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Intrinsic Properties Guide Proximal Abducens and Oculomotor Nerve Outgrowth in Avian Embryos

Cynthia Lance-Jones^{1,*}, Veeral Shah², Drew M. Noden³, and Emily Sours¹

¹Department of Neurobiology and Center for Neuroscience, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261, USA

²Department of Ophthalmology, Eye and Ear Institute, University of Pittsburgh Medical Center, Pittsburgh, PA 15261, USA

³Department of Biomedical Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853-6401, USA

Abstract

Proper movement of the vertebrate eye requires the formation of precisely patterned axonal connections linking cranial somatic motoneurons, located at defined positions in the ventral midbrain and hindbrain, with extraocular muscles. The aim of this research was to assess the relative contributions of intrinsic, population-specific properties and extrinsic, outgrowth site-specific cues during the early stages of abducens and oculomotor nerve development in avian embryos. This was accomplished by surgically transposing midbrain and caudal hindbrain segments, which had been pre-labeled by electroporation with an EGFP construct. Graft-derived EGFP+ oculomotor axons entering a hindbrain microenvironment often mimicked an abducens initial pathway and coursed cranially. Similarly, some EGFP+ abducens axons entering a midbrain microenvironment mimicked an oculomotor initial pathway and coursed ventrally. Many but not all of these axons subsequently projected to extraocular muscles that they would not normally innervate. Strikingly, EGFP+ axons also took initial paths atypical for their new location. Upon exiting from a hindbrain position, most EGFP+ oculomotor axons actually coursed ventrally and joined host branchiomotor nerves, whose neurons share molecular features with oculomotor neurons. Similarly, upon exiting from a midbrain position, some EGFP+ abducens axons turned caudally, elongated parallel to the brainstem, and contacted the lateral rectus muscle, their originally correct target. These data reveal an interplay between intrinsic properties that are unique to oculomotor and abducens populations and shared ability to recognize and respond to extrinsic directional cues. The former play a prominent role in initial pathway choices, whereas the latter appear more instructive during subsequent directional choices.

Keywords

extraocular; abducens; oculomotor; specification; motoneuron

INTRODUCTION

Many transcription factors and signaling systems involved in specifying the position and identity of developing vertebrate spinal and cranial branchiomotor neurons are known, and links between these intrinsic identities and later axonal responses to environmental guidance

cues have been established (see for review Guthrie, 2007; Butler and Tear, 2007; Dasen and Jessell, 2009). However, motoneurons that project to extrinsic eye muscles pose a unique situation. Unlike spinal motoneurons, which originate in longitudinally-arrayed columns, or branchiomotor neurons, which develop in repeating hindbrain segments, extraocular motoneurons are located in discrete, widely separated regions of the brain (Fig. 1). Moreover, both the developmental and evolutionary histories of these motoneuron pools and their target muscles are quite different from their branchial counterparts.

Oculomotor (cranial nerve III) motoneurons arise within the ventral midbrain (Levi-Montalcini, 1963). Upon exiting the brain, their axons project perpendicular to the floor of the midbrain and extend ventrally into periocular mesenchyme, where they innervate the dorsal (superior), medial and ventral (inferior) rectus muscles (DR, MR, VR) and the ventral oblique (VO) muscle (Figs. 1A–B and Carpenter, 1906; Chilton and Guthrie, 2004). Trochlear (cranial nerve IV) neurons originate within and immediately caudal to the midbrain-hindbrain boundary (isthmus) (Sohol, et al., 1985; Gilland and Baker, 2005). Their axons grow dorsally within the wall of the isthmus before emerging from the roof and coursing ventrally beside the contralateral wall of the brain then arching dorsal to the optic vesicle to reach the dorsal oblique (DO) muscle. Abducens motoneurons (cranial nerve VI) develop within hindbrain rhombomeres 5 and 6 (r5-6). Upon exiting the floor within multiple rootlets, abducens axons turn sharply and elongate cranially through mesenchyme located beneath the floor of the hindbrain to reach the lateral rectus (LR) primordium, which initially is located medial to the trigeminal ganglion (Fig. 1A–D and Carpenter, 1906; Wahl et al., 1994). Subsequently, this muscle and the abducens nerve shift cranially and laterally, coming to lie close to the temporal equatorial margin of the eye (reviewed in Noden and Francis-West, 2006).

There is substantial information on the initial induction of these motor nuclei. Similar to the situation for spinal and branchial motoneurons, the dorsoventral localization of progenitor zones for extraocular motoneurons depends on graded Sonic hedgehog signals originating from the notochord and floorplate of midbrain (Agarwala et al., 2001; Agarwala and Ragsdale, 2002; Sanders et al., 2002; Blaess et al., 2006; Bayly et al., 2007) and hindbrain regions (Ericson et al., 1997; Pattyn et al., 2003; Takahashi and Osumi, 2002; Pachikara et al., 2007). Oculomotor and trochlear neurons additionally require the expression of a *Phox2* gene for their generation (Coppola et al., 2005; Pattyn et al., 2000; Pattyn et al., 1997; Hasan et al., 2010), as do hindbrain branchiomotor but not abducens neurons.

The axial position and spatial identity of oculomotor and trochlear motoneurons is determined by signals emanating from the isthmus organizer at the midbrain-hindbrain boundary. Evidence from misexpression studies suggests that FGF8 and/or its downstream effectors are essential components of this patterning process (Crossley et al., 1996; Martinez et al., 1999; Irving and Mason, 2000; Fedtsova and Turner, 2001; Agarwala and Ragsdale, 2002; Irving et al., 2002). Transplantation studies in avian embryos suggest that the ventral positioning of midbrain neuronal populations is fixed shortly after neural tube closure (Martinez et al., 1995; Puelles, 2007; Perez-Balaguer et al., 2009). For example, ventral midbrain tissue taken from an embryo at early neural tube stages (stages 10–11) develops predominantly with a midbrain phenotype despite being grafted to the forebrain (Martinez et al., 1991, 1995) or hindbrain regions (Grapin-Botton et al., 1997). How oculomotor vs. trochlear identities are defined is not known, but it is suggested that the expression of the transcription factor *Otx2* by midbrain progenitors may confer a differential competence to respond to FGFs (Puelles, 2007; Simeone et al., 2009).

