Review

Evolution and development of extra-ocular nerves and muscles in vertebrates.

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Abstract: Extraocular eye muscles (EOMs) are innervated by axons of three ocular motor nuclei, the oculomotor (CNIII), trochlear (CNIV), and abducens (CNVI) neurons. The purpose of this review is to analyze the origin of ocular motor neurons, define the pattern of innervation of nerve fibers that project to the EOMs, provide an overview of vestibular pathway inputs to ocular motor nuclei, and describe congenital disorders that alter the development of ocular motor neurons. Oculomotor neurons originate in the midbrain and innervate the ipsilateral orbit, except for the superior rectus and the levator palpebrae which are contralaterally innervated. Trochlear motor neurons originate at the midbrain-hindbrain junction and innervate the contralateral superior oblique muscle. Abducens motor neurons originate variously in the rhombomeres r4-6 and innervate the posterior (or lateral) rectus muscle, and innervate the retractor bulbi. Genes allow yielding a distinction between special somatic (CNIII, IV) and somatic (CNVI) ocular motor neurons. The ocular motor neurons are innervating somites, which receive innervates vestibular nuclei that connects with the brainstem motor neurons. Development of ocular motor neurons and their axonal projections to the EOMs may be derailed by various genetic causes, resulting in the congenital cranial dysinnervation disorders.

Keywords: oculomotor motor neurons; trochlear motor neurons; abducens motor neurons; extraocular eye muscles; vestibular nuclei connections

1. Introduction

1.1. Ocular nerve anatomy in different species

The extraocular muscles (EOMs) control eye movements, innervated by three pairs of cranial nerves known as the oculomotor nerve (CNIII), trochlear nerve (CNIV), and abducens nerve (CNVI). These nerves arise from their respective motor nuclei in the brainstem and travel through the skull to innervate specific somites, the EOMs. In tetrapods, the oculomotor nerve (CNIII) controls the superior rectus, inferior rectus, medial rectus, and inferior oblique muscles (Table 1). The trochlear nerve (CNIV) innervates the superior oblique muscle, and the abducens nerve (CNVI) innervates the lateral rectus muscle (LR) and has retractor bulbi. The coordinated action of these EOMs allows precise eye movements such as tracking a moving object or maintaining stable gaze during head movements, provided by the vestibulo-ocular reflect (VOR; [1-5]). In tetrapods, oculomotor special somatic motor neurons **[6]** project their axons through cranial nerve III to form an inferior division that innervates the ipsilateral inferior rectus (IR), medial rectus (MR), and inferior oblique (IO), and a superior division that innervates the contralateral superior rectus (SR). Trochlear special somatic motor neurons project their axons through cranial nerve IV to innervate the contralateral superior oblique muscle (SO; Table 1). Abducens somatic motor neurons innervate through cranial nerve VI to reach the lateral rectus muscle (LR).

The extraocular muscles are innervated by the same three cranial nerves and are typically considered identical across vertebrates **[7,8]**. That said, oculomotor, trochlear and abducens targets can vary across species. Lampreys have only three cranial branches to the extraocular muscles whereas gnathostomes have four cranial nerves (Table 1). The targets of cranial nerve III include the nasal rectus (NR) that is innervated by a dorsal ramus comprising only contralateral fibers in

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chondrichthyans and lungfish. In contrast, the medial rectus (MR) is innervated by a ventral ramus which originates from ipsilateral fibers in bony fish, *Latimeria* and tetrapods **[9-17]**. Moreover, unique to mammals, is the levator palpebrae superioris (LPS) muscle that elevates the eyelid **[18,19]**. Trochlear motor neurons send their axons to exit the brainstem dorsally and then cross the midline to innervate the contralateral IO **[9,10,15,20]**. The abducens nucleus (cranial nerve VI) innervates two extraocular muscles in lampreys (the caudal and ventral rectus) but is restricted to a single muscle, the lateral rectus, in gnathostomes (Table 1). Basicranial muscles are likely present in all sarcopterygians (lobe-finned fishes); these muscles evolved to form the retractor bulbi in most tetrapods **[21-28]**. The retractor bulbi (Table 1) are reduced or absent in derived microchiropters and primates **[29,30]**.

Overall, the three ocular cranial nerves and associated extraocular muscles develop in a stereotyped fashion that is best understood from research in mice **[17,31,32]**.

Table 1. Cranial nerves and extraocular muscles.

	Lampreys	**Chondrichthyes**	**Osteognathostomes**
Extraocular muscles	Cranial nerves and mode of innervation		
Superior Rectus	CNIII, contralateral, dorsal	CNIII, contralateral, dorsal	CNIII, contralateral, dorsal
Nasal rectus	------	CNIII, contralateral, dorsal	------
Levator palpebrae	------	------	CNIII, contralateral, dorsal
Inferior (rostral) rectus	CNIII, ipsilateral, ventral	CNIII, ipsilateral, ventral	CNIII, ipsilateral, ventral
Inferior (rostral) oblique	CNIII, ipsilateral, ventral	CNIII, ipsilateral, ventral	CNIII, ipsilateral, ventral
Medial rectus	------	------	CNIII, ipsilateral, ventral
Superior (caudal) oblique	CNIV, contralateral	CNIV, contralateral	CNIV, contralateral
Lateral (ventral) rectus	CNVI, ipsilateral, medial	CNVI, ipsilateral, medial	CNVI, ipsilateral, medial
Caudal rectus	CNVI, ipsilateral, lateral	------	------
Retractor bulbi	------	------	ipsilateral, lateral (tetrapods)
Basicranial muscles	------	------	ipsilateral, lateral (coelacanths)

The three cranial nerves innervation patterns are highlighted. The retractor bulbi is likely homologous to the basicranial muscle fibers of the coelacanths and the caudal rectus of lampreys.

1.2. Ocular nerve growth and guidance

The growth and guidance of axons is a complex process that involves the coordinated action of many genes, and any disruptions or mutations in these genes can lead to abnormal development of the nervous system and related disorders. Several developmental defects are associated with the *Sema3* gene family, involving genes that interact with neuropilins (*Npn1/2*) and *Plexina1/2* to control trochlear decussation **[33,34]**. In addition, *Netrin*-1 repels the trochlear axons and *Unc5* **[35-37]**. The loss of genes *Slit1/2* and *Robo1/2* can likewise result in aberrant connections of trochlear axons **[38-40]**. Defects in axon guidance are associated with genes *Robo3, Col25a1,* and there are also various *Tubb* gene-related defects and gain-of function mutations **[34,38,41]**.

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1.3. Vestibular inputs to the motor neurons which innervate the extraocular muscles.

Epithelial mesodermal coeloms are believed to represent serial homolog of somites to generate three extraocular muscles [32]. The ocular motor neurons are themselves innervated by vestibular nuclei **[1,5,42]**. Vestibular inputs to ocular motor neurons display different patterns of connections in lampreys, chondrichthyans, and Osteichthyes **[11,21,43,44]**. Among teleosts, frogs, birds, and mammals each have a specific pattern of innervation by vestibular inputs **[1,4,17,42,45]**. By contrast, studies in chondrichthyans (cartilaginous fishes) revealed a different pattern with a combination of ipsilateral and contralateral nerve fibers **[11,12]**. Many details of vestibular relationships are incomplete in lampreys (which lack a horizontal canal) but can be compared to the pattern of connections in gnathostomes **[42,43,46-48]**.

1.4. Perturbation of the extraocular muscles.

Among these genes are those controlling the projections that can be the source of developmental defects. In mice, certain mutations result in the reduction or gain-of-function of these two muscles, resulting in ptosis due to the inability to lift the eyes and lids **[19]**. Perturbations in the growth and guidance of ocular cranial nerves can cause paralytic strabismus, which is one of a number of congenital cranial dysinnervation disorders (CCDDs;**[49]**). In addition, axons can make targeting errors implicating aberrant cell body migration and axon extension **[34]**.

Connection of three ocular motor neurons that innervate distinct EOMs will be described in Section 2 and 3. Next, the genetic basis of how and why different ocular motor neuron innervate the ocular muscles in vertebrates will be provided (Section 4 and 5). Vestibular nuclei connections differ between the eyes and ears across vertebrates (Section 6). Finally, an overview of axonal defects in mutant mice and humans is provided, detailing the congenital cranial dysinnervation disorders (Section 7).

2. Extraocular muscles are innervated by three cranial nerves.

Three groups of ocular motor neurons develop that innervate cranial nerve III+IV+VI to reach out the six EOMs.

