Eye Movements, Strabismus, Amblyopia and Neuro-Ophthalmology

Loss of CXCR4/CXCL12 Signaling Causes Oculomotor Nerve Misrouting and Development of Motor Trigeminal to Oculomotor Synkinesis

Mary C. Whitman,1–3 Elaine H. Nguyen,1,3 Jessica L. Bell,1,3 Alan P. Tenney,3–5 Alon Gelber,3,4 and Elizabeth C. Engle1–6

1Department of Ophthalmology, Boston Children’s Hospital, Boston, Massachusetts, United States
2Department of Ophthalmology, Harvard Medical School, Boston, Massachusetts, United States
3F. M. Kirby Neurobiology Center, Boston Children’s Hospital, Boston, Massachusetts, United States
4Department of Neurology, Boston Children’s Hospital, Boston, Massachusetts, United States
5Department of Neurology, Harvard Medical School, Boston, Massachusetts, United States
6Howard Hughes Medical Institute, Chevy Chase, Maryland, United States

Correspondence: Mary C. Whitman, Boston Children’s Hospital, Fegan 4, 300 Longwood Ave, Boston, MA 02115, USA; mary.whitman@childrens.harvard.edu.

Submitted: July 3, 2018
Accepted: September 20, 2018

Citation: Whitman MC, Nguyen EH, Bell JL, Tenney AP, Gelber A, Engle EC. Loss of CXCR4/CXCL12 signaling causes oculomotor nerve misrouting and development of motor trigeminal to oculomotor synkinesis. Invest Ophthalmol Vis Sci. 2018;59:5201–5209. https://doi.org/10.1167/iovs.18-25190

PURPOSE. Proper control of eye movements is critical to vision, but relatively little is known about the molecular mechanisms that regulate development and axon guidance in the oculomotor system or cause the abnormal innervation patterns (oculomotor synkinesis) seen in developmental disorders and after oculomotor nerve palsy. We developed an ex vivo slice assay that allows for live imaging and molecular manipulation of the growing oculomotor nerve, which we used to identify axon guidance cues that affect the oculomotor nerve.

METHODS. Ex vivo slices were generated from E10.5 IslMN-GFP embryos and grown for 24 to 72 hours. To assess for CXCR4 function, the specific inhibitor AMD3100 was added to the culture media. Cxcr4cko/cko: Isl-Cre: ISLMN-GFP and Cxcl12KO/KO: ISLMN-GFP embryos were cleared and imaged on a confocal microscope.

RESULTS. When AMD3100 was added to the slice cultures, oculomotor axons grew dorsally (away from the eye) rather than ventrally (toward the eye). Axons that had already exited the midbrain continued toward the eye. Loss of Cxcr4 or Cxcl12 in vivo caused misrouting of the oculomotor nerve dorsally and motor axons from the trigeminal motor nerve, which normally innervate the muscles of mastication, aberrantly innervated extraocular muscles in the orbit. This represents the first mouse model of trigeminal-oculomotor synkinesis.

CONCLUSIONS. CXCR4/CXCL12 signaling is critical for the initial pathfinding decisions of oculomotor axons and their proper exit from the midbrain. Failure of the oculomotor nerve to innervate its extraocular muscle targets leads to aberrant innervation by other motor neurons, indicating that muscles lacking innervation may secrete cues that attract motor axons.

Keywords: oculomotor, synkinesis, congenital cranial dysinnervation disorder, Marcus Gunn jaw winking, axon guidance, eye movements
lacking EOMs show normal nerve projections to the orbit, while muscle-derived cues are necessary for formation and maintenance of terminal nerve branches. The chemokine receptor CXCR4 promotes CN3 axon growth in vitro and Cxcr4 knockout mice have dorsal misprojection of oculomotor axons within the midbrain and a thin CN3 in the periphery. Studies of axon guidance in the ocular motor system have been hampered by technical limitations. Traditional in vitro axon guidance assays, such as stripe assays, require more cells than can be harvested efficiently from ocular motor nuclei. These approaches also remove the axons from the microenvironment that may influence their responses to exogenous cues along their trajectory. In vivo approaches, such as knockout mouse models, are time-consuming and expensive to use as a screening mechanism. To address these limitations and identify pathways important for oculomotor axon guidance, we developed an embryonic slice culture technique, described here, that allows time-lapse imaging of developing CN3 while maintaining the surrounding structures and microenvironment. We show that the inhibition of CXCR4 signaling causes oculomotor axons to grow dorsally rather than ventrally (toward the eye) in the slice assay. We further show that this phenotype is recapitulated in mice lacking Cxcr4 or its ligand Cxcl12 and that there is aberrant innervation of the EOMs by fibers from CN5m, providing a mouse model of trigeminal-oculomotor synkinesis.

