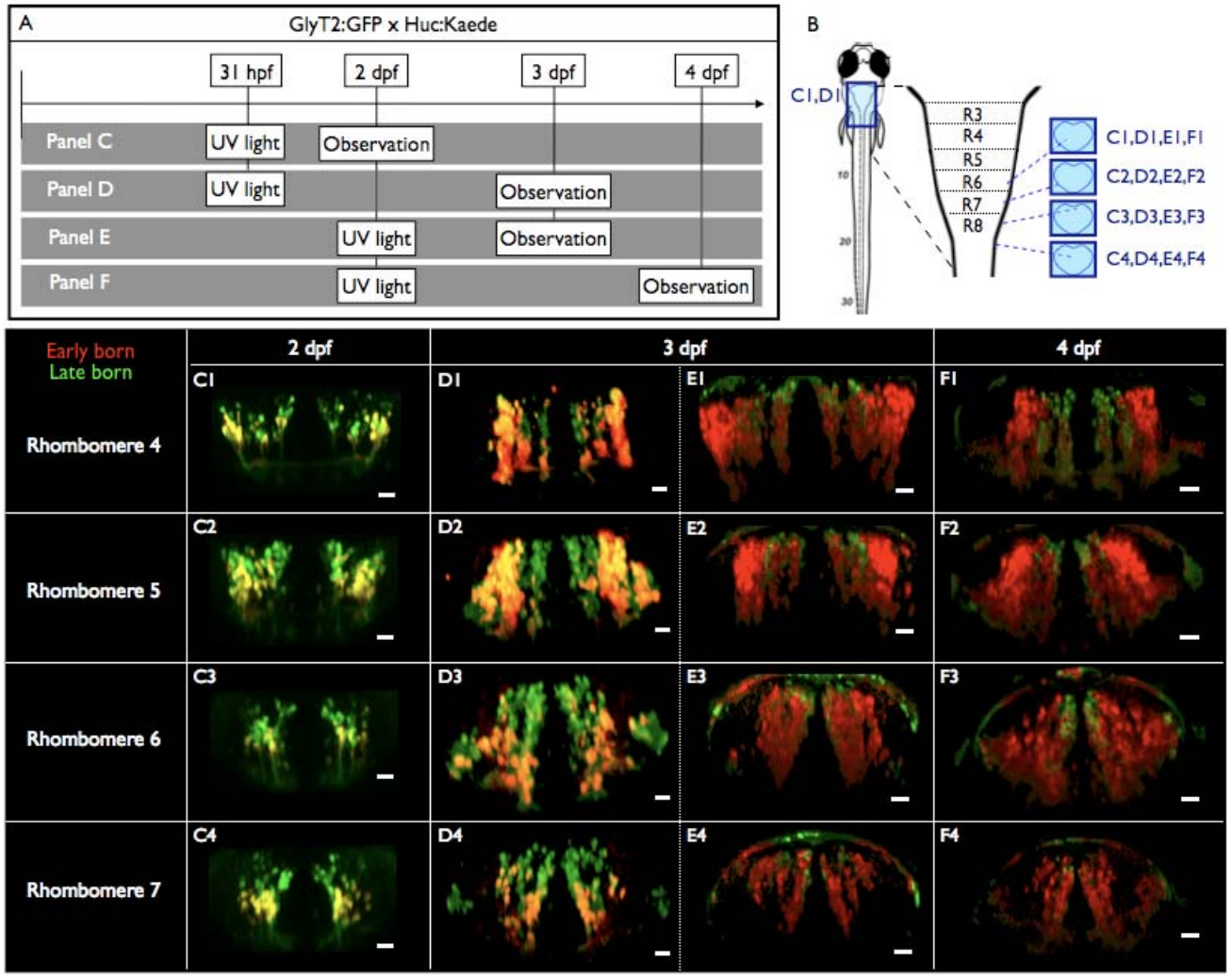

A
F
cross sections
C1
D1
C2
C3
C4
G
D2
R3
R4
R5
R6
R7
R8
E1
E2
E3
E4
B
GlyT2:GFP x Huc:Kaede
UV light
Observation
UV light
Observation
Huc:Kaede
GlyT2:GFP
31 hpf
2 dpf
Huc:Kaede (old: before 29 hpf)
Huc:Kaede (new) & GlyT2:GFP
First image
Second image
2 dpf
2 dpf
Huc:Kaede (all)
GlyT2:GFP
Early born glycinergic neurons (before 31 hpf)
Later born glycinergic neurons (between 31 hpf and 2 dpf)
Spinal cord
Hindbrain
C1
C2
C3
C4
Old
Young
Old
Young
Young
Old
Young
Old
Spinal cord
Hindbrain
D1
D2
D3
E1
E2
E3
E4
r4
r5
r6
r7
Spinal cord
F
Hindbrain
G

stripe regions across hindbrain (cross sections in figure 2.4C5-8, 2.4D3-4). Therefore, the age-related patterning that transforms from a mediolateral patterning in spinal cord to a dorsoventral patterning within hindbrain (figure 2.1) is also evident for glycinergic neurons in both spinal cord and in hindbrain, where stripes of glycinergic neurons are present (figure 2.5D1-4, red/green arrows below images indicate the transformation of the age-related patterning from mediolateral in spinal cord to dorsoventral in hindbrain). This transformation is shown in figure 2.5D-E, where the mediolateral segregation by age in spinal cord (figure 2.5D1-3, N=3) is similar to the dorsoventral segregation by age within hindbrain (figure 2.5E1-4, N=2) and in figure 2.5F-G, where the dorsoventral segregation in spinal cord (figure 2.5G, N=3) is similar to the mediolateral segregation in hindbrain (figure 2.5F, N=2). In each case, a rotation in the age-patterning between spinal cord and hindbrain is evident.

This age-related patterning of neurotransmitter stripes in hindbrain persists after 2 dpf. Figure 2.6A shows the timing of photoconversions and imaging of 2-4 dpf GlyT2:GFP x Huc:Kaede transgenic fish for the images shown in the figure. Cross sections within rhombomere 4 (figure 2.6C1,D1,E1,F1), rhombomere 5 (figure 2.6C2,D2,E2,F2), rhombomere 6 (figure 2.6C3,D3,E3,F3), and rhombomere 7 (figure 2.6C4,D4,E4,F4) are shown for these different times of both photoconversion and imaging. In figure 2.6C-D, GlyT2:GFP x Huc:Kaede transgenic fish were photoconverted at 31 hpf and imaged at either 2 dpf (figure 2.6C) or 3 dpf (figure 2.6D). At both 2 (N=2) and 3 dpf (N=2), glycinergic stripes across multiple hindbrain segments maintain an orderly patterning based on age with the oldest neurons ventral and younger ones filling in more dorsally.

In figure 2.6D-E, GlyT2:GFP x Huc:Kaede transgenic fish were converted at either 31 hpf (figure 2.6D) or 2 dpf (figure 2.6E) and imaged at 3 dpf. A comparison of the results of the two different conversion times persists with the oldest neurons

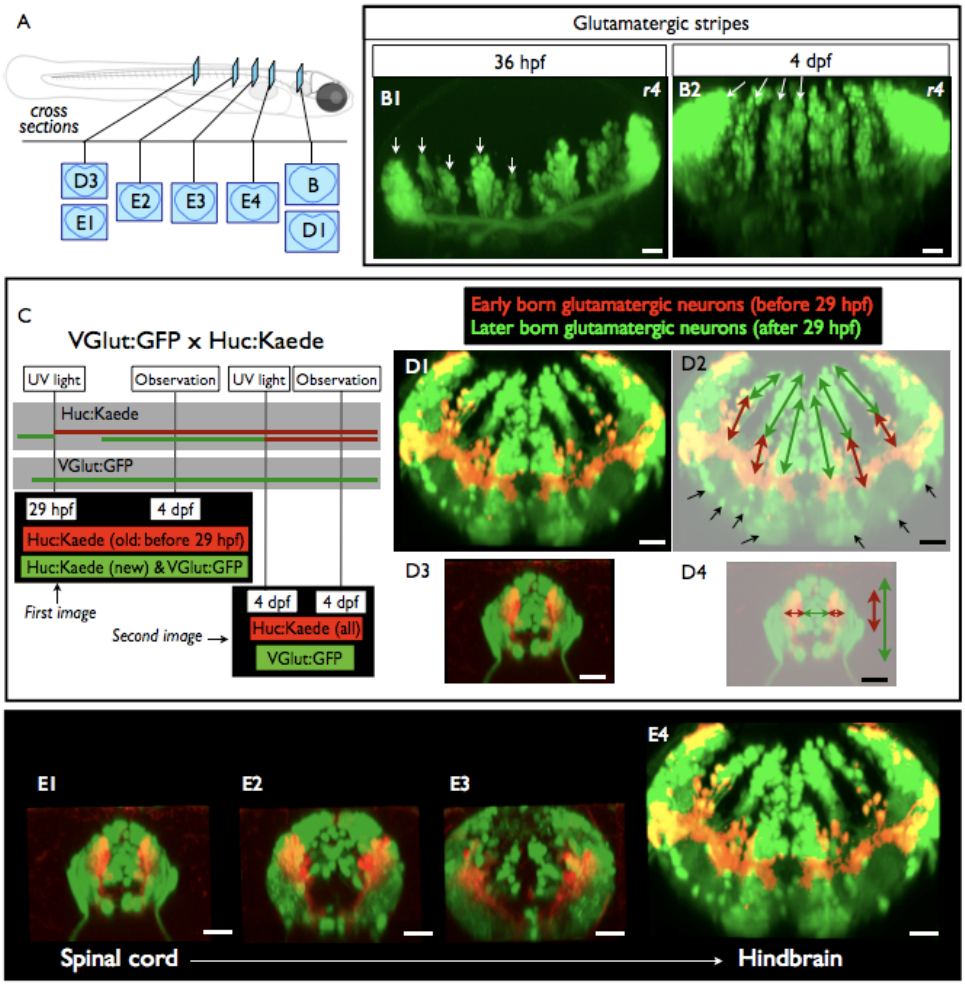
Figure 2.6 Age-related patterning of stripes of glycinergic neurons at 2 dpf, 3 dpf, and 4 dpf. **(A)** Legend for the timing of experiments in panels **C-F**. For all images, GlyT2:GFP x Huc:Kaede fish were photoconverted and imaged at the times indicated in the legend. On the day of imaging, fish were imaged a first time. In this first image, red cells are the oldest neurons. A second photoconversion was performed to photoconvert all Huc:Kaede to red and a second confocal image was collected. In this second image, all green cells are glycinergic interneurons. These two images were collected within a 2 hour imaging session. Green expression from the first image and red expression from the second image indicate the regions of all neurons within the hindbrain (see schematic in 5B). These two channels were used to perform an image registration into one frame of reference. In this new image-registered volume, colocalization of the red channel from the first image (all early born neurons) with the green channel from the second image (all glycinergic interneurons) revealed the oldest glycinergic neurons; these colocalized neurons are red in all images. Green neurons represent the colocalization of GlyT2:GFP expression colocalized with Huc:Kaede red expression (i.e. glycinergic neurons with expression in the Huc:Kaede transgenic line) in panels C and D. In panels E and F, fish were photoconverted at a time when most neurons express Kaede; therefore, most glycinergic neurons have red expression. To more easily see the exclusively green expressing neurons in these cross sections, green expression was masked out from regions where red expression was present, and green expression represents exclusively green neurons. **(C-F)** Age-related patterning at 2 dpf (**C**), 3 dpf (**D**-**E**), and 4 dpf (**F**). In each panel, cross sections are shown for rhombomere 4 (**C1**,**D1**,**E1**,**F1**), rhombomere 5 (**C2**,**D2**,**E2**,**F2**), rhombomere 6 (**C3**,**D3**,**E3**,**F3**), and rhombomere 7 (**C4**,**D4**,**E4**,**F4**). In each cross section, older (red) neurons are predominately ventrally positioned within stripes to younger (exclusively green in **C**-**D** and green in **E**-**F**) neurons, indicating that an age-related patterning of glycinergic stripes is present at 2 dpf and persists at 3 and 4 dpf. Scale bars = 20 um.


A
GlyT2:GFP x Huc:Kaede
31 hpf
2 dpf
3 dpf
4 dpf
Panel C
UV light
Observation
Panel D
UV light
Observation
Panel E
UV light
Observation
Panel F
UV light
Observation
B
C1,D1
R3
R4
R5
R6
R7
R8
C1,D1,E1,F1
C2,D2,E2,F2
C3,D3,E3,F3
C4,D4,E4,F4
Early born
Late born
2 dpf
3 dpf
4 dpf
Rhombomere 4
Rhombomere 5
Rhombomere 6
Rhombomere 7
C1
D1
E1
F1
C2
D2
E2
F2
C3
D3
E3
F3
C4
D4
E4
F4

ventral and younger neurons more dorsal for neurons added both after 31 hpf and much later, after 2 dpf (photoconverted at 31 hpf, N=2, photoconverted at 2 dpf, N=1). In figure 2.6E-F, GlyT2:GFP x Huc:Kaede transgenic fish were photoconverted at 2 dpf and imaged on either 3 dpf (figure 2.6E, N=1) or 4 dpf (2.6F, N=3). In both cases, the age-related patterning is orderly within stripes with old to young from ventral to dorsal. Therefore, an age-related patterning is present within glycinergic stripes and persists until 4 dpf, a time when zebrafish are freely swimming.

Glutamatergic neurons also form stripes in hindbrain that are segregated mediolaterally. We used transgenic lines where either GFP or DsRed is selectively expressed in glutamatergic neurons to examine where early differentiating neurons are located and when stripes become evident. During development, glutamatergic stripes are present by at least 36 hpf and initially populate ventral regions in a similar fashion as glycinergic stripes (figure 2.7B1, compare to figure 2.4). At 4 dpf, glutamatergic stripes have filled in and neurons are arrayed into stripes that progress to more dorsal hindbrain regions (figure 2.7B2). We again crossed Huc:Kaede transgenic fish with a neurotransmitter transgenic line (VGlut:GFP) and used two photoconversions, image registration, and colocalization to identify the old and young glutamatergic neurons in spinal cord and hindbrain. Image registration and colocalization to determine the oldest glutamatergic neurons relative to all glutamatergic neurons were performed as described for the GlyT2:GFP x Huc:Kaede experiments shown in figures 2.5-2.6 (figure 2.7C shows a legend for the experiments in this figure). In the hindbrain, ventral stripe regions contained older neurons (figure 2.7D1, red arrow regions in 2.7D2), and dorsal stripe regions contained younger neurons (figure 2.7D1, green arrow regions in 2.7D2) (N=5). Although most of the hindbrain followed the ventral to dorsal age-related patterning, the black arrows in figure 2.7D2 mark ventral,

Figure 2.7 Age-related patterning in the spinal cord and hindbrain of glutamatergic neurons. **(A)** Orientation of cross section images shown here. **(B)** Development of glutamatergic stripes. At 36 hpf, glutamate stripes are present as shown in **B1** where a cross section from a confocal image of the VGlut:DsRed transgenic line indicates short, ventrally positioned stripe regions. By 4 dpf, the stripes are filled in as shown in **B2** where a cross section of a confocal image of the VGlut:GFP at 4 dpf indicates stripes arrayed mediolaterally and filled in throughout the dorsoventral extent of hindbrain. Note the large number of cells ventrally positioned within the neuropil at 4 dpf (**B2**) not seen at 36 hpf (**B1**). **(C)** Photoconversion experiments performed for D-E. Age related-patterning of glutamatergic stripes is shown by image registration of two images from a single fish photoconverted once to reveal old neurons (red in first image) and photoconverted a second time to reveal all glutamatergic neurons (green in second image). **(**Huc:Kaede fish were crossed with VGlut:GFP fish and photoconverted at 24 or 29 hpf. Fish were imaged a first time and red cells in the first image were the older population of all neurons. The fish was then fully photoconverted again so that Huc:Kaede no longer had green expression. In this second image, green cells are uniquely VGlut:GFP cells. Colocalization of the red cells from the first image and the green from the second reveal the oldest glutamatergic neurons. Red cells in all images are colocalized cells as described above and green cells are VGlut:GFP labeled cells (N=6). **(D1)** Cross section in hindbrain indicates that old glutamatergic neurons (red) are ventrally positioned relative to younger (green) cells. In **D2**, green and red arrows indicate regions of green (young) and red (old) glutamatergic neurons within stripe regions. Black arrows indicate regions within the neuropil where younger cells are present. In **D3**, a cross section in spinal cord shows that old glutamatergic neurons (red) are lateral while younger glutamatergic cells (green) are more medially positioned. In **D4**, regions of red and green expression are indicated for dorsoventral and mediolateral positions by red and green arrows. Rohon-Beard cells have been digitally removed from the dorsomedial region of spinal cord to determine the patterning of glutamatergic neurons that do not undergo apoptosis early. Excluding these Rohon-Beard neurons, glutamatergic neurons in spinal cord follow an age-related patterning similar to both all neurons (figure 1) and glycinergic neurons (figure 5). Scale bars = 20 um.


A
cross sections
D3
E1
E2
E3
E4
B
D1
Glutamatergic stripes
36 hpf
4 dpf
B1
B2
r4
r4
C
VGlut:GFP x Huc:Kaede
UV light
Observation
UV light
Observation
Huc:Kaede
VGlut:GFP
29 hpf
4 dpf
Huc:Kaede (old: before 29 hpf)
Huc:Kaede (new) & VGlut:GFP
First image
Second image
4 dpf
4 dpf
Huc:Kaede (all)
VGlut:GFP
Early born glutamatergic neurons (before 29 hpf)
Later born glutamatergic neurons (after 29 hpf)
D1
D2
D3
D4
E1
E2
E3
E4
Spinal cord
Hindbrain

younger neurons that do not fit the ventral to dorsal age-related patterning and reside within neuropil regions ventral to stripes and below older neurons.

In the spinal cord at 4 dpf, older glutamatergic neurons tend to be lateral (figure 2.7D3, positions of old and young neurons indicated by red/green arrows in 2.7D4, N=4), as observed in glycinergic neurons in previous figures (see figure 2.5C-D). The transformation of the age-related patterning of glutamatergic neurons resembles that of glycinergic neurons. In spinal cord (figure 2.7E1), the oldest glutamatergic neurons are laterally positioned with younger neurons more medially located (N=5). More rostrally, gaps are present between glutamatergic populations (figure 2.7E2-3) that might indicate segregation between stripes (N=2). The oldest neurons are laterally positioned as in more caudal regions, but gaps and clustering of glutamatergic neurons have appeared. In more rostral regions within the spinal cord-hindbrain transition, the oldest populations are in ventrolateral positions and more gaps and clustering are present (figure 2.7E4, N=2). In hindbrain, shown in figure 2.7E4, stripes are clearly present and ventral neurons within stripes are the oldest glutamatergic neurons (N=5). There is a continuous transition of the age-related pattern in spinal cord to that in hindbrain as well as an emergence of a stripelike patterning of neurons by neurotransmitter phenotype. However, at 4 dpf, some younger glutamatergic neurons are present ventral to older ones, in hindbrain, disrupting the age-related patterning. In contrast, for glycinergic interneurons, an age-related patterning was present for all stripes at multiple stages from 2-4 dpf for multiple ages of neurons.

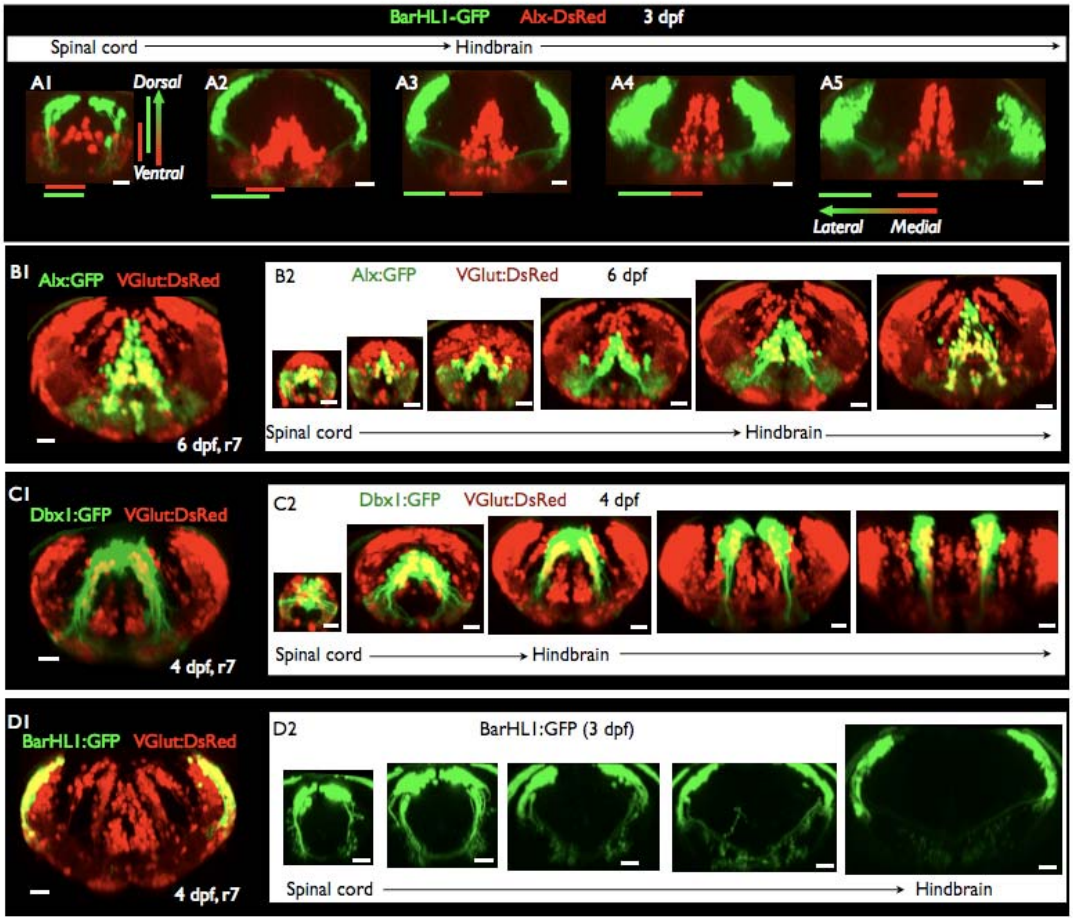
Organization of transcription factor stripes from spinal cord to hindbrain

An age-related patterning exists for stripes of neurons sharing a neurotransmitter phenotype within hindbrain, but the intermingling of neurons within

spinal cord hinders the observation of the transformation from spinal cord to a stripe-like pattern in hindbrain. The ability to observe individual stripes in isolation would allow for an observation of single stripe transformations in positioning and age-related patterning from spinal cord to hindbrain. Because of considerable evidence that the development of spinal cord is directed by the expression of bands of transcription factors as well as evidence of transcription factor patterning in hindbrain [99, 100, 108-134], one possibility was that individual hindbrain stripes would be marked by particular transcription factors. These might allow for the study of the differentiation of a particular stripe and a more clear view of the transformation from spinal cord to hindbrain. Here we examine the transcription factor patterning in relation to the transmitter stripes as a prelude to looking at age-related patterning in relation to transcription factor expression.

Within the spinal cord, transcription factors are expressed in bands across spinal cord stacked from ventral to dorsal early in development [3]. Later, the interneurons that arise from different progenitor zones do not maintain as obvious a dorsoventral patterning. However, by using transgenic lines expressing GFP or DsRed under the control of promoter regions for two transcription factors expressed far apart during development, we were able to observe a segregation of the transcription factor bands even at 3 dpf. We used the promoter for alx, a more ventral transcription factor to drive DsRed and the promoter for BarHL1, a very dorsal transcription factor, to drive GFP. When these are viewed in the spinal cord of dual expressing transgenic lines at 3 dpf there was still a clear dorsoventral segregation of the two groups of labeled cells, with the alx one more ventral (figure 2.8A1). More rostrally, this patterning rotates to a combination of dorsoventral and mediolateral segregation (figure 2.8A2); and finally, within hindbrain, the segregation is a mediolateral one as

Figure 2.8 Topological transformation of transcription factor maps from spinal cord to hindbrain. All images are cross sections of single or dual expression transgenic fish. Dorsal is top for all images. **(A)** Transcription factor patterning from spinal cord to hindbrain for two transcription factors, BarHL1 (BarHL1:GFP) and Alx (Alx:DsRed) at 3 dpf. In spinal cord, these transcription factors define cell types originating from progenitor zones at opposite dorsoventral extremes. In differentiated neurons, segregation is still present between these types (**A1**), where BarHL1:GFP neurons are dorsal to Alx:DsRed neurons. In the hindbrain, this dorsoventral segregation is present mediolaterally (**A3-5**), and, in the progression rostrally from spinal cord to hindbrain this segregation is continuously present and is transformed gradually from a dorsoventral to a mediolateral segregation (**A1-3**). **(B,C,D)** Transcription factor patterning is also stripe-like within hindbrain. In **A**, BarHL1 and Alx were shown to have a stripe-like patterning segregated mediolaterally. These transcription factor stripes, as well as Dbx1, align with neurotransmitter stripes. This is shown in **B1**,**C1**, and **D1** where dual expression transgenic fish were imaged and cross sections of these VGlut:DsRed x (Alx:GFP,Dbx1:GFP, or BarHL1:GFP) show that each green transcription factor stripe overlaps partially or completely with one distinct glutamatergic stripe. In **B2**, **C2**, and **D2**, the transformation of each individual transcription factor patterning from spinal cord to hindbrain is shown in cross sections from left to right. Alx neurons form a horizontal band of ventrally positioned glutamatergic neurons in spinal cord (**B2**, left) and form a tilted stripe more rostrally (**B2**, middle) and finally form a stripe of medially located neurons in hindbrain (**B2**, right). A similar progression is seen for Dbx1:GFP neurons in **C2**. In D2, from left to right, BarHL1:GFP neurons form a dorsal band in spinal cord that gradually transforms into a lateral stripe in hindbrain. Scale bars = 20 um.


BarHL1-GFP Alx-DsRed 3 dpf
Spinal cord
Hindbrain
A1
Dorsal
Ventral
A2
A3
A4
A5
Lateral
Medial
B1
Alx:GFP VGlut:DsRed
6 dpf, r7
B2
Alx:GFP
VGlut:DsRed
6 dpf
Spinal cord
Hindbrain
C1
Dbx1:GFP VGlut:DsRed
4 dpf, r7
C2
Dbx1:GFP
VGlut:DsRed
4 dpf
Spinal cord
Hindbrain
D1
BarHL1:GFP VGlut:DsRed
4 dpf, r7
D2
BarHL1:GFP (3 dpf)
Spinal cord
Hindbrain

observed for neurotransmitter stripes in hindbrain (figure 2.8A3-5). This transformation indicates that the dorsoventral transcription factor map of spinal cord transforms into a mediolateral one within hindbrain (figure 2.8A1-5).

Since both the transcription factor and neurotransmitter patterning within hindbrain are aligned dorsoventrally with mediolateral segregation in positions, we compared the positions of these two patterns by crossing transgenic lines for three different transcription factors (alx:GFP, Dbx1:GFP, BarHL1:GFP) with a transgenic line driving DsRed expression in glutamatergic neurons. We found that single transcription factor stripes overlap with single glutamatergic stripes (figure 2.8B1,C1,D1). This overlap in patterning is discussed in more detail in chapter 5. We compared the position of transcription factor patterns from spinal cord to hindbrain to discern how these stripes arise in the transition from spinal cord to hindbrain.

In the hindbrain, alx neurons form a medial stripe of neurons (figure 2.8B1) that aligns with the medial glutamate stripe. In more caudal regions, this stripe of alx neurons is rotated continuously in the progression from rostral to caudal from hindbrain to spinal cord until, in spinal cord, alx neurons are ventrally positioned (figure 2.8B2, right to left for hindbrain to spinal cord).

Dbx1 expressing neurons also form a stripe of neurons that overlaps with a glutamatergic stripe (figure 2.8C1) in hindbrain. In chapter 5, we show that Dbx1 marks two stripes that are mediolaterally segregated with the more medial regions of the Dbx1 stripe containing glutamatergic neurons. Therefore, observations of Dbx1 neuronal patterning from hindbrain to spinal cord indicate how two neurotransmitter stripes with shared transcription factor expression are patterned. Within hindbrain, Dbx1 neurons form a medial stripe that extends dorsoventrally (figure 2.6F5). The progression from hindbrain to spinal cord of the Dbx1 stripe is similar to the transition

observed for Alx neurons (compare figure 2.8B2 and 2.8C2, both from right to left for hindbrain to spinal cord transition).

The BarHL1 transcription factor is expressed in neurons in the lateral extreme of the lateral glutamate stripe (figure 2.8D1) in hindbrain. Caudal to hindbrain, this stripe is shifted dorsally in a continuous fashion from hindbrain to spinal cord (figure 2.8D2, right to left for hindbrain to spinal cord). This dorsal progression is a continuous transformation from hindbrain to spinal cord (figure 2.6H5 to 2.6H2) until neurons are dorsally positioned within spinal cord (figure 2.8D2, left) in comparison to their lateral position within hindbrain (figure 2.8D2, right).

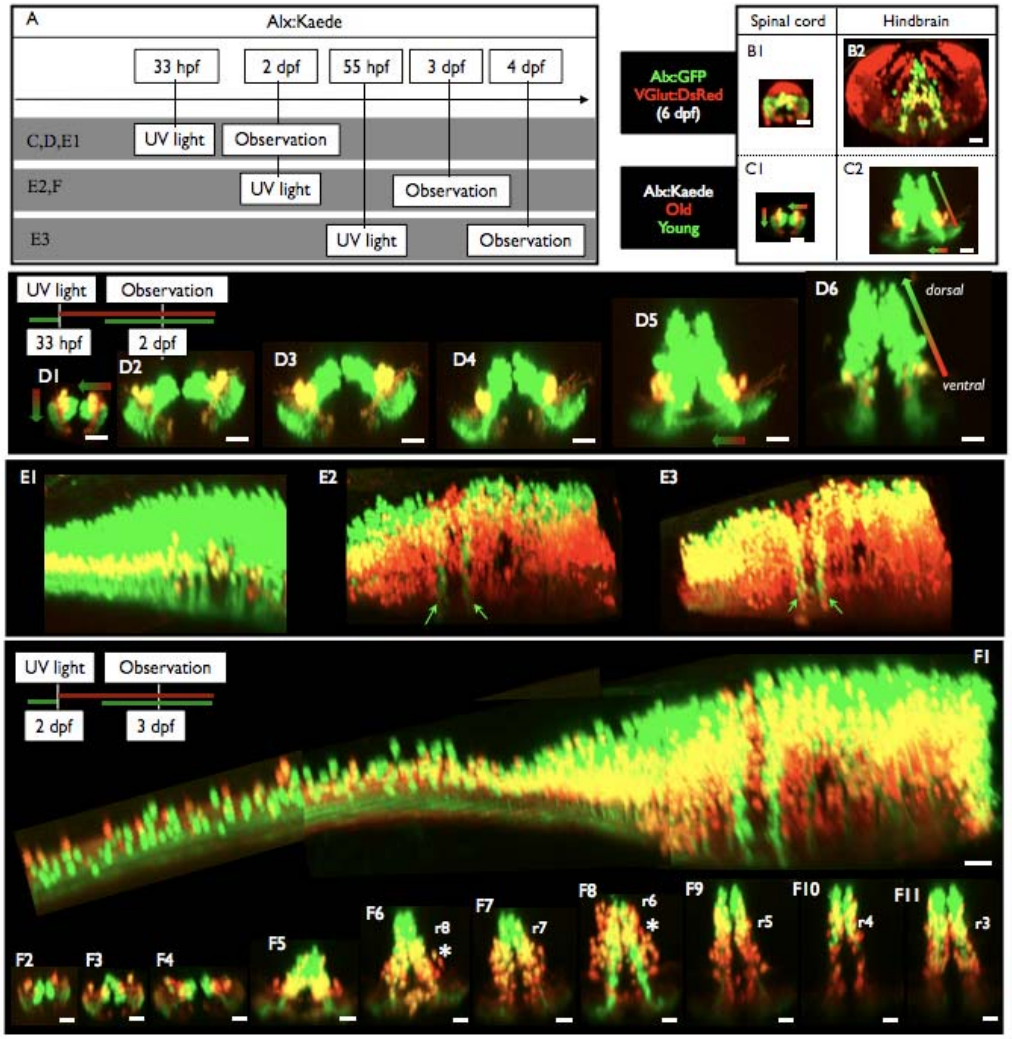
Age-related patterning of transcription factor organization

Alx transcription factor

Because alx neurons are located in specific positions within both spinal cord and hindbrain, we investigated how these neurons are organized by age in both regions. In the spinal cord, alx neurons are ventrally positioned glutamatergic interneurons (figure 2.9B1), and in the hindbrain, alx neurons form a medial stripe of glutamatergic interneurons (figure 2.9B2). Since neurotransmitter stripes are intimately related to transcription factor patterning (see figure 2.8), we expected that age-related patterning of transcription factor organization should also be present within the hindbrain.

Previous studies have shown that an age-related patterning of alx neurons exists within spinal cord using alx:Kaede transgenic fish [18]. Here we explored the age-related patterning of alx neurons in hindbrain using this transgenic line. We photoconverted alx:Kaede fish at different times and imaged within both spinal cord and hindbrain to compare the age-related patterning of these regions (see legend in figure 2.9A). We found that the oldest alx neurons in hindbrain are ventrally

Figure 2.9 Age-related patterning of alx neurons from spinal cord to hindbrain **(A)** Legend for the timing of experiments in panels **C-F**. Alx:Kaede transgenic fish were photoconverted at 33 hpf (**C**,**D**,**E1**), 2 dpf (**E2**,**F**), and at 55 hpf (**E3**), and imaged at 2 dpf (**C**,**D**,**E1**), 3 dpf (**E2**,**F**), or at 4 dpf (**E3**). In **B**, alx neurons (Alx:GFP) are positioned within a horizontal band in spinal cord (**B1**) and within a vertically oriented stripe in hindbrain (**B2**). In **C**, alx neurons in spinal cord are mediolaterally segregated by age (**C1**), while, in hindbrain, alx neurons are dorsoventrally segregated. Red/green arrows indicate direction of age-related patterning in both regions. **(D)** Cross sections from spinal cord (**D1**) to hindbrain (**D6**) indicate the transformation of age-related patterning between these regions. Alx:Kaede transgenic fish were photoconverted at 33 hpf and imaged at 2 dpf (left). In spinal cord, older neurons were laterally positioned relative to younger Alx neurons (**D1**). More rostrally, a tilted stripe of alx neurons is evident in **D2**-**4**. In the hindbrain, older neurons were ventral to younger Alx neurons (**D6**). This transition is continuous between spinal cord and hindbrain, both the positions of alx neurons transform as shown in **B1**-**2** as well as the age-related patterning shown in **C1**-**2**. Also noteworthy is a flipping of the age-related map dorsoventrally between spinal cord (**D1**) and hindbrain (**D6**). Red/green arrows indicate direction of age-related patterning in both spinal cord (**D1**, above, left) and hindbrain (**D6**, right). **(E-F)** Rhombomere-specific migrations of some Alx neurons. In **E1**-**3**, lateral views of fish photoconverted at 33 hpf (**E1**), 2 dpf (**E2**), or 55 hpf (**E3**) and imaged at 2 dpf (**E1**), 3 dpf (**E2**), or 4 dpf (**E3**) are shown. In **E1**, all older Alx neurons (red) are ventrally positioned (dorsal is top, rostral to the right in image). In **E2** and **E3**, red indicates the oldest cells and green indicates the youngest (i.e. green expression in red cells has been subtracted using a mask of the red image to subtract green expression within the mask using Imaris software). Two dorsoventral bands of young cells are present at slightly different rostrocaudal positions, indicated by arrows. In **E2**, green neurons in these bands extend dorsoventrally; however, in **E3**, when fish are photoconverted later and imaged later, green neurons are more ventral than older (red) neurons. **(F)** In **F1**, a lateral view from spinal cord to hindbrain for an Alx:Kaede transgenic fish photoconverted at 2 dpf and imaged at 3 dpf is shown (top). Below this image, cross sections from spinal cord (**F2**) to hindbrain (**F11**) are shown. Rhombomeres are identified from rhombomere 8 (r8) to rhombomere 3 (r3). Asterisks in rhombomere 8 and rhombomere 6 indicate unusual patterning in comparison to other hindbrain regions for age-related patterning and for stripe patterning as well. In rhombomere 8, a medial cluster of older Alx neurons is present in ventral regions outside of the stripe. In rhombomere 6, a green band of younger neurons is present on either side of hindbrain across the dorsoventral extent of the Alx stripe. This region is shown in A2-3, where two bands of migrating cells are shown. Scale bars = 20 um.


A
Alx:Kaede
33 hpf
2 dpf
55 hpf
3 dpf
4 dpf
C,D,E1
UV light
Observation
E2,F
UV light
Observation
E3
UV light
Observation
AbcGFP
VGlut:DsRed
(6 dpf)
Spinal cord
Hindbrain
B1
B2
Abc:Kaede
Old
Young
C1
C2
UV light
Observation
33 hpf
2 dpf
D1
D2
D3
D4
D5
D6
dorsal
ventral
E1
E2
E3
UV light
Observation
2 dpf
3 dpf
F1
F2
F3
F4
F5
F6
r8
F7
r7
F8
r6
F9
r5
F10
r4
F11
r3

positioned (figure 2.9C2). This is surprising since the oldest alx neurons in spinal cord are dorsally positioned (figure 2.9C1).

We examined how a dorsoventral age axis in hindbrain relates to the age-related organization in spinal cord. Alx neurons in hindbrain form a dorsoventral stripe with the oldest neurons ventrally positioned (figure 2.9D6). More caudally, the oldest neurons are displaced laterally and dorsally as the alx stripe becomes tilted (figure 2.9D5). Near spinal cord, this displacement gradually progresses and shifts from a dorsoventral age-related segregation in hindbrain to a mediolateral segregation (figure 2.9D1). Within spinal cord, the displacement of the oldest alx neurons is enough to displace them dorsally above the younger neurons and flip the dorsal ventral age map from hindbrain to spinal cord (figure 2.9D1). In the spinal cord, dorsal to ventral and lateral to medial define axes for an age-related patterning of old to young neurons (figure 2.9D1). Thus, the transition from hindbrain to spinal cord is a rotation and displacement of alx neurons (figure 2.9D, right to left for the hindbrain to spinal cord transition). This might be a consequence of the narrow mediolateral extent of spinal cord. Neurons arising at the neuroepithelium might be displaced laterally and then dorsally as more and more are generated.

Within hindbrain, most regions of alx neurons along the rostrocaudal axis of the brain exhibit an orderly patterning based on age. Lateral views of alx:Kaede transgenic fish photoconverted and imaged at different times (see legend in figure 2.9B) are shown in figure 2.9E. Across hindbrain, most alx neurons are positioned in an orderly manner based on age with the oldest neurons ventrally positioned relative to more dorsal, younger neurons. In one rostrocaudal region of hindbrain, alx neurons clearly do not follow this pattern. In this region, young neurons are ventrally positioned while older neurons are more dorsally positioned (figure 2.9E2-3). This

disruption of an age-related patterning is not present at 2 dpf (figure 2.9E1), but is present at both 3 and 4 dpf (figure 2.9E2-3).

To determine the rhombomere regions of this patterning as well as whether any other anomalies of age-related patterning of alx neurons are present, we looked more carefully along the rostrocaudal extent of these photoconverted fish. In figure 2.9F1, a lateral view of a fish photoconverted at 2 dpf and imaged at 3 dpf is shown, and in figure 2.9F2-11, cross sections from spinal cord to hindbrain are shown from left to right. Figure 2.9F1 is an overlay of lateral projections of 3 confocal stacks along the rostrocaudal extent of hindbrain and rostral regions of spinal cord. In the hindbrain, two bands of disrupted age-related ordering of the alx neurons are evident in mid-rostrocaudal hindbrain regions in this fish.

In the regions where the age-related patterning was disrupted, structural changes to stripe patterning were evident as well. For example, in rhombomere 8, shown in cross section in figure 2.9F6, older neurons have migrated to more medial regions and formed a cluster outside of the primary stripe region. The other example of a disruption in alx patterning occurs within rhombomere 6. This segment contains the two bands of young ventral neurons, which are evident in the cross section shown in rhombomere 6 in figure 2.9F8. In figure 2.9E2-3, lateral views indicate that these, potentially migrating, neurons are more ventrally positioned than all of the other alx neurons within hindbrain. The ventral green neurons shown in lateral view in figure 2.9E2-3 and in cross section in figure 2.9F8 in rhombomere 6 reside at the ventral edge of hindbrain.

Therefore, an age-related topography exists for most alx neurons in hindbrain, both at early times (2 dpf) and at times when zebrafish are freely swimming (4 dpf). There are two anomalies of age-related patterning, one in rhombomere 8 where older neurons were medially displaced out of stripe regions and one in rhombomere 6 where

young neurons are displaced to ventral regions past the ventral edge of the alx stripe through the rostrocaudal extent of hindbrain. Both anomalies were shown to be correlated with structural changes of the patterning of alx neurons in hindbrain. The bulk of the alx stripe region still maintains an age-related patterning, even at 4 dpf when some glutamatergic neurons may have shifted (see figure 2.7).