In marked contrast to the situation in the midbrain, the abducens nucleus develops in a region where motoneuron identities are influenced by retinoic acid (RA) derived from

paraxial mesoderm. (reviewed in Gavalas and Krumlauf, 2000, Schilling and Knight, 2001; Moens and Prince, 2002; Glover et al., 2006, Guthrie, 2007). In response to a gradient of RA, unique patterns of Hox expression are established within individual rhombomeres, with *Hox3* paralogues being critical for the development of abducens motoneurons in r5 and r6 (Gaufo et al., 2003; Guidato et al., 2003a and b). Abducens progenitors and postmitotic neurons share some features with spinal somatic motoneurons, including a dependence on Olig2 (Zhou and Anderson, 2002; Zannino and Appel 2009) and the Mnx proteins Hb9 and MNR2 (Tanabe et al., 1998; Thaler et al., 1999; William et al., 2003) for their determination and stabilization. Similar to oculomotor neurons, abducens motoneurons within r5-6 are committed to an abducens fate at early neural tube stages. These motoneurons are distinct from nearby branchiomotor neurons in that they express Hb9, and maintain this phenotypic marker when transplanted to r3 at stage 10 but not at stage 9 (Guidato et al., 2003a). Further, exposure of r5-6 tissues to isthmus signals, either via rhombomere transplantation or direct exposure to FGFs, does not lead to a reprogramming to a ventral midbrain phenotype (see for review, Hidalgo-Sanchez et al., 2005).

The issues of how these molecular differences are translated into stereotyped, site-specific patterns of axonal egress from the CNS and subsequent projection to individual extraocular muscles are not well understood. Several molecules have been shown to promote directed outgrowth and/or fasciculation of trochlear and oculomotor axons, including FGFs (Irving et al., 2002, trochlear only), Semaphorin 3F (Chen et al., 2000; Giger et al., 2000; Sahay et al., 2003; Watanabe et al., 2004), HGF (Caton et al., 2000), and SDF-1 (Lerner et al., 2010). The question of whether these factors influence abducens axons in a similar or different manner has not been specifically addressed. There is, however, evidence of abducens axon response to other putative guidance molecules. In vitro assays demonstrate that abducens axons are repelled by semaphorin 3A, whereas oculomotor axons are not (Colomarino and Tessier-Lavigne, 1995; Varela-Echavarria et al., 1997). Also, slit1 and slit2 appear to repel trochlear but not abducens motoneurons (Hammond et al., 2005).

These studies leave fundamental questions about normal pathway and target choice by oculomotor and abducens axons unanswered. What features of axonal behavior can be attributed primarily to intrinsic differences between motoneuron subsets as apposed to differential responses to unique environments encountered at their different sites of emergence from the brain. For example, oculomotor and abducens might differ in their complement of guidance receptors and thus respond differently to similar outgrowth microenvironments. We define these as intrinsically based or population-specific behaviors. In contrast, some unique aspects of abducens and oculomotor axon behavior may be directed by local differences in the array of extracellular signals each population encounters upon exiting the brain. We define these as extrinsically based or site-specific behaviors.

We have used classical embryonic microsurgical techniques in the avian embryo in combination with labeling by EGFP-transfection to broadly distinguish between intrinsic, population-specific and extrinsic, outgrowth site-specific guidance mechanisms involved in the formation of stereotyped oculomotor and abducens axon patterns. These two nerves were specifically chosen because their sites of origin are widely separated, they both normally exit ventromedially from the CNS, and they exhibit dramatically different and distinctive fasciculation and elongation patterns (Kuratani et al., 1988; Kuratani and Tanaka, 1990). GFP-labeled CNS segments containing developing oculomotor or abducens motoneurons were transposed at stages after they are known to have acquired population-specific molecular differences. The subsequent tracing of axon pathways provides evidence that intrinsic differences between oculomotor and abducens motoneurons play a prominent but not exclusive role in proximal pathway choice and fasciculation patterns.

METHODS

Experimental animals and surgery

Fertilized chick and quail eggs were obtained from local farms and incubated in a forced-draft incubator at 98°F. The production of experimental embryos involved the reciprocal transplantation of midbrain and caudal hindbrain segments in avian embryos. In most cases, donor tissue came from a chick embryo in which the neural tube had been transfected with an EGFP construct (Fig. 2A). In a small number of cases, quail embryos were used as a source of donor tissue; these provide a replicating cell marker. Control transplants consisted of transplanting EGFP-transfected chick midbrain (oculomotor) or r5-6 (abducens) segments in the identical location of a host chick embryo. Techniques used for identification, removal and transplantation of avian midbrain tissue and rhombomere segments were those of Guthrie (Guthrie et al., 1992; Guidato et al., 2003a) with the exceptions that we used application of a 1% neutral red solution to visualize the embryo and we transplanted fully intact rather than half neural tube segments. Embryos were staged according to Hamburger and Hamilton (1951).

Most surgeries were performed at stages 10–12 (9–16 somites), after the period when oculomotor and abducens motoneurons are reportedly specified. Midbrain tissue was identified by reference to the isthmus constriction; rhombomeres (r) 5–6, by reference to the first somite (located at r7) and inter-rhombomeric sulci. At stages 10–12, the neural tube is longer and wider at midbrain than at r5–6 levels (Guidato et al., 2003a; Grapin-Botton et al., 1997). To accommodate these longer midbrain pieces at hindbrain levels, we removed parts of r4 or r7 or transplanted only the caudal 2/3 of the midbrain. Slight lateral (outward) pressure was applied to mesodermal tissue in host embryos in order to accommodate the wider graft. Transplantation of r5–6 pieces to midbrain levels occasionally resulted in small gaps between host and donor neural tissue unless rostral midbrain tissue was left intact or parts of r4 or r7 were included. No differences in axon outgrowth patterns were found with these slight differences in transplant length, connection to donor tissue, or inclusion/exclusion of underlying notochordal tissue.

Two variations of the basic surgical procedures were carried out in smaller subsets of embryos: transplants of younger (stage 8–9, 4–8 somites) r5–6 pieces to midbrain levels, using the basic procedures of Grapin-Botton et al., (1995), and removal of host midbrain tissue in conjunction with midbrain to caudal hindbrain transplantations (Fig. 2D). The rationale for each of these variations will be discussed in the results section.

In ovo electroporation

Techniques for *in ovo* electroporation were those of Misra et al., 2009. Chick neural tubes were microinjected at midbrain and hindbrain levels with the pMES vector (provided by C. Krull), consisting of pCAX (Kobayashi) with the addition of the *ires-egfp* fragment from pIRES2-EGFP (Clontech). A β -actin promoter drives gene expression ubiquitously in progenitor and postmitotic neural cells. DNA was diluted to 1.25 μ g/ μ l with Tris-EDTA (pH 8.0) and 0.05% Fast green was added for visualization during injection. Immediately after injection, embryos were bathed in sterile saline and electroporated. Gold 0.5 mm electrodes were positioned on either side of the embryo to provide for transfection of the cranial neural tube on one side. Current was delivered by a square pulse electroporator (BTX) in three 50 ms pulses with a charging voltage of 17V. Neural tube transplantations were performed immediately after electroporation.