2.1. Ocular motor neurons (CNIII)

Ocular motor neurons projects ipsilaterally, with the exception of the oculomotor superior division, in which they project contralaterally [50]. In all vertebrates (Fig. 1), a contralateral motor neuron population arises to join the ipsilateral cranial nerve CNIII [17,36,51-53]. In addition to the contralateral projections to the superior rectus (SR) is a bilateral innervation of the levator palpebrae superior [LPS; [19]]. Although all axonal projections exit the brainstem as multiple small bundles, their axons coalesce into a single nerve to form cranial nerve CNIII (Fig. 1). Lampreys have only three branches that innervate the extraocular muscles (Fig. 1): the superior rectus is innervated from contralateral cranial nerve III whereas two ipsilateral branches innervate the rostral rectus and rostral oblique [9,16,53]. In contrast to gnathostomes, chondrichthyans and lungfish (Fig. 1) show a dorsal branch around the optic nerve to innervate the superior rectus and the nasal rectus (NR) whereas only two ventral branches form the inferior rectus and inferior oblique innervation [11-13,16,54]. Among tetrapods, Latimeria and teleosts exhibit one branch that passes dorsal to the optic nerve to reach the superior rectus [9,16,19,31], which is innervated from the contralateral cranial nerve CNIII [19,36,52,55,56]. In addition, three ventral branches innervate the inferior rectus (IR), medial rectus (MR), and inferior oblique (IO) through ipsilateral projections in cranial nerve III.

2.2. Trochlear motor neurons (CNIV)

Trochlear motor neurons have contralateral projections that extend dorsally to cross near the cerebellum and target a single extraocular muscle, the SO/CO (Fig. 1). In lampreys, trochlear motor

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neurons have a unique dorsal position that develops next to the cerebellum from which they innervate largely bilateral neurons to reach the CO **[9,16,53]**. These motor neurons also have long dendrites to extend close to the ocular motor neurons. Whether the trochlear nucleus develop close to the cerebellum or migrates there from more ventral origins is debated **[53,57]**. In gnathostomes, trochlear motor neurons form a largely contralateral projection which crosses near the cerebellum and innervates the SO rostrally. The bundle of CNIV can be demonstrated to cross over the contralateral CNIII by dye tracing and other means such as tubulin antibodies [4,19,31,36,38,56,58]. Studies of mutants and lesions to the trochlear have shown a mix of ipsi- and contralateral fibers. For example, cutting the nerve produces a redirection that interacts with adjacent CIII fibers to innervate the SO in frogs [59-62] and chicken [63,64].

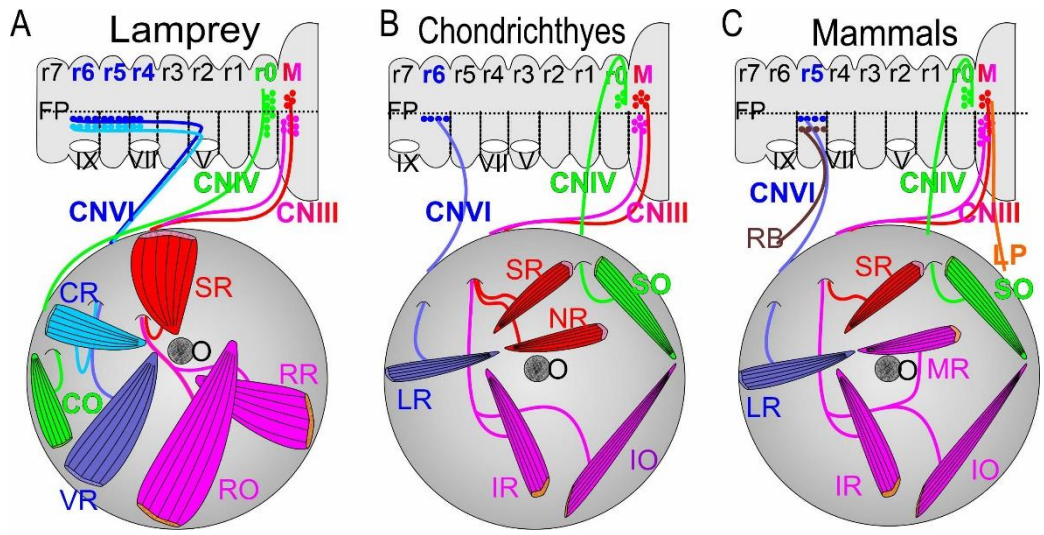

Figure 1. Patterns of innervation from cranial nerves III, IV and VI. Oculomotor (CNIII) and trochlear (CNIV) cranial nerves are derived from r0/midbrain, whereas the abducens (CNVI) develops in r4-6 (lamprey; A), with variable contribution from r5 (mammals, amphibians, lungfish; C), r5-6 (chickens, teleosts) and chondrichthyans (r6; B). The ocular motor neurons always have a contralateral projection to the superior rectus (SR) whereas two ipsilateral innervations are present in all vertebrates (lilac colors). In addition, there is a separate contralateral projection to the nasal rectus (NR) in chondrichthyans and lungfish and additional ipsilateral fibers innervate the medial rectus (MR) in tetrapods, teleosts, and the coelacanth *Latimeria*. The trochlear nerve muscle (CNIV) projects from the oblique muscle (CO) in lampreys, whereas it is shifted to innervate the superior oblique muscle (SO) in all gnathostomes. In lamprey, the CNIV develops next to the cerebellum to exit the crossing fibers bilaterally, whereas in gnathostomes it develops next to the ocular motor neurons and forms long fibers that cross near the cerebellum. The abducens (CNVI) innervates two extraocular muscles in lampreys but is reduced to a single innervation from LR in gnathostomes. In addition, *Latimeria* has a unique and very large innervation that is not connected to the eye muscles, whereas tetrapods have a variable retractor bulbi (RB). CO, caudal oblique; FP, floor plate; IO, inferior oblique; IR, inferior rectus; LR, lateral rectus; MR, medial rectus; NR, nasal rectus; O, optic nerve; r0-7, rhombomeres; RB, retractor bulbi; RO, rostral oblique; SR, superior rectus. Note that the color helps to trace the EOM innervation. Compiled from [8,9,13,16,19,22].

2.3. Abducens (CNVI)

The abducens projects ipsilaterally to innervate the lateral rectus (LR; also referred to the posterior rectus; PR), which is equivalent to the ventral rectus (VR) in lampreys (Fig. 1; Table 1). In addition, lampreys have a second extraocular muscle that is referred to as the caudal rectus (CR). It appears that the equivalent to the CR was lost in chondrichthyans, lungfish and actinopterygians [11,13]. In contrast, *Latimeria* and tetrapods have a unique innervation of a separate abducens extraocular muscle, the basicranial rectus (BR), and the retractor bulbi (RB; Fig. 2). In the sarcopterygian

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coelacanth *Latimeria,* the BR has no connection to the eye [22,25,65,66]. This innervation provides a very large muscle that likely is common across basal sarcopterygians [23], including the famous prehistoric sarcopterygian *Eusthenopteron* [67]. Unfortunately, details of CNVI organization in *Latimeria* are unclear [68] and would require modern labeling methods such as antibodies with tubulin [41,69]. The retractor bulbi (RB) in tetrapods has a ventral position that is comparable to the CR in lampreys (Fig. 1). Among tetrapods, the RB can be very large (as in chickens, Fig. 2; [24]), whereas the RB is absent in microchiroptera and primates [29,34]. Moreover, a unique formation, known as the tentacular retractor muscle, which is equivalent to the retractor bulbi in amniotes [21], is present in caecilians [27,28]. The distribution of the constituent neurons differs between vertebrates: they extends from r5 to 6 and parts of r4 in lampreys [8,43,70], derive exclusively from r6 in chondrichthyans [8], and are derived from r5 and 6 in sauropsids and teleosts [8] but exclusively from r5 in lungfish, amphibians and mammals [8,20,45].

3. Extraocular muscles are derived from several sources

Extraocular muscles (EOMs) are in many ways unique compared to other skeletal muscles [32,71,72]. Head cavities, or epithelial mesodermal coeloms in the head, are thought to represent serial homologs of somites (Fig. 2). Three pairs of head cavities form each pharyngeal pouches thereby showing a metamerical arrangement that was the basis of three cranial somites [8,16,73]. Extraocular muscles may diverge from different muscle precursors that are under the influence of different extrinsic signals [71]. The extraocular muscles are not affected from Duchenne muscular dystrophy but are differentially affected by other conditions, including myasthenia gravis [71]. The extraocular muscles differ in composition from branchial and trunk muscles as they have distinctive fibers and a unique pattern of innervation that allows fine control of movement [4].