Dbx1 transcription factor

The alx transcription factor expression marks all neurons within the medial glutamatergic stripe in hindbrain (figure 2.8). Examination of a dual expression transgenic line where glutamatergic neurons express DsRed and Dbx1 positive cells express GFP shows that the Dbx1 transcription factor expression, like alx, is oriented dorsoventrally in a stripe in hindbrain (figure 2.10B2). This patterning is not evident in spinal cord (figure 2.10B1). VGlut:DsRed x Dbx1:GFP transgenic fish imaged at 4 dpf are shown in cross section in spinal cord (figure 2.10B1) and hindbrain (figure 2.10B2). In the spinal cord, Dbx1 neurons overlap most regions where glutamatergic neurons are present (figure 2.10B1). In hindbrain, Dbx1 neurons overlap one glutamatergic stripe (figure 2.10B2).

During development, Dbx1 expression forms a continuous band that extends rostrocaudally throughout hindbrain (figure 2.10C, 1 dpf, dorsal view). In cross section, Dbx1 expression forms a band at a specific dorsoventral position in spinal cord (figure 2.10D1) and at a specific position within hindbrain as well (figure 2.10D2). At 3 dpf, Dbx1 expression is distributed more broadly in spinal cord (figure 2.10D3) but remains localized within hindbrain (figure 2.10D4).

To determine the age-related patterning of this stripe, we used Dbx1:GFP x Huc:Kaede transgenic fish as described earlier for VGlut:GFP and GlyT2:GFP x Huc:Kaede. In this case, fish were photoconverted at 29 hpf and imaged at 2 dpf (see

Figure 2.10 Transcription factor stripe, Dbx1, maintains an orderly patterning of old to new from ventral to dorsal. **(A)** Orientation of images in this figure. **(B)** Patterning of Dbx1 neurons within spinal cord and hindbrain. Cross sections of dual expressing Dbx1:GFP x VGlut:DsRed transgenic fish indicate that Dbx1 neurons are distributed throughout dorsal regions of spinal cord (**B1**) and form a stripe of neurons that overlaps a middle glutamate stripe within hindbrain (**B2**). **(C)** At 1 dpf, Dbx1:GFP expression forms a continuous band rostrocaudally across hindbrain. Dorsal view, bright spots on either side are otoliths. **(D)** Early in development, Dbx1 expression marks a band in a specific dorsoventral position within spinal cord (**D1**) and a titled band in hindbrain (**D2**). After most neurons have differentiated (3 dpf), this patterning is no longer present within spinal cord (**D3**) but persists as a stripe of Dbx1 expressing neurons within hindbrain (**D4**). **(E)** Timing of photoconversion experiments performed here. Huc:Kaede fish were crossed with Dbx1:GFP fish and photoconverted at 29 hpf. Fish were imaged a first time and red cells in the first image were the older population of all neurons. The fish was then fully photoconverted again so that Huc:Kaede no longer had green expression. In this second image, green cells are Dbx:GFP cells. Colocalization of the red cells from the first image and the green from the second reveal the oldest Dbx neurons. Red cells in all images are colocalized cells are described above and green cells are Dbx:GFP labeled cells. **(F)** Development of the Dbx stripe. **(F1)** Lateral view indicates that old Dbx neurons (red) are ventral to younger (green) cells (N=1). In cross section (**F2**), this dorsoventral patterning by age is also visible. In a dorsal view (**F3**), there is also a mediolateral segregation with more lateral Dbx neurons older than more medial Dbx cells. Green/red arrows in each image indicate regions of green (young) and red (old) cells. Scale bars = 20 um.

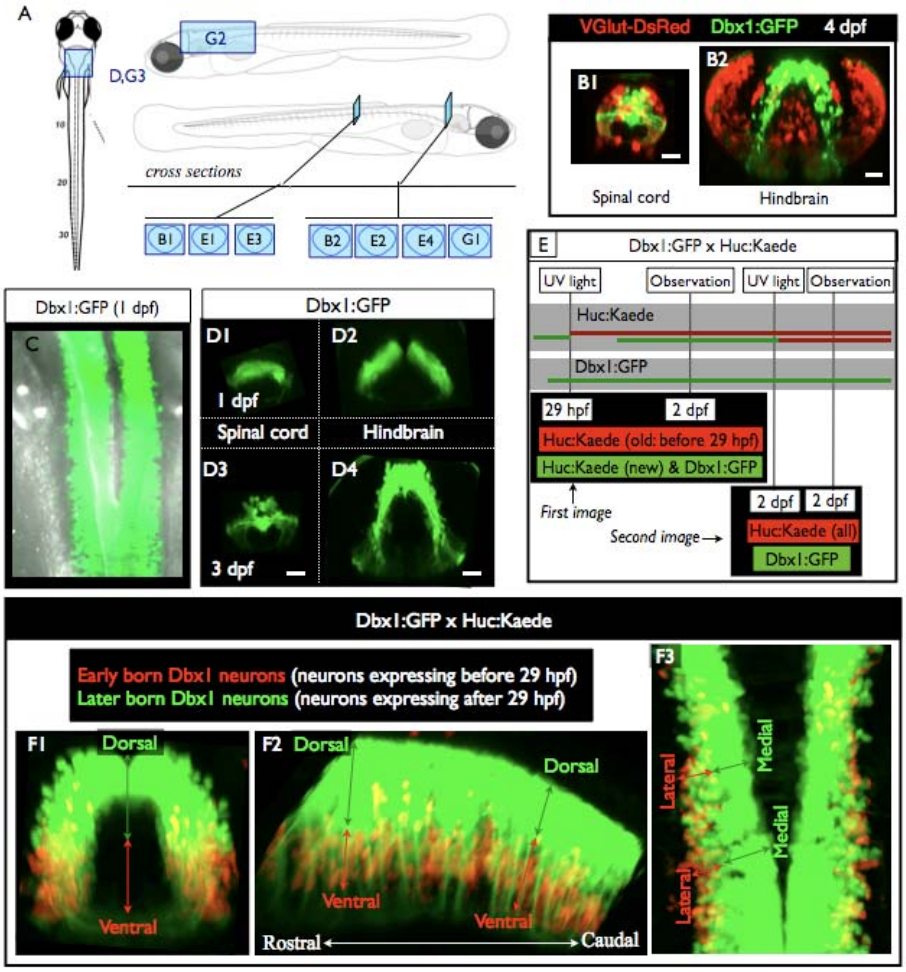

A
G2
D,G3
cross sections
B1
E1
E3
B2
E2
E4
G1
VGlut-DsRed
Dbx1:GFP
4 dpf
B1
B2
Spinal cord
Hindbrain
E
Dbx1:GFP x Huc:Kaede
UV light
Observation
UV light
Observation
Huc:Kaede
Dbx1:GFP
29 hpf
2 dpf
Huc:Kaede (old: before 29 hpf)
Huc:Kaede (new) & Dbx1:GFP
First image
Second image
2 dpf
2 dpf
Huc:Kaede (all)
Dbx1:GFP
Dbx1:GFP (1 dpf)
C
Dbx1:GFP
D1
D2
1 dpf
Spinal cord
Hindbrain
D3
D4
3 dpf
Dbx1:GFP x Huc:Kaede
Early born Dbx1 neurons (neurons expressing before 29 hpf)
Later born Dbx1 neurons (neurons expressing after 29 hpf)
F1
Dorsal
Ventral
F2
Dorsal
Dorsal
Ventral
Ventral
Rostral
Caudal
F3
Lateral
Medial
Medial
Lateral

figure legend in 2.10E). In a cross section view of hindbrain, older (red) Dbx1 neurons are ventrally positioned and younger (green) Dbx1 neurons are more dorsally positioned (figure 2.10F1, red/green arrows indicate regions of red and exclusively green expressing neurons, N=1). In figure 2.10F2, a lateral view indicates that this dorsoventral patterning by age of Dbx1 neurons extends rostrocaudally throughout hindbrain in a continuous band (N=1). In figure 2.10F3, a dorsal view indicates that lateral neurons are older than more medial ones (red/green arrows again indicate regions of red and exclusively green expressing neurons).

BarHL1 transcription factor

In figure 2.8, we showed the transformation from spinal cord to hindbrain for three transcription factors: Alx, Dbx1, and BarHl1. Alx and Dbx1 are medial stripes in hindbrain that are more ventrally oriented in spinal cord. BarHL1 expression differed from these two transcription factors in that BarHL1 neurons are dorsally positioned in spinal cord and progressively rotate into more lateral positions in hindbrain (figure 2.11B1-3). In the hindbrain, BarHL1 neurons overlap a portion of the lateral glutamate stripe (figure 2.11B4). At 1 dpf, in hindbrain cross section, BarHL1:GFP neurons are laterally positioned with circumferentially projecting axonal growth cones reaching the midline (figure 2.11C1-3). In dorsal view, BarHL1 neurons are distributed throughout hindbrain rostrocaudally with some gaps (figure 2.11C4). At 3 dpf, BarHL1:GFP expression forms a complete lateral stripe in all hindbrain segments (figure 2.11D1-6). In dorsal view, these cells form a continuous band that extends rostrocaudally throughout hindbrain and caudally within spinal cord as well (figure 2.11D7).

Since this stripe differs from Alx and Dbx1 in its position in spinal cord and hindbrain, we used BarHL1:GFP x Huc:Kaede transgenic fish to determine the age

Figure 2.11 Transcription factor stripe, BarHL1, maintains an orderly patterning of old to new from ventral to dorsal and from medial to lateral. **(A)** Orientation of images in this figure. **(B1-3)** Cross sections of BarHL1:GFP x Alx:DsRed at 3 dpf show the emergence of a lateral stripe from spinal cord to hindbrain. (**B4**) Cross section of VGlut:DsRed x BarHL1:GFP at 3 dpf. BarHL1 neurons are laterally positioned and coincide with the lateral stripe of glutamatergic interneurons. **(C)** Development of BarHL1 stripe. Confocal images of BarHL1:GFP transgenic fish imaged at 1 dpf when few BarHL1 neurons are present. In **C4**, a dorsal view of BarHL1 neurons shows that segmental gaps with no BarHL1 neurons are present at different rostrocaudal positions. BarHL1 neurons are laterally positioned and axon growth cones are approaching the midline. **(C1-C3)** Cross sections indicate that all BarHL1 neurons are positioned in the lateral most hindbrain regions throughout hindbrain. Axon growth cones are circumferentially approaching the midline. **(D)** BarHL1:GFP imaged at 3 dpf. BarHL1 neurons form a lateral stripe throughout hindbrain (cross sections **D1-6**). In dorsal view, BarHL1 neurons for a continuous band that rostrocaudally extends throughout hindbrain (**D7**). **(E,F)** Age related patterning of BarHL1 neurons. **(E1,F1)** Timing of photoconversion experiments performed here. Huc:Kaede fish were crossed with BarHL1:GFP fish and photoconverted at 29 hpf (**D**) and at 2 dpf (**E**). Fish were imaged a first time and red cells in the first image were the older population of all neurons. The fish was then fully photoconverted again so that Huc:Kaede no longer had green expression. In this second image, green cells are solely BarHL1:GFP cells. Colocalization of the red cells from the first image and the green from the second reveal the oldest BarHL1 neurons. Red cells in all images are colocalized cells as described above and green cells are BarHL1:GFP labeled cells. **(E,F)** Age-related organization of the BarHL1 stripe. Early born (red) neurons are both ventrally and medially positioned (**E2-5**) relative to younger (green) cells (N=5). By 2 dpf, most BarHL1 neurons have differentiated and the youngest exclusively green neurons are laterally positioned (**F6**: dorsal view, **F2-5**: cross sections indicate a similar medial to lateral age patterning across multiple rhombomeres) (N=1). Scale bars = 20 um.

A
D7,E5,F6
B2-4,C1-3,D1-6,E2-4,F2-5
B1
BarHL1:GFP
1 dpf
3 dpf
C1
C2
C3
C4
D1
D2
D3
D4
D5
D6
D7
r3
r4
r5
r6
r7
r8
BarHL1:GFP (3 dpf)
Abz-DsRed
VGlut:DsRed
Spinal cord
Hindbrain
B1
B2
B3
B4
BarHL1:GFP x Huc:Kaede
Early born BarHL1 neurons
Later born BarHL1 neurons
E1
UV light
Observation
28 hpf
2 dpf
Huc:Kaede (old: before 28 hpf)
Huc:Kaede (new) and BarHL1:GFP
Huc:Kaede (all)
BarHL1
F1
3 dpf
E2
E3
E4
E5
F2
F3
F4
F5
F6
r3
r4
r5
r6
r7

related patterning of this stripe for comparison with more medial ones (see figure legend in 2.11E1,F1). The earliest born BarHL1 neurons are medially and somewhat ventrally positioned relative to younger neurons (figure 2.11E2-5, N=5). The latest born BarHL1 neurons are laterally positioned relative to older BarHL1 neurons (figure 2.11F2-6, N=1). This old to young, medial to lateral patterning of the BarHL1 stripe is somewhat different from the age-related patterning of the Alx and Dbx1 stripes.

Disruption of age-related patterning at later times

Zebrafish at 2 dpf are still relatively immature, so we wished to know how organized the patterning of neurons by age is at 4 dpf, a time when zebrafish are freely swimming. We photoconverted Huc:Kaede fish at 24 hpf again, but, this time, imaged at 4 dpf. We found a largely similar patterning with red cells ventrally positioned and green cells more dorsally positioned (figure 2.12C1,D1-5); however, a population of younger cells was present ventrally in some regions of hindbrain (figure 2.12C1-lower green arrow, figure 2.12D1,D3-5). Despite these differences, the majority of the oldest neurons (expressing Kaede by 24 hpf) remained ventrally positioned at 4 dpf.

We next observed the patterning of neurons slightly younger than the oldest cells by photoconverting Huc:Kaede fish at 30 hpf and again imaging at 4 dpf. In this case, a much larger number of neurons contained red Kaede expression. In lateral view, these neurons populate more ventral regions than exclusively green neurons (figure 2.13C1, red/green arrows). In dorsal view, the 2 dpf patterning of old neurons in the center on either side of hindbrain with younger cells filling in both medially and laterally is still observed at 4 dpf (figure 2.13C2, red/green arrows). In cross section, individual rhombomeres differ slightly from one another. For instance, in rhombomeres 4-6, there are few if any young (green) cells ventral to the oldest ones (figure 2.13D1-3). However, in rhombomeres 7 and 8, a larger cluster of young cells

Figure 2.12 Positions of the earliest born neurons (by 24 hpf) from spinal cord to hindbrain at 4 dpf. **(A)** Timing of experiments shown in this figure. Photoconversion of Huc:Kaede was performed at 24 hpf and confocal images of fish were acquired at 4 dpf. **(B)** Orientation of images in this figure. **(C1)** Lateral view of hindbrain and spinal cord regions. Most young cells in hindbrain at 4 dpf are dorsal (green) and most old cells are ventral (red). In spinal cord, young (green) cells are located at both dorsal and ventral extremes with old (red) neurons in the mid-dorsoventral region. Red/green arrows (hindbrain) and bars (spinal cord) indicate regions of red/green cells in hindbrain and spinal cord (right of image). **(C1)** Dorsal view of 4dpf fish. In hindbrain young cells are both medial and lateral with the oldest (red) cells in-between. In the spinal cord, old cells (red) are laterally positioned relative to younger (green) cells. Red/green arrows and bars indicate regions of red/green cells in hindbrain and spinal cord (below image). **(D1-D6)** Cross sections of rhombomere 4 (R4) through rhombomere 8 (r8) and spinal cord (sc). On the right, red bars indicate regions of old neurons both dorsoventrally and mediolaterally. Green bars indicate regions of younger neurons. White arrows (left) and asterisks (right) indicate regions of younger ventral cell present in R4, R6, R7 and R8. Scale bars = 20 um

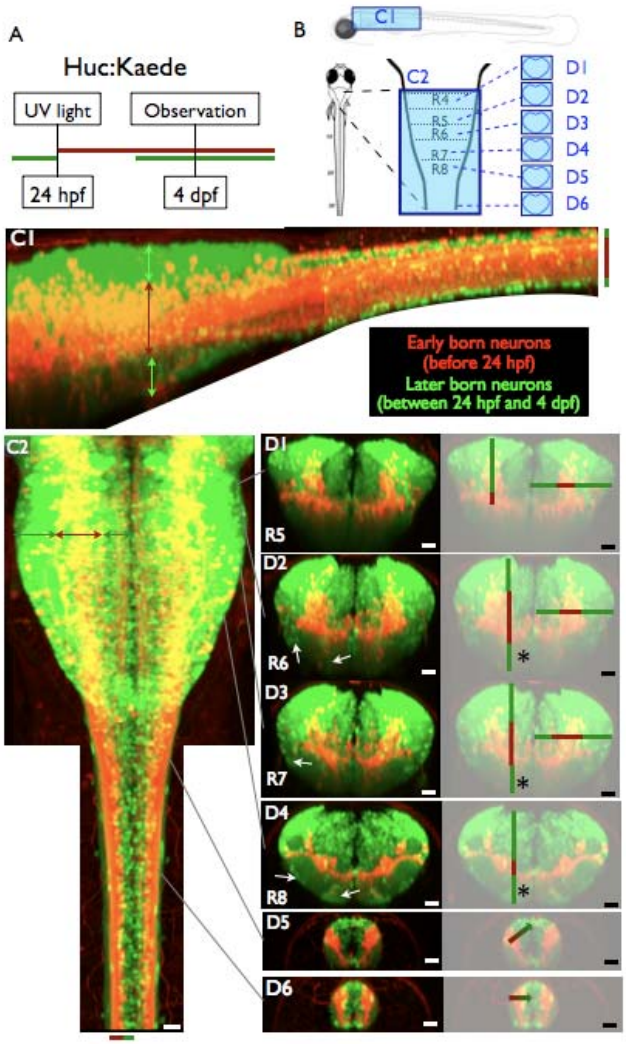

A
Huc:Kaede
UV light
Observation
24 hpf
4 dpf
B
C1
C2
R4
R5
R6
R7
R8
D1
D2
D3
D4
D5
D6
C1
Early born neurons
(before 24 hpf)
Later born neurons
(between 24 hpf and 4 dpf)
C2
D1
R5
D2
R6
D3
R7
D4
R8
D5
D6

Figure 2.13 Details of the patterning of early born neurons (by 30 hpf) in the hindbrain at 4 dpf. **(A)** Timing of experiments shown in this figure. Photoconversion of Huc:Kaede was performed at 30 hpf and confocal images of fish were acquired at 4 dpf. **(B)** Orientation of images in this figure. **(C1)** Lateral view of hindbrain and spinal cord regions. Most young cells in hindbrain at 4 dpf are dorsal (green) and most old cells are ventral (red). Red/green arrows indicate regions of red/green cells in hindbrain. In caudal hindbrain, there is a region of ventral young cells present. **(C2)** Dorsal view of 4 dpf fish. In hindbrain young cells are both medial and lateral with older (red) cells in between. Red/green arrows indicate regions of red/green cells in hindbrain. **(D1-D5)** Cross sections of rhombomere 4 (R4) through rhombomere 8 (R8). On the right, red bars indicate regions of old neurons both dorsoventrally and mediolaterally. Green bars indicate regions of younger neurons. White arrows (left) and asterisks (right) indicate regions of younger ventral cell present in R7 and R8.

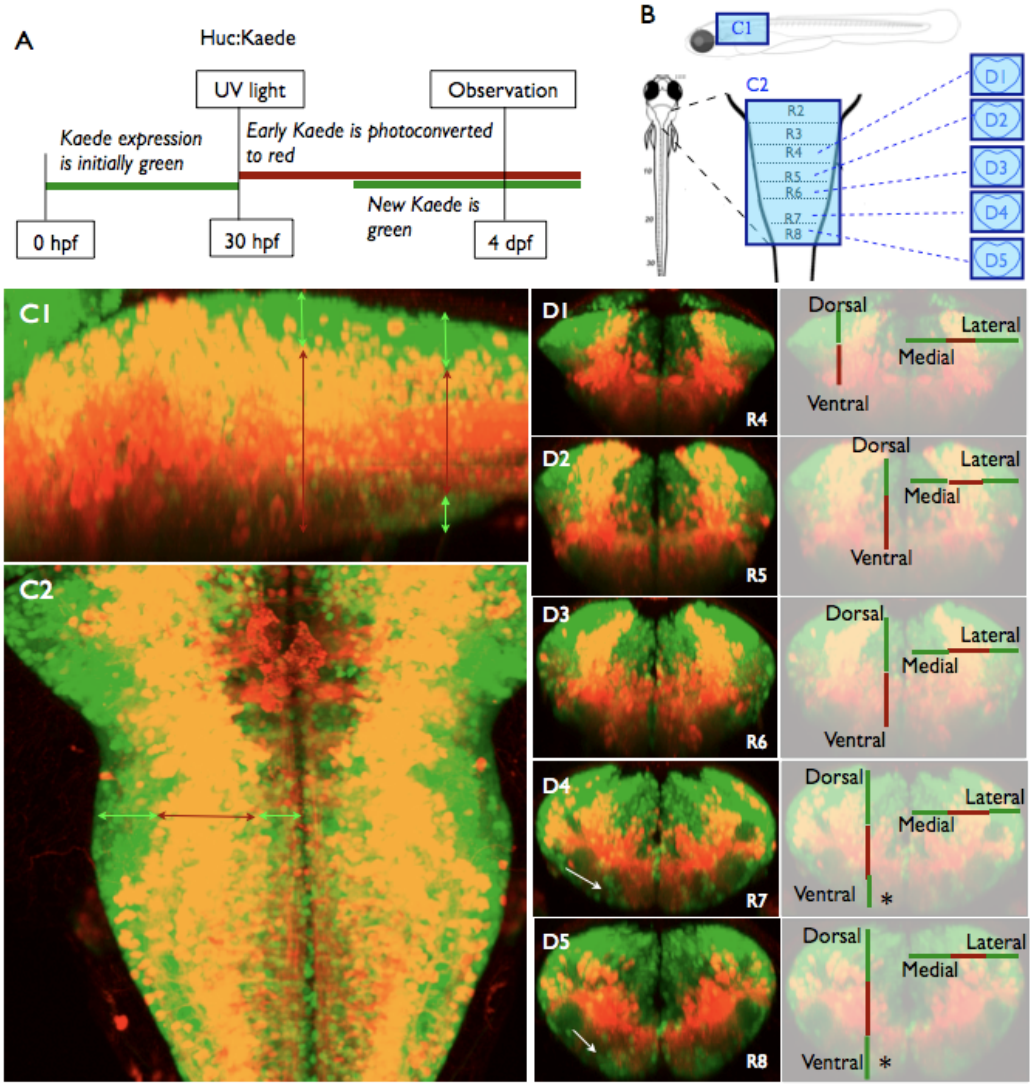


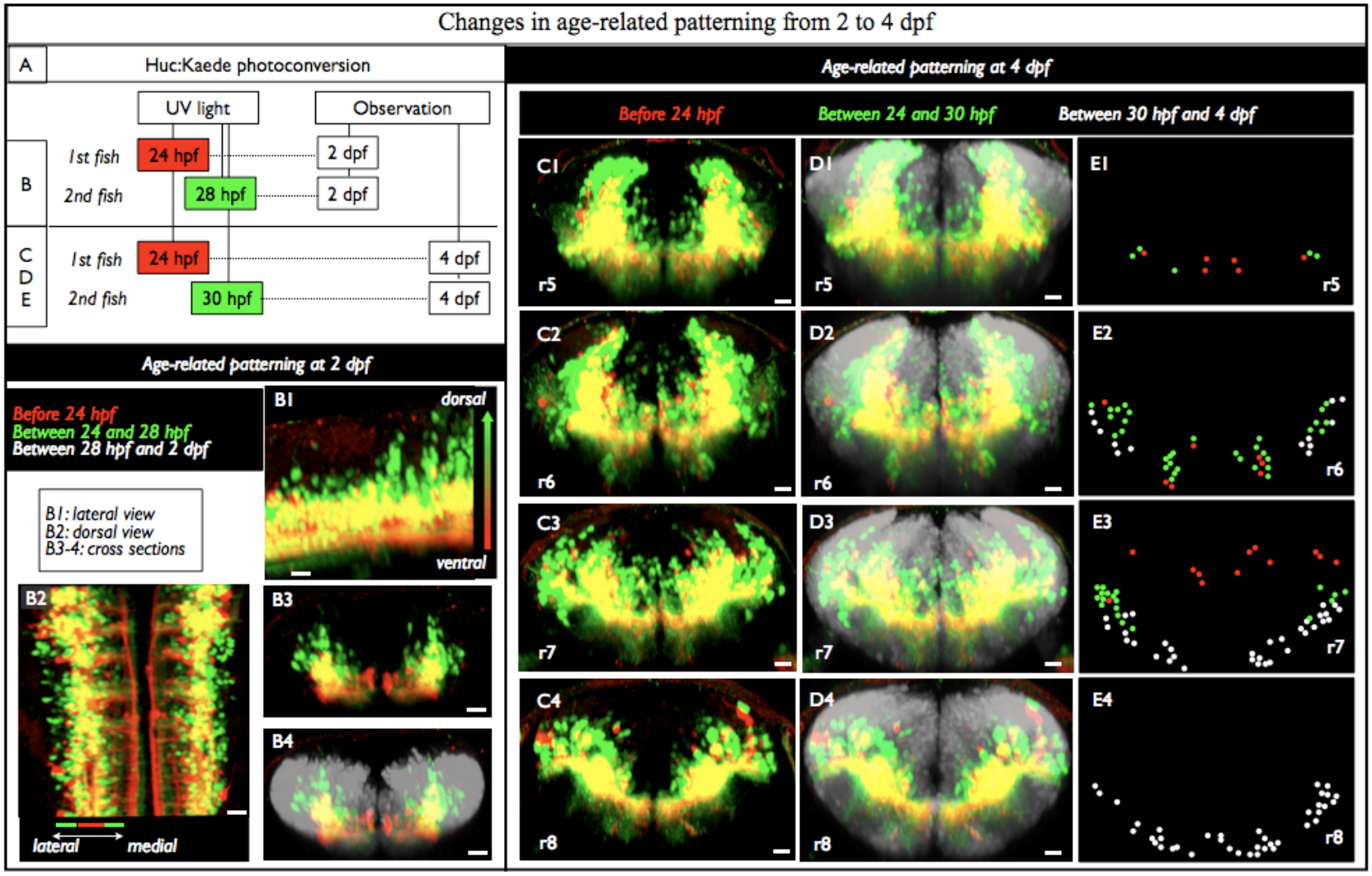
Figure 2.13 Early born neurons in hindbrain at 4 dpf

is present ventrally (figure 2.13D4-5), in a patterning that differs from the typical one where the oldest neurons are the most ventral.

To be able to determine relative positions at these three different stages (24 hpf, 30 hpf and 4 dpf), we again used image registration. We aligned the green Kaede channels of photoconverted transgenic fish flashed at different times but, this time, imaged at 4 dpf. The oldest cells were colored red (expression by 24 hpf), the younger cells were colored green (expression by 30 hpf) and the youngest were colored white (expression by 4 dpf, the time of imaging) and the three populations represent the majority of hindbrain neurons at three different time points for differentiation (figure 2.14B). In figure 2.14C, we show the relative positions of the youngest cells in rhombomeres 5-8. In rhombomeres 5 and 6, a small population of green cells is present ventral to the older red ones (figure 2.14C1-2). In rhombomere 7 and 8 (figure 2.14C3-4), and for the majority of the older neurons labeled in rhombomeres 5 and 6 (figure 2.14C1-2), green cells are dorsally positioned relative to the older red cells as observed for all neurons in a similar experiment described earlier at 2 dpf and shown again here for comparison in figure 2.14B. When comparing the positions of these older red (expression by 24 hpf) and green (expression by 30 hpf) to younger neurons (expression at 4 dpf) colored in white, the patterning in rhombomere 5 and 6 is orderly in that younger (white) neurons are more medial and more lateral than red and green neurons as neurons in the hindbrain progressively filled in from 30 hpf to 4 dpf. Therefore, the ventrally displaced younger cells in rhombomeres 5 and 6 are only slightly younger than the oldest cells since we have shown that they are present by 30 hpf and few if any cells after 30 hpf are displaced to very ventral regions.

In rhombomeres 7 and 8, however, where we observed no displacement of the patterning of cells of similar ages (green/red labeled), at 4 dpf there is a large population of the youngest cells that are ventrally displaced relative to older neurons

Figure 2.14 Changes in age-related patterning from 2 to 4 dpf**. (A)** Timing of photoconversion and imaging for **B-E**. In **B**, image registration for two fish imaged at 2 dpf is shown. The first fish was photoconverted at 24 hpf and the second fish slightly later at 28 hpf. In **C-E**, image registration for two fish imaged at 4 dpf is shown. The first fish was photoconverted at 24 hpf and the second at 30 hpf. Colored boxes indicated the color of the neurons obtained from the two different fish after image registration. Red cells in **B-E** represent the oldest neurons (those photoconverted in the first fish) while green cells represent slightly younger cells (those photoconverted in the second fish). All neurons present at either 2 dpf (**B**) or 4 dpf (**C-E**) are colored white. (**B**) Age-related patterning is orderly at 2 dpf; this was shown earlier in figure 2. Here we show the age-related patterning at 2 dpf for comparison to that at 4 dpf. In **B1**, a lateral view with rostral to the right is shown; in **B2**, a dorsal view is shown; and in **B3-4** cross sections are again shown. See figure caption for figure 2 for the description of the patterning. **(C-E)** Age-related patterning at 4 dpf. At the top, a legend indicates the color of neurons of different ages, with red cells represent the oldest population, green cells slightly younger, and white cells representing the youngest neurons in hindbrain. **(C1-C4)** Cross sections at different rhombomere segments showing positions of the oldest (before 24 hpf) cells in red and slightly younger (between 24 and 30 hpf) cells in green. In r5 and r6 **(C1,C2)**, small medial regions of green expressing cells are ventrally located relative to red cells, indicating a difference in the patterning of hindbrain segments solely by age between 2dpf and 4 dpf. A population of lateral green cells was also ventral to red cells in r7 **(C3)**. **(D1-D4)** Comparing positions of the youngest (white) cells to older neurons (red and green) in different rhombomere segments. In r7 **(D3)** and r8 **(D4)**, a large number of the youngest neurons were ventrally positioned in both medial and lateral regions. In r6 **(D2)**, a ventral population of white cells was present laterally that was ventral to both red and green cells. **(E1-E4)** Summary of cells of different ages that are positioned outside of a ventral to dorsal age-related patterning of cell bodies. In r5, no youngest white cells are positioned ventrally, only the older neurons are shifted into the neuropil region (red and green circles). In r6 **(E2)**, on the left side a medial cluster is organized by age with red cells ventral to green but this patterning is not seen at this time on the right side in this segment. In r7 **(E3)**, a large number of red neurons (before 24 hpf) are dorsally positioned relative to all of the green cells (24 hpf to 30 hpf) and some of the white cells (30 hpf to 4 dpf). In r8 **(E4)**, no old cells (red or green) are located in the ventralmost regions. However, a large number of younger cells are ventrally positioned (white circles). Although the patterns of r5 **(E1)** and r8 **(E4)** are dramatically different as shown here, it is important to note the larger number of neurons already present at early time points in r5 **(C1)** in comparison to r8 **(C4)**. Scale bars = 20 um.


Changes in age-related patterning from 2 to 4 dpf
A
Huc:Kaede photoconversion
UV light
Observation
B
1st fish
24 hpf
2 dpf
2nd fish
28 hpf
2 dpf
C D E
1st fish
24 hpf
4 dpf
2nd fish
30 hpf
4 dpf
Age-related patterning at 2 dpf
Before 24 hpf
Between 24 and 28 hpf
Between 28 hpf and 2 dpf
B1: lateral view
B2: dorsal view
B3-4: cross sections
B1
dorsal
ventral
B2
lateral
medial
B3
B4
Age-related patterning at 4 dpf
Before 24 hpf
Between 24 and 30 hpf
Between 30 hpf and 4 dpf
C1
D1
E1
r5
C2
D2
E2
r6
C3
D3
E3
r7
C4
D4
E4
r8

colored green and red (figure 2.14D3-4). Since the age of a neuron identifies functional distinctions within a class of cells in spinal cord [18], it is possible that the displacement of cells of different ages shown to exist between rhombomeres 5-6 and rhombomeres 7-8 could represent the generation of different populations/nuclei with functional differences. However, eventually determining the identities of these cells is crucial for understanding whether an age difference in these two populations (rhombomeres 5-6 and rhombomeres 7-8 displaced cells) might be functionally relevant.

To summarize, in figure 2.14E, we show positions of the cells at 4 dpf that do not follow the earlier simple patterning of old to young from ventral to dorsal seen at 2 dpf (figure 2.14B). Colored circles represent cells of the different ages described above (see legend in figure 2.14A) that are displaced relative to the age-related patterning shown for the majority of hindbrain neurons. In rhombomeres 5 and 6, displaced cells are predominately older (figure 2.14E1-2), with expression of Huc:Kaede present at either 24 hpf or 30 hpf. In rhombomere 6, younger cells are present in lateral regions (figure 2.14E2, white cells), but these might follow the medial and lateral filling in of this hindbrain segment (see figure 2.14D2). In rhombomeres 7 and 8, there are no obvious displacements of old cells of slightly different ages; however, a large number of younger cells are present at the ventral edges of hindbrain (figure 2.14E3-4). A possibly significant difference between rhombomeres 5-6 and rhombomeres 7-8 is that the displaced cells in rhombomeres 7-8 appear to be clustered and positioned at the ventral edge of hindbrain (figure 2.14D3-4), but in rhombomeres 5-6, the older displaced cells (relative to rhombomeres 7-8) are not as ventrally displaced (figure 2.14D1-2).

We examined the positions of glycinergic and glutamatergic neurons to determine which neurotransmitter phenotype these displaced cells might possess. At

Figure 2.15 Changes in stripe organization at early and late developmental times. **(A-B)** Development of glutamatergic stripes. Cross sections at 36 hpf (**A1-2**) and 4 dpf (**B1-2**) are shown for rhombomeres 4 (**A1**,**B1**) and 8 (**A2**,**B2**). At 36 hpf, glutamatergic neurons are clustered ventrally into short stripes that are mediolaterally segregated. Projections from neuron clusters project ventrally into a thin neuropil region. At 4 dpf, stripes extend throughout the dorsoventral extent of the cell body layer. Some glutamatergic neurons are present in neuropil regions as well, indicated by arrows in **C1-2**. **(C-D)** Displacement of some glycinergic neurons by 5 dpf. Cross sections of GlyT2:GFP transgenic line at 61 hpf (**D1-2**) and at 5 dpf (**E1-2**) are shown. At 61 hpf, neurons are present in clusters from medial to lateral. Their processes project down to a small neuropil region. At 5 dpf, stripes are still present, neuropil regions are enlarged, and some glycinergic neurons are present within neuropil regions, indicated by black arrows in **F1-2**. Scale bars = 20 um.

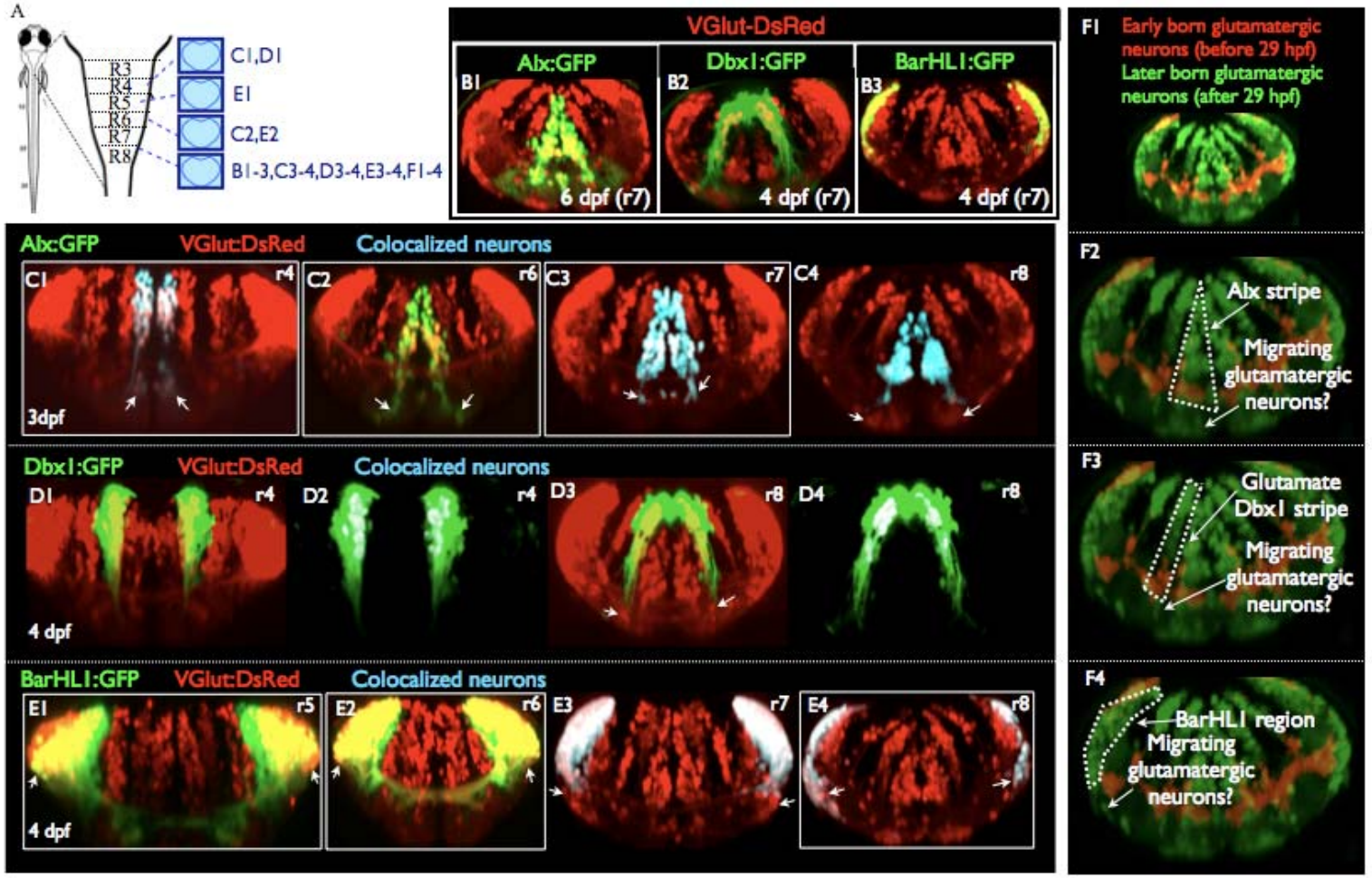
Glutamatergic stripes
36 hpf
4 dpf
A1
B1
C1
A2
B2
C2
Rhombomere 4
Rhombomere 8
Glycinergic stripes
61 hpf
5 dpf
D1
E1
F1
D2
E2
F2
Rhombomere 4
Rhombomere 8

1.5 dpf, glutamatergic neurons are not located in ventral neuropil regions (figure 2.15A1-2). At 4 dpf, a large number of glutamatergic neurons are present in these ventral regions (figure 2.15B-C, black arrows in figure 2.15C indicate neurons within neuropil regions).

At 61 hpf, glycinergic neurons are mid-dorsoventrally positioned within hindbrain with no neurons ventrally positioned within neuropil regions where displaced neurons are located (figure 2.15D1-2). In figure 2.15D-E, cross sections of GlyT2:GFP transgenic fish indicate that glycinergic interneurons are present in small numbers within neuropil regions at 5 dpf. In figure 2.6, we showed that most glycinergic neurons have differentiated by 2 dpf; however, in figure 2.15 C-D, stripes have dorsoventrally elongated and neurons have shifted to ventral neuropil regions at times after most neurons have differentiated (after 61 hpf and before 5 dpf). It is possible that they have migrated to these more ventral positions. Therefore, a small number of glycinergic neurons and a larger pool of glutamatergic neurons are located in this ventral region where age-related patterning is disrupted (see figures 2.10-12).

Because a large population of glutamatergic neurons are ventrally displaced relative to earlier times, we compared the transcription factor expression (figure 2.16B1-3) with the glutamatergic neuron patterning in this region to determine what zone might have given rise to the glutamatergic cells. Alx neurons overlap with the medial glutamate stripe. In rhombomere 4, Alx is expressed in all medial glutamatergic neurons (figure 2.16C1, white arrows indicate ventral stripe regions with Alx expression); however, in this rhombomere segment, medial glutamatergic neurons are not as ventrally displaced as in more caudal hindbrain segments. In rhombomere 6, alx neurons are located at the ventral edge of hindbrain and identify all medial glutamatergic neurons (figure 2.16C2). Earlier, we showed this region to be unique with the ventral-most Alx neurons in hindbrain located here and the age-related

Figure 2.16 Neuronal migration that disrupts overall age-related patterning occurs for glutamatergic neurons that are not expressing Alx, Dbx1, or BarHL1. **(A)** Orientation of images in this figure. **(B)** Cross sections of VGlut:DsRed x Transgenic lines (Alx:GFP for **B1**, Dbx1:GFP for **B2**, and BarHL1:GFP for **B3**) indicate the overlap of transcription factor patterning with glutamatergic stripe patterning. **(C)** Alx neurons encompass all medial glutamatergic neurons only within rhombomere 6 (**C2**). In rhombomere 8, a large number of medially and ventrally positioned glutamatergic neurons reside ventral to the Alx stripe but do not express Alx (**C4**). **(D)** Dbx1 neurons do not overlap with ventral glutamatergic neurons within neuropil regions that reside ventral to the Dbx1 stripe (**D3** cross section). White arrows indicate ventral neurons that do not possess Dbx1:GFP expression. **(E)** BarHL1 neurons represent some displaced neurons (**E4**, rhombomere 8), but not the ventralmost displaced glutamatergic neurons. In **F**, regions of transcription factor expression and regions where neurons have possibly migrated are shown (**F2** for Alx expression, **F3** for Dbx1 expression, and **F4** for BarHL1 expression).


