MULTIPLE ROLES FOR *OLIG2* IN THE HINDBRAIN: OLIGODENDROCYTE AND

ABDUCENS MOTOR NEURON SPECIFICATION AND FACIAL MOTOR NEURON

MIGRATION

By

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To my parents, Tom and Lisa Zannino,

for their infinite love and support

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CHAPTER I

INTRODUCTION

Development of the Hindbrain

"The primitive segmentation of the vertebrate brain is a problem which has

probably attracted as much of the attention of morphologists as any one of the great,

unsettled questions of the day, and many views have been advanced which have, it is

true, reached one important point of agreement; namely, that the primitive brain was

undoubtedly a segmented structure. But beyond this, in regard to the character of these

segments and the number of segments of which the brain originally consisted, I think it

can be said with perfect freedom that nothing whatever has been definitely proved."

~Charles F. W. McClure 1890

Morphological observations and significance

The hindbrain, or brain stem, controls many fundamental physiological functions

essential for survival, such as respiration and heart beat, as well as consciousness,

attention and coordination. Furthermore, all fiber tracts relaying information between the

spinal cord and the brain must pass through the hindbrain, indicating its significance. In

addition, eight of the twelve cranial nerves arise from the hindbrain, which provide the

motor and sensory innervation for the face, neck and tongue. The unique pattering of the

hindbrain has long fascinated embryologists because of the well-conserved

morphological changes it undergoes during development. Shortly after the neural tube

forms the presumptive hindbrain transiently segments along the anterioposterior axis

into seven or eight bulges, first described by von Baer in 1828 (von Baer, 1828) and

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originally termed "neuromeres" (Orr, 1887). Subsequent work described the appearance and distribution of neuromeres in various species (McClure, 1890; Neal, 1918) and eventually the term rhombomere was adopted. Early studies aimed to establish the significance of hindbrain segmentation to determine whether or not a functional relationship existed between specific rhombomeres and specific neurons, with supporters on each side (Neal, 1918; Streeter, 1933; Källén, 1956; Vaage, 1969). Ultimately, these rhombomeres were found to be the result of intrinsic neural segmentation and not merely a mechanical artifact of the neural tube and adjacent mesoderm (Neal, 1918; Keynes and Lumsden, 1990). More recent work demonstrated that cellular and molecular differentiation underlies the observed morphological pattern (Lumsden, 1990; Wilkinson and Krumlauf, 1990) and that this organization is conserved across vertebrates (Gilland and Baker, 1993). Additionally, each rhombomere contains restricted cell lineages with most cells incapable of migrating across rhombomeric boundaries (Fraser et al., 1990; Guthrie and Lumsden, 1991). This restriction persists resulting in each rhombomere giving rise to different structures and cell types and thus having a unique identity (Keynes and Krumlauf, 1994; Lumsden, 2004; Moens and Prince, 2002).

Conservation of segmentation across species

The reiterated pattern long observed in the hindbrain is reminiscent of the segmental patterning in *Drosophila,* which is established by the segmental expression of a group of related genes, termed homeotic genes (Harding et al., 1985; reviewed by Gehring and Hiromi, 1986). Mutations in these genes cause the transformation of one body structure into another, a process termed "homeosis" (reviewed by Gehring and Hiromi, 1986; Lawrence and Morata, 1994). Two main clusters of homeotic genes, the bithorax complex (BX-C) and the *Antennapedia* complex (ANT-C) (Lewis, 1978;

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Kaufman et al., 1980) confer segment identity of the *Drosophila* body. BX-C and ANT-C

belong to a family of genes containing a conserved DNA sequence, the homeobox

(McGinnis et al., 1984; Scott and Weiner, 1984; Gehring, 1985). Whereas most genes

have no observed relationship between location within the genome and location of

expression in the body, the anterioposterior (AP) organization of BX-C and ANT-C gene

expression within the body corresponds to their chromosomal arrangement (Lewis,

1978; Harding et al., 1985). Homeobox genes encode transcription factors that contain a

highly conserved domain designated "homeodomain" and they, in turn, regulate the

expression of many other genes (Levine and Hoey, 1988; Thali et al., 1988; Gehring and

Hiromi, 1986; Lawrence and Morata, 1994). Thus a relatively small number of genes,

termed master control genes, are capable of regulating the developmental pathways that

will establish the identity of each body segment (reviewed by Gehring and Hiromi, 1986).

As the function of the *Drosophila* homeotic genes, collectively called *HOM-C*,

were being uncovered, researches learned that functionally similar and related genes

were expressed in the mouse hindbrain, which also contain a homeobox and were thus

named *Hox* genes (Wilkinson et al., 1989b; Murphy et al., 1989). The term "*Hox* gene" is

reserved for only the vertebrate homeobox genes related to the *Drosophila HOM-C*

(Scott, 1992). Whereas two clusters of homeotic genes regulate *Drosophila*

segmentation, most vertebrates possess four clusters of *Hox* genes (Graham et al.,

1989; Duboule and Dolle, 1989; reviewed by Hunt and Krumlauf, 1992; McGinnis and

Krumlauf, 1992). These vertebrate clusters are named A-D with 13 subfamilies called

paralogous groups in each cluster (Scott, 1992), though not all 13 paralogs are present

in each cluster. Similar to the insect *HOM-C* genes, mutations in the *Hox* genes cause

homeotic transformations (Balling et al., 1989; Kessel et al., 1990; Chisaka and

Capecchi, 1991; Lufkin et al., 1991; Le Mouellic et al., 1992; Morgan et al., 1992;

Marshall et al., 1992) and they posses the same relationship between AP body

expression and location on the chromosomes (Graham et al., 1989; Duboule and Dolle, 1989; Wilkinson et al., 1989b; reviews by: Hunt and Krumlauf, 1992; Keynes and Krumlauf, 1994; McGinnis and Krumlauf, 1992). Additionally, the order of AP gene expression and chromosomal position is also related to the timing of gene expression with more anterior genes expressed earlier than more posterior genes (Graham et al., 1989; van der Hoeven et al., 1996). The similarities between the HOM-C and the vertebrate *Hox* genes suggested that these genes were homologues of a common ancestor (Akam, 1989; Graham et al., 1989; Duboule and Dolle, 1989; Gaunt, 1991; Krumlauf, 1992). Evidence supports the hypothesis that *Hox* genes arose by two duplication events (Kappen et al., 1989), however the relationship between *HOM-C* and *Hox* genes is not one to one, suggesting independent tandem duplications (Krumlauf, 1992; Hunt and Krumlauf, 1992). Similarly, some species such as teleost fishes have extra *Hox* clusters due to duplication events, as well as many gene losses (Amores et al., 1998; Moens and Prince, 2002).

Molecular mechanisms leading to segmentation

With the great discovery of *Hox* genes and their importance in hindbrain patterning came another crucial question: what is upstream of *Hox* genes and how are they regulated? Shortly after researchers established that a *Hox* combinatorial code was necessary for a properly segemented hindbrain came the finding that retinoic acid (RA), a metabolic derivative of vitamin A, can regulate *Hox* gene expression both in culture (Simeone et al., 1990) as well as *in vivo* (reviews by Keynes and Krumlauf, 1994; Glover et al., 2006). This advance correlates with earlier work demonstrating that excess exposure to vitamin A during fetal development can cause teratogenic changes to the hindbrain (Morriss, 1972) in a stage dependent manner. Similar effects are seen with other retinoid (naturally or synthetically related to vitamin A) exposure causing the

affected hindbrain to be shorter due to a loss of proper segmentation; consistent with

this many anteriorly expressed segmental genes are altered to a more posterior

phenotype (Morriss-Kay et al., 1991; Conlon and Rossant, 1992; Marshall et al., 1992;

Sundin and Eichele, 1992; reviewed by Glover et al., 2006). These results, in addition to

the finding that RA is enriched in the primative streak (Hogan et al., 1992), led to the

hypothesis that RA is a morphogen *in vivo* and plays a role in generating proper *Hox*

gene expression boundaries (Keynes and Krumlauf, 1994). Accordingly, some of the

molecular mechanisms of RA function in the hindbrain were soon uncovered; retinoic

acid receptors (RAR) and retinoid receptors (RXR) transduce the retinoid signal and

many *Hox* genes contain retinoic response elements (RAREs) (Giguere et al., 1987;

Kastner et al., 1997; reviewed by Chambon, 1996; Langston and Gudas, 1992; Melton et

al., 2004).

In addition to RA, fibroblast growth factor (FGF) can activate *Hox* genes (Isaacs

et al., 1998; Pownall et al., 1998; Partanen et al., 1998). Subsequent work demonstrated

the existence of two classes of *Hox* genes, one of which was responsive to retinoids: the

3' end of clusters encompassing paralogous groups 1-4, and the other sensitive to

FGFs: the 5' end including paralogous groups 5-9 (Melton et al., 2004). These two

initiation cues, as well as Wnt signaling necessary for primative streak formation (Liu et

al., 1999) where RA is enriched, encompass the first of three phases of *Hox* gene

regulation, as identified by Deschamps (1999). Following the initiation phase of *Hox*

gene expression is establishment and maintenance (Deschamps et al., 1999). During

the establishment phase, which is a continuous process with the initiation phase, the

Hox expression domains expand along the AP axis until they reach their anterior-most

positions (Gaunt and Strachan, 1996; Deschamps et al., 1999). The *Hox* domains are

further modulated and refined by RA and other transcription factors (Kessel and Gruss,

1991; Deschamps et al., 1999), some of which are necessary for the specification of

individual rhombomeres. For example, *Krox20* (Wilkinson et al., 1989a)*,* a zinc-finger

transcription factor (Chavrier et al., 1988) present in every species examined, specifies

r3 and r5 (Schneider-Maunoury et al., 1993; Giudicelli et al., 2001) by regulating the

expression of *Hoxb2* and *Hoxa2* (Sham et al., 1993; Vesque et al., 1996; Nonchev et al.,

1996). Similarly, *Hoxb3* and *Hoxa3* are activated by *Kreisler,* a basic domain-leucine

zipper of the c-*maf* proto-oncogene family (Cordes and Barsh, 1994; Frohman et al.,

1993), expressed and required in r5 and r6. *Kreisler* is also known as *valentino/mafB* in

fish (Moens et al., 1996). In the maintenance phase of *Hox* gene expression auto, cross

and para-regulatory mechanisms interact to fine tune and sustain the proper expression

patterns (Melton et al., 2004). The collective coordination of *Hox* genes and other

transcription factors, such as *Krox20* and *Kreisler,* results in a segmentally organized

hindbrain, which will serve as a blueprint for the specification of sensory ganglia, cranial

motor neurons, and glial cell types that make up the central and peripheral nervous

systems.

<center>Motor Neuron Development in the Hindbrain</center>

Neuronal organization of the zebrafish hindbrain

The organization of many neuronal cell types is correlated with segmentation of

the rhombomeres. By the 18 somite stage in zebrafish, the hindbrain divides into five

prominent bulges, which are numbered r2-r6 (Kimmel et al., 1995; Moens and Prince,

2002). Similar to early morphological observations in the chick hindbrain (Vaage, 1969),

the anterior-most region can be subdivided into a r0 and a smaller r1, which, in fish,

have distinct markers and fates (Moens and Prince, 2002; Koster and Fraser, 2001). The

rhombomeric boundaries do not arise in an anterior to posterior fashion, but instead they

develop in a seemingly irregular, though very stereotypical, fashion. The first

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rhombomere to form is r4, consistent with its role as an early signaling center, followed by the r3/r4 boundary, r4/r6, r1/r2, r2/r3, r6/r7 and finally the r5/r6 boundary (Moens and Prince, 2002). Consistent with chick, no r7/r8 boundary exists in zebrafish, either by morphology or by rhombomeric boundary markers (Clarke and Lumsden, 1993; Kimmel et al., 1985; Hanneman et al., 1988). The zebrafish hindbrain, as well as other species like chick, exhibits a two-segment periodicity. Earlier work demonstrate that while cells do not migrate across neighboring rhombomeric boundaries, cells from even-numbered rhombomeres can mix with other even-numbered rhombomeres, and the same is true for odd-numbered rhombomeres (Guthrie et al., 1993). Consistent with this two-segment organization, the reticulospinal neurons located in even-numbered have a more lateral position while those in the odd-numbered rhombomeres are medially situated (Mendelson, 1986a; Mendelson, 1986b; Metcalfe et al., 1986) (Fig. 1.1). Additionally, commissural neurons and the cranial motor neurons exhibit a segmented arrangement (Gilland and Baker, 1993; Chandrasekhar et al., 1997; Moens and Prince, 2002) that is fairly conserved across species. The functional connections made by these neurons at these early developmental timepoints will persist into adulthood, long after the segmental organization of the hindbrain ceases.

The patterning of the hindbrain also determines in which rhombomeres cranial motor neurons are born. There are three classes of cranial motor neurons: somatic motor neurons, visceral motor neurons, and branchiomotor (Kandel et al., 2000) (Fig. 1.1). All three classes are found in the hindbrain, with some somatic motor neurons also found in the midbrain. Somatic motor neurons provide the innervation for eye and tongue movements, visceral motor neurons innervate parasympathetic neurons, which will in turn innervate various glands and the smooth muscle of the pulmonary, cardiovascular, and gastrointestinal systems, whereas the branchiomotor neurons innervate all of the muscles derived from the pharyngeal arches, which includes those required for jaw and

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facial movements, as well as the larynx and pharynx (reviewed by Chandrasekhar, 2004). The branchiomotor neurons leave the hindbrain through common exit points, located in the even-numbered rhombomeres, whereas all of the somatic motor neurons, except cranial nerve IV (nIV, trochlear), exit the brain via ventral rootlets (Guthrie, 2007; Chandrasekhar, 2004) (Table 1.1). For example, the cell bodies of the trigeminal cranial nerve (in the branchiomotor class) arise in r2 and r3 and all of their axons collectively exit out of r2 (Chandrasekhar et al., 1997); and the abducens cranial nerves (of the somatic motor neuron class) occupy r5 (mouse) or r5 and r6 (chick and zebrafish) and their axons exit ventrally out of these same rhombomeres (Guthrie, 2007; Chandrasekhar, 2004). Visceral motor neurons have not been definitively identified in zebrafish (Higashijima et al., 2000), nor have the hypoglossal motor neurons and their targets (Wullimann et al., 1996) (Fig. 1.1).

Neuronal specification in the hindbrain

The hindbrain is home to many different neuronal populations that arise and reside in particular rhombomeres. With the knowledge that each rhombomere has a unique combination of *Hox* genes, a logical hypothesis is that these *Hox* genes play a role the specification of neurons within specific rhombomeres (reviewed by Glover, 2001). Indeed, manipulation of various *Hox* genes does affect the differentiation of neurons (Bell et al., 1999; Grapin-Botton et al., 1995; Jungbluth et al., 1999; Kessel, 1993). However, due to the variation of location of neurons across species, as well as the presence of species-specific specialized populations, all-encompassing conclusions or correlations are hard to make. One of the best-studied neuronal populations is the motor neuron, due to the ability to trace their projections using retrograde and anterograde

Table 1.1. Cranial Motor Neuron Summary

Cranial Nerve	Class	Cell Body Location (in Zebrafish)	Axonal Exit Point
I. Olfactory	Sensory	Forebrain	Forebrain
II. Optic	Sensory	Midbrain	Midbrain
III. Oculomotor	Somatic/Visceral	Midbrain	Ventral Rootlets
IV. Trochlear	Somatic	r0	Ventral Rootlets/r0
V. Trigeminal	Branchial	r2 and r3	Dorsal r2
VI. Abducens	Somatic	r5 and r6	Ventral Rootlets/r5 and r6
VII. Facial/OLe	Branchial/Visceral	r6 and r7	Dorsal r4
VIII. Vestibulocochlear	Sensory	r6 and 7	Dorsal r4
IX. Glossopharyngeal	Branchial/Visceral	r7	Dorsal r6
X. Vagus	Branchial/Visceral	Caudal Hindbrain	Caudal Hindbrain
XI. Spinal Accessory	Branchial	Caudal Hindbrain (mouse)	Caudal Hindbrain (mouse)
XII. Hypoglossal	Somatic	Caudal Hindbrain (mouse)	Caudal Hindbrain (mouse)

Reticulospinal Neurons
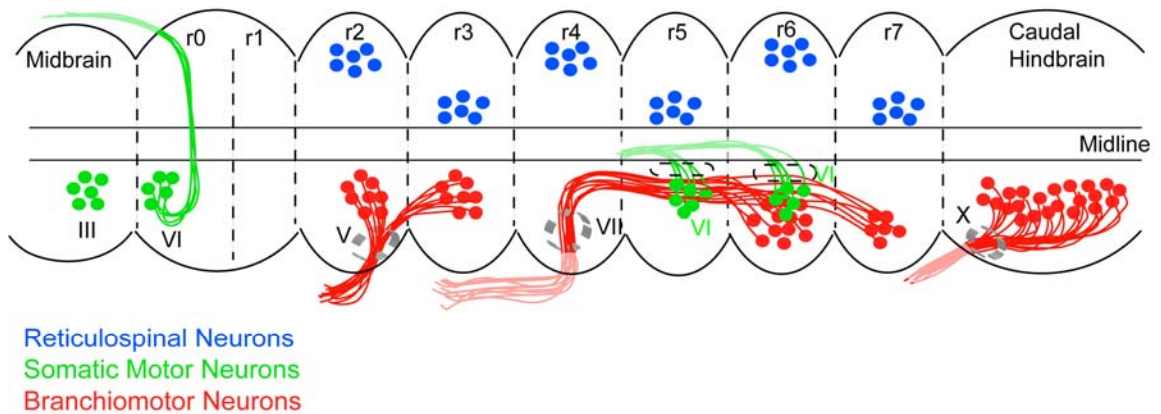**Somatic Motor Neurons**
Branchiomotor Neurons

Figure 1.1. Schematic of the rhombomeric organization of neurons in the zebrafish hindbrain. The reticulospinal neurons are located medially in odd rhombomeres and more laterally in even rhombomeres.The axons of the branchiomotor neurons exit dorsally out of even rhombomeres whereas somatic motor neuron axon exit out of ventral rootlets.

dyes. In general, the relative rhombomeric location of these cranial nerves is the same across species, though there are exceptions. Although limited, relationships between some of these cranial nerves and various *Hox* genes are being established. For example, the facial motor neurons require *Hoxb1* for their specification and subsequent migration across most species (Goddard et al., 1996). *Hoxb3,* expressed in r5 and r6, is required for the abducens motor neurons, which are born in those rhombomeres (Gaufo et al., 2003), whereas the trigeminal motor neurons require *Hoxa2* (Jungbluth et al., 1999). Because the expression of *Hox* genes is very dynamic over time and many motor neurons migrate from their rhombomere of birth, very careful and comprehensive studies are needed to understand the relationship between these genes and particular neuronal groups.

In addition to *Hox* genes, many other transcription factors necessary for the induction of hindbrain motor neurons have been identified, including a few familiar players: *Krox20* and *Kreisler,* which are required for the identity of the rhombomeres in which they are expressed (Moens and Prince, 2002). In the absence of these genes' functions the corresponding rhombomeres and subsequent neuronal specification are lost, or functionally altered. For example, *Kreisler* is expressed in r5 and r6, the same rhombomeres in which the abducens motor neurons will arise. In *valentino/Kreisler* mutant fish the abducens motor neurons are lost (Chandrasekhar, 2004). These affects are likely indirect as the entire rhombomere is affected in mutant *Krox20* or *Kreisler/valentino* animals.

Although the direct relationship between specific *Hox* genes and the specification of neuronal populations is limited, many necessary morphogens and transcription factors have been identified. One of the most broadly acting morphogens is Sonic Hedgehog (Shh), a glycoprotein which is expressed first in the notochord and then in the floor plate of the spinal cord and also induces the differentiation of the floor plate (Ericson et al.,

1995). Shh undergoes unique post-translational processing in which the protein is proteolytically cleaved and a cholesterol is then added to the C-terminal end and a palmitoyl group is added to the N-terminal (Bumcrot et al., 1995; Porter et al., 1996a; Porter et al., 1996b; Chamoun et al., 2001; Pepinsky et al., 1998). These two modifications are necessary for Shh to properly signal and act as a morphogen (reviewed by Marti and Bovolenta, 2002). Because motor neurons arise in the ventral neural tube, which is in part patterned by the floor plate, Shh was an attractive candidate for the specification of motor neurons. Indeed, researchers found that Shh can induce motor neurons both in vitro (Marti et al., 1995; Roelink et al., 1995; Tanabe et al., 1995) and *in vivo* (Ericson et al., 1996). Additionally, motor neurons are completely lost in Shh knockout mice (Chiang et al., 1996; Litingtung and Chiang, 2000). Similar effects are seen in zebrafish, however there are multiple hedgehog signals and once all hedgehog signals are removed motor neurons are almost completely lost (Krauss et al., 1993; Ekker et al., 1995; Currie and Ingham, 1996; Chandrasekhar et al., 1998; Beattie et al., 1997; Bingham et al., 2001).

Shh functions by binding to its receptor, a twelve-pass transmembrane receptor Patched, which relieves inhibition of a seven-pass transmembrane protein Smoothened, with the ultimate result of transcriptional control of the Gli family of zinc finger transcription factors (reviewed by Lum and Beachy, 2004). There are three Gli proteins regulated by Shh activity, and they can have activator or repressor functions (Jacob and Briscoe, 2003). Work in fish demonstrates the requirement of Gli activator function for the induction of motor neurons (Vanderlaan et al., 2005). Additionally, some apparent differences exist between hindbrain and spinal cord motor neuron specification, as evident by the gene *detour* that is required for cranial motor neurons but not for spinal cord motor neurons. For example, d*etour* is thought to be involved with the hedgehog pathway (Chandrasekhar et al., 1999).

Shh also functions to establish domains along the dorsoventral axis by either

inducing or repressing many homeodomain transcription factors, which will be described

more in depth in a later section. These transcription factors will then specify and regulate

neuronal and glial specification depending on their dorsoventral position. For example,

Nkx6.1, Nkx2.2 and Pax6 are required for motor neuron induction, both in the spinal cord

as well as the hindbrain (Muller et al., 2003; Pattyn et al., 2003; Briscoe et al., 1999;

Ericson et al., 1997b). In the hindbrain, the different classes of motor neurons have

unique sets of the following transcription factors: Pax6, Nkx6.1, Nkx6.2, Nkx2.2, Nkx2.9,

Phox2a and Phox2b (Ericson et al., 1997b; Osumi et al., 1997; Muller et al., 2003;

Pattyn et al., 2003; Briscoe et al., 1999; Pattyn et al., 1997; Pattyn et al., 2000).

Another class of homeobox genes required for motor neuron specification and

development in a combinatorial code is the LIM family, which in addition to its DNA

binding domain also contains two tandem repeats of the LIM domain to facilitate protein-

protein interactions (Arber and Caroni, 1996; Tsuchida et al., 1994). These genes are

involved in both cranial and spinal motor neuron specification and development (Varela-

Echavarria et al., 1996; Korzh et al., 1993; Appel et al., 1995). While each class of

cranial motor neuron has its own unique combination of essential transcription factors,

one common feature exists, they all require and express Isl1, another member of the

LIM family (Ericson et al., 1992; Appel et al., 1995; Varela-Echavarria et al., 1996; Pfaff

et al., 1996).

Somatic motor neuron specification

Four of the cranial nerves are in the somatic motor neuron class; the oculomotor

(nIII) in the midbrain; trochlear (nIV) in r0/r1 in zebrafish, mouse and chick; abducens

(nVI) in r5 in chick or r5 and r6 in zebrafish and mouse; and the hypoglossal (nXII)

located in r8/caudal hindbrain in chick and mouse but not identified yet in fish

(Chandrasekhar, 2004; Guthrie, 2007). Most studies examining the specification of somatic motor neurons tend to investigate either the more posterior neurons together (nVI and XII) or the more anterior neurons (nIII and nIV). While functionally the abducens, trochlear and oculomotor neurons are similar to each other, innervating the various muscles controlling eye movement; these neurons arise in different origins along the anterioposterior axis, which indicates that different factors are necessary for their specification. This is consistent with the overall patterning of the hindbrain by *Hox* genes in a very specific anterioposterior organization. For example, *Hoxb3* is required for abducens motor neuron specification, but is not expressed more anteriorly where the oculomotor and trochlear motor neurons are born (Gaufo et al., 2003; Guidato et al., 2003). Similarly, the bHLH transcription factor *olig2,* first expressed in r5 and r6, is required for the specification of the posterior somatic motor neurons (Lu et al., 2002; Zhou and Anderson, 2002; Gaufo et al., 2003). However, these genes are not expressed in the anterior hindbrain and thus do not play a role in the specification of the oculomotor or trochlear motor neurons.

No *Hox* genes are expressed in the anterior-most hindbrain where trochlear motor neurons are born (Jungbluth et al., 1999). Instead, different genes and organizing centers are involving in the patterning of the midbrain and anterior hindbrain. The boundary between the midbrain and hindbrain is called the isthmus or the midbrain/hindbrain organizer (MHO) and is an essential signaling center for this region of the CNS (Prakash and Wurst, 2004). FGF8 is a key gene involved with the organizing activity of the MHO and, because the trochlear motor neurons arise just posterior to this boundary, FGF8 regulates the development of the trochlear motor neurons (Prakash and Wurst, 2004; Irving et al., 2002). The oculomotor neurons rise just anterior to the MHO and require Wnt-1 for their specification, along with the trochlear motor neurons (Fritzsch et al., 1995; Prakash and Wurst, 2004). Although these differences in anterioposterior

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specification exist, some genes are common to all somatic motor neurons, such as Nkx6.1 and Isl1 (Muller et al., 2003; Ericson et al., 1992; Varela-Echavarria et al., 1996). Thus, some transcription factors are necessary for the somatic motor neuron class as a whole whereas the requirement of other factors dependents upon the anterioposterior position.

Facial motor neuron specification

The facial motor neuron provides innervation to the muscles controlling facial expressions. Their cell bodies are born in r4 in zebrafish, mouse and chick and, consequently, genes required for the proper patterning of r4, such as *Hoxb1,* are also essential for the specification of the facial motor neurons (Goddard et al., 1996; Chandrasekhar, 2004). Additionally, Phox2b is also essential for the specification of facial motor neurons; knockout mice for either *Phox2b* or *Hoxb1* fail to form facial motor neurons (Pattyn et al., 2000; Goddard et al., 1996). Another factor necessary for the proper development and maturation of the facial motor neurons is LIFRβ, a receptor complex necessary for signaling from the IL-6-cytokine family member LIF (Alfonsi et al., 2008; Bauer et al., 2007). Similar to other cells with ventral origins, facial motor neurons require Shh for their specification (Chandrasekhar, 2004).