**Materials and Methods**

**Mouse Strains**

Transgenic islet motor neuron, green fluorescent protein (ISL1<sup>transgenic</sup>, GFP) reporter mice (Mouse Genome Informatics number J:132726, gift of Sam Pfaff) were used for slice cultures and were crossed to all other strains used. ISL1<sup>transgenic</sup>, GFP reporter mice express a noncytotoxic farnesylated GFP that localizes to the membrane of motor neurons and axons, allowing for visualization of motor nerves during development.

\[
\text{C}xcr4^{cko/cko} (B6.129P2-Cxcr4<sup>tm2Yzo</sup>/J, stock number 008767), 10 \text{C}xcl12^{cko/cko} (B6(FVB)-Cxcl12<sup>tm1(cre)Sev</sup>/J, stock number 021775), 14 \text{Is}l1-cre (STOCK Isl1<sup>tm1(cre)serf</sup>/J, stock number 024242), and Ella-cre (B6(FVB)-Tg(EIIa-cre)[C5379Mdgf], stock number 00724) mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Isl1-cre mice express cre recombinase under the lslet1 promoter, which is expressed in motor neurons. Ella-cre mice express cre recombinase under the control of the adenovirus Ella promoter that targets expression to the early mouse embryo, allowing germline deletion of floxed genes. All animal work was approved and performed in compliance with Boston Children's Hospital Institutional Animal Care and Use Committee protocols and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Slice Culture Assay**

The oculomotor ex vivo slice culture assay was adapted from a protocol for peripheral nerve outgrowth studies reported by Brachmann and Tucker. Embryos from Isl<sup>transgenic</sup>, GFP mice were removed from the uterine horn at embryonic day (E) 10.5, immersed in 4°C low-melting temperature agarose, oriented so the oculomotor nucleus and eye would be in the same slice, and sliced at 400- to 450-μm thickness on a vibratome in slicing buffer (Hanks’ balanced salt solution without Ca<sup>2+</sup> and Mg<sup>2+</sup>) supplemented with 1 mM HEPES and penicillin/streptomycin (Fig. 1). The slices were grown on Millicell cell culture inserts over media (FlouroBright Dulbecco’s modified Eagle’s medium with 25% Hanks’ balanced salt solution, 25% fetal bovine serum, 0.5% glucose, 1 mM glutamine, and 2.5 mM HEPEs) in 6-well plates on a microscope stage incubator (37°C and 5% CO<sub>2</sub>). Fluorescent images were taken every 30 minutes for up to 3 days by using a Nikon Ti Perfect Focus microscope (Tokyo, Japan). To assess for Cxcr4 function, AMD3100 (Sigma-Aldrich Corp., St. Louis, MO, USA) (dissolved in culture media) or vehicle was added to the culture media for a final concentration of 1 μg/ml (1.26 μM). A dose response curve showed that concentrations of 0.5 μg/ml and 0.1 μg/ml of AMD3100 had minimal effects (Supplementary Fig. S1).

**In Situ Hybridization**

To verify the specific loss of Cxcr4 and Cxcl12, chromogenic in situ hybridization was performed using standard protocols on 20-μm sections of fresh frozen tissue. Adjacent tissue sections were probed for Isl1 to identify motor neurons and either Cxcr4 or Cxcl12 on knockout animals and littermate controls (CXCR4<sup><sup>cko/cko</sup></sup> Isl1-cre, n = 3; wild type controls, n = 3; Cxcl12<sup><sup>cko/cko</sup></sup>, n = 3; wild type controls, n = 3). Probes were generated using the following primers: Cxcl12 forward 5’CCATGGACGCCAAGGTGCG, Cxcl12 reverse 5’T7seq-TAAATCTGGGTCAATGCACACT, CXCR4 forward 5’ATGGACCAGGCTAGGTGATTGTA, and Cxcr4 reverse 5’T7seqATGCTCTCGAAGTCAACATCC. Images were acquired using brightfield on a Nikon Ti Perfect Focus microscope.

**Frozen Section Immunohistochemistry**

Immunohistochemistry for Cxcr4 and Isl1 was performed on 20-μm frozen sections. Antigen retrieval was performed using a citrate-based buffer (Antigen Unmasking Solution H-3300; Vector Laboratories, Burlingame, CA, USA) and steam for 20 minutes. Sections were then blocked in 2% normal donkey serum in 0.1% Triton X-100 in PBS, incubated in primary antibody overnight at 4°C, washed, incubated in secondary antibody for 1 hour at room temperature, and washed and mounted in FluoroGold mounting media. Images were acquired on a Zeiss LSM 700 series laser scanning confocal microscope (Carl Zeiss Meditec, Oberkochen, Germany).