Immunocytochemistry

Embryos were sacrificed at stages 20–29, with most embryos being sacrificed at stages 24–27 (5–7 days of incubation), which is after the period when oculomotor and abducens axons have normally reached their target muscles. Embryos showing a clear malpositioning of the transplant or an absence of EGFP+ cells were excluded from further analysis.

Cranial tissues were fixed in 4% paraformaldehyde, cryoprotected in 30% sucrose, embedded in 50:50 30% sucrose:OCT, frozen, and sectioned at 14 μ m. Serial sagittal or transverse sections were placed on two or three sets of slides in an alternating pattern to permit processing of adjacent sections with different antibody combinations. The following antibodies were used at the indicated dilutions: mouse anti-MNR2/Hb9 (Developmental Studies Hybridoma Bank, DSHB), 1:200; rabbit anti-EGFP (Invitrogen), 1:1500; mouse anti-Isl1/2 (DSHB), 1:100; mouse anti-neurofilament (3A10, DSHB), 1:100; mouse anti-quail (QCPN, DSHB), 1:100; and mouse anti-myosin (MF20, DSHB), 1:100. Cy2- and Cy3-conjugated secondary antibodies were used according to manufacturer's recommendations. (Jackson Immunoresearch). Tissues from some quail-chick chimeras were fixed in Serra's and processed through paraffin embedding. Alternating sets of 6–8 sections (10 μ m each) were mounted on separate slides for staining with anti-quail (QCPN) or anti-neurofilament (3A10) antibodies using standard DAB protocols and an ABC kit (Vector Laboratories). Fluorescent and bright-field microscopy was carried out using a Nikon Eclipse E600 compound microscope and a Q Imaging Retiga 2000R camera.

Embryo numbers

Sections from all embryos with EGFP-transfected transplants were stained with anti-EGFP and anti-neurofilament antibodies in order to trace the course of graft-derived nerve fibers, to visualize gross nerve patterns, and to identify axon termination sites. Sections from a subset of each experimental group were stained with anti-MNR2/Hb9 and anti-Isl1/2 to identify abducens and oculomotor cell bodies using molecular profiles. Here, the numbers per experimental group ranged from 4–11 embryos, in each case representing at least 1/3 of the total number of embryos in that experimental group. To visualize extraocular muscle primordia, selected sections were stained with MF-20.

RESULTS

Abducens and oculomotor axon trajectories in normal and control transplant embryos

We first examined projections in homotopic transplant embryos (midbrain to midbrain and r5-6 to r5-6) in order to assess the extent of EGFP labeling and to determine if neural tube excision alone would influence anatomical patterns of the oculomotor and abducens nerves. Examination of embryos at stages 25–28 indicated that the basic pattern of projections matched those found in normal embryos (Fig. 1).

In embryos with midbrain to midbrain transplants (n=9), the graft region is identified by staining with anti-EGFP (Figs. 1E–H). Cells within the graft region express the pan-motoneuron marker, Isl1/2 (Fig. 1G). They do not express Hb9 (Fig. 1H), a marker normally expressed by abducens and hypoglossal motoneurons, but not by oculomotor neurons (see Guidato et al., 2003a). As in a normal embryo, oculomotor axons exit medially as a tight fascicle and course ventrally to the periocular region (Figs. 1F and J). Staining with anti-neurofilament (NF) and anti-EGFP indicate that many but not all NF+ axons are also EGFP+. In assessing axonal distribution in this and subsequent experiments, we scored only those projections that were EGFP+. This is because the ramification of the nearby trigeminal sensory nerve fibers is extensive and confounds the tracing of peripheral axons labeled with anti-NF alone. In midbrain transplant embryos, EGFP+ axons extend to muscle primordia

normally innervated by the oculomotor (DR, MR/VR, and VO), and not to the LR, the normal target of the abducens (Figs. 1A, B and I, see also Table 1).

In embryos with r5-6 to r5-6 transplants (n=8), EGFP+ CNS regions contain both Isl1/2+ and Hb9+ cells, indicating an abducens-like somatic motoneuron identity (Figs. 1M–N). Medially exiting, EGFP+ abducens axons emerge as small fascicles that turn cranially beneath the hindbrain and coalesce into a single abducens nerve, a pattern similar to that in normal embryos (Fig. 1K–L). The abducens nerve normally contains two populations of axons, those that innervate the LR but also accessory abducens axons that project to the pyramidalis and quadratus muscles (P-Q, Wahl et al., 1994). As this mixed nerve approaches the common primordium of the LR and P-Q, it splits into ventral and dorsal branches that project to LR and P-Q regions, respectively (Fig. 1C). In control transplant embryos, the EGFP+ abducens nerve shows a similar bifurcation and terminations within the LR/P-Q primordium (Figs. 1O–Q, see also Table 1).

Axon trajectories following midbrain to hindbrain transplants

As with control transplants, transposition of EGF-transfected midbrain tissue to a caudal hindbrain position was done at stages 10–12. In a subset of embryos (n=9/24), host midbrain tissue was also removed (Fig 2D). Since oculomotor nuclei are normally substantially larger than abducens nuclei, our surgeries would be expected to increase the ratio of motoneurons to targets at early stages of axon outgrowth. Removal of host midbrain tissue, thus, provided a test of the hypothesis that target availability limits pathway and/or target choice by donor oculomotor neurons. In fact, axon projections were very similar $\pm$ host midbrain removal (see Table 1) and outcomes are described together.

At sacrifice (stages 24–29), embryos show a conspicuous midbrain vesicle at caudal hindbrain levels (Figs. 2B and 2E arrows, 3A). The rostrocaudal position of the EGFP+ transplant varies $\pm$ 1 rhombomere segment from the r5-6 level, such that the cranial border of the transplant impinges upon the r4 level in some embryos. Isl1/2+ motoneurons are readily visible in the transplant region but no Hb9+ cells were detected, indicating that an abducens-like cellular phenotype is not induced within the transplanted midbrain tissue (Figs. 3B–C).