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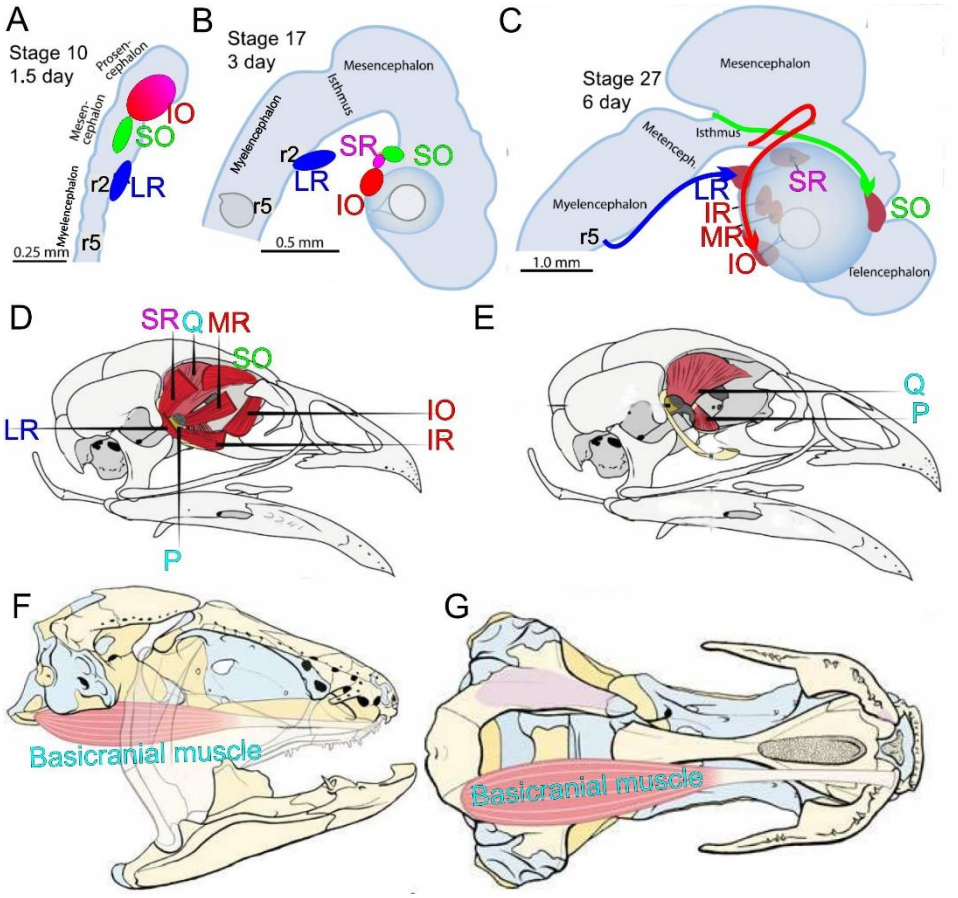

Figure 2. Patterns of extraocular muscles compared. (A-C) Chicken extraocular muscles start out as three paraxial mesoderm areas located near the telencephalon, the midbrain and the myelencephalon (A), coded here in three colors [blue for lateral rectus (LR), green for superior oblique (SO), red/lilac for inferior oblique (IO) and the other four extraocular muscles (SR,IR,MR). *Myf5* is the first gene expressed in the mesoderm region (A, B) that will migrate and expand into muscle fibers later (C). The pattern of cranial nerve innervation is indicated in colors for CNIII, CNIV and CNVI. (D, E) Adult chickens have six eye muscles that converge in the eye bulb and two additional muscles that interact with the nictitating membrane: the quadratus (Q) and the pyramidalis (P; E). (F, G) *Latimeria* has a unique basicranial muscle that is innervated by the abducens, CNVI. The muscle is inserted into a tendon that will move the intracranial joint, when contracted. After [24,32,65].

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Paraxial mesoderm was proposed as somitomeres that were transformed into epithelial somites [74]. More recently, it was demonstrated that myoblasts form in paraxial mesoderm that will differentiate into distinct muscle [32]. Extraocular muscles form relatively close to the midbrain (CNIII), midbrain-hindbrain (CNIV) and hindbrain (CNVI; Fig. 2). Muscle fibers in the chicken will split into 4 extraocular muscles that innervate CNIII, will migrate a long distance to innervate the dorsal SO (CNIV) and develop into CNVI innervated by lateral rectus precursors close to the trigeminal ganglion and next to rhombomere 2 (Fig. 2; [31,32,38]). In addition, CNVI also provides the retractor bulbi and innervation. *Myf5* and *Pitx2* are sequentially active and are essential for myoblasts for all ocular muscles that do not differentiate in a mutation in mice [31,38].

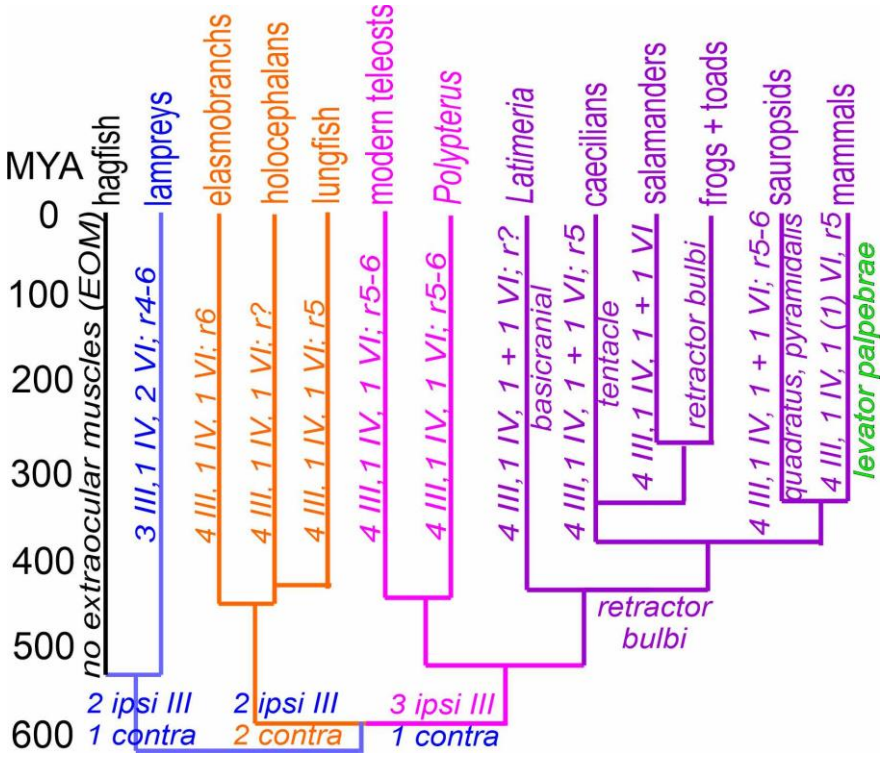

Figure 3. Cladistic analysis of vertebrate extraocular eye muscles and their cranial innervation, shown over 520 million of years (MYA). Hagfish lack EOMs; lampreys have three CNIII (blue color), whereas four CNIII are common in gnathostomes. Two ipsi- and two contralateral fibers form in chondrichthyans and lungfish, including a unique nasal rectus (NR) muscle (orange color). Three ipsi- and one contralateral innervation from Osteichthyes, inclduing the medial rectus (MR) muscle (lilac color). In addition, lampreys have two abducens muscle fibers whereas only a single abducens is found to the lateral rectus (LR) in all gnathostomes (lilac and orange). A variation of the rhombomeres shows three origins, chondrichthyans is from r6, lungfish, amphibians, and mammals provide r5 CNVI neurons and sauropsids and teleost have two rhombomeres, r5 + 6. *Latimeria* have a very large abducens, the basicranial muscle, which requires proper investigation. The retractor bulbi is equivalent to one of the two lamprey eye muscles. Basicranial and tentaculate muscles, as well as quadratus/pyramidalis muscles, seem to be derived from retractor bulbi muscles. A split into the levator palpebrae from the superior rectus is a unique late segregation in mammals. The ages of different lines are indicated as approximations in millions of years. Assembled from [4,8,9,11-13,20,22,23,27,31,32,71,75,76].

Traditionally, almost all extraocular muscles are suggested to be nearly identical across vertebrates [32]. However, a few additional or unique extraocular muscles and their innervation are known. Lampreys have three CNIII, a CNIV and two extraocular muscles that are innervated by

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CNVI (Fig. 1,3; [9]). In contrast, gnathostomes have four CNIII fibers that split into two combinations: two ipsi- and two contralateral branches of CNIII supplies, inclduing the contralateral nasal rectus (NR; chondrichthyans, lungfish), whereas bony fishes, *Latimeria* and tetrapods have one contralateral and three ipsilateral CNIII branches, including projection to ipsilateral medial rectus (MR; Fig. 3). The CNIV in lampreys and gnathostomes is likely equivalent between the CO and SO and migrates late from the midbrian/hinbrain into a different, rostral position in gnathostomes (Fig. 2,3). Whether one CNVI evolved in lampreys to become the retractor bulbi in sarcopterygians remains to be seen (Fig. 2,3). In addition, the retractor bulbi in tetrapods converts into two muscles in birds, the pyramidalis and quadratus, which drive the nictitating movement in almost all amniotes (Fig. 2,3; [24,77]). A unique tentacular retractor muscle in amphibians [27,28] derives from the retractor bulbi. The basicranial muscle is innervated by the abducens, which is not connected with the eyes (Fig. 2,3; [22,23,25,65,66]). Chondrichthyans, teleosts and lungfish do not have an 'accessory' muscle but several cases of unique branches of new extraocular muscles are reported [10,78]. An additional branch is a nictitating membrane in elasmobranchs, which evolved independently in sauropsids and most mammals [77,79,80]. The nictitating membranes are replaced by eyelids in chiroptera and primates [29,30], which evolved into a muscle unique to mammals, the levator palpebrae (LP; [18,19]), which is innervated by contralateral cranial nerve III [34].