**Fluorescent Whole-Mount Embryo Immunohistochemistry**

**BABB Method.** Whole-mount E11.5 or E12.5 embryos were prepared as previously described. Embryos were fixed overnight in 4% paraformaldehyde, dehydrated through a methanol series, fixed in 1:3 parts dimethyl sulfoxide (DMSO):methanol, and then rehydrated through a methanol series. They were then blocked in a solution of 5% goat serum and 20% DMSO in PBS for 3 hours, placed in primary antibody overnight at 4°C, washed, blocked in secondary antibody for 1 hour at room temperature, and washed and mounted in FluoroGold mounting media. Images were acquired on a Zeiss LSM 700 series laser scanning confocal microscope (Carl Zeiss Meditec, Oberkochen, Germany).

**iDISCO Method.** Whole-mount E13.5 embryos were prepared using the iDISCO+ method as previously described, with longer (7 days) incubations in primary and secondary antibodies, which we found improved antibody penetration. Briefly, embryos were fixed overnight in 4% paraformaldehyde, dehydrated through a methanol series, bleached overnight in a 5% H<sub>2</sub>O<sub>2</sub> in methanol, and then rehydrated through a methanol
Series and washed in PBS with 2% Triton-X. They were incubated in permeabilization solution for 2 days and then in blocking solution for 2 days. They were then incubated with primary antibody diluted in a solution of 2% Tween-20 and heparin in PBS (PTwH), 5% DMSO, and 3% donkey serum for 7 days, washed with PTwH for one day, and placed in secondary diluted in a solution of PTwH with 3% donkey serum for 7 days. Once stained, embryos were washed in PTwH, dehydrated through a methanol series, and incubated in a solution of dichloromethane and methanol. They were then incubated in dibenzyl ether to clear. Cleared samples were mounted on coverslips with dibenzyl ether. Embryos were imaged on a Zeiss LSM 700 series laser scanning confocal microscope. Images were acquired using Zen Software (Carl Zeiss MicroImaging GmbH, Göttingen, Germany) and manipulated in three dimensions and quantified using Imaris software (Bitplane, Zurich, Switzerland).

Quantification. The proportion of CN5m axons projecting along sensory or motor pathways was quantified using Imaris software (Bitplane). The three-dimensional image was thresholded to create a “surface” along each nerve branch at two locations (just after exit from the brainstem and where axons turned toward the orbit) and the volume of each surface projecting along motor or sensory tracks was calculated. Results are reported as percentage (by volume) of axons following the sensory pathway.

Antibodies. Mouse Anti-Actin, α-Smooth Muscle-Cy3 (1:750, RRID AB 476856; MilliporeSigma, Burlington, Massachusetts, USA), rabbit anti-GFP (1:500, RRID AB221569; Thermo Fisher Scientific, Boston, Massachusetts, USA), and rabbit anti-GFP-Alexa488 (1:500, RRID AB 162553; Thermo Fisher Scientific) primary antibodies and the Alexa-Fluor 647 goat anti-rabbit (1:1000, RRID AB 141663; Invitrogen, Carlsbad, California, USA) secondary antibody were used to stain prepared whole-mount embryos. Rabbit anti-CXCR4 (1:500, RRID AB10775635; Abcam, Cambridge, MA, USA) and guinea pig anti-Isl1 (1:2000, gift of S. Morton and T. Jessell) primary antibodies and Alexa-Fluor 546 goat anti-rabbit (1:1000, RRID AB2534093; Invitrogen) and Alexa-Fluor 647 donkey anti-guinea pig (1:1000, catalog no. 706-605-148; Jackson ImmunoResearch, West Grove, PA, USA) secondary antibodies were used on frozen sections.

RESULTS

Oculomotor Axons Advance Toward the Orbit in Slice Culture

In E10.5 wild-type mice, oculomotor axons emerged from the nucleus in the midbrain and some exited the midbrain into the mesenchyme. The first GFP-positive oculomotor axons reached the orbit/eye over the next 18 to 24 hours, and then began branching to their final targets. Within the Midbrain and Stall

Inhibition of CXCR4 Signaling in Slice Culture Causes Oculomotor Axons to Grow Dorsally Rather Than Ventrally

As proof of principle for the slice assay, we examined the inhibition of CXCR4 signaling by adding 1 μg/ml (1.26 μM) of the soluble CXCR4 small molecule inhibitor AMD3100 directly to the culture media immediately after the slice culture was set up. Monitoring growth over 24 to 48 hours revealed that oculomotor axons exited the oculomotor nucleus dorsally rather than ventrally and grew away from the orbit. By contrast, axons that had already exited the midbrain ventrally at E10.5 continued toward the orbit.