Shortly after exiting the hindbrain, EGFP+ axons take two quite separate trajectories, projecting either cranially (Fig. 3, orange arrows, grey bars in 3L) or ventrally and laterally (Fig. 3, red arrows, black bar in 3L). A cranially directed branch is present in 21/24 embryos. In the majority of cases, this branch follows a course typical of a normal abducens nerve with EGFP+ axons terminating in the LR (63% of the total number of embryos, Figs. 3D–E and 3L). However, in a quarter of all embryos, EGFP+ axons also project further rostrally as branches that fasciculate with host oculomotor nerve axons and subsequently course to target regions normally innervated by the oculomotor (Figs. 3F–G and 3L). A unique feature of this latter set of transplant embryos is that the site of implantation and healing is consistently slightly more rostral, including part of the r4 territory. This observation suggests that proximity to the host oculomotor nerve or the target field may play a role in pathway choice. In the remaining cases (25%, Fig. 3L), cranially directed nerve fascicles appear to end within mesenchyme beneath the hindbrain, thus failing to reach extraocular muscle primordia.

In all midbrain to hindbrain transplant embryos, some or all EGFP+ axons project ventrally and laterally after exiting from the transplant (Fig. 3, red arrows). These axons fasciculate with branchiomotor nerves and project to branchial arch (BA) tissue. Although not quantified, ventrolateral directed branches usually appear larger than cranial directed fascicles emerging from the same transplant regions (Figs. 3D, H, and J). Whether the

ventrolaterally directed branches fasciculate with CN V, CN VII, or CN IX, appears to reflect slight differences in the rostrocaudal position of the transplant. In the case shown in Figs. 3H–I, the transplant is positioned at approximately the r4–6 level and EGFP+ axons course ventrally as well as slightly cranially to fasciculate with CN V. In contrast, EGFP+ axons emerging from more caudally positioned transplants often fasciculate with more caudal branchiomotor nerves (Figs. 3J–K). These observations raise the question of whether proximity and limited space in the cranial route led displaced oculomotor axons to fasciculate with nearby BA nerves. However, the findings of embryos with a BA-directed nerve but no cranially directed branch (n=3) as well as embryos with abnormally large cranial directed nerves (Fig. 3F) do not support this hypothesis.

The variable ratio of abducens-like and inappropriate branchiomotor projections might be due to varying amounts of mesenchyme included with or derived from the transplant. To address this issue, midbrain to hindbrain transplants were performed using quail embryos as a source of donor tissue (n=6). By staining with the quail cell-specific antibody, QCPN, we could then identify quail cells derived from contaminating mesoderm or neural crest that emigrated from the transplant after surgery. To track axon patterns, alternating sets of serial sagittal sections were stained with anti-NF. QCPN+ cells are sparsely distributed in mesenchyme directly beneath the brainstem but generally are absent from the periocular region (Figs. 4A–D). They are variably found along peripheral nerves (Fig. 4C) and within BAs, suggesting that neural crest emerged from the roof of the transplant or its borders (Sechrist et al., 1995; Couly et al., 1996). No correlation, however, is found between the amount of donor derived mesenchyme and outcome. Large ventrally directed nerves course from transplanted CNS tissue to BAs, regardless of whether BAs contain some (n=3) or no (n=3) QCPN+ mesenchymal cells. In addition, all chimeras show a cranially directed branch projecting from the transplant region (Figs. 4C–E).

In sum, transposed oculomotor axons show a combination of behaviors not obviously influenced by the presence of donor-derived non-CNS tissue (see summary schematic, Fig. 4F). Since most axons appear to course ventrally in an oculomotor-like manner upon exit from the CNS, intrinsic identity is prominently implicated in proximal pathway choice. However, the additional presence of small cranially directed, abducens-like, nerve fascicles and the behavior of axons within the periocular region more readily suggest responses to site-specific signals.

Axon trajectories following hindbrain to midbrain transplants

Transposition of EGF-transfected r5–6 in place of midbrain tissue was first carried out at stages 10–12 and embryos sacrificed at stages 25–29. As can be seen in Figs. 2C and 5A, the gross morphology of transplanted r5–6 tissue reflects its original axial location. Clearly identifiable clusters of Hb9+ cells are present in the EGFP+ transplant region, indicating an abducens-like somatic motoneuron identity (Figs. 5B–C). R5–6 contains not only abducens motoneurons, whose axons exit medially, but also glossopharyngeal neurons, whose axons exit laterally. In transplanted r5–6 tissue, axonal segregation is maintained and both exit routes are used (Figs. 5C–D), in line with prior data indicating that differential responses to guidance cues in the transverse and dorsoventral planes within the CNS dictate the unique exit sites of somatic and branchial/visceral populations (see Guthrie, 2007). For this study, we only traced the course of those EGFP+ axons that exited medially.

As with midbrain to r5–6 transplants, medially exiting graft-derived axons take two quite separate proximal trajectories, projecting either ventrally, perpendicular to the brainstem (Fig. 5E), or caudally beneath the brainstem (Fig. 5H). Ventral projections were evident in most r5–6 to midbrain transplant embryos (n=19/21, see Table 1) suggesting that site-specific (midbrain level) features play a prominent role in defining proximal trajectory.

Many of these axons continue to mimic the trajectory normally followed by oculomotor neurons (Chilton and Guthrie, 2004) and course to DR (91% of cases) and MR/VR (38% of cases) muscles (Figs. 5F–G and 5K, see also Table 1). However, graft-derived axons are less tightly fasciculated than a normal oculomotor nerve (see Fig. 5E) and, in 43% of embryos, EGFP+ axons deviate from the site-appropriate ventral pathway and terminate in the LR muscle primordium. These axons either project independently to the LR or join the host abducens nerve as it enters the LR (Fig. 5F–G). These patterns raise the possibility that cues specific to the LR or the host abducens nerve are recognized by some graft-derived axons. However, the loose fasciculation of ventral projections and the broad spectrum of their termination sites are equally compatible with the hypothesis that graft-derived axons projected non-specifically to both site specific and origin specific targets.

In nearly half of embryos with r5-6 to midbrain transplants (n=10/21), some graft-derived axons never take a proximal ventral course but instead turn immediately caudally (Fig. 5H) and elongate parallel to the brainstem, following a trajectory matching the position, albeit not the direction, of the normal abducens nerve. In 19% of cases, EGFP+ axons fasciculate with the host abducens nerve and project into the LR/P-Q complex (Figs 5I–J, see also summary schematic, Fig. 6H). In the remaining cases (29%), axons terminate within mesenchyme without entering the periocular region (Fig. 5K and Table 1). The presence of these trajectories suggests that some graft-derived abducens axons recognize and respond to proximal guidance cues in a manner strikingly different from oculomotor axons.

