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REVIEW

Transcriptional regulation of guidance at the midline and in motor circuits

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Abstract Axon navigation through the developing body of an embryo is a challenging and exquisitely precise process. Axonal processes within the nervous system harbor extremely complicated internal regulatory mechanisms that enable each of them to respond to environmental cues in a unique way, so that every single neuron has an exact stereotypical localization and axonal projection pattern. Receptors and adhesion molecules expressed on axonal membranes will determine their guidance properties. Axon guidance is thought to be controlled to a large extent through transcription factor codes. These codes would be responsible for the deployment of specific guidance receptors and adhesion molecules on axonal membranes to allow them to reach their targets. Although families of transcriptional regulators as well as families of guidance molecules have been conserved across evolution, their relationships seem to have developed independently. This review focuses on the midline and the neuromuscular system in both vertebrates and *Drosophila* in which such relationships have been particularly well studied.

Keywords Axon guidance · Transcription · Motoneuron · Commissural neuron

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Introduction

The identity of a neuron is defined, among other factors, by the specific connections it establishes, its neurotransmitter phenotype, and specific surface receptor expression. In the first half of the 1990s, there was a major breakthrough in the field of axon guidance with the discovery of most of the families of guidance receptors known to us today. A common theme was the conservation of these families of receptors and ligands across evolution, highlighting the importance of their role. In fact, some guidance decision in vertebrates and invertebrates such as crossing the midline are dependent on the very same cues. Not long after these discoveries, several components of the complex regulatory network of transcription factors required for neuronal specification were also identified. Several transcription factor families and specific transcriptional codes were also shown to be responsible for the trajectory of specific axonal projections. Here again, the function of some of the families of transcription factors seemed to have preserved their function in evolution. It was then clear that there had to be a link between transcriptional codes and the expression of specific receptors and cell adhesion molecules. Work in the last few years has started to reveal an increasing number of examples where transcriptional regulators control the expression of guidance molecules in specific subpopulations of neurons.

The most thoroughly studied systems have been the midline and the neuromuscular system, because of their stereotypical projections and the relatively ease of analysis of guidance phenotypes in these systems. Clearly, nervous systems of vertebrates and invertebrates are very different although some structures may share the same evolutionary origin. A common theme from recent studies is that, while both systems are present in vertebrates and invertebrates

and guidance molecules and transcriptional regulators are shared, transcriptional networks differ among different organisms. In this review, we summarize the different transcriptional codes regulating guidance receptors that have been identified at the midline and the neuromuscular system of vertebrates and insects.

The midline

In organisms with bilateral symmetry, the midline is the symmetry axis that divides the developing organisms into a right and a left side and coincides with the floor plate (FP) in vertebrates. At early developmental stages, axons are presented with the choice of whether to cross the midline (contralateral axons) or project towards their targets on the same side, hence no crossing (ipsilateral axons). Ipsilateral axons avoid the midline by interpreting repellent signals from the FP. Contralateral axons sense attractive cues from the midline and are lured towards it. As they cross the midline, they form commissural bundles connecting both sides of the central nervous system (CNS). At the midline, commissural axons start to respond to repellents to crossing to the contralateral side and avoid recrossing. The differential response of commissural axons before, while, and after crossing the midline depends on the combination of receptors they express on their growth cones [1, 2]. Transcriptional regulation of the expression of such receptors is, to a large extent, a decisive factor in specifying the responsiveness of axons at the midline.

To cross or not to cross the midline in the vertebrate spinal cord

In the vertebrate spinal cord, commissural axons sense attractive guidance cues from the FP and move towards the midline. Attractants secreted from the FP are Netrin-1 [3], Sonic Hedgehog (Shh) [4], and VEGF [5]. Before crossing, commissural axons are not sensitive to repulsive cues secreted from the FP, but as they cross the midline they become sensitive to repellents from the Slit [6, 7] and Semaphorin [8–10] families. Commissural axons also switch their response to Shh upon crossing the midline from attraction along the dorsoventral axis to repulsion along the anterioposterior axis [11, 12]. The floor plate is, therefore, the source of a variety of signaling molecules including morphogens, attractants, and repellents whose activities are translated through membrane receptors present in the navigating axons. Attraction by Netrin and Shh is mediated through their respective receptors, DCC and Boc [13, 14]. Slits and Sema-3B repulsion is mediated by their respective receptors, Robos and PlexinA1 with its Neuropilin2 coreceptor [6, 7, 10, 15]. Three Robo receptors are expressed in vertebrate spinal cord Robo1, Robo2, and Robo3/Rig1 [16, 17]. Alternative splicing of Robo3/Rig1 pre-mRNA results in the formation of two isoforms, Robo3.1 and Robo3.2, with differential expression [9]. Robo3.1 is expressed in pre-crossing axons while Robo3.2 is expressed in post-crossing axons. Further investigations revealed that Robo3.1 inhibits Slit-Robo repulsion in precrossing commissural axons through an unknown mechanism [9, 18]. Repulsion away from the FP mediated by Plexin-A1 and its Neuropilin2 coreceptor is also prevented in precrossing commissural axons through proteolysis of Plexin-A1 [10].