A
R3
R4
R5
R6
R7
R8
C1,D1
E1
C2,E2
B1-3,C3-4,D3-4,E3-4,F1-4
VGlut-DsRed
Alx:GFP
Dbx1:GFP
BarHL1:GFP
B1
B2
B3
6 dpf (r7)
4 dpf (r7)
4 dpf (r7)
Alx:GFP
VGlut:DsRed
Colocalized neurons
C1
r4
C2
r6
C3
r7
C4
r8
3dpf
Dbx1:GFP
VGlut:DsRed
Colocalized neurons
D1
r4
D2
r4
D3
r8
D4
r8
4 dpf
BarHL1:GFP
VGlut:DsRed
Colocalized neurons
E1
r5
E2
r6
E3
r7
E4
r8
4 dpf
F1
Early born glutamatergic neurons (before 29 hpf)
Later born glutamatergic neurons (after 29 hpf)
F2
Alx stripe
Migrating glutamatergic neurons?
F3
Glutamate Dbx1 stripe
Migrating glutamatergic neurons?
F4
BarHL1 region
Migrating glutamatergic neurons?

patterning flipped relative to the rest of hindbrain. In regions within rhombomere 7, Alx neurons overlap with medial glutamatergic neurons, but little or no glutamatergic expression is found at the ventral edge (figure 2.16C3). In rhombomere 8, a large number of ventral glutamatergic neurons are present medially that do not express the Alx transcription factor (figure 2.16C4). Therefore, the age-related patterning is not disrupted since these neurons are a cluster separate from the Alx stripe. This was expected since the age-related organization of the alx stripe region in rhombomere 8 was shown earlier to maintain an orderly patterning (see figure 2.9F6).

The Dbx1:GFP expression does not overlap with the ventral neurons just ventral to the Dbx1 stripe (figure 2.16D1-4). However, Dbx1 expression is transient, and it is possible that these neurons had expressed Dbx1 at earlier times. BarHL1:GFP expression largely does not overlap with more ventral neurons ventral to the BarHL1 stripe with the exception of a population within rhombomere 8 (figure 2.16E1-4). Therefore, the neurons within ventral neuropil regions did not obviously arise from the transcription factor stripes described here and could represent a special pool of neurons migrating from another region than those identified by Alx, Dbx1, and BarHL1 (figure 2.16F1-4). These neurons are predominately glutamatergic with a smaller population of glycinergic neurons as well. The patterning of transcription factor stripes relative to these displaced cells is shown in figure 2.16F1-4, where the regions are delineated within the age-related map described for glutamatergic neurons in figure 2.5.

Although the orderly patterning by age observed at 2 dpf is disrupted at 4 dpf, those neurons that do not follow the pattern do not possess transcription factor expression localized to all neurons within the stripes dorsal to these potentially migrating neurons. The age-related organization of neurons by transcription factor type might persist in larval zebrafish. If neurons slowly migrate into a nuclear

organization, hints of an age-related patterning of cell types within a nucleus might still be present if the migration if their migration is orderly.

We have shown that there is a continuous patterning between spinal cord and hindbrain identified by neurotransmitter, transcription factor, and age-related organization. In each case, a topological transformation of the patterning occurs. A mediolateral segregation of old and young cells in spinal cord transitions into a dorsoventral segregation in hindbrain. A dorsoventral segregation between transcription factor and neurotransmitter patterning in spinal cord transforms into a mediolateral segregation in hindbrain. In the hindbrain, this segregation persists in the form of mediolaterally segregated stripes identified by both neurotransmitter and transcription factor phenotypes and persists in larval zebrafish. We have shown that the age-related segregation of the stripes is maintained at 4 dpf, a time when zebrafish are freely swimming. This pattern by age, transcription factor, and neurotransmitter expression extends rostrocaudally through hindbrain at a time when hindbrain circuits are functional, indicating that this patterning could have broad functional significance as has been shown previously for spinal neurons where neurons of different ages in the same transcription factor class are involved in the coordination of movements of different strengths [18].

Discussion

In this study we have shown that stripes of interneurons defined by both neurotransmitter and transcription factor expression exist at the earliest times when few neurons are present and persist into larval stages (5 dpf). We have shown that the age-related patterning of all neurons is orderly at 2 dpf and that some cells have moved around by 4 dpf. By using photoconvertible proteins to track cells of different ages within stripes, we found that, despite the changes in cell positions of some

neurons, stripes maintain an age-related organization at 4 dpf, and, when age-related patterning is disrupted, stripe patterning is disrupted as well. Disruption of age-related patterning exists within the alx stripe only within rhombomeres 6 and 8. In all other regions, the age-related organization from ventral to dorsal for old to young within a stripe is present across multiple rhombomere segments, suggesting a broad organization for interneurons within a stripe based on their dorsoventral position and age.

Spinal cord and hindbrain are linked by age-related patterning and transcription factor organization: principles of spinal organization may shape the organization of neural circuits in hindbrain

Previous studies have shown that alx neurons in spinal cord form a functional topography based on dorsoventral position [18, 19] and age [18]. In a recent study describing a functional topography for excitatory and inhibitory cell types within spinal cord, recruitment patterns of excitatory and inhibitory interneurons are opposed, with excitatory neurons recruited from ventral to dorsal for slow to fast swimming and inhibitory neurons recruited from dorsal to ventral for slow to fast swimming [19]. Alx neurons, an excitatory cell type present in this functional topography, are recruited also by age, with young (ventral) neurons recruited during weak movements and older (dorsal) neurons recruited during stronger movements [18]. Unpublished work shows that the age-related functional topography extends to other cell types as well [D McLean and J Fetcho].

Since neurotransmitter stripes in hindbrain extend dorsoventrally, we predicted that an age-related patterning might exist within a stripe along its dorsoventral extent because an age-related order in spinal cord was shown to exist along a dorsoventral direction for alx neurons. Here we showed that alx neurons, as well as other

transcription factor types and neurotransmitter types, are organized by age in both spinal cord and hindbrain. In the hindbrain stripes, the oldest neurons are ventral and younger ones are stacked above them. It is possible that this age-related order for neurotransmitter stripes might reflect a functional organization within a stripe, where the oldest (ventral) neurons participate in the coordination of stronger movements and younger (dorsal) neurons within a stripe coordinate weaker behaviors. Thus, it is possible that alx hindbrain neurons maintain functional differences based on age as seen in spinal cord [18]. If an age-related recruitment occurred across all of the different hindbrain stripes, then both excitatory and inhibitory neurons would be recruited in the same direction as all hindbrain stripes are arranged with the oldest neurons located ventrally. This recruitment pattern would, then, differ from that of spinal cord, where dorsoventral recruitment of excitatory and inhibitory neurons is opposed [19].

Since the patterning both by age and transcription factor organization is continuous, although with a transformed topography, between spinal cord and hindbrain, it is possible that other principles of organization for spinal circuits are present in hindbrain. For example, a functional topography for spinal interneurons was previously shown to exist continuously across cell types where excitatory neurons involved in slow swimming are young and ventrally positioned, and, at faster speeds, a continuous shift occurs as a more dorsal, morphologically distinct cell type is engaged when that animal moves at faster speeds [29]. Since spinal networks show a switch in the neuronal classes involved in the slowest versus faster speeds of swimming, a similar switch from one stripe to another might be expected in the hindbrain, as each stripe contains morphologically different neurons (see chapter 3). Determining how motor behaviors are initiated and coordinated by the hindbrain in terms of recruitment

within and across stripes will allow direct tests of predictions based upon a hypothesis of a similar functional organization in the two regions.

Rhombomere-specific alterations in age-related patterning

In some cases, we found that the age-related organization was disrupted, such as the age-related flipping of alx neurons in rhombomere 6. When age-related patterning was disrupted, it was isolated to specific rhombomere segments and disrupted the structure of the stripe in that local region. In rhombomere 6, the younger alx neurons are located ventrally, perhaps as a result of migration. In this rhombomere, the stripe extends to the ventral edge of hindbrain, far more ventral than the ventral edge of the alx stripe throughout the rest of hindbrain. In contrast, in rhombomere 8, old neurons are ventromedially positioned and disrupt stripe patterning by forming a medially cluster of alx neurons outside of stripe regions. Therefore, there are rhombomere-specific changes in the structure and age-related organization of alx neurons.

For the other transcription factors studied, BarHL1 and Dbx1, transcription factor expression is downregulated, and thus, fluorescent proteins driven by the transcription factor promoters are downregulated as well, making it hard to decipher where the oldest neurons are positioned. It is possible that more changes in stripe patterning occur for other transcription factor types. Evx2 antibody staining, marking excitatory Dbx1 neurons, has indicated that these neurons, originating in the middle glutamatergic stripe, shift to more lateral positions outside of the Dbx1 stripe of origin (data not shown). Evx1/2 (V0 excitatory neurons) have been shown to migrate in a specific pattern within spinal cord as well [145]. We showed, in this study, that age-related patterning is orderly at 2 dpf for Dbx1 neurons, but this is perhaps not surprising since neurons remained ordered by age at this time.

We found that BarHL1 neurons remain ordered by age from medial to lateral for old to young neurons within the stripe at 2 and 3 dpf. By 3 dpf, some cells have moved around; yet we found no dramatic changes in age-related order as shown in specific rhombomeres for alx neurons. It is possible that BarHL1 expressing neurons that have downregulated expression of this transcription factor have shifted positions, but these cells were not found in this study. In spinal cord, BarHL1 neurons are dorsally positioned [146], but follow a migratory path to more ventral spinal positions [S Higashijima, unpublished]. In the hindbrain, BarHL1 expression is also present in anterior and posterior rhombic lips that give rise to external and internal cerebellar granular cells and to the deep nuclei [146]; therefore, hindbrain BarHL1 neurons might be subdivided into a population resembling spinal interneurons and one involved in cerebellar circuits. A dramatic increase in the number of BarHL1 neurons in more rostral hindbrain regions indicates that this might be the case (see figures 2.11D and 2.16E); however, age-related patterning was maintained at 3 dpf across these different hindbrain segments. Determining whether stripes undergo similar changes in age-related patterning, or changes in structural patterning, in a rhombomere-specific way, as observed for alx neurons, might indicate specific programs for the formation of hindbrain nuclei comprised of neurons expressing distinct transcription factor types.

Stripes maintain an age-related organization into larval stages

We have shown that, at 2 dpf, neuropil regions are minimal, and, by 4 dpf, have increased in size dramatically. Some neurons have migrated to the neuropil regions as well. Since an age-related patterning of stripes persists in most regions at 4 dpf when the fish are freely swimming, it seems likely that neurons are arrayed into orderly stripes while circuits are wired, and, subsequently, migrations occur for the

formation of nuclei. Determining the precise timing of wiring and migration for a single cell type will reveal if this is the case. An age-related organization within a stripe would also allow for an orderly wiring of neurons by age (see chapter 4). For example, the escape circuit is the first functional hindbrain circuit, and, it is likely that escape circuit interneurons are old. The Mauthner cell and its homologues initiate startle responses and are ventrally positioned within rhombomeres 4-6 [147, 148]. An age-related organization by speed would predict that all escape circuit interneurons are present in ventral regions of stripes throughout hindbrain since the oldest neurons are ventrally positioned within a stripe. For example, spiral fiber neurons are located within rhombomere 3 [149, 150], feedforward and feedback inhibitory cells are located within more caudal rhombomeres (rhombomeres 4-6) [M Koyama, unpublished], and cranial relay neurons, also termed T reticular interneurons, are located even more caudally in rhombomeres 7-8 [151]. We would predict that these cell types, extending rostrocaudally through hindbrain from rhombomeres 3-8, are both old and ventrally positioned within stripes. Also, if all escape circuit interneurons are old, then old neurons would project to old neurons to form appropriate connections within the escape circuit. Connections within the escape circuit might be located within the region of these ventral cell bodies or in a local neuropil region in which these ventral cells project. Therefore, we suspect that at least some circuits might be built by connecting neurons of similar ages drawn from similar dorsoventral locations in different stripes.

Predictions for an age-related patterning in hindbrain nuclei

Stripe patterning by neurotransmitter and transcription factor expression has been shown to extend rostrocaudally across hindbrain; however, the organization of hindbrain circuits is segmental in nature [51, 53, 70, 75, 77, 152-157], with specialized

cell types [45, 63, 64, 77, 79, 80, 85, 87, 158-160] and hindbrain nuclei [83, 87-90, 92, 93, 97, 99-106, 113, 114, 144, 160-169] localized to particular segments. Previous studies have shown that transcription factor patterning extends rostrocaudally across hindbrain in many vertebrates for multiple transcription factors [99, 100, 108-134]. Understanding how stripe patterning is organized within both rhombomere segments, and within a rhombomere into specific hindbrain nuclei is an important next step.

Recent studies have shown that hindbrain nuclei are comprised of multiple cell types, each potentially expressing different transcription factors [98-100]. Although the organization by age within hindbrain has been studied [103-106, 169], age-related organization within a single transcription factor type has not been shown in hindbrain. We have shown that stripes identify unique transcription factor types and remain ordered by age in most hindbrain segments at 4 dpf. Since stripes are mediolaterally segregated, stripe patterning must be disrupted to form hindbrain nuclei with distinct transcription factor types present in a single nucleus.

Determining how and when this transition occurs could reveal new principles of organization within hindbrain nuclei. For example, if migration out of stripes is orderly, at least two possible scenarios could be envisioned. One possibility is that neurons from the same stripe regions, and thus the same age, could migrate to a new location outside of a stripe. If this is the case, then nuclei are formed by cells of similar ages that are present in the same dorsoventral regions of stripes. This has been shown in rhombomere 1/cerebellar regions in chicks, where old neurons migrate to rhombomere 1 and younger neurons migrate to the cerebellum [104, 169]. One study indicated that subnuclei within the vestibular nucleus, defined by their difference in projections within hindbrain, develop at slightly different times, and thus, each subnucleus might contain neurons of similar ages within the subnucleus and different ages across different subnuclei [106]. Another possibility is that neurons across the

dorsoventral extent of the stripes migrate to a single nucleus. In this case, cells of different ages would be present within a nucleus and an age-related order could be present as has been shown within the rat inferior olivary complex but not for individual cell types [105]. Determining how stripes, whose patterning remains ordered by age at a time when zebrafish are freely swimming, are dismantled to form nuclei could indicate how neurons within a stripe become parceled out for different functions.

Methods

Confocal imaging

Confocal imaging was performed as described previously [16]. Transgenic zebrafish were anaesthetized in 0.02% MS222 and embedded in 1.4% low melting point agar, typically with the dorsal side down and resting against a glass coverslip floor of a small petri dish. Agar was covered with 0.02% MS222 solution in 10% Hanks to prevent desiccation. Images were collected using an inverted Zeiss LSM confocal microscope. Green fluorescence was excited using 488 nm laser light and red fluorescence using a 543 nm laser. Green fluorescence emission was collected using a filter to collect light from either 505-530 nm or 505-550 nm and red fluorescence emission was collected using a long pass 560 nm filter. Single image stacks were collected throughout the dorsoventral extent of hindbrain or spinal cord and generally collection last 40 minutes to 1.5 hours. To prevent photo damage, images were collected with high gain settings and averaged either twice or four times. Fish remained healthy and anaesthetized during this time.

Photoconversion

Huc:Kaede and alx:Kaede transgenic embryos were illuminated with UV light using a mercury bulb source for 10-40 seconds within their chorion. Immediately afterwards, photoconversion was confirmed by observation of a presence of red expression and an absence of red expression using green and red filters.

Image registration

To compare the positions of neurons within two different confocal stacks, either in the same fish for Kaede photoconversion experiments, or in different fish at the same day, the general patterning of neurons from each confocal stack was used to perform an image registration. Either the position of all neurons (Huc:Kaede) or a subset (GlyT2:GFP) that were present in a single channel in each image were registered together using the spm routine in Matlab. Registration was performed as a rigid body rotation and no rescaling or morphing of either image was necessary to align two confocal stacks. Image registration was considered successful when alignment of the two channels reached a criterion of less than 10 um error in all directions. In most cases, image registration was well within this limit. To generate a single image of both confocal stacks, the image registered stacks were added as channels to the reference image using Imaris software.

Fluorescent in situ hybridization

Animals were anaesthetized in 0.02% MS222 and subsequently fixed overnight in 4% formaldehyde in phosphate-buffered saline (1X). After fixation, in situ hybridization was carried out following a standard protocol [170]. cDNA probes for GLYT2 were used to label glycinergic interneurons as described previously [107]. Complementary RNA probes were tagged with digoxigenin (Dig, obtained from Roche) and Dig was

detected by alkaline phosphatase (AP) conjugated anti-Dig antibody (Roche). For Fluorescence detection, HNPP/Fast-Red was used as the substrate for AP, generating fluorescent products within a similar spectrum to rhodamine.

Colocalization

Confocal image stacks containing green and red channels were observed using Imaris software (BitPlane). Using the colocalization add-on, minimum thresholds were determined for red and green channels slightly above noise levels for each channel. We ensured that the dimmest cells were not thresholded out. A new channel was generated using the Imaris colocalization add-on that represented colabled signal. For image stacks with low noise levels in both channels, the automated colocalization feature in Imaris was adequate for our purposes, but, in most cases, thresholds required adjustments by hand due to high noise levels producing spurious results when observing fish in three-dimensional maximum intensity projections.

CHAPTER 3

A NOVEL PATTERNING IN HINDBRAIN REVEALS AN ORDERLY ARRANGEMENT OF GLYCINERGIC NEURONS BY TYPE

Abstract

The segmental organization of hindbrain has been revealed in previous studies describing the patterning of specialized cell types, transcription factors, and the presence of physical boundaries defining hindbrain segments named rhombomeres. We previously described a novel patterning that crosses these rhombomere boundaries [107]. Glycinergic interneurons form 3 stripes of cells in cross sections of hindbrain from rhombomere 3 to 8 that form continuous rostrocaudal columns that cross rhombomeric boundaries. Each stripe possesses neurons with shared morphological features, some of which directly correspond to features of inhibitory cell types recognized from studies of the spinal cord [17, 171, 172]. Medial stripe neurons are ipsilateral ascending neurons resembling Engrailed1 neurons within the spinal cord of mice [2], zebrafish [17], and xenopus [22]. Middle stripe neurons are contralateral branching neurons resembling CoBL neurons in zebrafish [16, 171, 172] and inhibitory V0 neurons in mice [21, 138]. Lateral stripe neurons are ipsilateral and contralaterally projecting neurons. Stripes of glycinergic interneurons also contain cell types with distinct projection patterns: medial and middle stripes project medially ipsilaterally and contralaterally respectively, while lateral stripes project more laterally. This stripelike arrangement might represent a simple underlying pattern of network organization in hindbrain.

Introduction

Recent work has shown that motor output from the spinal cord is coordinated by distinct classes of interneurons defined by transmitter phenotype, axonal projection, and transcription factor expression [1, 3, 7, 10, 14, 20, 21, 40]. These broad classes are shared among vertebrates with similar classes defined in mice, zebrafish and xenopus, and some of these classes also play restricted functional roles in spinal networks [1, 2, 10, 16-19, 21, 22, 26, 135-139, 171-173]. For example, Engrailed1 positive interneurons are ipsilateral inhibitory interneurons in mice, xenopus, and zebrafish that have been shown to play a role in regulating the speed of movements and in sensory gating [2, 17, 22].

While the foundation for the broad cellular composition of vertebrate spinal cord is reasonably well defined, the organization of broad classes of interneurons in hindbrain is less established. We previously described a novel organization in hindbrain, where interneurons form an array of stripes that alternate between excitatory (glutamatergic) and inhibitory (glycinergic) neurotransmitter phenotype [107]. This patterning is distinct from the segmental, or rhombomeric, patterning along the rostrocaudal axis of hindbrain conserved across vertebrates (reviewed in [51]). We set out to understand whether the stripes present within hindbrain have any significance for the organization of neural circuits. We hypothesized that this type of organization might reflect an orderly arrangement of cell types and connectivity in neural circuits in the hindbrain. To explore this, we addressed three questions: whether the stripes contained neurons with shared morphological features, whether systematic projection patterns exist between stripes, and how neurons within the stripes participate in a known neural network, the Mauthner escape circuit.

To examine the morphology of neurons we labeled single neurons and found that neurons within one stripe have shared morphological features. In some cases,

these features are similar to known functional cell types in spinal cord used to coordinate motor behavior such as swimming in zebrafish [17, 172] and left/right alternation during walking in mice [1]. To explore the possibility of stripes having orderly patterns of projections, we compared the projections of single neurons in different stripes and performed backfills in single stripes. Since mediolateral position is a unique feature of each stripe, we hypothesized that each stripe might project to a unique mediolateral location. We found that a mediolateral segregation of stripe projections is present and extends rostrocaudally throughout multiple hindbrain segments. To examine how this pattern maps into a known neural network, we focused on the well-studied network for the startle response that can be driven by the firing of the Mauthner neuron (reviewed in [143]). The massive Mauthner body and dendrites extend across all stripes. We found by targeted backfills that interneurons within the escape circuit follow the simple projection patterns of the stripes. We suggest that the stripes correspond to a simple set of cell types with orderly patterns of projection that form an underlying primitive wiring pattern that contributes to many hindbrain networks, including that of the Mauthner cell. This organization has many parallels to that in spinal cord, from which the hindbrain might have arisen.

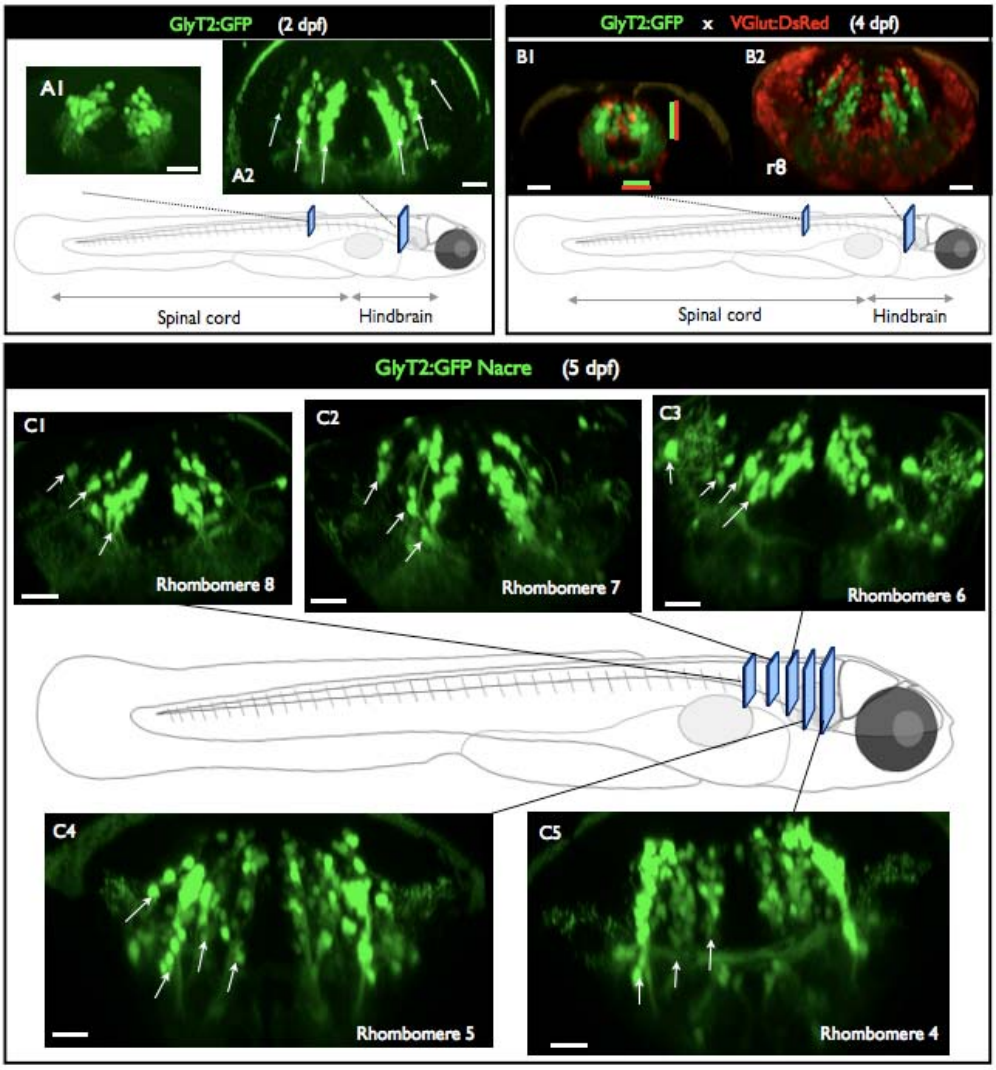
Results

Interleaved stripes of excitatory and inhibitory neurotransmitter phenotype are present in hindbrain

In a previous study, in situ hybridization using an mRNA probe for GlyT2 showed a stripe patterning of glycinergic interneurons exists in hindbrain from rhombomere 3 to 8 [107]. Examination of a BAC transgenic line in which glycinergic neurons are labeled with GFP under control of the glycine transporter 2 promoter (GlyT2:GFP, [19]) revealed a difference in the patterning of glycinergic neurons in the

spinal cord and hindbrain at 2 dpf (figure 3.1). In cross sections of spinal cord, the neurons were located in dorsal regions with no obvious patterning of the somata of the neurons (figure 3.1A1) while, in the hindbrain, the glycinergic neurons were arranged into a series of 3 stripes (figure 3.1A2). By using a dual transgenic line with GlyT2:GFP and the vesicular glutamate promoter driving DsRed expression (VGlut:DsRed) we were able to compare the positions of glycinergic and glutamatergic neurons within spinal cord and hindbrain. Figure 3.1B1 shows a cross section of spinal cord from GlyT2:GFP x VGlut:DsRed transgenic fish. Red and green bars to the right indicate regions of glutamatergic and glycinergic neurons dorsoventrally. The extent of glutamatergic and glycinergic neuron positions is similar dorsoventrally and mediolaterally as well (see red/green bars to the right and below figure 3.1B1). There was some clustering of neurons by transmitter phenotype in cross section, but some were intermixed as well. In contrast, an orderly patterning of interneurons by neurotransmitter phenotype was present in cross sections of hindbrain where interleaved stripes of glycinergic and glutamatergic neurons are present (figure 3.1B2). Glycinergic neurons formed stripes that extended both dorsoventrally in cross section and rostrocaudally in horizontal view across rhombomere boundaries. These are shown in figure 3.1A2, where each stripe is positioned in a specific mediolateral location and extends dorsoventrally. Examination of similar cross sections in which the glutamatergic neurons were also labeled with DsRed showed that the dark regions between glycinergic stripes in panel A2 are regions in which glutamatergic neurons are arrayed in stripes (figure 3.1B2). This organization is present even at 4 dpf when the fish is freely swimming and engaging in many behaviors to which hindbrain networks contribute.

Figure 3.1 Neurotransmitter stripes are present in hindbrain **(A)** Cross sections of confocal images of GlyT2:GFP Nacre fish at 2 dpf. In spinal cord **(A1)**, glycinergic interneurons are not obviously clustered. However, in hindbrain, glycinergic interneurons form stripes. Each stripe is positioned at a unique mediolateral position and extends dorsoventrally. **(B)** Cross sections of confocal images of GlyT2:GFP x VGlut:DsRed transgenic fish at 4 dpf. **(B1)** Glycinergic and glutamatergic neurons are present throughout similar dorsoventral (vertical red/green bars for glutamatergic/glycinergic neurons) and mediolateral regions (horizontal red/green bars) of spinal cord. **(B2)** In hindbrain, glutamatergic and glycinergic interneurons form stripes spread across the mediolateral extent of hindbrain. Neurotransmitter stripes in hindbrain alternate between excitatory and inhibitory phenotype from medial to lateral. **(C)** Glycinergic stripes across hindbrain rhombomere segments. **(C1-C5)** Cross sections of confocal images of GlyT2:GFP Nacre fish at 5 dpf. White arrows indicate stripe regions on the left side of hindbrain in each rhombomere. In rhombomeres 5 and 6 (**C4**,**C3**), a fourth stripe is present laterally that forms a lateral cluster of glycinergic interneurons. In more caudal rhombomeres (**C1-3**), stripes are present and project at an angle in comparison to the more dorsoventral stripes in rhombomere 4 (**C5**). All segments possess the three main stripes, but in caudal rhombomeres (**C1-2**), fewer neurons populate the lateral stripe. Scale bars = 20 um.


GlyT2:GFP (2 dpf)
A1
A2
Spinal cord
Hindbrain
GlyT2:GFP x VGlut:DsRed (4 dpf)
B1
B2
r8
Spinal cord
Hindbrain
GlyT2:GFP Nacre (5 dpf)
C1
Rhombomere 8
C2
Rhombomere 7
C3
Rhombomere 6
C4
Rhombomere 5
C5
Rhombomere 4

This striking patterning suggested a possible orderly arrangement of cell types in hindbrain. We explored this possibility by first looking at the stripe patterning along the rostrocaudal axis of hindbrain in different rhombomeres. Although there is variation in different rhombomeres in the angle, number, and shape of these stripes of glycinergic neurons, a stripe-like patterning is evident in multiple rhombomere segments in hindbrain (figure 3.1C). Each of these stripes is present at a different mediolateral position in all rhombomere segments (figure 3.1C1-5). In rhombomeres 5 and 6 and parts of rhombomere 4 a fourth lateral stripe is present and forms a lateral cluster of glycinergic interneurons (figure 3.1C4-5). The main gross difference between stripes across different rhombomere segments is the angle of the dorsoventral stripe projection. In rhombomere 4 the stripes are primarily segregated mediolaterally and each stripe projects dorsoventrally (figure 3.1C4). In more caudal rhombomeres, all three stripes are present but are oriented at an angle (figure 3.1C1-2). Although all three stripes are present in all rhombomeres, the number of cells within the stripes, and the discreteness of the columns of cells differ. In rhombomere 3, the most rostral region of the brain containing glycinergic interneurons, few glycinergic neurons are present, but these cells are mediolaterally ordered into stripes (data not shown). In rhombomere 4, a large number of glycinergic neurons are present in the lateral stripe (figure 3.1C5) while, in more caudal rhombomeres, a smaller population of neurons form the lateral stripe (figure 3.1C1-2). Despite these differences, the basic stripe patterning is present in all of these rhombomere segments.

Each stripe possesses glycinergic neurons with a distinct morphology

Because the stripe patterning is orderly and present in multiple rhombomere segments, we next asked whether neurons within a stripe were morphologically similar to one another and different from those in other stripes. To determine the

morphology of individual glycinergic interneurons, we stochastically labeled single neurons in the GlyT2:GFP Nacre transgenic line using the Gal4-UAS system driven by the GlyT2 promoter and driving membrane-targeted MCherry expression. In vivo imaging of these transgenic fish revealed the morphology of a single neuron labeled by mMCherry among all of the glycinergic neurons labeled with GFP so that we could determine the stripe position of this neuron. The three-dimensional morphology of these neurons was reconstructed by using Imaris filament tracing software. The morphology of single cells within a glycinergic stripe was examined and compared to others within the same stripe as well as those within different stripes. For the medial and lateral glycinergic stripes, neurons were successfully labeled from rhombomeres 3 through 8. For the middle glycine stripe, neurons were successfully labeled in rhombomeres 6-8. Figure 3.2 shows reconstructions of the neurons in each stripe. Figure 3.3 indicates the location, axon length, and both the ipsilateral and contralateral projections of each neuron.

Medial stripe neurons were predominately cells with ipsilateral and ascending axonal projections (Figure 3.2A). 13 out of 15 medial stripe neurons imaged had primary ipsilateral ascending axons. Most of these axons were over 100 um long (Figure 3.3A). Of these 15 medial stripe neurons, one had an ipsilateral descending axon and another possessed a commissural descending axon. Both of these cells were positioned in rhombomere 7 or 8 and possibly projected to spinal cord regions instead of to the hindbrains regions like the other 13 neurons in the same and more rostral segments.

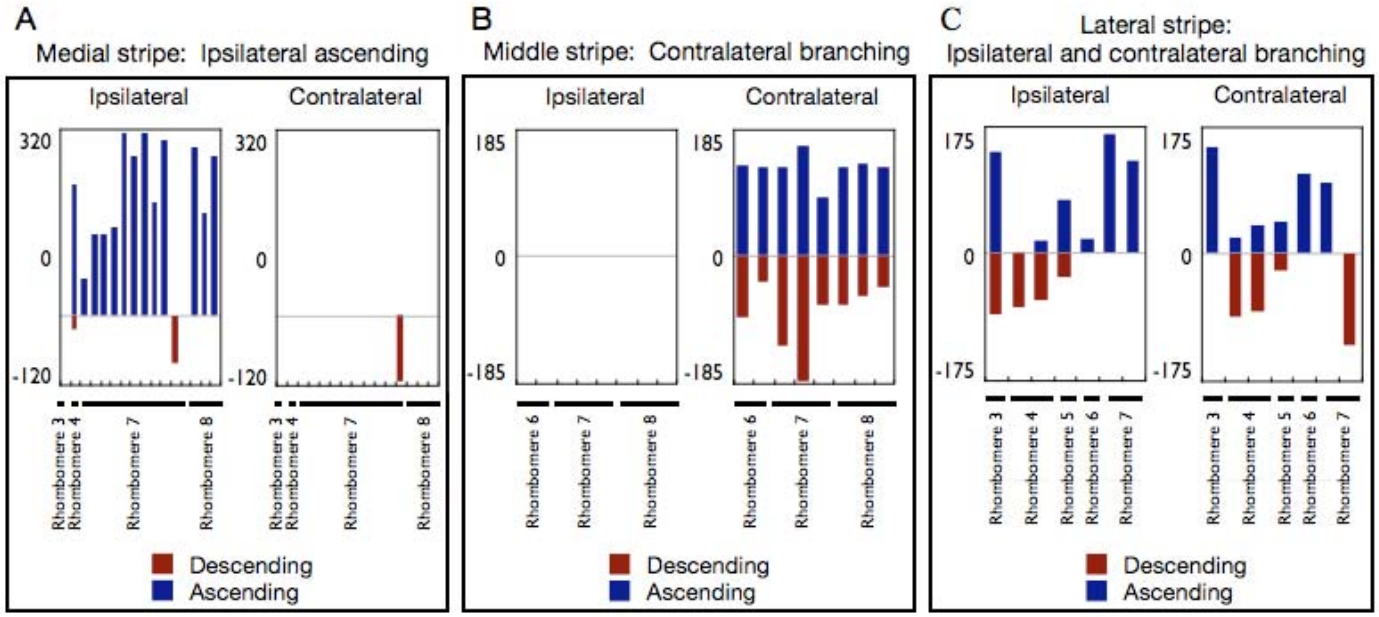
Figure 3.2 Glycinergic neurons within a stripe have a similar morphology In each panel, the top image **(A1,B1,C1)** is a cross section of a confocal image of GlyT2:GFP Nacre fish at 5 dpf with one neuron labeled by stochastic expression of GlyT2:Gal4, UAS:mMCherry and reconstructed using Imaris filament reconstruction. **A1** shows a cross section where an ipsilateral medial stripe neuron is labeled. **B1** shows a middle stripe neuron labeled that projects contralaterally. **C1** shows a contralaterally projecting neuron within the lateral stripe region. At the bottom of each panel are dorsal views of single cell reconstructions of neurons within the medial stripe **(A2)**, middle stripe **(B2)**, and lateral stripe **(C2)** in 5 dpf fish. **(A)** Medial stripe: All medial stripe neurons were ipsilateral ascending **(B)** Middle stripe: All middle stripe neurons labeled were contralateral and branching. **(C)** Lateral stripe: Lateral stripe neurons formed the most diverse class of the three stripes. Neurons all had some combination of ipsilateral and contralateral projections. Scale bars = 20 um.

A1
Medial stripe neurons
A2
Ipsilateral ascending
B1
Middle stripe neurons
B2
Contralateral branching
C1
Lateral stripe neurons
C2
Ipsilateral and contralateral branching

All eight neurons labeled in the middle stripe had contralaterally extending axons with both ascending and descending branches, often of similar length (figure 3.2B, 3.3B). When one process was longer, the tendency was for it to be the ascending branch. Labeled neurons were located in rhombomeres 6,7 and 8 and the length of their projections was not obviously dependent upon rhombomere position.

Neurons in the third (and sometimes fourth) lateral stripe were unique in that they possessed both ipsilateral and contralateral axonal projections (figure 3.2C). This stripe possessed the largest diversity in cell type. All seven neurons reconstructed did possess both ipsilateral and contralateral axonal projections. However, out of these seven: two neurons were ascending on both sides of hindbrain (rhombomere 6 and 7); one was predominately ascending with a short descending process on either side (rhombomere 5); one was descending (rhombomere 4) with a short ascending process on either side; one was ascending on both sides with a descending projection on the ipsilateral side as well (rhombomere 3); one was descending on both sides with a short ascending projection on the contralateral side (rhombomere 4); and one was ascending ipsilaterally and descending contralaterally (rhombomere 7) (figure 3.3C). Slightly more neurons had longer ascending processes than descending ones (figure 3.3C). There was diversity in the lengths of the ascending and descending projections on both the ipsilateral and contralateral side. In rhombomere 4, two cells were labeled, and in both cases, the longer process was descending. In rhombomeres 5 and 6, the longer process of labeled cells was ascending (N=2). This distinction could be explained if these cells are projecting to the same rhombomeres (rhombomeres 4-6). Neurons in either more rostral or more caudal rhombomere segments possessed longer projections than those in intermediate rhombomeres, and most lateral stripe neurons projected only in within hindbrain.

Figure 3.3 Single cell projections within a stripe. In each panel, the ipsilateral and contralateral projections of neurons within a stripe are shown. Blue bars indicate ascending processes and red bars indicated descending processes. The x-axis is divided into each individual cell measured and the y-axis is projection length in microns. Below each plot, the rhombomere segment that each cell belonged to is shown. In **A**, medial stripe neurons are predominately ipsilateral ascending. Out of 15 cells, one was ipsilateral descending and one was contralateral descending. Both of these cells were positioned in rhombomere 7 and could represent cell types associated with spinal cord circuits. In **B**, middle stripe neurons are shown to never have ipsilateral axonal projections; instead, they project contralaterally and branch. When ascending and descending processes are different sizes, the ascending process tends to be longer. These cells were present in rhombomeres 6, 7, and 8. In **C**, lateral stripe neurons are shown to represent a diverse class, but all neurons maintain some combination of ipsilateral and contralateral projections. In rhombomeres 4, the longer process is descending, while in rhombomeres 5 and 6, the longer process is ascending. This distinction could be explained if these cells are projecting to the same rhombomeres (rhombomeres 4-6). In rhombomere 3, one example is shown with longer ascending processes. In rhombomere 7, two cells are shown. One is ipsilateral and contralateral ascending and the other is ipsilateral ascending and contralateral descending.