In summary, muscle formation is derived from paraxial mesoderm, which generates cranial nerves III, IV and VI to innervate extraocular muscles, including the unique branch of lampreys, *Latimeria* and tetrapods. A detailed analysis of gains and losses of CNIII and CNVI requires additional work to elucidate the genetic and development of different muscle fibers and their pattern of innervation in vertebrates.

4. Development and axon guidance

Dating of rat cranial motor neurons using 3H-thymidine reveal that they form along a caudal-to-rostral gradient, with abducens emerging at E12 and oculomotor and trochlear motor neurons between E12 and E13 [81,82]. The extension of nerve fibers begins at about E10 in mice, reaches the extraocular muscles at about E11 and segregates to each of the six extraocular muscles by about E13 [19,31,38]. Despite overall similarities in the two special somatic motor neuron groups and the one somatic motor neuron group, each group is unique in its projections through cranial nerves CNIII, CNIV and CNVI. A detailed Edinger-Westphal complex develops in tetrapods [6,83]. A temporal progression of gene expression is evident for cranial nerves CNIII and CNIV in mice, zebrafish and chicken [55,83]. In contrast, abducens nerve development is independent of CNVI [84-86] but depends on Hox genes.

The long-established use of 3H-Thymidine as a marker for proliferation is now complemented by use of the synthetic thymidine analog bromodeoxyuridine (BrdU) [55]. BrdU expression starts in chicken at developmental stage 11 and ends at about stage 23 [55]. This pattern of proliferation is confirmed for the abducens in zebrafish [87]. A gradient is found whereby ocular motor neurons develop slightly later from trochlear motor neuron IV in zebrafish [17,75], whereas mammals have a temporal overlap of cranial nerve CNIII/IV/VI cell cycle exits [81,88]. Specifically, the innervation of extraocular muscles begins in zebrafish around 30 h postfertilization (hpf) and reaches all six eye muscles by about 54 hpf [75]. Fluorescent labeling to detail the generation of cranial nerves CNIII and CN IV has demonstrated an earlier projection [17].

Recent labeling of EdU [89] could help detail the origin of ocular motor neurons across vertebrates from lampreys through mammals and shows clear dorso-ventral progression in the chicken [55]. Extraocular muscles innervating neurons that develop spatial and temporal maturity to generate the three ocular motor neurons (CNIII, CNIV, CNVI). Only hagfish have no eye muscles and are lacking all ocular motoneurons [90], unique among vertebrates [45]. Cranial nerve III innervates the contralateral superior rectus and ipsilateral inferior oblique, inferior rectus and medial rectus (Fig. 1) in teleosts, chicken, mice [31,38,55,75,91] and lampreys [53,57]. Cranial nerve III fibers require 1-3 days (zebrafish, mammals) to develop, with a pause prior to expanding to innervate the four extraocular muscles [38]. Mice show delayed innervation compared to chickens thanks to the superior

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rectus that migrates across the floor plate to innervate the superior rectus (and levator palpebrae [31]). Development of the superior rectus depends on the *Kif21a* mouse mutant that eventually becomes reduced by cell death in the contralateral neurons, as documented by using caspase immunofluorescent labeling [19]. In addition, instead of extending to the superior rectus as in control mice, the axons prematurely exit from the dorsal root but do extend completely to the superior rectus (or levator palpebrae). Consequently, the elevation of levator palpebrae and upward eye movements caused by the superior rectus are reduced and there is partial degeneration of the extraocular muscles [19]. Moreover, EOMs depend on genes that affect the muscle innervation [92].

4.1. Ocular motor neurons

Deletion of roundabout guidance receptor (*Robo)* and slit guidance ligand (*Slit)* causes derailed innervation after the incomplete contralateral superior rectus of cranial nerve III [40]. Mice that are null for *myogenic factor 5 (Myf5)* do not have any extraocular muscle formation [31]. The initial patterns of innervation by ocular nerves develop nearly normally but, in the absence of their EOM targets, the nerves have begun to degenerate by E14.5 [31]. Loss of *paired-like homeodomain transcription factor 2 (Pitx2)* results in disoriented fibers and delayed innervation [38]. Functions are needed for S*emaphorin3f (Sema3f),* which interacts with *Neuropilin* 2 (*Nrp2),* which requires defasciculating of cranial nerve III [33,93]. *Sema3a* interacts with *Nrp1* to regulate mouse innervation; it requires detailing the ocular motor innervation [94]. *Sema3a/c* ligands signal via *Plexin a1* (*Plxna1*) and *α*2-*chimaerin* (*Chn1*), and can result in defasciculating [75,95-97], which involves a double null for *Sema3a/f* and *Nrp1/2* [93,94], including *Plxna1* and *Chn1*. To fully understand the various ligands that repel or attract different nerve ocular fibers that have an additional role for *Nova2* in various peripheral innervation [98] requires detailing its effect on extraocular muscles' innervation.

In summary, three patterns of ocular motor neurons form with three branches to reach from one contra- and two ipsilateral branches in lampreys, two innervate from ipsi- and two contralateral branches in chondrichthyans and lungfish, whereas one contra- and three ipsilateral branches are found in teleosts, *Latimeria* and tetrapods (Fig. 3). In mice, the cranial nerve III axons begin to extend by E10, interacts with extraocular muscles by E11, and develops branches around E13 which proceed to innervate four extraocular muscles. Absence of *Wnt1* or *Phox2a* results in the loss of innervation of extraocular muscles. The genes *Robo, Slit, Nrp2, Sema3f, Plxn1, Chn1, Myf5* and *Pitx2* influence fiber branching, which is specifically affected by *Kif21a* superior rectus and levator palpebrae.

4.2. Trochlear fibers

Trochlear fibers are involved in several genes controlling projections that plays a role for dysplasia [99,100]. *Netrin1* (*Ntn1*) acts as a bifunctional guidance cue that simultaneously attracts some axons to the floor plate while steering others away [35]. *α*- and *β-tubulin* monomers of different tubulin are near normal in terms of loss-of-function mutations, but there can be a missense mutation in *Tubb3* [41] that results in the misrouting of CNIII and CNIV. Downstream, *Ntn1* interacts with *Dcc* to create attractive or repulsive signaling. *Unc5 netrin receptor c (Unc5c)* displays an incomplete and variable projection to reach the SO bilaterally [36,37] and interacts with *Fgf8* [101,102]. *Nrp2* must interact with *Sema3f* because null mutants show loss or absence of CNIV [34,93]. Interestingly, exuberant branches form that innervate CNIII after perturbations of *Myf5* and *Pitx2* [31,38]. Incomplete crossing results if *α2-chimaerin (Chn1*$^{KI/KI}$) mutants are combined with *Epha4*$^{KO/KO}$, pointing to reversed and bidirectional interactions. A loss-of-function of *Chn1*$^{KO/KO}$ with *Epha4*$^{KO/KO}$ [88] documents the interaction with *ephrin A5* (*Efna5*). Misexpression of *Smo* using *Pax2* results in redirection of trochlear fibers to exit ipsilaterally, adjacent to CNIII [36]. Unfortunately, *Pax2-cre; Smo* overexpressing mice die early around E15 before the extraocular muscles have fully developed, meaning that the pattern of innervation in a redirected ipsilateral SO innervation is not known.

In summary, the trochlear nerve innervates the CO/SO from dorsal (lamprey) or ventral (gnathostomes) origins.

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4.3. Abducens fibers

Abducens fibers depend on *Shh* and other genes for differentiation of neurons in r5 of mice [69,84,103-106]. Downstream, *Sall4* is found in mice [97,107]. *Robo3* in CNVI show exuberant crossings and combine with ipsilateral projections [34,108,109]. Downstream of *Mafb* are several cadherins that lead to misdirections in *Pcdh17* null mutant zebrafish [110]. Several factors interact with semaphorins, plexins and neuropilins and participate in guidance of the abducens fibers [93,94]. Abducens defasciculating occurs after loss of *Npr1* [33,34]. In addition, *α2-chimaerin* abducens' guidance in the absence of loss or gain of this gene is described *(Chn1*$^{KO/KO}$*; Chn1*$^{KI/KI}$). Moreover, *Eph receptor A4* (*Epha4*$^{KO/KO}$) with *Efna5* interacts to signal forward and reverse; both are critical for abducens signaling, pointing to a bidirectional interaction [34,88].