Regulation of floor plate signals

The Netrin/UNC-6 family of secreted molecules constitutes a group of well-studied signaling molecules expressed in the ventral neural tube of vertebrates and invertebrates. In vertebrates, Netrin-1 attracts commissural axons navigating along the DV axis towards the FP [3, 19–21]. Early studies of *netrin* expression in zebrafish revealed that hedgehog signaling is required for *netrin-1* expression in the neural tube [22]. Ectopic expression of any of the hedgehogs present in the FP, *sonic hedgehog*, *tiggy-winkle hedgehog*, or *echidna hedgehog*, is sufficient to induce *netrin-1*, although in a patchy and non-uniform pattern, in the brain and spinal cord [22, 23]. Hedgehog signals are transduced in target cells through down-regulation of protein-kinase A (PKA) activity [24]. Similar to *shh*-injected embryos, over-expression of a dominant negative regulatory subunit of mouse PKA in zebrafish embryos is sufficient to induce ectopic expression of Netrin-1 in the brain of injected embryos [23]. Interestingly, *shh* misexpression causes ectopic expression of the winged-helix transcription factor Foxa2 [23], and *foxa2* can induce expression of *netrin-1*, although other *shh*-independent pathways also induce *netrin-1* expression via *foxa2* in the FP [25]. Regulation of *netrin-1* by *foxa2* seems to be evolutionarily conserved, since a zebrafish *netrin-1* enhancer responsive to *foxa2* also drives expression of a reporter in the FP of mouse and chicken [25]. The other FP attractant, Shh, is secreted from the notochord and induces *foxa2* in the FP, which in turn induces the expression of Shh in the FP [26]. This regulation is probably mediated through a direct interaction of Foxa2 with regulatory elements within the S*hh* locus [26]. Thus, *foxa2* plays a central role in the regulation of secreted guidance cues from the FP, a role that it also plays at the mouse midbrain FP where it directly regulates *Shh* and *Slit2* [27] (Table 1).

Regulation of guidance receptors in commissural neurons

In the developing mouse, dorsal spinal cord post-mitotic neurons emerge from the ventricular zone and migrate in

Table 1 Guidance molecules and their transcriptional regulators

	Guidance molecule	Place of action	Transcription factor	References
Vertebrate	Netrin-1	Floor plate	Foxa2	[25]
	Sonic Hedgehog	Floor plate	Foxa2	[26]
	Slit2	Floor plate	Foxa2	[27]
	Robo3/Rig1	dl1 commisural neurons	Lhx9 and Lhx2	[38]
	EphB1	Ipsilateral retinal ganglion cells	Zic2	[65, 66]
	EphB1	LMC(m)	Isl1	[76]
	EphA4	LMC(l)	Lim1	[124]
	Ephrin-B2	Dorsal limb	Lmx1b	[124]
	Ephrin-A5	Dorsal limb	Lmx1b	[76]
	FGF receptor 1	MMC neurons	Lhx3	[134]
	Robo2	SACM	Nkx2.9	[137]
Drosophila	Slit	Lateral neurons	Midline	[58]
		Midline glia	Lola	[59]
	Frazzled	Commisural neurons	Engrailed	[56]
		CNS	Midline	[58]
	Roundabout	CNS	Midline	[58]
		CNS	Lola	[59]
	Fasciclin III	Ventral motoneurons	Nkx6 (lim3/Isl)	[81].
	Unc-5	Dorsal motoneurons	Even-skipped	[91]
		Dorsal motoneurons	Grain	[93]
	Toll	Muscle M12	Tey	[97]

a very precise manner. Early-born neurons (prior to E11.5) migrate either ventrally or ventrolaterally [28, 29]. Early-born dorsal interneurons are classified into six groups (dl1–6) and each group can be identified by their unique transcription factor expression profile [30–33]. Their fate is specified by cross-repressive activity between Lim-homeodomain (LIM-HD) proteins [34, 35] and bHLH transcription factors. The HLH transcription factor *Math1* (mouse atonal homolog 1) is necessary and sufficient for the generation of dl1. Whereas ectopic expression of *Math1* leads to an increase in the number of dl1 cells, these cells together with a subset of commissural neurons are lost in *Math1* knock-out mice [36, 37]. The dl1 neurons comprise two major classes with distinct axonal trajectories: dl1i (ipsilateral) extend their axons in the ipsilateral funiculus, and dl1c (contralateral) project towards the FP, cross the midline, and extend rostrally in the contralateral funiculus [38]. Each subgroup of dl1 neurons can be clearly identified after their migratory phase by their differential expression of transcription factors from the LIM family. Ipsilateral dl1i exclusively express *Lhx9* and commissural dl1c express both *Lhx9* and *Lhx2* although *Lhx9* at low levels [38]. *Math1* also regulates two bar-class homeobox genes *Mbh1* (mammalian bar homeobox 1 gene) and *Mbh2* in dl1 interneurons [39, 40], and *Mbh1* is known to be a direct target of *Math1* [39]. Misexpression of either *Mbh1* or *Mbh2* by electroporation of E11.5 mouse embryo spinal cord induces dl1 fate, expression of both *Lhx2* and *Lhx9*, and makes some of the misexpressing neurons project their axons ventrally towards the floor plate, becoming commissural [33, 38, 40]. Hence, a linear cascade of transcriptional regulators *Math1- > Mbh1*, *Mbh2- > Lhx2*, *Lhx9* seems to be regulating the guidance behavior of commissural dl1 neurons. Electroporation of *Mbh1* or *Mbh2* in mouse embryonic spinal cords also induces the expression of *DCC*, *Nrp2*, *Tag1*, and *Rig1* [33, 40]. However, electroporation of *Lhx2* only induces expression of *Rig1* and *Lhx9* fails to induce expression of any of those guidance receptors [40]. Nevertheless, both *Lhx2* and *Lhx9* work together to mediate commissural guidance of dl1c axons, since mice lacking either *Lhx2* or *Lhx9* show no obvious commissural defects, while in double mutants, commissural axons fail to cross the midline and project ipsilaterally. Further analyses revealed that axons of the double mutant animals lack *Robo3/Rig1*. Interestingly, Wilson et al. [38] showed that the *Robo3/Rig1* regulatory region has consensus Lhx2 and Lhx9 binding sites and is subject to transcriptional control, probably through direct binding of these two factors in dl1 commissural neurons. However, dl1c neurons in these *Lhx2/Lhx9* double mutants still express DCC, suggesting a parallel pathway downstream of *Mbh1* or *Mbh2* to regulate other guidance receptors [38] (Fig. 1). Ipsilaterally projecting interneurons need to repress *Robo3/Rig1* to prevent midline crossing. This process is controlled through the action of the PAS domain transcription factors Sim1, Sim2, and Arnt2 in different populations of neurons in the brain