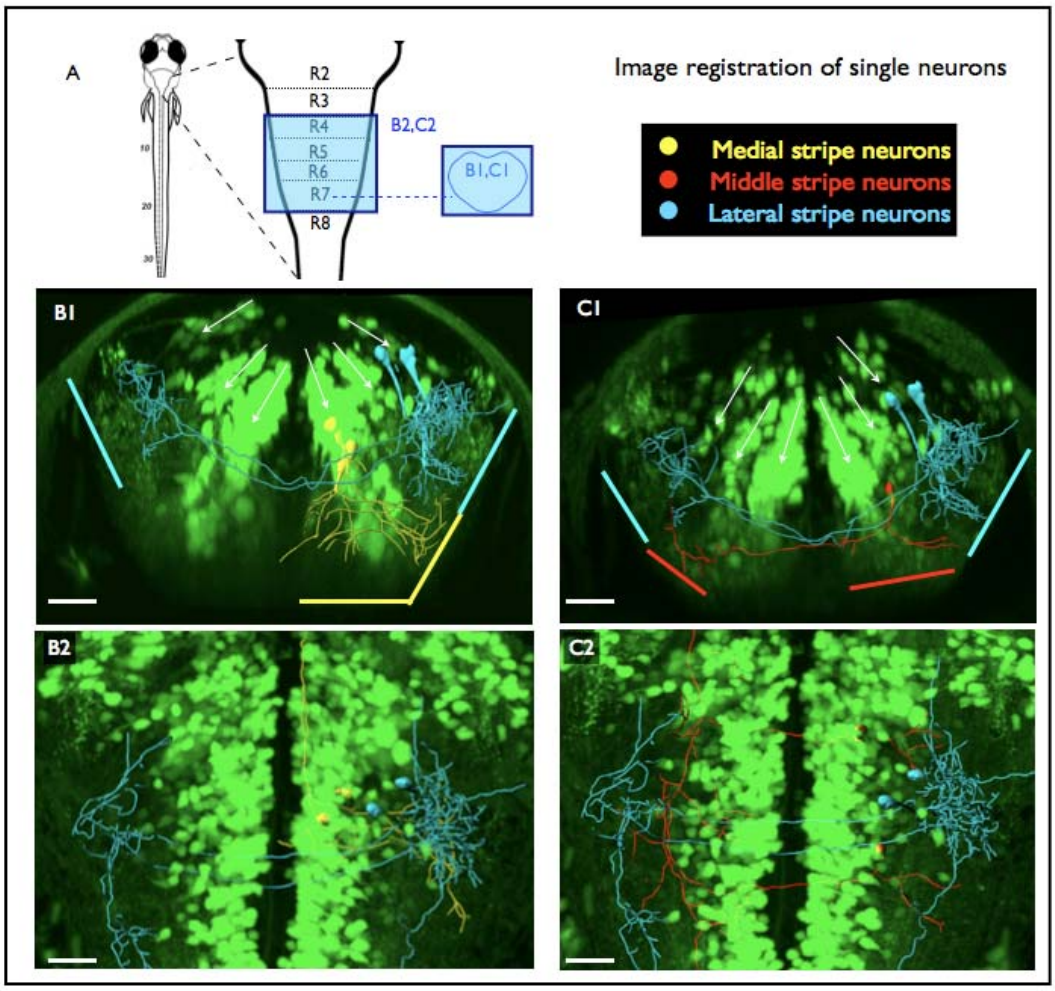

A
Medial stripe: Ipsilateral ascending
Ipsilateral
Contralateral
320
0
-120
Rhombomere 3
Rhombomere 4
Rhombomere 7
Rhombomere 8
B
Middle stripe: Contralateral branching
185
-185
Rhombomere 6
Rhombomere 7
Rhombomere 8
C
Lateral stripe:
Ipsilateral and contralateral branching
175
-175
Rhombomere 3
Rhombomere 4
Rhombomere 5
Rhombomere 6
Rhombomere 7
Descending
Ascending

Although these thirty single cells represent only a small fraction of the glycinergic neurons in hindbrain, they do reveal a general pattern consistent with the idea that cells within a stripe share axonal projection patterns. Medial stripe neurons are largely ipsilateral ascending, middle stripe neurons are contralateral branching, and lateral stripe neurons are both ipsilaterally and contralaterally projecting. Therefore, the stripes of glycinergic neurons may reflect regions of inhibitory neurons with different morphologies, some of which resemble classes of spinal neurons defined by transcription factor expression, morphology and functional roles ([1, 2, 17, 22, 172], see discussion). More evidence supporting the idea that stripes contain neurons with common morphological features is presented below.

Mediolateral segregation in stripe projections of single cells

To examine the projection patterns of cells located in different stripes of glycinergic neurons, we took advantage of the similarity of stripe patterning across different fish and used image registration of the GlyT2:GFP expression to align neurons from different fish together into one frame of reference. This allowed us to overlay reconstructions of single neurons that were in the same rhombomere in different fish to examine their relative projection patterns. The most dramatic difference we noted in the projection patterns in cross sectional view was between lateral stripe neurons and both medial and middle stripe neurons. Lateral stripe neurons projected laterally on both sides of hindbrain relative to both medial stripe neurons and middle stripe neurons (figure 3.4). Ipsilateral medial stripe projections were medial to all lateral stripe neurons within a segment where image registration was performed (Figure 3.4B, N=3 for medial stripe neurons and N=2 for lateral stripe neurons, all neurons were located in rhombomere 7). Both ipsilateral middle stripe processes (dendritic based upon the branching pattern and process tapering) and

Figure 3.4 Neurons within different stripes project to different mediolateral locations. **(A)** Orientation of images in this figure. **(B)** Legend for stripes: Medial stripe neurons are yellow, middle ones are red, and lateral stripe neurons are light blue. In both **(B)** and **(C)**, image registration was utilized with the GlyT2:GFP expression as a template for aligning neurons from different fish. **(B1-B2)** Cross section **(B1)** and dorsal **(B2)** view of lateral stripe (light blue) and medial stripe (yellow) neurons image registered to one frame of reference. Green expression is GlyT2:GFP expression from the fish used as a template for aligning all other fish. In cross section **(B1)**, Medial stripe neurons project more medially on the ipsilateral side than lateral stripe neurons. **(C1-C2)** Methods are similar as described above, but here middle stripe neurons are in red and lateral stripe neurons are in light blue. Middle stripe neurons project more medially on the contralateral side than lateral stripe neurons. This is clear in both cross section **(C1)** and dorsal view **(C2)**. Scale bars = 20 um.


A
R2
R3
R4
R5
R6
R7
R8
B2,C2
B1,C1
Image registration of single neurons
Medial stripe neurons
Middle stripe neurons
Lateral stripe neurons
B1
C1
B2
C2

contralateral middle stripe axonal projections were medially positioned relative to processes from lateral stripe neurons (Figure 3.4C, N=1 for middle stripe neurons and N=2 for medial stripe neurons, all neurons were located in rhombomere 7).

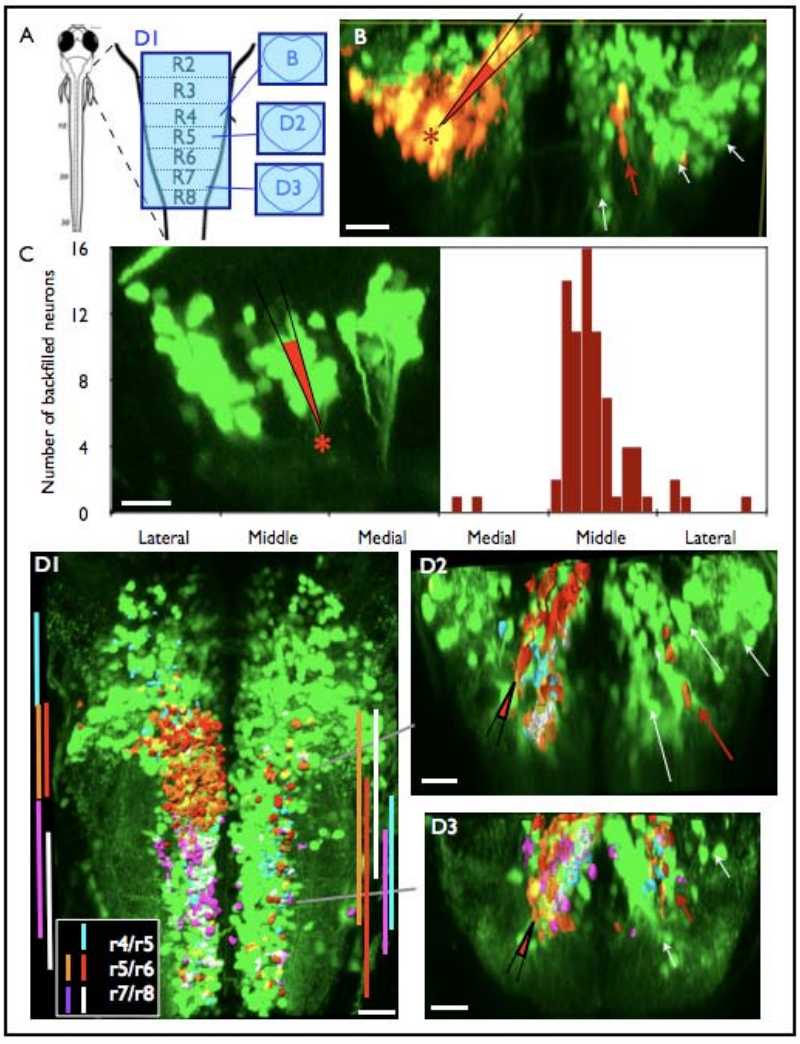
Backfilling confirms patterns observed on the single cell level

The single cell labeling pointed to both shared morphologies within stripes as well as differences in the mediolateral projection patterns of the different stripes. These examples are important because they provide detailed projection patterns, but they are limited by a small sample size from a large number of neurons within each stripe. To determine whether broader patterns of morphology and projection are present and consistent with our single cell labeling, we targeted different regions within or near stripes for backfilling by injection of rhodamine dextran (3000 molecular weight) and examined the distribution of backfilled neurons outside of the targeted region. We were interested in glycinergic neurons only, so filled cells were colocalized with the GlyT2:GFP expression to determine which cells were glycinergic neurons. Their position in the stripes was then easily determined.

In 10 GlyT2:GFP Nacre larval zebrafish (5 dpf), we backfilled in the ventral cell body area of a middle stripe in different rhombomeres and found that a large majority of labeled glycinergic neurons on the opposite side were located in the middle stripe (Figure 3.5A-C). Within these 10 fish backfilled in ventral middle stripe regions, 77 neurons were labeled on the contralateral side with 71 located in the middle stripe, 4 in the lateral stripe, and 2 in the medial stripe (figure 3.5C).

In figure 3.5D, an image registration of backfilled neurons from 5 different fish projecting to middle stripe regions is shown. To show the cell bodies within each fish, the surfaces were rendered in different colors for each, and, surfaces representing a

Figure 3.5 Middle stripe neurons project to contralateral middle stripe regions. **(A)** Orientation of images in this figure. **(B)** Example of middle stripe backfill. Cross section of rhodamine-dextran backfill in ventral region of middle stripe on left side. Glycine neurons colocalized with rhodamine dye on right project to middle stripe region on left. Arrows on right indicate different stripe regions. Red arrow indicates middle stripe that contained contralaterally backfilled neurons. **(C)** Number of neurons backfilled in 10 different fish at 5 dpf. Backfill in ventral middle stripe region illustrated on left side of plot. On the right, contralaterally backfilled neurons were predominately located in the middle stripe. Middle stripe neurons project contralaterally to middle stripe regions, neurons that project to middle stripe regions belong predominately to the middle stripe. **(D1-D3)** Dorsal view of backfills at different rostrocaudal regions of the brain indicate the patterning extends through hindbrain. Image registration using GlyT2:GFP expression in five 5 dpf fish backfilled on left side. Colored lines on left side indicate regions for each backfill. Legend indicates rhombomere regions for each of these backfills. On the right, colored bars indicate regions of contralaterally backfilled neurons for each backfill. Cell bodies are colored for each backfill as indicated in legend. Rostral **(D2)** and caudal **(D3)** cross sections of **(D1)** shows stripe position of ipsilateral and contralateral backfilled glycinergic neurons. In each cross section, backfills were targeted on the left within ventral middle stripe regions and contralaterally labeled neurons were mostly positioned in middle stripe (red arrow). White arrows indicate ventral areas of other stripes on right. Scale bars = 20 um.


A
D1
R2
R3
R4
R5
R6
R7
R8
B
D2
D3
B
C
Number of backfilled neurons
16
12
8
4
0
Lateral
Middle
Medial
Medial
Middle
Lateral
D1
D2
D3
r4/r5
r5/r6
r7/r8

large enough volume to be considered a cell body were considered labeled cells. The dorsal view in figure 3.5D1, shows that the backfilled neurons, on the right, are in a column throughout hindbrain suggesting an orderly arrangement of projections in different hindbrain segments. Backfills registered together spanned rhombomere 4 through rhombomere 8 for targeting and contralaterally labeled neurons spanned rhombomere 6 through rhombomere 8.

In dorsal view, the positions of these backfilled neurons within stripes are difficult to determine. In figures 3.5D2-3, cross section views at two different rostrocaudal points indicate that contralateral middle stripe neurons were, predominately, the contralaterally backfilled neurons in these 5 fish. One of the few exceptions is a contralaterally backfilled dorsal cell in the medial stripe in figure 3.5D3 (the two smaller surfaces medially present are too small to be considered labeled cells and are not present within stripe regions). This projection pattern of neurons in the middle stripe based on these backfills is consistent with the single cell labeling data, and indicates that the middle glycinergic stripe contains commissural neurons that project to more medial regions on the contralateral side of hindbrain.

To label neurons projecting to lateral stripe regions, we backfilled in ventral locations of the lateral glycinergic stripe where the neuropil is located. These backfills of lateral stripe regions labeled lateral stripe glycinergic neurons on the contralateral side (Figure 3.6B1-2). In three 4-5 day old fish, 20 neurons were backfilled on the contralateral side and all 20 neurons belonged to the lateral glycinergic stripe (figure 3.6D1). Dorsal views of three backfills in figures 3.6C1-3 show that this pattern is present from rhombomere 3 through rhombomere 7. Figure 3.6D2 shows a direct comparison of the distribution of labeled cells from the lateral and middle stripe labeling, showing the clear differences in the distribution of labeled neurons with middle stripe neurons projecting to middle stripe regions and lateral stripe neurons

Figure 3.6 Lateral stripe neurons project to contralateral lateral stripe regions in 5-6 dpf transgenic fish. **(A)** Orientation of images in this figure. **(B1-B2)** Example of lateral stripe backfills. Cross sections of rhodamine-dextran backfill in ventral region of lateral stripe on left side. Glycine neurons colocalized with rhodamine dye on right project to lateral stripe region on left. Arrows on right indicate different stripe regions. Red arrow indicates lateral stripe that contained contralaterally backfilled neurons. **(C1-C3)** Dorsal view of 3 lateral stripe backfills. Backfills in different rostrocaudal regions (r3 in **C1**, R4/r5 in **C2**, and r6/r7 in **C3**) indicate that lateral stripe neurons project laterally in multiple rhombomere segments. **(D1-D2)** Number of backfilled neuron in different fish. In **D1**, three fish were backfilled in ventral regions of the lateral stripe and only lateral stripe neurons on the contralateral side were labeled. In **D2**, lateral stripe backfilled are blue and middle stripe backfills are red, a clear segregation is visible where middle stripe neurons project more medially than lateral stripe neurons on the contralateral side. Scale bars = 20 um.

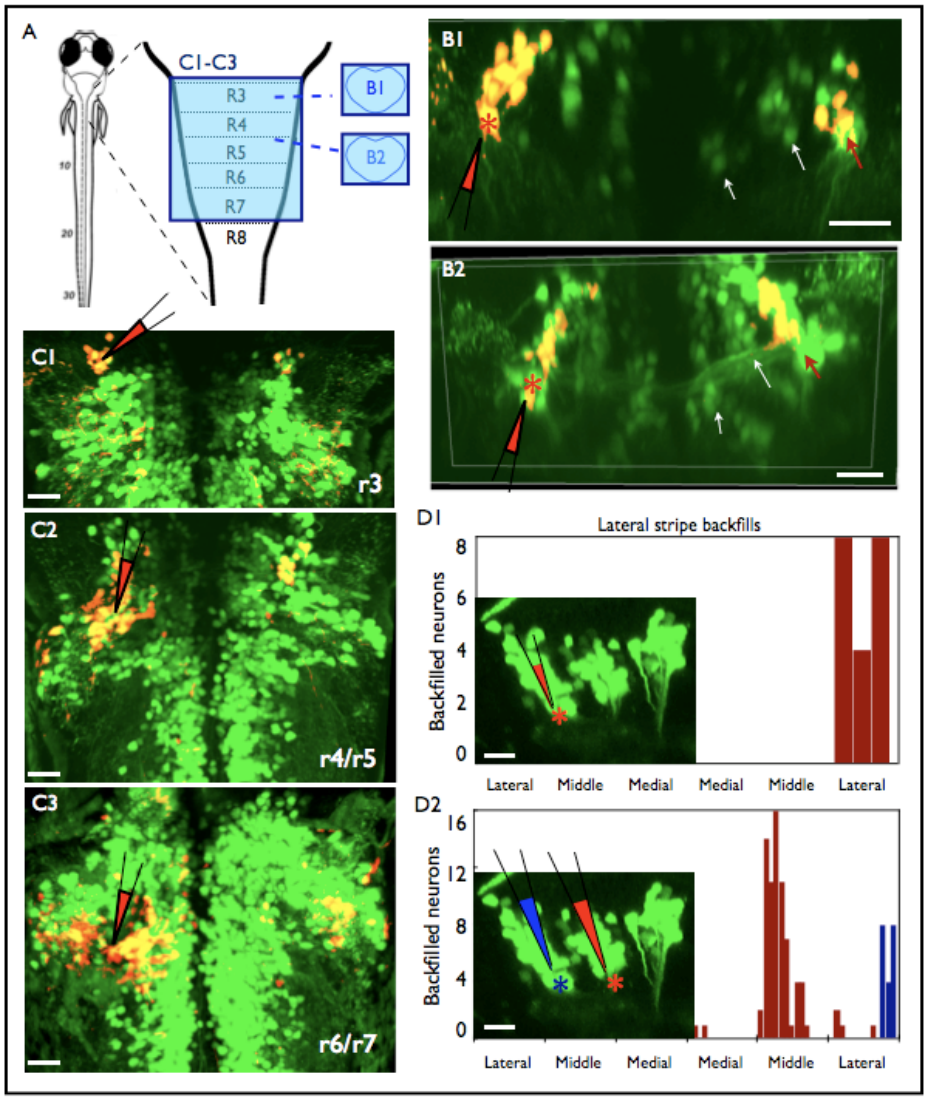
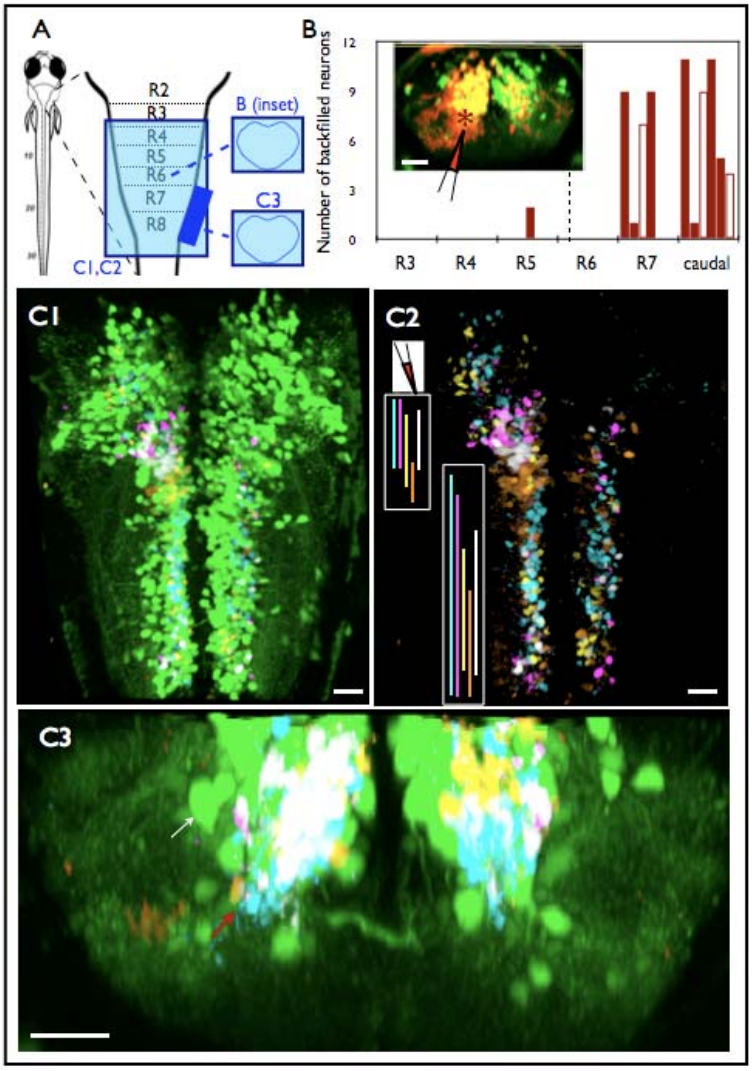

A
C1-C3
R3
R4
R5
R6
R7
R8
B1
B2
B1
B2
C1
r3
C2
r4/r5
C3
r6/r7
D1
Lateral stripe backfills
Backfilled neurons
0
2
4
6
8
Lateral
Middle
Medial
Medial
Middle
Lateral
D2
Backfilled neurons
0
4
8
12
16
Lateral
Middle
Medial
Medial
Middle
Lateral

Figure 3.7 Medial stripe neurons project to ipsilateral medial stripe regions. **(A)** Orientation of images in this figure. **(B)** In the inset image, the targeted region for backfills in r6 is shown. Medial stripe regions were targeted in a single rhombomere segment and neurons on the same side in other rhombomere segments were counted. Most neurons belonged to the medial stripe and were caudal to the backfill site, indicating that they are ipsilateral ascending neurons. Solid red bars indicate neurons in different rhombomere segments across four different fish with backfill sites at r6. White bars with a red outline are neurons from two fish with more caudal backfill sites. **(C1-C3)** In **C1**, a dorsal view of medial stripe backfills image registered into one frame of reference is shown. GlyT2:GFP expression from one fish is shown and glycinergic neurons colocalized with rhodamine from each backfill are shown in different colors. In **C2**, colocalized backfilled neurons from different fish are shown (same as in **C1**). Colored bars indicate backfilled neurons from different fish. The bars below the dye-filled pipette indicate the segment targeted. The bars in the right panel were observed in other segments than the targeted one. **(C3)** Cross section of caudal region of **C1**. Neurons in this cross section are located in caudal segments to the backfill site. Neurons on the left are ipsilateral to the backfill site and backfilled neurons are located in the medial stripe. Neurons on the opposite side were located in the middle stripe. Red arrow indicates medial stripe and white indicates middle stripe. The lateral stripe is dorsal and out of frame. Scale bars = 20 um.


A
R2
R3
R4
R5
R6
R7
R8
B (inset)
C3
C1,C2
B
Number of backfilled neurons
12
9
6
3
0
R3
R4
R5
R6
R7
caudal
C1
C2
C3

projecting to lateral stripe regions, both on the contralateral side of hindbrain.

Single cell labeling described earlier indicated that medial stripe neurons are ipsilateral ascending (figures 3.2 and 3.3). In the middle and lateral stripe backfills, only 2 of the 97 neurons contralaterally filled were located within the medial glycinergic stripe (figures 3.5 and 3.6). We looked at backfilled neurons on the ipsilateral side in six 4-5 dpf GlyT2:GFP Nacre fish in which ventral regions of the medial stripe were targeted for backfills. In these 6 fish, 69 neurons within the medial stripe were backfilled outside of the targeted region (figure 3.7B). Backfills were predominately located within rhombomere 6 in medial stripe regions and labeled more caudal medial stripe neurons on the ipsilateral side. Image registration of backfilled fish indicates that this pattern extends across multiple hindbrain segments (Figure 3.7C).

In figure 3.7C2, a dorsal view of this image registration from these medial backfilling experiments shows the colocalized, backfilled neurons from 5 different fish in different colors. Targeted backfilled regions for 5 different fish are shown by colored bars to the far left, and bars to the right of these indicate backfilled neurons outside of the targeted region. Backfills spanned regions from rhombomere 6 through rhombomere 7 and ipsilateral neurons labeled outside of the targeted region belong to the medial stripe in caudal regions (rhombomeres 7-8) indicating that they are ipsilaterally projecting ascending neurons. In figure 3.7C3, a cross section view indicates that ipsilaterally projecting neurons belong to the medial stripe and contralaterally projecting neurons belong to the middle stripe.

Thus, the backfills indicate that medial stripe neurons are ipsilaterally projecting with predominantly medial projections, middle stripe neurons are commissural, with projections in middle/medial regions of contralateral hindbrain, and lateral stripe neurons have commissural projections to lateral regions of hindbrain.

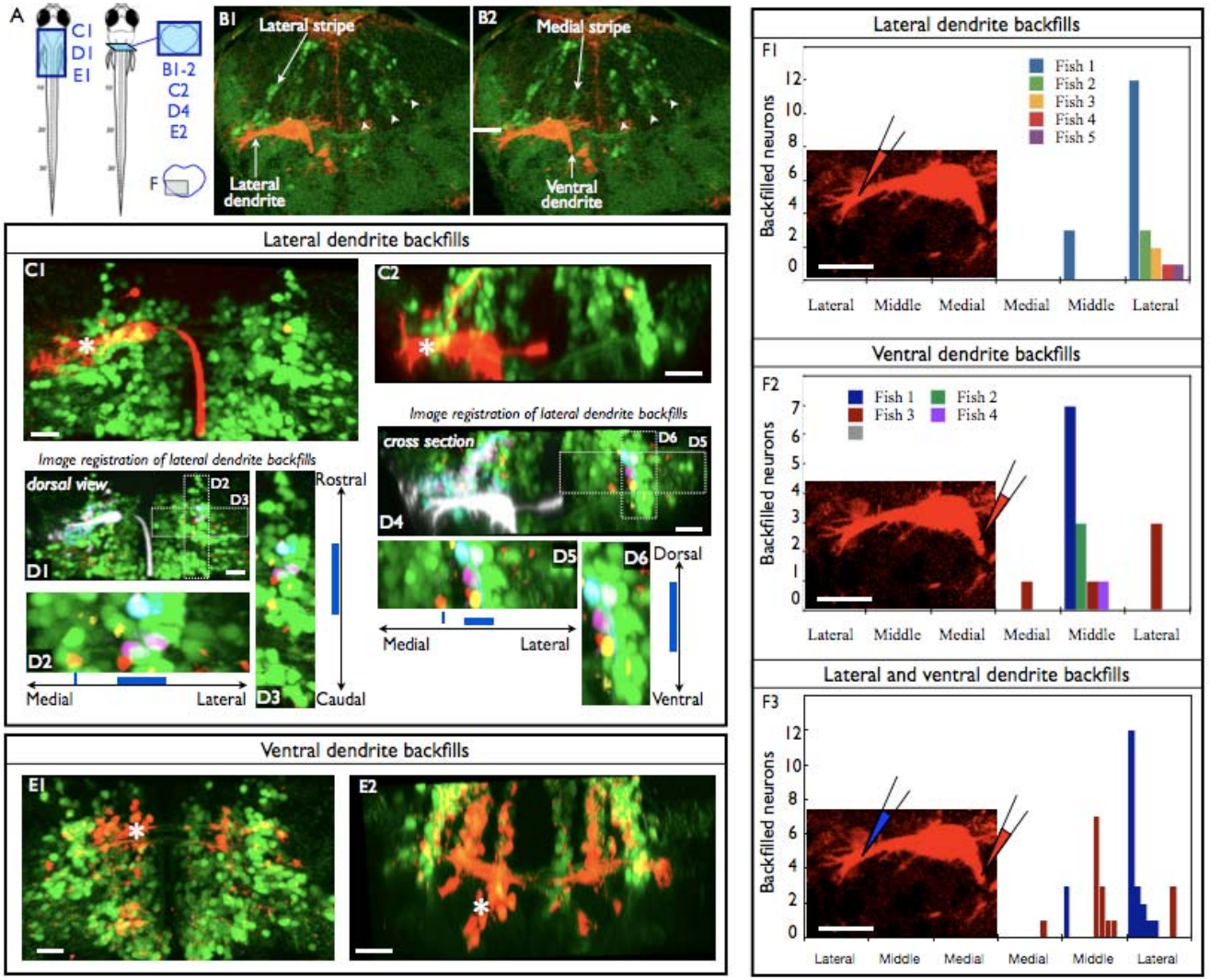
Taken together, the single cell and backfilling data support the conclusion that each glycinergic stripe at 5 dpf, after the larvae are free swimming, contains neurons that share key morphological features and broad projection patterns.

Mauthner dendrites have different mediolateral positions and receive projections from different glycinergic stripes

The previous data indicate that a broad patterning of stripes of glycinergic neurons and their projections extends across multiple rhombomeres. Thus far, however, our experiments have looked at broad patterning without consideration for how a particular circuit might map onto this pattern. Since there are circuits for many different behaviors in these regions where stripe patterning is present, the implication is that a similar orderly patterning of cell types and projections might underlie a variety of circuits for different behaviors. To investigate this possibility, we explored the organization of neurons involved in the escape circuit that project to the Mauthner neuron, which initiates the initial rapid turn of the escape. We used information from other studies of the network [143, 148, 149, 158, 174-205] to recognize specific cell types in this circuit.

The massive Mauthner cell extends mediolaterally across a large region of the hindbrain, with portions extending across all stripes (figure 3.8B). Its axon hillock is located medially in the ventral region of the medial stripe. In goldfish, glycinergic interneurons project to the axon cap of the Mauthner cell to prevent the Mauthner cell from firing a second time and initiating a second, energetically costly movement [148, 149, 176, 182, 184-187, 193, 197, 205]. Inhibitory interneurons are also known to project to regions of the cell body and onto the lateral dendrite [143, 176-178, 187, 196, 197, 206], which extends out to the ventral region of the lateral glycine stripe

Figure 3.8 Glycinergic stripes map onto the Mauthner escape circuit. **(A)** Orientation of images in this figure. **(B)** Cross section of fixed GlyT2:GFP transgenic fish with Mauthner cell labeled in red. Arrowheads indicate stripes on the contralateral side of the Mauthner cell. The lateral dendrite of the Mauthner cell is located in the ventral region of the lateral stripe (**B1**). The ventral dendrite is located in the ventral region of the medial stripe (**B2**). **(C,D,F)** Backfills of lateral and ventral dendrite regions. Both sets of backfills were performed and 5 dpf and confocal images were collected at 6 dpf. Asterisks in images indicate targeted backfill region. **(C)** Lateral dendrite backfills. **C1** shows a dorsal view of a GlyT2:GFP Nacre transgenic fish where the lateral dendrite of the Mauthner cell was targeted. The Mauthner cell was labeled with dye in this case and a cell on the contralateral side is shown to be backfilled as well (red). **C2** shows a cross section of this same backfill. The Mauthner cell is on the left and the backfilled neuron on the contralateral side is a glycinergic neuron in the lateral stripe. In **F1**, backfilled glycinergic neurons from five fish are shown. The majority of contralateral backfilled glycinergic neurons were located in the lateral stripe. **(D)** Image registration of lateral stripe neurons projecting to the lateral dendrite. **(D1)** Dorsal view of GlyT2:GFP expression with different backfills targeting the lateral dendrite registered together using GlyT2:GFP expression. **D2** and **D3** show magnified mediolateral and rostrocaudal regions of **D1**. In each case, neurons form a local cluster within the lateral stripe. Blue bars below (**D2**) and to the right (**D3**) indicate the extent of cell body positions mediolaterally and rostrocaudally respectively. A small vertical bar below **D2** shows one more medially positioned middle stripe cell not within the cluster. **(D4)** Cross section view of image registration. **D5** and **D6** show magnified mediolateral and dorsoventral regions of **D4**. In each case, neurons form a local cluster within the lateral stripe. Blue bars below (**D5**) and to the right (**D6**) indicate extent of cell body positions mediolaterally and dorsoventrally. A small vertical bar below **D5** shows one ventrally positioned middle stripe cell not within the cluster (same cell as in **D2**). **(E)** Ventral dendrite backfills. **E1** shows a dorsal view of a GlyT2:GFP Nacre transgenic fish where the ventral dendrite of the Mauthner cell was targeted. The red cells in this case are backfilled neurons that were colocalized with GFP (glycinergic neurons). **E2** shows a cross section of this same backfill. Targeting of the Mauthner cell is shown on the left and backfilled neurons on the right side predominately belonged to the middle stripe. In **F2**, backfilled glycinergic neurons from four fish are shown. The majority of labeled glycinergic neurons that projected to the ventral dendrite region were located in the middle stripe. In **F3**, backfilled neurons from lateral dendrite backfills are shown in blue and for ventral dendrite in red. There is a clear segregation in the projections of middle and lateral stripe neurons with middle stripe neurons predominately projecting to ventral dendrite regions on the contralateral side and lateral stripe neurons projection to lateral dendrite regions on the contralateral side. Medial stripe neurons were previously shown to be ipsilateral and only one ventral (neuropil region) medial stripe neuron was found to contralaterally project in these 9 backfilled fish. Scale bars = 20 um.


A
C1
D1
E1
B1-2
C2
D4
E2
F
B1
Lateral stripe
Lateral dendrite
B2
Medial stripe
Ventral dendrite
Lateral dendrite backfills
C1
C2
Image registration of lateral dendrite backfills
dorsal view
D1
D2
D3
Rostral
Caudal
Medial
Lateral
Image registration of lateral dendrite backfills
cross section
D4
D5
D6
Medial
Lateral
Dorsal
Ventral
Ventral dendrite backfills
E1
E2
Lateral dendrite backfills
F1
Backfilled neurons
Fish 1
Fish 2
Fish 3
Fish 4
Fish 5
Lateral
Middle
Medial
Medial
Middle
Lateral
Ventral dendrite backfills
F2
Backfilled neurons
Fish 1
Fish 2
Fish 3
Fish 4
Lateral
Middle
Medial
Medial
Middle
Lateral
Lateral and ventral dendrite backfills
F3
Backfilled neurons
Lateral
Middle
Medial
Medial
Middle
Lateral

(figure 3.8B1). In goldfish, these neurons are involved in determining whether the Mauthner cell fires in response to a sensory stimulus [143, 176, 177, 197]. The ventral dendrite is medially positioned and extends ventrally from the soma (figure 3.8B2) and receives visual information in goldfish [201]. From the broad mediolateral patterning of projections for neurons belonging to different stripes of glycinergic neurons, we postulated that lateral stripe neurons would project to the lateral dendrite, middle stripe neurons would project contralaterally to more medial regions of the Mauthner cell, and medial stripe neurons would project ipsilaterally to the axon hillock.

We explored the pattern of projections of neurons within the glycinergic stripes to different regions of the Mauthner cell by injections of rhodamine dextran directly and very locally onto portions of the cell body or dendrites. Backfilled glycinergic neurons were found by colocalization of GFP and rhodamine dextran. When the lateral dendrite, which lies in the region of the lateral glycinergic stripe, was targeted for backfills in 5 dpf fish and examined at 6 dpf, labeled neurons on the contralateral side consisted predominantly of neurons in the lateral glycinergic stripe (figure 3.8C1-2,F1, N=5). A dorsal view of a fish targeted for a lateral dendrite backfill on the left side is shown in figure 3.8C1. A colocalized glycinergic neuron is present on the right side. In cross section, the glycinergic neuron is shown to reside within the lateral stripe (figure 3.8C2). Backfills from 5 different fish labeled a total of 22 neurons contralaterally and 19 of the 22 were located in the lateral stripe and 3 were located within the middle stripe (figure 3.8F1). All 3 middle stripe neurons were located in 1 fish and might reflect an injection error. These commissural lateral stripe neurons are most likely the feedforward inhibitory neurons described in prior studies of escape networks [143, 158, 176-178, 187, 196, 197].

In figure 3.8D1-6, an image registration of those fish with backfills near the lateral dendrite reveals that lateral dendrite projecting neurons are clustered within the lateral glycinergic stripe. In figure 3.8D1, a dorsal view is shown, and in figures 3.8D2 and 3.8D3 a magnification across mediolateral and rostrocaudal regions indicates that these neurons are clustered in both directions (blue bars below panel 3.8D2 and to the right of 3.8D3 show mediolateral and rostrocaudal regions of the neurons and a small horizontal bar below 3.8D2 indicates the position of one middle stripe neuron not within the cluster). This indicates that these neurons are rostrocaudally clustered and present only in rhombomeres 4 and 5. In figure 3.8D4, a cross section indicates that these cells form a cluster of relatively ventrally positioned cells within the lateral stripe. In figure 3.8D5, a magnification of the mediolateral region in cross section shows the mediolateral clustering (see blue bar below this image), and in figure 3.8D6, a magnification of the dorsoventral extent indicates that these neurons are dorsoventrally clustered within the lateral stripe (see blue bar to right of image). Figure 3.8D6 also reveals that these neurons are potentially clustered in the ventromedial region of the lateral stripe. This suggests that a local ventromedial portion of the lateral stripe might possess glycinergic neurons projecting to the lateral dendrite of the Mauthner neuron on the contralateral side.

We next targeted ventral dendrite regions for backfills and again looked for contralaterally labeled glycinergic neurons that might project to this region. In figure 3.8E1, a dorsal view of a ventral dendrite backfill on the left side reveals contralaterally labeled neurons localized to rhombomere 4. In figure 3.8E2, a cross section shows that a large number of neurons are labeled within the middle stripe and 1 neuron is labeled in both the medial and lateral stripe within ventral stripe regions. Since the ventral dendrite is medially positioned, targeted backfills might disrupt axons of passage and label more laterally projecting neurons as well. We found lateral

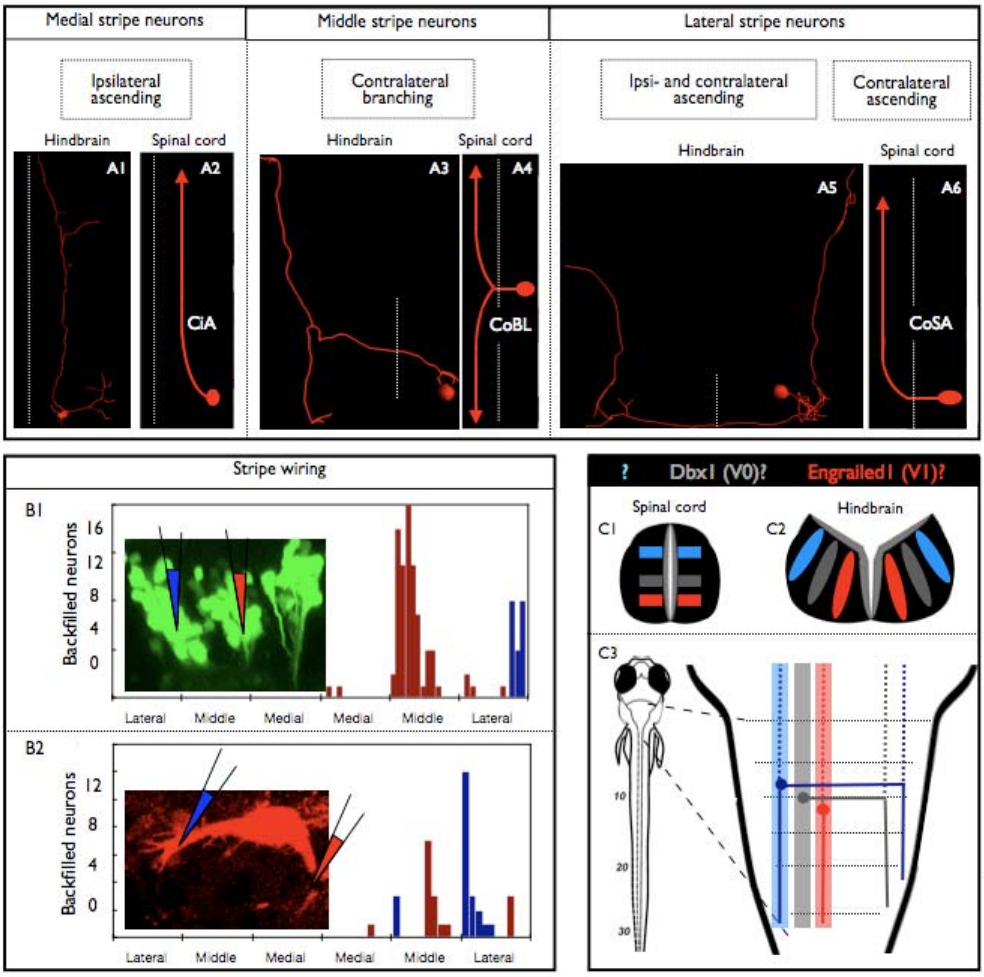
and medial stripe neurons to be labeled in only one of 4 fish. In 4 fish, 16 neurons were labeled on the contralateral side. Of these 16 neurons, 12 were positioned within the middle stripe, 3 within the lateral stripe and 1 was located in the middle stripe (figure 3.8F2). It is most likely that neurons from the middle stripe project to the ventral dendrite, a medial/middle region of the Mauthner cell, on the contralateral side. We postulate that these neurons might play a role in the gating of visual input onto the Mauthner cell [175, 195, 201]. The projections to the region of the ventral dendrite of the Mauthner cell are again consistent with the broad organization of mediolateral projection patterns of neurons within glycinergic stripes.

In sum, glycinergic interneurons that we presume are involved in aspects of the escape circuit follow the broad organization identified here for stripes of glycinergic interneurons in hindbrain. Lateral stripe neurons were found to project to the contralateral lateral dendrite, and middle stripe neurons were found to project to the more medially located ventral dendrite. Thus, neurons in a known network might superimpose upon the broad stripe-like patterning of organization in hindbrain. If other networks do the same, this suggests that many behaviors might be built from a simple, underlying cellular patterning in hindbrain.