Facial Motor Neuron Migration

Overview of the phases in facial motor neuron migration

In addition to determining the location of neuron specification, the organization of the rhombomeres also influences the migration of neurons. One of the best-studied examples of neuronal cell body movement is the facial motor neuron, which undergoes a very interesting and complex migration. This migration occurs in almost every species

examined and the facial motor neurons will give rise to the internal genu of the facial

nerve, a characteristic bend in axons of this tract (Altman and Bayer, 1982; McKay et al.,

1997). The cell bodies migrate from r4, where they are born, into r5 and r6 (mouse and

chick) or r6 and r7 (zebrafish) (Lumsden and Keynes, 1989; Chandrasekhar et al.,

1997). In zebrafish and mice, the facial motor neuron cell bodies begin their journey by

migrating posteriorly out of r4 in close proximity and parallel to the floor plate into their

target rhombomeres (Altman and Bayer, 1982; Ashwell and Watson, 1983; Auclair et al.,

1996; Chandrasekhar, 2004; Song, 2007). As they migrate caudally, the facial motor

neurons leave behind their axonal projections, which will ultimately exit the hindbrain

from r4. Once in their target rhombomeres the neurons will then migrate laterally and

later radially to ultimately reside in the dorsal neural tube (Song, 2007). Thus, there are

three separate directional movements, each requiring a unique set of genes:

anterioposterior from r4 into r6 and r7 (zebrafish), mediolateral (or just called lateral),

and radial towards the pial surface (Chandrasekhar, 2004; Song, 2007). Fig1.2 depicts

the migration of the facial motor neurons through the neural tube and some of the genes

they express.

Caudal migration

The initial caudal migration of facial motor neurons out of r4 involves a

combination of transcription factors and members of the non-canonical Wnt pathway.

Facial motor neurons require *Nk6.1,* which they normally express throughout their

journey, to initiate caudal migration out of r4, however their lateral movement is

maintained, albeit in r4 instead of the proper rhombomere (Muller et al., 2003; Pattyn et

al., 2003). Loss of *Phox2b* signaling, also expressed within facial motor neurons

throughout their migration, causes a similar phenotype, with very minimal caudal

migration prior to a lateral turn (Coppola et al., 2005). Another intrinsic gene involved

with the caudal migration is Ebf1, a helix-loop-helix (HLH) transcription factor initially

found in B lymphocytes and adipocytes (Garel et al., 2000; Hagman et al., 1995). While

similar to Nkx6.1 and Phox2b in its expression throughout facial motor neuron migration,

only a subset of facial motor neuron fails to complete their caudal migration and instead

prematurely migrate radially in r5 (Garel et al., 2000).

The rhombomeric environment also affects aspects of facial motor neuron

migration. Studer demonstrated, using transplantation studies, that local cues in r5 and

r6 were capable of initiating the caudal migration of facial motor neurons (Studer, 2001).

However facial motor neurons are still capable of short caudal migrations in the absence

of r5, such as in *Krox20* and *Kreisler* mutant mice, into r6 followed by the correct radial

migration (McKay et al., 1997; Manzanares et al., 1999; Garel et al., 2000). Therefore,

while r5 and r6 contain cues sufficient to initiate the caudal migration of facial motor

neurons, they may not be solely necessary.

Many members of the PCP pathway have been implicated for the caudal

migration of facial motor neurons. Many of these genes, best studied in zebrafish, are

broadly expressed, such as *trilobite*, a mutant of Strabismus/Van Gogh-like 2 (Jessen et

al., 2002), *off-limits/frizzled3a,* in which a putative Wnt receptor is lacking (Wada et al.,

2005; Wada et al., 2006), *land-locked/scribble1* (Wada et al., 2005)*, off-road/celsr2*

(Wada et al., 2006) and *colgate/hda1* (Nambiar et al., 2007)*.* Cell-transplantation studies

demonstrate that these PCP members primarily function non-autonomously, or outside

of the cell which expresses them (Carreira-Barbosa et al., 2003; Jessen et al., 2002;

Wada et al., 2005; Wada et al., 2006). However, one PCP component, *prickle1b (pk1b),*

functions cell-autonomously, being required for that cell's identity, and appears to be

specifically expressed within migrating facial motor neurons and weakly expressed

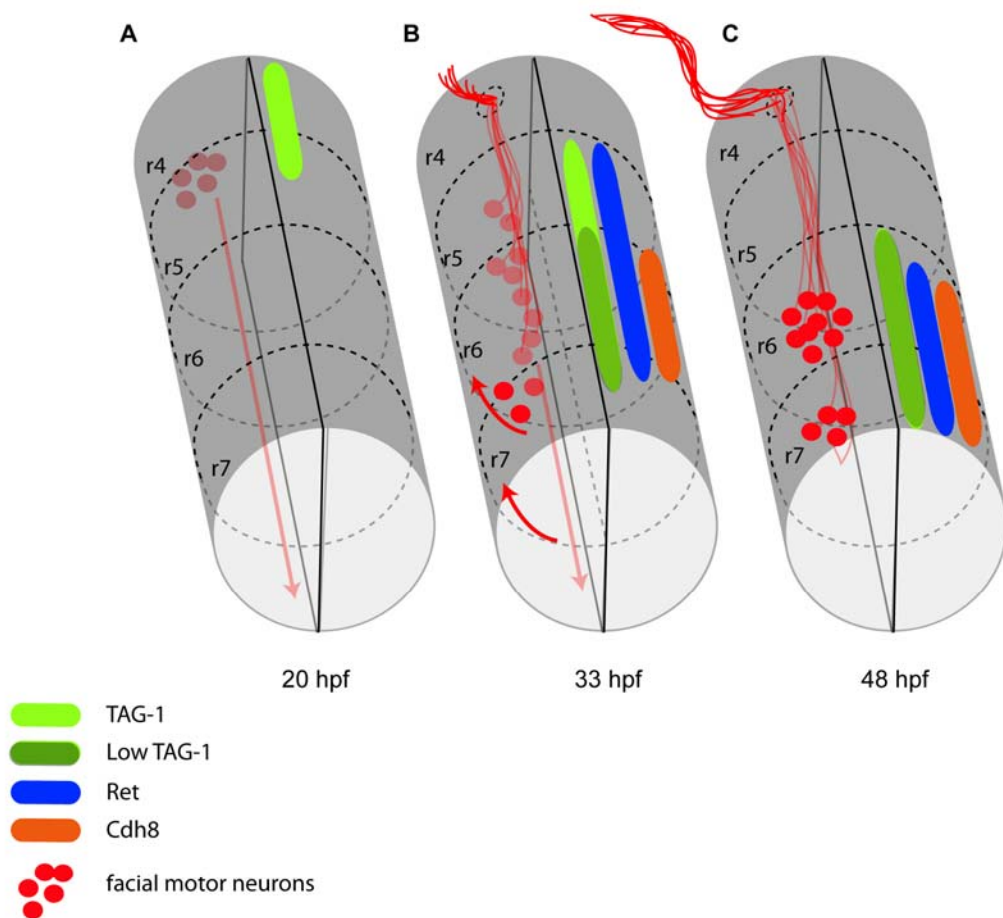within r4 (Rohrschneider et al., 2007).

Figure 1.2. Schematic of facial motor neuron migration in the zebrafish hindbrain. A. Facial motor neurons are born around 18 hpf in ventral r4 along the midline and will be gin to migrate caudally. While in r4 they express TAG-1. B. By 33 hpf the facial motor neurons have left r4, begin downregulating TAG-1 and turn out Ret. Once the reach r5 and r7 the being to migrat out laterally and radially. At this point they express Cdh8. C. At 48 hpf the facial motor neurons are in their final location, in organized clusters in r6 and r7. Only a few express TAG-1 at this stage. Throughout their migration the facial motor neurons extend their axonal projections anteriorlly, which will exit out of r4.

Lateral migration

Of the three phases of facial motor neuron migration, the least is known about lateral migration by itself, as it is most often indistinguishable from radial migration. All branchiomotor neurons, other than facial motor neurons, undergo a simple dorsolateral migration and a few factors have been identified for this type of migration in general. Two such factors include Nk6.1 and Tbx20. The trigeminal motor neurons in animals mutant for either of these two genes are capable of initiating normal migration, but fail to complete their lateral migration (Muller et al., 2003; Pattyn et al., 2003; Song et al., 2006).

Radial migration

The radial migration of neurons is best studied in the neuroepithelium that gives rise to the six layers of the cerebral cortex. Neurons migrate from the ventricular zone (VZ) along a scaffold consisting of radial glia fibers that span from the VZ to the pial surface (Kandel et al., 2000). One of the best-characterized mutant mouse models for perturbations in this migration is the *reeler* mouse, in which the order of the six layers is reversed (Frotscher, 1998). The *reeler* mouse is lacking proper *reelin* signaling (D'Arcangelo et al., 1995). Subsequent studies demonstrated that deficits in *reelin* or in *reelin* signaling, such as *cdk5* or *scrambler* mice, also affected the radial migration of facial motor neurons in their target rhombomeres, while the caudal migration was normal (Ohshima et al., 2002; Rossel et al., 2005). Interestingly, it appears that two members of the Wnt/PCP pathway, Frizzled3a and Celsr2, function to inhibit premature radial migration during the caudal migration in more anterior regions and this inhibition is only relieved once the facial motor neurons reach their proper target rhombomere (Wada et al., 2006).

Intrinsic factors regulating facial motor neuron migration

While investigators who study mutant animals uncovered many genes involved in facial motor neuron migration, less is know about the underlying intrinsic mechanisms. Three proteins expressed during specific phases of migration shed light on how theses neurons adopt such complicated and precise migration. TAG-1 and cadherin 8 (cdh8), implicated in axonal outgrowth and cell adhesion, are cell surface molecules while Ret, which is expressed in other migrating cells, is a GDNF-receptor subunit (Furley et al., 1990; Redies and Takeichi, 1996; Pachnis et al., 1993). TAG-1 is expressed in facial motor neurons as they begin their migration in r4 and persists until r6 (mouse) where it is down regulated as the facial motor neurons turn to migrate dorsally. Facial motor neurons turn on Ret as they are exiting r4 and maintain this expression for the duration of their migration, while cdh8 is only expressed during the final dorsal phase of migration (Garel et al., 2000; Muller et al., 2003). Garel proposed that the facial motor neurons may follow along longitudinal fibers from r4 into r6 (mouse) where they interact with radial glia and that such changes in direction would require modifications in the appropriate adhesion molecules (Garel et al., 2000). TAG-1 interacts with L1, another cell adhesion molecule expressed on longitudinal fibers (Dodd et al., 1988; Kuhn et al., 1991), and thus may mediate caudal migration of facial motor neurons (Garel et al., 2000). Meanwhile, because cdh8 is expressed specifically in r6 (mouse) it might mediate the radial migration (Garel et al., 2000). Even in Nkx6.1, Krox20, and Ebf1 mutants, which perturb normal facial motor neuron migration, TAG-1, Ret and Cdh8 are expressed with their associated migration (Muller et al., 2003; Garel et al., 2000), further supporting this hypothesis.

Cranial motor neuron disorders

Although rare, there are developmental disorders affecting the proper cranial motor neuron specification and organization. Patients with Duane syndrome have limited or absent ability to move their eyes outward towards the ear (abduction) and often impaired ability to move their eyes inward toward the nose (adduction) (Duane, 1996). These symptoms arise from the absence of the abducens motor neuron, confirmed by MRI (Parsa et al., 1998), and its innervation of the lateral rectus muscle and often one branch of the oculomotor neuron improperly innervates the medial rectus muscle (Miller et al., 1982; Hotchkiss et al., 1980). The cause of Duane Syndrome is not known, but environmental factors may be involved at 3-8 weeks of gestation during which the cranial nerves and the ocular muscles are developing. Because of the long distance the abducens motor neuron axons travel, many external factors can damage the axons and cause palsy, such as elevated intracranial pressure, subarachnoid space lesions, metabolic or vascular problems, infections, congenital (eg, Duane Syndrome) or trauma (Denis et al., 2008; Calisaneller et al., 2006; Dwarakanath et al., 2006). Another disorder affecting cranial motor neurons is Möbius Syndrome, first described in a case of facial diplegia in 1880 by von Graefe and later defined by Paul Julius Möbius in 1888 and 1892. These patients have congenital facial weakness and often abnormal ocular abduction due to problems with the abducens and facial motor neurons (Briegel, 2006). Interestingly, patients with Möbius Syndrome have a higher incidence of autistic spectrum disorders (Rodier et al., 1996; Briegel, 2006). As with Duane Syndrome, there is no known cause and no cure, only treatments. Whereas linkage studies have implicated some genes underlying Duane and Möbius Syndromes, the etiology of these disorders is largely unknown. By uncovering the genes necessary for the specification and migration of cranial motor neurons, we can better understand disorders that affect their normal development.

Oligodendrocyte Specification in the Spinal Cord

"Some accept as though it were established fact that neuroglial fibers form a passive support network to simply fill out and bind the tissue in a matrix that is swelled with nutritive substance. Any observer who wishes to form a ration opinion of the function of neuroglia must begin by dispelling such notions which might hamper his thinking."

Santiago Ramón y Cajal 1952

The presence of non-neuronal cells types, or glial cells, in the central nervous system (CNS) were first identified over a century and a half ago. Though some controversy persisted over who truly first identified glial cells, Virchow's term "Nervenkitt" meaning nerve glue, stuck (Virchow, 1858). He found a "connective substance" that forms a "sort of putty in which the nervous elements are embedded" (p. 890 in Virchow, 1856). Another early researcher who identified glial calls was Deiters, defining them as any cell in the CNS without an axon (Deiters, 1865). However, the first scientist who perhaps truly identified glial cells was Golgi, with his novel staining technique (reviewed by Somjen, 1988). Cajal and Lugaro were the first to accurately begin describing, or even suggesting, the function of glial cells: serving to insulate nerve fibers, regulating the interstitial fluid, and removing ("chemically split or take up") the substances by which nerve cells excite each other thus ending their action (Ramón y Cajal, 1909; Lugaro, 1907). Although initially described as putty, scientists soon established glial cells as more than support, indeed they comprise up to 90% of the cells in the human brain (Rowitch, 2004), implying a much more critical function that simply the glue that holds neurons together.

Glial cells are divided into three principle classes: oligodendrocytes, astrocytes, and microglia. Astrocytes provide structural support, play a major role in ion homeostasis in the CNS, maintain the blood-brain barrier (Kandel et al., 2000) and have even been implicated in cell-cell signaling via calcium flux, modulating synaptic transmission and neuropeptide production (Bennett et al., 2003; Newman, 2003; Ubink et al., 2003). Similar to phagocytes in the immune system, microglia clear debris and mediate inflammation of the CNS (Ling and Wong, 1993). Oligodendrocytes intimately interact with neurons in the CNS and produce the myelin sheaths that insulate axonal projections allowing for salutatory conduction. While many parallels exist between the specification of oligodendrocytes and astrocytes, both being born early in development from the neural tube, it is clear that they then follow distinct routes (Rowitch, 2004).

A considerable amount of research has focused on the development of oligodendrocytes in the spinal cord. The predominate view prior to the 1990s was that all regions of the VZ could and did give rise to oligodendrocytes (Altman, 1966). Additionally, some researchers also believed that radial glia could trans-differentiate into oligodendrocytes (Choi and Kim, 1985; Hirano and Goldman, 1988). However, soon much evidence arose demonstrating restricted ventral origins for oligodendrocyte precursors cells in the embryonic spinal cord (Warf et al., 1991; Pringle and Richardson, 1993; Noll and Miller, 1993; Timsit et al., 1995; Lu et al., 2000; Takebayashi et al., 2000; Zhou et al., 2000). Once born, OPCs migrate away from the VZ laterally and dorsally to populate the all parts of the CNS (Rowitch, 2004). These oligodendrocytes can be identified early in their development by the expression of platelet-derived growth factor receptor α (PDGFRα) (Hall et al., 1996; Pringle and Richardson, 1993). PDGF, secreted from type-1 astrocytes, is involved with the proliferation and migration of OPCs (Noble and Murray, 1984; Noble et al., 1988). As they develop oligodendrocytes begin expressing other genes such as *Sox10*, an HGM-box transcription factor (Kuhlbrodt et

al., 1998); 04 (Bansal et al., 1989); *Plp/Dm20,* a proteolipid protein involved with myelination (Timsit et al., 1995), and myelin basic protein (MBP) (Brosamle and Halpern, 2002).

Oligodendrocyte origins

Early transplantation studies further confirmed the ventral origin of OPCs and demonstrated the requirement of the notochord and subsequent Shh signaling for OPC development. The notochord, located ventral to the neural tube, is essential not only for the for the dorsoventral patterning of the neural tube (van Straaten et al., 1989) but also the specification of specific cell types, OPCs (Orentas and Miller, 1996; Pringle et al., 1996; Trousse et al., 1995) and motor neurons (Yamada et al., 1991). Moreover, the notochord is also necessary for OPC production for when it is ablated or absent OPCs fail to form (Maier and Miller, 1997; Pringle et al., 1996). Interestingly, there is a temporal window for the ability of the notochord to induce OPCs (Orentas and Miller, 1996). The main signal mediating the effects of the notochord is Shh (Echelard et al., 1993; Roelink et al., 1994). in vitro studies confirmed the role of Shh from the notochord to induce the patterning of DV domains by a concentration gradient via regulation of homeodomain transcription factors (Roelink et al., 1994; Roelink et al., 1995; Jessell, 2000). Moreover OPCs and motor neurons require similar concentrations of Shh (Orentas et al., 1999; Pringle et al., 1996), which may function by inducing the transcription factor *olig2* expressed in both cell types (Lu et al., 2000; Zhou et al., 2000). Both Shh and *olig2* will be discussed at greater lengths in following sections. Thus, both OPCs and motor neurons arise in the ventral neural tube and require Shh signaling indicating a link between the two cell types.

While unequivocally shown to have a ventral origin, debates still existed over possible dorsal origins for OPCs as well as their relationship to other cell types: motor

neurons versus astrocytes. Based on a *lacZ* reporter for a myelin proteolipid protein

(*Plp*) some argued for the presence of dorsal origins (Spassky et al., 1998; Spassky et

al., 2000). However, others supposed that *Plp* may not always be restricted to the

oligodendrocyte lineage (reviewed by Richardson et al., 2006). Even seemingly similar

transplant studies gave different results with one group concluding both dorsal and

ventral origins and other supporting ventral-only origins (Cameron-Curry and Le Douarin,

1995; Pringle et al., 1998; Richardson et al., 2006). However, three recent labs provide

convincing evidence for the existence of both dorsal and ventral origins (Cai et al., 2005;

Vallstedt et al., 2005; Fogarty et al., 2005). Two of these papers used double knockout

mice for the transcription factors Nkx6.1 and Nkx6.2, which will be discussed more

below (Cai et al., 2005; Vallstedt et al., 2005). Furthermore, fate mapping of the dorsal

spinal cord with the Cre-*lox* system for *Dbx1* expression demonstrated dorsal OPCs

(Fogarty et al., 2005). Combining these works, it appears that OPCs arise from the dP5

domain of the dorsal spinal cord (Richardson et al., 2006). These dorsal origins may

arise independent of Shh, as dorsal oligodendrocytes are still specified when all

hedgehog signaling is blocked (Chandran et al., 2003; Kessaris et al., 2004). Though

there is no evidence yet to support it, many have suggested that the different origins for

oligodendrocytes could lead to functional differences in the mature populations.

Another controversy involves the relationship of OPCs to other cell types, namely

whether they arise from precursors that also give rise to motor neurons or if they arise

from precursors that are restricted to a glial lineage. Work in primary cell culture

revealed the presence of glial restricted precursors (GRP), which generated

oligodendrocytes and astrocytes, but not motor neurons (Rao et al., 1998; Liu and Rao,

2003). These GRPs were isolated from many different parts of the neuroepithelium,

however their in vitro competence is likely exaggerated as the local environment in the in

vivo setting could constrain the identity of their progeny. For example, only or primarily

oligodendrocytes generated in some locations (like the ventral spinal cord) and only astrocytes in other locations (Rowitch et al., 2002; Richardson et al., 2006). Additionally, in culture studies the growth factor FGF2 was found to perturb the dorsoventral patterning and only then allowing oligodendrocytes, astrocytes and motor neurons to arise from the same stem cells. The following paragraphs will provide in vivo evidence of the relationship between OPCs and motor neurons in the ventral spinal cord.

Sonic hedgehog signaling

Previous work showed that the mitogen Shh is required for the generation of both motor neurons (Jessell, 2000) and oligodendrocytes (Orentas et al., 1999; Alberta et al., 2001). Based upon the concentration gradient of Shh, with antagonistic interactions with BMP signaling from the roof plate, dorsoventral domains are established (Rowitch, 2004). These domains will then give rise to specific neural cell types (Jessell, 2000). It is thought that Shh initially patterns these domains by regulating lineage genes (Pringle et al., 1996) and then cross-repression among homeodomain proteins further maintain and refine the domains. Motor neurons and oligodendrocytes arise in one of these domains, the pMN domain, which is located in the ventral spinal cord (Jessell, 2000; Briscoe et al., 2000). Shh is not only present but required for the appearance of oligodendrocytes in the spinal cord (Orentas et al., 1999; Alberta et al., 2001) and it is both necessary and sufficient for oligodendrocyte induction (Pringle et al., 1996; Alberta et al., 2001). In addition, a prolonged exposure to Shh signaling is required for oligodendrocytes to arise from the spinal cord (Orentas et al., 1999). This indicates that Shh is not only required for specification but also plays a role later in differentiation. Explant studies have also shown that Shh can induce the formation of oligodendrocytes, and that similar Shh concentrations are required for oligodendrocyte and motor neuron induction (Orentas et al., 1999). In addition to these inductive properties, there is a continued dependence for

Shh (Park et al., 2004) possibly for cell survival and proliferation signals (Davies and

Miller, 2001). Inhibition of hedgehog signaling, including Shh, by cyclopamine

suppresses the formation of OPCs in both the spinal cord and the telencephalon (Tekki-

Kessaris et al., 2001). Shh signaling, therefore, seems to be required at several levels in

the development of oligodendrocytes.

Homeodomain protein code

As mentioned above, one function of the Shh concentration gradient is to

regulate the expression of homeodomain proteins that will then define domains along the

dorsoventral axis of the spinal cord (Figure 2). After their initial expression, these

proteins will undergo cross-repressive interactions to further define and then maintain

the domains (Briscoe et al., 2000). For example, the homeodomain proteins Pax7, Pax3,

Pax6, Dbx1, Dbx2, and Nkx2.2 are ultimately expressed by ventral progenitor cells

(Melton et al., 2004; Briscoe et al., 2000). The homeodomain proteins can be organized

into two classes, those repressed by Shh (class I: Pax7, Irx3, Dbx1, Dbx2, and Pax6)

and those induced by Shh (class II: Nkx6.1 and Nkx2.2 (Clarke and Lumsden, 1993).

The combination of these proteins establishes five domains in the ventral neural tube

(Briscoe et al., 2000), named p3, pMN, p2, p1, and p0 respectively from ventral to

dorsal. Each of these domains will give rise to a particular neural cell type (Jessell, 2000)

(Fig. 1.3).

The pMN domain is located in the ventral spinal cord, just dorsal to the

expression of Nkx2.2 which has the most ventral expression in the neural tube (Briscoe

et al., 2000; Jessell, 2000). This domain is characterized by the expression of Pax6,

Nkx6.1, Olig2, and Isl1 (Melton et al., 2004). Nkx6.1, Nkx2.2 and Irx3 work together to

restrict motor neuron generation to the pMN domain, with Nkx2.2 expression ventral and

Irx3 expression dorsal to the pMN domain (Rowitch, 2004). Based on these various
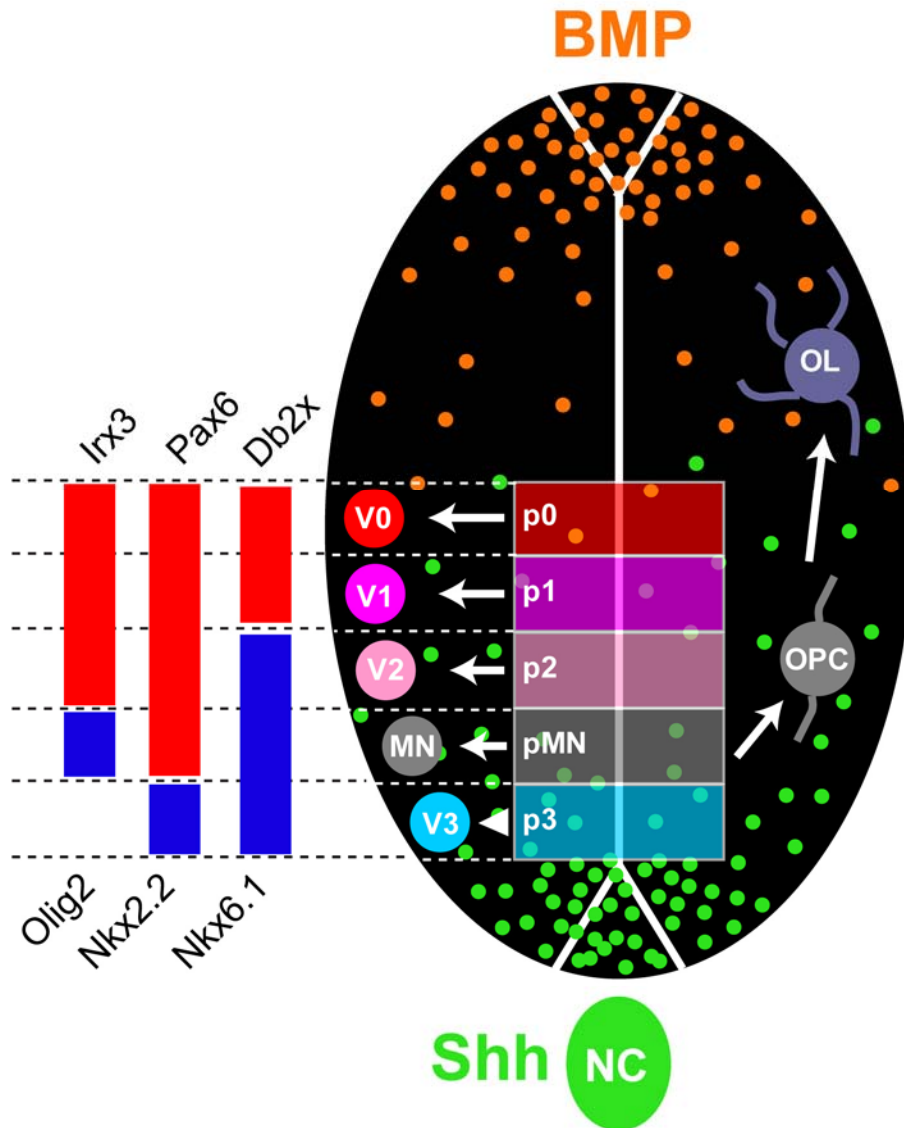
27

Figure 1.3. Schematic of dorsoventral patterning in the vertebrate spinal cord. Concentration gradients of sonic hedgehog (Shh) ventrally from the notochord (NC) and floorplate and bone morphogenic protein (BMP) dorsally help establish various domains. Each of these domains is defined by a unique combination of homeodomain transcription factors and will give rise to particular neural cell type. The pMN domain gives rise first to motor neurons and then to oligodendrocyte progenitor cells (OPC) and oliogdendrocyte lineage cells (OL).

homeodomain interactions, v3, v2, v1 and v0 interneurons will arise from the p3, p2, p1, and p0 domains (Briscoe et al., 2000). Many of the homeodomain proteins shown to be involved with motor neuron and oligodendrocyte development in the spinal cord are also involved with similar processes in the hindbrain, suggesting that these precursor cells are similar in each region. However, these defined expression domains seen in the spinal cord have not been established in the hindbrain.