In contrast to CNVI, innervation of CNIII is unaffected by *Hoxa3/b3* and *Mafb* [69,97,103]. Likewise, *Hoxa1* loss-of-function deletions results in aberrant innervation of the abducens [111] interacting with *Sall4* [105,107]. Conversely, loss of *Lmx1b, Wnt1* and *Phox2a,* which eliminates CNIII and CNIV, results in an expansion of CNVI to innervate the denervation of remaining extraocular muscles [85,86,112], showing a plasticity in the residual innervation.

In summary, CNVI is unique in its size (from r4-6, just r6, just r5 or both r5 and 6) and gives rise to major abducens projections and additional extraocular muscles like retractor bulbi.

Overall, gains and losses are evident in lampreys, chondrichthyans and sarcopterygians, which added two different ocular motoneurons (nasal and medial rectus), whereas the caudal rectus of lampreys evolved into the retractor bulbi in tetrapods. Fiber guidance is presented as a variation of the same theme for the three ocular motoneuron group with certain guidance genes in CNVI compared to CNIII and IV that may lead to dysplasia of innervation [92]. The split into CNIII and VI requires additional work to elucidate the origin of the additional motoneurons.

5. Molecular properties of ocular III, IV and VI motor neurons.

In vertebrates, sonic hedgehog (*Shh*) acts as a long-range morphogen to define three different ventral lineages (V1-3) and all motor neurons. Oculomotor and trochlear motor neurons are special somatic motor neurons (SSM) while abducens are somatic motor neurons (SM) that generate next to the branchial motor neurons, (BM; **[6,113-116]**). Special somatic motor neurons develop next to the isthmus (between the r0 and the midbrain) to generate ocular motor neurons (cranial nerve III) and trochlear motor neurons (cranial nerve IV) whereas somatic motor neurons are found in the abducens (cranial nerve VI). In addition, somatic motor neurons are continuous with the spinal cord and hypoglossal somatic motor neurons **[114,117]**. Following a discussion of the general role of *Shh,* I will detail the genetics for CNIII/IV, followed by CN VI.

5.1. Ocular III + IV are dependent on a unique set of genes.

The sonic hedgehog protein (*Shh)* interacts with several genes: Patched (*Ptch*), Smoothened (*Smo*) and GLI-Kruppel family member *Gli1-3* **[36,118]**. The neurectoderm homeobox genes *Otx2*/*Gbx2* define the isthmic region that specifies the midbrain-hindbrain barrier, including the CNIII/IV in vertebrates **[119-121]**. Modifying the concentration equilibrium between *Gbx2* and *Otx2* define the location of the midbrain-hindbrain boundary **[116,121-124]**. Several transcription factors, including *En1/2, Foxa1/2, Lmx1a/b, Nurr1,* and *Pitx3,* interact with growth factors or morphogens such as *Shh, Fgf8, Tgf,* and *Wnt1* **[102,116,121,125,126]**.

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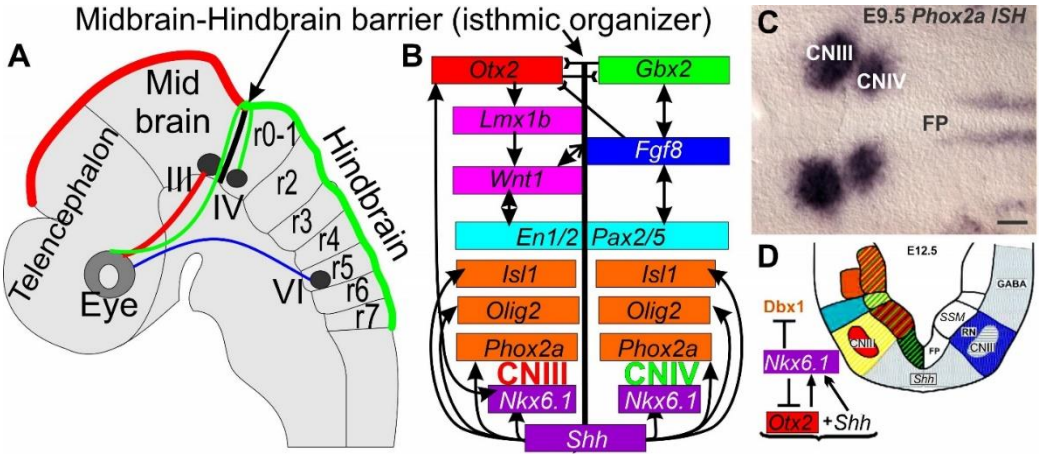

Figure 4. Defining the isthmic organizer in the mouse. (A) The Midbrain-Hindbrain barrier is established by reciprocal inhibition of *Otx2* (red) and *Gbx2* (green) to regulate the isthmic organizer, including cranial nerves CNIII (midbrain, red) and CNIV (rhombomere 0, green) and cranial nerve VI at rhombomere 5 (blue). (B) Downstream is controlled by *Otx2* such as *Lmx1b* and *Wnt1*, whereas *Gbx2* is downstream of *Fgf8*. *Shh* is needed for *Nkx6.1, Phox2a, Olig2* and *Isl1* upregulation and interaction with *Otx2*. (C) *Phox2a* is expressed in the two adjacent groups of special somatic motor neurons located in cranial nerves (CN) III and IV. (D) Expression of *Shh* is needed for upregulation of *Nkx6.1*. Modified after **[36,121,127-129]**.

In addition to *Wnt1* (Fig. 4), other transcription factors such as *Notch-Delta*, *Shh*, transforming growth factor 13 (*TGF-13*), and bone morphogenetic protein (*BMP*), play a role in development **[123]**. The transcription factor *Wnt1* is highly conserved across cnidarians, flies, and vertebrates and shows frizzled (*Fzd*) and lipoprotein receptor-related protein (*Lrp5/6).* β-catenin (*β-cat*) activates transcription factors *Wnt, Dvl, Axin, β-cat, β-TrCP, Cki, Gsk, Apc* and proteasome degrade *β-cat*, which will be blocked by transcription factors through binding with *Graucho* **[123]**. Previous work showed the absence of cranial nerves III and IV after *Wnt1* loss **[85,130]** and reduced proliferation of CNIII and IV **[102,123,125]**.

Downstream of *Shh* in mammals is the transcription factor Nkx6 homeobox 1 (*Nkx6.1*), which interacts with oligodendrocyte transcription factor 1/2 (*Olig1/2*) in special and somatic motor neurons [117,131,132]. Specifically, *Nkx6.1* is a transcriptional repressor that is required for the development of somatic motor neurons and special somatic motor neurons in the brainstem and the spinal cord [127]. *Nkx6.1* expression requires derepression of the *Shh* to develop brain homeobox 1 and 2 (*Dbx1* and *Dbx2*) and fate-switching of motor neurons into interneurons [127]. *Otx2* participates in a reciprocal interaction with *Nkx6.1* to develop cranial nerve III (Fig. 4).

Upstream of ocular and trochlear motor neurons III and IV is the boundary of *Otx2*/*Gbx2* expression that defines the isthmic region [36,129]. Once the *Otx2*/*Gbx2* boundary is established (Fig. 4), the regulatory genes of *Shh*/*Fgf8*/*Lmx1b*/*Phox2a*/*Wnt1*/*En2* interact with each other to develop the isthmic region [119,124]. In the absence of *Otx2* there is reduced expression of *Nkx6.1, Olig2* and *Isl1* in cranial nerves III and IV. Furthermore, *Nkx6.1* is needed for *Phox2a* to develop these cranial nerves while *Olig2*, *Isl1* and *Pou4f1* are reduced after *Nkx6.1* null mutation [112,127].

Otx2 is upstream of *Lmx1b,* which is required for cranial nerve III to develop special somatic motor neurons [128]. *Lmx1b* is required for the earliest expression of *Phox2a*, suggesting that *Phox2a* acts downstream of *Lmx1b* [112,119,128]: *Lmx1b* null mice show near complete loss of cranial nerves CNIII and CNIV in *Phox2a* null mice [112,115]. Moreover, *Lmx1b* is lost in initially expressed *Wnt1, En1* and *Fgf8* [128], suggesting a dependence on the isthmic region that allows the formation of cranial nerves III and IV [36,119,124]. *Fgf8* expression is dependent on *Lmx1b* [128], which is absent after *Fgf8* null mice [102,121,129]. Interestingly, early upregulation of *Nkx6.1* is evident in *Lmx1b* null mice, suggesting partial independence from *Nkx6.1* and *Lmx1b* [112]. The expression of *Phox2a, Olig2* and *Isl1* is likely downstream of *Shh, Lmx1b* and *Wnt1 [112,127,132]*. The expression of *Lmx1* in lampreys is controlled by a single gene, which could function as both *Lmx1a/b* [119,133]. To know how many *Wnts*

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are present in lampreys, and which could have a similar expression in the isthmus, requires more work to define the midbrain-hindbrain barrier in lampreys [36,124]. The trochlear motor neurons likely depend on *Nkx6.1* and *Phox2a,* but studies using *in situ* hybridization are required to demonstrate gene expression.