In a subset of r5-6 to midbrain transplant embryos (29%), remnants of the midbrain and some host-derived oculomotor axons were present. However, no correlation is found between the presence of host midbrain tissue and the variable pathways of donor-derived axons. We also produced quail/chick hindbrain to midbrain chimeras to assess mesenchymal contamination (n=5). At proximal levels, QCPN+ cells are variably present in peripheral nerves, the notochord, and perichordal mesenchyme beneath the transplant region (Figs. 6A–B). We did not identify any correlation between the amounts or positions of contaminating mesenchymal cells and variations in axonal projections. While it might be expected that axonal projections parallel to the brainstem (abducens-like) would be present in those embryos with substantial donor-derived mesenchyme, morphological evidence of a caudal longitudinal nerve and a proximal connection between donor and host abducens nerves is found even in cases with very few donor-derived QCPN+ mesenchymal cells (Figs. 6A–D).

We did find that the presence of a caudally projecting proximal nerve is more frequent in transplants using donors that were slightly older (62% of stage 11–12 donor embryos) compared to younger (39% of stage 10 donor embryos). Previous studies (Guidato et al., 2003a) suggest that abducens motoneuron identity is fixed at stage 10 (10 somites) but not earlier. We reasoned that if commitment to a specific identity is required in order to form a caudal, abducens-like projection, then this feature might be absent in embryos with donor tissue taken at an earlier stage. We, therefore, performed hindbrain to midbrain transplants using stage 8–9 embryos (4–8 somites) as a source of donor tissue (n=5, Figs. 6E–G). Unexpectedly, we again find Hb9+ cells present in the transplant region of these embryos (Figs. 6E–F), suggesting that commitment to an abducens phenotype occurs before stage 10. This discrepancy with the data of Guidato et al. (2003a) may be related to differences in transplant size (our whole vs. Guidato's 1/2 segments) or greater position disparities (our midbrain implant site vs. Guidato's in the cranial hindbrain). While the Hb9 marker is consistently present, a caudal projection from the graft was identified in only 1/5 embryos in this series. This finding is compatible with the hypotheses that the presence of a proximal caudal projection reflects intrinsic identity and that this identity is gradually solidified between stages 8–10.

DISCUSSION

The stereotyped pathway choices made by growing motor axons are the result of interactions between axons and their extracellular environment that broadly fall into two categories: those dependent on unique intrinsic identities of motoneuron subsets, and those that are position-specific, reflecting temporal or spatial heterogeneity in the local environment. These categories are relative rather than absolute, and the balance may change as axons elongate from their sites of emergence towards their target tissues. The experiments described here were undertaken to define the roles of intrinsic identities in the pathway choices made by developing oculomotor and abducens axons.

Intrinsic identities and proximal pathway choice

Our results demonstrate the presence of unique, intrinsic identities in the choice of proximal pathways. In a normal chick embryo, abducens axons originating from r5-6 turn sharply cranially upon exiting the brainstem and elongate cranially beneath the brainstem to the common primordium of the LR and P-Q. In approximately ½ of our r5-6 to midbrain transplant embryos, some transplant-derived abducens axons also abruptly turned and coursed beneath the brainstem in an abducens-like manner rather than projecting perpendicular to the midbrain floor like oculomotor nerve fibers. Some of these axons ultimately fasciculated with the normal (host) abducens nerve as it approached the LR primordium. This donor site-appropriate behavior was most frequent with stage 11–12 donors (62%) than in stage 10 (39%) or stage 9 (20%) donors, suggesting a progressive commitment to the abducens identity.

This feature may represent the maintenance of transient projections that are normally made by abducens axons at early stages. In normal stage 19–20 embryos, presumptive somatic motoneurons exit medially from r5-r8 as a continuous array of rootlets that are interconnected by a longitudinal nerve (Fig. 7, double asterisks and Kuratani et al., 1988). Some fibers within the longitudinal nerve may be abducens axons that transiently turn and elongate caudally rather than in the normal cranial direction. By stage 25, this pattern is remodeled. The intervening rootlets and connecting portions of the longitudinal nerve are lost, fully separating the abducens nerve, which arises from r5-6, from the hypoglossal nerve whose most cranial roots are at the level of r8. At early stages of axon outgrowth, abducens axons are often loosely fasciculated as they project towards their target primordia (Fig. 7, asterisk). This failure to tightly fasciculate was similarly manifest when graft-derived abducens fibers exited from a midbrain position and projected ventrally towards periocular mesenchyme (see Fig. 5E). Whether this behavior is due to a failure to recognize extrinsic factors promoting fasciculation at midbrain levels or an inability to execute is unknown.

Observations made in embryos with midbrain-to-hindbrain transplants also suggest identity-based behavior at proximal levels. In this group of embryos, the majority of EGFP+ axons failed to recognize or respond to nearby longitudinal pathways and instead reached then joined with ventrally oriented branchial nerves V, VII, and/or IX, and extended into branchial arches. This proved to be true even in cases where the host oculomotor nerve was depleted or absent as a consequence of the removal of midbrain tissue at the time of transplantation.

A possible explanation for this behavior is that oculomotor axons responded to local guidance cues in a manner reflecting intrinsic similarities to branchiomotor nerves. Several prior studies indicate that the ontogenetic profile of oculomotor neurons resembles that of branchiomotor neurons. Unlike hindbrain abducens (and hypoglossal) motoneurons, oculomotor and branchiomotor neurons lack Hb9 and Lim3 expression (Varela-Echevarria et al., 1996; Sharma et al., 1998), require a Phox2 gene for generation (Pattyn et al., 1997

and 2000; Muller et al., 2003; Coppola et al., 2005) and are dependent on Nkx6.1 for migration and axon outgrowth (Pattyn et al., 2003; Prakash et al., 2009). Moreover, forced expression of Phox2b within spinal cord motoneurons causes their proximal axonal trajectories and fasciculation patterns to more closely resemble those of branchiomotor axons, indicating a link between Phox2 expression and proximal axon pathway choices (Hirsch et al., 2007).

Intrinsic differences also define the paths of branchiomotor neurons originating from different segments as well as axonal subsets within cranial motor nerves. For example, when r2 is transfected with Hoxb1, a gene normally expressed by more caudal rhombomeres, motor axons emerging from r2 diverge and project to branchial musculature normally innervated by the facial nerve, in accord with their new Hox identity (Bell et al., 1999). In addition, branchiomotor and visceromotor neurons within the facial nerve appear to be differentially regulated by specific combinations of class 3 semaphorins and their neuropilin/plexin receptors that are independent of final target-derived cues (Schwarz et al., 2008).