Olig2: A motor neuron and oligodendrocyte link

  A pair of bHLH proteins, *olig1* and *olig2*, were found to identify OPCs prior to the expression of PDGFRα (Zhou et al., 2000; Lu et al., 2000), the earliest known marker for OPCs (Noble et al., 1988; Pringle and Richardson, 1993). The expression of these genes is similar to many of the other oligodendrocyte lineage genes, such as *Sox10* (an HGM-box transcription factor) (Kuhlbrodt et al., 1998), 04 (Bansal et al., 1989), and *Plp/Dm20* (proteolipid protein involved with myelination) (Timsit et al., 1995). *Olig1/2* double knockout mice exhibit an almost complete lack of oligodendrocytes as marked by PDGFRα, *Sox10*, *plp/dm20* and MBP (myelin basic protein) in the spinal cord (Zhou and Anderson, 2002; Takebayashi et al., 2002), while ectopic *olig2* expression induces ectopic *Sox10* expression (Fu et al., 2002). In addition, the precursor cells in the pMN domain of *olig1/2$^{-/-}$* mice give rise to v2 neurons and then astrocytes (Zhou and Anderson, 2002) instead of motor neurons and oligodendrocytes. Shh also plays a role in *olig1/2* expression (Park et al., 2002) being both necessary and sufficient for *olig1/2* expression (Lu et al., 2000). The location of *olig1/2+* cells in the ventral ventricular zone in close proximity to Shh and PGFRα expression regions suggests a common lineage between motor neurons and oligodendrocytes (Zhou and Anderson, 2002; Lu et al., 2002; Takebayashi et al., 2002; Park et al., 2002). Motor neurons are largely absent in

olig2-/- mice (Zhou and Anderson, 2002; Takebayashi et al., 2002) while astrocytes are not affected (Zhou and Anderson, 2002) (Zhou and Anderson, 2002) supporting the hypothesis of a common motor neuron-oligodendrocyte precursor population excluding astrocytes. In fact, Park found, using *in vivo* single cell labeling, that *olig2*^{+} cells can produce both motor neurons and oligodendrocytes and that the precursor cells are temporally and spatially controlled, by Shh (Park et al., 2004).

While the expression pattern of *olig1* and *olig2* largely overlaps (Zhou et al., 2000; Zhou and Anderson, 2002) these two bHLH proteins play different roles during oligodendrocyte development. *olig1* is involved with the maturation of oligodendrocytes whereas *olig2* is required for specification (Lu et al., 2002). However, both genes function as transcriptional repressors to regulate neurogenin2 (Ngn2), a bHLH factor in the pMN domain that can drive cell cycle exit in neurons, and with Nkx2.2 promote oligodendrocyte formation (Zhou et al., 2001). The Olig genes are unlike other known bHLH proteins in that they couple neuron glia specification while other bHLH factors control the neuron versus glia decision (Zhou et al., 2000).

The homeodomain proteins also function in correlation with *olig2* to determine cell fate. Nkx2.2, which has the most ventral expression pattern in the spinal cord (Briscoe et al., 2000), is vital for sensing the Shh gradient and subsequently affecting neural identity (Briscoe et al., 1999). Early in the establishment of dorsoventral domains, *Nkx2.2* expression is ventral to *olig2* expression (Briscoe et al., 2000). These two domains then begin to overlap concurrent with a decrease in *ngn2* expression, and give rise to oligodendrocytes (Fu et al., 2002; Zhou et al., 2001). The coexpression of Olig2 and Nkx2.2 was sufficient to induce ectopic oligodendrocyte development, but was blocked by forced ngn1 expression (Zhou et al., 2001). The requirement for Nkx2.2 in dorsally derived oligodendrocytes has not been established.

Nkx6.1, which is expressed in the ventral neural tube (Briscoe et al., 2000), is involved in motor neuron production (Pattyn et al., 2003; Liu et al., 2003) similar to its role in the hindbrain (Pattyn et al., 2003; Vallstedt et al., 2005). Recently, however, it has been implicated in oligodendrocyte induction. Nkx6.1 regulates Olig gene expression, and thus can influence oligodendrocyte development in the spinal cord, however not in the hindbrain (Liu et al., 2003). Recent evidence has found a dorsal population of cells expressing the olig genes independent of Nkx6 and Shh function (Cai et al., 2005). This population could be similar to the dorsal hindbrain populations discussed above.

Oligodendrocyte Generation from the Hindbrain

As described above, the development of neurons has been well studied in the hindbrain, whereas the generation of oligodendrocytes is not well understood. Oligodendrocyte generation is best characterized in the spinal cord and additional data have implicated many similarities between oligodendrocyte development in the spinal cord and hindbrain. This section will discuss what is known about hindbrain oligodendrocyte development compared to what is known in the spinal cord. Additional information about oligodendrocyte development in the spinal cord will be discussed below.

Origins

Early studies have identified both ventral and dorsal origins for OPC in the hindbrain. Ventral OPCs arise from the midline of the ventricular zone (VZ) (Ono et al., 1997) and later in development dorsal OPCs arise (Davies and Miller, 2001) possibly from the alar plate (Vallstedt et al., 2005). These domains, like other hindbrain components, are not continuous along the anterior/posterior axis as is seen in the spinal

cord. In fact, some studies show that avian oligodendrocytes remain in the rhombomere from which they arose, myelinating axons within their region (Olivier et al., 2001). These oligodendrocytes were identified by examining *plp/dm20* expression, which is known to mark OPCs (Perez Villegas et al., 1999). In addition, they are responsive to Sonic Hedgehog (Shh) signaling (Davies and Miller, 2001), which is required to generate OPCs in the spinal cord (Orentas et al., 1999; Alberta et al., 2001; Davies and Miller, 2001). Similar to the spinal cord, this OPCs also seem to disperse in a ventral to dorsal manner (Ono et al., 1997). Although previously a controversy, recent work seems to confirm the earlier data identifying dorsal origins for oligodendrocytes (Davies and Miller, 2001; Vallstedt et al., 2005). Through a series of quail-chick chimera experiments, oligodendrocytes were found, *in vivo,* to arise from both the ventral and dorsal neural tube (Cameron-Curry and Le Douarin, 1995). In addition, the oligodendrocytes from each region were capable of becoming mature oligodendrocytes. While the bulk of these studies focused on the spinal cord, Vallstedt et al have recently shown that dorsal hindbrain explants can produce oligodendrocytes (Vallstedt et al., 2005). They also provided *in vivo* evidence for dorsal oligodendrocyte generation in the hindbrain, and spinal cord, which is dependent upon *olig1/2* expression (*olig2* discussed below). This population arises subsequent to ventral OPC production and may be influenced by the decreased levels of BMP (bone morphogenic protein) signaling that occurs with an expanding hindbrain. BMP is expressed dorsally and is known to antagonize ventral signals (Lee and Jessell, 1999), such as Shh. In fact, BMP was found to affect many stages of oligodendrocyte development (Grinspan et al., 2000). The origins of oligodendrocytes are beginning to be understood and it appears that, similar to motor neurons in the hindbrain, oligodendrocytes may also be segmentally regulated.

Genes

There are several homeodomain proteins that are needed to pattern the spinal cord that are also required for the hindbrain, such as Nkx2 and Nkx6. These proteins have complementary roles in the production of visceral and somatic motor neurons (vMN and sMN) in both the hindbrain and spinal cord (Pattyn et al., 2003). Nkx2.2 functions upstream to Phox2b, a vMN determinant, while Nkx6 proteins prevent vMNs from adopting a different neuronal fate. These two homeodomain proteins are also involved in oligodendrocyte development in the spinal cord (Liu et al., 2003; Fu et al., 2002). *Hoxa3* and *Hoxb3* are involved in the specification of sMN through a *Pax6/Olig2* regulatory pathway (Gaufo et al., 2003). *olig2*, a basic helix-loop-helix (bHLH) transcription factor known to play a role in both motor neuron and oligodendrocyte development (Zhou and Anderson, 2002; Takebayashi et al., 2002; Park et al., 2002), functions in this case to promote sMN development by inhibiting its inhibitor (Pattyn et al., 2003). *olig1/2* knockouts completely lack OPC formation in the hindbrain (Zhou and Anderson, 2002). Therefore, *olig2* appears to be involved in both motor neuron and oligodendrocyte formation in both the spinal cord and the hindbrain. However, *olig2* appears to be differentially regulated in the hindbrain and spinal cord. Nkx6 is required for spinal cord expression of *olig2* while inhibiting hindbrain expression (Pattyn et al., 2003; Vallstedt et al., 2005). However, all oligodendrocytes appear to require *olig2* expression (Rowitch, 2004). While there are many similarities between spinal cord and hindbrain oligodendrocyte development, other aspects are differentially regulated.

CHAPTER II

OLIG2[+] PRECURSORS PRODUCE ABCUENS MOTOR NEURONS AND OLIGDOENDROCYTES IN THE ZEBRAFISH HINDBRAIN

Abstract

During development, a specific subset of ventral spinal cord precursors called pMN cells produces first motor neurons and then oligodendrocyte progenitor cells (OPCs), which migrate, divide and differentiate as myelinating oligodendrocytes. pMN cells express the Olig2 transcription factor and Olig2 function is necessary for formation of spinal motor neurons and OPCs. In the hindbrain and midbrain, distinct classes of visceral, branchiomotor and somatic motor neurons are organized as discrete nuclei and OPCs are broadly distributed. Mouse embryos deficient for Olig2 function lack somatic motor neurons and OPCs, but it is not clear whether this reflects a common origin for these cells, similar to spinal cord, or independent requirements for Olig2 function in somatic motor neuron and OPC development. We investigated cranial motor neuron and OPC development in zebrafish and found, using a combination of transgenic reporters and cell type specific antibodies, that somatic abducens motor neurons and a small subset of OPCs arise from common *olig2*[+] neuroepithelial precursors in rhombomeres r5 and r6, but that all other motor neurons and OPCs do not similarly develop from shared pools of *olig2*[+] precursors. In the absence of *olig2* function r5 and r6 precursors remain in the cell cycle and fail to produce abducens motor neurons, and OPCs are entirely lacking in the hindbrain. These studies therefore reveal both common and independent roles for *olig2* in development of somatic motor neurons and oligodendrocytes of the hindbrain.

Introduction

In the spinal cord of vertebrate embryos, motor neurons and most oligodendrocytes, the myelinating cell type of the central nervous system, have a common origin. Dividing, neuroepithelial precursors that occupy ventral spinal cord and express the transcription factor Olig2 produce first motor neurons and then oligodendrocyte progenitor cells (OPCs), as well as some ventral interneurons, astrocytes and ependymal cells (Lu et al., 2000; Takebayashi et al., 2000; Zhou et al., 2000; Park et al., 2004; Masahira et al., 2006). These precursors, called pMN cells, are specified by graded distribution of the morphogen Sonic Hedgehog (Ericson et al., 1997a; Briscoe and Ericson, 1999; Jessell, 2000), and Olig2 function is necessary for development of both motor neurons and OPCs (Park et al., 2002; Lu et al., 2002; Zhou and Anderson, 2002; Takebayashi et al., 2002). In mouse embryos deficient for *Olig2* and the related *Olig1* gene, pMN precursors instead produce V2 interneurons and astrocytes (Zhou and Anderson, 2002). Some OPCs also arise within dorsal spinal cord, and in dorsal hindbrain, independent of motor neuron origins (Vallstedt et al., 2005; Cai et al., 2005; Sussman et al., 2000; Cameron-Curry and Le Douarin, 1995; Fogarty et al., 2005).

The developmental relationship of motor neurons and oligodendrocytes in the hindbrain has not been investigated as thoroughly as in spinal cord. Hindbrain, or cranial, motor neurons form three classes, visceral, somatic and branchiomotor, based upon their synaptic targets and columnar organization, and develop as groups within segmentally iterated rhombomeres (r) (Guthrie, 2007; Chandrasekhar, 2004). Many hindbrain oligodendrocytes arise from ventral neuroepithelium (Ono et al., 1997; Davies and Miller, 2001; Vallstedt et al., 2005), but these cells may originate from discrete focal clusters rather than from a continuous longitudinal domain as in the spinal cord (Spassky et al., 1998; Miller, 2002; Perez Villegas et al., 1999). The correlation of these putative

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focal clusters to hindbrain motor neuron origins is not known. Notably, somatic cranial motor neurons fail to develop in *Olig2* mutant mouse embryos, or in embryos lacking functions of *Olig2* and the related *Olig1* gene, whereas other motor neuron classes appear to form normally (Zhou and Anderson, 2002; Lu et al., 2002). *Olig1;Olig2* mutant mice also lack all hindbrain oligodendrocytes (Zhou and Anderson, 2002). Whether hindbrain oligodendrocytes arise from *Olig*[+] precursors that also produce somatic motor neurons, or somatic motor neurons and oligodendrocytes have independent requirements for *Olig* gene function has not been resolved.

Here we report an investigation of hindbrain motor neuron and oligodendrocyte development using zebrafish as a model system. Through a combination of gene expression analysis, fate mapping and time-lapse imaging, we determined that abducens motor neurons and some oligodendrocytes arise from common *olig2*[+] precursors in r5 and r6, but that all other hindbrain motor neurons and oligodendrocytes appear to develop independently of each other. In the absence of *olig2* function, r5 and r6 *olig2*[+] precursors are not specified for neuronal or glial fates but maintain their neuroepithelial characteristics.

Materials and Methods

Wild-type and transgenic zebrafish

Wild-type and transgenic fish were raised either in the Vanderbilt University Zebrafish Facility or the University of Colorado Denver Facility and embryos collected from pair matings. The embryos, raised at 28.5°C, were staged according to morphological criteria (Kimmel *et al.,* 1995) and hours post-fertilization (hpf). We used the following transgenic lines: *Tg(olig2:egfp)*[vu12] (Shin et al., 2003)*, Tg(isl1:egfp)* (Higashijima et al., 2000)*, Tg(olig2:DsRed2)*[vu19] (Kucenas et al., 2008) and

Tg(nkx2.2a:megfp)^{vu17} (Kirby et al., 2006)*. The Tg(olig2:Kaede)*^{VU85} transgenic line was produced using the same strategy that created the *Tg(olig2:olig2)* and *Tg(olig2:DsRed2)* lines (Shin et al., 2003; Kucenas et al., 2008).

<p align="center">In situ RNA hybridization</p>

The following previously described RNA probes were used: *egr2b* (also known as *krox20*) (Oxtoby and Jowett, 1993), *ngn1* (Blader et al., 1997), *isl1* (Inoue et al., 1994), *sox10* (Dutton et al., 2001), *olig2* (Park et al., 2002), *plp/dm20* (Park et al., 2002), *mbp* (Brosamle and Halpern, 2002), *hoxb1a* (McClintock et al., 2002), *hoxb3a* (Hadrys et al., 2004), *hoxd4a* (Moens and Prince, 2002)*, gfp, sdf1a* and *sdf1b*. Embryos were fixed in 4% paraformaldehyde (PFA) and then stored in 100% methanol at –20°C. The in situ RNA hybridization was performed as previously described (Hauptmann and Gerster, 2000) followed by a color reaction with BM purple (Roche Diagnostics). For double RNA labeling probes were labeled with either digoxygenin or fluorescein. The first probe was detected with the appropriate antibody conjugated to alkaline phosphotase and followed by a color reaction with BM purple. Washing the embryos with 0.1M glycine, pH 2.2, followed by a 20 min incubation with 4% PFA inactivated the first antibody and the appropriate second antibody was then applied and developed with a solution of INT/BCIP (Roche Diagnostics). Once developed, the embryos were dissected from the yolk and mounted in 70% glycerol for whole mount imaging on bridged cover-slips. All images were captured using Openlab software (Improvision) using an Olympus AX70 microscope equipped with DIC optics and a Retiga Exi 1300 color digital camera. Once captured, images were imported into Adobe Photoshop and adjustments were limited to contrast, levels, color matching settings and cropping.

Immunocytochemistry

Primary and secondary antibodies used

For immunocytochemistry we used the following primary antibodies: mouse anti-Isl (39.4D5, 1:100; Developmental Studies Hybridoma Bank (DSHB)), mouse anti-Zn8 (1:1000; DSHB), mouse anti-BrdU (G3G4, 1:200; DSHB), rabbit anti-Sox10 (1:500) (Park et al., 2004), rabbit anti-Pax2 (1:100; Berkeley Antibody Company), mouse anti-Pax7 (1:25 DSHB), mouse anti-Nkx6.1 (1:1000; DSHB), rabbit anti-GABA (1:1000; Chemicon International INC), mouse anti-GFAP (1:400; Sigma), mouse anti-Zrf1 (1:250; DSHB), mouse anti-HuC (1;100; Molecular Probes) and rabbit anti-Calretinin (1:1000; Swant). For fluorescent detection, the following Alexa Fluro secondary antibodies were used: 568 goat anti-mouse, 568 goat anti-rabbit, 647 goat anti-mouse, and 647 goat anti-rabbit (all at 1:200; Molecular Probes).

Single antibody or two antibodies raised in different species on section

Embryos were fixed in 4% AB fix (4% paraformaldehyde (PFA)), 8% sucrose, 1x PBS) for 2 hr at room temperature (RT) or overnight at 4°C. Embryos for sectioning were embedded in 1.5% agar/5% sucrose, frozen with 2-methyl-butane chilled by immersion in liquid nitrogen, and sectioned using a cryostat microtome (10 μm). Sections were re-hydrated with 1x PBS and pre-blocked for 30 min in 2% sheep serum/BSA-1x PBS. The sections were incubated with primary antibody overnight at 4°C, washed extensively with 1x PBS and incubated with the appropriate fluorescent secondary antibody for 2 hr at RT. Once the secondary antibody was washed off sections were covered with Vectashield (Vector Laboratories) and then cover-slips.

Whole mount antibody labeling

Embryos for whole mount imaging were fixed as above and pre-blocked with 10% sheep serum/BSA-1x PBS for 1 hr at RT. The embryos were incubated in primary antibody for 24 hr at 4°C, washed semi-continuously with 1x PBS with 0.2x Trition (PBSTx) for 2 hr at RT, and then incubated with the secondary antibody for 12 hr at 4°C, followed by 3 hr of semi-continuous washes with 1x PBSTx. These embryos were then dissected from the yolk and mounted on bridged cover-slips in 70% glycerol for imaging.

Two antibodies raised in the same species

Embryos were fixed as described above. The strongest antibody was applied first and a 568 Alexa Fluro secondary in the appropriate animal was used. Each washing step was increased in time to insure all unbound antibody was removed; for labeling on sections the primary antibody incubation is followed by at least an hour wash with 1x PBS and for whole mount this wash is at least 3 hours with 1x PBSTx. The wash following the secondary antibody labeling was at least 45 min for sections and at least 4 hours for whole mount embryos. The sections or whole mount embryos were imaged and processed as described below.

Whole mount antibody labeling following in situ RNA hybridization

Embryos for whole mount in situ RNA hybridization followed by whole mount antibody labeling were fixed as above in 4% AB fix for 2 hrs at RT. The in situ RNA hybridization was performed as described in the previous section, however the embryos were not treated with 100% methanol. Once the color reaction with BM purple concluded the embryos were washed with 1x PBS with % Tween (PBSTw) for two 5 min washes.

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The embryos were then fixed in 4% PFA for 20 min to de-active the alkaline phosphotase. The fix was washed off with 1x PBSTx followed by 10% block solution for 1 hr. The rest of the whole mount antibody was performed as described above. Following in situ RNA labeling and antibody labeling, embryos were dissected from the yolk and mounted on bridged cover-slips in 70% glycerol for imaging. The DIC in situ RNA hybridization image was overlapped with the fluorescent image in Photoshop. Images were exported and analyzed as described below.

Imaging

All imaging was conducted on a Zeiss Axiovert 200 inverted microscope equipped with either a 40x oil immersion objective (NA=1.3) or 20x dry objective (NA=0.75), mounted on a Piezo drive, and a PerkinElmer Ultraview ERS Live Cell Imager. Images were exported and analyzed using Volocity (Improvision) and Adobe Photoshop. Image adjustments were limited to level settings, contrast and cropping.

Bromodeoxyuridine (BrdU) labeling

Embryos were manually dechorionated and incubated with 10 mM BrdU in 10% DMSO in embryo medium for 20 min on ice. For longer incubations, a 20 min pulse on ice was followed by incubation with 10 mM BrdU solution in embryo medium at 28.5°C. The embryos were then fixed using 4% PFA in PBS and sectioned as described above. Prior to anti-BrdU immunocytochemistry the sections were treated for 30 min with 2M HCl. All sections were imaged using the Zeiss Axiovert 200 microscope described above.

For BrdU labeling in conjunction with another primary antibody labeling, embryos were fixed and treated with BrdU as described above. For the primary antibody labeling

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step a second primary antibody was added at the appropriate concentration. The rest of the labeling procedure was conducted and imaged as described above.

Antisense morpholino oligonucleotide injections

An antisense morpholino (MO) oligonucleotide with the sequence 5'-ACACTCGGCTCGTGTCAGAGTCCAT-3' (Gene Tools, LLC) was designed to the *olig2* translation start site. We also used a Standard Control MO (Gene Tools, LLC). Both morpholinos were re-suspended in distilled water for a stock solution of 3 mM. The stock solution was further diluted with water and phenyl red and 1-2 ng was injected into the yolk of one- to two-cell stage embryos.

Conditional expression experiments

The plasmid *p(hsp70l:olig2)Tol2* was constructed by subcloning *olig2* cDNA into a vector carrying the zebrafish *hsp70l* heat-responsive promoter (Shoji et al., 1998) and recognition sequences for Tol2 transposase (Kawakami, 2004). Newly fertilized eggs produced by *Tg(olig2:egfp)* or *Tg(olig2:DsRed2)* adults were injected with a solution containing 0.1 µg/µl of the plasmid and 0.3 µg/µl in vitro synthesized Tol2 mRNA. Expression was induced by transferring embryos at selected stages to egg water prewarmed to 39°C for 30 min.

Photoconversion

Tg(olig2:Kaede) embryos were grown in embryo medium, containing 0.003% phenyl-thiourea (PTU) to prevent the formation of dark pigment, at 28.5C. They were kept in the dark to minimize photoconversion of the Kaede protein by ambient light. To photoconvert the Kaede protein, embryos were lightly anesthetized with 3-aminobenzoic acid ethyl ester (Tricaine) and immersed in 0.8% low-melting temperature agarose. They

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were then mounted in either lateral or dorsal orientations in glass-bottom 35 mm Petri

dishes. Using a Zeiss Axioskop 2 FS microscope equipped with a nitrogen pulsed laser,

405 nm dye cell, yellow beamsplitter, 40x water immersion lens and GFP filter, small

clusters of Kaede$^+$ cells were photoconverted with ~4s laser pulses. The live embryos

were then imaged using the confocal microscope described above.

Time-lapse imaging

The embryos were manually dechorionated at 24 hpf and transferred to embryo

medium containing PTU. Embryos for time-lapse imaging were anesthetized using

Tricaine and immersed in 0.8% low-melting temperature agarose. They were then

mounted in either lateral or dorsal orientations in glass-bottom 35mm Petri dishes.

Images were captured using a 20x dry (NA=0.75) objective mounted on the confocal

microscope described above. A heated stage and chamber kept the embryos at 28.5°C

and Z image stacks were collected every 5-15 min. The data sets were analyzed using

Volocity software and exported as QuickTime files to create movies.

Results

olig2 expression reveals common precursors for abducens motor neurons and
oligodendrocytes

We initiated our studies by using in situ hybridization to determine the distribution

of *olig2* RNA (Park et al., 2002) in the hindbrain of zebrafish embryos. *olig2*$^+$ cells were

first evident by 22 hours post fertilization (hpf) at the level of the otic vesicle (data not

shown). By 28 hpf *olig2*$^+$ cells were located in two distinct bilateral clusters at the midline

of the posterior hindbrain (Fig. 2.1*A*). Using *egr2b* (previously known as *krox20*) RNA

expression as a marker for r3 and r5 (Oxtoby and Jowett, 1993), we determined that

these *olig2⁺* clusters occupy r5 and r6 (data not shown). By 40 hpf a third *olig2⁺*

population appeared just posterior to the original two (Fig. 2.1*B*). Subsequently, *olig2*

expression expanded anteriorly and posteriorly along the midline of the hindbrain (Fig.

1*C,D*) and by 3 d̲ays p̲ost f̲ertilization (dpf) appeared throughout the hindbrain (data not

shown).

Enhanced Green Fluorescent Protein (EGFP) driven by *olig2* regulatory DNA

precisely recapitulates *olig2* expression in the spinal cord (Shin et al., 2003). To

compare reporter gene expression to endogenous *olig2* expression in the hindbrain, we

performed in situ hybridization to detect *egfp* RNA in *Tg(olig2:egfp)* transgenic embryos.