Loss of CXCR4 in Developing Motor Neurons In Vivo Causes Oculomotor Axons to Project Dorsally Within the Midbrain and Stall

To validate the phenotype seen in the slice cultures and to then examine the consequences of a misrouted oculomotor nerve on innervation of the EOMs, we next examined the in vivo loss...
of CXCR4 function in mice. CXCR4 is expressed in developing oculomotor neurons \(^1\) (Fig. 3A, 3B, 3E, 3F), and E12.5 Cxcr4 null embryos were reported to have aberrant dorsal projections from the oculomotor nucleus and an apparently small CN3,\(^2\) but technical limitations prevented specific labeling of oculomotor axons and an examination of the full course of CN3 to its final targets. To assess the cell-autonomous effects of CXCR4 signaling in oculomotor neurons and to follow the full trajectory of mutant oculomotor axons to their EOM targets, Cxcr4 was selectively deleted in motor neurons by using Isl1-cre, in which cre recombinase was expressed under the Islet1 promoter. Motor neuron loss of Cxcr4 in Cxcr4\(^{cko/cko}:\) Isl-cre:IslMN-GFP mice was confirmed by in situ hybridization for Cxcr4 and immunohistochemistry for CXCR4 (Figs. 3C–F).

Examination of whole mounts of Cxcr4\(^{cko/cko}:\) Isl-cre:IslMN-GFP mutant embryos at E11.5 to E13.5 showed misrouting of CN3, which projected dorsally within the midbrain and stalled (Fig. 4), recapitulating the phenotype seen in the slice cultures. One of six E11.5 embryos had bilateral CN3s that projected ventrally and exited the midbrain correctly but were then misguided in the periphery. Four of seven E12.5 and E13.5 embryos had very thin unilateral remnants of CN3 in the periphery, three of which reached the orbit and one of which was misrouted in the periphery. On the contralateral side of those embryos, all axons projected dorsally and stalled within the midbrain. In all mutants, the trochlear nerve followed the wildtype trajectory: it exited the nucleus laterally, turned and projected dorsally and caudally, crossed the midline, and then projected to the superior oblique muscle in the contralateral orbit. We were not able to visualize CN6 in Cxcr4 mutant animals.

**Aberrant Innervation of EOMs by Trigeminal Motor Axons**

In mouse models of DRS, in which CN6 axons do not reach the orbit, the target lateral rectus muscle is aberrantly innervated by fibers from CN3.\(^5\)^\(^,\)\(^8\) We, therefore, hypothesized that if, in the absence of CXCR4, CN3 (and possibly CN6) does not reach the orbit, there may be aberrant innervation of its target muscles by other motor nerves. Indeed, we identified aberrant innervation of the EOMs by CN5m axons. In E11.5 and E12.5 wild-type embryos, axons of the trigeminal motor nucleus exited the hindbrain, skirted around the trigeminal sensory ganglion,\(^2\) and extended toward the jaw to innervate muscles of mastication. In Cxcr4\(^{cko/cko}:\) Isl-cre:IslMN-GFP embryos, GFP-positive CN5m axons exited the hindbrain, skirted around the trigeminal sensory ganglion,\(^2\) and extended toward the jaw to innervate muscles of mastication. In Cxcr4\(^{cko/cko}:\) Isl-cre:IslMN-GFP embryos, GFP-positive CN5m axons exited the hindbrain normally, but then 43% ± 16% (n = 12 sides from seven embryos) of the axons followed the trajectory of the maxillary (V2) and, to a lesser extent, the ophthalmic (V1) branches of the trigeminal sensory nerve. The remainder of the axons projected normally toward the muscles of mastication. As the misrouted CN5m axons passed inferior to the orbit, 48% ± 16% of these misrouted axons left the trigeminal sensory trajectory, entered the orbit, and contacted EOMs (Fig. 5). At E11.5, the EOMs are still a muscle anlage, and individual muscle targets could not be distinguished. By E12.5, however, individual muscles could be distinguished, and the aberrant CN5m fibers contacted variable combinations of oculomotor targets, including the superior
sections. In Cxcr4cko/cko:Isl1-cre shows Cxcr4 projections from ventral motor neurons (vMNs) in the spinal D equals 100 hybridization on wild-type tissue at E12.5 for Cxcl12 in bar Boxed regions indicate the position of the right-sided Isl1 expression in the mesenchyme (M). In Cxcl12KO/KO mice (J) Cxcl12 expression is absent. Arrows indicate area of CN3 nucleus. Scale bar in D equals 100 μm for A-D. Scale bar in G equals 100 μm for E, G. Scale bar in H equals 50 μm for E, F. Scale bar in J equals 100 μm for J, I, J.

rectus, inferior rectus, medial rectus, and inferior oblique muscles, as well as the lateral rectus muscle normally innervated by CN6. No two orbits had the same combination of muscles contacted. In the orbits where a unilateral remnant of CN3 was present, there was no aberrant innervation from CN5m axons.

Other Motor Neurons

Cranial motor nerves 7, 9, 10, and 12 had grossly normal trajectories (see Fig. 4). As reported in full knockouts,23 the projections from ventral motor neurons (vMNs) in the spinal cord were aberrant, both within the spinal cord and in the periphery, with relatively few motor axons exiting the neuroepithelium.