Discussion

In this study, we show that stripes of glycinergic interneurons exist, spanning multiple hindbrain segments and defining an orderly patterning of interneurons by morphological type and projection. Each stripe contains cells with shared morphological features: medial stripe neurons are ipsilateral ascending, middle stripe neurons are contralateral branching, and lateral stripe neurons are ipsilateral and contralaterally projecting (figure 3.9A). These stripes project in orderly ways in the

Figure 3.9 Summary of mapping of cell types and wiring within glycinergic stripes **(A)** Examples of neurons showing the morphology that is associated with neurons within the medial, middle, and lateral stripes. Medial stripe neurons are ipsilateral ascending (**A1**) and resemble CiA neurons in spinal cord (**A2**). These spinal neurons have previously been shown to express the Engrailed1 transcription factor and correspond to V1 interneurons in mice. Middle stripe neurons are contralateral branching (**A3**) and resemble CoBL neurons within spinal cord (**A4**). It is possible that these neurons express the Dbx1 transcription factor and are related to V0 inhibitory neurons described in mice. Lateral stripe neurons are ipsilaterally and contralaterally projecting in hindbrain (**A5**). The closest morphological class in spinal cord might be the CoSA interneuron class (**A6**), but these neurons only project contralaterally. A transcription factor identity for these cells has yet to be determined. **(B)** Summary of the mediolateral segregation in stripe projections shown for both general stripe wiring (**B1**) and within the Mauthner escape circuit (**B2**). In both cases, middle and lateral stripe neurons project contralaterally, but lateral stripe neurons project laterally and middle stripe neurons project more medially. **(C)** Schematic showing the general patterning found to be unique for neurons within different stripes and how this might relate to spinal patterning. In **C1**, bands of dorsoventrally segregated progenitor zones that give rise to distinct transcription factor class are shown. In the hindbrain, it is possible that this spinal dorsoventral transcription factor map is a lateral to medial map with each stripe containing neurons of a unique transcription factor type (**C2**). These transcription factor types are expressed in neurons of distinct morphologies and this corresponds with the morphologies described here (**C3**). Along with the morphology of the neuron differing between stripes, the mediolateral projection patterning differs as well, setting up a wiring template across multiple hindbrain segments.


Medial stripe neurons
Middle stripe neurons
Lateral stripe neurons
Ipsilateral ascending
Contralateral branching
Ipsi- and contralateral ascending
Contralateral ascending
Hindbrain
Spinal cord
A1
A2
CiA
A3
A4
CoBL
A5
A6
CoSA
Stripe wiring
B1
Backfilled neurons
16
12
8
4
0
Lateral
Middle
Medial
B2
? Dbx1 (V0)? Engrailed1 (V1)?
C1
C2
C3
10
20
30

hindbrain. Medial and middle stripes project medially and lateral stripes project more laterally on both sides of the hindbrain, both broadly, and within the Mauthner escape circuit (figure 3.9B). Since these stripes represent a patterning of all glycinergic interneurons, the implication is that circuits may be built via a simple set of component elements wired into networks via an early, possibly topographic, wiring template (figure 3.9C).

Morphological classes in hindbrain resemble spinal neuron types

Some of the neuronal types in hindbrain have morphological features similar to types in spinal cord. Neurons within the medial glycine stripe are predominately ipsilateral and ascending (dorsal view of one neuron shown figure 3.9A1). This type of neuron in hindbrain closely matches the morphology of CiA interneurons in spinal cord ([16], illustrated in figure 3.9A2). These CiA interneurons have been identified as Engrailed1 interneurons and were shown to participate in sensory gating and in the coordination of fast swimming in zebrafish and xenopus [17, 22]. In mice, Engrailed interneurons (V1) maintain the same morphological features and functional roles [2]. Understanding the role that medial stripe neurons play in hindbrain neural circuits will indicate whether they share common functional roles with Engrailed1 neurons in spinal cord.

Middle stripe neurons were consistently found to be contralateral and branching (figure 3.9A3). This morphology closely resembles CoBL neurons within the spinal cord ([16], illustrated in figure 3.9A4). CoBLs are also contralateral bifurcating neurons of glycinergic type and have been shown to participate in the coordination of swimming and struggling within zebrafish [172]. In mice, contralaterally projecting inhibitory neurons involved in the coordination of motor output are named V0 interneurons and express the Dbx transcription factor [1]. We

suggest that CoBL interneurons in zebrafish spinal cord belong to the V0 inhibitory interneuron class since they are both contralaterally projecting inhibitory neurons involved in motor coordination in the spinal cord of mice and zebrafish respectively. If this is the case, then the middle stripe might contain neurons with similarities to inhibitory V0 spinal neurons and express the Dbx transcription factor. In chapter 5, we discuss the expression of the Dbx1 transcription factor, a homologue of the mouse Dbx transcription factor, in larval zebrafish. It is expressed in a middle glutamatergic stripe and in the region of the middle glycinergic stripe, so this relationship between middle glycinergic stripe neurons in hindbrain and V0 neurons in spinal cord is supported by the transcription factor expression pattern.

The lateral stripe of glycinergic neurons was found to contain the most diverse collection of morphological phenotypes of the 3 glycinergic stripes. However, all neurons showed some combination of ipsilateral and contralateral projections in single cell labeling experiments (with indications of contralateral projections also shown in lateral stripe backfills). Although no known spinal neuron type exactly matches this morphology, other work in the lab has shown that some neurons within the lateral stripe in hindbrain are contralaterally projecting and ascending (M. Koyoma, unpublished). A cell type within spinal cord has been previously described with this morphology; CoSA interneurons have been shown to be contralaterally projecting and ascending ([16], see illustration in figure 3.9A6) and to be active during escape, swimming, and struggling [172].

Within spinal cord, no evidence of a segregation of CiA (Engrailed1), CoBL, and CoSA interneurons has been described in swimming larvae, although there is segregation of the precursor zones that give rise to similar cell types in vertebrate spinal cords [3]. We have found, in contrast, that neurons with morphology similar to these spinal types are segregated into stripes in the hindbrain. Medial stripe neurons

resemble CiA neurons, middle stripe neurons resemble CoBLs, and lateral stripe neurons are heterogeneous, but some might correspond to CoSAs. This relatively simple organization is present even after the larva is swimming and engaging in many of the behaviors involving the hindbrain. This suggests that circuits are built and functional at a time when the neurons are arranged in a simple, orderly pattern in hindbrain.

Heterogeneity of cell types within a stripe

Although the broad features of morphology were shared by neurons within each of the three stripes, there was some variation, and a few exceptions to the general rules were present in the lateral and the most medial stripes. Lateral stripe neurons were found to be a combination of ascending/descending of both ipsilateral and contralateral types. The heterogeneity within the lateral stripe might indicate functional heterogeneity as well. Determining the functional role of lateral stripe neurons and whether different functional classes can be defined within the lateral stripe would indicate whether this is the case.

Morphological heterogeneity could also arise from neurons in different rostrocaudal positions projecting to the same region; for instance, the descending axon of a rostral neuron and the ascending axon of a caudal neuron might project to the same region within hindbrain and be functionally similar despite the difference in ascending/descending projections. Neural circuits in hindbrain are known to be rostrocaudally localized, unlike in the spinal cord, so it might be important for neurons in different rhombomeres to converge on the same region within hindbrain. At this point we cannot determine whether the differences in lateral stripe neurons represent functional differences or not.

Within the medial stripe, out of 15 neurons, 2 did not follow the ipsilateral ascending morphology of the other 13. In one case, a medial stripe neuron was ipsilateral descending. In a second case, a medial stripe neuron projected contralaterally and descended - the opposite morphology of most medial stripe neurons. In both cases, the neurons were positioned in caudal rhombomeres. These are rare, but we cannot rule out the possibility that both of these cells define cell types present either within rhombomere 7/8 exclusively, or present throughout hindbrain. We also did find rare cases where medial stripe neurons in caudal rhombomeres projected contralaterally in our backfill experiments that were present in caudal rhombomeres (1 cell in each of 2 different fish out of the 10 backfilled fish). While some fraction of neurons within each stripe might not match the general patterning described here, given the rarity of finding these cell morphologies that differ from the primary one for each stripe when we performed backfilling experiments, these neurons must either be very rare or possess fine processes that were not labeled.

Stripes in hindbrain represent a regular arrangement of cell types similar to progenitor zones described in spinal cord.

We have drawn parallels between spinal cord Engrailed1 (V1) neurons and medial stripe hindbrain neurons as well as Dbx (V0) neurons and middle stripe hindbrain neurons (figure 3.9A1-4). In figure 3.9C1, the regions of progenitor zones of these two types are shown within spinal cord [3]. In hindbrain, this patterning persists in larval zebrafish that are freely swimming in the form of stripes of differentiated neurons. In figure 3.9C2, the stripes are colored for their potential transcription factor expression based on the morphology of neurons within each stripe. The dorsoventral patterning of spinal cord progenitor zones (panel 3.9C1) may be present in hindbrain in the form of mediolaterally-segregated stripes (panel 3.9C2).

Within the hindbrain, we showed that stripes of glycinergic neurons have systematic patterns of mediolateral projections. Medial stripe neurons project medially on the ipsilateral side (figure 3.7). Lateral and middle stripe neurons both project contralaterally, but lateral stripe neurons project more laterally than middle stripe neurons (figure 3.6, panel 3.9B1). The combination of unique morphology (figure 3.9A) and mediolateral projection (figure 3.9B1) for each stripe may represent an orderly wiring pattern for inhibitory neurons in hindbrain (figure 3.9C3). Perhaps, for example, ipsilateral ascending inhibitory neurons projecting medially, involved in any neural circuit in hindbrain, are located within the medial stripe. Also, if a neuron within a hindbrain circuit either excited or inhibited this cell type, then it would project to medial regions as well. Therefore, projections from and to these stripes of glycinergic neurons could form a simple wiring template that might underlie neural circuits of very different types.

Broad patterning is present in a specific neural circuit

To understand how the broad wiring pattern in hindbrain might superimpose on a particular neural circuit, we examined projections of glycinergic interneurons projecting near the Mauthner cell. We found that lateral stripe neurons projected to the lateral dendrite of the contralateral Mauthner cell and middle stripe neurons projected to the medial ventral dendrite of the contralateral Mauthner cell (figure 3.9E2). This difference in projections onto the Mauthner cell suggests a difference in functional roles for different stripes within the escape circuit. It is possible that lateral stripe neurons play a role in feedforward inhibition since they project to the lateral dendrite, a region of the Mauthner cell where direct inputs from the auditory (VIIIth) nerve reside [200]. In goldfish, commissural inhibitory neurons driven by auditory inputs provide inhibition of both the ipsilateral and contralateral Mauthner neurons

[143, 158, 176-178, 187, 196, 197]. The backfilled lateral stripe neurons are most probably this cell type in larval zebrafish.

Middle stripe neurons in ventral stripe regions that project to the ventral dendrite of the Mauthner cell might play a role in the gating of visual information onto the Mauthner cell [175, 195, 201] since it receives input from the optic tectum onto its ventral dendrite in goldfish [201]. If so, we would expect that the neurons would be driven by visual input. Although we did not try targeting backfills for ipsilaterally projecting neurons since the angle of approach to the Mauthner cell might influence which ipsilateral cells were labeled, we expect that some medial stripe neurons might form the known ipsilateral feedback inhibitory neurons in goldfish that receive excitation from neurons driven by the Mauthner cell and act to block repetitive firing of the Mauthner neuron. Medial glycinergic neurons that provide feedback inhibition on the Mauthner cell have been found in pair-patch clamp experiments in our lab (M. Koyama, unpublished).

In sum, the stripes of glycinergic neurons contain neurons that could project to unique locations on or near the Mauthner cell. The location of their projections could determine their function within the escape circuit. These observations suggest how specific circuits might be constructed by components drawn from portions of the orderly stripelike patterning in hindbrain.

Stripes represent a novel organization of interneurons in hindbrain (relationship to earlier work)

Stripes of glycinergic interneurons represent unique morphological cell types with mediolateral segregation of their wiring. These stripes form continuous bands that extend rostrocaudally from rhombomere 3 through 8. This continuous rostrocaudal patterning differs from previous descriptions of hindbrain organization,

where distinct cell types are present in different rhombomere segments and provide unique identities to each segment (reviewed in [51]). For instance, reticulospinal neurons and cranial motor neuron pools are present in specific rhombomere segments[51]. There is, however, a serial repetition of these cell types. For instance, the Mauthner cell is present in rhombomere 4, but homologues of the Mauthner cell with similar morphology and function (Mid2cm and Mid3cm) are present in rhombomeres 5 and 6 [148].

Previous studies have indicated a serial repetition of cell types within the hindbrain of other vertebrates [58, 111, 151]. Here, we have shown that serially repeated interneurons are arrayed into stripes that extend both rostrocaudally and dorsoventrally. Given the conservation of cellular organization in the rhombomeres and spinal cord of vertebrates, we expect that the pattern we describe here will be present in other vertebrates as well, where it may form the developmental substrate for the many networks that arise in hindbrain.

In many vertebrates, a banded patterning of transcription factors has been described that extends rostrocaudally in hindbrain [99, 100, 108-134]. However, the significance of these bands for the structural and functional organization of hindbrain has not been clear. A striking band of cholinergic neurons has been described that forms an arcuate pattern from hindbrain to midbrain [207], but the projection patterns or functional identities of these cells are unknown. Another study has linked the projection patterns of a population of neurons to bands of transcription factors of the Lim homeodomain class [111]. This study focused on the specialized reticulospinal neurons that define a small population of hindbrain neurons and did not describe similarities beyond morphology of the neurons that neurons in the transcription factor bands might share. Here we show that all glycinergic neurons form a patterning of

cell types arrayed into stripes based upon both morphology and projection patterns and we place the pattern in the context of a known neural network.

Predictions about the organization of hindbrain neural circuits

Since these stripes represent a novel patterning of interneurons in hindbrain, there appears to be a previously undescribed orderly patterning of interneurons that extends rostrocaudally throughout most of hindbrain. Within the Mauthner escape circuit, neurons with different functional roles are present in different stripes. We suggest that neurons performing different functions within other neural circuits, such as the oculomotor integrator, acoustic nuclei, the equivalent of the pre-Botzinger circuit, and many others, might be drawn from particular stripes. For example, a circuit with reciprocal inhibition between the two sides of the brain might involve commissural glycinergic neurons arising from the middle hindbrain stripe. This stripe might then contribute the reciprocal inhibitory components to many different hindbrain networks.

Within the Mauthner escape circuit, most backfilled neurons were confined to regions within rhombomere 4-6 and were usually the ventral cells in the stripes in these areas (see figure 3.8). In chapters 2 and 4, we show that ventral stripe neurons are the oldest ones. This suggests that old cells might participate in fast circuits, as they do in spinal cord [18, 208]. We predict that some ventral cells in the stripes in other regions might play a role in other aspects of the coordination of escape behaviors such as eye movements, pectoral fin flexion, and jaw closure, or could participate in networks for other faster motor behaviors or higher threshold movements.

Since the oldest neurons within hindbrain might form neural circuits involved in the fastest motor behaviors, we suggest that younger neurons might be involved in slower movements such as the coordination of swimming. If this is the case, then a

functional topography of interneurons within and across classes of interneurons might be present as is the case in spinal cord [19]. Two levels of organization could then be present. Across stripes, a stripe might contain a neuron type with a particular functional role in different hindbrain circuits. Within a stripe, the location of the contributing neurons along the axis of the stripe could reflect the recruitment threshold of that neuron, and, in turn its contribution to fast or slow or weak or strong movements.

Although the type of recruitment described in spinal networks [18, 19] has not been described within sensory processing centers in the brain, we suggest that an age-related recruitment could also be present in the form of an orderly recruitment of sensory neurons based upon their location within a stripe. For instance, the lateral line sensory system produces or alters movements based upon sensing water flow along the body. It is possible that the oldest neurons within the lateral line circuits are involved in sensory control or initiation of fast movements and younger ones are involved in controlling slower movements or initiating weaker, more local motor responses.

Because cell types within the stripes share properties of cell types within the spinal cord, hindbrain neural circuits might represent a larger and more specialized form of the spinal cord, but with much the same core wiring patterns arising from a simple toolbox of cells. These may be more evident in hindbrain because of the organized proliferation of cells in the brain. The hindbrain may thus offer a fruitful avenue for exploring the structural and functional principles that underlie the development of a variety of different networks from a simple early topography. The most immediate question is whether the orderly age and position related recruitment seen in spinal cord is also present within the hindbrain stripes, as suggested by the ventral location of the oldest, and fastest, Mauthner escape network.

Methods:

Neuronal tracing

Neurons were traced using the filament reconstruction feature provided in the Imaris software package (Bitplane). For image stacks with low noise levels, automated reconstructions were performed choosing a diameter for a starting point (cell body) and a minimum diameter for end points along projections from the starting point. A threshold was used to adjust the number of start and end points for optimal tracing of single neurons. In cases where noise levels were high, the autodepth feature of the Imaris filament software was used to trace neurons by hand in two-dimensions and allowing the program to determine the depth of the tracing. Reconstructions were constantly verified to be accurate representations of the labeled cell and were used in this study since fine processes were hard to depict in two-dimension projections of confocal image stacks.

Stochastic labeling

Stochastic labeling was performed as described previously in [209]. Briefly, single cell embryos were positioned in wells within an agar plate (1.5% DNA-grade agarose in 10% Hanks with methylene blue). To label early born neurons, injections of small volumes of constructs (30ng/uL for each construct when injecting two simultaneously) were performed into single cells within 45 minutes of fertilization when eggs remained at the single cell stage. To label later differentiating neurons, embryos were injected at approximately the 1000 cell stage into the yolk. Embryos were screened using a Leica fluorescent dissecting scope at either 3 or 4 dpf for embryos with only 1-3 cells labeled in the brain. Fish were imaged using a confocal microscope at 5 dpf.

Image registration

To compare the positions of neurons within two different confocal stacks, either in the same fish for Kaede photoconversion experiments, or in different fish at the same day, the general patterning of neurons from each confocal stack was used to perform an image registration. Either the position of all neurons (Huc:Kaede) or a subset (GlyT2:GFP) that were present in a single channel in each image were registered together using the spm routine in Matlab. Registration was performed as a rigid body rotation and no rescaling or morphing of either image was necessary to align two confocal stacks. Image registration was considered successful when alignment of the two channels reached a criterion of less than 10 um error in all directions. In most cases, image registration was well within this limit. To generate a single image of both confocal stacks, the image registered stacks were added as channels to the reference image using Imaris software.

Targeted backfills

Fish were anaesthetized in 0.02% MS222 in 10% Hanks solution and embedded in a small Petri dish in 1.5% low melting point agar in 10% Hanks solution. Anesthetic was pipetted into the dish after 1-2 minutes, and the embedded fish was positioned on an Olympus fluorescent dissecting scope. For stripe backfills, GFP fluorescence was used to determine backfill regions, and for Mauthner cell backfills, the DIC image of the Mauthner cell was used to determine regions on the Mauthner cell body for local backfills. A glass pipette filled with 5 um or rhodamine dextran 3000MW in 20% extracellular solution was positioned above the fish and diagonally oriented to pierce through the skin and minimize movements of cells within the brain. When the targeted region was reached, a 20-40V pulse for 20 seconds was applied as the tip was moved slightly around the local region by using a micromanipulator. The voltage

allowed for both the diffusion of the dye into the region as local processes were disrupted and for a focal electroporation. Afterwards, fish were dissected out of the agar and placed in a dish of 10% Hanks to recover and were imaged the following day on a confocal microscope to detect backfilled neurons.

Colocalization

Confocal image stacks containing green and red channels were observed using Imaris software (BitPlane). Using the colocalization add-on, minimum thresholds were determined for red and green channels slightly above noise levels for each channel. We ensured that the dimmest cells were not thresholded out. A new channel was generated using the Imaris colocalization add-on that represented colabled signal. For image stacks with low noise levels in both channels, the automated colocalization feature in Imaris was adequate for our purposes, but, in most cases, thresholds required adjustments by hand due to high noise levels producing spurious results when observing fish in three-dimensional maximum intensity projections.

Confocal imaging

Transgenic zebrafish were anaesthetized in 0.02% MS222 and embedded in 1.4% low melting point agar, typically with the dorsal side down and resting against a glass coverslip floor of a small petri dish. Agar was covered with 0.02% MS222 solution in 10% Hanks to prevent desiccation. Images were collected using an inverted Zeiss LSM confocal microscope. Green fluorescence was excited using 488 nm laser light and red fluorescence using a 543 nm laser. Green fluorescence emission was collected using a filter to collect light from either 505-530 nm or 505-550 nm and red fluorescence emission was collected using a long pass 560 nm filter. Single image stacks were collected throughout the dorsoventral extent of hindbrain or spinal cord

and generally collection last 40 minutes to 1.5 hours. To prevent photo damage, images were collected with high gain settings and averaged either twice or four times. Fish remained healthy and anaesthetized during this time.

CHAPTER 4

ALX NEURONS IN HINDBRAIN FORM A STRIPE WITH AN AGE-RELATED MAP OF RESISTANCE AND PROJECTIONS

Abstract

A novel organization of interneurons is present in the hindbrain of larval zebrafish. Stripes identified by neurotransmitter phenotype are present throughout hindbrain and alternate between excitatory and inhibitory phenotypes [107]. We show here that neurotransmitter stripes align with transcription factor stripes. These transcription factors are some of the same ones also present in the spinal cord of vertebrates [1, 10, 18, 21, 119, 136, 137, 139]; here we show that a topological transformation of the early dorsoventral transcription factor map of spinal cord exists in hindbrain as a mediolateral array of neurotransmitter stripes. We show for one stripe that hindbrain neurons are morphologically similar to spinal cord neurons sharing the same transcription factor type (alx in zebrafish [18, 139], Chx10 in mice [10, 136, 137]). We show that the stripe axis identifies an orderly patterning based on age, input resistance, and possibly recruitment during swimming. We also describe an age related organization of the projections of the neurons that occurs in both the hindbrain and spinal cord. These age-related projections might have functional implications since the age of a neuron determines its participation in different speeds of swimming. The stripe patterning by neurotransmitter and transcription factor expression represents a simple pattern of organization of neural circuits in hindbrain that is present before the clustering into hindbrain nuclei occurs, but during a time when zebrafish are freely swimming.

Introduction

In the spinal cord, two principles of organization have recently been described for interneurons. During development, progenitor cells, as defined by patterns of expression of transcription factors, are arrayed in an orderly manner where progenitor cells of a certain type are positioned at a specific dorsoventral position and produce specific types of neurons [3]. For example, Nkx6.1 progenitor cells are located in the mid-dorsoventral region of ventral spinal cord and give rise to Chx10 (alx in zebrafish [18, 139]) and Gata3 interneurons [40, 136, 137, 139]. The Chx10 neurons arising from this domain have been established in multiple species to be ipsilateral excitatory interneurons involved in the coordination of motor behaviors [10, 18] and whose absence disrupts motor behavior [10]. Despite the presence of an orderly transcription factor map defining cell classes early in development, interneurons marked by different transcription factor types are somewhat intermingled by migrations within the spinal cord [210].

A second principle involves an orderly pattern of organization of the neurons according to the functional roles they play during behavior. A functional topography exists in the spinal cord of swimming larval zebrafish, in which both the speed at which neurons are recruited and their input resistances map onto their position along the dorsoventral axis of spinal cord [19]. For example, neurons expressing the alx transcription factor, CiDs (Circumferential ipsilateral Descending) in zebrafish, are ipsilateral descending glutamatergic interneurons recruited during swimming, but the dorsal (low resistance) alx neurons are recruited only during the fastest movements and the more ventral (high resistance) are recruited at slower swimming speeds [19].

The presence of simple principles of organization of interneurons in spinal cord raises the question of whether there might be similar patterns of organization in the brain. In the hindbrain of zebrafish, we have recently described a novel patterning

of interneurons where stripes of alternating excitatory and inhibitory neurotransmitter phenotype exist [107]. We were intrigued by this novel stripe patterning of hindbrain, and we suspected that it might relate to the functional organization established within the spinal cord since the hindbrain is a continuation of spinal cord [43].

A segmental (rhombomeric) organization has been recognized as a central feature of hindbrain organization for decades (reviewed in [51]). Segmental organization has been observed physically [57, 75, 211], by molecular patterning [45, 52, 66, 69-71, 81, 82, 93, 96, 126, 152-157, 212], and by specialized cell types [45, 63, 64, 77, 79, 80, 85, 87, 158-160]. However, the stripes of neurons we identified by neurotransmitter phenotype are present throughout the rostrocaudal extent of hindbrain and do not follow a rhombomere-specific organization [107]. This patterning suggested to us a possible patterning of interneurons throughout hindbrain that may provide a template contributing distinct cell types to different neural circuits in hindbrain.

Within the hindbrain, there is evidence for the existence of interneurons expressing the same transcription factor types as in the spinal cord in xenopus, zebrafish, chick, and mice [99, 100, 108-134]. However, these studies have not addressed how these transcription factor expression patterns relate to the general organization of neural circuits in hindbrain. We first set out to characterize transcription factor patterning in hindbrain in relation to the neurotransmitter stripes previously described. Specifically, we hypothesized that cells with a distinct morphology and transcription factor type might reside in one neurotransmitter stripe. This possibility was suggested to us by the observation that stripes of distinct transcription factor expression also exist in hindbrain and cross rhombomere segments. We found that all transcription factor stripes maintain a relationship to distinct neurotransmitter stripes, some on a 1:1 basis. The ventral to dorsal spinal cord

transcription factor map transiently present within progenitor cell populations in spinal cord (but not in differentiated neurons) aligns directly with the medial to lateral transcription factor patterning of interneurons in the hindbrain of larval zebrafish.

Given that the transcription factor organization in hindbrain showed parallels with that in spinal cord, we were interested in examining whether the functional topography for recruitment during different speeds of swimming present within a transcription factor class in spinal cord [19] was also evident in the hindbrain. The functional topography of spinal cord was observed along the dorsoventral axis, and because stripes extend dorsoventrally we predicted that a difference in input resistance and participation during different speeds of swimming might be present along the dorsoventral extent of a stripe. We focused on the alx transcription factor because alx interneurons in spinal cord are known to play an important role in the coordination of motor behaviors for zebrafish [18, 19, 29, 135].

Our data show striking parallels between the organization in spinal cord and hindbrain. The time of differentiation of a neuron maps on the axis of a stripe in the hindbrain, with the young neurons typically more dorsal in a stripe. Although the orientation of this age map is different from that in spinal cord, the organization by age corresponds both to a map of input resistance and differences in the pattern of recruitment of neurons similar to that in spinal cord [18, 19]. Older, lower resistance neurons activated in faster movements lie ventrally in a stripe, with increasingly higher resistance, slower movement ones located more dorsally. We conclude that there is evidence of a simple structural and functional order in hindbrain at a time when the fish is already freely swimming, but before the more complex arrangements of nuclei have arisen. This may reflect a simple underlying patterning that forms a foundation for the development of many of the networks underlying the many

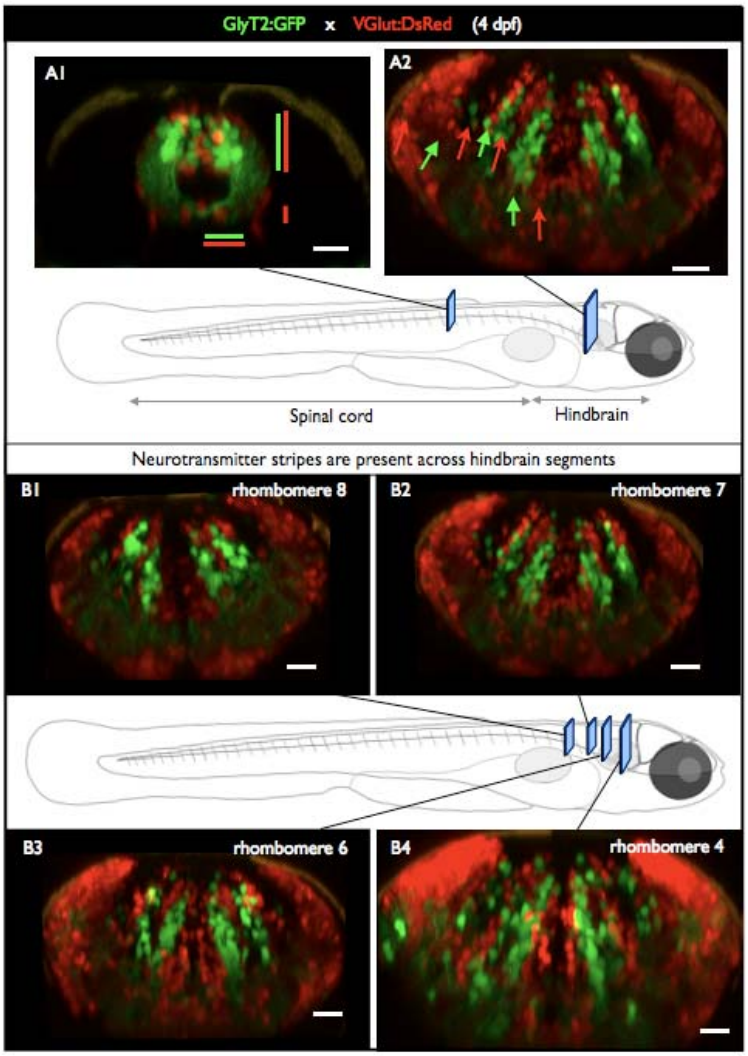
behaviors produced by the hindbrain, but that is obscured by the later migration and reassortment of neuronal types.

Results

Interleaved stripes of excitatory and inhibitory interneurons are present in hindbrain

In the spinal cord, excitatory and inhibitory neurons are intermingled. This is evident in cross section in a 4 dpf transgenic fish expressing GFP under the control of the glycine transporter 2 promoter as well as DsRed under the control of the vesicular glutamate promoter (GlyT2:GFP x VGlut:DsRed) in figure 4.1A1. Although there is some clustering of cells by transmitter type they overlap in position and are sometimes well intermixed. Glycinergic and glutamatergic interneurons are present throughout similar dorsoventral and mediolateral regions of spinal cord as indicated by red (glutamatergic neurons) and green (glycinergic neurons) bars in figure 4.1A1. In the hindbrain, however, neurons are organized into stripes that alternate between glutamatergic and glycinergic phenotypes [107]. In cross sections, these stripes are organized in an alternating fashion from medial to lateral (figure 4.1A2). The medial stripe of neurons is glutamatergic and extends dorsoventrally; the next more lateral stripe is glycinergic and also extends dorsoventrally. This alternation between excitatory and inhibitory neurotransmitter stripes continues to the lateral edge of hindbrain. This stripe patterning is present in all rhombomere segments possessing both glycinergic and glutamatergic interneurons [rhombomere 3 (r3) through rhombomere 8 (r8) with r4, r6, r7, and r8 shown in figure 4.1B1-4].

Figure 4.1 Interleaved stripes of neurons by neurotransmitter phenotype are present in hindbrain. **(A)** Cross sections of dual expression VGlut:DsRed x GlyT2:GFP transgenic fish imaged at 4 dpf. Dorsal is top for all images. **(A1)** In the spinal cord, glycinergic and glutamatergic neurons are present in similar regions. Vertical red/green bars indicate dorsoventral regions of glutamatergic and glycinergic interneurons. Horizontal red/green bars indicate mediolateral regions of glutamatergic and glycinergic neurons. Both dorsoventrally and mediolaterally, glutamatergic and glycinergic interneurons are present in similar regions. Glutamatergic neurons span slightly larger regions in both directions and also identify a ventral population where glycinergic neurons are not present indicated by the ventral, isolated red bar to the right of the cross section image. **(A2)** In the hindbrain, glycinergic and glutamatergic neurons form interleaved stripes that alternate neurotransmitter phenotype from medial to lateral. Red/green arrows indicate ventral regions of each stripe of glutamatergic and glycinergic neurotransmitter phenotype. **(B)** Neurotransmitter stripes are present throughout hindbrain. Cross sections of VGlut:DsRed x GlyT2:GFP transgenic fish imaged at 4 dpf. Images show stripe patterning in rhombomere 4 (**B1**), rhombomere 6 (**B2**), rhombomere 7 (**B3**), and rhombomere 8 (**B4**). Within each rhombomere, the same patterning of stripes alternating between glutamatergic and glycinergic neurotransmitter phenotype from medial to lateral is present. Scale bars = 20 um.


GlyT2:GFP x VGlut:DsRed (4 dpf)
A1
A2
Spinal cord
Hindbrain
Neurotransmitter stripes are present across hindbrain segments
B1
rhombomere 8
B2
rhombomere 7
B3
rhombomere 6
B4
rhombomere 4

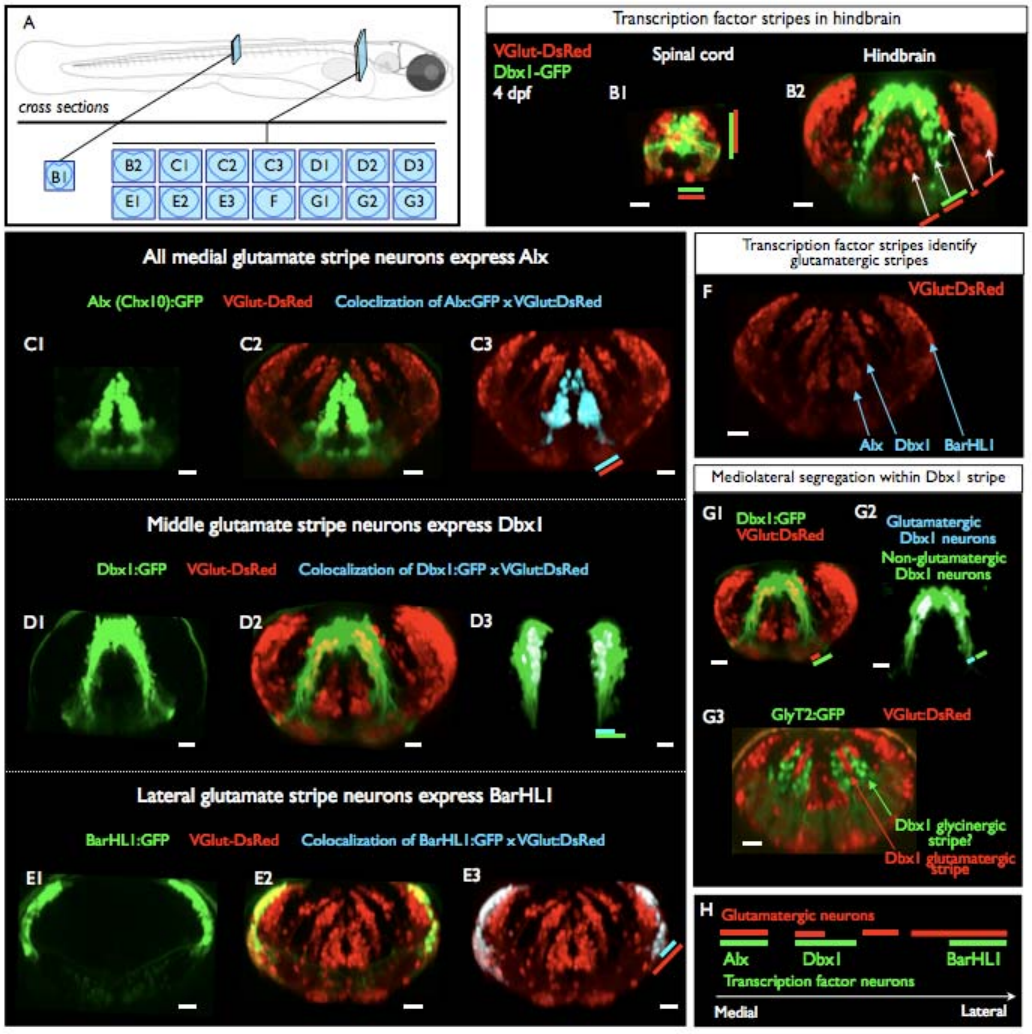
Transcription factor stripes align with neurotransmitter stripes in hindbrain

The discovery of an interleaved stripe-like organization of neurons by neurotransmitter phenotype suggests that a level of organization of interneurons in hindbrain may exist that has not been previously observed. To determine whether the stripes may identify structural as well as functional subtypes of neurons we were interested in the expression patterns of transcription factors in relation to this neurotransmitter phenotype expression. Interneurons with distinct structure and functional roles in spinal cord have been identified and express different transcription factors [1-3, 10, 15, 17, 18, 22]; however, a broad organization of all interneurons by either transcription factor or neurotransmitter phenotype has not been revealed for hindbrain.

Transcription factor expression does overlap with the neurotransmitter stripes as evident in dual transgenic lines expressing GFP under control of various transcription factor promoters and DsRed in glutamatergic neurons (figure 4.2, 3-4 dpf). The alx (Chx10) transcription factor is expressed in medial neurons in the hindbrain, and all medial stripe glutamatergic neurons express alx (figure 4.2C1-3). The Dbx transcription factor is expressed in spinal cord in progenitors of the V0 domain, which lies dorsal to the Chx10 domain in spinal cord. This zone gives rise to commissural spinal interneurons which are also labeled by Dbx after differentiation [21]. In hindbrain, Dbx1 expression was observed more lateral and dorsal to alx expression (figure 4.2D1).

Because the V0 interneurons labeled by Dbx in spinal cord are known to be of both glycinergic and glutamatergic neurotransmitter phenotypes, we explored the relationship between Dbx1 expression and the transmitter stripes in dual expression transgenic lines. Colocalization of Dbx:GFP with VGlut:DsRed delineated a region in

Figure 4.2 Transcription factor stripes align with neurotransmitter stripes. **(A)** Orientation of images shown in this figure. All images are reconstructed cross section of confocal images collected from anaesthetized transgenic fish. **(B)** Transcription factor stripes are present in hindbrain. Images in spinal cord and hindbrain of VGlut:DsRed x Dbx1:GFP at 4 dpf. In the spinal cord **(B1)**, Dbx1 neurons populate most regions where glutamatergic neurons are present. Red/green bars indicate mediolateral (below image) and dorsoventral (right of image) regions of VGlut:DsRed (red) and Dbx1:GFP (green) expression in spinal cord. In the hindbrain **(B2)**, Dbx1 neurons are located in a single stripe region throughout hindbrain that includes a stripe of glutamatergic neurons. Red bars indicate glutamatergic stripes mediolaterally distributed across hindbrain with arrows indicate ventral regions of each stripe. A single green bar indicates regions of Dbx1 expression. **(C-E)** Transcription factor stripes overlap single glutamatergic stripes. **(C)** Alx neurons populate the medial glutamate stripe. All images are of 3 dpf transgenic fish (VGlut:DsRed x Alx:GFP). Colocalization of Alx:GFP and VGlut:DsRed shows that all medial stripe glutamate neurons expression the Alx transcription factor (**C3**). **(D)** Some Dbx1 neurons populate the middle glutamate stripe. All images are of 4 dpf transgenic fish (VGlut:DsRed x Dbx1:GFP). Colocalization of Dbx1:GFP and VGlut:DsRed shows that all middle stripe glutamate neurons express the Dbx1 transcription factor (**D3**). **(E)** BarHL1 neurons populate lateral regions of the lateral glutamate stripe. All images are of 4 dpf transgenic fish (VGlut:DsRed x BarHL1:GFP). Colocalization of BarHL1:GFP and VGlut:DsRed shows that the lateral neurons within the lateral glutamate stripe express the BarHL1 transcription factor (rhombomere 8, **E3**). **(F)** Image of 3 dpf VGlut:DsRed transgenic fish indicating transcription factors expressed in different glutamate stripes. **(G)** Mediolateral segregation of neurotransmitter types with the Dbx1 transcription factor stripe. **(G1)** Cross section of Dbx1:GFP x VGlut:DsRed transgenic fish at 4 dpf. Red bar indicates region of glutamatergic neurons within the region of Dbx1:GFP expression shown by green bar. **(G2)** Colocalization of Dbx1:GFP and VGlut:DsRed indicated a medial region of the Dbx1 stripe of glutamatergic neurons with non-glutamatergic cells more laterally positioned within the Dbx1 stripe. In **G3**, a cross section of GlyT2:GFP x VGlut:DsRed shows the region of glutamatergic neurons expression Dbx1. Lateral to this region is a glycinergic stripe that potentially could express Dbx1. **(H)** Summary of transcription factor expression in glutamatergic stripes. Scale bars = 20 um.


A
cross sections
B1
B2 C1 C2 C3 D1 D2 D3
E1 E2 E3 F G1 G2 G3
Transcription factor stripes in hindbrain
VGlut-DsRed
Dbx1-GFP
4 dpf
Spinal cord
Hindbrain
B1
B2
All medial glutamate stripe neurons express Alx
Alx (Chx10):GFP
VGlut-DsRed
Coloclization of Alx:GFP x VGlut:DsRed
C1
C2
C3
Middle glutamate stripe neurons express Dbx1
Dbx1:GFP
VGlut-DsRed
Colocalization of Dbx1:GFP x VGlut:DsRed
D1
D2
D3
Lateral glutamate stripe neurons express BarHL1
BarHL1:GFP
VGlut-DsRed
Colocalization of BarHL1:GFP x VGlut:DsRed
E1
E2
E3
Transcription factor stripes identify glutamatergic stripes
F
VGlut:DsRed
Alx
Dbx1
BarHL1
Mediolateral segregation within Dbx1 stripe
G1
Dbx1:GFP
VGlut:DsRed
G2
Glutamatergic Dbx1 neurons
Non-glutamatergic Dbx1 neurons
G3
GlyT2:GFP
VGlut:DsRed
Dbx1 glycinergic stripe?
Dbx1 glutamatergic stripe
H
Glutamatergic neurons
Alx
Dbx1
BarHL1
Transcription factor neurons
Medial
Lateral

which glutamatergic Dbx and non-glutamatergic, Dbx positive neurons were present (figure 4.2D). The glutamate positive Dbx neurons were in the medial part of the Dbx zone (figure 4.2D3, 4.2G1-3). The more lateral part of the zone included the region of a glycinergic stripe (figure 4.2D2-3, 24.G1-3), suggesting that the Dbx area included both a medial glutamatergic domain and a more lateral, potentially glycinergic one.