Similar to *olig2, egfp* RNA was first evident in two distinct clusters in r5 and r6 and then

along the anterioposterior length of the hindbrain (Fig. 2.1*E-H*). Therefore, as in the

spinal cord, transgenic reporter gene expression is an accurate indicator of hindbrain

olig2 expression.

To compare the distribution of *olig2⁺* cells to the distribution of hindbrain motor

neurons, we examined the expression of *isl1* RNA, a motor neuron marker (Korzh et al.,

1993). *isl1* was first detected at 16 hpf (data not shown) in the anterior hindbrain. By 28

hpf distinct clusters of *isl1⁺* cells were located along the anterioposterior axis,

corresponding to cranial nerves V, VII, and X (Fig. 2.1*I*), as previously described

(Higashijima et al., 2000; Chandrasekhar et al., 1997). Cranial nerves V and X arise and

remain in the rhombomeres of their birth, r2/3 and caudal hindbrain respectively,

whereas the cell bodies for cranial nerve VII are born in r4 and subsequently migrate into

r6/r7 (Chandrasekhar, 2004). At 40 hpf cranial nerve VI was present in r5 and r6 and we

observed the cell bodies of cranial nerve VII along the midline as they migrated from r4

to r6 and r7 (Fig. 2.1*J*). Migration was completed soon after and the final pattern of
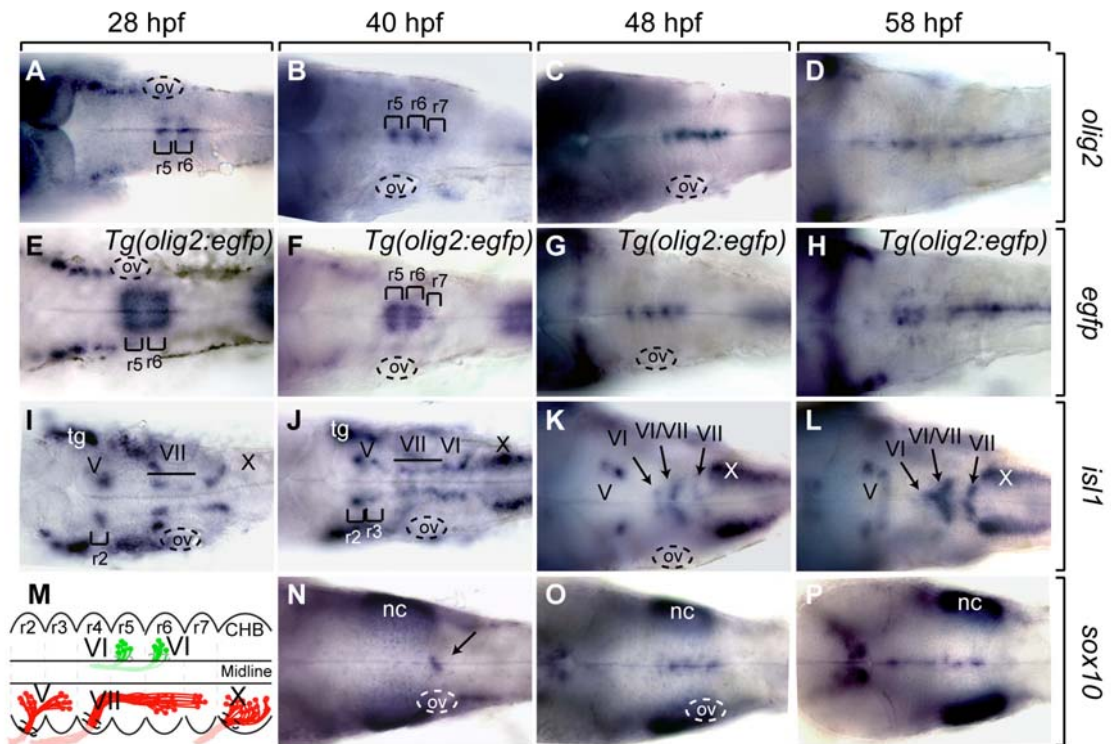
Figure 2.1. RNA expression profiles of hindbrain motor neuron and OPC markers. All panels show dorsal views of whole embryos, anterior to the left. A-D, *olig2* expression begins in rhombomeres (r) 5 and 6 at the midline and extends posteriorly and then anteriorly. E-H, *egfp* expression in *Tg(olig2:egfp)* embryos is similar to *olig2* expression. I-L, *isl1* expression marks the cell bodies of cranial nerves V, VII, X at 28 hpf and cranial nerves VI and IX beginning at 40 hpf. *isl1* also labels trigeminal ganglion cells (tg). M, Schematic of hindbrain motor neuron organization. Branchial motor neurons are depicted in red, somatic motor neurons in green. N-P, sox10 expression (arrow) begins at 40 hpf at the midline near the otic vesicle (ov) and extends along the anterioposterior axis by 48 and 58 hpf. CHB-caudal hindbrain. *sox10* expression also marks neural crest (nc) cells migrating over the surface of the hindbrain.

hindbrain motor neurons, revealed by *isl1* expression, was evident at 48 and 58 hpf (Fig. 2.1*K-M*).

We also used in situ RNA hybridization to detect the expression of *sox10,* which specifically marks oligodendrocyte lineage cells in the CNS, including oligodendrocyte progenitor cells (OPCs), dividing and migratory cells that give rise to myelinating oligodendrocytes (Kuhlbrodt et al., 1998). At 28 hpf, no *sox10* expression was evident indicating that, like spinal cord, OPCs are produced after motor neurons (data not shown). By 40 hpf, a small cluster of *sox10*$^+$ cells was apparent at about the position of r5 (Fig. 2.1*N*). Subsequently, *sox10* expression expanded anteriorly and posteriorly similar to *olig2* expression (Fig. 2.1*O,P*).

The correlation of *olig2, isl1* and *sox10* expression in r5 and r6 raised the possibility that these rhombomeres are sites of common precursors for oligodendrocytes and motor neurons. As a first test of this possibility, we examined the morphology of *olig2*$^+$ cells marked by EGFP expression in *Tg(olig2:egfp)* embryos. EGFP fluorescence was evident by 33 hpf in two prominent clusters occupying r5 and r6 (Fig. 2.2*A*). This pattern is similar to that revealed by in situ RNA hybridization, but somewhat later, reflecting the time necessary to translate and fold EGFP. Transverse and sagittal sections revealed that *olig2*$^+$ cells were ventral and medial within the hindbrain and extended fine processes to the pial surface, characteristic of neuroepithelial cells (Fig. 2.2*E,I*). By 48 hpf, *olig2*$^+$ cells appeared at the hindbrain midline both anterior and posterior to the r5 and r6 clusters and some cells with elongated morphologies occupied more lateral positions (Fig. 2.2*B*). High magnification views of sections showed some cells in the r5/r6 clusters with processes extending to the pial surface and others with thicker processes that exited the hindbrain ventrally and turned anteriorly, suggestive of axonal projections (Fig. 2.2*F,J*). Other cells, located outside the clusters, had very fine
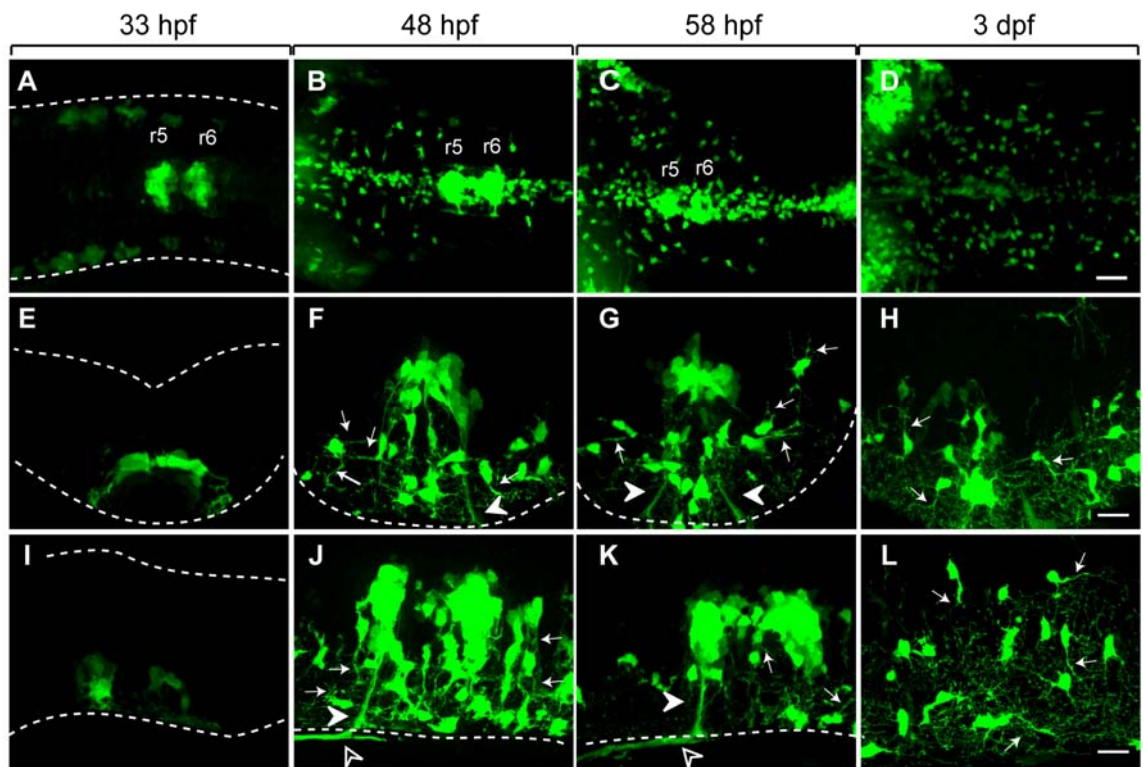
45

| | 33 hpf | 48 hpf | 58 hpf | 3 dpf |

Figure 2.2. Morphologies of hindbrain *olig2*^{+} cells revealed by transgenic reporter expression. All images show EGFP fluorescence in *Tg(olig2:egfp)* embryos. A-D, Dorsal views of whole embryos, anterior to the left. r5 and r6 indicate EGFP expression at the midline in r5 and r6. Dashed lines mark edges of neural tube. Scale bar, 48 μm. E-H, Transverse sections through the hindbrain, dorsal up, either 30 μm (F) or 10 mm (E,G,H) thick. At 33 hpf (E) EGFP[+] cell bodies are clustered at the midline and extend processes to the pial surface. From 48 hpf onward (F-H) cells with fine membrane protrusions characteristic of OPCs are present (arrows). Arrowheads indicate thick, ventrally extending processes. Scale bar, 24 μm. I-L, Sagittal sections, dorsal up, anterior to the left, either 30 μm (J) or 10 μm (I,K,L) thick. Dotted lines mark the edge of the neural tube. Open arrowheads indicate axon-like extensions that exit the hindbrain ventrally and turn anteriorly. Scale bar, 24 μm.

processes characteristic of OPCs. By 58 hpf, the r5 and r6 clusters were less distinct

and more *olig2*$^+$ cells occupied lateral positions (Fig. 2.2*C*). Fewer neuroepithelial cells

were evident within the clusters but cells with ventrally and anteriorly extending

processes were still present (Fig. 2.2*G,K*). By 3 dpf, the r5 and r6 *olig2*$^+$ clusters were no

longer visible but individual cells were dispersed throughout the hindbrain (Fig. 2/2*D*).

High magnification views showed that *olig2*$^+$ cells had OPC-like morphologies, extending

numerous fine processes to form a dense meshwork (Fig. 2.2*H,L*).

The static images presented above show that *olig2*$^+$ cells are first at the ventral

medial neural tube and then occupy the entire hindbrain. To better understand how

olig2$^+$ cells become distributed, we performed time-lapse imaging. This revealed that

some *olig2*$^+$ cells with OPC morphologies migrated from the r5 and r6 clusters and

confirmed that other cells within the clusters were the source of apparent axons that

extended ventrally and then anteriorly (Supplementary Movie 1). Additionally, many

other *olig2*$^+$ OPC-like cells emerged individually along the anterioposterior axis of the

hindbrain, both within the ventral and dorsal neural tube, and subsequently migrated

along the dorsoventral and anterioposterior axes (Supplementary Movies 1 and 2).

These observations suggested that *olig2*$^+$ neuroepithelial precursors in r5 and r6

produce some OPCs and motor neurons but that many OPCs arise from *olig2*$^-$

precursors throughout the remainder of the hindbrain.

By morphology, hindbrain *olig2*$^+$ cells appeared to include both motor neurons

and OPCs. To obtain supporting evidence and determine the motor neuron identities we

labeled *Tg(olig2:egfp)* embryos with various cell type specific markers. At 40 hpf, no

cells were labeled with anti-Sox10 antibody (Park et al., 2005) indicating that OPCs were

not yet specified (Fig. 2.3*A*). Sox10$^+$ cells appeared by 48 hpf, and each cell was also

olig2$^+$ (Fig. 2.3*B*). All *olig2*$^+$ multiprocess cells outside the r5 and r6 clusters were

Sox10$^+$, confirming their identity as OPCs. Notably, some r5 and r6 cluster cells also
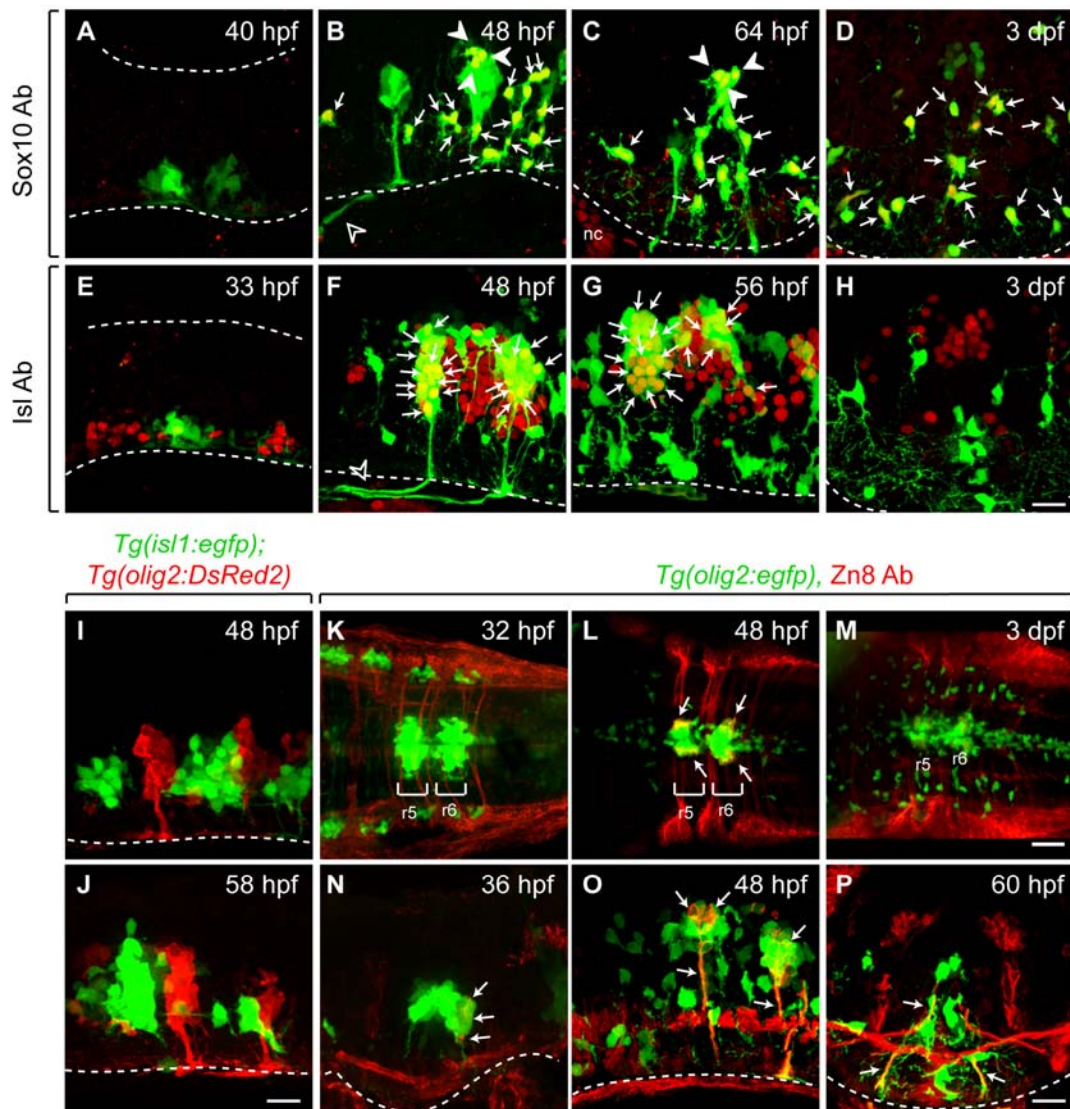
Figure 2.3. OPCs and abducens motor neurons express *olig2*. A-D, Anti-Sox10 immu-nohistochemistry to mark OPCs in sagittal (A,B) and transverse (C,D) sections of *Tg:(olig2:egfp)* embryos. Arrows and arrowheads mark *olig2*+ Sox10+ cells outside and within *olig2*+ r5 and r6 clusters, respectively. Open arrowheads mark axonal projections. E-H, Anti-Isl antibody immunohistochemistry, shown in red, to mark motor neurons in sagittal (E-G) and transverse (H) sections of *Tg:(olig2:egfp)* embryos. Arrows mark *olig2*+ Isl+ motor neurons and open arrowhead points to axonal projections. I,J, Sagittal sections of *Tg(isl1:egfp); Tg(olig2:DsRed2)* embryos. The transgenic reporters mark distinct cell populations. K-P, Zn8 immunohistochemistry, shown in red, to mark abducens motor neurons in *Tg:(olig2:egfp)* embryos. Whole embryos viewed from dorsal, anterior to the left (K-L) and transverse (N,P) and sagittal sections (O), dorsal to the top. Arrows mark *olig2*+ Zn8+ abducens motor neurons and projections. Dashed lines indicate neural tube boundary. Scale bar, 24 μm (A-J, N-P) and 48 μm (K-M).

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expressed Sox10 (Fig. 2.3*B*), consistent with the possibility that neuroepithelial cells within the clusters are precursors for OPCs. By 64 hpf, nearly every *olig2*[+] cell in the hindbrain was

Sox10[+], with only a few cells with long, ventrally extending processes remaining Sox10^{−} (Fig. 2.3*C*). By 3 dpf, *olig2* and Sox10 expression completely coincided (Fig. 2.3*D*) indicating that all hindbrain Sox10^{+} OPCs express *olig2.*

We next used anti-Isl antibody to label all motor neuron cell bodies in the hindbrain of *Tg(olig2:egfp)* embryos (Chandrasekhar et al., 1997). Whereas no *olig2*^{+} cells expressed Isl at 33 hpf, numerous cells within the r5 and r6 *olig2*[+] clusters were labeled by anti-Isl antibody at 48 hpf (Fig. 2.3*E,F*). However, many Isl^{+} cells in r5 and r6 did not express *olig2* at 48 hpf and 56 hpf (Fig. 2.3*F,G*), nor did any other Isl^{+} motor neurons in midbrain or hindbrain express *olig2* between 24 hpf and 3 dpf (Supplemental Fig. 2.1*A-D*), consistent with the observation that, in mice, *Olig2* function is necessary for development of only a subset of motor neurons (Lu et al., 2002; Zhou and Anderson, 2002; Pattyn et al., 2003; Gaufo et al., 2003). By 3 dpf double labeling was no longer evident (Fig. 2.3*H*), suggesting that, as in the spinal cord, motor neurons down-regulate *olig2* expression.

Isl protein is localized to nuclei and so anti-Isl labeling does not reveal other features of cell identity except for position within the neural tube. Consequently, to more definitively identify *olig2*^{+} motor neurons, we examined *Tg(isl1:egfp); Tg(olig2:DsRed2)* transgenic embryos. EGFP expressed by the *isl1* transgene is cytoplasmic, permitting visualization of motor neuron cell bodies and their axons (Higashijima et al., 2000). Although this particular transgenic reporter labels most cranial motor neurons, it does not label abducens motor neurons (Higashijima et al., 2000), which are located in r5 and r6 in zebrafish (Moens et al., 1996). In contrast to our anti-Isl antibody labeling results, at 48 hpf and 58 hpf the transgenes were not expressed in the same cells (Fig. 2.3*I-J*). This

raised the possibility that the r5 and r6 *olig2*⁺ motor neurons are abducens motor neurons. To test this, we next used Zn8 antibody, which recognizes the cell surface protein Neurolin, also known as Dm-Grasp (Kanki et al., 1994; Chandrasekhar et al., 1997). Zn8 did not label any *olig2*⁺ cells at 32 hpf, however, by 36 hpf some double labeling was evident (Fig. 2.3*K,N*). At 48 and 60 hpf, Zn8 clearly labeled a subset of *olig2*⁺ cells, including their ventral projections, within the r5 and r6 clusters (Fig. 2.3*L,O,P*). Consistent with the Isl antibody labeling, very little double labeling with Zn8 persisted to 3 dpf (Fig. 2.3M,*P*). Therefore, *olig2*⁺ r5 and r6 cells include abducens motor neurons. Altogether, these data indicate that, in the zebrafish hindbrain, *olig2* expression marks all OPCs but only abducens motor neurons.

  The close proximity of *olig2*⁺ abducens motor neurons and some OPCs in r5 and r6 raised the possibility that they arise from common populations of dividing, *olig2*⁺ neuroepithelial precursors. To test this, we treated *Tg(olig2:egfp)* embryos with the thymidine analog BrdU to label cells in S phase. *olig2*⁺ BrdU⁺ cells were evident in r5 and r6 at 30 and 33 hpf, but by 48 hpf significantly fewer cells incorporated BrdU throughout the hindbrain (Fig. 2.4*A–C*), reflecting a dramatic reduction in the dividing cell population as previously reported (Lyons et al., 2003). By 56 hpf, we found no *olig2*⁺ BrdU⁺ cells in ventromedial r5 and r6, which coincides with the loss of *olig2*⁺ cells with neuroepithelial morphology noted above. Instead, the only *olig2*⁺ cells labeled by BrdU at this time were OPCs (Fig. 2.4*D*). These data show that *olig2*⁺ r5 and r6 cells divide, consistent with the possibility that these cells are precursors for both abducens motor neurons and OPCs.

  We next used fate mapping to directly investigate the fates of *olig2*⁺ r5 and r6 cells. To do so we created a *Tg(olig2:Kaede)* transgenic line, which expresses the Kaede photoconvertable fluorescent protein under the control of *olig2* regulatory DNA. As expected, photoconversion of either r5 or r6 clusters labeled cells with axonal projections characteristic of abducens motor neurons (Fig. 2.5*A–D,E–H*). Additionally, by
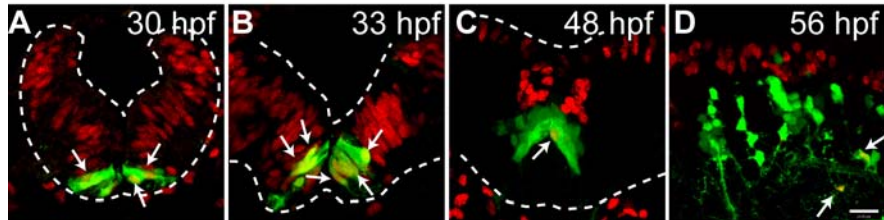
Figure 2.4. *olig2⁺* neuroepithelial cells and OPCs are mitotically active. Anti-BrdU immunohistochemistry, shown in red, to reveal S-phase cells in *Tg(olig2:egfp)* embryos. A-C, Transverse sections obtained from 30, 33 and 48 hpf embryos reveal *olig2⁺* BrdU⁺ neuroepithelial cells (arrows). D, Sagittal section. By 56 hpf, *olig2⁺* neuroepithelial cells are no longer evident and only OPCs incorporate BrdU (arrows). Dotted lines mark the edge of the neural tube. Scale bar, 24 μm.
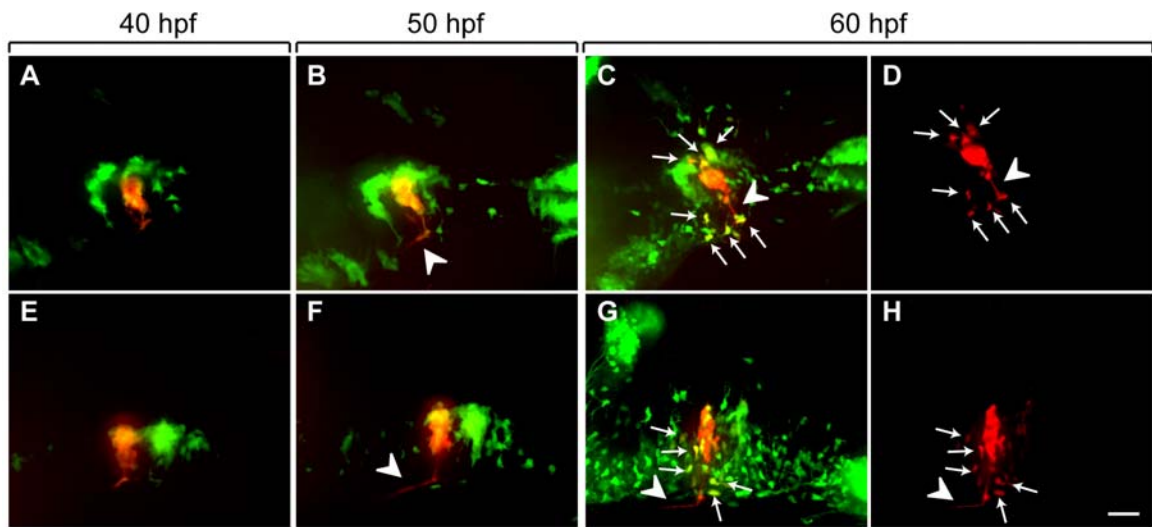
Figure 2.5. Fate mapping reveals that abducens motor neurons and some OPCs arise from r5 and r6 olig2+ cells. A-D and E-H show images from two different Tg(olig2:Kaede) embryos. In both cases, olig2+ cells where photoconverted at 40 hpf and imaged at 50 hpf and 60 hpf. D and H show only the red channel, revealing the photoconverted cells. Arrows indicate photoconverted cells that migrated from their origins and arrowheads mark abducens motor axon projections. Scale bar, 48 μm.