Loss of the CXCR4 Ligand CXCL12 Also Causes Oculomotor Axons to Project Dorsally, With Aberrant Innervation of EOMs by Trigeminal Motor Axons

CXCL12 is expressed in the mesenchyme surrounding the ventral spinal cord, hindbrain, and midbrain, as well as in the orbit10,23 (Fig. 3G). To determine whether loss of the CXCR4 ligand results in a similar phenotype to the loss of CXCR4 in motor neurons, Cxcl12−/− embryos were generated by crossing Cxcl12−/− mice to Ella-cre mice for two generations, and the loss of Cxcl12 was confirmed by in situ hybridization (Fig. 3H). Indeed, full loss of Cxcl12 recapitulates the Cxcr4 motor neuron phenotype, with the dorsal rather than ventral projection of the oculomotor axons and aberrant innervation of EOMs by axons originating from CN5m nucleus (Fig. 6). The proportion of misrouted axons was similar, with 49% ± 9% of CN5m axons projecting along the V2 (and some V1) sensory pathways.

Discussion

A fundamental question of developmental neurobiology is how developing axons traverse long distances and synapse on correct targets. The ocular motor system has many advantages for studying axon guidance mechanisms. It is a relatively simple system: three CNs exit the brainstem and traverse the mesenchyme along stereotypical trajectories to innervate seven target muscles. Eye movements provide a reliable, sensitive readout of function and can be perturbed by disorders of development and regeneration of these nerves. Historically, a disadvantage of the ocular motor system as a model for axon guidance has been the paucity of motor neurons in each nucleus, making it challenging to obtain enough cells for traditional in vitro guidance assays. Additionally, until recently, it was difficult to follow the full course of the nerves in vivo.

We describe here a new embryonic slice culture assay for studying axon guidance of CN3 and show that inhibition of CXCR4 signaling caused oculomotor axons to project dorsally rather than ventrally. The slice culture provides numerous benefits over traditional axon guidance assays. Nerve growth can be studied despite the small number of motor neurons in each nucleus. When oriented properly, the slice culture does not axotomize growing axons, as dissociated or explant cultures do, thus removing the confounding factors of damage and stress to the neurons and enabling assessment of axon outgrowth rather than regeneration. The slice culture also maintains more of the endogenous environment. Because neurons respond to multiple different cues along their trajectory, often in combination, this provides an opportunity to evaluate the effects of modifying one signaling pathway without disturbing other signaling pathways and to assess differential effects at various points along the axon trajectory.

As we demonstrate here, the slice culture can be manipulated by adding small molecule inhibitors to block receptor signaling. Other potential manipulations include the addition of axon guidance cues, growth factors, or receptor-activating or -blocking antibodies to the media, or placement of ligand-secreting beads on the slice to assess for directional effects. There are also challenges and limitations to the CN3 slice culture preparation. Preparation and orientation of CN3 slices require practice, and the ideal embryonic time window is
FIGURE 4. Conditional Cxcr4 deletion in motor neurons causes misrouting of CN3. (A) E12.5 maximum intensity projections of whole-mount imaging of Cxcr4cKO/cKO embryo with no cre recombinase shows the normal trajectory of the oculomotor (CN3), trochlear (CN4), abducens (CN6), motor trigeminal (CN5m), facial (CN7), glossopharyngeal (CN9), vagus (CN10), and hypoglossal (CN12) nerves. Midbrain enlarged in A and midbrain to orbit enlarged in A0. (B) E12.5 Cxcr4cKO/cKO:Isl1-cre embryo showing dorsal projection and stalling of CN3 in the midbrain, whereas CN4 projects normally. Fibers of CN5m aberrantly project along the trigeminal sensory pathway (arrows in B), and project into the orbit (arrows in B0). CN6 cannot be identified. CN7, CN9, CN10, and CN12 are thin but have grossly normal trajectories, whereas spinal cord axons project aberrantly. Midbrain enlarged in B and midbrain to orbit enlarged in B0. Images in A, A0, B, and B0 have been cropped in the z dimension to show only one side of the head. N = 12 Cxcr4cKO/cKO:Isl1-cre (5 E11.5, 5 E12.5, and 2 E13.5), and 21 controls (14 E11.5, 5 E12.5, and 3 E13.5). Mutant and control littermates were examined from nine independent litters. Scale bars: 500 μm (A, B); 200 μm (A0, B0).