In summary, a complex genetic interplay generates the midbrain-hindbrain boundary, where *Shh, Otx2, Gbx2, Nkx6.1, Lmx1b, Phox2a, Wnt1, Fgf8, Olig2 and Isl1* interact to develop the two special somatic motor neuron of cranial nerves III and IV (Fig. 4).

5.2. Abducens motor neurons develop independent of oculomotor and trochlear motor neurons.

In contrast to the isthmic organizer that defines cranial nerves CNIII and CNIV (Fig. 4), cranial nerve CNVI (Fig. 5) depends on Hox gene expression that ends in rhombomeres [114,134]. Obviously, *Shh* expression is required to develop motor neurons, including cranial nerve CNVI, to drive *Nkx6.1* [114,116]. As with the role for *Nkx6.1* in cranial nerves III and IV, the expression of cranial nerve VI depends on *Nkx6.1,* suggesting it acts like a typical somatic motor neuron, comparable to hypoglossal and spinal motor neurons [84,117]. Downstream are gene regulatory networks that regulate transcription factors that are specified by cis-regulatory elements to exert their functions [114]. In addition to requiring the gene *Shh,* there are several other genes that are needed for somatic motor neuron formation: *Pax6*, *Nkx6.1* and *Olig2* [84,103,127]. Superimposed on this arrangement is the *Hox* code control of the hindbrain by regional upregulation of rhombomeres r2-r11, defined by Puelles work [6,83,129,134].

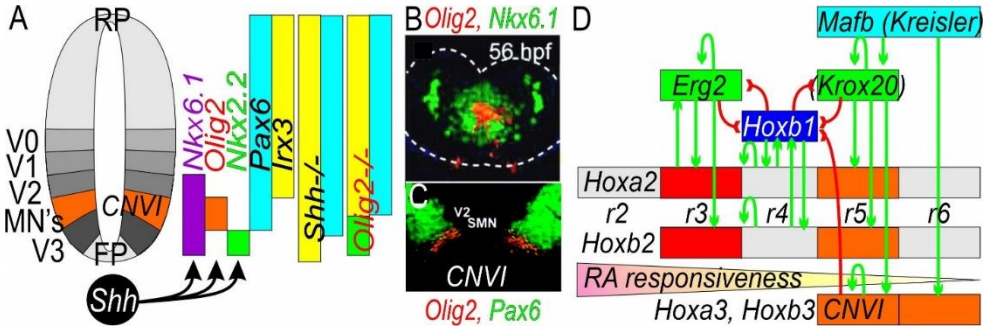

Figure 5. Abducens motor neuron development depends on *Nkx6.1* and *Olig2* in mice. (A) A schematic cross section of rhombomere r5 show dependence on *Shh* for *Nkx6.1* expression, which, in turn, is required for the *Olig2* gene to induce motor neurons (MNs). Adjacent bars show the expression of *Nkx2.2, Pax6* and *Irx3* genes to define the populations of somatic and branchial MNs. Absence of *Shh* will result in a loss of *Nkx6.1, Nkx2.2* and *Olig2* that is expanded by *Irx3* (further right). Absence of *Olig2* results in an expansion of *Nkx2.2 (*far right). (B) The gene *Olig2* (red) is expressed within rhombomeres r5/6 and embedded by *Nkx6.1* in a teleost (green). (C) Somatic MNs are positive for *Olig2* (red) in cranial nerve CNVI of mice of r5 that is adjacent to *Pax6* (green). (D) The reciprocal interaction is shown as upregulation (green arrows) and suppression (red arrows). Note that *Erg2* (*Krox20)* defines two rhombomeres (r3, r5) that interact with the caudal expression of *Mafb* (*Kreisler;* r5, r6) to develop r5 and cranial nerve CNVI. Responsiveness to retinoic acid (*RA*) is increased in more rostral regions and defines the sequential Hox code expression. FP, floor plate; V0-V3, ventral neurons; MNs, motor neurons; RP, roof plate. Modified after [87,103,114,134-136].

The *Hox* genes have four *Hox* clusters that each have a unique expression pattern (Fig. 5.; [134]). Complex feedback and feedforward pathways that regulates rhombomeres have been identified in fish and mammals. In mice, *early growth response 2* gene (*Egr2, aka Krox20*) is expressed in two bands in rhombomeres r3 and r5 and interacts with the *v-maf musculoaponeurotic fibrosarcoma oncogene (Mafb, aka Kreisler;* r5 and r6). Further, expression of *Hoxa3/b3* is needed to develop rhombomere r5 (Fig. 5). In addition, retinoic acid (Fig. 5) exerts its effects in a graded manner whereby concentration is typically higher close to the spinal cord, whereas RA are degraded rapidly in later development [135].

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While *Phox2a* is needed for the development of ocular and trochlear motor neurons CNIII and CNIV [112], abducens motor neurons and CNVI can form independently of *Phox2a*, suggesting a different molecular dependency of somatic motor neurons such as *Nkx6.1* [84]. In fact, branchial motor neurons could develop by *Phox2a/b,* which could control *Nkx6.1, Olig2* and *Isl1* expression in mice in the brainstem [84,103,115,117]. In r5, *Hoxa3*$^{-/-}$*: Hoxb3*$^{-/-}$ double mutant mice show a complete loss of somatic motor neurons including CNVI [103,137]. Therefore, it is suggested that genes *Hoxa3* and *Hoxb3* are required for generation of somatic motor neurons [103]. None of the genes *Pax6, Olig2, Isl1, Tuji* or *ChAT* are present in double null mutants and/or are reduced in the residual expression of abducent motor neurons CNVI [103]. All brainstem motor neurons in mice are absent after *Olig2* deletion [131,132]. The bHLH gene *Neurog2* is closely related to *Olig2* and is known to interact with *Olig2* to induce somatic motor neurons to differentiate [131,138], whereas *Olig2* interacts with *Phox2b* to regulate branchial motor neurons [112,115,131].

Hoxa3 gain-of-function in chicken suggests the formation of anterior somatic motor neurons in rhombomeres r1-4 [139]. In contrast, *Hoxb3*^{T8} mutants exhibit a possible r4 to r2 identity switch, but not an r4 to r5 identity switch [137]. One has to keep in mind that the chicken hindbrain is generated from two rhombomeres (r5+6) whereas in mammals is generated only in r5 [8,103]. Like chicken, bony fish have two rhombomeres – r5 and r6 (Fig. 2) – that are highly positive for *Olig2* and *Isl1* [87].

In summary, a combination of *Shh, Nkx6.1, Olig2, Isl1, Hoxa3* and *Hoxb3* genetic control is required for the development of the abducens neurons, CNVI (Fig. 5).

6. Vestibulo-ocular interactions across vertebrates.

The vestibulo-ocular reflex (VOR) is a crucial connection that helps vertebrates maintain stable vision during head movements; it involves a complex interplay between the vestibular system (which senses head motion) and the ocular motor system (which controls eye movements). Vertebrates, except for hagfish, have a vestibular connection that innervates - via three ocular motor neuron groups - six eye muscles to help move the eyes. Connections between the vestibular neurons and higher order connections form a three-neuron arc [3,4,44,45,140,141].

The first neuron in this arc receives input from the vestibular ganglion neurons to reach the brainstem, the vestibular nuclei. Vestibular nuclei are composed of four areas, the lateral, inferior, medial, and descending vestibular neurons (LVN, IVN, MVN, and DVN). The second leg of neurons crosses mostly to the contralateral side to reach the three oculomotor cranial nerves (III, IV and VI; [1,142]), the third leg. The VOR is a remarkable adaptation that enables vertebrates to maintain stable vision in a dynamic environment and highlights the complex interplay between different sensory and motor systems in the hindbrain [44,47].

The majority of tetrapods have been shown to receive predominantly contralateral excitatory connections [143] with the exception of the medial rectus (MR). The MR involves a contralateral projection of internuclear fibers with ipsilateral fibers of the ascending tract of Deiters (Fig. 6; [144]. A recent study has described a much simpler connection in a teleost, composed of ipsilateral and contralateral VOR pathways [17,42] that are consistent with the VOR connections in chondrichthyans [11]. Limited information on lamprey vestibular connections suggests receiving of a direct vestibular input into the dorsal cranial nerve IV that reaches out the contralateral cranial nerve III [42,43,47].

In summary, a detailed analysis shows at least three patterns of the vestibulo-ocular reflex: lampreys, chondrichthyans and mammals that split into primary antagonistic extraocular muscles of the left and right side of the eyes (Fig. 6).