60 hpf a small number of photoconverted cells with OPC morphologies were outside of

the original clusters (Fig. 2.5*C–D,G–H*). Notably, many OPCs expressed only the green

form of Kaede, indicating that they did not arise from a photoconverted cluster, even

when both clusters were photoconverted (data not shown). Therefore, r5 and r6 *olig2*[+]

precursors produce abducens motor neurons and a subset of hindbrain OPCs

olig2 is required for OPC and abducens motor neuron specification

　　　　To determine the requirement of *olig2* in OPC and motor neuron specification in

the hindbrain, we injected into newly fertilized *Tg(olig2:egfp)* eggs an *olig2* antisense

morpholino (MO) designed to block translation of endogenous *olig2* transcripts but not of

egfp mRNA driven by the transgene. This allowed us to assess the fates of cells that

express *olig2* in the absence of *olig2* function. As we showed previously (Park et al.,

2002), injected embryos produced few spinal cord motor neurons and oligodendrocytes

(data not shown). 33 and 48 hpf MO-injected embryos had few *olig2*^{+} cells in the

hindbrain outside of the r5 and r6 clusters (Fig. 2.6*A–D*), suggesting that they had a

deficit of OPCs. The r5 and r6 *olig2*[+] clusters were present in MO-injected embryos,

however, most of the cells appeared to have neuroepithelial morphologies, and axonal

morphologies characteristic of abducens motor neurons were not evident (Fig. 2.6*E–H*).

Labeling MO-injected embryos with anti-Sox10 antibody confirmed the near absence of

hindbrain OPCs, both within and outside of the r5/r6 *olig2*[+] clusters (Fig. 2.6*I–L*, Fig.

2.7*A*). Although the general pattern of all other Isl[+] cranial motor neurons appeared

unaffected, including the anterior somatic motor neurons, (Fig. 2.6*M–P,* Supplemental

Fig. 2.1*E,F*), MO-injected embryos had few Isl[+] *olig2*^{+} cells in r5 and r6, indicating the

absence of only abducens motor neurons. Consistent with this, MO-injected embryos

also had a deficit of Zn8^{+} *olig2*[+] r5 and r6 cells between 48 and 56 hpf (Fig. 2.6*Q–X,* Fig.

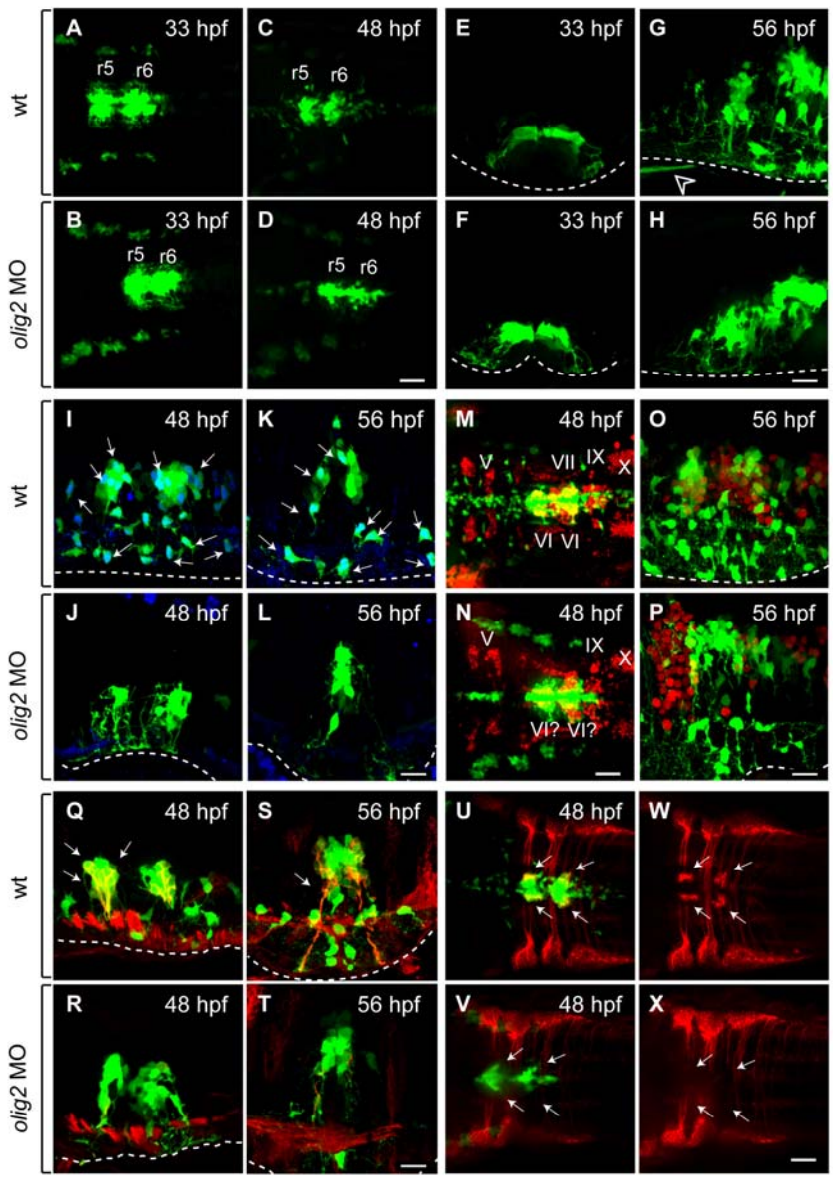2.7*B*). To further confirm the loss of OPCs and abducens motor neurons in MO-injected

Figure 2.6. OPC and abducens motor neuron development require *olig2* function. All panels show *Tg(olig2:egfp)* embryos. A-D, Dorsal views of whole wild-type (A,C) and *olig2* MO-injected (B,D) embryos, anterior to the left. Wild-type and MO-injected embryos appear similar at 33 hpf (A,B) but at 48 hpf MO-injected embryos have few dispersed OPCs compared to wild type (C,D). E,F, Transverse sections showing that *olig2*+ neuroepithelial cells appear similar in wild-type and MO-injected embryos at 33 hpf. G,H, Sagittal section of 56 hpf embryos. No *olig2*+ cells with OPC and motor neuron morphologies are apparent in the MO-injected embryo. Open arrowhead marks motor axon projections in wild type. I-L, Anti-Sox10 immunohistochemistry (blue) at 48 hpf (I,J, sagittal sections) and 56 hpf (K,L, transverse sections) reveals absence of OPCs in MO-injected embryos. Arrows point to *olig2*+ Sox10+ OPCs in wild type. M-P, Anti-Isl immunohistochemistry (red) at 48 hpf (M,N, dorsal view, whole embryos) and 56 hpf (O,P, sagittal sections). The general pattern of Isl+ cranial motor neurons is unaffected in MO-injected embryos (M,N) but MO-injected embryos have fewer *olig2*+ Isl+ motor neurons than wild type (N,P). Q-X, Zn8 immunohistochemistry (red) shown in transverse sections (Q,R), sagittal sections (S,T) and whole embryos from dorsal view (U-X). MO-injected embryos have a deficit of Zn8+ abducens motor neurons (arrows). Dotted lines mark the edge of the neural tube. Scale bar, 48 μm (A-D, M-P, U-X) and 24 μm (E-L, Q-T).
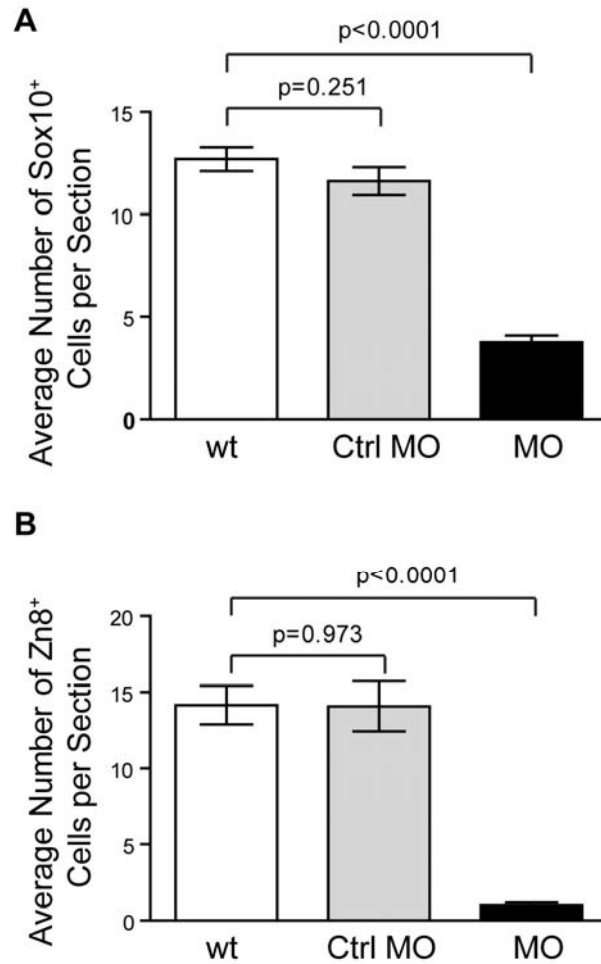
Figure 2.7. Quantification of OPCs and abducens motor neurons. A, Average number of *olig2*[+] Sox10^{+} OPCs per transverse section in wild-type, control MO-injected and *olig2* MO-injected embryos. B, Average number of *olig2*[+] Zn8^{+} abducens motor neurons per transverse section in wild-type, control MO-injected and *olig2* MO-injected embryos. Statistical significance was determined using the unpaired t-test. P values are shown for each treatment compared to wild type.

embryos, we used the *Tg(olig2:Kaede)* line to determine the fate of *olig2*$^+$ cells.

Consistent with the antibody labeling, we found an absence of photoconverted cells

outside of the original r5/r6 clusters and none of the photoconverted cells had axonal

projections characteristic of abducens motor neurons (Supplemental Fig. 2.2).

Therefore, *olig2* is required for specification of abducens motor neurons, in addition to

OPCs.

In mice, mutation of *Olig* genes alters dorsoventral patterning of the spinal cord

so that cells that would normally have pMN precursor identity produce v2 interneurons

and astrocytes instead of motor neurons and oligodendrocytes (Zhou and Anderson,

2002; Lu et al., 2002). Therefore, we sought to determine whether r5 and r6 *olig2*$^+$

precursors are similarly specified for alternative neuronal and glial fates in the absence

of *olig2* function. To test this, we first labeled embryos with anti-HuC antibody, which

marks all newly specified neurons (Marusich et al., 1994). Consistent with anti-Isl

labeling, a subset of r5 and r6 *olig2*$^+$ cells in wild-type embryos was HuC$^+$ at 56 hpf but

not at 33 hpf or 3 dpf (Fig. 2.8*A,C* and data not shown). By contrast, MO-injected

embryos had very few r5 and r6 *olig2*$^+$ HuC$^+$ cells at any stage (Fig. 2.8*B,D* and data not

shown). Next, we labeled wild-type and MO-injected embryos with Zrf1 antibody, which

marks radial glia (Trevarrow et al., 1990) and GFAP antibody, which marks radial glia

and astrocytes (Marcus and Easter, Jr., 1995). Neither antibody labeled *olig2*$^+$ cells in

wild-type or MO-injected embryos (Fig. 2.8*E,F* and data not shown). Therefore, in the

absence of *olig2* function, r5 and r6 *olig2*$^+$ precursors fail to differentiate as neurons or

glial cells.

We further investigated hindbrain patterning using antibodies that recognize

Pax2 and Nkx6.1, and the transgenic reporter *Tg(nkx2.2a:megfp)* as markers of

hindbrain cell populations. In wild-type embryos, *nkx2.2a* reporter expression marks

ventral hindbrain cells that border *olig2*$^+$ cells (Fig. 2.8*G,I*). Nkx6.1$^+$ cells surround *olig2*$^+$
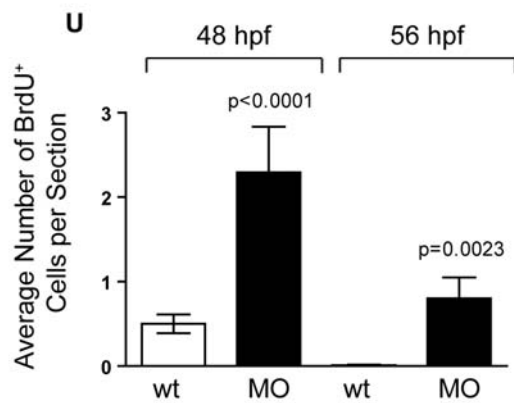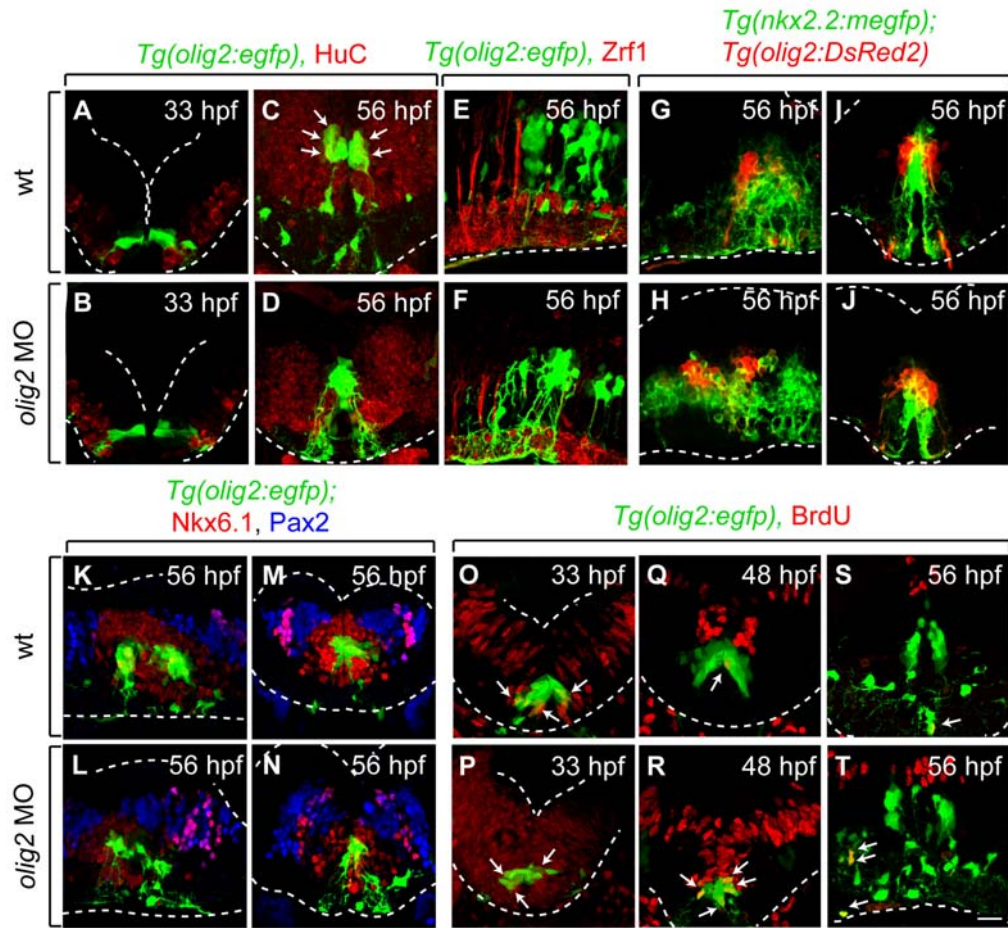
Figure 2.8. *olig2*+ cells retain neuroepithelial precursor characteristics in the absence of olig2 function. A-D, Anti-Hu immunocytochemistry (red) to reveal neurons in transverse sections. Wild-type and MO-injected embryos are similar at 33 hpf (A,B). At 56 hpf, MO-injected embryos have few *olig2*+ Hu+ neurons compared to wild type (C,D). E,F, Zrf1 immunohistochemistry (red) to reveal radial glia in sagittal sections. G-J, Sagittal (G,H) and transverse (I,J) sections of *Tg(olig2:DsRed2);Tg(nkx2.2a:megfp)* embryos. The general pattern of *nkx2.2a* expression (green) is unchanged in MO-injected embryos. K-N, Anti-Nkx6.1 (red) and anti-Pax2 (blue) immunocytochemistry on sagittal (K,L) and transverse (M,N) sections. O-T, Anti-BrdU immunohistochemistry (red) to reveal cells in S-phase. MO-injected embryos have more *olig2*+ BrdU+ neuroepithelial cells (arrows) at 48 and 56 hpf than wild type. U, Average number of *olig2*+ BrdU+ cells per tranverse section in wild-type and *olig2* MO-injected embryos. Statistical significance was determined using the unpaired t-test. P values are shown for each treatment compared to wild type. Dotted lines mark the edge of the neural tube. Scale bar, 24 μm.

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cells and, in turn, are surrounded by Pax2$^+$ cells (Fig. 2.8*K,M*). Although hindbrain patterning appears slightly perturbed in *olig2* MO-injected embryos, the relationship of these gene expression patterns to one another remained unchanged (Fig. 2.8*G–N*). The failure of r5 and r6 *olig2*$^+$ hindbrain cells to produce neurons and glial cells in the absence of *olig2* function raised the possibility that they remain as precursor cells.

To test this we treated wild-type and MO-injected embryos with BrdU to mark cells in S-phase. At 33 hpf, there was no difference in the number of *olig2*$^+$ r5 and r6 cells that incorporated BrdU in wild-type and MO-injected embryos (Fig. 2.8*O,P*). By contrast, whereas the number of *olig2*$^+$ cells that incorporated BrdU in wild type declined by 48 hpf and 56 hpf, the number of *olig2*$^+$ S-phase cells remained high in MO-injected embryos (Fig. 2.8*Q–U*). These data indicate that r5 and r6 neuroepithelial precursors require *olig2* function to exit the cell cycle and differentiate.

In chick spinal cord, ectopic expression of Olig2 in combination with Nkx2.2, but not alone, can promote premature and ectopic formation of oligodendrocytes (Zhou et al., 2001). Additionally, ectopic expression of Olig2 in chick and zebrafish spinal cord causes dorsal expansion of the motor neuron population (Mizuguchi et al., 2001; Novitch et al., 2001; Park et al., 2002). To test the capacity of *olig2* to promote formation of oligodendrocytes and motor neurons in the zebrafish hindbrain, we injected newly fertilized eggs with a plasmid that permits time-dependent expression of *olig2* controlled by a heat-responsive promoter. We induced expression in separate sets of embryos at 16 and 24 hpf, fixed them either 2, 12 or 16 hr later and performed Sox10 and Isl immunocytochemistry to detect OPCs and motor neurons, respectively. From a total of 66 experimental embryos, we never observed prematurely or ectopically produced OPCs or motor neurons (data not shown). Therefore, as in the spinal cord, *olig2* is necessary, but apparently not sufficient, for OPC and motor neuron specification in the hindbrain.

Discussion

olig2 expression identifies neural precursors that produce abducens motor neurons and a subset of hindbrain oligodendrocytes

The best characterized source of OPCs are pMN precursors, which occupy ventral spinal cord, express *Olig* genes and produce motor neurons, some interneurons, astrocytes and ependymal cells in addition to OPCs (Zhou and Anderson, 2002; Lu et al., 2002; Novitch et al., 2001; Masahira et al., 2006). Subsequently, OPCs migrate radially, dorsoventrally and longitudinally to reach their target axons, whereupon they differentiate as mature oligodendrocytes. A smaller number of oligodendrocytes also originates from dorsal spinal cord, indicating that neither *Olig* gene expression by neuroepithelial precursors nor shared lineage with motor neurons is obligatory for OPC formation (Sussman et al., 2000; Cai et al., 2005; Vallstedt et al., 2005; Fogarty et al., 2005).

The origins of hindbrain OPCs have not been as clearly defined as in spinal cord. Expression of the oligodendrocyte lineage cell marker *plp/dm20* suggested that OPCs arise from segmentally iterated domains along the hindbrain ventral ventricular zone of chick and mouse embryos, reflecting a rhombomeric organization (Perez Villegas et al., 1999; Le Bras et al., 2005; Timsit et al., 1995). Labeling of chick embryos with the O4 antibody, which also marks oligodendrocyte lineage cells (Ono et al., 1997; Orentas and Miller, 1996) likewise implicated a ventral ventricular zone origin of oligodendrocytes within the hindbrain as well as other more lateral and dorsal origins (Davies and Miller, 2001). At E13.5, mouse embryos express *Olig1* and *Olig2* within a ventral domain that extends along the entire anterioposterior axis of the hindbrain, with the exception of r1, and within the more dorsal alar plate (Vallstedt et al., 2005). Both ventral and dorsal

hindbrain explants produce oligodendrocytes, providing strong evidence that hindbrain oligodendrocytes have multiple origins (Davies and Miller, 2001; Vallstedt et al., 2005).

Whereas spinal motor neurons arise from pMN precursors to form columns along the length of the cord, cranial motor neurons have different dorsoventral origins and are organized as discrete nuclei within the midbrain and hindbrain (Guthrie, 2007; Chandrasekhar, 2004). Like spinal motor neurons, somatic cranial motor neurons emerge from the pMN precursor domain but branchiomotor and visceral cranial motor neurons originate from more ventral p3 precursors (Pattyn et al., 2003). Somatic motor neuron nuclei consist of three that innervate eye muscles, oculomotor, trochlear and abducens, and one, hypoglossal, that innervates tongue muscles (Guidato et al., 2003). The oculomotor and trochlear motor neurons occupy midbrain and r1, respectively, and abducens motor neurons are located in r5 of mice and r5 and r6 of chick and zebrafish (Guidato et al., 2003; Moens and Prince, 2002; Guthrie, 2007). Hypoglossal motor neurons form within r8 of mouse and chick but may be absent from zebrafish (Chandrasekhar, 2004).

These characterizations of oligodendrocyte and motor neuron origins reveal a potentially interesting difference between spinal cord and hindbrain. In the spinal cord apparently all spinal cord pMN precursors produce both OPCs and motor neurons (Zhou and Anderson, 2002; Lu et al., 2002; Masahira et al., 2006; Park et al., 2002; Park et al., 2004). In the hindbrain, some pMN precursors, for example those in r5 and r7 in mouse, might similarly produce somatic motor neurons and OPCs whereas others might give rise only to OPCs. Our investigation of cranial motor neuron and hindbrain OPC development in zebrafish now provides evidence for this possibility. In the spinal cord expression of *olig2* appears first in an uninterrupted column of dividing, neuroepithelial precursors and is then maintained by descendent OPCs and oligodendrocytes. By contrast, in the zebrafish hindbrain *olig2* RNA is first evident within two clusters of cells

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in r5 and r6. Only later do more anterior and posterior hindbrain cells begin to express

olig2. EGFP expression driven by an *olig2* transgene revealed differences in the

morphologies of cells along the anterioposterior hindbrain axis as they initiated *olig2*

expression. Only the cells that expressed *olig2* earliest in r5 and r6 had morphologies

characteristic of neuroepithelial cells, which we confirmed by showing that they

incorporate BrdU. EGFP$^+$ cells in all other rhombomeres had morphologies and

migratory behaviors characteristic of OPCs. Examination of transgenic reporter gene

expression and labeling by cell-type-specific antibodies revealed that, of the different

cranial motor neuron classes only abducens motor neurons express *olig2*. The close

association of abducens motor neurons and some OPCs to *olig2*$^+$ neuroepithelial cells in

r5 and r6 suggested that the latter serve as multipotent precursors. The results of our

fate mapping experiments, using photoconversion of a transgenic reporter, are

consistent with this although it is important to note that because we did not label single

cells we do not know if the same neuroepithelial precursor can produce motor neurons

and OPCs as they do in the spinal cord (Park et al., 2004). In all other regions of the

hindbrain, both ventral and dorsal, *olig2* expression is initiated in OPCs and not the

neuroepithelial precursors from which they arise. Therefore, our results indicate that the

timing of *olig2* expression within a cell lineage correlates with cell fate potential. Among

hindbrain neuroepithelial precursors, those that express *olig2* produce both somatic

motor neurons and oligodendrocytes whereas those that do not express *olig2* give rise

to OPCs but not somatic motor neurons. This raises the possibility that differences in

expression of *Olig2* genes underlie species-specific distributions of somatic motor

neurons.

olig2 is required for cell cycle exit and specification of motor neuron-OPC precursors

Olig1/Olig2 gene functions have already been shown to be necessary for development of oligodendrocytes and motor neurons, including hindbrain somatic motor neurons (Zhou and Anderson, 2002; Lu et al., 2002; Gaufo et al., 2003; Pattyn et al., 2003). In the spinal cord of mouse embryos lacking both gene functions, pMN precursors give rise to V2 interneurons and astrocytes rather than motor neurons and oligodendrocytes (Zhou and Anderson, 2002) suggesting that pMN precursors take the fate of more dorsal p2 precursors. By contrast, we found that in zebrafish embryos lacking *olig2* function, r5 and r6 precursors do not adopt alternative neuronal or glial fates, but instead continue to divide and maintain their neuroepithelial characteristics. Notably, overexpression of *Olig2* in chick reduced the number of spinal cord cells that incorporated BrdU, and increased the number that expressed the *p27* cyclin dependent kinase inhibitor and a pan-neuronal marker (Novitch et al., 2001). Additionally, *Olig1$^{-/-}$;Olig2$^{-/-}$* mouse embryos had BrdU$^+$ cells located outside the ventricular zone suggesting that pMN cells failed to exit the cell cycle prior to their migration to the marginal zone (Zhou and Anderson, 2002). Therefore, in both spinal cord and hindbrain *Olig* gene expression in neuroepithelial precursors promotes cell cycle exit and neurogenesis. This may occur through *Ngn2*, because pMN cells in *Olig1$^{-/-}$;Olig2$^{-/-}$* mouse embryos lack *Ngn2* expression (Zhou and Anderson, 2002), over-expression of *Olig2* promotes ectopic *Ngn2* expression (Novitch et al., 2001) and *Ngn2* promotes cell cycle exit and neurogenesis (Farah et al., 2000; Novitch et al., 2001). Formation of OPCs must then require that *Ngn2* function is repressed within a subset of *Olig$^+$* precursors. One likely mechanism is signaling mediated by Notch receptors, because Notch activity is reiteratively required during development for specification of OPCs from pMN precursors (Park and Appel, 2003; Kim et al., 2008).