FIGURE 5. In the absence of Cxcr4 expression in motor neurons, the motor branch of the trigeminal nerve projects aberrantly to the orbit. Maximum intensity projections of whole-mount imaging of (A) an E12.5 wild-type orbit shows CN3 approaching the developing SR, MR, IR, and IO muscles; CN4 projecting to the superior oblique (SO); and CN6 passing by retractor bulbi (RB) toward the lateral rectus (LR). CN5m projects to the muscles of mastication and CN7 begins branching in the periphery to innervate the muscles of the face. (B) In an E12.5 Cxcr4cKO/cKO:Isl1-cre orbit, CN4 projects normally to the SO, but CN3 is absent. 43% ± 16% of the motor division of the trigeminal nerve aberrantly projects along the path of the sensory nerves to the face (white arrows), and 48% ± 16% of those axons then branch into the orbit and contact EOMs. In this example, axons contact the LR, IR, and IO (yellow arrows), but there is variability between orbits, with combinations of SR, LR, MR, IR, and IO contacted. SO, superior oblique; SR, superior rectus; MR, medial rectus; IR, inferior rectus; IO, inferior oblique; RB, retractor bulbi; LR, lateral rectus. N = 7 Cxcr4cKO/cKO:Isl1-cre (5 E12.5 and 2 E13.5), and N = 8 controls (5 E12.5 and 3 E13.5, from 5 independent litters). Scale bar: 200 μm.
After E11.0, many axons have reached the orbit and the slice becomes less useful for studying intermediate guidance decisions. Ideal timing may differ for other CNs, based on their developmental time course. Although damage to the neurons and axons is minimized, there is injury to surrounding tissues that may alter the expression or release of signaling cues. A stage-top incubator is required, and imaging through 400 μm of uncleared tissue can be technically challenging. Finally, individual EOMs cannot be visualized in the slice preparation, so specific EOM targeting by oculomotor axons cannot be assessed.

The chemokine receptor CXCR4 and its ligand CXCL12 have multiple roles in nervous system development, including neuronal migration and axon guidance. In the spinal cord, CXCR4 signaling is required for initial pathfinding of motor neurons. CXCL12 increases the outgrowth of axons in midbrain explants containing oculomotor and trochlear nuclei and has a mild chemoattractant effect on oculomotor axons. In Cxcr4 knockout mice, oculomotor axons were reported to misproject centrally and the exiting CN3 was thin. We, therefore, hypothesized that inhibiting CXCR4 signaling in the slice culture would alter oculomotor guidance and provide proof-of-concept for the slice culture. Indeed, in both mouse models, there was aberrant innervation of EOMs by axons of the trigeminal motor nerve CN5m (see flowchart, Fig. 7).

Motor neurons in the hindbrain and spinal cord can be divided into vMNs and dorsal motor neurons (dMNs) based on their initial axon trajectory within the neural epithelium. vMNs, which include spinal, hypoglossal (CN12), and abducens (CN6) motor neurons, extend their axons through the ventral neural tube and exit into the mesenchyme via a ventral point. dMNs, which include the remaining hindbrain cranial
Loss of CXCR4 Causes Oculomotor Misrouting and Synkinesis

FIGURE 7. Flowchart of experiments, hypotheses, and results.

Ex vivo embryonic midbrain-orbit slice cultures

Inhibit CXCR4 signalling (AMD3100)

CN3 grows dorsally

What will happen to the innervation of CN3 target EOMs in the absence of CN3?

In vivo mouse models

CXCR4 (receptor) conditional knock-out in motor neurons

CXCL12 (ligand) full knock-out

CN3 grows dorsally (does not reach orbit)

trigeminal motor nerve contacts EOMs (oculomotor synkinesis)

nuclei, initially send axons dorsal-laterally within the ventral neural tube and exit into the mesenchyme near incoming sensory fibers. With the exception of CN5m axons, dMN axons invade nearby sensory ganglia as they project ventrally to peripheral targets.22-25,26 It was previously reported that the loss of Cxcr4 caused vMNs in the spinal cord to exit dorsally and often invade sensory pathways of the dorsal root ganglia, while the only hindbrain dMNs affected were a subset of CN5m, which aberrantly invaded the trigeminal sensory ganglia.25 Here, we show that in the absence of Cxcr4, motor axons of CN3, which normally have a ventral exit, acted similarly to vMNs of the spinal cord in the absence of Cxcr4 and extended dorsally. Tracking CN5m axons in the periphery, we found that while approximately half followed the correct trajectory toward the muscles of mastication, the other half followed the trajectory of the V2 and V1 sensory trigeminal branches, and about half of these then branched away from V2 to enter the orbit and contact variable combinations of EOMs. Although this aberrant innervation pattern is predicted to cause ocular synkinesis, these animals did not survive and their eye movements could not be assessed.