6.1. Lampreys have a different VOR.

Lampreys have a simpler vestibular organization that consists of two canal cristae, the anterior and the posterior cristae (Fig. 6; AC, PC; [46,48]), innervated by three populations of vestibular neurons: the anterior, inferior and posterior nuclei (AN, IN, PN; [42,43,53]). Selectively tracing the anterior crista shows fibers projecting mostly caudally whereas the posterior crista projects rostrally [43]. A direct link may exist between the dorsal CNIV and the posterior crista [9,53]. Contralateral

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vestibular fibers have been traced to project to three CNIII nuclei. Untangling the detailed pattern of connections between the vestibular neurons and the abducens nerves requires further work. Assuming the same configuration is true for gnathostomes one can expect the following pairing of antagonistic interactions: RR/SR, RO/CO, CR/VR (Fig. 6). Essentially, we can assume the same extraocular muscles are present in gnathostomes in terms of function despite the different origin and insertion [32] between the lamprey and gnathostomes [47]. Lampreys exhibit robust visuo-vestibular integration, suggesting that visual and vestibular inputs are integrated and respond to saccades and nystagmus. Thus, all basic components of the visuo-vestibular control of gaze have different ocular motor neurons to the eye and lack a horizontal canal [9,42,46,48]. Further investigations are needed that can explain the VOR in lampreys fully.

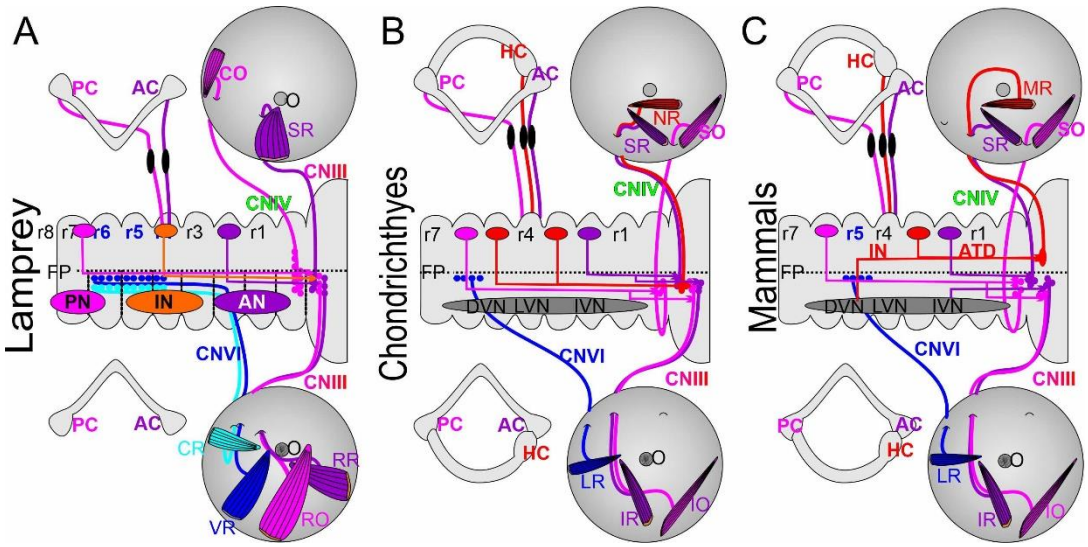

Figure 6. Vestibulo-ocular reflex varies in vertebrates**.** For convenience, we separate extraocular muscles into antagonistic innervation to the left and right side of the eyes, respectively, to receive the ear input: IR/SR (burgundy); IO/SO (lilac); MR/LR (red/blue). (A) Lampreys have only two vestibular canals (AC, PC) whereas three canals are present in gnathostomes (AC, PC, HC). Central vestibular projections in lampreys provides a direct connection from the posterior canal (PC) to the CNIV directly, whereas the anterior canal (AC) projects predominantly to the descending part. Connections form three vestibular nuclei: anterior, inferior, and posterior nuclei (AN, IN, PN). One branch of CNIII and bilateral dorsal located CNIV will receive the single contralateral projection; all four ipsilateral projections provide two CNIII (RR, RO) and two CNVI (CR, VR). (B) Chondrichthyans have a simple contralateral innervation of SR, NR and SO (upper) whereas the ipsilateral innervation is sending to two CNIII/IV (IO, IR) and the CNVI (LR) that receive second-order VOR inputs. (C) Mammals have a unique connection that projects as abducens internuclear neurons (IN) which combines the ascending tract of Deiters (ATD) to establish a connection to the MR. In contrast to these unique ipsilateral fibers, IR and IO serve as a counteraction to the other half of the innervation eye. Modified after [4,11,43,143].

6.2. Elasmobranchs and lungfish may have a unique connection of the VOR.

Chondrichthyans are well described and have the simplest connections between the vestibular output and the ocular motor neurons [11,12]. A few things are different about the vestibular organization and the utricle that indicates a separation between chondrichthyans and lungfish [13] and the remaining gnathostomes [44,45]; it evolved an anterior and a posterior branch within elasmobranchs [11,145]. Differences from the contralateral CNIII should be named the nasal retractor (NR). A contralateral projection receives from the PC to reach out predominantly to the IO/SO. Likewise, we can assume that a counteraction occurs between SR/IR whereas the counteraction should be between the NR/LR. The detail of this connection remains to be determined in chondrichthyans and lungfish [11-13].

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6.3. Osteichthyes (bony fishes) share a separate connection of the VOR.

Tetrapods, coelacanth and teleosts have a unique connection between the medial rectus (MR), compared to the equivalent to the NR [11], which counteracts with lateral rectus in Osteichthyes [1,5,42]. While most connections are identical among gnathostomes, despite some variation of the abducens origin between r5-6, the connection between the MR evolved differently: In part, the input is provided by abducens internuclear neurons (IN) that project contralaterally via the ipsilateral ascending tract of Deiters (ATD). Simply speaking, this unique arrangement in tetrapods combines a contralateral input, (the internuclear neurons) with an ipsilateral input (the ascending tract of Deiters), to drive the MR movement [4,11,143,146,147]. The synergistic muscle pairs are well understood; there is counteraction between lateral rectus and medial rectus extraocular muscles to act as abductors and adductors; a counteraction drives superior rectus and inferior rectus extraocular muscles that act as elevator and depressor; a counteraction of superior oblique and inferior oblique extraocular muscles' actions executes intorsion and extorsion [1]. In addition, we know that secondary and tertiary function are known among eye movements in mammals that depend on the position of the eye. For example, the superior rectus has primary action as elevator, secondary as adductor, and third as extorsion [4]. In contrast to mammals, teleosts have a direct connection of the VOR [17,148] without involving the ascending tract of Deiters, which has a comparison to elasmobranchs [42,143].

In summary, the VOR shows a common function to drive extraocular muscles in different directions. The six extraocular muscles are different and form into three major groups. Despite all the differences it appears that similar circuitry drives the VOR with the ocular motor neurons to generate coherent movements across vertebrates, as predicted over 70 years ago by Szentágothai [1,5,142].

7. Congenital cranial dysinnervation disorders (CCDD) in humans.

Eye alignment and movement requires the coordination of the six extraocular muscles (EOMs) which are innervated by the three ocular nerves CNIII, IV and VI in humans [49,105]. CNIII divides into a superior branch which innervates the superior rectus (SR) and levator palpebrae (LP) muscles; the motor neurons are born ipsilateral to the muscles they innervate, but then the SR motor neurons migrate across the floor plate and the levator palpebrae MNs migrate to the floor plate to innervate the contralateral SR or, bilaterally, the levator palpebrae muscles. The CNIII inferior branch extends from the ipsilateral motor neurons and innervates the ispilateral IO, IR, and MR muscles. Cranial nerve IV innervates the contralateral SO muscle; the motor neurons send their axons dorsally to exit the midbrain and the nerve then loop around the dorsal midbrain/cerebellum junction to innervate the muscles on the contralateral side. CNVI innervates the LR from ipsilateral neurons that form in r5 (Figs. 4,7). Cranial ocular innervation develops in a spatial pattern that aligns the six EOMs and the levator palpebrae.