Olig2 function is required for formation of all oligodendrocytes, whether they arise from *Olig*⁺ or *Olig*⁻ precursors. This suggests that, in addition to the role of *Olig2* in forming the pMN precursor domain and promoting the transition of dividing precursors to post-mitotic motor neurons, it has an independent function in promoting differentiation of oligodendrocyte lineage cells. Consistent with this, over-expression studies in chick indicated that Olig2 can work with the zinc finger transcription factor Zfp488 to promote precocious *Mbp* expression (Wang et al., 2006). The specific roles of *Olig2* genes in neural development are therefore likely to be determined both by transcriptional control elements that independently direct *Olig2* expression in neural precursors and OPCs and cell type specific cofactors that determine the regulatory targets of Olig2 proteins.
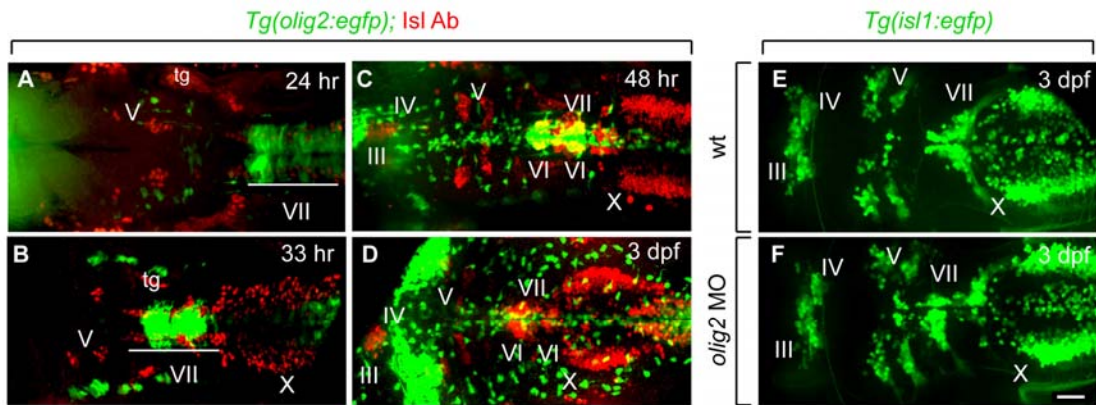
Supplemental Figure 2.1. Anterior somatic cranial motor neurons do not express *olig2* and are not affected by loss of *olig2* function. All panels show dorsal views of whole embryos. A-D, Anti-Isl antibody labeling (red) of *Tg(olig2:egfp)* embryos. Only the motor neurons of the VIth cranial nerve express *olig2*. E-F, Wild-type (E) and *olig2* MO-injected (F) *Tg(isl1:egfp)* embryos. Motor neurons revealed by transgenic reporter expression are unaffected by loss of *olig2* function. Scale bar, 24 μm.
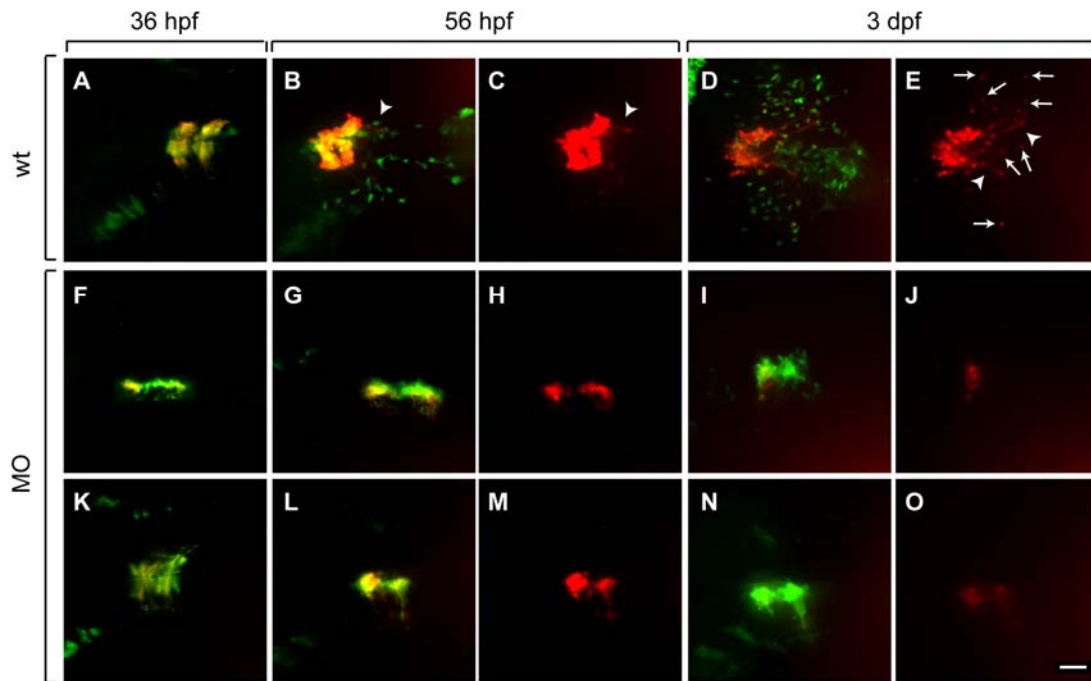
Supplemental Figure 2.2. Fate-mapping *olig2*+ cells in the absence of olig2 function. A-E, F-J and K-O show images of three different *Tg(olig2:Kaede)* embryos in which Kaede was photoconverted at 36 hpf. A-E, Photoconversion of Kaede in r5 and r6 (yellow) in wild-type embryos reveals migratory OPCs (arrows) and abducens motor axon projections (arrowheads). F-O, *olig2* MO-injected embryos. No cells with OPC or motor axon morphologies are present. C,E,H,J,M, and O show only the red channel, to show the photoconverted cells. Scale bar, 48 μm.
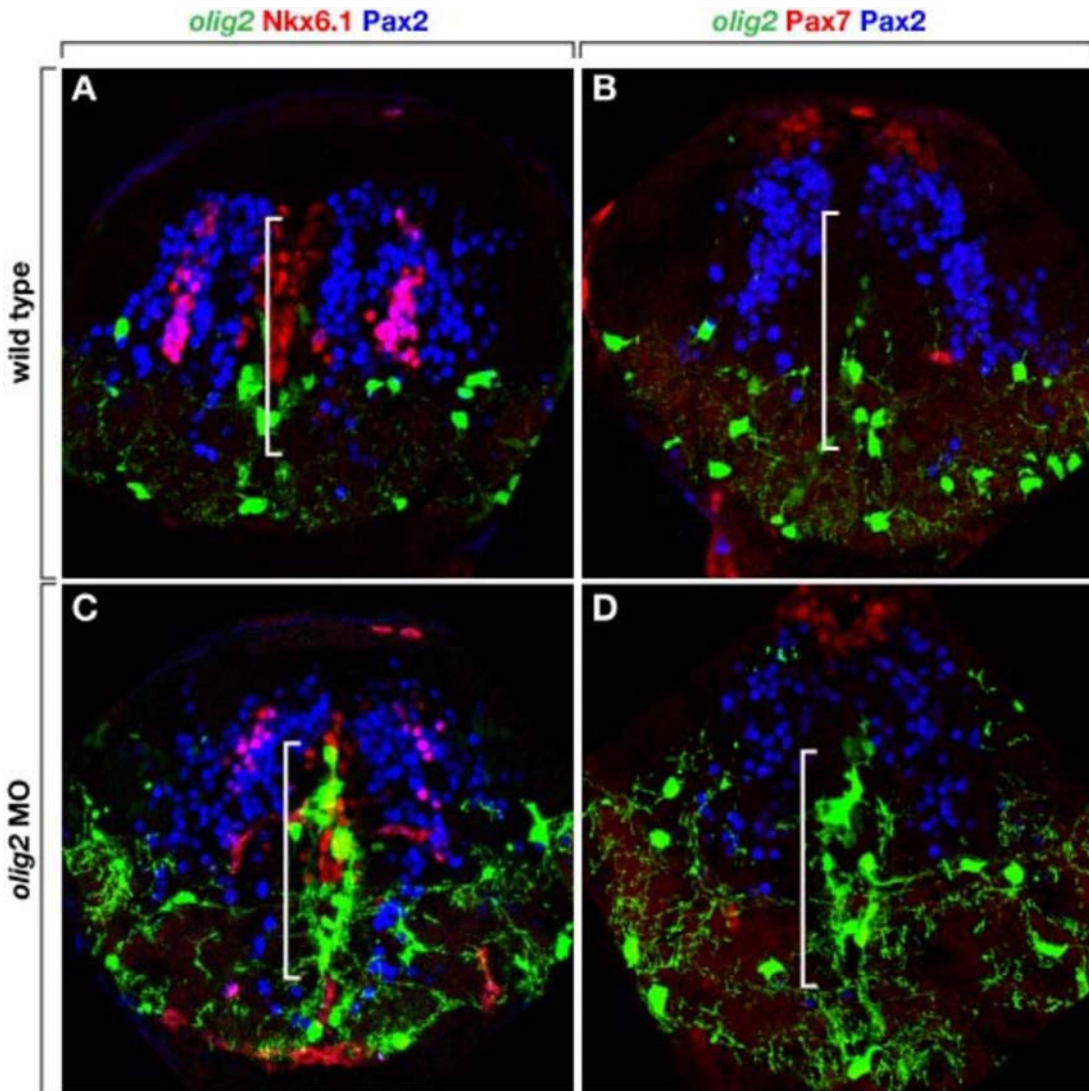
Supplemental Figure 2.3. In the absence of *olig2* function, *olig2*+ neuroepithelial cells remain at the hindbrain midline but dorsoventral patterning is normal. All panels show transverse sections through the hindbrains of 4 dpf *Tg(olig2:egfp)* larvae. A,C, Wild-type (A) and *olig2* MO-injected (C) larvae labeled with antibodies to Nkx6.1 (red) and Pax2 (blue). B,D, Wild-type (B) and olig2 MO-injected (D) larvae labeled with antibodies to Pax7 (red) and Pax2 (blue). *olig2*+ cells (green) with neuroepithelial morphologies persist at the midline of MO-injected larvae but not wild-type larvae (brackets).

CHAPTER III

THE ROLE OF *OLIG2* EXPRESSING CELLS IN FACIAL MOTOR NEURON

MIGRATION

Introduction

Forming a nervous system requires that cells not only be born at the right place and time, but often that they also migrate to their correct positions. This process, known as neuronal migration, begins with post-mitotic cells segregating from their progenitor neighbors, extending a leading process and then moving in a very stereotypical pattern to their target location to create the cytoarchitechture of the nervous system (Rakic, 1990; Rakic, 1999; Hatten, 1999; Marin and Rubenstein, 2003). Neuronal migration, which differs from axonal extension that usually occurs after a neuron reaches its final location, depends upon the interaction between migrating cells and the surface of their neighboring cells (Pearlman et al., 1998). Whereas many of the necessary transcription factors and cell surface proteins expressed within migrating cells (Hatten, 1999; Jurata et al., 2000; Song, 2007; Guthrie, 2007), as well as the environmental cues that act as attractants or repellants have been identified (Ackerman et al., 1997; Robinson et al., 1997; Birchmeier and Gherardi, 1998; Artigiani et al., 1999; Bloch-Gallego et al., 1999; Yee et al., 1999; Alcantara et al., 2000; Brose and Tessier-Lavigne, 2000), how the migrating cell actually integrates all this information to follow the correct path and make the appropriate directional changes is not well understood.

Among known populations of migrating neurons are facial motor neurons, which migrate from r4 into r6 and r7 (in zebrafish) (Chandrasekhar et al., 1997; Song, 2007). The overall trajectory of facial motor neurons can be broken down into three phases:

caudal, lateral, and radial migration, each requiring a unique set of transcription factors

and cell surface proteins (Song, 2007) (Fig. 1.2). The caudal migration begins with the

facial motor neuron cell bodes moving in a column located ventromedially from r4 into

their posterior target rhombomeres (Altman and Bayer, 1982; Ashwell and Watson,

1983; Auclair et al., 1996; Chandrasekhar, 2004; Song, 2007). At this point, the cell

bodies turn and migrate out laterally and radially to eventually reach the dorsal neural

tube (Chandrasekhar, 2004; Song, 2007; Guthrie, 2007). Thus, the facial motor neurons

must change directions from a caudal migration towards the spinal cord to a lateral

migration away from the midline and a radial migration toward the pial surface. The

lateral and radial migration is common among other branchiomotor neurons, such as the

trigeminal motor neurons, but the caudal migration is a distinctive feature to the facial

motor neurons. Another unique aspect to the facial motor neurons is that they extend

their axonal projections anteriorly concurrently with their cell bodies translocating

posteriorly (Guthrie, 2007; Song, 2007; Chandrasekhar, 2004). Although all facial motor

neurons are born in r4 and migrate caudally, their target rhombomeres can vary between

species (Guthrie, 2007; Chandrasekhar, 2004). For example, in zebrafish the facial

motor neurons migrate into r6 and r7 (Chandrasekhar et al., 1997; Higashijima et al.,

2000) whereas in mouse, shark, lizard and salamander they terminate primarily in r6

with a few cell bodies located in r5 (Guthrie, 2007; Barbas-Henry, 1982; Roth et al.,

1988; Gilland and Baker, 1993). One exception exists in avian embryos, in which the

facial motor neurons migrate laterally and radially within r4 (Lumsden and Keynes, 1989;

Szekely and Matesz, 1993) and only a small subset will migrate caudally into r5 (Jacob

and Guthrie, 2000). Nevertheless, many of the other aspects of their journey are

conserved across species.

Various studies have identified transcription factors, such as Nkx6.1 and Phox2b,

expressed within the facial motor neurons necessary for this migration (Muller et al.,

2003; Pattyn et al., 2000; Song et al., 2006). In addition, cell surface proteins, such as TAG-1 and Cadherin8, and many members of the non-canonical Wnt pathway are also required for proper facial motor neuron migration (Garel et al., 2000; Wada et al., 2006; Rohrschneider et al., 2007; Nambiar et al., 2007; Bingham et al., 2002). The majority of these non-canonical Wnt proteins function cell non-autonomously, with the exception of *Prickle1b* (Rohrschneider et al., 2007). On the other hand, other studies, using tissue transplantation experiments, demonstrate that still unknown factors from r5 and r6 are also sufficient for facial motor neuron migration in mouse (Studer, 2001). In addition, mutations that perturb theses rhombomeres, such as *valentino* in fish (Moens et al., 1996) (*kreisler* in mouse), cause aberrant facial motor migration (Chandrasekhar et al., 1997).

Interestingly, we have found that *olig2,* which is specifically expressed in r5 and r6 while the facial motor neurons are migrating, has a cell non-autonomous effect on facial motor neuron migration. The facial motor neuron in embryos injected with a MO blocking *olig2* translation fail to complete their migration into r6 and r7 and form the appropriate clusters in those respective rhombomeres. Instead some the facial motor neurons remain in r4 and r5 and appear disorganized. This lead us to hypothesize that either *olig2,* or the cells expressing *olig2,* serve as a guidepost for the facial motor neurons as they migrate through r5 and r6 or there is some chemoattractant downstream of *olig2* necessary for the caudal migration of the facial motor neurons.

Methods and Materials

Wild-type and transgenic zebrafish

Wild-type and transgenic fish were raised either in the Vanderbilt University Zebrafish Facility or the University of Colorado Denver Facility and embryos collected

from pair matings. The embryos, raised at 28.5°C, were staged according to morphological criteria (Kimmel *et al.,* 1995) and hours post-fertilization (hpf). We used the following transgenic lines: *Tg(olig2:egfp)*^{vu12} (Shin et al., 2003)*, Tg(isl1:egfp)* (Higashijima et al., 2000) and *Tg(olig2:DsRed2)*^{vu19} (Kucenas et al., 2008)*.*

In situ RNA hybridization

The following previously described RNA probes were used: *egr2b* (also known as *krox20*) (Oxtoby and Jowett, 1993), *isl1* (Inoue et al., 1994), *hoxb1a* (McClintock et al., 2002), *hoxb3a* (Hadrys et al., 2004), *hoxd4a* (Moens and Prince, 2002)*, sdf1a* and *sdf1b.* Embryos were fixed in 4% paraformaldehyde (PFA) and then stored in 100% methanol at –20°C. The in situ RNA hybridization was performed as previously described (Hauptmann and Gerster, 2000) followed by a color reaction with BM purple (Roche Diagnostics). For double RNA labeling probes were labeled with either digoxygenin or fluorescein. The first probe was detected with the appropriate antibody conjugated to alkaline phosphotase and followed by a color reaction with BM purple. Washing the embryos with 0.1M glycine, pH 2.2, followed by a 20 min incubation with 4% PFA inactivated the first antibody and the appropriate second antibody was then applied and developed with a solution of INT/BCIP (Roche Diagnostics). Once developed, the embryos were dissected from the yolk and mounted in 70% glycerol for whole mount imaging on bridged cover-slips. All images were captured using Openlab software (Improvision) using an Olympus AX70 microscope equipped with DIC optics and a Retiga Exi 1300 color digital camera. Once captured, images were imported into Adobe Photoshop and adjustments were limited to contrast, levels, color matching settings and cropping.

Immunocytochemistry

Primary and secondary antibodies used

For immunocytochemistry we used the following primary antibodies: mouse anti-Isl (39.4D5, 1:100; Developmental Studies Hybridoma Bank (DSHB)), mouse anti-Zn8 (1:1000; DSHB), mouse anti-HuC (A21271; 1;100; Invitrogen) and rabbit anti-GFP (A11122; 1:500, Invitrogen). For fluorescent detection, the following Alexa Fluro secondary antibodies were used: 568 goat anti-mouse, 568 goat anti-rabbit, 647 goat anti-mouse, and 647 goat anti-rabbit (all at 1:200; Invitrogen).

Single antibody or two antibodies raised in different species on section

Embryos were fixed in 4% AB fix (4% paraformaldehyde (PFA), 8% sucrose, 1x PBS) for 2 hr at room temperature (RT) or overnight at 4°C. Embryos for sectioning were embedded in 1.5% agar/5% sucrose, frozen with 2-methyl-butane chilled by immersion in liquid nitrogen, and sectioned using a cryostat microtome (10 μm). Sections were re-hydrated with 1x PBS and pre-blocked for 30 min in 2% sheep serum/BSA-1x PBS. The sections were incubated with primary antibody overnight at 4°C, washed extensively with 1x PBS and incubated with the appropriate fluorescent secondary antibody for 2 hr at RT. Once the secondary antibody was washed off sections were covered with Vectashield (Vector Laboratories) and cover-slips.

Whole mount antibody labeling

Embryos for whole mount imaging were fixed as above and pre-blocked with 10% sheep serum/BSA-1x PBS for 1 hr at RT. The embryos were incubated in primary antibody for 24 hr at 4°C, washed semi-continuously with 1x PBS with 0.2x Trition

(PBSTx) for 2 hr at RT, and then incubated with the secondary antibody for 12 hr at 4°C, followed by 3 hr of semi-continuous washes with 1x PBSTx. These embryos were then dissected from the yolk and mounted on bridged cover-slips in 70% glycerol for imaging.

Two antibodies raised in the same species

Embryos were fixed as described above. The strongest antibody was applied first and a 568 Alexa Fluro secondary in the appropriate animal was used. Each washing step was increased in time to insure all unbound antibody was removed; for labeling on sections the primary antibody incubation is followed by at least an hour wash with 1x PBS and for whole mount this wash is at least 3 hours with 1x PBSTx. The wash following the secondary antibody labeling was at least 45 min for sections and at least 4 hours for whole mount embryos. The sections or whole mount embryos were imaged and processed as described below.

Whole mount antibody labeling following in situ RNA hybridization

Embryos for whole mount in situ RNA hybridization followed by whole mount antibody labeling were fixed as above in 4% AB fix for 2 hrs at RT. The in situ RNA hybridization was performed as described in the previous section, however the embryos were not treated with 100% methanol. Once the color reaction with BM purple concluded the embryos were washed with 1x PBS with % Tween (PBSTw) for two 5 min washes. The embryos were then fixed in 4% PFA for 20 min to de-active the alkaline phosphotase. The fix was washed off with 1x PBSTx followed by 10% block solution for 1 hr. The rest of the whole mount antibody was performed as described above. Following in situ RNA labeling and antibody labeling, embryos were dissected from the yolk and mounted on bridged cover-slips in 70% glycerol for imaging. The DIC in situ

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RNA hybridization image was overlapped with the fluorescent image in Photoshop.

Images were exported and analyzed as described below.

Imaging

All imaging was conducted on a Zeiss Axiovert 200 inverted microscope

equipped with either a 40x oil immersion objective (NA=1.3) or 20x dry objective

(NA=0.75), mounted on a Piezo drive, and a PerkinElmer Ultraview ERS Live Cell

Imager. Images were exported and analyzed using Volocity (Improvision) and Adobe

Photoshop. Image adjustments were limited to level settings, contrast and cropping.

Antisense morpholino oligonucleotide injections

An antisense morpholino (MO) oligonucleotide with the sequence 5'-

ACACTCGGCTCGTGTCAGAGTCCAT-3' (Gene Tools, LLC) was designed to the *olig2*

translation start site. We also used a Standard Control MO (Gene Tools, LLC). Both

morpholinos were re-suspended in distilled water for a stock solution of 3 mM. The stock

solution was further diluted with water and phenyl red and 1-2 ng was injected into the

yolk of one- to two-cell stage embryos.

Conditional expression experiments

The plasmid *p(hsp70l:olig2)Tol2* was constructed by subcloning *olig2* cDNA into

a vector carrying the zebrafish *hsp70l* heat-responsive promoter (Shoji et al., 1998) and

recognition sequences for Tol2 transposase (Kawakami, 2004). Newly fertilized eggs

produced by *Tg(olig2:egfp)* or *Tg(olig2:DsRed2)* adults were injected with a solution

containing 0.1 µg/µl of the plasmid and 0.3 µg/µl in vitro synthesized Tol2 mRNA.

Expression was induced by transferring embryos at selected stages to egg water

prewarmed to 39°C for 30 min.

The embryos were manually dechorionated at 24 hpf and transferred to embryo medium containing PTU. Embryos for time-lapse imaging were anesthetized using Tricaine and immersed in 0.8% low-melting temperature agarose. They were then mounted in either lateral or dorsal orientations in glass-bottom 35mm Petri dishes. Images were captured using a 20x dry (NA=0.75) objective mounted on the confocal microscope described above. A heated stage and chamber kept the embryos at 28.5°C and Z image stacks were collected every 5-15 min. The data sets were analyzed using Volocity software and exported as QuickTime files to create movies.

Results

Improper location of facial motor neuron cell bodies in *olig2* MO-injected embryos

We initiated our studies by comparing Isl antibody labeling and *isl1* transgene expression in the hindbrain between 30 hpf and 3 dpf. The Isl antibody labels all motor neuron cell bodies (Korzh et al., 1993) whereas the *isl1* transgene labels all motor neurons, except for the abducens and glossopharyngeal motor neurons, and the motor neuron axonal projections (Higashijima et al., 2000). By 33 hpf the trigeminal, facial and vagal motor neurons are visible by both the Isl antibody and the *isl1* transgene. The two clusters of nV are located in r2 and r3 (Fig. 3.1B,C) and they are begin to extend their axonal projections towards their r2 exit point (Fig. 3.1CE). At this timepoint the facial motor neurons are migrating posteriorly and thus located in a stream at the midline between r4 and r6/7 (Fig. 3.1B,C). As they migrate caudally, they leave behind their axonal projections (Fig. 3.1C). At 50 hpf nV and nX remain largely in the same position while the facial motor neurons have finished their migration and now reside in large
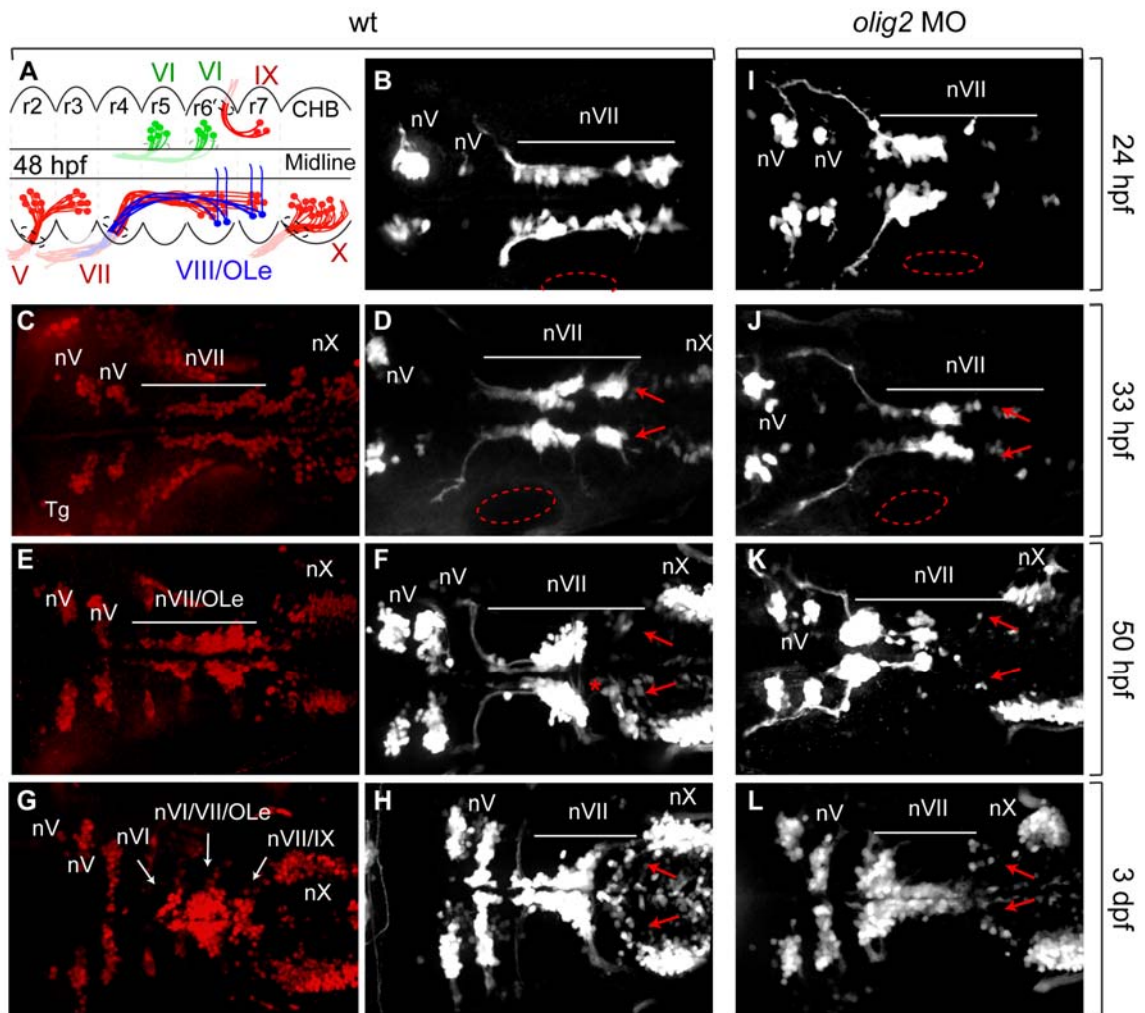
Figure 3.1. Isl expression in wild type and *olig2* MO-injected embryos**.** A. Schematic of motor neuron organization in the hindbrain. C,E,G. Isl antibody labeling in wild type embryps, dorsal whole mount, anterior to the left. B,D,F,H. *Tg(isl1:egfp)* whole mount embryos. I-L. *olig2* MO-injected embryos. Dorsal whole mount. Anterior to the left. Arrows indicated posteriorly migrated facial motor neurons. Asterisk labels contralateral OLe neurite porejctions. Otic vesivle outlined in red.

cluster in r6 and smaller, looser cluster in r7 (Fig. 3.1B,F). In addition, the octavolateralis

efferent or otic and lateral line efferent neurons (OLe) (cranial nerve nVIII) are located

intermixed with the facial motor neurons and their axons exit from r4 (Higashijima et al.,

2000; Chandrasekhar, 2004). The cell bodies of these two nerves are indistinguishable

from each other by molecular markers and both are labeled with retrograde dye when

applied at the r4 exit point (Chandrasekhar et al., 1997; Higashijima et al., 2000). The Isl

antibody, but not the *isl1* transgene, allows for visualization of the abducens motor

neurons at this timepoint in two clusters in r5 and r6 (Fig. 3.1D). Thus, the cluster of Isl$^+$

cells in r6 contains a mixed population of abducens and facial motor neurons. While the

expression pattern revealed by Isl antibody labeling looks the same at 3 dpf, the *isl1*

transgene allows the visualization of axonal projections from the vagal motor neurons,

as well as the trigeminal and facial motor neurons (Fig. 3.1F,G). Thus, both the antibody

and transgene accurately label the cranial motor neurons. Because the cell bodies and

their axonal projections can be observed with the *isl1* transgene and it fails to label the

abducens motor neurons which are located intermixed with the facial motor neurons, we

will primarily use this transgenic line to study the facial motor neuron migration

      We used *olig2* antisense morpholino oligonucleotides designed to block

translation of *olig2,* which is expressed within precursor cells in r5 and r6, and examined

the effects on facial motor neuron migration. The facial motor neurons are specified in

both wild-type and MO-injected embryos and begin extending their axonal projections

properly, however the position of the facial motor neuron cell bodies are more anterior in

the MO-injected embryo (Fig. 3.1A,H). The wild-type facial motor neurons migrate in an

almost single file line, bilaterally along the midline stretching from r4 into r6 and r7 (Fig.