The molecular determinants underlying the unique proximal behavior of oculomotor and abducens axons are unknown, although some possible candidates have been identified. Sema3A is expressed in mesenchyme adjacent to the notochord and beneath the brainstem (Anderson et al., 2003), and extending within periocular mesenchyme to the sites of DO and VO differentiation (Lance-Jones and Noden, unpublished data). Further, *in vitro* studies indicate that abducens, but not oculomotor, axons are repelled by Sema3A (Colomarinio and Tessier-Lavigne, 1995; Varela-Eschavarria et al., 1997). In conjunction with our data, these observations raise the possibility that Sema3A-Npn signaling may normally constrain abducens axons within a longitudinal path beneath the brainstem and prevent them from following a ventrolateral course to the branchial arches, a path not recognized as inappropriate by transplant-derived oculomotor axons. Alternatively, or in addition, marked differences in adhesive molecules and interactions among abducens vs. oculomotor axons may account for our observations. Prior studies also implicate cadherins in the regulation of branchiomotor and spinal motor axon outgrowth as well as axon sorting (Price et al., 2002; Nakao et al., 2005; Barnes et al., 2010). Thus, it would be of particular interest to define the distribution of these adhesion molecules on abducens and oculomotor axons.

Our experiments do not rule out the possibility that behaviors we are attributing to intrinsic motoneuron identity are due instead to signals emanating from non-motoneuron components of the transplant or contaminating mesenchymal cells. Possible candidates include the floorplate and isthmus tissue at the midbrain-hindbrain boundary, structures that have been implicated in the rostrocaudal polarity of axon growth within, but not outside, the CNS (Irving et al., 2002; Lyuksyutova et al., 2003; Bourikas et al., 2005; Yamauchi et al., 2009; Domanitskaya et al., 2010; Holz et al., 2010). Our analyses of quail-chick chimeras indicated the presence of some graft-derived neural crest cells along nearby peripheral nerves. These cells may include boundary cap cells which normally are derived from late migrating neural crest and appear to function as gate keepers at ventral motor axon exit points (see Bron et al., 2007; Feldner et al., 2007; Mauti et al., 2007). Here again, however, there is no evidence that boundary cap cells influence the direction of peripheral axons outgrowth. In addition, our analyses of projections in quail-chick chimeras did not indicate correlations between the presence or absence of quail-derived mesenchyme and variations in axon trajectories.

Site-specific cues and target choice

Our data indicate the presence of intrinsic identities in oculomotor and abducens nerve outgrowth, but it is clear that other pathway determinants are operating. In both series of mismatch transplantations, some EGFP+ transplant-derived axons formed distinctive, site-

specific projections. Small fascicles of donor abducens motoneurons projected ventrally from their new midbrain locations and followed a course resembling that of the normal oculomotor. In a comparable manner, the axons of transplanted oculomotor neurons projected cranially beneath the brainstem to the region of the LR, mimicking the normal abducens. These observations parallel data obtained from analyses of branchiomotor projections after rhombomere reversal (Warrilow and Guthrie, 1999), in which early projection patterns from reversed segments appeared to be site-specific.

Factors influencing the choice of site-specific vs. origin-specific pathways are not known. One possibility is that separate pathways were chosen by different subpopulations known to be present within both abducens and oculomotor neurons. For example, accessory abducens axons might be those axons that course caudally after transposition and fasciculate with the dorsal branch of the host abducens which normally contains accessory abducens axons. This possibility might be addressed by transplanting only r6, which lacks accessory abducens motoneurons.

Prior studies of cranial motor neuron outgrowth suggest that intrinsic identities can affect recognition of final muscle targets. Specific patterns of Eph receptors within motoneurons in local hindbrain regions and ephrins within final targets have been implicated in topographic matching between r2-3 motoneurons and branchial arch 1 muscles (Prin et al., 2005). Furthermore, recent reports suggest that there are differences in the responses of oculomotor and trochlear axons to SDF-1 and HGF as they begin to branch within periocular mesenchyme (Lerner et al., 2010). We found no clear evidence that axons originating from grafted oculomotor or abducens neurons responded to signals emanating from their normal targets. EGFP+ axons often projected to both the LR and the DR, despite the fact that these muscles are normally exclusively innervated by the abducens and oculomotor, respectively. We did find a small number of cases in which a transposed oculomotor nerve passed through the LR, fasciculated with the host oculomotor, and projected in accord with its origin to the rectus muscles. However, these findings are as likely explained by a selective fasciculation with like axons, as they are to be explained by the recognition of correct targets.

Our surgeries involved the transposition of motoneuron populations that normally reach their targets at quite different times. Abducens axons encounter the LR/P-Q and form separate abducens and accessory abducens branches early, before the LR/P-Q muscle primordium enters the periocular region (Wahl et al., 1994). Also, the LR demonstrates precocious molecular and cytological development when compared to other extraocular muscle primordia (Noden et al., 1999). Oculomotor neurons have two distinct phases of growth: one in which axons approach periocular mesenchyme and a second phase in which contact with targets is made (Chilton and Guthrie, 2004; see also Mar et al., 2005). In chick embryos, the latter occurs approximately a day later than abducens – LR contacts are established. Our findings of multiple incorrect projections to individual extraocular muscles after transposition may reflect the fact that target choice is normally based, in part, on the relative state of maturation of target primordia, features that are likely to have been mismatched by our surgeries.

Aberrant innervation and congenital ocular abnormalities

Though infrequent, congenital anomalies involving maldevelopment of the oculomotor and abducens nerve do occur. Marcus Gunn Jaw-Winking synkinesis (MGJWS) results from aberrant connections between the mandibular motor branches of the trigeminal nerve (CN V3) and the branch of the oculomotor nerve that innervates the levator superioris muscle (Pratt et al., 1984; Brodsky, 1998; Kodsi, 2000). We have found that branches of the oculomotor nerve invariably fasciculate with branchiomotor nerves, including the mandibular nerve, after surgical transposition. This observation, in conjunction with

recognized molecular features shared by oculomotor and branchiomotor neurons, suggests that MGJWS may reflect an unmasking of an inherent tendency for these nerves to fasciculate, an event that is normally prevented by other mechanisms.

Duane's Retraction Syndrome (DRS) is characterized by deficits in abducens nerve development and, subsequently, cross innervation of the LR by the oculomotor nerve (Alexandrakis and Saunders, 2001; Engle, 2007). Does cross innervation reflect responses to target-derived factors from aneural muscle during early stages of motor innervation or is it a late event? Wahl and Noden (2001) proposed the former, based on the occasional innervation of the LR muscle by oculomotor axons following lesions that blocked or misdirected abducens nerve outgrowth. To specifically model DRS in the chick embryo, Chilton and Guthrie (2004) unilaterally ablated r5-6 at stages 10–12. Although some regeneration and projections from the non-ablated side may follow, these authors found no evidence of cross innervation of the LR by the oculomotor up through stage 31.