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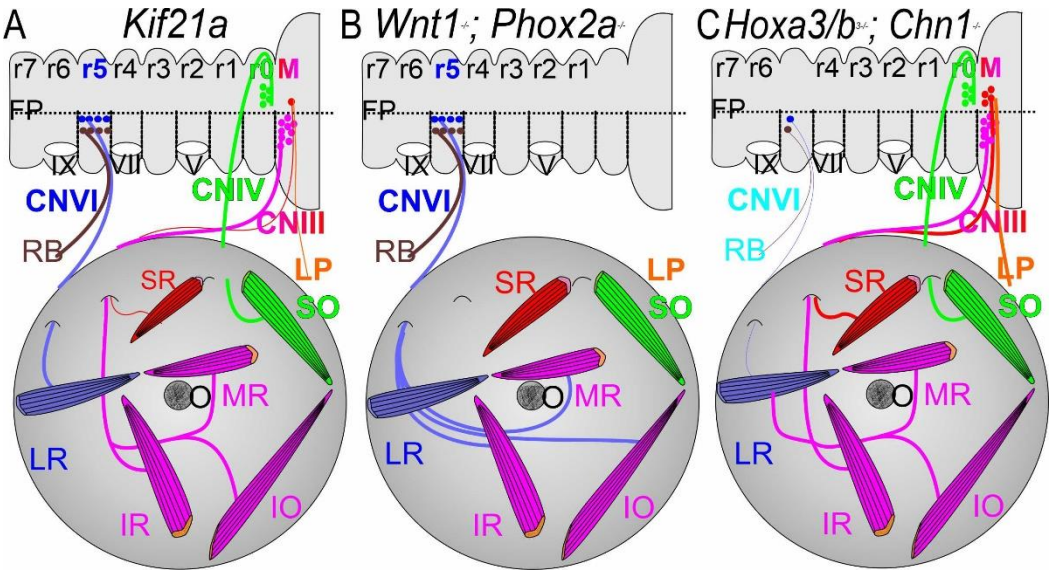

Figure 7. Aberrant extraocular muscle innervation in mice. (A) *Kif21a* mutations results in reduction of superior rectus (SR) and levator palpebrae (LP) fiber innervation and reduces the contralateral CNIII selectively. Superior rectus and levator palpebrae shrink in proper innervation and reduce upward movements. B) *Lmx1b, Wnt1* and *Phox2a* loses all cranial nerves III and IV and show an expansion of CNVI to innervate some of the denervated extraocular muscles and generate a Duane retraction syndrome effect. C) Several null mutations reduce the abducens (*Hoxa3/b3; Mafb; Nkx6.1*) and eliminate the CNVI neurons, whereas *Chn1* reduces and misroutes CNVI and causes an expansion of CNIII. Abbreviations are in Fig. 1. Modified after [19,69,84-86,88,97,103,112,127,149].

The human congenital cranial dysinnervation disorders (CCDD) include disorders in which children are born with restricted eye or facial movements secondary to defects in cranial motor neuron development or cranial axon growth or guidance. Most CCDDs are genetic, and many are inherited [97]. CCDDs that perturb oculomotor development (with or without additional perturbations of trochlear and abducens development) are referred to as congenital fibrosis of the extraocular muscles (CFEOM), while those that perturb abducens development are referred to as Duane syndrome or horizontal gaze palsy (Table 2).

7.1. CFEOM1: KIF21A

CFEOM1 is typically an isolated congenital eye movement disorder. Affected individuals have bilateral ptosis, eyes fixed in a downward position, and absent vertical and variably restricted horizontal eye movements. CFEOM1 results from monoallelic missense variants in *KIF21A* that can be inherited as a dominant trait or arise *de novo* [150]. *KIF21A* is widely expressed in developing and mature neurons and many other cell types, and it encodes an anterograde kinesin protein with a motor domain that interacts with microtubules, a flexible stalk domain, and a C-terminal WD40 domain. The protein has been shown to transport cargos along microtubules, as well as to regulate microtubule dynamics by serving as a 'brake' near the cell periphery [19,151-154]. KIF21A can exist in an autoinhibited conformation in which the stalk domain interacts with the motor domain or can exist in an active conformation in which two molecules dimerize and their motor domains interact with and use ATP to walk down microtubules. CFEOM1 missense variants are clustered in specific regions of the motor and stalk domain that are critical to KIF21A autoinhibition and the disease-causing variants attenuate autoinhibition *in vitro* [19,151]. Therefore, CFEOM1 results from an alteration rather than loss of KIF21A function.

Introduction of one of the most frequent human CFEOM1-*KIF21A* variants into a mouse model recapitulated the human eye phenotype (Fig. 7; [19,151,155]). Evaluation of affected embryonic mice revealed stalling of superior division axons within a bulge in the proximal oculomotor nerve; growth

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cones within the bulge were enlarged, had increased numbers of filopodia, and were randomly directed. These observations are reminiscent of the anatomy of decision region for axon turning or branching that, in this case, has formed prematurely. The SP and LPS muscles markedly attenuated innervation and appeared hypoplastic. It remains to be determined how attenuated KIF21A autoinhibition leads to this very selective error in oculomotor development.

7.2. CFEOM2: PHOX2A

CFEOM2 is an isolated autosomal recessive eye movement disorder that has been identified primarily in consanguineous pedigrees. Affected individuals have bilateral ptosis and their eyes are fixed in an exotropic position at rest with limited residual horizontal and vertical movements; magnetic resonance imaging reveals absent oculomotor nerves and, to the extent it can be detected, absent trochlear nerves [156]. CFEOM2 results from loss-of-function variants in *PHOX2A [157]*. In mice, PHOX2A is essential for oculomotor and trochlear motor neuron specification and, in its absence, oculomotor and trochlear nuclei and nerves do not form [85,112,115,157-159]. Further work detailing the complete absence or partial deletions of *PHOX2A* would be helpful.

Table 2.

CFEOM1	bilateral ptosis, infraduction, restricted eye movements	KIF21A	[19,150].
CFEOM2	bilateral ptosis, exotropia, restricted eye movements	PHOX2A	[157]
CFEOM3	variable ptosis, infraduction, restricted eye movements	TUBB3	[41]
DRS	restricted horizontal eye movements	CHN1	[69,88,160,161]

Abbreviations: CFEOM1-3, congenital fibrosis of the extraocular muscles; DRS, Duane retraction syndrome[105].

7.3. CFEOM3:TUBB3

Individuals with CFEOM3 can have a variable ocular motility phenotype with mild to severe ptosis and limited upgaze. It is a dominant disorder that results from missense variants in *TUBB3* [41] that alter amino acid residues in the tubulin beta III isotype, a building block of neuronal microtubules. Remarkably, while some *TUBB3* variants can cause isolated CFEOM, others cause CFEOM together with additional cranial nerve, spinal nerve, and central white matter maldevelopment [34,41]. These variants perturb the growth and guidance of oculomotor as well as other peripheral and central axons [41,162]. Moreover, a different set of missense variants can cause malformations of cortical development in the absence of CFEOM [49]. Additional mechanistic studies are necessary to understand the developmental etiologies of these phenotype-genotype correlations.

7.4. Duane Retraction Syndrome (DRS)

Duane retraction syndrome is a CCDD defined by limited abduction or both abduction and adduction, accompanied by globe retraction on attempted adduction. It is inherited in only the minority of cases but can result from dominant altered function variants in *CHN1* [88,160], dominant loss-of-function variants in *SALL4* [161] and *MAFB* [69], or recessive loss-of-function variants in *HOXA1* [111]. *CHN1* variants result in isolated DRS, while *MAFB* results in DRS with or without hearing loss, and *SALL4* in DRS with or without radial ray anomalies. By contrast, loss of *HOXA1* results in the most syndromic form of DRS. Mouse models of *MAFB* confirm that this form of DRS results from failure of abducens motor neuron specification, while a mouse model of *CHN1* revealed aberrant abducens axon growth and guidance. In both cases, later in embryonic development the abducens

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nerve is absent and the lateral rectus is secondarily innvervated by aberrant branches of the oculomotor nerve [69,88].

Notably, studies *in vitro* and in a *CHN1* knock-in mouse harboring a human DRS variant revealed that *CHN1* variants cause DRS through hyperactivation of the encoded alpha2-chimaerin protein. By contrast, *CHN1* and *Epha4* knock-out mice have abducens axon wandering distinct from the *CHN1* knock-in mice and do not have DRS. In addition, several guidance molecules help to fine tune abducens axon guidance, including *Epha2*, *Efna5*, various *Sema's*, *Nrp1* and *PlexinA*, among others [105]. Further work is needed to consolidate guidance across all ocular motoneurons.

8. Summary and Conclusions.

Ocular motor neurons are the basis of eye movements in vertebrates. Several additional muscles are innervated from the abducens and form the retractor bulbi in tetrapods and the basicranial muscle in *Latimeria*: the retractor may be derived from a single extraocular muscle in lamprey that was replaced by two independent muscles of gnathostomes: the medial rectus and the nasal rectus. The motor neurons form two groups that have molecularly distinct origins: A) Populations projecting via cranial nerves III and VI to originate at the midbrain-hindbrain boundary and are regulated from *Lmx1b, Wnt1, Nkx6.1, Phox2a, Olig2* and *Isl1*, which are factors controlling special somatic motor neuron development. B) A variable population is associated with r4-6 projects via cranial nerve V and is regulated by *Hoxa3/b3, Nkx6.1, Olig2* and *Isl1*, which are factors controlling the development of general somatic motor neurons (SM). The earliest development of extraocular muscles is independent of cranial nerves, which subsequently interact with the developing muscles to, yield complete differentiation. The circuitry serving the vestibulo-ocular reflex, which remains unclear in detail for lampreys and chondrichthyans, has been clarified in bony fish, frogs, and mammals. Predominantly contralateral vestibular neurons have projections via the internuclear neurons and the ascending tract of Deiters nuclei to innervate the medial rectus in mammals. Several syndromes have been linked to distinct genetic perturbations (mostly incomplete deletions) in specific groups of ocular motor neurons: the lack of one innervation target can allow other branches to sprout and cause misinnervation, leading to aberrant movements, for example the Duane retractor syndrome.

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