Aberrant innervation of EOMs in the absence of their neural tube and exit into the mesenchyme near incoming sensory fibers. With the exception of CN5m axons, dMN axons invade nearby sensory ganglia as they project ventrally to peripheral targets.22-25,26 It was previously reported that the loss of Cxcr4 caused vMNs in the spinal cord to exit dorsally and often invade sensory pathways of the dorsal root ganglia, while the only hindbrain dMNs affected were a subset of CN5m, which aberrantly invaded the trigeminal sensory ganglia.25 Here, we show that in the absence of Cxcr4, motor axons of CN3, which normally have a ventral exit, acted similarly to vMNs of the spinal cord in the absence of Cxcr4 and extended dorsally. Tracking CN5m axons in the periphery, we found that while approximately half followed the correct trajectory toward the muscles of mastication, the other half followed the trajectory of the V2 and V1 sensory trigeminal branches, and about half of these then branched away from V2 to enter the orbit and contact variable combinations of EOMs. Although this aberrant innervation pattern is predicted to cause ocular synkinesis, these animals did not survive and their eye movements could not be assessed.

Aberrant innervation of EOMs in the absence of their normal innervation suggests that uninnervated EOMs secrete factors attractive to motor axons. Consistent with this, we saw aberrant innervation of CN3 target muscles in the absence of CN3. Surprisingly, we also saw aberrant innervation of the lateral rectus with the loss of Cxcr4. This suggests that the lateral rectus is not fully innervated, and, indeed, we were unable to identify CN6 in Cxcr4 mutant animals. By contrast, Lieberam et al23 were able to identify the CN6 nucleus in full Cxcr4 knockouts on the HB9-GFP line, with rare aberrant projections within the neuroepithelium, but they did not trace the peripheral projection of the nerve. Thus, there may be variability in the degree to which CN6 is affected in Cxcr4 mice.

MGJW is believed to result from aberrant innervation of the LPS by CN5m axons, usually in the absence of CN3 innervation. Although we did not see direct innervation of the LPS by the aberrant axons in Cxcr4<sup>−/−</sup>/Islcre;Isth<sup>Cre</sup>/GFP mice, the LPS cannot be identified at early embryonic ages. Although it is unlikely that the loss of Cxcr4 causes isolated MGJW (because of the systemic phenotypes in Cxcr4 knockout mice), this may represent a general mechanism for the development of MGJW; mutations affecting a variety of axon guidance pathways could cause misrouting of trigeminal motor axons along the course of the sensory trigeminal nerve. Those misdirected motor axons may then be attracted to an uninnervated LPS.

Understanding mechanisms of axon guidance is important in both developmental disorders and aberrant regeneration. Cranial motor nerves are peripheral and can regenerate after injury. In adults, transient CN palsies are often caused by aneurysms or tumors.27 As the nerves regenerate, they can form aberrant connections that cause debilitating synkinetic symptoms for patients. Understanding the cues that guide initial CN trajectories could lead to treatments to prevent or alleviate aberrant regeneration.

Acknowledgments

Supported by the National Eye Institute (5K08EY027850), Harvard-Vision Clinical Scientist Development Program (5K12EY016355), the Knights Templar Eye Foundation (Career Starter Grant), and the Children’s Hospital Ophthalmology Foundation (Faculty Discovery Award). ECE is a Howard Hughes Medical Institute investigator.

Disclosure: M.C. Whitman, None; E.H. Nguyen, None; J.L. Bell, None; A.P. Tenney, None; A. Gelber, None; E.C. Engle, Amgen (R)

References

1. Davidson S, Quinn GE. The impact of pediatric vision disorders in adulthood. <i>Pediatrics</i>. 2011;127:334–339.

2. Nakano M, Yamada K, Fain J, et al. Homozygous mutations in ARIX (PHOX2A) result in congenital fibrosis of the extraocular muscles type 2. <i>Am J Hum Genet</i>. 2001;29:315–320.

3. Tischfield MA, Bosley TM, Salih MA, et al. Homozygous HOXA1 mutations disrupt human brainstem, inner ear, cardiovascular and cognitive development. <i>Neuron</i>. 2005;37:1035–1037.

4. Webb BD, Shaaban S, Gaspar H, et al. HOXB1 founder mutation in humans recapitulates the phenotype of Hoxb1<sup>−/−</sup> mice. <i>Am J Hum Genet</i>. 2012;91:171–179.

5. Park JG, Tischfield MA, Nugent AA, et al. Loss of MAFB function in humans and mice causes Duane syndrome, aberrant extraocular muscle innervation, and inner-ear defects. <i>Am J Hum Genet</i>. 2016;98:1220–1227.

6. Cheng L, Desai J, Miranda CJ, et al. Human CFEOM1 mutations attenuate KIF21A autoinhibition and cause oculomotor axon stalling. <i>Neuron</i>. 2014;82:334–349.

7. Tischfield MA, Baris HN, Wu C, et al. Human TUBB3 mutations perturb microtubule dynamics, kinesin interactions, and axon guidance. <i>Cell</i>. 2010;140:74–87.