BarHL1 neurons in spinal cord lie in very dorsal spinal cord regions [146]. Neurons expressing this transcription factor are located very laterally in hindbrain (figure 4.2E1). This region of BarHL1 neurons includes some of the neurons within the lateral crescent of glutamatergic neurons throughout hindbrain (figure 4.2E2), and colocalization indicates that, in some hindbrain regions, BarHL1 neurons are only a subset of glutamatergic neurons in this region (figure 4.2E3), with non-BarHL1 expressing cells located more medially within this lateral crescent.

In summary, each transcription factor stripe described includes stripes of neurons identified by transmitter phenotype (figure 4.2F). Alx neurons are the only neuron type in the medial glutamatergic stripe (figure 4.2C2-3). Dbx neurons are present in the middle glutamate and probably glycinergic stripes (figure 4.2D2-3, 4.2G). Finally, BarH1 neurons are laterally positioned and contain neurons in the lateral glutamatergic stripe/crescent (figure 4.2E1-3). The relationship between transcription factor stripes and glutamatergic stripes from medial to lateral is summarized in figure 4.2H.

Our data indicate that transcription factor stripes are positioned in specific locations relative to neurotransmitter stripes. Each of the three transcription factors described overlapped with only one glutamatergic stripe (figure 4.2F). The relationship of transcription factor stripes and neurotransmitter stripes was shown to be orderly with a patterning of one of three types: a 1:1 relationship (alx); a glutamate

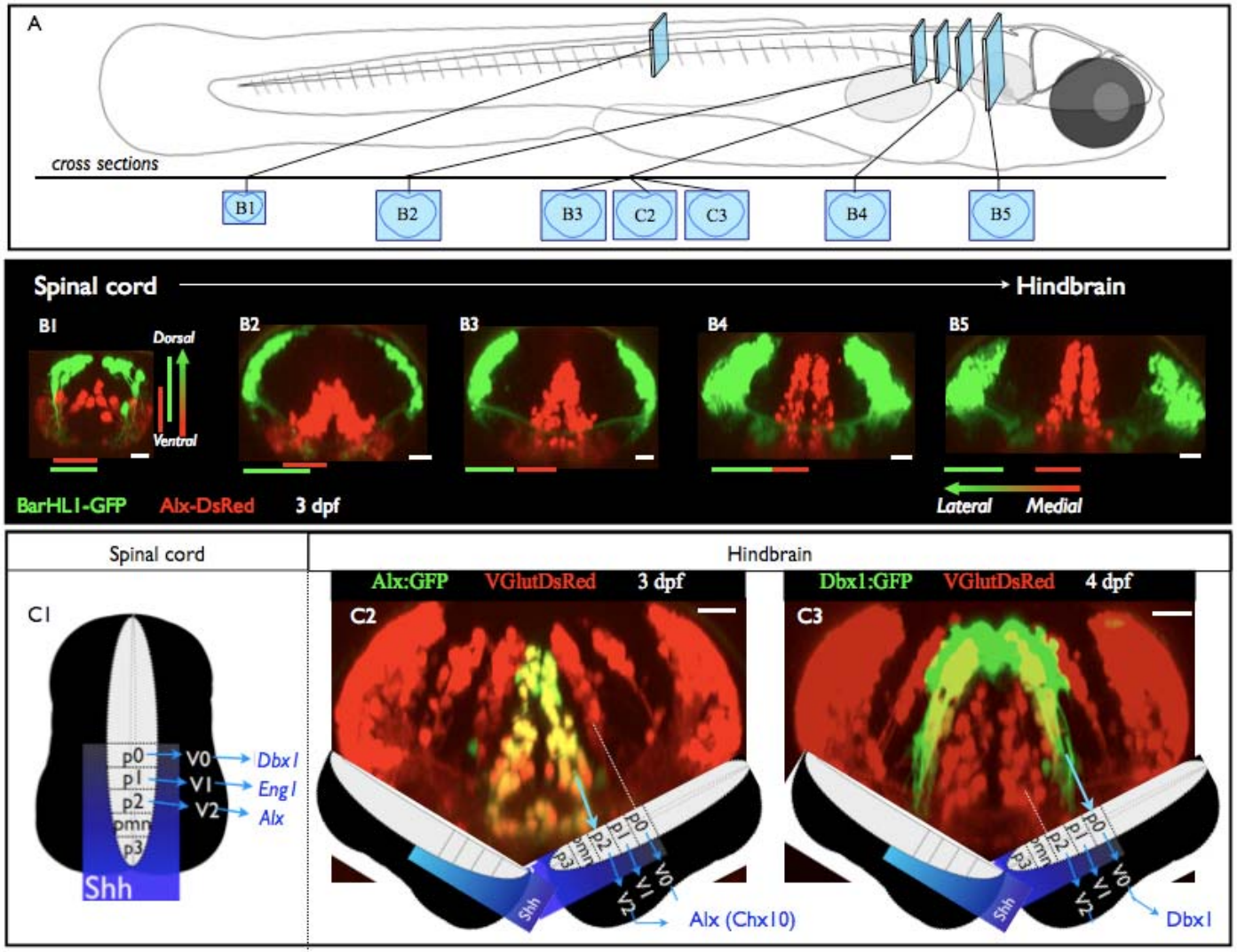
stripe occupied a portion of a transcription factor stripe (Dbx1); and a transcription factor marks a portion of a glutamate stripe (BarHL1) (figure 4.2H).

A topological transformation of transcription factor zones between spinal cord and hindbrain

We examined the transcription factor patterning in spinal cord in the transgenic lines to understand the relationships between the pattering there and that in hindbrain. In figure 4.3B, the transition from spinal cord to hindbrain for two transcription factors (alx and BarHL1) derived from dorsoventrally distant progenitor zones is shown. Alx neurons (alx:DsRed) and BarHL1 neurons (BarHL1:GFP) are shown. In the spinal cord, shown in cross section in figure 4.3B1, BarHL1 neurons (BarHL1:GFP) are dorsally positioned relative to the more ventrally positioned alx neurons (alx:GFP). Within hindbrain, BarHL1 neurons are laterally positioned relative to the more medially positioned alx neurons (figure 4.3B5). There is a topological transformation from spinal cord to hindbrain where the dorsoventral segregation of transcription factor types in spinal cord continuously changes into a mediolateral segregation within hindbrain from caudal to rostral (figure 4.3B1-5).

Figure 4.3C shows this transformation diagrammatically. In spinal cord, a dorsoventral map of progenitor zones that give rise to different classes of interneurons with unique transcriptional profiles is present (figure 4.3C1). In the hindbrain, this mapping appears split open as shown in figure 4.3C2-3. Alx neurons, ventral in spinal cord, are medially located in hindbrain (figure 4.3C2), and Dbx1 neurons, derived from a more dorsal progenitor zone in spinal cord, are more laterally positioned in hindbrain (figure 4.3C3). Each of these transcription factors aligns with a different stripe of glutamatergic neurons, indicating that neurotransmitter stripes represent

Figure 4.3 Topological transformation of transcription factor maps from spinal cord to hindbrain. **(A)** Orientation of images in spinal cord and hindbrain in this figure. All images are cross sections of dual expression transgenic fish. (B1-5) Cross sections of Barhl1:GFP and Alx:DsRed lines crossed together and imaged at 3dpf. Red/green bars indicate regions of Alx:DsRed and BarHL1:GFP expression (horizontal bars for mediolateral regions and vertical bars for dorsoventral regions). **(B1)** Spinal cord section shows that Barhl1 and Alx neurons, which are relatively far apart in the cord, are largely still dorsoventrally separated at 3 dpf, although there are rare cells in the same regions. Closer to hindbrain, dorsoventral segregation disappears and is replaced by a mediolateral segregation of neurons of these two transcription factor types (B2-3). In hindbrain **(B3-B5).** a mediolateral segregation is present and in **B5** the two transcription factors types populate opposite mediolateral extremes in hindbrain. **(C1)** Cartoon illustration the dorsoventral segregation of progenitor zones within spinal cord. In the hindbrain, this pattern is cracked open to reveal a mediolateral segregation of transcription factor types (C2-3). Alx:GFP neurons identify all neurons in the medial glutamate stripe (3C2, rhombomere 7, 3 dpf), while Dbx1 neurons identify neurons in the next more lateral glutamate stripe (3C3: rhombomere 8, 4 dpf). Scale bars = 20 um.

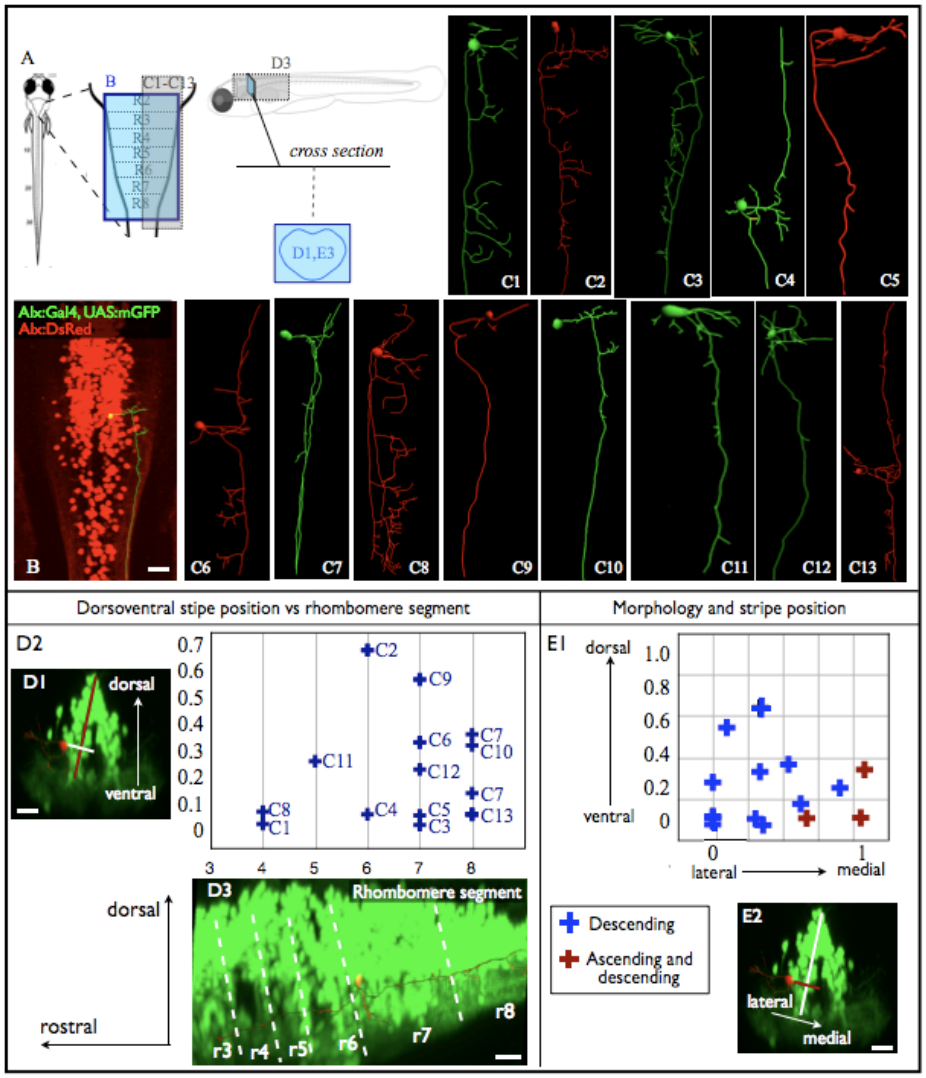

A
cross sections
B1
B2
B3
C2
C3
B4
B5
Spinal cord
Hindbrain
B1
B2
B3
B4
B5
Dorsal
Ventral
BarHL1-GFP
Alx-DsRed
3 dpf
Lateral
Medial
Spinal cord
Hindbrain
C1
p0
p1
p2
pmn
p3
V0
V1
V2
Dbx1
Eng1
Alx
Shh
Alx:GFP
VGlutDsRed
3 dpf
C2
Dbx1:GFP
VGlutDsRed
4 dpf
C3
Alx (Chx10)
Dbx1

classes of interneurons with different transcriptional profiles. The mediolateral segregation of transcriptional profiles indicates that there is a transformation of the dorsoventral spinal transcription factor map into a mediolateral one in hindbrain.

Alx neurons in hindbrain maintain the morphological phenotype of those in spinal cord

Since single neurotransmitter stripes possess unique transcription factor phenotypes, we were interested in whether the morphological features of neurons expressing a particular transcription factor in spinal cord are also present for the neurons expressing that transcription factor in the stripes in hindbrain. We examined this for the alx expressing neurons. Alx (or Chx10) neurons in the spinal cord of zebrafish are known to be excitatory ipsilateral neurons that play a role in swimming and escape behaviors [18, 19, 29, 135]. To label small numbers of neurons to allow easier examination of their morphology, we stochastically expressed either alx:Gal4/UAS:mGFP or alx:Gal4:UAS/mMCherry in alx:RG and alx:GFP lines respectively [18] and looked for doubly labeled cells. All neurons were imaged at 5 dpf and reconstructed using Imaris software. All 14 labeled alx neurons imaged in hindbrain had ipsilateral axons (figure 4.4) and so were ipsilateral, glutamatergic neurons based upon the glutamatergic phenotype of all alx neurons. Despite differences in dorsoventral, mediolateral, and rostrocaudal positions within the hindbrain alx stripe (Figure 4.4D2), all neurons maintained the descending axon characteristic of neurons expressing the alx transcription factor within spinal cord. They were not all morphologically identical, however, because some also had an ascending axon branch (figure 4.4E1).

Figure 4.4 Alx neurons in hindbrain have similar morphology to spinal cord Alx neurons **(A)** Orientation of images in this figure **(B)** Example of stochastic labeling with Alx:Gal4, UAS:mGFP in Alx:DsRed transgenic line. One Alx cell is labeled here with mGFP and is ipsilateral descending. **(C1-C13)** Examples of Alx cells labeled with this method. All hindbrain Alx neurons are either ipsilateral descending or ipsilateral ascending and descending as seen in the spinal cord across species. **(D)** Cells were labeled in multiple rhombomere segments and within each segment at multiple dorsoventral positions relative to the Alx stripe. In **D1** the normalized dorsoventral position of an Alx neuron is illustrated. This method for calculating dorsoventral position defines the y-axis for **D2** and **E1**. In **D3**, the rhombomere boundaries are illustrated in a lateral view showing how rhombomere identity is assigned within the Alx line. The rhombomere segments were originally found by reticulospinal array positions relative to the Alx stripe (not shown). The rhombomere position of each cell is plotted along the x-axis in **D2**. Cells were found to possess the typical Alx morphology through rhombomere segments and dorsoventral positions within a segment (see **D2**). **(E)** Position of cells within a stripe and their morphology is depicted in the plot in **E1**. The mediolateral position of the cell within the stripe is measured by determining the width at the cell's dorsoventral stripe position and then the distance from the lateral edge of the width is the cells mediolateral position within the stripe. Each position is normalized by dividing by the width at the position of measurement. Cells that are positioned with no mediolateral neighbors were given a value of zero. The cross section in **E2** shows an example of the mediolateral measurement that is plotted along the x-axis in **E1** against the dorsoventral position along the y-axis to show the normalized position within the stripe. Cells plotted with blue crosses are descending and those with red crosses are both ascending and descending. Scale bars = 20 um.

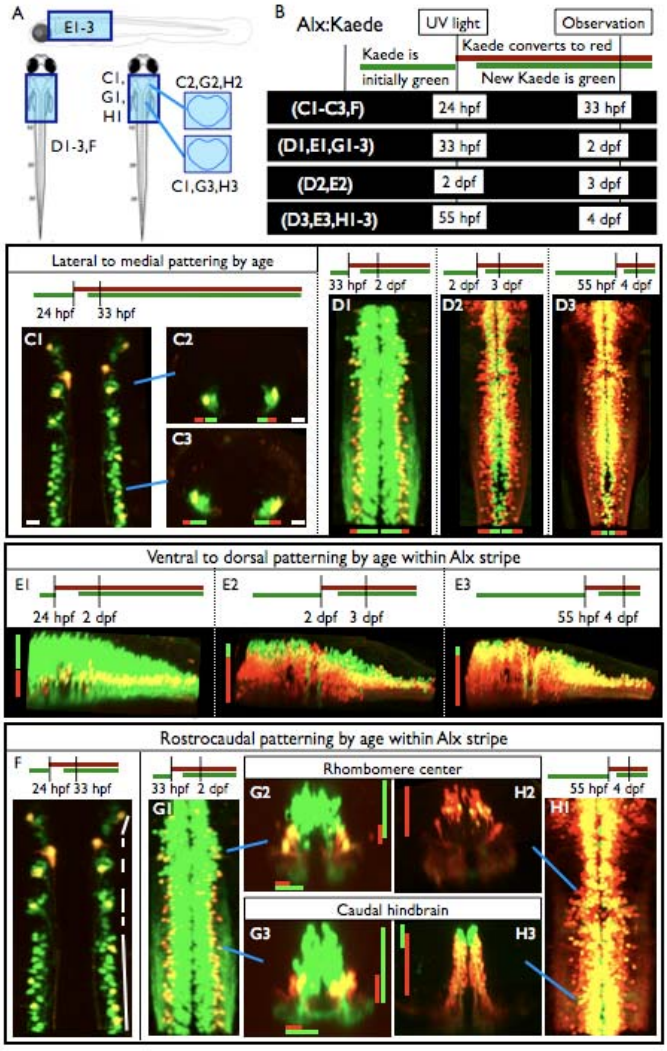

A
B
C1-C13
D3
cross section
D1,E3
C1
C2
C3
C4
C5
AbcGal4, UAS:mGFP
AbcDsRed
B
C6
C7
C8
C9
C10
C11
C12
C13
Dorsoventral stipe position vs rhombomere segment
Morphology and stripe position
D2
D1
dorsal
ventral
C2
C9
C6
C7
C10
C11
C12
C7
C8
C1
C4
C5
C3
C7
C13
D3
Rhombomere segment
dorsal
rostral
r3
r4
r5
r6
r7
r8
E1
dorsal
ventral
lateral
medial
Descending
Ascending and descending
E2
lateral
medial

Age-related patterning of alx neurons

Spinal alx neurons show an age related map of position, with the youngest neurons lying ventrally and older and older ones stacked above them. The neurons of different ages participate in swimming at different frequencies. We examined the alx stripe in hindbrain to look for any age related order. We used the alx:Kaede line [18], photoconverted the fish at different time points during development to convert earlier differentiating neurons to red and then imaged these fish at later times to identify the location of older (red) and younger (green) neurons.

We found that the earliest born neurons (expression by 24 hpf) were laterally positioned relative to those just slightly older (expression by 33 hpf, figure 4.5C1-3). By photoconverting at 33 hpf and imaging much later at 2 dpf (figure 4.6B1, 4.6B3), we found that the oldest neurons were positioned ventrolaterally at the bottom of the stripe of alx neurons, with younger ones stacked above them along the axis of the stripe (figure 4.5G2-3). In the ventral regions of the stripe, the neurons differentiated by 33 hpf neurons were laterally located while younger neurons were medially positioned at 2 dpf (figure 4.5G2-3), consistent with the data from flashing at 24 hpf and examination at 33 hpf. By photoconverting at 2 dpf and imaging at 3 dpf, a small population of some very late born neurons were located at the extreme dorsal end of the stripe (figure 4.5H3). At this time point, all neurons within rhombomere center regions were red, indicating that neurons present in those areas of the rhombomeres at 3 days had all differentiated by 2 dpf (figure 4.5H2). Rhombomere center stripe areas maintain wider stripes (figure 4.5G2,5H2) in comparison to other hindbrain regions (figure 4.5G3), but the overall developmental patterning from lateral to medial and ventral to dorsal was consistent across the hindbrain for alx neurons, with the exception of one local region discussed later.

Figure 4.5 Alx neurons in hindbrain maintain an age-related stripe patterning **(A)** Orientation of images in this figure **(B)** Timing of experiments shown in this figure. Photoconversion for images (**C1-C3,F**) was at 24 hpf, for (**D1,E1,G1-3**) at 33 hpf, for (**D2,E2**) at 2 dpf, and for (**D3,E3,H1-3**) at 55 hpf. Images were collected at 33 hpf (**C1-C3,F**), 2 dpf (**D1,E1,G1-3**), 3 dpf (**D2,E2**), and at 4 dpf (**D3,E3,H1-3**). **(C-D)** Mediolateral patterning by age within Alx stripe. Red/green bars below images indicate mediolateral regions of either red or exclusively green Kaede expression. In C1-3, images of a transgenic fish photoconverted early and imaged 7 hours later shows few cells present in a dorsal view (**C1**). In cross section (**C2-3**), old (red) neurons are laterally positioned relative to younger (exclusively green) neurons. In **D1-3**, dorsal views of 3 fish photoconverted at different stages are shown. In each case, red neurons are laterally positioned relative to exclusively green neurons. For later photoconversions (**D2-3**), medial regions of exclusively green cells are narrowed but the mediolateral age-related patterning remains indicating that this patterning is present for multiple ages of neurons. **(E)** Ventral to dorsal patterning by age. In **E1-3**, lateral views with rostral to the left are shown for multiple photoconversion times. Red/green bars to the left of each image illustrate dorsoventral regions of either red or exclusively green neurons. In each case, red (old) neurons are ventral to exclusively green (younger) neurons. As in **D**, later photoconversions (**E2-3**) reveal the same patterning with fewer young neurons present with progressively later photoconversions (compare **E1**, **E2** and **E2**, **E3**). **(F-H)** Rostrocaudal patterning by age within Alx stripe. **(F)** Early in development, Alx neurons are clustered rostrocaudally with gaps in-between clusters. White bars to the right of F indicate regions rostrocaudally where Alx neurons reside. **(G-H)** Neurons in some rostrocaudal regions fill in from ventral to dorsal for old to young earlier than in other hindbrain regions. Within rhombomere centers **(H2)** all neurons are present by 55 hpf. Within other regions **(H3)**, small populations of Alx neurons express Kaede protein after 55 hpf and are dorsally positioned. Red bars indicate regions of red expression and green bars indicate regions of green expression in the absence of red expression dorsoventrally. Despite slight differences in the complete differentiation of neurons across rostrocaudal regions, all regions maintain an orderly patterning by age (with the exception of rhombomere 6, see figure 13).


A
E1-3
C1, G1, H1
C2,G2,H2
D1-3,F
C1,G3,H3
B
Alx:Kaede
UV light
Observation
Kaede is initially green
Kaede converts to red
New Kaede is green
(C1-C3,F)
24 hpf
33 hpf
(D1,E1,G1-3)
33 hpf
2 dpf
(D2,E2)
2 dpf
3 dpf
(D3,E3,H1-3)
55 hpf
4 dpf
Lateral to medial pattering by age
24 hpf 33 hpf
C1
C2
C3
33 hpf 2 dpf
D1
2 dpf 3 dpf
D2
55 hpf 4 dpf
D3
Ventral to dorsal patterning by age within Alx stripe
E1
24 hpf 2 dpf
E2
2 dpf 3 dpf
E3
55 hpf 4 dpf
Rostrocaudal patterning by age within Alx stripe
F
24 hpf 33 hpf
33 hpf 2 dpf
G1
Rhombomere center
G2
H2
55 hpf 4 dpf
H1
Caudal hindbrain
G3
H3

This old to young, ventral to dorsal age-related patterning is opposite the age-related order in spinal cord (figure 4.6C2-3). We examined the transition from the pattern in hindbrain to that in spinal cord by using the alx:Kaede line to compare the positions of neurons of different ages in optical cross sections along the rostrocaudal axis of the fish from hindbrain to spinal cord. The spinal neurons are not arranged in orderly stripes like the hindbrain, so we also explored the basis for this difference. Alx:Kaede fish were photoconverted at 2 dpf and imaged at 3 dpf from the hindbrain to the spinal cord (figure 4.6D). In the hindbrain, as described earlier, ventral (red) neurons were older than more dorsal (green) neurons (figure 4.6D1). At the hindbrain-spinal cord boundary the age-related segregation dorsoventrally is not present (figure 4.6D3). Within spinal cord, the hindbrain pattern is even more disrupted such that the oldest neurons are displaced laterally and displaced dorsally to be more dorsal than the youngest (green) alx neurons positioned more medially (figure 4.6D4). From hindbrain to spinal cord, a curling of the alx stripe is observed where, within spinal cord regions, the stripe has curled over itself to the extent that the oldest cells are now dorsal to the younger cells (figure 4.6E1-4), and thus, the regions of old and young neurons have flipped from spinal cord to hindbrain.

Age-related wiring of alx neurons in hindbrain and spinal cord

Although the position of alx cells based on age has been shown to determine their participation in different frequencies of swimming within spinal cord [18], no age related patterning of the processes of these cells has been described in spinal cord in prior studies. We looked for such patterning in both spinal cord and hindbrain, as its presence might suggest possible age related wiring in networks. Using alx:Kaede fish photoconverted at different times during development and imaged later, we found that, in the hindbrain, processes of older ventral, red neurons are located dorsally in the

Figure 4.6 Age-related patterning is flipped in hindbrain relative to spinal cord. **(A)** Orientation of images in this figure. **(B)** Timing of experiments shown in this figure. Photoconversion for images **C1-3** was at 33 hpf and for **D1-D4** at 2 dpf. Images were collected at 2 dpf **(C1-3)** and 3 dpf **(D1-D4)**. **(C)** Alx:Kaede transgenic fish photoconverted at 33 hpf and imaged at 2 dpf shows age related patterning flips between hindbrain and spinal cord. In **C1**, a lateral view of hindbrain and spinal cord regions shows that ventral cells in hindbrain and dorsal cells in spinal cord are the oldest (red). To the left and right, patterning of hindbrain and spinal cord are illustrated indicating a flip in the dorsoventral positions of old and young cells between these regions. In a cross section of hindbrain **(C2)**, early born neurons are ventrally positioned (red expression) while in spinal cord **(C3)** old neurons are dorsally positioned (red). To the left and right of **C2** and **C3**, dorsoventral regions of red and exclusively green expressing neurons are shown by red/green bars. **(D1-D4)** Cross sections of Alx:Kaede fish photoconverted at 2 dpf and imaged at 3 dpf with dorsal to the top for all cross section images. Red/green lines indicate dorsoventral and mediolateral regions of red and solely green neurons with asterisks indicating a separation for either dorsoventral (vertical red/green bars) or mediolateral (horizontal red/green bars) patterning. Alx stripe in hindbrain in **D1** is oriented from ventral to dorsal for old (red) to new neurons (green only). In more caudal regions in the hindbrain/spinal cord boundary, there is no longer a dorsoventral map of Alx neurons based on age **(D3)**. However, old neurons are more laterally positioned than new ones. In spinal cord **(D3)**, old neurons are displaced laterally and dorsally to be dorsal to the later born neurons (green) and flipped relative to the age-related map in hindbrain. **(E1-E3)** Confocal images of Alx:GFP fish imaged at 5 dpf showing the alx stripe in cross section in hindbrain **(E1)** and the caudal transition to spinal cord curling the stripe and flipping the age-related map of cell bodies **(E2-E3)**. In spinal cord **(E4)**, the stripe is curled and old neurons are laterally positioned and flipped by age when compared to hindbrain. Scale bars = 20 um.

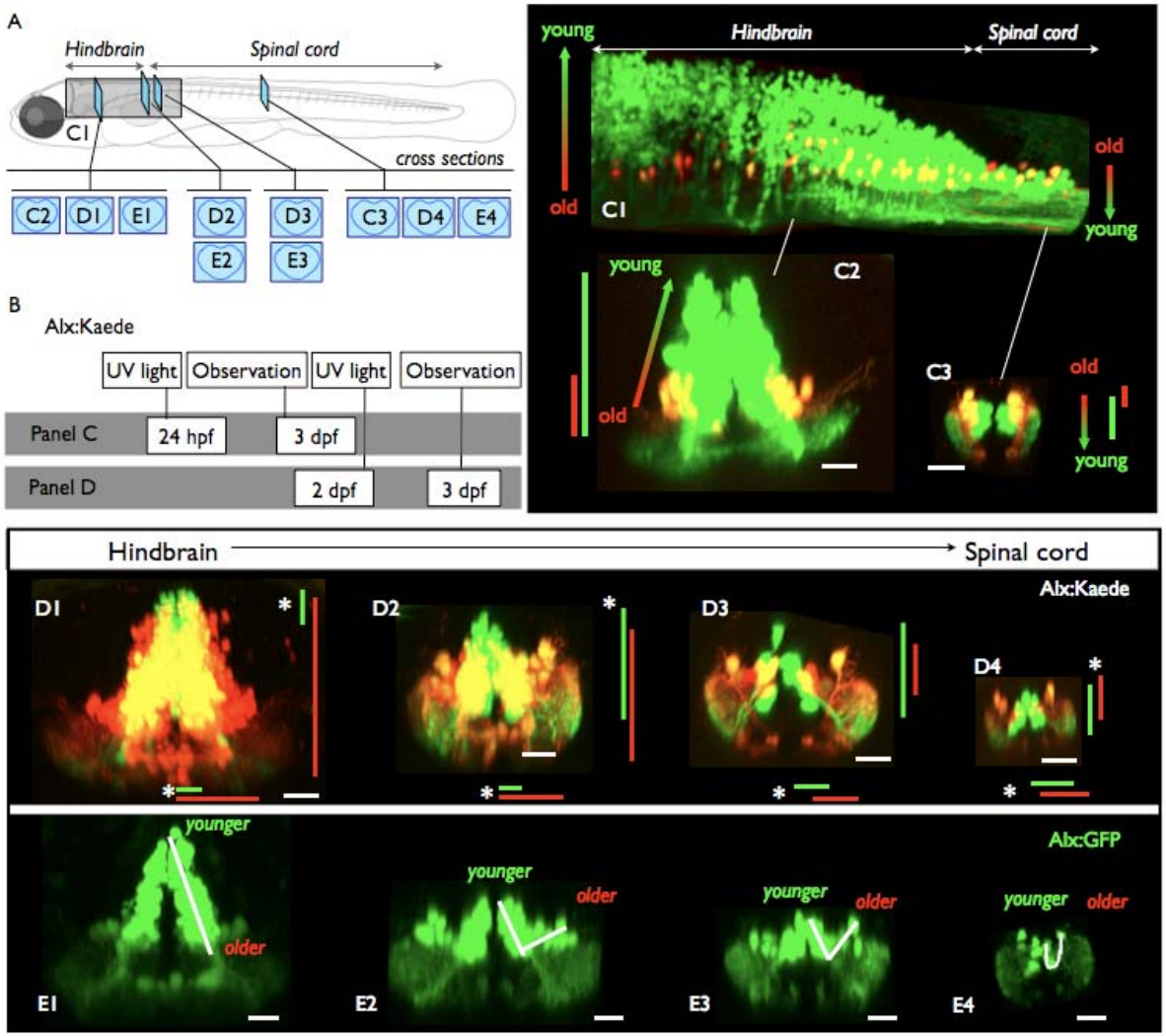
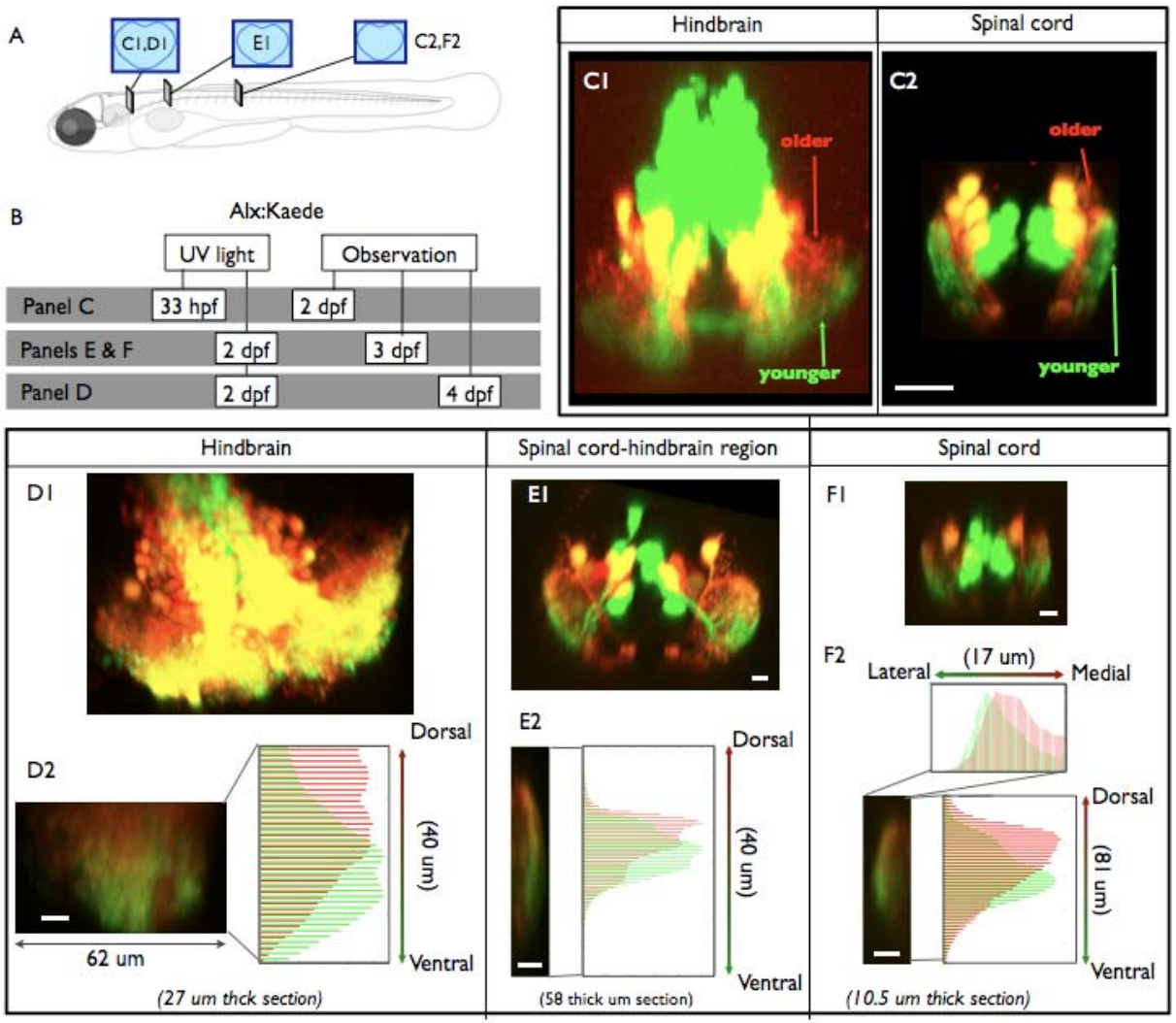

A
Hindbrain
Spinal cord
C1
cross sections
C2
D1
E1
D2
E2
D3
E3
C3
D4
E4
B
Alx:Kaede
UV light
Observation
UV light
Observation
Panel C
24 hpf
3 dpf
Panel D
2 dpf
3 dpf
young
Hindbrain
Spinal cord
old
C1
old
young
young
C2
old
C3
old
young
Hindbrain
Spinal cord
Alx:Kaede
D1
D2
D3
D4
younger
older
younger
older
younger
older
younger
older
Alx:GFP
E1
E2
E3
E4

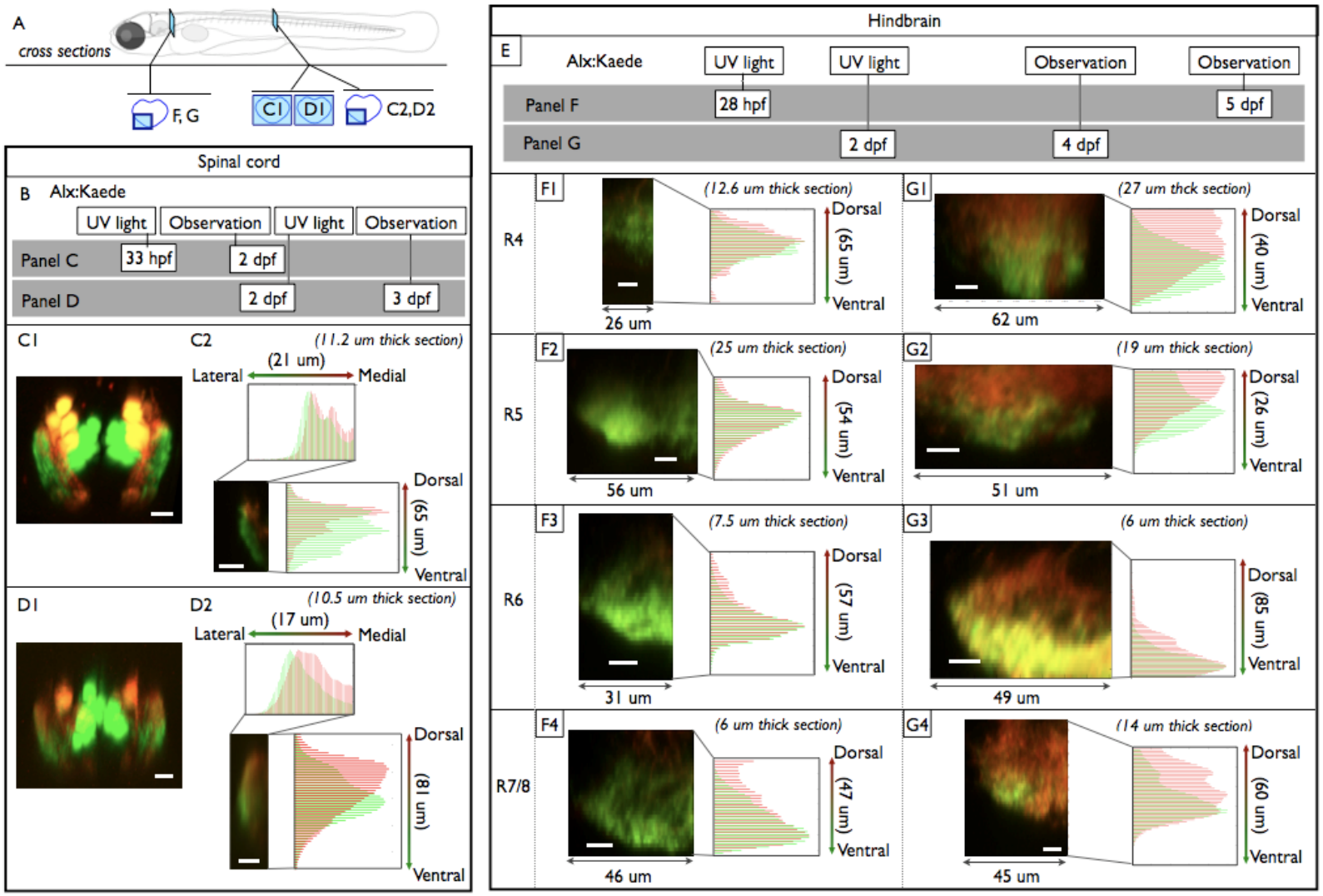
Figure 4.7 Alx neurons in hindbrain and spinal cord possess age-related neuropil segregation **(A)** Orientation of images in this figure **(B)** Timing of experiments shown in this figure. Photoconversion for images **C1-2** was at 33 hpf and for **D-f** at 2 dpf. Images were collected at 2 dpf (**C1-2**), 3 dpf (**E1**,**F1**) and 4 dpf **(D1)**, **(C1-2)** Age-related segregation of neuropil is present for Alx neurons in both hindbrain and spinal cord. In both hindbrain (**C1**) and spinal cord (**C2**), neuropil for older neurons (red) is dorsal and ventral for younger neurons (green). **(D-F)** Quantification of red/green expression within neuropil regions from hindbrain to spinal cord. In **D1**,**E1**, and **F1**, cross section images are shown in hindbrain, a region between hindbrain and spinal cord where the dorsoventral age map is not present, and in spinal cord respectively. In each confocal image stack, a neuropil volume was extracted and is shown in cross section in **D2**, **E2**, and **F2**. These images are raw sums of red/green neuropil expression normalized by the number of cross section frames in each case. (**D1**,**E1**, and **F1** are maximum intensity projection images obtained using Imaris software). To the right of **D2**, **E2**, and **F2**, normalized sums across the dorsoventral (top to bottom) axis for red and green expression are shown. In each case, red expression is shifted dorsally relative to more ventral green expression; thus, neuropil regions from older neurons are dorsal to younger neuron neuropil regions. This is surprising since the cell body age-related map of Alx neurons is flipped between hindbrain and spinal cord. In figure **F2**, the mediolateral segregation of red/green expression is shown for spinal cord. Scale bars = 10 um.