Evidence from studies of Wnt-1 null mice, which lack oculomotor and trochlear motoneurons, is most compatible with the hypothesis that an expansion of innervation by remaining nerves is a late event as there is little indication of aberrant abducens innervation of muscles prior to E18.5 (Fritzsch et al., 1995; Porter and Baker, 1997). In mid-stage chick embryos (stages 25–28), we did not find an increase in axon projections to extraocular muscles from transplanted donor oculomotor nerves despite removal of host midbrain tissue and the subsequent depletion of host oculomotor nerve branches. While DRS may be accompanied by other cranial abnormalities (see Kohlhasse et al., 2002), our data are most compatible with the hypothesis that cross or aberrant innervation of extra-ocular muscles may be a late event in vertebrates occurring well after the initial establishment of axonal projections, or may require disruptions within the periocular environment that secondarily affect axonogenesis.

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Figure 1. Oculomotor and abducens pathways in normal and control transplant embryos at stages 25–28

A. Schematic of cranial motor nerve pathways in a normal stage 28 (5.5-day) chick embryo. Sites of root emergence, pathways, and nerve target locations are shown for extra-ocular (DR, LR, MR, VR, dorsal, lateral, medial and ventral rectus muscles; DO, VO, dorsal and ventral oblique muscles) and branchial arch (BA1, BA2) muscle primordia. The LR mass includes the primordia of the pyramidalis and quadratus muscles, but is labeled as LR alone for simplicity. Nerves: nIII, oculomotor; nIV, trochlear; nV, trigeminal; nVI, abducens; nVII, facial.

B–D. Representative immunostained (red, myosin; green, neurofilaments) sagittal sections showing the normal course of the oculomotor nerve (B), bifurcation of the abducens as it approaches the LR (C), and ramification of the abducens within the LR complex (D). CG, ciliary ganglion; nII, optic nerve.

E–J. Immunostained sagittal sections from control midbrain to midbrain transplant embryos (EGFP+ midbrain in place of unlabeled midbrain). E. Low magnification image showing EGFP labeling in ventral transplant regions. The CNS is outlined with a dashed line in this and subsequent low magnification figures. Boxed area corresponds to image shown in F. As in a normal embryo, EGFP+ axons project ventrally within a large NF+ nerve trunk (F and J). G–H. EGFP+ axons exit beneath an Isl1/2 positive, but Hb9 negative cluster of motoneurons. I–J. In the periocular region, EGFP+ axons contact the DR and VR/MR muscle primordia, but do not project to the LR.

K–Q. Immunostained sagittal sections from control r5-6 into r5-6 (hindbrain to hindbrain) transplant embryos. K. Low magnification image showing well-delineated EGFP labeling in a hindbrain position. Boxed area corresponds to image in L, which shows the abducens roots and nerve. M–N. Graft-derived axons emerge from an EGFP+ transplant region containing both Isl1/2+ (M) and Hb9+ (N) cells. As in a normal embryo, the abducens nerve turns cranially upon exiting the floor of the brainstem (L, N) then bifurcates as it approaches the LR (O). The two branches enter the LR complex at caudal and dorsolateral sites (P). Q. Ramification of EGFP+ axons within the LR.

Calibration bars: B–D, I–J = 0.2 mm; E and K = 1 mm; F–H, L–Q = 0.1 mm.

Figure 2. Stage 11 surgery and whole mount views of stage 26–27 transplant embryos
 A. Schematic representations of EGFP microinjection and electroporation sites (green) at midbrain or caudal hindbrain (r5-6) levels and sites of neural tube transplantation to age-matched hosts. B. Midbrain (m) to r5-6 (r) transplant embryo at sacrifice; white arrow, donor midbrain vesicle. C. R5-6 to midbrain transplant embryo at sacrifice. Note the absence of a midbrain vesicle. D. Embryo immediately after transplantation of midbrain to r5-6 (black arrow) and host midbrain removal (white arrow). E. Embryo with midbrain to r5-6 transplant and host midbrain removal at sacrifice; white arrow, donor midbrain vesicle.

Figure 3. Midbrain to hindbrain transplants using EGFP-transfected donor tissue

A–K. Sagittal sections from stage 24–27 embryos with EGFP-transfected tissue transplants. A. Low magnification image showing transplant region. B. EGFP+ axons exit beneath an Isl1/2+ cluster of motoneurons. C. Section adjacent to that shown in B. As in a normal embryo, cells within the oculomotor nuclei lack Hb9 expression. D–K. Proximal trajectories and termination sites of EGFP+ axons. Upon exit from the CNS, EGFP+ axons projected cranially (orange arrows) and ventrally (red arrows). The cranial branch, which is usually smaller than the ventral branch (see D, H, and J), takes an abducens-like path to terminate within the LR (D–E, orange arrows). F–G. Sections from an

embryo in which the cranial branch continues past the LR, and follows a distal oculomotor-like course to DR and MR/VR muscle regions.

H–K. Ventral branches (red arrows) are large and associated distally with host branchiomotor nerves V (H–I) or VII (J–K), independent of the presence (in K) or absence (in I) of a substantial host oculomotor nerve (nIII).

L. Histogram showing the percentages of experimental (n=24) and control (n=9) embryos with different EGFP+ axonal terminations sites. The midbrain to r5-6 transplant group includes embryos $\pm$ midbrain removal. White bars indicate percentages obtained for control transplants. Grey and black bars indicate percentages for experimental cases in which EGFP + axons originated from cranially directed and ventrally directed proximal branches, respectively (see key). Refer to Table 1 for the number of embryos in each group.

Calibration bars: A, F = 1 mm; D, F, H–K = 0.5 mm; B–C = 0.1 mm; E, G = 0.2 mm.

Figure 4. Midbrain to hindbrain transplants using quail neural tube as donor tissue and schematic summary diagram

A–C. Sagittal sections from a stage 25 quail-chick chimera. A. Low magnification image showing the quail (dark QCPN+) transplanted tissue. Box corresponds to region shown in B. B. QCPN+ mesenchymal cells are present beneath the transplant and around the exiting donor oculomotor nerve (nIII, arrow). C. A more lateral section just proximal to the bifurcation of the graft-derived oculomotor nerve into cranially (orange arrow) and ventrally (red arrow) oriented branches. Quail derived neural crest cells are evident in the large ventral nerve branch that ultimately courses to BA tissue.