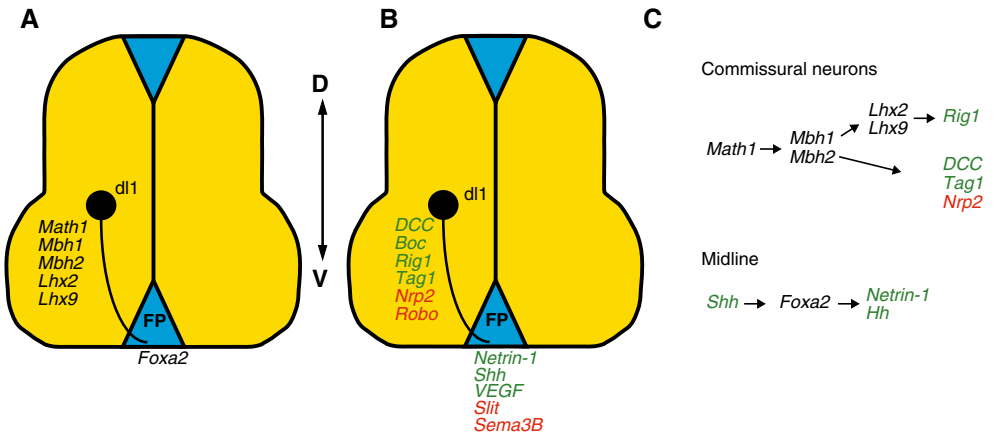

Fig. 1 Transcriptional regulation of guidance at the vertebrate spinal cord. Schematic representation of the spinal cord and commissural neurons (*dl1*) crossing to the contralateral side through the floor plate (*FP*). Transcription factors present in commissural neurons or in the floor plate (**a**) or guidance molecules (**b**) are represented. Guidance receptors present on neurons or ligands at the floor plate are color coded in *green* if they mediate attraction or in *red* if they mediate repulsion. The relationships between transcriptional regulators and receptors in commissural neurons or ligands in the floor plate are also presented (**c**)

[41, 42] although it is not clear if the same transcriptional mechanism plays a role in spinal interneurons.

Guidance at the *Drosophila* midline

In the *Drosophila* ventral nerve cord (VNC), the segmentally repeated arrangement of axonal projections is defined by two commissures present on each segment commissures, an anterior (AC) and a posterior commissure (PC). Similar to vertebrates, in the fly VNC, neuronal projections organize into ipsilateral and contralateral projections and some of the guidance cues and receptors are evolutionarily conserved. *frazzled* (*fra*) [43], the *Drosophila DCC* homologue, is expressed by commissural axons and interprets the Netrins (NetA and NetB) [44, 45], secreted by midline glial cells, as attractive cues. The phenotype of Frazzled mutants strongly mimics that of Netrin mutants and is manifested mainly as lack of or defective PCs [43]. The 3 Robo receptors present in *Drosophila* [46–48] are able to bind midline secreted Slit [6]. Of the three receptors, Robo1 seems to be the major determinant that keeps axons away from the midline while Robo2 can also play a positive role in commissure formation that may be similar to Rig1 [49, 50]. In addition, there is a mechanism in *Drosophila* to neutralize Slit-Robo1 repulsion not found in vertebrates, commissureless (Comm) [51], an endosomal receptor that targets Robo1 for degradation in precrossing commissural axons allowing them to cross [52, 53]. *Comm* expression seems to be transcriptionally regulated by *fra* in a Netrin-independent way [54].

In a study using chromatin immunoprecipitation (ChIP) aimed at recognizing direct targets of the homeodomain (HD) transcription factor Engrailed (En), *fra* was identified as one of them [55]. In *en* mutants, PCs are thinner and *fra* mRNA expression is reduced [56]. Immunostaining of Frazzled in wild-type embryos reveals a uniform expression in both ACs and PCs, but it appears much lower in PCs of *en* mutant embryos. This confirms a correlation between lack of Frazzled expression in PCs and thinner-PCs phenotype observed in engrailed mutants, and also indicates a role for this transcription factor in the formation of PCs through transcriptional regulation of Frazzled [56]. Another segmentation gene, *gooseberry-Neuro* (*gsbN*), works together with *en* in the formation of PCs [57] and might be a candidate to regulate *fra* together with *en*. Thinning of commissures and interrupted longitudinal axons is observed in *midline* (*mid*) mutants [58]. Mid is a T-box transcription factor, a *Drosophila* homolog of *Tbx20*, expressed in the CNS, and its mutant phenotype mimics a combination of *fra* and *robo* mutant phenotypes [58]. *mid* mutants show a significant reduction of *fra*, *robo*, and *Slit* mRNA and protein expression in the CNS of that is restored by pan-neural expression of a *mid* transgene. This regulation is likely to be direct since mutations of Mid binding sites in their regulatory regions abolish the expression of a reporter construct. Furthermore, ChIP with an anti-Mid antibody resulted in an enrichment of their regulatory regions which contain Mid consensus binding sites [58]. Regulation of *Slit*, however, is not in the midline glial cells but in some lateral neurons [58] (Fig. 2). In addition to Mid, Lola, another transcription factor containing BTB/POZ-like Zinc finger domains, also regulates the expression of *robo* and *Slit* [59]. In *lola* mutants, axons exhibit multiple aberrant midline crossings; however, only follower axons seem to be affected. Slit protein expression in the midline glia neurons is reduced to nearly 50 % in