A
C1,D1
E1
C2,F2
B
Alx:Kaede
UV light
Observation
Panel C
33 hpf
2 dpf
Panels E & F
2 dpf
3 dpf
Panel D
2 dpf
4 dpf
Hindbrain
Spinal cord
C1
older
younger
C2
older
younger
Hindbrain
Spinal cord-hindbrain region
Spinal cord
D1
D2
Dorsal
(40 um)
Ventral
62 um
(27 um thck section)
E1
E2
Dorsal
(40 um)
Ventral
(58 thick um section)
F1
F2
(17 um)
Lateral
Medial
Dorsal
(81 um)
Ventral
(10.5 um thick section)

neuropil, which lies below the stripe, while younger, more dorsal, green neurons have processes located more ventrally in the neuropil (figure 4.7C1). In the spinal cord, processes of older neurons are located medial to those of younger neurons (figure 4.7C2). This patterning is present continuously from hindbrain to spinal cord (figure 4.7D1,E1,F1).

To verify this organization in hindbrain, we quantified the intensities of red and green by extracting a series of cross sections. Normalized voxels were summed for each dorsoventral position and distributions were normalized to look for regions of dominant red/green expression, and, in each case, red dominated dorsally and green ventrally. Examples are shown from hindbrain to spinal cord in figure 4.7D-F. Dorsal regions, in this case, have virtually no green neuropil but possessed significant amounts of red neuropil. We looked at this pattern for flashes and imaging at different times in both spinal cord (figure 4.8C-D) and hindbrain (figure 4.8E-F), and in hindbrain, over different rhombomeres (figure 4.8E2-5 and 4.8F3-6). This patterning was found within spinal cord for photoconversion at 33 hpf and imaging at 2 dpf (figure 4.8C1-2) as well as photoconversion at 2 dpf and imaging at 3 dpf (figure 4.8D1-2). In hindbrain regions, when a small number of neurons are photoconverted red early in development and the rest are green (differentiated between 28 hpf and 5 dpf), a dorsal region within the neuropil in multiple hindbrain segments shows only red expression (indicating projections from the few old alx neurons present, figure 4.8E2-5). When only a small number of alx neurons possess solely green expression (photoconversion at 48 hpf and imaged at 4 dpf), most rhombomere regions observed showed a difference in the dorsoventral distribution of red and green neuropil (figure 4.8F3,4,6). Rhombomere 6, however, still showed a region of exclusively red neuropil dorsally (figure 4.8F5). This is surprising since many neurons in this case

Figure 4.8 Age-related segregation within alx neuropil is present across regions of spinal cord and hindbrain at multiple times. **(A)** Orientation of images in this figure. **(B,E)** Timing of photoconversion/imaging in spinal cord (**B**) and in hindbrain (**E**). From confocal image stacks, a neuropil volume was extracted and is shown in cross section within spinal cord (**C2**,**D2**) or in hindbrain within rhombomere 4 (**F1**,**G1**), rhombomere 5 (**F2**,**G2**), rhombomere 6 (**F3**,**G3**), or rhombomere 7/8 (**F4**,**G4**). These images are raw sums of red/green neuropil expression normalized by the number of cross section frames in each case. (**C1** and **D1** are maximum intensity projection images obtained using Imaris software). To the right of each neuropil image, normalized sums across the dorsoventral (top to bottom) axis for red and green expression are shown. In **C2** and **D2**, the mediolateral segregation of red/green expression is shown above the neuropil images for spinal cord. **(C-D)** Spinal age-related segregation within neuropil regions is present at different time points. In **C1** and **D1**, cross sections of the spinal cord of alx:Kaede transgenic fish photoconverted and imaged at different times (see **B**) are shown. In **C1**, a mediolateral segregation of red/green expression within the lateral neuropil is evident visually, while, in **D1**, a dorsoventral segregation of red/green expression is evident. In **C2** and **D2**, a cross section image of a neuropil region is shown. To the right of this image, a plot of the dorsoventral normalized intensity of red/green expression shows a dorsoventral age-related segregation within neuropil. For both **C2** and **D2**, red and green distributions are shifted with red distributions slightly more dorsal than green distributions. Above each image, mediolateral distributions of red/green normalized intensities also show an age-related segregation within neuropil regions, this time, with red expression shifted more medial to the distribution of green expression. **(E-F)** Age-related segregation within alx neuropil in hindbrain is present within multiple rhombomeres and for different ages of neurons. In panel F, plots of dorsoventral distributions of red/green expression are shown for rhombomeres 4 (**F1**), 5 (**F2**), 6 (**F3**), and 7/8 (**F4**). In each case, red expression is present in more dorsal regions where green expression is not obviously present. In panel **G**, quantification was performed on fish photoconverted at a later time, and, in this case, the distribution of red expression is shifted dorsally to the distribution of green expression. In each case shown in this image, red/green expression show distinct patterns of distribution within neuropil regions, with red expression present both dorsal and medial to green expression in spinal cord and red expression more dorsal to green expression across multiple rhombomeres in hindbrain. Scale bars = 10 um. Dimensions of neuropil images quantified are shown in each panel.


A
cross sections
F, G
C1
D1
C2,D2
Spinal cord
B Alx:Kaede
UV light
Observation
UV light
Observation
Panel C
33 hpf
2 dpf
Panel D
2 dpf
3 dpf
C1
C2
(11.2 um thick section)
(21 um)
Lateral
Medial
Dorsal
(65 um)
Ventral
D1
D2
(10.5 um thick section)
(17 um)
Lateral
Medial
Dorsal
(81 um)
Ventral
Hindbrain
E
Alx:Kaede
UV light
UV light
Observation
Observation
Panel F
28 hpf
5 dpf
Panel G
2 dpf
4 dpf
R4
F1
(12.6 um thick section)
Dorsal
(65 um)
Ventral
26 um
G1
(27 um thck section)
Dorsal
(40 um)
Ventral
62 um
R5
F2
(25 um thick section)
Dorsal
(54 um)
Ventral
56 um
G2
(19 um thick section)
Dorsal
(26 um)
Ventral
51 um
R6
F3
(7.5 um thick section)
Dorsal
(57 um)
Ventral
31 um
G3
(6 um thick section)
Dorsal
(85 um)
Ventral
49 um
R7/8
F4
(6 um thick section)
Dorsal
(47 um)
Ventral
46 um
G4
(14 um thick section)
Dorsal
(60 um)
Ventral
45 um

express both red and green Kaede protein because even converted neurons can continue to express new green protein if the gene is not yet turned off. This age related segregation suggests possible age-related wiring and the possibility of structural segregation of the wiring of functionally distinct networks because the age of a neuron in spinal cord has been shown to determine the frequency of swimming movements in which it participates.

We examined this pattern further at the single cell level by reconstructing neurons described earlier in the stochastic expression experiments for fish in which two neurons with axons projecting into spinal cord were labeled in hindbrain. We found that the projections of these cells were mediolaterally segregated within spinal cord (N=3 pairs, figure 4.9A1,B1,C1), with the axon of the older cell medial to the younger one. In the hindbrain, the processes of the oldest neuron were located dorsally (figure 4.9A3,B4,C5) to those of the younger neuron. In the spinal cord, a mediolateral segregation was present for all 3 pairs (figure 4.9A6,B5-7,C8). For 2 of the 3 fish, a dorsoventral segregation of projections was observed (4.9A6,C8). In summary, single cell projections and alx:Kaede photoconversion experiments indicate that an age-related segregation of projections is present for alx neurons in hindbrain projecting within both hindbrain and spinal cord.

Age-related resistance map exists in hindbrain

Since the hindbrain showed a similar, but more organized, patterning of interneurons than that of spinal cord, we suspected that any functional patterns in spinal cord might also be present in hindbrain. Along with the dorsal neurons in spinal cord being the earliest born [18], they are also involved in the fastest movements only [18, 19, 135] and have the lowest input resistances of all alx neurons

Figure 4.9 Age-related segregation within Alx neuropil is observed at the single cell level. **(A-C)** 3 examples of Alx transgenic fish where 2 neurons are labeled and their projections are shown in 2 different colors. Fish of either Alx:GFP (**B,C**) or Alx:DsRed (**A**) transgenic lines were stochastically labeled using either Alx:Gal4,UAS:mMCherry (**B,C**) or Alx:Gal4,UAS:mGFP (**A**). Single Alx cells positioned dorsally or medially within Alx stripe are reconstructed and colored blue. Single Alx cells within local ventral or lateral stripe position are colored either green **(A)** or red **(B,C)**.**(A1,B1,C1)** Mediolateral segregation of single cell projections. In each panel (**A1,B1,C1**), a dorsal view is shown, and, in each case, a mediolateral segregation of projections is seen through a significant rostrocaudal extent of hindbrain and spinal cord. In each case, medial projections are found for either more ventral (**A2**) or more laterally (**B3,C4**) positioned neurons that would be older based on the age map depicted in figure 5. Lateral projections arise from potentially younger cells (**A3,B4,C5**), consistent with the observations using the Alx:Kaede transgenic line. (**A2-6,B3-7,C3-8)** In these panels, cross sections throughout the rostrocaudal extent of the images in **A1**,**B2**, and **C3** are shown. For each pair of cells in these three fish, the two cells labeled in each fish project into spinal cord and are mediolaterally segregated. In each case, the ventral (**A**:green) or lateral (**B,C**:red) neuron projects to medial neuropil regions in spinal cord; dorsal **(A)** and medial **(B,C)** neurons (light blue projections) project laterally in spinal cord neuropil (**A6,B5-7,C8**, colored bars below each cross section indicate mediolateral extent of axonal projections for the cell of that color). There is segregation by age within the hindbrain (**A4,C6**) as well as in regions in the transition between hindbrain and spinal cord (**A5,B4,C7**). In most cases, a dorsoventral ordering is present as well, as indicated by colored bars to the right of each image illustrating the dorsoventral extent of projections for each neuron.

Alx:DsRed
Ventral neuron (old)
Dorsal neuron (young)
A1
A2
A3
A4
A5
A6
Medial
Lateral
Alx:GFP
Lateral neuron (old)
Medial neuron (young)
B1
B2
B3
B4
B5
B6
B7
Medial
Lateral
Alx:GFP
Lateral neuron (old)
Medial neuron (young)
C1
C2
C3
C4
C5
C6
C7
C8
Medial
Lateral

[19]. Younger, more ventral neurons in spinal cord are recruited first, are involved in slower movements and have higher input resistances [19].

We examined whether there was an age related map of input resistance along the alx stripe axis in hindbrain. By using an exposed brain preparation of the alx:GFP transgenic line at 5 dpf, we targeted alx:GFP neurons for patching and examined their input resistances in relation to their normalized locations along the axis of the stripe obtained after fixation and reconstruction. One example is shown in figure 4.10C. We sampled neurons from one rostrocaudal region in hindbrain across the dorsoventral extent of the alx stripe at 5 dpf. Neurons positioned very dorsally were very young with longer duration action potentials and a higher resting membrane potential. We sampled resistance from some of these, but focused primarily on more mature alx neurons. Neurons with the lowest input resistance values were consistently located in the ventralmost stripe regions at 5 dpf. Two examples of low input resistance alx neurons in ventral stripe regions are shown in figure 4.10D2-3. There was a significant ($p < 0.0001$) relationship between the position of a neuron along the stripe axis and its input resistance, with input resistance lowest at the bottom of the stripe and increasing more dorsally. This map is flipped from the input resistance map in spinal cord but agrees with the flipped dorsoventral age map of these neurons relative to those in spinal cord (Figure 4.10E).

Resistance map is altered in a hindbrain region where the typical age map is not evident

In rhombomere six, there are two regions of neurons just rostral and caudal to the segment center in which the developmental pattern of these neurons is altered relative to the rest of hindbrain; young neurons are present ventrally and older ones dorsally. In figure 4.11C1-2 and 4.11D3, red neurons represent the older population

Figure 4.10 Input resistance of alx hindbrain neurons at 5 dpf. **(A)** Orientation of images in this figure **(B)** Example of current injections in Alx neuron. Small hyperpolarizing current injections produced small hyperpolarizing changes in cell without rebound firing. These current injections were use to calculate input resistance for each Alx neuron. (**C1-C2)** Cross section (**C1**) and dorsal view (**C2**) of neuron patched from (**B**). Neurons were patched using an exposed brain prep and filled with sulforhodamine B (Invitrogen). Fish were fixed and imaged following the recordings to determine cell's position within Alx stripe. **(D1)** Lateral view of Alx:Kaede fish photoconverted at 33 hpf and imaged at 2 dpf showing ventralmost cells as the old Alx neurons. In (**D2**,**D3**) two examples of cells patched either in 5 dpf Alx:GFP fish (**D2**) or 5 dpf Alx:Kaede fish (**D3**) show ventral oldest cells have low input resistance values (429 MOhm in **D2** and 227 MOhm in **D3**. The cell in **C1** is ventral and has input resistance of 624 MOhm). (**E**) Input resistance of each cell plotted against its normalized dorsoventral stripe position (calculated at shown in cross section in **E**). Ventral neurons consistently had the lowest resistance and dorsal neurons had much higher resistances within stripe.

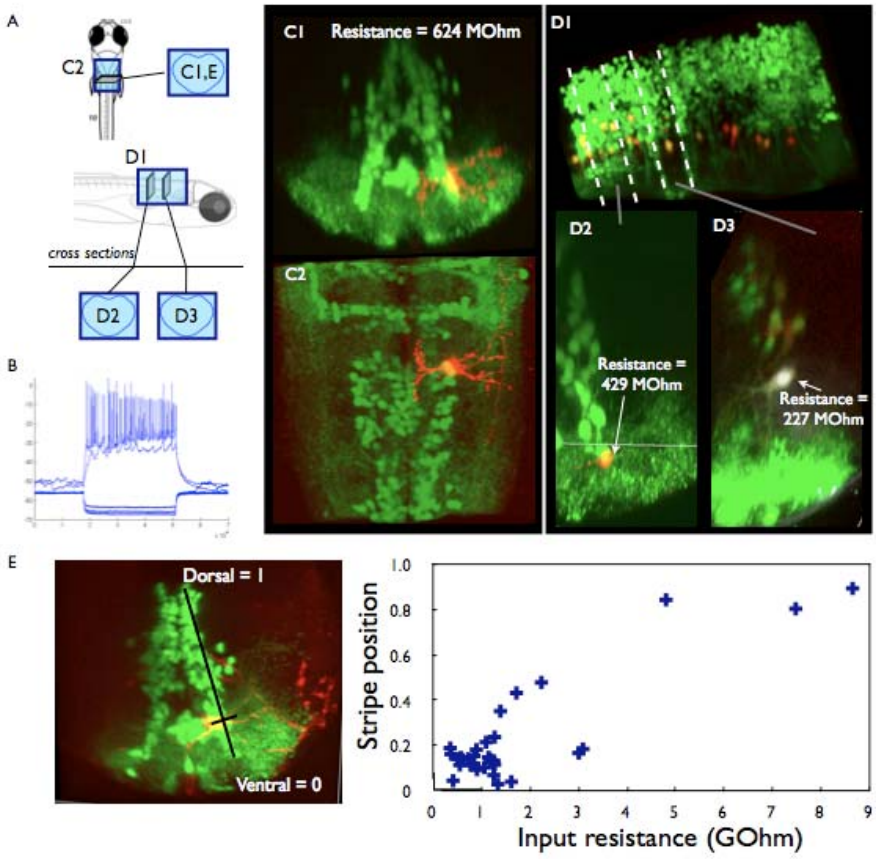
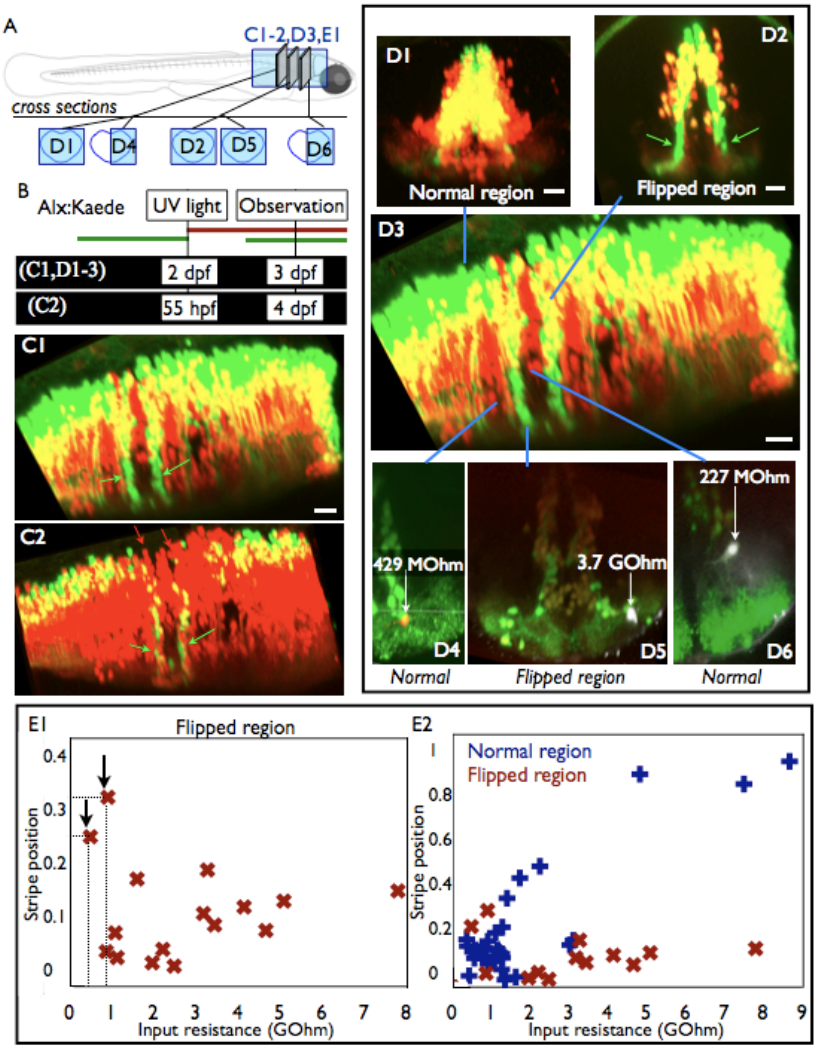


Figure 4.10 Input resistance of alx hindbrain neurons

Figure 4.11 Regions of rhombomere 6 contain Alx neurons that are developmentally flipped along the dorsoventral axis. **(A)** Orientation of images in this figure. **(B)** Timing of experiments shown in this figure. Photoconversion for images was at 2 dpf **(C1,D1-3)** and at 55 hpf **(C2)** Imaging of fish was performed at 3 dpf **(C1,D1-3)** and at 4 dpf (**C2**). **(C1,C2)** Lateral views of fish photoconverted at two late times during development. In C1, most young Alx neurons in hindbrain are dorsal (green) while older (red) neurons are ventral. In rhombomere 6, 2 bands at the rostrocaudal edges contain new neurons extending to the ventral part of the Alx stripe (green arrows). In **C2**, Alx:Kaede was photoconverted at 55 hpf, slightly later than the 2 dpf photoconversion shown in **C1**. Here, the oldest cells again are ventral (red) and younger cells are positioned only at the dorsal tip of the stripe through hindbrain. In rhombomere 6, the oldest cells in the region of bands described in **C1** are dorsal (red arrows) and the youngest ventral (green arrows). In **D3**, the same lateral view is shown as in **C1**, here indicating regions where cross sections were taken for **D1-2** and **D4-6**. In **D2**, the dorsal tips are green, while in **D3**, a population dorsal and ventral is shown to be young. At the later time point shown in **C2**, these dorsal green cells no longer appear in this rhombomere 6 flipped region. **(D4-6)** The flipped region contains high input resistance cells ventrally as compared to normal regions of the alx stripe. In **D4** and **D6**, ventral cells in normal regions have low input resistance values. **D4** shows a ventral cell patched in rhombomere 7 in the alx:GFP line at 5 dpf in a cross section view. **D6** shows a neuron on the right side of the brain in rhombomere 6, in-between the two bands of age-flipped alx cells. In both **D4** and **D6**, these ventral neurons had some of the lowest input resistance values of cells patched throughout the dorsoventral extent of the stripe (429 MOhm for rhombomere 7 cell in **D4** and 227 MOhm for rhombomere 6 center region cell in **D6**). **D5** shows the results for a ventral neuron in the flipped region in an alx:Kaede fish photoconverted at 2 dpf and patched at 5 dpf. This ventral cell had a high input resistance value of 3.7 GOhm, unlike the ventral cells in non-flipped regions. In **E1**, the input resistance of 16 Alx cells in the bands of age-related flipped cells is shown. All neurons were positioned in the ventral half of the stripe (y-axis dorsoventral values extend from 0 to 0.4), and many high input resistance cells were found in this region. Some of the lowest input resistances were found in the dorsalmost patched cells and are indicated by arrows in the plot. In **E2**, plots of input resistance vs. dorsoventral stripe position are shown for both the flipped and normal regions of the Alx stripe. In the ventral region, input resistance values for the flipped region were found to be more than twice the values of input resistance for normal cells in this area. There was no significant correlation ($p = 0.3633$) between input resistance and position in the flipped region, unlike the significant correlation in regions outside this area ($p < 0.0001$)


A
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D1
D4
D2
D5
D6
B
Alx:Kaede
UV light
Observation
(C1,D1-3)
2 dpf
3 dpf
(C2)
55 hpf
4 dpf
C1
C2
D1
Normal region
D2
Flipped region
D3
429 MOhm
D4
3.7 GOhm
D5
227 MOhm
D6
Normal
Flipped region
Normal
E1
Flipped region
Stripe position
Input resistance (GOhm)
E2
Normal region
Flipped region
Stripe position
Input resistance (GOhm)

of alx neurons and green expression represents the youngest. When alx:Kaede fish were photoconverted at 2dpf and imaged one day later at 3 dpf, two bands of green expression are seen over the dorsoventral extent of the stripe in rhombomere 6 (figure 4.11C1). Arrows in figure 4.11C1-2 indicate bands of cells not following the age-related patterning of other regions. In figure 4.11C1, green cells are present along the dorsoventral extent of the stripe in these two bands. In figure 4.11C2, conversion was performed slightly later in development (55 hpf) and fish were imaged at 4 dpf. In this case, a smaller number of green cells are present, representing the youngest population of hindbrain alx neurons. In rhombomere 6, these younger cells are ventrally positioned, disrupting the age-map in this local region since older cells are more dorsally positioned. In cross section, the normal regions of hindbrain only have exclusively green expression dorsally, but, in the flipped region, the ventral cells are exclusively green (figure 4.11D1-3).

The significance of this flipped pattern is unknown, but it offered the possibility to examine whether the input resistance of a neuron was related to its age or to its location because in this region young neurons were located ventrally where typically older cells are found. When we recorded from ventral neurons, there was a clear difference between this region and other regions of hindbrain. The ventral-most neurons in the flipped region possess far higher input resistances (figure 4.11D5), equivalent only to the dorsal most neurons in other hindbrain regions (figure 4.11D4, 4.11D6). There was no correlation between the dorsoventral position and input resistance in this flipped region ($p = 0.3633$). We did, however, find 2 low input resistance neurons found dorsal to the higher resistance neurons in the developmentally flipped region (arrows in figure 4.11E1). Therefore, the age of a neuron, and not necessarily its dorsoventral position, is correlated with the input resistance for alx cells in hindbrain.

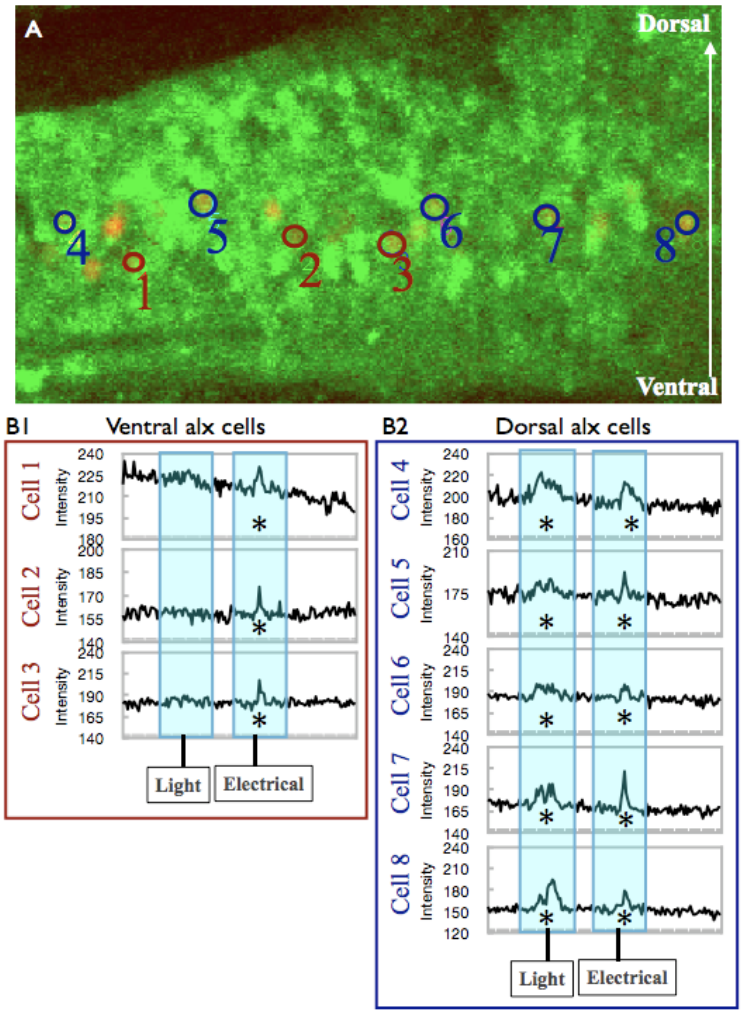
Recruitment during swimming aligns with age and resistance topography in hindbrain as in spinal cord

In spinal cord, both age [18] and input resistance [19] within the population of alx neurons are correlated with recruitment during different speeds of swimming. If the hindbrain is organized like spinal cord, we would expect that the old ventral neurons would be involved in fast movements and the more dorsal ones in slower movements. We examined this by using blastomere-stage injections of Calcium Green Dextran 3000 [213] into the yolk of alx:RG transgenic embryos and performing calcium imaging on paralyzed fish at 5 dpf. The alx neurons could be identified on the red channel and their calcium responses monitored on the green one. In one fish with adequate CGD3000 labeling, we found that more dorsal alx cells in hindbrain responded during both light and electrical stimuli (N=5) while ventral cells responded only to electrical stimuli in the same trial (N=3) (figure 4.12). Light induces swimming at slow speeds, while electrical stimuli leads to escape and fast swimming. This early data suggests the possibility of an orderly recruitment from dorsal to ventral of alx cells in hindbrain as movements change from slow to fast. This pattern, if it exists, would be consistent, although flipped, like the age and resistance map, relative to the ventral to dorsal recruitment of alx cells in spinal cord.

Discussion

Our understanding of the organization of hindbrain has been dominated by the segmental organization present across vertebrates [51]. In a previous study, stripes of neurons by neurotransmitter phenotype were discovered that form continuous rostrocaudal bands of interneurons throughout hindbrain [107]. In this study, we examined the patterning of these neurotransmitter stripes in vivo using transgenic zebrafish to identify features of stripe organization. We found that neurotransmitter

Figure 4.12 Calcium imaging of alx neurons indicate a dorsoventral recruitment for different strengths of sensory stimuli that lead to different speeds of movement. (**A**) Lateral view of 5 dpf Alx:DsRed (red) transgenic fish labeled with Calcium Green Dextran 3000 (green) using blastomere injections. Blue circles/numbers indicate more dorsally positioned neuron where calcium transients were evoked during sensory stimuli. Red circles/numbers indicate ventral most cells in different rostrocaudal positions within the Alx stripe that also showed calcium responses to sensory stimuli. **(B1-B2)** Calcium-induced fluorescence changes of Calcium Green Dextran during presentations of sensory stimuli. In **B1**, 3 ventral neurons are shown and shaded blue boxes indicate regions of sensory stimulation using both light and electrical stimuli. In previous work in spinal cord, it has been shown that light induces bouts of swimming that are slower than those produced by electrical stimulation. In some cases, electrical stimuli will induce an escape bout followed by fast swimming. In **B1**, the 3 ventral neurons responded only to electrical stimuli. However, in **B2**, neurons from the same experiment are shown that are positioned more dorsally than neurons shown in **B1**. These more dorsal neurons showed responses to both light and electrical stimuli in the same experiment where ventral neurons responded only to ventral stimuli. Asterisks indicate times of increased calcium-induced fluorescence changes in individual cells. This difference in response to sensory stimuli in dorsal and ventral cells may indicate a difference in recruitment for different swimming frequencies that relates to age and position in a stripe, similar to an age-related (dorsoventral) recruitment seen in alx cells in spinal cord [18, 19].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Light
Electrical
B2
Dorsal alx cells
Cell 4
Cell 5
Cell 6
Cell 7
Cell 8
Intensity
Light
Electrical

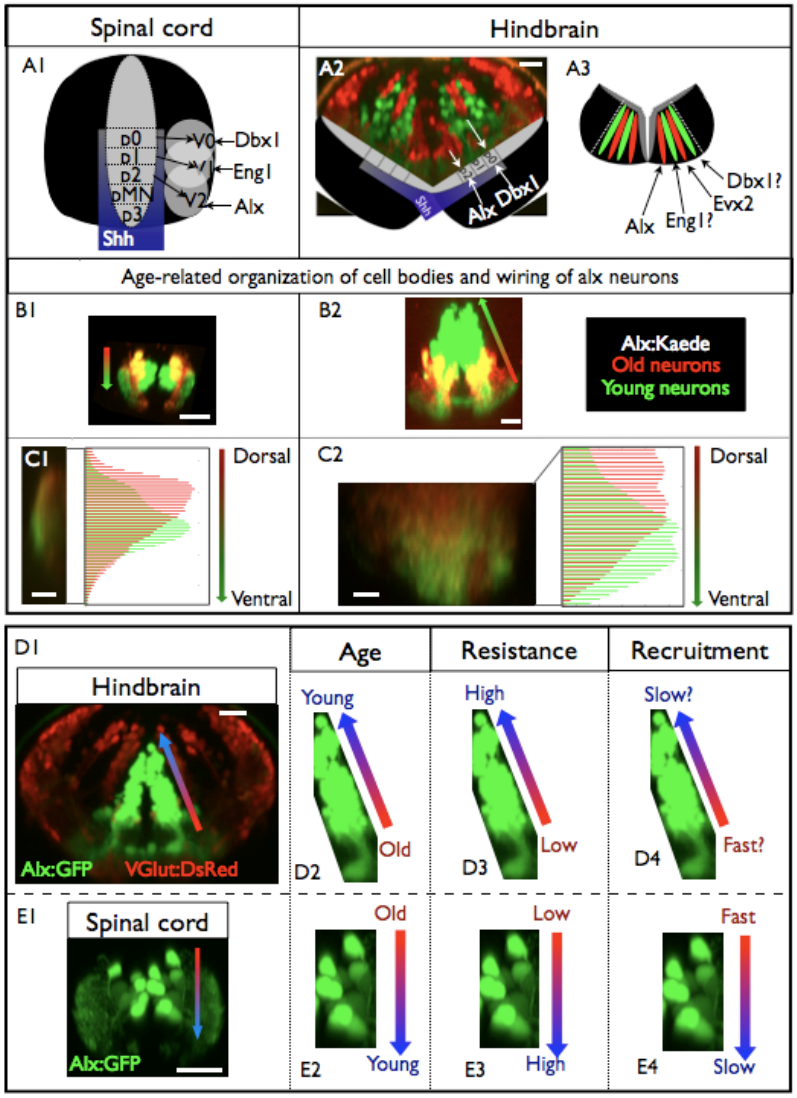
stripes in hindbrain align with single transcription factors that are known to identify functional classes of spinal interneurons. Medial glutamatergic stripe neurons express Alx, middle glutamatergic stripe neurons express Dbx1, and subsets of neurons within the lateral glutamatergic stripe express BarHL1. This orderly patterning of interneurons in hindbrain both by neurotransmitter and transcription factor phenotype reveals a novel organization of all excitatory and inhibitory interneurons. In recent studies across vertebrates, continuous transcription factor patterning has been shown to exist rostrocaudally across multiple rhombomere segments in the hindbrain of xenopus, zebrafish, chicks, and mice [99, 100, 108-134]. For this reason, it is likely that a stripelike organization of hindbrain is present in vertebrates, but might be obscured later in life by neuronal migration. If this were the case then, within a transcription factor type, an organization by age, input resistance, and involvement in the coordination of different strengths of behavior or threshold of recruitment as observed in zebrafish might be present across hindbrain neural circuits in vertebrates.

A shared transcription factor map exists in spinal cord and hindbrain

The importance of these neurotransmitter stripes possessing unique transcription factor identities for individual stripes arises from the understanding of spinal cord organization. In figure 4.13A1, an illustration of the patterning of ventral spinal cord is shown. Progenitor zones are present in dorsoventrally isolated regions and express unique patterns of transcription factor expression [3]. These zones give rise to cells of different types, three of which, V0, V1, and V2, are illustrated in figure 4.13A1 along with their unique transcription factor expression.

The organization of neurons by transcription factors that is transiently present in spinal cord persists in the hindbrain of larval zebrafish in the form of transcription factor stripes that align with neurotransmitter stripes. Within hindbrain, we have

Figure 4.13 Summary figure (**A**) Transformation of the dorsoventral spinal map of progenitor zones into a mediolateral map of differentiated neurons in hindbrain. **(A1)** A map of spinal cell types is depicted with progenitor domains for ventral spinal types shown. Progenitor domains for ventral cell types are arrayed from dorsal to ventral for p0,p1,p2,pMN, and p3 zones. V0 neurons arise from the p0 domain that expresses the Dbx1 transcription factor; V1 (Eng1) arise from the p1 domain, and a subset of V2 neurons express Alx (Chx10) and arise from the V2 domain. Within the 5 dpf spinal cord, the neurons arising from these dorsoventrally segregated progenitor zones are intermingled. **(A2)** In hindbrain, the positions of Alx and Dbx1 populations shown in this study suggest that the spinal map is cracked open to reveal a mediolateral patterning of spinal types within hindbrain. A confocal image of VGlut:DsRed x GlyT2:GFP 4 dpf transgenic fish is shown in cross section with a spinal transcription factor map overlaid and split open. We have shown that Alx neurons (subset of V2 in spinal cord) are present in the medial glutamate stripe. Dbx1 expression was found in neurons in the middle glutamate stripe. **(A3)** Illustration of the possible transcription factor patterning present in hindbrain. Dotted white lines indicate a boundary between basal and alar derived neurons. Red and green ovals represent glutamatergic and glycinergic stripes respectively. Alx and Dbx1 expression align with the two medial glutamatergic stripes. Based on the spinal transcription factor map within progenitor zones, it is possible that the medial glycine stripe expresses Engrailed1 and that the middle glycine stripe is derived from Dbx1 expressing progenitor cells. **(B-C)** Age-related organization of alx neurons in spinal cord and hindbrain. In the spinal cord, old neurons are dorsal and younger neurons are ventral (**B1**, [18]). In the hindbrain, the age-related patterning is flipped from that of spinal cord (**B2**). In both spinal cord (**C1**) and hindbrain (**C2**) age-related patterning is present in the alx neuropil, with older (red) neuropil shifted dorsally to younger (green neuropil). **(D-E)** Summary of the ventral to dorsal patterning within hindbrain that corresponds to a dorsal to ventral patterning within spinal cord. Alx neurons are patterned into a stripe projecting dorsoventrally in hindbrain (**D1**), while in spinal cord alx neurons are present in a mid-dorsoventral region (**E1**). In the hindbrain, alx neurons are aligned from ventral to dorsal for age (**D2**), input resistance (**D3**), and possibly recruitment during swimming (**D4**). In spinal cord, alx neurons are organized from dorsal to ventral for age (**E2**, [18]), and input resistance (**E3**, [19]), and for recruitment during swimming (**E4,** [18, 29]). Scale bars = 10 um (**C1-2**), 20 um (**A**,**B**,**D**,**E**).


Spinal cord
Hindbrain
A1
A2
A3
p0
p1
p2
pMN
p3
V0
V1
V2
Dbx1
Eng1
Alx
Shh
Alx Dbx1
Dbx1?
Evx2
Alx
Eng1?
Age-related organization of cell bodies and wiring of alx neurons
B1
B2
Alx:Kaede
Old neurons
Young neurons
C1
C2
Dorsal
Ventral
D1
Hindbrain
Alx:GFP
VGlut:DsRed
Age
Resistance
Recruitment
Young
Old
D2
High
Low
D3
Slow?
Fast?
D4
E1
Spinal cord
Alx:GFP
Old
Young
E2
Low
High
E3
Fast
Slow
E4

shown that the medial glutamate stripe is identified by the alx transcription factor and that the middle glutamate stripe arises from the Dbx1 progenitor zone (see figure 4.2). In the hindbrain, this establishes a medial to lateral patterning of the ventral to dorsal transcription factor map of spinal cord as illustrated in figure 4.13A1-2. Therefore, a patterning by transcription factor type persists in the hindbrain of zebrafish at a time when they are freely swimming. Based on the spinal pattern of progenitor zones, we would predict that medial and middle glycinergic stripes might express Engrailed1 and Dbx1 respectively as illustrated in figure 4.13A3. This organization, present rostrocaudally across hindbrain, suggests that different neural circuits might be formed from the same early toolkit of cell types in both spinal cord and hindbrain.

Age-related patterning

Since spinal alx neurons exhibit an age-related topography that is correlated with a dorsoventral map of swimming recruitment [18], we examined the transition caudal to rostral from spinal cord to hindbrain. We have shown here that the Alx stripe is rotated in spinal cord (figure 4.6). This rotation of the stripe actually transforms the age-related patterning previously described for spinal cord to flip relative to hindbrain (figure 4.13B1-2), where the oldest alx neurons in spinal cord are dorsal while those within hindbrain are ventrally positioned within the alx stripe. This age-related flipping is correlated with a dorsoventral flipping of input resistance and swimming recruitment maps between spinal cord and hindbrain (figure 4.13D-E). A dorsoventral rotation of the stripe gradually occurs in the transition from spinal cord to hindbrain until the stripe is dorsoventrally aligned and medially positioned relative to the other stripes in hindbrain (figure 4.13B1-5). Since the transition between spinal cord and hindbrain is continuous, understanding how patterns of functional

organization within spinal cord transform within hindbrain to map onto stripes could reveal new principles of organization of hindbrain. For example, determining how other patterns such as the functional topography within spinal cord that crosses transcription factor classes [19] as well as the shifting from one neuron class to another for different speeds of swimming [29] are reflected in hindbrain could reveal wiring principles within and across other transcription factor stripes than just alx.

Age-related wiring might indicate functional segregation within neuropil regions

We have described here another striking patterning based on the age of a neuron. In the hindbrain, ventral (older) neurons project within dorsal regions of the neuropil while dorsal (younger) neurons project to more ventral neuropil regions (figure 4.13C2). This patterning is present continuously across both hindbrain and spinal cord (figure 4.13C1). Since the age of a neuron denotes the speed/strength of the behavior in which it is recruited, then this age-related neuropil segregation could indicate a functional segregation of wiring for cells within the same transcription factor class, but of different ages.

In the hindbrain, this age-related wiring could be more broadly present within other stripes as well. If this is the case, then an orderly patterning of projections could exist for stripes in two levels. First, cells within different stripes could project to different regions; for example, here we have shown that all alx neurons are predominately ipsilateral descending. Alx projections are also medially positioned within neuropil regions (data not shown). This might create a mediolateral segregation within neuropil regions for projections from different stripes. Within a stripe, age-related wiring would subsequently divide neuropil regions dorsoventrally as was shown in hindbrain for alx neurons. This sectioning of neuropil regions could enable an orderly patterning within and across stripes with mediolateral neuropil

regions allowing wiring to different stripes and dorsoventral neuropil regions across the mediolateral axis allowing cells to wiring within a stripe to cells of different ages. This simple organization of transcription factor and neurotransmitter stripes might enable orderly wiring to occur for all different types of neural circuits in hindbrain. One possibility is that a stripelike organization allows for an orderly patterning of projections within and across stripes and this, followed by a nuclear organization, is a stepwise approach to organizing neural circuits where cells are fated and wired in an orderly stripe-like manner, and then, cells move into nuclei to minimize wiring costs or maintain wiring locally for a specific nucleus.

Are stripes a transiently present wiring motif?

We have provided evidence in this study that the hindbrain of larval zebrafish is divided into rostrocaudally and dorsoventrally projecting stripes. However, our understanding of hindbrain organization is shaped by a segmental organization where segments across hindbrain produce distinct cell types that later coalesce into nuclei that perform distinct functions within sensorimotor circuits. If stripes represent an orderly patterning of neural circuits, then hints of the orderly arrangement might persist after nuclei have formed (in the same way that segmental organization persists after physical segmental boundaries disappear).

In one region of hindbrain, rhombomere 6, the age-related patterning was shown to be flipped relative to the rest of hindbrain. Not only are the youngest born cells in this region ventral, but they are also the ventral most alx cells in all of hindbrain. These late born cells have potentially migrated a significant distance to be displaced at the ventral edge of hindbrain; however, we showed that the age-related map of input resistance is preserved in this region (figure 4.11). This suggests that some principles of hindbrain organization, such as the correlation between the age of a

neuron and its input resistance, are preserved even after neurons have migrated to their final positions in the brain. Understanding how many of these principles such as age-related wiring are preserved when a nuclear organization is established could provide insight into a functional organization within hindbrain nuclei. We conclude that there is evidence of a simple structural and functional order in hindbrain at a time when the fish is already freely swimming, but before the more complex arrangements of nuclei have arisen that may provide insight into a shared patterning from which all hindbrain circuits might arise.