3.1A). However, the facial motor neurons in MO-injected embryos are clustered together

primarily in r4 and r5 with only a few cell bodies located more posteriorly. The trigeminal

motor neurons appear unaffected in the MO-injected embryos as compared to the wild-

type embryos (Fig. 3.1A,H). By 33 hpf the wild-type facial motor neurons are no longer in r4 and are beginning to form two clusters of cells, in r6 and r7. Some of the facial motor neurons in MO-injected embryos at this time point still remain in r4 and few reach r7, instead most remain more anteriorly (Fig. 3.1C,I). Wild-type facial motor neurons, at 50 hpf, are in their final locations; a prominent cluster in r6 and a looser cluster in r7 (Fig. 3.1E). In addition, some of the cells within these clusters project neurites contralaterally. These contralateral projections are thought to be from the OLe neurons (Higashijima et al., 2000; Chandrasekhar, 2004). However, the facial motor neurons in the MO-injected embryos remained more anteriorly, many still within r4 and very few reaching to r6 and r7 (Fig. 3.1J). This failure of facial motor neurons in MO-injected embryos to properly reach r6 and r7 persists through 3 dpf (Fig. 3.1G,K).

Normal expression of anterioposterior markers and the location of the facial motor neuron migration defect

We used in situ RNA hybridization to detect expression of *krox20, Hoxb1a,* and *Hoxb4a* in *Tg(isl1:egfp)* embryos to ensure that the patterning of the AP axis was not perturbed with the *olig2* MO and to more precisely determine where along the this axisthe deficit in facial motor neuron migration occurred. We then conducted immunocytochemistry to detect GFP expressed by the *isl1:egfp* transgene. In both wild-type and MO-injected embryos the facial motor neurons are born around 19 hpf in r4, as seen with the *Hoxb1a* in situ RNA hybridization, which specifically labels r4 at this timepoint in both wild-type and MO-injected embryos (Fig. 3.2A,B). Whereas expression of *krox20* at 22 hpf was normal, the facial motor neurons in MO-injected embryos displayed a deficit in their migration, residing mostly in r4 and r5 based on the *krox20* expression specifically in r3 and r5, as compared to wild-type embryos in which the facial motor neurons have progressed into r6 (Fig 3.2I,J). At 26 hpf the stream of facial motor
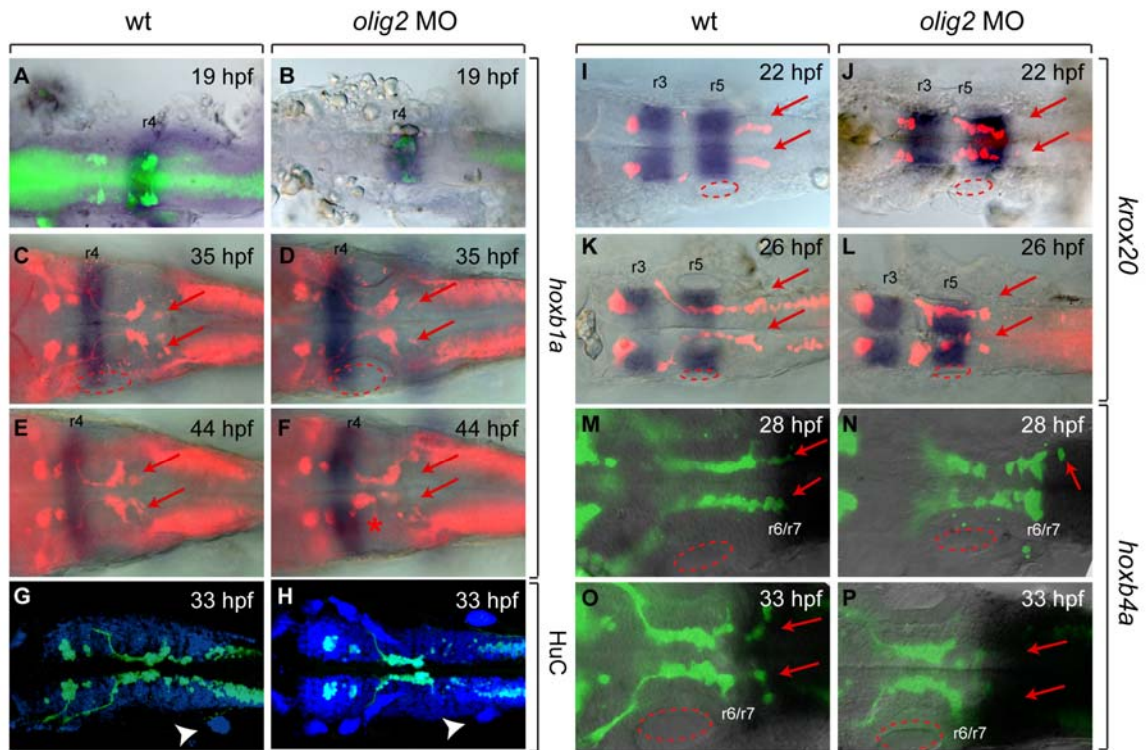
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Figure 3.2. Expression of anterioposterior markers in both wild type and *olig2* MO-injected dorsal whole mount *Tg(isl1:egfp)* embryos. *isl1:egfp* expression amplified using an anti-GFP antibody and either an Alexa 488 or 568 seconday antibody. A-F, *hoxb1a* expression marks r4. Asterisk indicates improperly migrated facial motor neurons. G-H, HuC antibody labeling to examine lateral line, arrowhead. I-L, *krox20* expression labels r3 and r5. M-P, *hoxb4a* expression begins at the r6/r7 boundary and extends posteriorly. Arrows indicate region where facial motor neurons normally migrate. Approximate location of the otic vesicle outlined with red oval.

neurons in wild-type embryos has left r4 and extends from r5 into r7, arching out laterally

and into clusters in r6 and r7 (Fig 3.2K,L). However, the facial motor neurons in MO-

injected embryos were predominately present in r4 and r5 with only a few cells in r6.

Nevertheless, they also arch out laterally despite their too-anterior position. To

determine how far posteriorly the facial motor neurons migrated we used *hoxb4a,* which

is expressed from the r6/7 boundary and extends posteriorly. This *hox* gene is also

expressed normally in both wild-type and MO-injected embryos (Fig. 3.2M-P). At 28 hpf

only an occasional cell reaches r7 in the MO-injected embryo, as compared to several in

wild-type embryos (Fig. 3.2M,N). The line of migrating facial motor neurons in wild-type

embryos extends into r7 at 28 hpf while only an occasional facial motor neuron cell body

in MO-injected embryos crosses the r6/7 boundary. Additionally, the wild-type facial

motor neurons are elongated along the AP axis while the facial motor neurons in MO-

injected embryos fail to have this morphology. By 33 hpf the wild-type facial neurons

have formed a small cluster in r7 but still few facial motor neurons in the MO-injected

embryos are located in r7 (Fig. 3.2O,P). Using *hoxb1a* to mark r4, we found that at 35

and 44 hpf wild-type facial motor neuron cell bodies are absent from r4 and only their

axonal projections are present, exiting the neural tube from this rhombomere. Instead

the cell bodies are located in r6 and r7 (Fig. 3.2C-F). A few cell bodies of the facial motor

neurons from MO-injected embryos are still located in r4 at these timepoints and while

they are also present in r6 and r7 they are reduced in number.

As a control to ensure the effects we saw were not due to overall developmental

delay, we examined the lateral line using an anti-HuC antibody. The cells contributing to

the lateral line also migrate in a posterior direction and therefore their migration should

also be affected if the *olig2* MO was causing an overall developmental delay in the

embryos. At 33 hpf both wild-type and MO-injected embryos the migration of the lateral

line was in the normal location (Fig 3.2G,H).

Quantification of migration defect

To quantify the facial motor neuron migration defect in MO-injected embryos we created a scale describing the various steps of migration at 33 and 50 hpf (Table 3.1). Using this scale the MO-injected embryos could be quantitatively compared to the wild-type embryos and using a Chi Square the statistical significance was determined. At 33 hpf, the facial motor neurons are mid-way through their migration, so the scale only has six points. By 50 hpf the migration is more complicated and thus there are more points on the scale. Each point represents one step of the wild-type migration path. At 33 hpf the MO-injected embryos exhibited a trend towards being statistically different from the wild type, with a chi square score of 15.1. With 12 degrees of freedom and a 0.05 confidence interval, this is less than the chi probability of 20.0, and thus not statistically significant. However, at 50 hpf, when wild-type facial motor neurons are in their final locations, the MO-injected embryos displayed a statistically significant difference from the wild-type embryos with a chi square score of 78.8, which is much higher than the chi probably of 33.9. Graph in Fig. 3.3. represents the mean score for each timepoint.

Timelapse imaging reveals facial motor neurons fail to complete their caudal migration and form clusters in r6 and r7 in the absence of *olig2* function

In addition to fixed images and the quantification described above, we also performed time-lapse imaging to better understand where and when the migration defect occurs in MO-injected embryos. In wild-type embryos, facial motor neurons are first visible by 19 hpf using the *isl1:egfp* transgene (Fig. 3.4A). Over the next several hours more facial motor neurons are born and they all begin to migrate posteriorly in a line

Table 3.1. Facial Motor Neuron Migration Scale

33 hpf
1) Facial motor neurons present in stream from r5 into r6/7
2) Cluster of neurons present in r7
3) Normal relative size of r7 cluster
4) Cluster in r7 distinct from r6
5) Lateral migration occurred
6) Posterior lateral line present

50 hpf
1) Facial motor neuron cell bodies in r6
2) Facial motor neuron cell bodies in r7
3) Normal clustering of neurons in r6
4) Normal clustering of neurons in r7
5) Normal relative size of r6 cluster
6) Normal relative size of r7 cluster
7) Cluster in r7 distinct from r6 cluster
8) Lateral migration
9) Posterior lateral line present
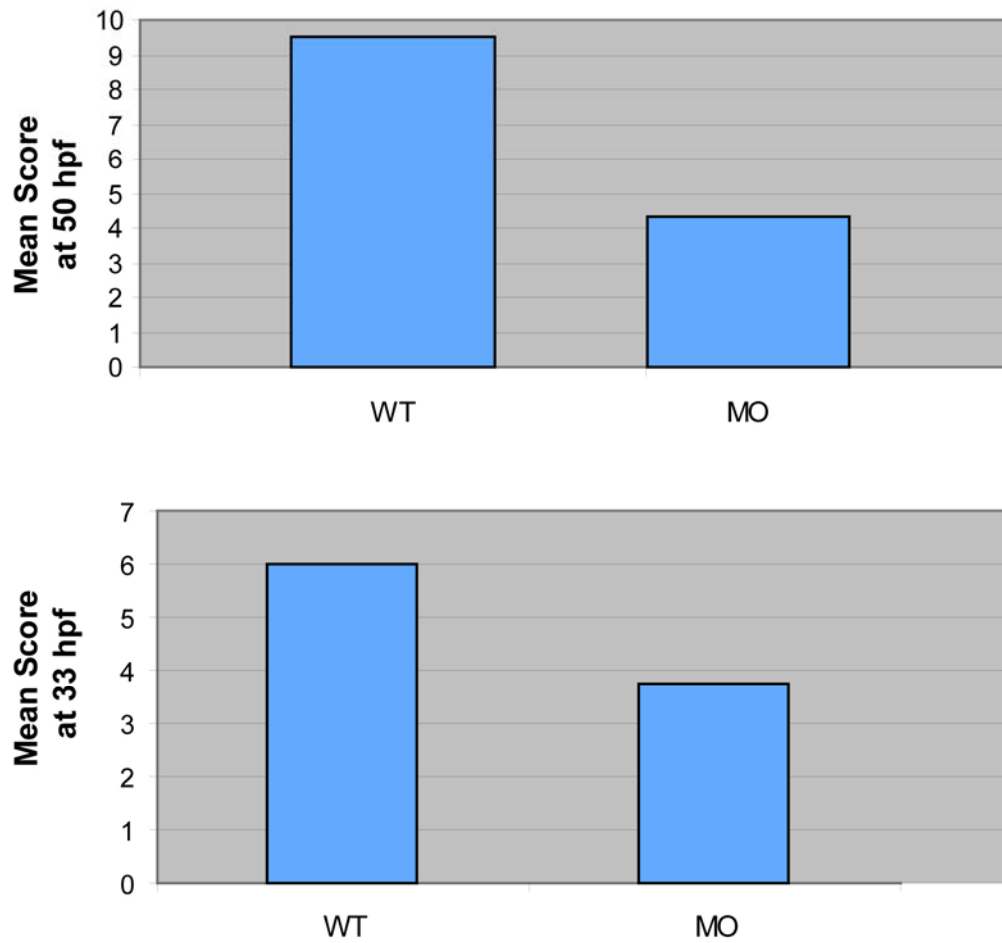10) Contralateral neurites from nVII/OLe

Figure 3.3. Facial motor neuron migration scale. Maximum score at 50 hpf is 10 point and at 33 hpf is 6 points. Statistical significant at 50 hpf with a Chi Square score of 78.8, The Chi Square probability for P=0.05 and 22 degrees of freedom is 33.92. At 33 hpf the Chi Square score is 15.2, with a Chi probabilty of 21.0 at P=0.05.

along the midline of the neural tube (Fig. 3.4B-F). Around 23 hpf the facial motor

neurons' axonal bundle is visible extending anteriorly from the migrating cells (Fig. 3.4E

arrow). This projection continues to extend and will exit the neural tube from r4 to reach

its target tissue (Fig. 3.4). Within a few hours the facial motor neurons begin to enter r6,

where they turn laterally (Fig. 3.4F). By 25 hpf the vagal motor neurons begin to be

visible, in the caudal hindbrain (Fig. 3.4G) and shortly after that the few facial motor

neurons that arrive in r7 begin to form a cluster of cells first visible around 27 hpf (Fig.

3.4I arrowhead). Over the next 10 hours the facial motor neuron cell bodies in r4 and r5

continue to migrate caudally into r6 and r7, where they take a lateral turn while their

axons extend rostrally (Fig. 3.4K-R). During this time the number of vagal motor neurons

also increases. At approximately 34 hpf contralateral projections from the OLe neurites

are visible (Fig. 3.4P, open arrowhead). Over the following 12 hours the facial motor

neurons continue to move out of r4 and r5 and into r6 and r7 (data not shown).

Similar to wild-type embryos, the facial motor neurons in MO-injected embryos

are first visible by *isl1:egfp* by 19 hpf, on either side of the midline (Fig. 3.5A). Over the

next few hours some of these cells begin to migrate posteriorly, however they do not

extend as far posteriorly as in the wild-type embryos (fig 3.5A-F). At 24 hpf the facial

motor neuron axon bundle begins to extend anteriorly, as in wild-type embryos (Fig.

3.5F). While a small number of cells are able to continue their caudal migration (Fig. 3.5J

asterisk), the majority of facial motor neuron cell bodies remain clustered in r4 and r5

(Fig. 3.5G-R). Around 29 hpf the first vagal motor neurons become apparent and

continue to increase in number in the following hours, as in wild-type embryos (Fig.

3.5K-R). At the end of this timelapse, at 36 hpf, the majority of facial motor neuron cell

bodies remain in r4 and r5 with only a few able to migrate into r6 and r7. However, they

correctly extend their axonal projections, which properly exit from r4 (Fig. 3.5)

Figure 3.4. Timelapse of wild type *Tg(isl1:egfp)* embryo. Cranial motor neurons labeled (n). Arrow indicates facial motor neuron (nVII) axonal projection. Arrowhead indicates cluster of nVIII in r7. Open arrowhead to label contralateral OLe neurite porjections.
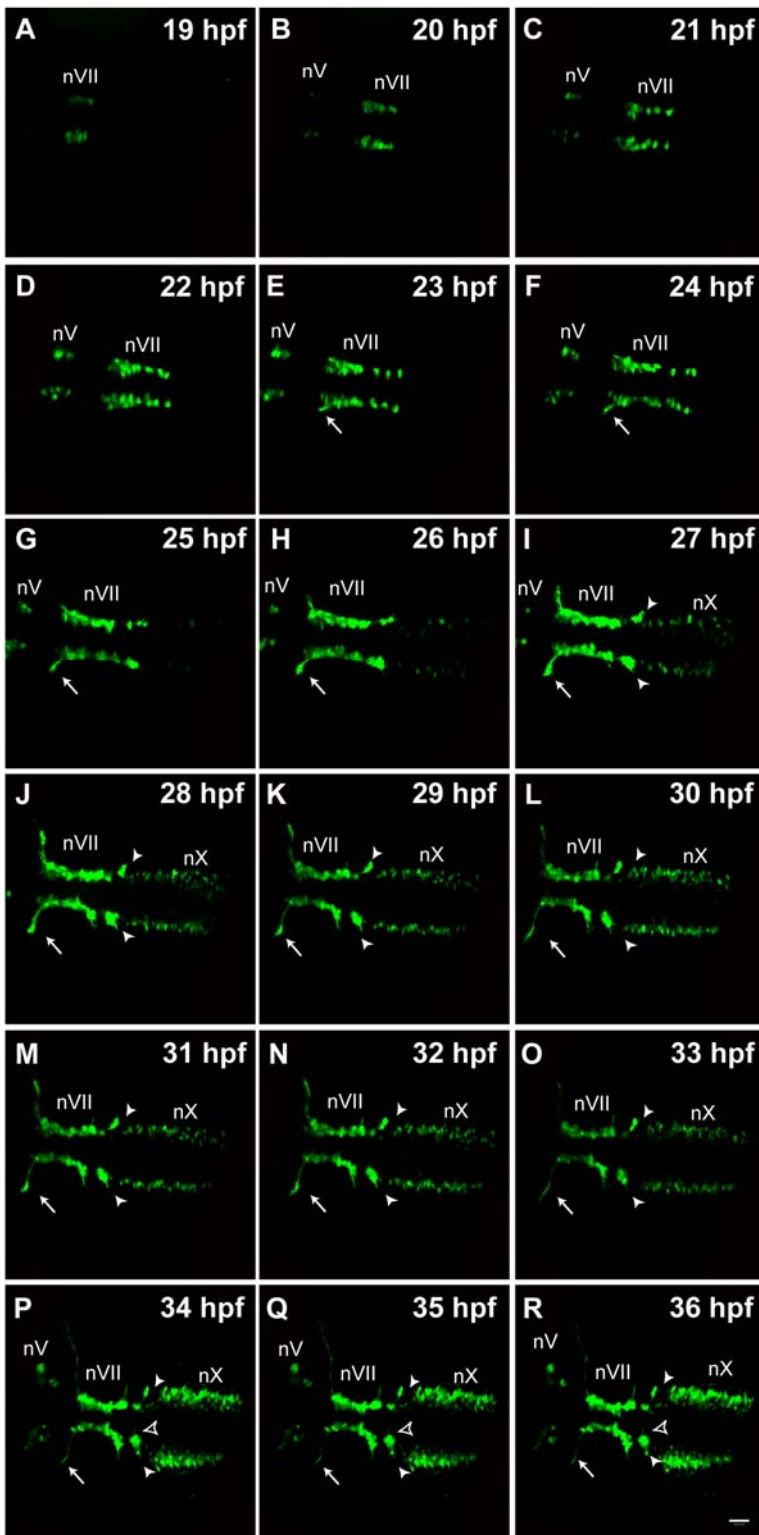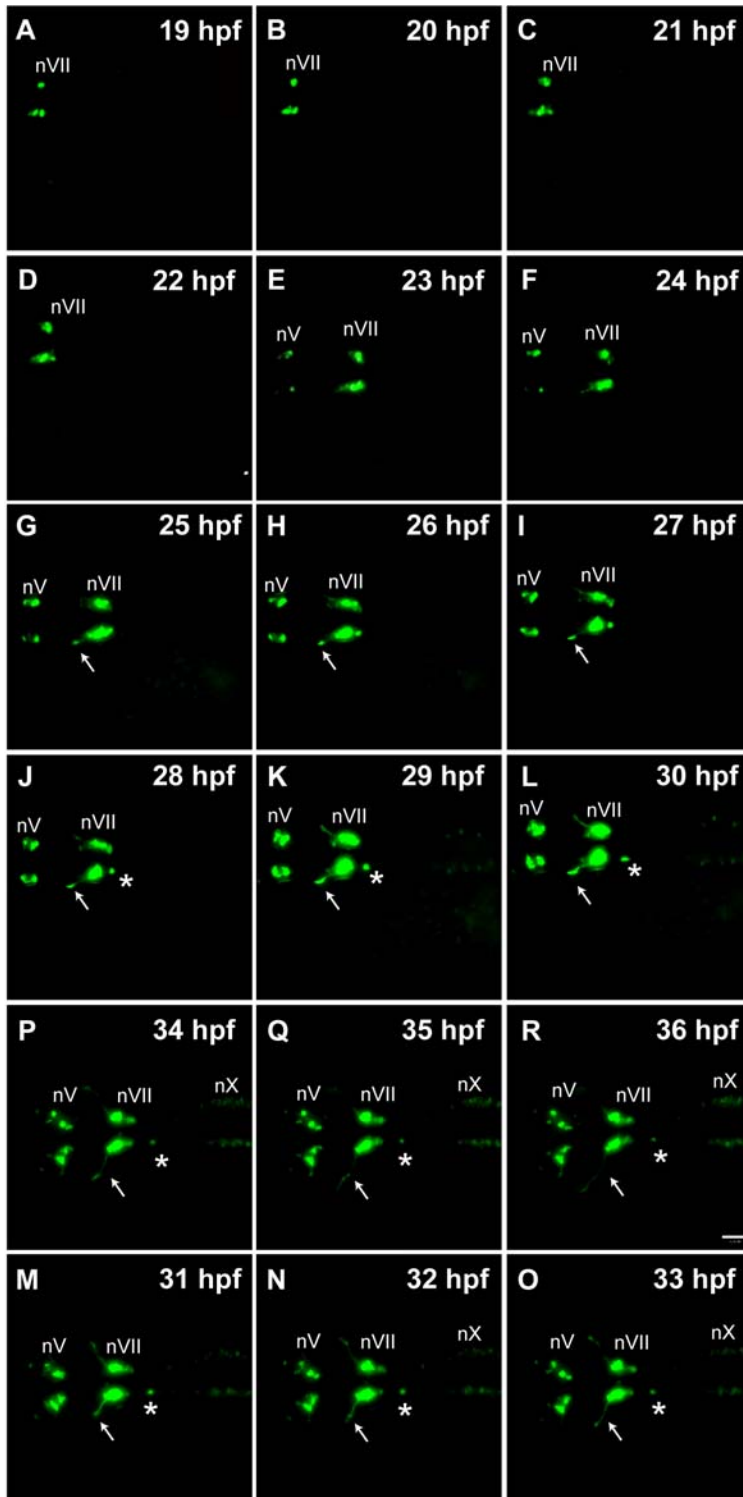
Figure 3.5. Timelapse of *olig2* MO-injected *Tg(isl1:egfp)* embryo. Cranial motor neurons labeled (n). Arrow indicates facial motor neuron (nVII) axonal projection. Astrick indicates small number of cells migrating caudally.