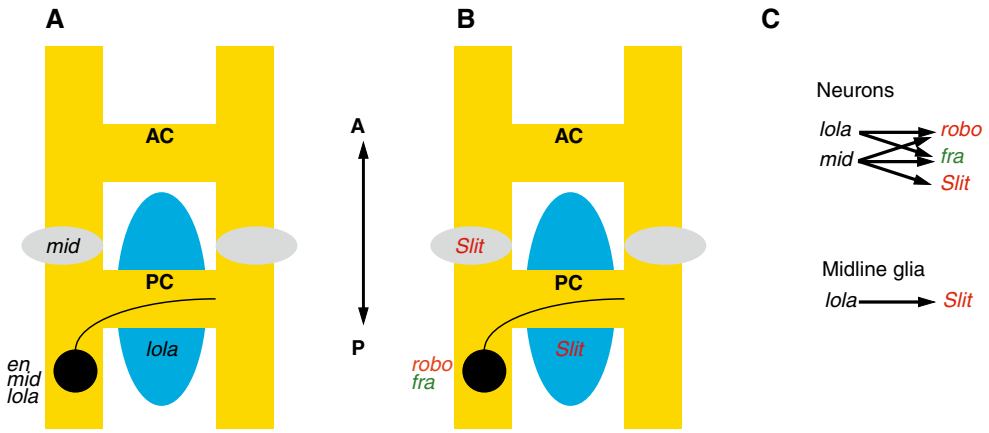

Fig. 2 Transcriptional regulation of guidance at the *Drosophila* midline. Schematic representation of the Drosophila midline and a commissural neuron crossing to the contralateral side through the midline Transcription factors present in commissural neurons or in the midline glia (**a**) or guidance molecules (**b**) are represented. Guidance receptors present on neurons or ligands at the midline glia are color coded in *green* if they mediate attraction or in *red* if they mediate repulsion. The relationships between transcriptional regulators and receptors or ligands are also presented (**c**). *AC* anterior commissure, *PC* posterior commissure

lola mutants, and misexpression of *lola* in the developing midline of *Drosophila* led to ectopic expression of *Slit* at both mRNA and protein levels. Similarly, immunostaining of Robo also showed a decrease compared to wild-type animals. In addition, Robo is relocalized to commissural axon tracts in *lola* mutants from which it would normally be excluded [59] (Fig. 2).

Transcriptional regulation of midline crossing at the optic chiasm

Proper development of binocular vision is dependent on the correct routing of retinal ganglion cells (RGCs) axons at the optic chiasm to the appropriate hemisphere. During development, RGC axons make the critical decision of whether or not to cross at a midline point (optic chiasm) in the ventral diencephalon, which establishes the foundation of binocular vision and spatial positioning perception. The degree of binocularity in each species depends on the percentage of RGCs axons remaining ipsilateral. Positioning of the RGCs cell bodies in the retina is an important factor in making the decision at the midline as RGCs in the ventrotemporal retina project their axons ipsilaterally [60–62].

Several guidance cues play a role in optic chiasm formation, in particular ephrins from the B class [63]. The EphB1 receptor expressed by RGC axons mediates repulsion away from the ephrinB2 present in the radial glia at the chiasm [64]. Mice lacking *EphB1* show higher numbers of crossing axons [64], and forced expression of *EphB1* in dorsal RGCs causes more axons to project ipsilaterally [65–67], indicating the importance of this receptor for proper routing of ipsilateral RGCs. Zic2, a zinc finger transcription homolog of the *Drosophila* gene *odd-paired*, is expressed in RGCs from the ventrotemporal retina that will remain ipsilateral [68]. Interestingly, this transcription factor expression in the retina correlates with the degree of binocularity in different species [68]. Compelling gain of function and loss of function experiments show that this factor is not only necessary but also sufficient to prevent RGCs axons from crossing to the contralateral side at the chiasm through the regulation of EphB1 in RGCs [65, 66, 68].

The LIM family member Islet 2 (Isl2) is exclusively expressed in RGCs whose axons will project to the contralateral side [69]. *Isl2*-deficient mice present a higher number of RGCs co-expressing Zic2 and EphB1 and an increase in the number of ipsilateral axons [69]. Therefore, *Isl2* is likely to act upstream of *Zic2* and inhibit its expression in projecting contralaterally. The membrane guidance molecules that *Isl2* regulates in those RGCs are currently unknown, but *Nr-CAM* and *Plexin-A1* are some of the candidates since they are required to promote axonal growth in response to glial-expressed *Sema6D* at the chiasm [70]. Other regulators and putative guidance receptors are definitely important for guidance at the chiasm [71]; however, our description here is limited to examples where a clear connection between them has been established (Fig. 3).

Transcriptional regulation of guidance in motor neurons

During development, motor axons are instructed to navigate to their target muscles by simultaneously integrating multiple extracellular signals along the pathway; migrating axons continuously adapt their response by modulating the expression of guidance receptors and their intracellular

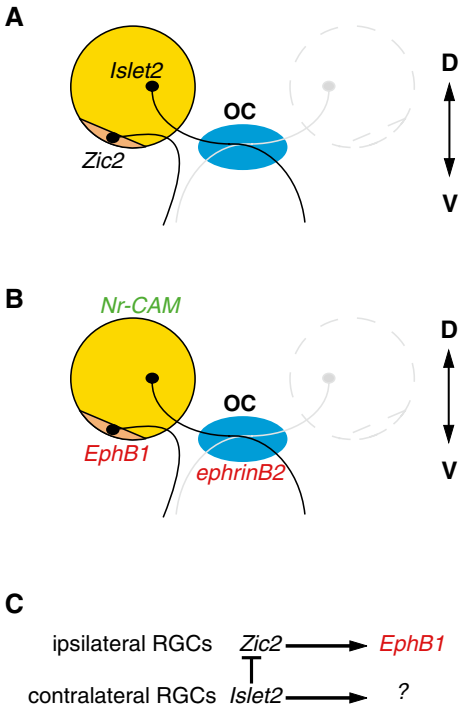

Fig. 3 Transcriptional regulation of guidance at the optic chiasm. Some axons of retinal ganglion cells from the ventral retina will remain ipsilateral wile axons crossing to the contralateral side through the optic chiasm (*OC*) originate in a dorsal domain. The ratio of ipsilateral versus contralateral axons varies depending of the extent of binocular vision of each organism. The transcription factors *Zic2* and *Islet2* are present in ipsilateral and commissural neurons, respectively (**a**). Guidance receptors or ligands are also represented (**b**). Guidance receptors present on neurons or ligands at the optic chiasm are color coded in *green* if they mediate attraction or in *red* if they mediate repulsion. The relationships between transcriptional regulators and receptors in retinal ganglion cells are also presented (**c**). *Arrows* represent a positive regulation and *bars* a negative regulation