Methods

Confocal imaging

Transgenic zebrafish were anaesthetized in 0.02% MS222 and embedded in 1.4% low melting point agar, typically with the dorsal side down and resting against a glass coverslip floor of a small petri dish. Agar was covered with 0.02% MS222 solution in 10% Hanks to prevent desiccation. Images were collected using an inverted Zeiss LSM 510 confocal microscope. Green fluorescence was excited using 488 nm laser light and red fluorescence using a 543 nm laser. Green fluorescence emission was collected using a filter to collect light from either 505-530 nm or 505-550 nm and red fluorescence emission was collected using a long pass 560 nm filter. Single image stacks were collected throughout the dorsoventral extent of hindbrain or spinal cord and generally collection last 40 minutes to 1.5 hours. To prevent photo damage, images were collected with high gain settings and averaged either twice or four times. Fish remained healthy and anaesthetized during this time.

Colocalization

Confocal image stacks containing green and red channels were observed using Imaris software (BitPlane). Using the colocalization add-on, minimum thresholds were determined for red and green channels slightly above noise levels for each channel. We ensured that the dimmest cells were not thresholded out. A new channel was generated using the Imaris colocalization add-on that represented colabled signal. For image stacks with low noise levels in both channels, the automated colocalization feature in Imaris was adequate for our purposes, but, in most cases, thresholds required adjustments by hand due to high noise levels producing spurious results when observing fish in three-dimensional maximum intensity projections.

Stochastic labeling of neurons

Stochastic labeling was performed as described previously in [209]. Briefly, single cell embryos were positioned in wells within an agar plate (1.5% DNA-grade agarose in 10% Hanks with methylene blue). To label early born neurons, injections of small volumes of constructs (30ng/uL for each construct when injecting two simultaneously) were performed into single cells within 45 minutes of fertilization when eggs remained at the single cell stage. To label later differentiating neurons, embryos were injected at approximately the 1000 cell stage into the yolk. Embryos were screened using a Leica fluorescent dissecting scope at either 3 or 4 dpf for embryos with only 1-3 cells labeled in the brain. Fish were imaged using a confocal microscope at 5 dpf.

Photoconversion of Kaede

5-6 embryos between 24 hpf and 55 hpf (all of one age) were placed in drop of 10% Hanks solution within a glass-bottomed well in a small Petri dish. The petri dish was positioned on the inverted confocal microscope and a mercury bulb with a DAPI filter

was used to photoconverted fish. UV exposure typically lasted between 10 seconds and 40 seconds.

Targeted whole-cell patching of alx neurons in exposed brain preparation

Whole-cell patch in the exposed brain was performed as described previously in [214]. For our experiments, however, cells were targeting using GFP or Kaede expression in transgenic lines. To perform fluorescence targeting, we used low intensity excitation to avoid photodamage. An integrating CCD camera controlled by IPLabs software was used for the acquisition of fluorescence images integrated over 500 msec to detect fluorescent neurons. Switching between visible light and fluorescent allowed for the targeting of cells in the transgenic line. When in the proximity of a possible target, fluorescence was verified one final time before whole-cell patching was attempting, but most of the targeting was performed using visible light to avoid damage from increased exposure to excitation light.

Input resistance measurements

Both of these methods were performed as described previously in [19]. Briefly, small hyperpolarizing pulses that resulted in changes < 10 mV were applied for 5 different current values, each averaged over 5 pulses (500 msec each). IV plots indicated that these pulses maintained a linear current-voltage relationship and the slope of this was measured to determine the input resistance of each cell.

Kaede quantification

Confocal image stacks were rotated by 90 degrees using Imaris software (Bitplane). Neuropil regions were cropped in cross section and a series of tiffs was generated. A series of tiffs within a local region where the dorsoventral and mediolateral extent of

the neuropil region was similar was analyzed using Matlab (generally 15-100 slices). To generate an image of this region, tiffs were summed and divided by the number of sections to show a raw image of red and green intensities. To quantify the dorsoventral extent of red and green expression, voxels at the same dorsoventral position were summed across all dorsoventral positions. Each value was divided by the maximum value of the distribution to generate a normalized distribution since the absolute intensity differences between red and green expression might be related to imaging conditions and not differences in the distribution of red and green.

Neuronal tracing

Neurons were traced using the filament reconstruction feature provided in the Imaris software packed (Bitplane). For image stacks with low noise levels, automated reconstructions were performed choosing a diameter for a starting point (cell body) and a minimum diameter for end points along projections from the starting point. A threshold was used to adjust the number of start and end points for optimal tracing of single neurons. In cases where noise levels were high, the autodepth feature of the Imaris filament software was used to trace neurons by hand in two-dimensions and allowing the program to determine the depth of the tracing. Reconstructions were constantly verified to be accurate representations of the labeled cell and were used in this study since fine processes were hard to depict in two-dimension projections of confocal image stacks.

Calcium indicator labeling and imaging

Calcium indicator labeling was performed as described previously [213]. Briefly, embryos were collected and sorted into wells formed within an agar plate (made using a plastic mold) approximately 2 hours after fertilization. During the blastomere stage,

a 0.01-0.05% Calcium Green Dextran 3000 MW (Invitrogen) solution in 0.2M KCl was injected into the yolk of approximately 100 alx:DsRed embryos. Although the volume injected was not measured, we used approximately a 30-70 ms pulse at 30-40 PSI with an approximate tip size of 1-5 microns. Larval fish were screened at either 4 or 5 dpf for bright green label, and those fish were selected for calcium imaging experiments.

At 5 dpf, fish were anaesthetized and then immobilized in alpha-bungarotoxin (Sigma-Aldrich) dissolved in extracellular solution. After 2 minutes in an alpha-bungarotoxin solution, fish were transferred to a petri dish with extracellular solution for 10 minutes. Fish were embedded in 1.5% low melting point agar dissolved in extracellular solution. Using blue light and a stimulating electrode positioned against the skin of the fish near the tail, bouts of activity were elicited while a time series of images of both Calcium Green Dextran and DsRed channels were collected. ROIs (regions of interest) were hand drawn in the LSM5 software and text files were exported for further analysis in Matlab.

CHAPTER 5

CONCLUSIONS

Stripes represent an organization of interneurons in hindbrain

In this thesis, I have shown a new patterning of interneurons in the form of stripes in the hindbrain. In chapter 2, I showed that these stripes are present at the earliest times and that an age related organization is present within a stripe where old neurons are ventral and younger ones fill in more dorsally within each stripe. This age-related organization persists, in most cases, at 4 dpf, a time when zebrafish are freely swimming, and thus, hindbrain circuits are functional. In chapter 3, I showed that these stripes represent a wiring template for glycinergic interneurons, both broadly across multiple rhombomere segments, and more specifically within the startle response circuit. In chapter 4, I addressed the organization within a single stripe and found that, along with the age-related organization of cell bodies, there is an age-related segregation in the projections of these neurons where older neurons are ventrally positioned and project dorsally within hindbrain neuropil regions and both dorsally and laterally within spinal regions and younger neurons are more dorsally positioned and project ventrally within hindbrain neuropil and more laterally within spinal cord. I also showed that the age-related organization is correlated with a map of input resistance, where older, ventral cells have low input resistance values and younger, dorsal cells have higher input resistances and that this could correspond to differences in recruitment where older, ventral cells are recruited only during the strongest movements and younger, more dorsal cells are recruited during weaker movements, as observed previously within spinal circuits [19].

This organization described in chapters 2-4 was shown to be present continuously across multiple rhombomere segments. This suggests that the orderly stripe patterning might represent a toolkit for a diverse array of hindbrain neural circuits. For example, we have predicted that some of the oldest neurons across stripes might participate in the coordination of the escape behavior, where oculomotor circuits must adjust to the rapid startle movement, pectoral fins must move in towards the body, and the gating of sensory inputs such as the lateral line must occur to both initiate and maintain a fast escape maneuver away from a predator. If this is the case, then some of the more dorsal, younger neurons might participate in the sensorimotor circuits involved in the coordination of slower movements.

The functional implications of an age-related order and an input resistance map present dorsoventrally within a stripe were suggested to us by the simple principles recently described for spinal interneurons coordinating different speeds of swimming. In the next section, we directly compare our results to the organization of spinal circuits. We use principles of spinal organization to make new predictions about the broad patterning of stripes.

Principles that can be derived from the relationship between spinal cord and hindbrain

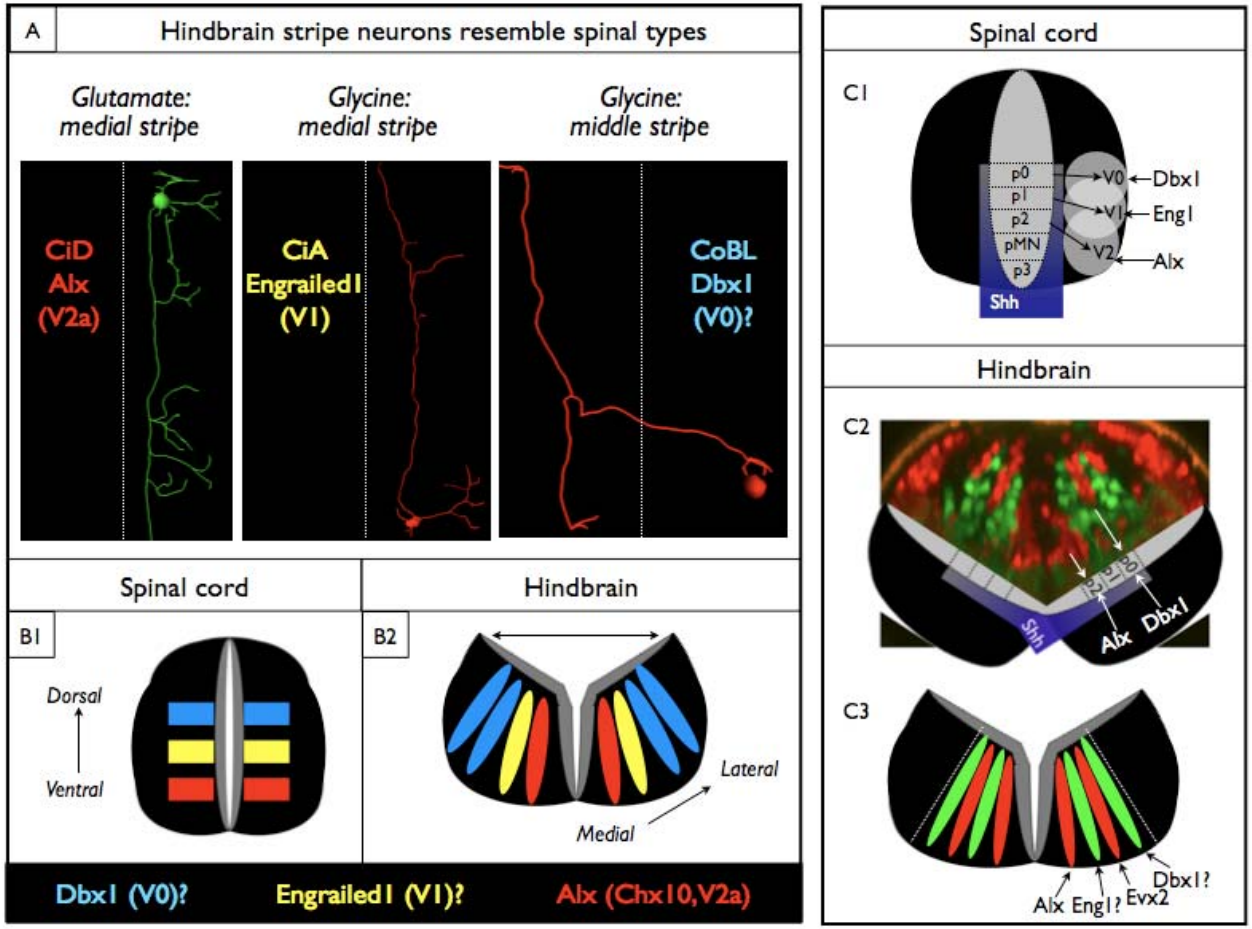
Neurotransmitter stripes have been shown in this study to define morphological classes of hindbrain interneurons. In figure 5.1A, three examples of neuron reconstructions in dorsal views are shown that represent the morphological classes of three different stripes. In the left panel, a neuron within the medial glutamate stripe indicates the key features of this stripe: ipsilateral and predominately descending (chapter 4). In chapter 4, I showed that this medial glutamate stripe expresses the alx transcription factor. In the spinal cord, alx neurons are also

glutamatergic and ipsilateral and predominately descending [18]. Neurons in this transcription factor class are active during motor behaviors in both zebrafish and mice [10, 18, 19, 29, 135]. Previous studies have shown that these neurons are derived from a ventral progenitor zone termed p0 (see bottom left panel in 1A) and express the transcription factor Nkx6.1 [3, 18].

In the middle panel of figure 5.1A, a neuron within the medial glycinergic stripe is shown that is ipsilateral and ascending. In chapter 3, we indicate that CiA neurons within spinal cord are also glycinergic interneurons with ipsilateral and ascending morphologies. CiA neurons in zebrafish and xenopus express the Engrailed1 transcription factor and play a role in both sensory gating and motor coordination of swimming [17, 22, 135]. Engrailed neurons are derived from the p1 domain, just dorsal to the p2 domain where progenitors producing alx neurons are positioned (see figure 5.1A bottom left, red bars indicate p2 zones that produce alx neurons and yellow bars indicate p1 zones that exclusively produce Engrailed1 neurons), that expresses the Pax6 transcription factor. In mice and chicks, Engrailed1 neurons form a class of ipsilateral glycinergic interneurons termed V1 interneurons that are more heterogeneous in their morphologies than those of zebrafish and xenopus. In mice, these V1 neurons were evidently subdivided so that different types perform the functions present in a single zebrafish Engrailed1 cell type [2, 26].

In figure 5.1A, the right panel shows an example of a middle glycine stripe neuron. These neurons are contralateral and branching (see chapter 3) and resemble CoBL neurons in spinal cord (see discussion in chapter 3). CoBLs are inhibitory spinal neurons that project contralaterally and bifurcate [16] . These neurons are active during swimming and struggling [172] and might represent the inhibitory population homologous to V0 neurons in mice. In mice, V0 neurons play a role in motor coordination [1] and are derived from the p0 domain that expresses Dbx

Figure 5.1 Topological transformation of transcription factor organization from spinal cord to hindbrain **(A)** Hindbrain cell types resemble spinal cell types. In **A**, three reconstructions are shown for neurons belonging to different stripes (top). In chapter 4, we showed that Alx neurons populate the medial glutamatergic stripe and are predominately ipsilateral descending neurons, resembling CiDs in spinal cord that express the Alx transcription factor. An example of an alx neuron is shown in the top left panel of **A**. Alx is homologous to Chx10 in mice, known to be expressed in V2a neurons, also ipsilateral glutamatergic neurons. Medial glycinergic neurons were shown in chapter 3 to be ipsilateral ascending cells and resemble CiA neurons in spinal cord. These spinal neurons have previously been shown to express the Engrailed1 transcription factor and correspond to V1 interneurons in mice. An example of this cell in hindbrain is shown in the top, middle panel of **A**. Middle stripe neurons are contralateral branching cells and resemble CoBL neurons within spinal cord (**A,** top, middle panel, example in hindbrain). This spinal neuron class might express Dbx1 and be homologous to inhibitory V0 neurons in mice. In the bottom panel in A, an illustration of the progenitor zones that give rise to the three cell types described above is shown on the left for spinal cord. These zones are dorsoventrally segregated. In the hindbrain, the neurons shown above populate different neurotransmitter stripes in freely swimming zebrafish (bottom, right). The medial to lateral patterning of hindbrain neurons corresponds to the ventral to dorsal patterning of progenitor zones by transcription factor previously described for spinal cord (bottom) panel. In **C1**, the spinal cord patterning is illustrated with progenitor zones orderly patterned from ventral to dorsal, but differentiated neurons arising from these different zones become intermingled. In the hindbrain, differentiated neurons maintain a patterning of these cell types, but a medial to lateral one, as if the spinal cord map were split open (**C2**). In **C2**, white arrows indicate positions of Alx and Dbx1 neurons described in chapters 2 and 4. Based on the positions of these neurons, it is possible that the medial glycinergic stripe expresses Engrailed1 and the middle glycinergic stripe expresses Dbx1 (illustrated in **C3**).


A
Hindbrain stripe neurons resemble spinal types
Glutamate:
medial stripe
Glycine:
medial stripe
Glycine:
middle stripe
CiD
Alx
(V2a)
CiA
Engrailed1
(V1)
CoBL
Dbx1
(V0)?
Spinal cord
Hindbrain
B1
B2
Dorsal
Ventral
Lateral
Medial
Dbx1 (V0)?
Engrailed1 (V1)?
Alx (Chx10,V2a)
Spinal cord
C1
p0
p1
p2
pMN
p3
Shh
V0
V1
V2
Dbx1
Eng1
Alx
Hindbrain
C2
Shh
Alx
Dbx1
C3
Alx
Eng1?
Evx2
Dbx1?

[21, 138]. The p0 domain is located dorsal to both the p1 and p2 domains as illustrated by the bottom left panel in figure 5.1A.

In the bottom right panel of figure 5.1, the three stripes are color coded for their possible transcription factor expression patterns based on their morphology. The red medial stripe is identified by the alx transcription factor and these hindbrain neurons have similar morphologies to the spinal type (see chapter 4). This medial hindbrain stripe pattern maps to a ventral progenitor domain in spinal cord (figure 5.1A bottom left). Just dorsal to the alx progenitor domain in spinal cord is the Engrailed1 progenitor domain (5.1A, bottom left). Similar neurons in hindbrain are laterally positioned relative to alx neurons and form a stripe (5.1A, bottom right). Dorsal to the Engrailed1 domain is the Dbx domain in spinal cord (5.1A, bottom right). Neurons in hindbrain that express Dbx1 are positioned laterally to alx neurons (chapter 4). In chapter 4, we also showed that medial Dbx1 neurons were glutamatergic. Neurons with a similar morphology to inhibitory V0 neurons were shown to be positioned within the middle glycine stripe (chapter 3). This pattern is illustrated by two blue stripes laterally positioned to the alx and potentially Engrailed-1 expression stripes in the bottom right panel of figure 5.1A.

The bottom panel of figure 5.1A illustrates the topological transformation between spinal cord and hindbrain. In spinal cord (dorsal), a ventral to dorsal map of progenitor domains is evident (alx to Engrailed1 to Dbx1 from ventral to dorsal). In the hindbrain, a medial to lateral map of neurotransmitter stripes (alx to possibly Engrailed1 to Dbx1 from medial to lateral) is present that appears to correspond with the ventral to dorsal progenitor domains in spinal cord. Therefore, the transformation from spinal cord to hindbrain of the transcription factor map is from horizontal domains to more vertical ones. In the spinal cord, the map is transiently present in the form of a progenitor mapping, but is quickly obscured as the neurons arising from the

different domains migrate and intermingle. In contrast, the organization persists within hindbrain in the form of neurotransmitter stripes of alternating excitatory and inhibitory neurotransmitter phenotype from medial to lateral even at an age when larval zebrafish are freely swimming.

We have revealed three important findings that allow us to compare the spinal cord organization to that of hindbrain to make predictions about functional classes of neurons within hindbrain: cell types within hindbrain resemble spinal types by both morphology and transcription factor expression (figure 5.1A); the patterning between spinal cord and hindbrain is continuous for both transcription factor and neurotransmitter expression patterns (see chapter 2); and the patterning by transcription factor and neurotransmitter expression persists in larval zebrafish hindbrain in the form of stripes that are mediolaterally segregated (see chapters 2 and 4).

In figure 5.1C, we directly compare the ventral to dorsal transcription factor map of progenitor cells to the medial to lateral patterning of neurotransmitter stripes. In figure 5.1C1, the horizontal domains of progenitor pools that give rise to different progenitor domains is shown, and in figure 5.1C2, the vertical domains of stripes of differentiated neurons is shown. In figure 5.1C3, we predict the transcription factor identity of stripes where this has not been identified by comparing the positions of known transcription factors between spinal cord (figure 5.1C1) and hindbrain (figure 5.1C2).

We predict that the medial glycinergic stripe that is positioned in-between these domains in hindbrain expresses the Engrailed1 transcription factor (figure 5.1C3) and suggest that these neurons might play similar roles in hindbrain circuits of sensory gating and the coordination of behaviors at different speeds. The next stripe of unknown transcription factor identity is the middle glycinergic stripe that contains

contralaterally branching neurons (figure 5.1A, top right) that resemble CoBLs in spinal cord that could be related to inhibitory V0 neurons in mice. Therefore, we predict that the middle glycinergic stripe expresses Dbx1 (figure 5.1C3) and could be involved in the left-right coordination of motor behaviors.

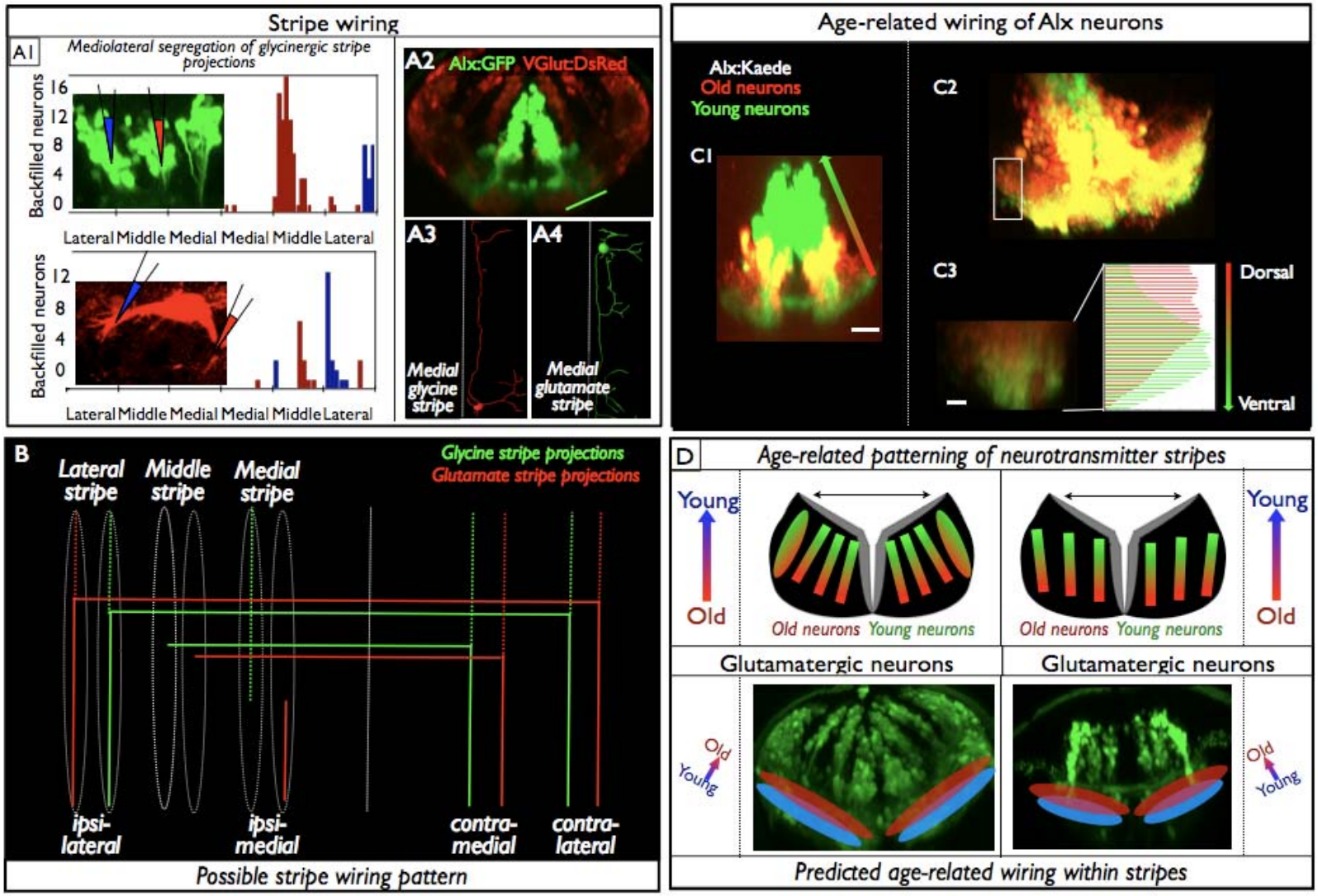
From this mapping, we can predict that the medial glutamatergic stripe contains V2a neurons, the medial glycinergic stripe contains V1 neurons, and the middle glutamatergic and glycinergic stripes contain V0 neurons. All of these neuron types perform specific roles in motor coordination within spinal cord (see chapter 1 for a review), and we predict that these hindbrain neurons might be involved in similar functional roles. If this is the case, then not only do stripes identify unique morphological and transcription factor classes, but they identify functional subtypes within hindbrain for a variety of neural circuits involved in motor control.

Predictions for orderly wiring within and across neurotransmitter stripes

Since these neurotransmitter stripes identify neurons with unique morphologies, I next investigated whether they form the foundation for orderly wiring within hindbrain. In chapter 3, I showed that this was the case for glycinergic neurons, where medial stripes project more medially and lateral stripes project more laterally, both broadly (figure 5.2A1, top) and locally within the Mauthner escape circuit (figure 5.2A1, bottom).

In figure5. 2A2, a cross section of a dual expression larval zebrafish shows the medial alx transcription factor stripe (green) that aligns with the medial glutamatergic stripe (red). The green bar indicates neuropil regions of alx neurons and shows that this medial glutamatergic stripe maintains medial projections, as was the case for medial glycinergic stripes. In figure 5.2A3-4, examples of medial glycine and

Figure 5.2 Patterns of orderly projections of hindbrain stripes. **(A-B)** Stripes identify populations with unique projection patterns in hindbrain. In **A1**, results described in chapter 2 are shown that revealed a mediolateral segregation of the projections of neurons in different glycinergic stripes. Middle stripe neurons project medially on the contralateral side, and lateral stripe neurons project laterally on the contralateral side both within stripe regions (top) and within the Mauthner escape circuit (bottom). **(A2)** A cross section of a dual expression transgenic fish of Alx:GFP x VGlut:DsRed shows that Alx neurons populate the medial stripe. The green bar indicates neuropil regions of this medial stripe. Note the medial position of the green bar. **(A3-4)** Medial stripe neurons of both glycinergic and glutamatergic neurotransmitter types are ipsilaterally projecting. In chapter 3, we show that the medial glycinergic stripe projects medially and in **A2** it is clear that the medial glutamatergic stripe projects medially as well. In **B**, an illustration of a possible wiring template of neurotransmitter stripes is shown in a dorsal view. On the left, stripe regions are illustrated with white ovals. Glycinergic projections are shown in green bars and glutamatergic stripe projections are illustrated with red lines. Solid vertical lines are descending projections and dotted lines are ascending projections and horizontal lines indicate contralateral projections. If the middle and lateral glutamatergic stripes project in a similar fashion to the middle and lateral glycinergic stripes as described in **A2-4** for the medial stripe of both neurotransmitter types, then an orderly wiring of excitatory and in inhibitory projections could create a wiring template that follows simple rules. **(C-D) Age**-related wiring within a stripe. In **C1**, a cross section on an Alx:Kaede transgenic fish photoconverted at 33 hpf and imaged at 2 dpf shows a segregation within the neuropil based on age (red projections are from older neurons than exclusively green projections). In **C2-3**, an illustration of the quantification of red and green distributions within a neuropil region (**C3**, left) indicates a dorsoventral segregation within the neuropil with older projections dorsal to younger ones (**C1** and **C3**, right). These results are discussed in more detail in chapter 4. In the top panels in **D**, an illustration of the age-related patterning of cell bodies is shown for both glutamatergic and glycinergic stripes (discussed in chapter 2). The medial glutamate stripe expresses Alx; therefore, all stripes maintain an orderly patterning of cell bodies of old to young from ventral to dorsal. If all stripes maintain an age-related order of projections then the neuropil would be segregated dorsoventrally by age as shown in the bottom panel of **D**.


Stripe wiring
A1
Mediolateral segregation of glycinergic stripe projections
Backfilled neurons
16
12
8
4
0
Lateral Middle Medial Medial Middle Lateral
Backfilled neurons
12
8
4
0
Lateral Middle Medial Medial Middle Lateral
A2
Alx:GFP VGlut:DsRed
A3
Medial glycine stripe
A4
Medial glutamate stripe
Age-related wiring of Alx neurons
Alx:Kaede
Old neurons
Young neurons
C1
C2
C3
Dorsal
Ventral
B
Lateral stripe
Middle stripe
Medial stripe
Glycine stripe projections
Glutamate stripe projections
ipsi-lateral
ipsi-medial
contra-medial
contra-lateral
Possible stripe wiring pattern
D
Age-related patterning of neurotransmitter stripes
Young
Old
Old neurons Young neurons
Old neurons Young neurons
Young
Old
Glutamatergic neurons
Glutamatergic neurons
Old
Young
Old
Young
Predicted age-related wiring within stripes

glutamate stripe neurons are shown, and in both cases, these neurons are ipsilaterally projecting. Therefore, medial glutamate and glycinergic neurons project medially on the ipsilateral side.

One possibility, then, is that glutamatergic stripes follow the orderly wiring template of glycinergic stripes. This is shown in figure 5.2B in an illustration shown in dorsal view. Green lines indicate projections of glycinergic neurons that were discussed in chapter 3, and red lines indicate predictions for the wiring of medial, middle, and lateral glutamatergic stripes based on the patterning of the medial, middle, and lateral glycinergic stripes. White ovals on the left indicate stripe regions of each neurotransmitter type. We suggest that one possibility is that, like the relationship between medial glycinergic and glutamatergic stripes shown in figure 5.2A2-4, there is a relationship for both middle glycinergic/glutamatergic and more lateral glycinergic/glutamatergic stripes in their projections. If this is the case, then an orderly patterning of excitatory and inhibitory projections exists across hindbrain and is organized in a stripe like manner as illustrated in figure 5.2B. This could allow for a wiring template of interneurons to exist across multiple hindbrain segments that would contribute to a variety of hindbrain neural circuits.

I also investigated the patterning of projections within a single stripe in chapter 4. We showed by photoconversion experiments using the alx:Kaede transgenic line that an age-related patterning of projections exists. In figure 5.2C1, a cross section of an alx:Kaede fish photoconverted at 33 hpf and imaged at 2 dpf is shown. Dorsal neuropil regions are red and ventral neuropil regions are green, indicating that projections from older (ventral) neurons are dorsal to those of younger (more dorsal) neurons. An example of the quantification of red and green distributions across the dorsoventral extent of a neuropil region is shown in figure 5.2C2-3 (see chapter 4 for more details).

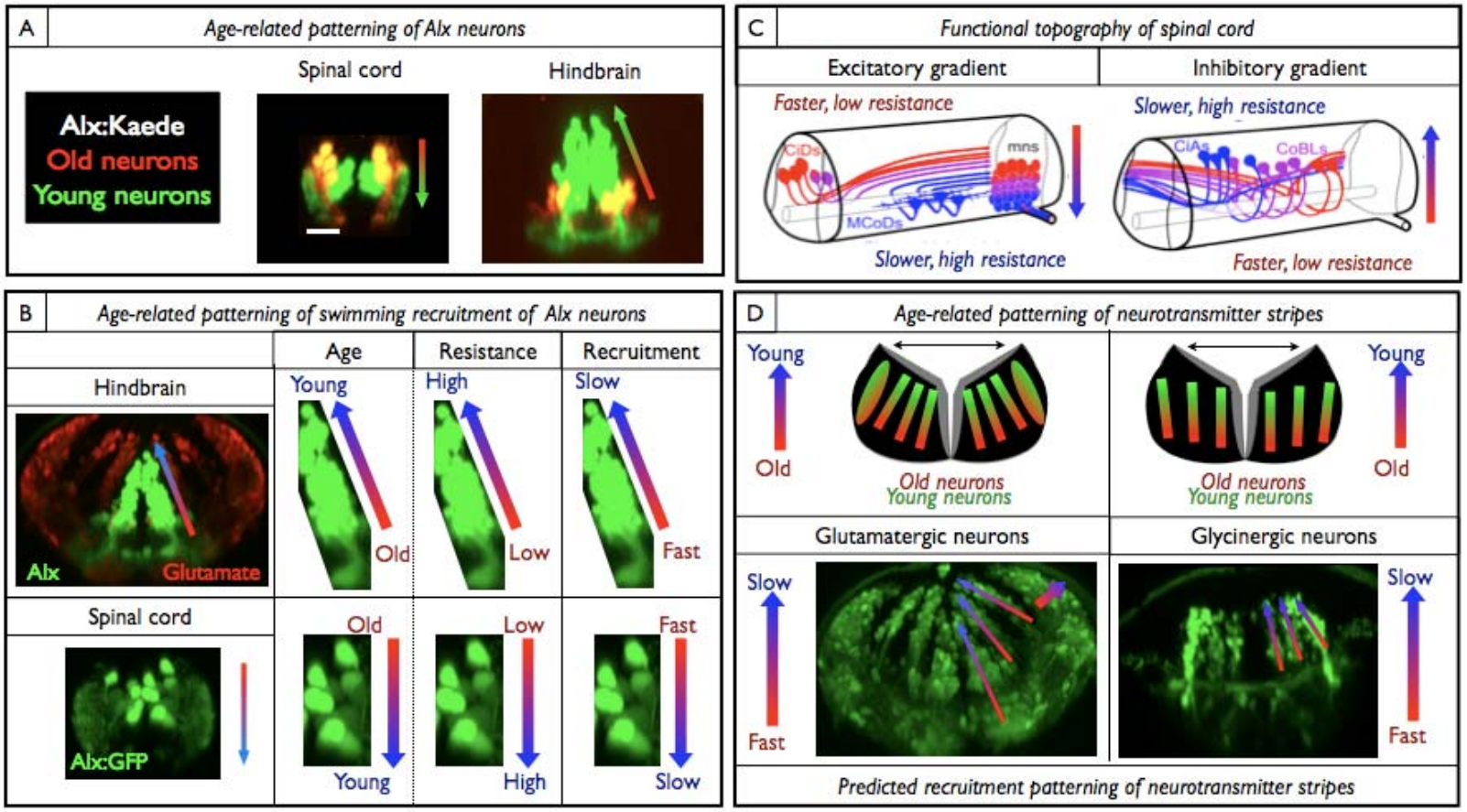
In chapter 2, I showed that, like the alx stripe shown in figure 5.2C1, all neurotransmitter stripes of both excitatory and inhibitory neurotransmitter phenotypes exhibit an age-related patterning of cell bodies with older neurons in a stripe more ventral to younger neurons within the same stripe. This is illustrated in the top panel of figure 5.2D. Since there is an organization by age of cell bodies within a stripe, it is possible that, like the alx stripe, there is an organization by age of cell projections. In the bottom panel of figure 5.2D, we illustrate this possibility for glutamatergic (left) and glycinergic (right) neurons. This would create an age-related segregation of wiring throughout the neuropil with projections from older neurons more dorsal to those from younger neurons.

If there is indeed an orderly wiring pattern of neurons based on both their stripe membership (figure 5.2B) and their dorsoventral position within a stripe, and thus their age relative to other neurons within the same stripe (figure 5.2D), then an orderly wiring template would exist across the mediolateral extent of hindbrain that is dorsoventrally segregated by age. Since the age of a neuron was shown to be correlated to its recruitment during different speeds of swimming (and its input resistance) within hindbrain (see chapter 4), this segregation of projections by age could represent a functional segregation of wiring across the dorsoventral extent of the neuropil rostrocaudally throughout hindbrain.

Predictions for a functional organization within neurotransmitter stripes

Studies within the spinal cord have revealed two principles of functional organization where both the age and input resistance of a neuron form a topographic map within and across transcription factor types (an illustration from one of these studies is shown in figure 5.3C) [18, 19]. For alx spinal neurons, older neurons have

Figure 5.3 Functional organization within hindbrain stripes **(A)** In chapters 2 and 4, the age-related map of Alx neurons was described. In the spinal cord, old neurons are dorsal and younger neurons are ventral [18] (left). In the hindbrain, the age-pattern is flipped from that of spinal cord and a stripe of alx neurons projects dorsoventrally in a straight line with old neurons (red) ventral and younger ones (green) more dorsal (right). In **B**, a summary of the ventral to dorsal patterning within hindbrain (that corresponds to a dorsal to ventral patterning within spinal cord) described in chapter 4 is shown. Alx neurons are patterned into a stripe projecting dorsoventrally in hindbrain (left panel, top), while in spinal cord, alx neurons are present in a mid-dorsoventral region (left panel, bottom). In the hindbrain, alx neurons are aligned from ventral to dorsal for age, input resistance, and recruitment during swimming (top). In spinal cord, Alx neurons are aligned from dorsal to ventral for age [18], input resistance [29], and recruitment during swimming [18, 29] ((bottom). Note that the pattern is flipped between hindbrain and spinal cord for each type of organization. In **C**, an illustration adapted from a former study of spinal cord is shown [29]. This study reported a functional topography within and across cell types in spinal cord consisting of a continuous gradient of input resistance and recruitment during swimming is present. This pattern is opposite for excitatory (left) and inhibitory neurons (right). In **D**, an illustration of the results described in chapter 2 for the development of neurotransmitter stripes is shown. Neurotransmitter stripes maintain the age related patterning of hindbrain Alx neurons with the oldest neurons in a stripe ventrally positioned and younger neurons more dorsally positioned (**D**, top). Since the age of a neuron is correlated with the input resistance and recruitment for Alx neurons in spinal cord and in hindbrain, then a possibility for the functional organization of other stripes in hindbrain is a ventral to dorsal organization for each stripe for fast to slow recruitment within hindbrain neural circuits (**D**, bottom).


A
Age-related patterning of Alx neurons
Spinal cord
Hindbrain
Alx:Kaede
Old neurons
Young neurons
B
Age-related patterning of swimming recruitment of Alx neurons
Age
Resistance
Recruitment
Hindbrain
Young
High
Slow
Alx
Glutamate
Old
Low
Fast
Spinal cord
Old
Low
Fast
Alx:GFP
Young
High
Slow
C
Functional topography of spinal cord
Excitatory gradient
Inhibitory gradient
Faster, low resistance
Slower, high resistance
CiDs
mns
MCoDs
CiAs
CoBLs
Slower, high resistance
Faster, low resistance
D
Age-related patterning of neurotransmitter stripes
Young
Old
Old neurons
Young neurons
Young
Old
Old neurons
Young neurons
Glutamatergic neurons
Glycinergic neurons
Slow
Fast
Slow
Fast
Predicted recruitment patterning of neurotransmitter stripes

lower input resistances, dorsal positions, and are recruited only during the fastest movements while younger alx neurons have high input resistances, are ventrally positioned, and are recruited during slow swimming [18, 19].

In chapters 2 and 4, we showed that the age-related map of cell bodies is dorsoventrally flipped between spinal cord and hindbrain. This is visible in figure 5.3A where cross sections in spinal cord and hindbrain of alx:Kaede transgenic fish photoconverted at 33 hpf and imaged at 2 dpf are shown. In chapter 4, we showed that a patterning of swimming recruitment and input resistance also exists along the dorsoventral direction within the alx stripe and are flipped as well. These three patterns between hindbrain and spinal cord are summarized in figure 5.3B.

Since there is good evidence for the oldest neurons having functional roles in the strongest movements, such as primary motoneurons and the dorsal alx transcription factor neurons in spinal cord [18, 76, 208] , we suggest that a functional topography, if present in hindbrain, would be correlated to the age-related patterning within neurotransmitter stripes. In figure 5.3C, the functional topography shown in a previous study indicates that excitatory and inhibitory neurons are recruited in opposite directions within spinal cord. However, the age-related patterning of cell bodies within hindbrain is in the same direction for glutamatergic and glycinergic neurons (see chapter 2) and is illustrated in the top panel of figure 5.3D. Our prediction is that a functional topography, if it were to exist in neurotransmitter stripes other than the medial, alx glutamate stripe, would be oriented in the same direction for all neurotransmitter stripes. The ventral (oldest) neurons within a stripe would be recruited for their roles in the strongest movements (or higher threshold functions) and the dorsal (younger) neurons would be recruited during slower behaviors (or lower threshold functions), as illustrated in the bottom panel of figure 5.3D.

The work described here indicates an orderly patterning of interneurons in hindbrain, where neurotransmitter stripes identify unique cell types for both morphology and mediolateral projections. Organization within a stripe was shown to be present for age, wiring, input resistance, and possibly recruitment during swimming. Organization across stripes was shown to be present by transcription factor expression, morphological features, and mediolateral wiring. We suggest that these stripes form a foundation for neural circuits in hindbrain, where interneurons are arrayed early into stripes and, later, are distributed into hindbrain nuclei. Determining how this transition from stripes to hindbrain nuclei occurs might reveal shared patterns of organization of interneurons within and across hindbrain nuclei.

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