Discussion

Like many neuronal populations, facial motor neurons migrate from the location at which they are born to populate another region of the CNS. This migration requires the coordination of many transcription factors and cell surface molecules. Some of these function cell autonomously and others cell non-autonomously. Facial motor neurons express and require Nkx6.1 and Phox2b to begin their caudal migration out of r4 (Muller et al., 2003; Pattyn et al., 2003; Coppola et al., 2005), as well as the cell surface protein TAG-1, which is thought to help mediate their caudal migration (Garel et al., 2000). Various members of the non-canonical Wnt pathway also play a role in the caudal migration of the facial motor neurons, such as *trilobite* (Bingham et al., 2002)*, frizzeled3a* (Wada et al., 2005; Wada et al., 2006)*, scribble1* (Wada et al., 2005)*, celsr2* (Wada et al., 2006), *colgate/hda1* (Nambiar et al., 2007) and *prickle1b* (Rohrschneider et al., 2007)*.* However, only *prickle1b* appears to function cell autonomously. Once the facial motor neurons reach their target rhombomere (r6 and r7 in zebrafish or r6 and some in r5 in mouse), they turn and migrate both laterally and radially to reside in the dorsal neural tube (Guthrie, 2007; Song, 2007). At this point in their journey, the facial motor neurons have down-regulated TAG-1 and turned on Ret, a GDNF-receptor subunit (Pachnis et al., 1993) and begin to express cadherin 8 (cdh8) (Garel et al., 2000). Additionally, the gene *reelin,* essential for radial migration in the developing cortex, is also involved in the radial migration of the facial motor neurons.

In addition to these specific genes, other work demonstrates that the proper rhombomeric environment in r5 and r6 is essential for facial motor neuron migration. Transplantation of these rhombomeres is sufficient to induce facial motor neuron migration (Studer, 2001) and the mutations *kreisler* and *valentino (val),* which perturb r5 and r6 (McKay et al., 1994; Moens et al., 1996), cause the facial motor neurons to

migrate aberrantly (McKay et al., 1997; Manzanares et al., 1999; Garel et al., 2000; Chandrasekhar et al., 1997). However, what specific molecules are necessary in these rhombomeres for proper facial motor neuron migration is not clear. Interestingly, our work indicates that *olig2,* a basic helix-loop-helix (bHLH) transcription factor (Lu et al., 2000; Zhou et al., 2000) expressed in r5 and r6 and necessary for the specification of the abducens motor neurons and oligodendrocytes from the same rhombomeres, is required for facial motor neurons to reach their target rhombomeres and form the appropriate clusters. The facial motor neurons in embryos injected with an *olig2* morpholino fail to completely migrate into r6 and r7 and form their stereotypical clusters in zebrafish. A similar, though more severe, phenotype occurs in *valentino* mutant embryos (Chandrasekhar et al., 1997). *Valentino* (*val*) functions to subdivide a protosegment "rX" into r5 and r6, and in its absence a region one rhombomere's length with an identity developmentally earlier persists in between r4 and r7 (Moens et al., 1996). The facial motor neurons in *val⁻* embryos are specified, but fail to complete their caudal migration and form organized clusters (Chandrasekhar et al., 1997). In addition, many of the facial motor neuron axons in *val⁻* embryos are defasciculated, however they still properly turn at r4 to exit the neural tube. This defasciculation is not seen in the embryos injected with *olig2* MO.

One hypothesis is that the effects of the *valentino* mutation on facial motor neuron migration is due, in part, to the presumed loss of *olig2* expression. In addition to the defects in facial motor neuron migration, the *val⁻* embryos fail to form the abducens motor neurons in r6 and r7 and glossopharyngeal branchiomotor neurons in r7 (Chandrasekhar et al., 1997). The *olig2*-MO situation is less severe, with only the abducens motor neurons and some oligodendrocytes absent, while the glossopharngeal motor neuron is unaffected and the facial motor neuron axons are largely fasciculated. One way to test this hypothesis is to attempt to rescue the facial motor neuron migration

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phenotype in *val* embryos by either driving *olig2* expression with a posterior hindbrain promoter, such as *hoxb4, hoxd3* or *hoxb3* which remain expressed in "rX" in *val* embryos (Prince et al., 1998), or by transplanting *olig2*⁺ cells into the posterior *val* hindbrain. It is also possible that *olig2* has un-identified interactions with other factors in r5 and r6 to regulate facial motor neuron migration.

Several controls are needed to ensure the observed phenotype is specific to the absence of *olig2* and not general developmental delay. First, we are examining the migration of other migratory cells in *olig2* MO-injected embryos, such as the lateral line using antibody labeling with HuC. An alternative to using a MO to knock down the expression of *olig2,* laser ablation can be used to specifically ablate *olig2*⁺ cells in the hindbrain at the appropriate timepoints. This technique, when performed on one half of the hindbrain, allows for an internal control on the un-ablated side. In this situation we would expect the facial motor neurons on the ablated side to behave similar to the facial motor neurons in *olig2* MO-injected embryos.

In addition to loss-of-function studies, it would be beneficial to examine the effects of miss or over-expression of *olig2* on facial motor neuron migration. Injection of *olig2* mRNA at the single cell stage will cause global over-expression. There are a few ways to induce ectopic *olig2* expression. One is to make use of a heat-inducible promoter to drive *olig2,* which allows for temporal control of expression. This construct can be used either transiently, resulting in random ectopic expression, or can be made into a stable line. Alternatively, transplanting *olig2*-expressing cells into a wild-type embryo can cause ectopic *olig2* expression in the hindbrain. In either of these instances, the effect of *olig2*-expressing cells outside of the normal r5 and r6 location on facial motor neuron migration can be determined. Two possibilities exist; one is that *olig2*-expressing cells have a chemoattractant role in which case ectopic *olig2* expression would attract the facial motor neurons to their ectopic location. Alternatively, the *olig2*⁺

cells, or some factor secreted or expressed on their surface, could serve as a guidepost for the facial motor neurons.

The biggest outstanding question is what is the relationship between *olig2,* a transcription factor expressed in r5 and r6 but not in facial motor neurons, and the migration of facial motor neurons? Most of the studies looking at the molecular interactions of *olig2* focus on its role in astrocyte development and demonstrated that *olig2* interacts with p300, a transcriptional coactivator, to prevent it from forming a complex with STAT3, signal transducer and activator of transcription 3 (Fukuda et al., 2004; Fukuda and Taga, 2005). The p300/STAT3 complex is necessary for the development of astrocytes (Nakashima et al., 1999). In addition, *olig2* must be sequestered by ID4 (inhibitor of differentiation) to allow for the generation of astrocytes (Samanta and Kessler, 2004). However, one candidate gene that might bridge the gap between *olig2* and facial motor neuron migration is SDF1, which is already known to be required on the facial motor neuron end (Sapede et al., 2005). To test this hypothesis, we need to examine the expression of SDF1 in both wild-type and *olig2* MO-injected embryos at the appropriate times.

Another candidate gene to link *olig2* and facial motor neuron migration is *Ebf1* (early B cell factor) (Garel et al., 2000), which is a helix-loop-helix (HLH) transcription factor (Hagman et al., 1991; Hagman et al., 1993; Wang and Reed, 1993) expressed and required in facial motor neurons for their proper migration (Garel et al., 1997; Pattyn et al., 2000). In Ebf1-/- mice, a small population of the facial motor neurons migrates out laterally prematurely in r5, and concurrently they express Ret and Cdh8 as they would in r6 (Garel et al., 2000). A similar situation is seen in the striatum, where the differentiating neurons in the absence of *Ebf1* fail to down-regulate those genes expressed earlier, leading to a discrepancy between the gene expression pattern and the neurons location (Garel et al., 1999). The authors hypothesize that the facial motor neurons are unable to

properly interpret their environment and migrate out laterally too soon. Or more specifically, that *Ebf1* functions to block an r6 like phenotype while the facial motor neurons are in r5 and that some signal in r6 releases this inhibition (Garel et al., 2000). However, this mechanism maybe redundant with another system as only a subset of the facial motor neurons is affected.

Ebf1/Olf1 was found in mouse B-lymphocyte development and independently in the regulation of the rat *olfactory marker protein* gene (Hagman et al., 1993; Travis et al., 1993; Kudrycki et al., 1993; Wang and Reed, 1993). The expression of *Ebf1* is not limited to facial motor neurons, but is also found in other differentiating cells, such as motor neurons and commissural axons (Garel et al., 1997; Wang et al., 1997). Activation of *Ebf1* requires dimerization and while it contains a unique HLH motif and novel zinc coordination motif, it also has limited homology with the bHLH sequence (Hagman et al., 1993; Travis et al., 1993; Hagman et al., 1995). Interestingly, *olig2* is a bHLH transcription factor (Lu et al., 2000; Zhou et al., 2000; Takebayashi et al., 2000) and therefore, if expressed in the same cells, could function to activate *Ebf1.* Other bHLH transcription factors, such as *ngn1* and *ngn2,* are involved with *Ebf1* to promote neurogenesis and this interaction can be blocked with an Id inhibitor of bHLH proteins (Garcia-Dominguez et al., 2003). The current model puts *Ebf1* downstream of the proneural genes required for cell cycle exit, with some putative positive feedback loops, and upstream of various components necessary for neuronal differentiation and migration, such as cadherins, neurofilaments and Lim proteins (Garcia-Dominguez et al., 2003). While a link between *Efb1/Ole* and bHLH proteins is best characterized in the mouse immune system (Sigvardsson et al., 1997; Sigvardsson, 2000; Gisler and Sigvardsson, 2002), other data also indicate bHLH binding sites in the *Ebf1/Ole* promoter (Smith et al., 2002). Taken together, *Ebf1* is clearly involved in neurogenesis,

and differentiation and migration of neurons however, possible interactions with *olig2*

have not been examined.

CHAPTER IV

CONCLUSIONS

One of the fundamental questions in neural development is how a single layer of neuroepithelium gives rise to such a wide array of neuronal and glia cell types and form intricate structures, such as the hindbrain. By studying the fates of early precursor populations and the genes that regulate specification, we can begin to understand this process. One class of genes found to regulate many aspects of neural development is the basic helix-loop-helix (bHLH) transcription factors (Lee, 1997). While many bHLH members are specific for directing neurogenesis, *olig2* also plays a role in glial development (Lu et al., 2000; Zhou et al., 2000; Takebayashi et al., 2000). This role is best studied in the spinal cord pMN domain where *olig2* is required for both OPC and motor neuron specification (Lu et al., 2002; Zhou and Anderson, 2002; Park et al., 2002; Takebayashi et al., 2002). However the function of *olig2* in the hindbrain had not been as carefully studied, moreover, the relationship between cranial motor neurons, hindbrain oligodendrocytes and *olig2* had not been established.

Multiple Origins of OPCs and the Role of *olig2*

The origins of hindbrain oligodendrocytes have not been as clearly defined as in the spinal cord. Work in the hindbrain primarily focused on the expression of the oligodendrocyte lineage gene *plp/dm20* and the labeling with O4 antibody, which also marks oligodendrocyte lineage cells. Reflecting a rhombomeric organization, *plp/dm20* expression data indicated that hindbrain oligodendrocytes arose in segmentally iterated domains from the ventricular zone of mouse and chick embryos (Timsit et al., 1995; Perez Villegas et al., 1999; Le Bras et al., 2005). Labeling with the O4 antibody also

supported ventricular zone origins for oligodendrocytes in the hindbrain (Orentas and

Miller, 1996; Ono et al., 1997), as well as lateral and dorsal origins (Davies and Miller,

2001). Additionally, explant studies demonstrated that both ventral and dorsal regions of

the hindbrain give to oligodendrocytes (Davies and Miller, 2001; Vallstedt et al., 2005).

Olig function appears to be necessary both in the spinal cord and hindbrain for all

oligodendrocyte development, for in *olig1/2*$^{-/-}$ double knockout mice all OPCs in both

regions are absent (Zhou and Anderson, 2002). However, it is important to point out that

the dorsal explants described above (Vallstedt et al., 2005) were harvested prior to

dorsal *olig2* expression and therefore it remains unclear whether the resulting

oligodendrocytes arose from *olig2*$^{+}$ precursors or if they turned on *olig2* only after being

specified from an *olig2*$^{-}$ precursor population. Taken together, these data support the

hypothesis of multiple origins of oligodendrocytes in the hindbrain

In the spinal cord, the *olig2*$^{+}$ precursors of the pMN produce all of the motor

neurons, as well as many OPCs and some interneurons, astrocytes and ependymal cells

(Lu et al., 2002; Zhou and Anderson, 2002; Novitch et al., 2001; Masahira et al., 2006).

This ventral domain is continuous along the anterioposterior axis of the spinal cord

(Briscoe et al., 2000; Jessell, 2000) and all regions are equally capable of giving rise to

both motor neurons and OPCs (Lu et al., 2002; Zhou and Anderson, 2002; Park et al.,

2002; Park et al., 2004; Masahira et al., 2006). However, in the hindbrain, possible

regional differences exist along the anterioposterior axis with the pMN precursors in r5

and r7 of mouse capable of giving rise to both somatic motor neurons and

oligodendrocytes but other pMN precursors only giving rise to oligodendrocytes. While

our work examining cranial motor neuron and OPC development in the zebrafish

hindbrain supports this hypothesis, many questions remain. Why do somatic motor

neurons, and not other motor neurons, share a lineage with OPCs? Moreover, only two

of the somatic motor neuron populations appear to have this relationship, the abducens

and hypoglossal, which is not identified in fish, whereas the trochlear and oculomotor arise from yet other precursor populations.

Several disorders, such as Duane's Syndrome and Mobius Syndrome, specifically affect the development of the abducens motor neurons. In some instances, the abducens motor neuron is simply absent. Given that the abducens motor neurons arise from a precursor population that also gives rise to many OPCs, it would be interesting to know if patients diagnosis with these disorders had any other symptoms related to deficit in OPC specification, such as a lack of complete myelination.

In contrast to the continuous expression of *olig2* along the spinal cord anterioposterior axis, the initial expression in the zebrafish hindbrain is limited to r5 and r6. Subsequent expression is seen in other anterior and posterior regions, but limited to OPCs as determined by morphology, migratory behavior and double labeling with anti-Sox10 antibody. Only the early *olig2* expressing cells in r5 and r6 had neuroepithelial-like morphologies, confirmed by their ability to incorporate BrdU, similar to the pMN precursors of the spinal cord. Using cell specific antibody labeling and fate mapping techniques, we determined that these *olig2*^{+} neuroepithelial cells give rise to both OPCs and the abducens motor neurons. However, because we did not fate map individual cells, we cannot determine if the same precursor cell can produce both OPCs and motor neurons as shown in the spinal cord (Park et al., 2004). Interestingly, other motor neurons and many OPCs arise from independent origins. Thus, it appears that there are several different ventral domains that will give rise to OPCs in the hindbrain, those that express *olig2* and also give rise to the abducens motor neurons, and those that do not express *olig2* or share a lineage with abducens motor neurons. Little is known about potential *olig2*[-] precursors that are ventrally located and give rise to OPCs. What are the characteristics of this putative population, such as gene expression, precise dorsoventral location, and cell fate? It would be interesting to determine if these *olig2*[-] precursors give

rise to other motor neurons in the hindbrain in addition to OPCs, similar to the

relationship seen with the r5 and r6 *olig2*^{+} pMN cells. In a larger scope, is a neuron/glial

lineage relationship a common theme repeated throughout the CNS? In the spinal cord

interneurons from other dorsoventral domains share a lineage with astrocytes

(Muroyama et al., 2005; Hochstim et al., 2008). Nevertheless, it appears that *olig2*

expression determines where along the anterioposterior axis abducens motor neurons

arise and thus might underlie species-specific distribution of somatic motor neurons.

While the r5 and r6 pMN domain appears functionally similar to the spinal cord

pMN domain, expressing *olig2* and giving rise to both OPCs and motor neurons,

significant differences exist between the two domains. In mouse embryos lacking *olig1/2*

function, the pMN precursors adopted a more dorsal identity and gave rise to V2

interneurons and astrocytes (Zhou and Anderson, 2002). However, our studies showed

that in the zebrafish hindbrain lacking *olig2* function, the r5 and r6 *olig2*[+] precursors did

not adopt a different fate but instead maintained their neuroepithelial state and continued

to divide. *Olig2* overexpression in chick embryos reduces the number of cells in the

spinal cord that incorporate BrdU (Novitch et al., 2001), whereas in *olig1/2*[-/-] double

knockout mice BrdU^{+} cells were located outside of the ventricular zone, suggestive of

pMN cells that failed to exit the cell cycle before migrating to the marginal zone.

Therefore, it appears that in both the spinal cord and hindbrain *olig* genes function, in

part, to promote neuroepithelial precursors to exit the cell cycle and undergo

neurogenesis. This switch from a proliferative precursor state to neurogenesis likely

involves *ngn2,* for the pMN cells in *olig1*^{-/-} or *olig2*^{-/-} knockout mice lack *ngn2* expression

(Zhou and Anderson, 2002) and overexpression of *olig2* can cause ectopic *ngn2*

expression (Novitch et al., 2001). In addition, *ngn2* can also promote cell cycle exit and

neurogenesis (Farah et al., 2000; Novitch et al., 2001). Subsequently, *ngn2* function

must be repressed within a subset of *olig2*^{+} cells to allow for OPC specification. One

candidate mechanism for this switch is the Notch signaling pathway, which is required

for the specification of OPCs from the spinal cord pMN cells (Park and Appel, 2003; Kim

et al., 2008).

The organization of motor neurons was known to differ between the spinal cord

and hindbrain, with the former arising from columns along the length of the spinal cord in

the pMN domain and the latter organized as discrete nuclei in the hindbrain

(Chandrasekhar, 2004; Guthrie, 2007). Not only are there differences in the organization

of spinal and cranial motor neurons, but also in gene requirements, such as the gene

detour that is required for cranial but not spinal neurons (Chandrasekhar et al., 1999). In

addition, there are distinctions between the various cranial motor neurons. Similar to

spinal motor neurons, somatic motor neurons arise from the pMN domain and require

some of the same ventral homeodomain proteins, whereas the visceral and

branchiomotor neurons arise more ventrally from the p3 domain (Pattyn et al., 2003).

Consistent with their dorsoventral origins and similar to spinal motor neurons, only the

somatic cranial neurons require *olig2* for their specification (Gaufo et al., 2003; Lu et al.,

2002; Zhou and Anderson, 2002; Pattyn et al., 2003). Thus, somatic, but not visceral or

branchial, motor neurons appear to be specified similar to spinal motor neurons.

While some of the aspects and characteristics of the ventral OPCs domains are

being uncovered, much less is known about the dorsal origins. It is thought that OPCs

arise later in the dorsal neural tube concurrent with decrease in dorsal BMP signaling,

which inhibits OPC specification (Vallstedt et al., 2005). These dorsal OPCs do not

require Shh or Nk6 for their specification (Vallstedt et al., 2005). However they do

express the homeobox gene, *Dbx1,* which is located in four precursor domains around

the dorsoventral boundary of the neural tube, p1, p0, dP6 and dP5 (Fogarty et al., 2005;

Richardson et al., 2006). When these data are combined with studies with *Nkx6*

knockout mice (Cai et al., 2005), it can be concluded that the dorsal OPCs arise from the

dP5 domain and express the dorsal marker, Pax7 (Cai et al., 2005; Vallstedt et al., 2005). Given the various dorsoventral and anterioposterior origins for OPCs in the hindbrain, it would be intriguing to determine whether any functional difference existed between the different origins.

Regardless of dorsoventral or anterioposterior origin, all OPCs express and require *olig2*. This suggests that *olig2,* in addition to regulating cell cycle exit and promoting neurogenesis from the pMN domain, it also has an independent function in the differentiation of oligodendrocytes. These multiple roles for *olig2* likely are determined by both transcriptional control elements in neural precursors and OPCs and cell type specific cofactors that will determine the regulatory targets of Olig2 proteins.

Other interesting questions remain about the migratory behaviors of OPCs. All OPCs are migratory often migrate from ventral origins into the dorsal neural tube and visa versa (Cameron-Curry and Le Douarin, 1995; Kirby et al., 2006; Rowitch, 2004). However, it is not known how far OPCs migrate along the anterioposterior axis and if particular origins populate only the neighboring regions. In addition, if OPC specification is blocked or lost from one location, can other origins compensate for the deficit? For example, can the dorsally derived OPCs divide and migrate enough to sufficiently fill the neural tube in the absence of ventral OPCs? Or, if the r5 and r6 *olig2*$^+$ precursors were ablated in the hindbrain, could other OPC precursor populations make up for the loss? Work in zebrafish indicates that OPCs interact with one another to evenly distribute along the spinal cord and when ablated OPCs from neighboring hemi-segments divide and migrate in to fill the void (Kirby et al., 2006). Of course, this brings back the question of any functional differences between the various origins.

Facial motor neurons are one of many populations of neurons that migrate from where they are born to occupy other regions of the CNS. The trajectory of facial motor neurons is unique in that it begins in a caudal direction until the cell bodies reach the right region if the hindbrain and then they turn and migrate laterally radially to occupy the dorsal neural tube (Chandrasekhar, 2004; Guthrie, 2007). As the cell bodies migrate, they extend axonal projections anteriorly. While significant progress has been made, we are only beginning to understand the various steps and factors necessary for facial motor neuron migration.

While examining embryos injected with a translation blocking MO for *olig2,* we discovered a problem in facial motor neuron migration. Many of the cell bodies in MO-injected embryos, instead of migrating into r6 and r7 and forming clusters in each rhombomere, remained more anterior in r4 and r5 and the overall appearance of the cell bodies was disorganized. However, the cell bodies that reach r6 and r7 do appear to migrate out laterally and radially as the wild-type embryos. In addition, the facial motor neuron axonal projections in MO-injected embryos were fasciculated and correctly exited the hindbrain in r4. Thus, it appears that *olig2* plays some role in the migration, but not specification or axonal guidance, of facial motor neurons.

This phenotype brings to light many questions, the most important being the molecular mechanism by which *olig2* affects facial motor neuron migration. *olig2* is a bHLH transcription factor expressed in precursors cells located in r5 and r6, but it is not expressed in facial motor neurons. Thus, *olig2* has a cell non-autonomous effect on facial motor neuron migration. The downstream targets of *olig2* have not been identified and so it is hard to determine the exact molecular mechanism. However, some candidate genes linking *olig2* function and facial motor neuron migration exist, SDF1 and *Ebf1.*

SDF1 is a chemokine necessary for facial motor neuron migration, as well as lateral line migration (Sapede et al., 2005). Because the facial motor neurons migrate caudally within the neural tube of the hindbrain and the lateral line neurons migrate in the periphery down the length of the body, it would be logical for each to require different sources of SDF1 for their migration. Previous work demonstrates that both SDF1a and SDF1b are expressed in the hindbrain posterior to r4 (Knaut et al., 2005), where facial motor neurons are born, before and during facial motor neuron migration. Thus, these sources of SDF1 are present at an appropriate time and location to help direct facial motor neuron migration. In addition, SDF1 is expressed in the same rhombomeres that express *olig2*. Therefore, one hypothesis is that SDF1 is downstream of *olig2* and the link between *olig2* function and facial motor neuron migration. An easy test of this idea is to examine the expression of SDF1 in wild-type embryos and *olig2* MO-injected embryos. Furthermore, we would expect that driving the expression of SDF1 in r5 and r6 would rescue the *olig2* MO phenotype.

Another candidate to link *olig2* function and facial motor neuron migration is the HLH transcription factor *Ebf1*. Though expressed in facial motor neurons, *Ebf1* is also expressed in other cell populations that have not been well identified (Garel et al., 1997; Wang et al., 1997). Interestingly, *Ebf1* activation requires dimierzation (Hagman et al., 1995) and appears to have bHLH binding sites in its promoter (Smith et al., 2002). *olig2* is a bHLH transcription factor, and therefore, if expressed in the same cells, could function to activate *Ebf1*. Additionally, *Ebf1* is known to interact with other bHLH proteins, best characterized in the mouse immune system (Sigvardsson et al., 1997; Sigvardsson, 2000; Gisler and Sigvardsson, 2002). *Efb1* is upstream of several component required for neuronal differentiation and migration, such as cadherins, neurofilaments and Lim proteins (Garcia-Dominguez et al., 2003). One cadherin, cdh8, is expressed in facial motor neurons as they reach their target rhombomere and turn to

migrate laterally and radially. The hypothesis is that *Ebf1* enables the facial motor

neurons to interpret their environment and turn on the correct genes at the correct time

(Garel et al., 2000). However, *Ebf1* also regulates the chromogranin A and SCG10

promoters (Persson et al., 2004). Chromogranin A is a glycoprotein with multiple

functions in the CNS, many of which depend upon its location and post-translational

modifications (Woulfe et al., 1999; Xie et al., 2008). SCG10 is GAP that is neural

specific, developmentally regulated and capable of being induced by nerve growth factor

(Stein et al., 1988a; Stein et al., 1988b; Mori and Morii, 2002). In addition, it functions in

neural cytoskeletal rearrangement (Mori and Morii, 2002).

Other intriguing questions about the disruption of normal facial motor neuron

migration concern the effect this perturbation has on the local connectively and

ultimately behavior. Many studies demonstrate that regardless of abnormal cell body

location, the axonal projections correctly exit the hindbrain from r4 (Garel et al., 2000;

Pattyn et al., 2003; Chandrasekhar, 2004). However, do the cells that project onto facial

motor neurons make their correct connections if the cell bodies are in the wrong

location? Moreover, are there behavioral consequences to this possible miswiring?

Finally, one of the most interesting possibilities raised by the multiple roles of *olig2* in the

hindbrain is a link between facial motor neurons and abducens motor neurons. Several

disorders, including Mobius Syndrome, affect both the abducens and facial motor

neurons. Given that these two neuronal populations arise at different times, from

different areas of the hindbrain and require different genes, it is perplexing that the two

would often be affected simultaneously. *olig2* is required for the specification of one and

the migration of the other, thus in its absence, both abducens and facial motor neurons

are affected.

Given the scarcity of work focused on oligodendrocyte development in the

hindbrain and the relationship with motor neurons, this work provides novel and

important information. We confirmed that oligodendrocytes arise from multiple origins in the hindbrain, only some of which express *olig2.* The precursors that do express *olig2* also give rise to motor neurons, the abducens motor neurons, as described in the spinal cord. However, unlike the spinal cord, *olig2* function is necessary for the r5 and r6 pMN precursors to exit the cell cycle and differentiate. In addition to known specification and cell cycle roles, we also observed that facial motor neuron migration requires *olig2* function. This is perhaps the most novel and interesting finding, indicating that *olig2* has many roles in the development of the nervous system

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