D–E. QCPN and anti-NF stained sagittal sections from a stage 23 chimera. Few QCPN+ cells are present in BA or periocular regions. In this embryo, a small host-derived nerve joins donor-derived NF+ axons that project to the LR.

F. Schematic summary of axon paths in midbrain to hindbrain transplant embryos. The transplant and graft derived axons are shown in green. Image at right corresponds to boxed area shown at left. Abbreviations as in Fig. 1A.

Calibration bars: A = 1 mm; B–C = 0.1 mm; D–E = 0.2 mm.

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Figure 5. Hindbrain to midbrain transplants using EGFP-transfected donor tissue

A–J. Sections from stage 24–28 embryos with EGFP-transfected tissue transplants. A. Low magnification image showing sagittal view of transplant. B. High magnification of the same transplant as A, but from an adjacent sagittal section. Immunostaining reveals Hb9+ cells in the transplant region (see also C). C–D. Transverse sections through transplant regions. As from normal hindbrain segments, axons leave r5-6 transplants via medial (C) and dorsolateral (D) exit sites (arrows). Only EGFP+ axons exiting medially were traced. In the image shown in D, Isl1/2 staining is evident in a cranial nerve ganglion as well as motoneurons in the transplant region.

E–J. Sagittal sections illustrating representative trajectories of medially exiting EGFP+ axons. E. Some EGFP+ axons follow a proximal oculomotor-like ventral pathway but are less tightly fasciculated than a normal oculomotor (arrows, E, compare to Fig. 1J). F–G.

Adjacent sections showing terminations of ventrally projecting EGFP+ axons. These axons usually project to oculomotor targets, such as the DR, but also in some cases to the LR, an abducens target muscle (arrow in F), fasciculating with the host abducens within the muscle primordium (arrow in G).

H–J. In a subset of embryos, EGFP+ axons turn immediately upon exiting the transplant and project caudally beneath the brainstem (H, arrow), fasciculate with the host abducens (I, arrow) and then enter the LR muscle primordium (J). In the embryo illustrated, EGFP+ axons are also present in the DR. These axons reached the DR after taking a ventral trajectory from the transplant region.

K. Histogram showing the percentages of experimental (n=21) and control (n=8) embryos with different EGFP+ axonal terminations sites. White bar indicates percentage obtained for control transplants. Grey and black bars indicate percentages for experimental cases in which EGFP+ axons originated from ventrally directed and caudally directed proximal branches, respectively (see key). Refer to Table 1 for the number of embryos in each group. Calibration bars: A = 1 mm; H = 0.2 mm; all others = 0.1 mm.

Figure 6. Hindbrain to midbrain transplants: quail-chick chimeras, transplants with young donor tissue and summary schematic

A–B. Low (A) and high (B) magnification images of a QCPN-stained sagittal section from a stage 20 quail-chick chimera in which r5-6 had been transplanted to midbrain levels at stage 10. Box in A corresponds to region shown in B. The transplant region (donor r5-6) and some cells along a peripheral nerve are QCPN+. The nerve shown in B is the abducens of the host embryo. C–D. Low and high magnification views of a nearby section from the same embryo stained for NF. Box in C corresponds to region shown in D. Arrow in D shows an anastomosis between host and donor abducens (nVI) nerves.

E–G. Images of sagittal sections from a stage 25 embryo, in which transfection and transplantation were performed using younger (stage 9 vs. stage 10–12) donor tissue. Box in E corresponds to region shown in F. Hb9+ cells are present in both the host abducens nucleus and in the donor transplant region. G. Originating from this hindbrain to midbrain transplant were EGFP+ axons that took a ventral course to the DR, but also sent branches to the LR.

H. Schematic summary of axon paths in hindbrain to midbrain transplant embryos. The transplant and its medially exiting axons are shown in green. In normal and experimental embryos, axons exit the hindbrain from both medial and lateral sites. Laterally exiting (branchiomotor and visceromotor) axons originating from transplanted hindbrain tissue were not traced in our studies and are not shown in this schematic. Abbreviations as in Fig. 1A. Calibration bars: A, C, E = 1 mm; B, D, F, G = 0.1 mm, all others = 0.1 mm.

Figure 7. Oculomotor and abducens axon pathways in the normal stage 20 chick embryo
 Composite image of anti-NF stained sagittal sections. At stage 20, oculomotor axons form a tight fascicle as they exit from the midbrain. In the periocular region, these axons have begun to defasciculate (arrow) but have yet to reach individual extraocular muscle primordia (Chilton and Guthrie, 2004). Abducens axons emerge medially from r5-6 as multiple small fascicles and form a conspicuous longitudinal connective with axons from more caudal rhombomeres (double asterisks, see also Kuratani et al., 1988). Although not visible in this image, the abducens has reached the LR/P-Q by stage 20 and begun to branch within it (Wahl et al., 1994). Calibration bar: 0.2 mm.

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Table 1

Summary of EGFP+ axonal projections in control and experimental embryos.

Surgery ¹ (total number of embryos)	Proximal branch trajectory ²	DR ³	MR/VR	VO	LR/P-Q	Mesenchyme	BA
Control: midbrain to midbrain (9)	Ventral (9)	9	9	8	0	0	0
Control: r5-6 to r5-6 (8)	Cranial (8)	0	0	0	8	0	0
Midbrain to r5-6 (15)	Cranial (13)	3 (cr)	2 (cr)	2 (cr)	10 (cr)	3 (cr)	0
	Ventral (15)	0	0	0	0	0	15 (v)
Midbrain to r5-6 with host midbrain removal (9)	Cranial (8)	2 (cr)	2 (cr)	2 (cr)	5 (cr)	3 (cr)	0
	Ventral (9)	0	0	0	0	0	9 (v)
R5-6 to midbrain (21)	Ventral (19)	19 (v)	8 (v)	0	11 (v)	0	0
	Caudal (10)	0	0	0	4 (ca)	6 (ca)	0
R5-6 to midbrain: stage 8–9 donor (5)	Ventral (5)	4 (v)	3 (v)	2 (v)	3 (v)	0	0
	Caudal (1)	0	0	0	0	1 (ca)	0

¹ All surgeries were performed at stages 10–12, unless noted.

² Proximal branch trajectory refers to the initial course taken by EGFP+ axons after exiting the transplant region. Number of embryos with each branch type is given in parentheses.

³ Termination sites: DR, dorsal rectus; MR/VR, medial and ventral rectus; VO, ventral oblique; LR/P-Q, Lateral rectus/pyramidalis-quadratus; Mesenchyme, termination in mesenchyme above eye; BA, branchial arch. Letters in parentheses refer to origin from a cranial (cr), ventral (v), or caudal (ca) proximal branch.