signaling cascades. The subset of transcription factors expressed in different motor neurons is crucial not only for neural identity but also for axonal innervation at the appropriate muscles [72–75]. Some remarkable similarities in motoneuron specification and guidance have been found between vertebrates and invertebrates, although particularities from each system seem to prevail.

Specification of MN identity and axonal projections in *Drosophila*

The segmented VNC of *Drosophila* is composed of two identical left and right sides known as hemisegments (half segment), each of which contains 36 motor neurons (MNs) innervating 30 different body wall muscles. Based on the route that they choose, the somatic MNs assemble into three main nerve branches: the intersegmental nerve (ISN), the segmental nerve (SN), and a minor branch, the transverse nerve (TN). This division into ISN and SN/TN motor neuron–muscle units in *Drosophila* is somewhat comparable to the musculature of vertebrate limbs where dorsal and ventral muscle groups are innervated by motor neurons projecting through divergent routes [76, 77].

Drosophila MNs that project axons though common trajectory pathways, share a similar set of transcriptional regulators, and unique combinatorial codes are responsible for expression of different set of cell surface receptors in each distinct MN subclass [78]. In *Drosophila*, Zfh1, a zinc finger homeobox protein, is expressed postmitotically by all MNs, and in *zfh1* mutants, motor axon guidance in both ISN and SN is severely impaired [79]. On the other hand, ectopic expression of *zfh1* in some subsets of commissural interneurons leads to lateral projection of their axons out of the CNS. The authors propose *unc-5* as one of the genes that may be regulated by *zfh-1* [79].

Ventral motoneurons

A concerted action of *dHB9* and *Nkx6* is required for specification of ventral MNs (vMNs) [80–82], partly through the expression of the LIM family members *lim3* and *islet* within those neurons [81]. Dorsal motor neuron (dMNs) identity is defined by yet another homeobox gene called *even-skipped* (*eve*) [83–85]. Interestingly, there are cross-repressive interactions between dorsal and ventral fate determinants, so that parallel collaboration of *Nkx6* and *dHB9* restricts *eve* expression to dorsal MNs and pan-neural misexpression of *eve* represses *dHB9* and *Nkx6* [80–82]. Nkx6, dHb9, and Eve contain structural domains that, in vertebrate homeodomain proteins, interact with the Groucho co-repressor, suggesting that they function as transcriptional repressors [82, 83, 86, 87]. Two LIM-HD proteins, Islet (Isl) and Lim3, are involved in specification as well as axon guidance of groups of vMNs [88]. Deciphering the target genes regulated by these transcriptions factors is a prerequisite to better understand how these combinatorial codes specify neural identity and axonal projections, but good candidates are Beat-Ia, Fra [43, 89], and other receptors required for vMN guidance. Among adhesion molecules, the neural cell adhesion molecule Fasciclin III (*FasIII*) has been shown to be regulated by *nkx6*; however, it is still unknown whether this regulation is direct or through Lim3 and Isl [81].

Dorsal motoneurons

Even-skipped (Eve) and Grain (Grn), a HD and a GATA family transcription factor, respectively, are specifically expressed in the most dorsally projecting dMNs (ISN^D) [83, 84, 90]. In dMNs *eve* alone is necessary and sufficient for many aspects of dMN specification including guidance

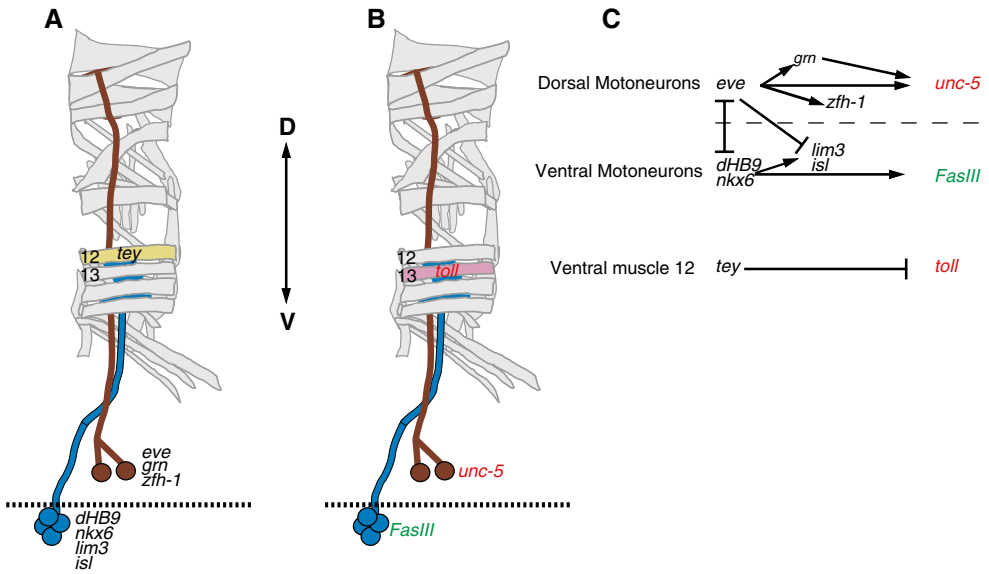

Fig. 4 Transcriptional regulation of guidance at the *Drosophila* neuromuscular system. Motoneurons project to dorsal muscles (in *green*) or ventral muscles (in *blue*) as well as the body wall musculature are schematically represented with the transcription factors (**a**) or guidance receptors and cues (**b**) expressed on motoneurons or muscles. Guidance receptors and cues are color coded in *green* if they mediate attraction or in *red* if they mediate repulsion. The relationships between transcriptional regulators and receptors in dorsal and ventral motoneurons as well as muscle 12 are also presented (**c**). *Arrows* represent a positive regulation and *bars* a negative regulation