8. Nugent AA, Park JG, Wei Y, et al. Mutant alpha2-chimaerin signals via bidirectional ephrin pathways in Duane retraction syndrome. <i>J Clin Invest</i>. 2017;127:1064–1082.
9. Michalak SM, Whitman MC, Park JG, Tischfield MA, Nguyen EH, Engle EC. Ocular motor nerve development in the presence and absence of extraocular muscle. *Invest Ophthalmol Vis Sci*. 2017;58:2388–2396.

10. Lerner O, Davenport D, Patel P, Psatha M, Lieberam I, Guthrie S. Stromal cell-derived factor-1 and hepatocyte growth factor guide axon projections to the extraocular muscles. *Dev Neurobiol*. 2010;70:549–564.

11. Ferrario JE, Baskaran P, Clark C, et al. Axon guidance in the developing ocular motor system and Duane retraction syndrome depends on Semaphorin signaling via alpha2-chimaerin. *Proc Natl Acad Sci U S A*. 2012;109:14669–14674.

12. Lewcock JW, Genoud N, Lettieri K, Pfaff SL. The ubiquitin ligase Phr1 regulates axon outgrowth through modulation of microtubule dynamics. *Neuron*. 2007;56:604–620.

13. Nie Y, Waite J, Brewer F, Sunshine MJ, Littman DR, Zou YR. The role of CXCR4 in maintaining peripheral B cell compartments and humoral immunity. *J Exp Med*. 2004;200:1145–1156.

14. Greenbaum A, Hsu YM, Day RB, et al. CXCL12 in early mesenchymal progenitors is required for hematopoietic stem-cell maintenance. *Nature*. 2013;495:227–230.

15. Yang L, Cai CL, Lin L, et al. Isl1Cre reveals a common Bmp pathway in heart and limb development. *Development*. 2006;133:1575–1585.

16. Lakso M, Pichel JG, Gorman JR, et al. Efficient in vivo manipulation of mouse genomic sequences at the zygote stage. *Proc Natl Acad Sci U S A*. 1996;93:5860–5865.

17. Brachmann I, Tucker KL. Organotypic slice culture of GFP-expressing mouse embryos for real-time imaging of peripheral nerve outgrowth. *J Vis Exp*. 2011;2309.

18. Schaeren-Wiemers N, Gerfin-Moser A. A single protocol to detect transcripts of various types and expression levels in neural tissue and cultured cells: in situ hybridization using digoxigenin-labelled cRNA probes. *Histochemistry*. 1993;100:451–440.

19. Huber AB, Kania A, Tran TS, et al. Distinct roles for secreted semaphorin signaling in spinal motor axon guidance. *Neuron*. 2005;48:949–964.

20. Renier N, Adams EL, Kirst C, et al. Mapping of brain activity by automated volume analysis of immediate early genes. *Cell*. 2016;165:1789–1802.

21. Lewellis SW, Nagelberg D, Subedi A, et al. Precise SDF1-mediated cell guidance is achieved through ligand clearance and microRNA-mediated decay. *J Cell Biol*. 2013;200:337–355.

22. Zhang L, Yoshimura Y, Hatta T, Otani H. Reconstructing the pathway of the tensor veli palatini motor nerve during early mouse development. *Anat Embryol (Berl).* 2000;201:235–244.

23. Lieberam I, Agalliu D, Nagasawa T, Ericson J, Jessell TM. A Cxcl12-CXCR4 chemokine signaling pathway defines the initial trajectory of mammalian motor axons. *Neuron*. 2005;47:667–679.

24. Li M, Ransohoff RM. Multiple roles of chemokine CXCL12 in the central nervous system: a migration from immunology to neurobiology. *Prog Neurobiol*. 2008;84:116–131.

25. Sharma K, Sheng HZ, Lettieri K, et al. LIM homeodomain factors Lhx3 and Lhx4 assign subtype identities for motor neurons. *Cell*. 1998;95:817–828.

26. Jacob J, Tiveron MC, Brunet JF, Guthrie S. Role of the target in the pathfinding of facial visceral motor axons. *Mol Cell Neurosci*. 2000;16:14–26.

27. Sibony PA, Lessell S, Gittinger JW Jr. Acquired oculomotor synkinesis. *Surv Ophthalmol*. 1984;28:382–390.

**Supplementary Materials**

**Supplementary Video S1.** CN3 outgrowth in slice culture. Time-lapse imaging of slice culture from E10.5 Isl-GFP embryo. Images taken every 30 minutes over 42 hours in culture. CN3 (*green*) grows from the nucleus (*top*) toward the eyes and branches extensively. CN4 can also be seen ventral to CN3.

**Supplementary Video S2.** Mistargeting of CN3 with inhibition of CXCR4 signaling. Time-lapse imaging of slice cultures from E10.5 Isl-GFP embryos over 48 hours in culture. When 1 μg/ml AMD3100 (specific inhibitor for CXCR4) is added directly to the media, CN3 axons that are not yet in the periphery exit the oculomotor nucleus dorsally (*left*) rather than ventrally (*right*).