[83–85]. In *eve* mutants, dMNs fail to exit the CNS and reach their dorsal muscle targets, and *eve* misexpression induces vMNs to project dorsally, indicating that it is likely to regulate the guidance receptors present in dMNs. The mechanism employed by *eve* to control dMNs projections is not yet clear, since it has been shown that it behaves as a repressor in dMNs [83]. Nevertheless, it also works genetically upstream of *zfh-1* and *grn* promoting their expression [90]. These later transcriptional regulators may in turn directly regulate different guidance receptors. In fact, Unc-5, a repulsive receptor for Netrin, is also expressed in two dMNs and is required for proper guidance of their motor axons [91, 92]. Arzan Zarin et al. [93] found that *eve* and *grn* independently induce *unc-5* transcription in dMNs. Whether *eve* regulates *unc-5* through a direct binding to its regulatory region or through *zfh-1* is still unknown (Fig. 4).

Transcriptional regulation of guidance cues in *Drosophila* muscles

Accurate axon guidance of motor neurons is achieved by the selective responsiveness to environmental cues in parallel regulation of such cues in the environment in which axons. Target muscle selection is achieved through attraction to the target cells and repulsion from non-target cells [76, 94–96]. An invertebrate example of such regulation is the transcriptional repression of a repulsive cue in a group of *Drosophila* muscles [97]. Tey is a putative DNA binding transcription factor, the expression of which is confined to a single muscle, M12, among the 30 muscles in the body wall. Tey is involved in negative regulation of transcription and inhibits the expression of its target genes (i.e. *toll*) [97]. Toll is a transmembrane receptor of the leucin-rich repeat family specifically expressed on muscles, and acts as a repulsive cue in the development of *Drosophila* neuromuscular junctions [98]. Toll is differentially expressed in M13 and some other surrounding muscles but not in the neighboring M12; by acting as a repellant, it locally prevents the innervation of M12-specific motor neuron axons onto M13. The inhibitory function of Toll is negatively suppressed in M12 by the transcription factor Tey, which works as a transcriptional repressor. In *tey* mutant animals, *toll* is ectopically induced in M12 and synapse formation on M12 is impaired [97]. On the other hand, ectopic *tey* in M13 prevents *toll* expression in the muscle, and M13 is innervated by ectopic motor axons. This is an excellent example of how a target cell can be specified via repression of an inhibitory signal in just one among a group of target cells expressing the signal (Fig. 4).

Identity of *Drosophila* body wall muscles is determined by several transcription factors that are differentially expressed in subsets of muscle and/or their progenitor cells [99]. Furthermore, various target recognition molecules, including homophilic cell adhesion molecules, secreted factors, and heterophilic ligands or receptors are expressed in specific muscles [100] making them very good targets for the transcription factor identified.

Establishment of generic motor neuron identity in vertebrates

In vertebrates, motor neurons and interneurons in the ventral spinal cord are created in response to graded extrinsic signals of sonic hedgehog and retinoic acid, which act along the dorsoventral axis of the neural tube. The bHLH transcription factors Olig2, Ngn1, and Ngn2 and the homeodomain factors Pax6, Nkx6.1, and Nkx6.2 are expressed in the progenitor domain that gives rise to motor neurons [101–103]. After mitosis, motor neurons express a set of homeodomain transcription factors (Hb9, Lhx3, Lhx4, Isl1, and Isl2) that are responsible for common features of all spinal motor neurons and are also involved in later aspects of motor neuron subtype specification [104–108]. In order to adjust with differences in peripheral targets throughout the body, MN number, identity, and axon trajectory vary drastically along the rostrocaudal axis of the spinal cord. Based on anatomic positioning, MN cell bodies are organized into different column classes.

Phrenic motor column

Phrenic motor columns (PMC) are located in rostrocervical segments of the spinal cord and innervate diaphragm [109]. They can be distinguished by selective expression of Hoxa5 and Hoxc5, and the exclusion of other Hox factors as well as their accessory factor FoxP1. Continuous Hox5 (Hoxa5 and Hoxc5) function is needed for different aspects of PMC neurons, including motor neuron migration, clustering, axon projection towards the diaphragm, and branching [109]. The netrin receptor Unc5c is a likely target of the Hoxa5 and/or Hoxc5 factors, as this receptor is required for normal projection of phrenic motor neurons, and phrenic axons fail to reach the diaphragm in mice homozygous for null mutation in *Unc5c* [110]. Furthermore, the cell adhesion molecule ALCAM, known to regulate the guidance and fasciculation of motor and retinal axons [111] and the NgR ligand, NogoA, is also implicated in the visual cortex plasticity [112] are downregulated in *Hoxa5*, *Hoxc5* double mutants and could also be directly regulated by them [109].

Lateral motor columns

Lateral motor columns (LMCs) are generated only at brachial and lumbar limb levels of the spinal cord and innervate limb muscles. Cross-repressive interactions between Hox6, Hox9, and Hox10 proteins are required for the refinement of Hox expression profiles along the rostrocaudal axis, whereas their activator functions determine their identities as well as their peripheral target connectivity [113–115]. Within LMCs, MNs innervating a dedicated target muscles in the limb are clustered into around 50 distinct pools of MNs whose identities are also controlled by Hox proteins and cofactors [114]. Interestingly, in animals lacking Hox cofactor, Foxp1, motor neurons lack the expression of molecular markers of LMC divisional and pool identities, including LMC transcription factors (e.g., Lhx1, Pea3, Nkx6.1) as well as axon guidance and synaptic specificity molecules (EphA4, Sema3E, Cad20) [116].

Combinatorial codes of LIM proteins specify MN diversity enabling different classes of MNs to choose their appropriate projection routes in vertebrates [108, 117, 118]. LIM codes confer MNs with such ability apparently by controlling the genes involved in responsiveness to either attractive or repulsive signals from midway environment or final target muscles. In the chick and rodent spinal cord, LMCs can be further split into medial, LMC(m), and lateral, LMC(l), divisions projecting to the ventral and the dorsal part of the limb, respectively [119–121]. LMC(m) neurons express Lim proteins Isl1 and Isl2 whereas LMC(l) neurons express Isl2 and Lim1 [122]. Columnar Hox (*Hox6/10*) partition the LMC into medial and lateral divisions inducing *Isl1* in LMC(m) and *Lim1* in LMC(l) [75, 123]. Most of the LIM-proteins downstream targets remain unknown; however, members of the Eph-receptor family and their membrane-bound ligands, the ephrins, are known to be regulated by Lim proteins [76, 124]. *Isl1* induces the expression of *EphB1* in LMC(m) and *Lim1* promotes *EphA4* expression in LMC(l) (Fig. 5).

Along with forward signaling, ephrin-dependent reverse signaling is important for LMC axon guidance. In contrast to EphA- and EphB-mediated forward signaling which leads to repulsive response in LMC axons, ephrin-A and ephrin-B reverse signaling, which exists in lateral and medial LMC neurons, respectively, results in motor axon attraction [125]. For example, EphA4 and EphA7 are expressed by dorsal limb mesenchyme where they act as "ligands" for ephrin-As present in LMC(l) axons, leading to attraction and growth of LMC(l) to dorsal limb muscles via reverse signaling [125–127].

Limb-derived growth factors such as glial cell line-derived neurotrophic factor (GDNF) are also pivotal in motor axon navigation. In addition to participating in ephrin-A reverse signaling, Ret mediates GPI-anchored GFRα1 signaling in response to GDNF. GDNF binds to a receptor complex composed of GPI-anchored GFRα1 receptor (Gfrα1) and Ret [128]. GDNF is expressed at the dorsoventral trajectory choice point within the hind limb, whereas Ret and GFRα1 are expressed by limb-innervating motor neurons [129]. Previous studies have reported that motor axons expressing Ret and GFRα1 are attracted to gradients of GDNF [130], and that *Gdnf*, *Gfrα1*, and *Ret* mutants are defective in peroneal nerve projection [129, 131]. There is also synergistic interaction between GDNF

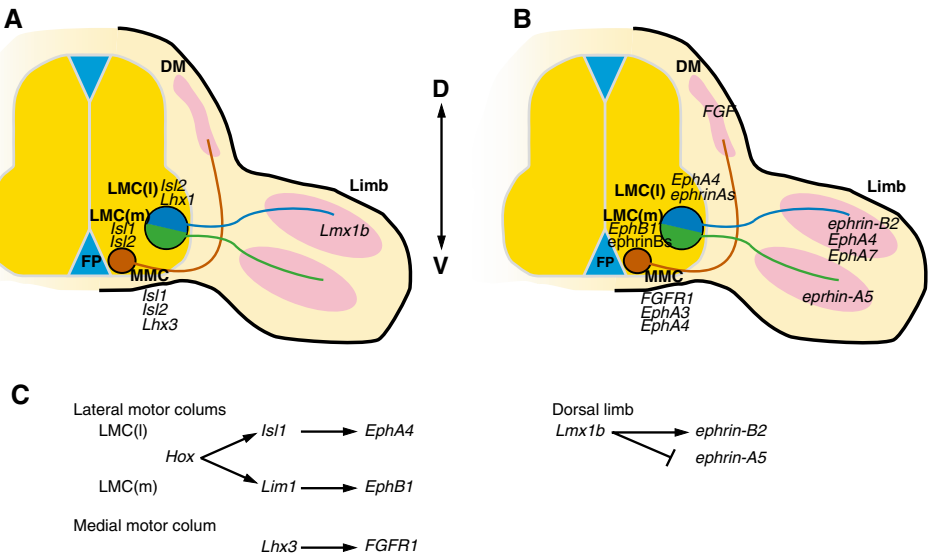

Fig. 5 Transcriptional regulation of motor guidance in vertebrates. Schematic representation of the spinal cord and different types of motoneurons arranged in different motor columns: lateral motor column lateral and medial subset (LMC(l) or LMC(m), respectively and medial motor column (MMC). LMC(l) and LMC(m) neurons project their axons towards the dorsal and ventral side of the limb mesenchyme respectively. MMC neurons project their axons towards the dermomyotome (DM). Transcription factors (**a**) or guidance receptors and cues (**b**) expressed on motoneurons limb mesenchyme or dermomyotome are presented. The relationships between transcriptional regulators and receptors in dorsal and ventral motoneurons as well as muscle 12 are also indicated (**c**). *Arrows* represent a positive regulation and *bars* represent negative regulation

and EphA signals where GDNF might potentiate ephrin-A reverse signaling [126, 127].

Genetic experiments in mouse and chick also demonstrate the contribution of repulsive Sema3/Neuropilin signaling in LMC axon navigation. Some medial LMC axons expressing Npn-2 receptor are repelled from Sema3F ligands in the dorsal limb leading to their ventral diversion [132]. In addition, interactions between Sema3A and its receptor Npn-1 expressed in all brachial LMC neurons control the timing of motor axon limb innervation as well as the extent of fasciculation of both lateral and medial LMCs [132, 133]. Sema3/Neuropilin may act in parallel or perhaps synergistically via direct interactions with ephrin/Eph signaling components to increase the robustness of dorsoventral navigation at the base of the limb, and they may very well also be regulated by the same LIM-HD codes. LIM-HD factors also play a role in the regionalization of the limb mesenchyme. *Lmx1b* is expressed in the dorsal mesenchyme and its elimination leads to a ventralization of the limb [122]. *Lmx1b* induces *ephrin-B2* and represses *ephrin-A5* in the dorsal limb, leading to expression of *ephrin-B2* in the dorsal limb and *ephrin-A5* in the ventral limb. As a result, EphA4-expressing LMC(l) neurons are guided to the dorsal limb due to repulsion from ephrin-A5, and EphB1-expressing LMC(m) neurons are repelled from ephrin-B2 projecting to the ventral limb [76, 124] (Fig. 5).

Medial motor column

In contrast to segmentally restricted columns described above, motor neurons innervating axial muscles are located in the medial motor column (MMC), that span all segments of the spinal cord, and Lim proteins also play a pivotal role in their guidance [118]. The LIM factor Lhx3 selectively triggers expression of FGF receptor 1 (FGFR1) in MMC neurons making their motor axons attractive to fibroblast growth factors (FGFs). FGF is expressed in dermomyotome acting as a secreted long-range chemoattractant. The dermomyotome is a temporary structure that subsequently becomes axial musculature, the target for MMC neurons. Interestingly, forced expression of *Lhx3* in LMC MNs reprograms their identity to MMC cells and induces *FGFR1* expression [134]. This reprograming of LMC into MMC motor neurons is associated with increased projections to towards the FGF-expressing dermomyotome [134]. MMC motor neurons also express EphA3 and EphA4 receptors and are repelled by ephrin-As expressed by sensory neurons of the dorsal root ganglion (DRG), highlighting the crucial role of heterotypic trans-axonal signaling and interaction between sensory and motor axons in proper assembly of sensory-motor circuits. Likewise, ephrin-As are expressed in ventral mesenchyme, preventing MMC axons from innervating limb muscles [135]. In double mutants for *EphA3* and *EphA4*, MMC axons aberrantly

project into the DRG, a phenotype also observed in *FGFR1* mutants [134, 135]. It is likely that *EphA3* and *EphA4* will be regulated in MMC neurons in the same way as *FGFR1*, but their regulation by *Lhx3* has not yet been established (Fig. 5).

Spinal accessory motoneurons

Spinal accessory motoneurons (SACM) are a population of neurons located at the cervical level in the spinal cord. They project their axons towards a dorsal exit point and assemble into a spinal accessory nerve (SAN) that projects anteriorly and innervates neck and back muscles. The HD transcription factor Nkx2.9 is required for SACM to exit the spinal cord [136]. In *Nkx2.9* mutants, SAN are truncated and project ectopically within the spinal cord. Among the guidance receptors expressed in SACM is the Robo2 receptor, which is downregulated in *Nkx2.9* mutants [137]. Additionally, *Robo2* mutants present SACM exit phenotypes that phenocopy those of *Nkx2.9* mutants, strongly supporting the role of *Nkx2.9* in the regulation of Robo2 [137]. The Slit ligand is expressed in the SCAM exit point and *Slit* mutants present similar phenotypes, indicating that Slit/Robo signaling is essential in this process [137].

Conclusions

Axon guidance is a process that is to a large extent transcriptionally regulated. Transcription factors control axonal projections by regulating the expression of cell surface molecules involved in axon guidance. Despite all the effort devoted to understanding the transcriptional programs that govern axon guidance, our knowledge is still relatively limited. In most cases, transcriptional codes have been assigned to specific projections and have been correlated with the expression of individual guidance molecules by genetic means. Nevertheless, there is no example available in which a particular transcription factor has been shown to regulate the expression of a guidance receptor in vivo through a direct binding to its regulatory region, although several studies provide suggestive evidence for such processes [38, 138, 139].

The relatively limited availability of transcriptional regulators encoded in the genome raises the question of how they can account for the various axonal pathfinding decisions in each neuron. Some evolutionarily conserved mechanisms are starting to emerge. There is a hierarchical organization of different transcriptional cascades directing axon guidance. For example, homeodomain transcription factors such as Nkx6 or Even-skipped play an early role in guidance during neuronal specification through repression [86]. These transcriptional programs tightly linked with neuronal specification are also responsible for the regulation of an array of guidance receptors and adhesion molecules in specific neurons. They may play a direct role preventing their expression or through the regulation of other transcriptional regulators. At a later stage, and in postmitotic neurons, distinct codes of transcription factors including LIM-HD proteins define the axon-outgrowth pathways for different neuronal subtypes. These codes confer neurons with such ability by controlling the genes involved in responsiveness to either attractive or repulsive signals through a direct regulation. While epigenetic regulation has not been considered in this review, early events of epigenetic control will definitely determine different patterns of receptor expression regulating the accessibility to their regulatory sequences and increasing variability among different neuronal populations. Transcriptional regulators with broad expression, not exclusive to the nervous system, may also mediate cell-specific regulation through the same mechanism. Thus, regulation of the expression of the particular footprint of guidance receptors that determines the path-finding properties of a neuron likely starts before the neuron is specified and is not solely dependent on specific transcriptional codes.

It is obvious that axons on their path respond to multiple cues. Related neurons whose axons assemble into nerves will express a very similar footprint of receptors and adhesion molecules on their membranes and many of them will be common. Nevertheless, while specific transcriptional codes have been identified for different nerve branches, in particular motoneurons, receptor codes have not been or they have not been linked to the particular transcriptional code. It is possible that, in a similar way as selector genes co-regulate batteries of genes involved in common neuronal processes [140, 141], individual transcriptional codes co-regulate particular guidance receptor footprints. Novel approaches such as cell-specific chromatin immunoprecipitation will aid the understanding of how different transcriptional codes regulate selectivity in axon guidance decisions to reveal the actual targets of specific transcriptional codes.

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