Transcriptional regulation of vertebrate axon guidance and synapse formation

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Abstract | The establishment of functional neural connections requires the growth of axons to specific target areas and the formation of synapses with appropriate synaptic partners. Several molecules that regulate axon guidance and synapse formation have been identified in the past decade, but it is unclear how a relatively limited number of factors can specify a large number of connections. Recent evidence indicates that transcription factors make a crucial contribution to the specification of connections in the nervous system by coordinating the response of neurons to guidance molecules and neurotransmitters.

Zinc-finger transcription factor
A protein motif consisting of two antiparallel β-strands and an α-helix forming a binding pocket for a zinc ion that is crucial for the stability of this domain type.

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One of the important challenges of modern neurobiology is to understand how neurons are instructed to project to specific regions of the brain. This has been one of the most intensely studied areas of developmental neurobiology in the past decade, and a number of important molecules that regulate axon guidance and synapse formation have been identified[1–3]. The focus of many of these studies has been on proximal mediators — signals that act directly on growth cones to regulate the development of axons and synaptic terminals. Over the past two decades, significant progress has been made in the identification of the extracellular cues, their corresponding receptors and the intracellular effectors that mediate the precise guidance of axons from their origin to their target area[1–3]. However, the establishment of functional circuits requires that different neuronal populations respond selectively to guidance and synaptogenic cues at particular times and at specific locations. This spatial, temporal and cell-type-specific regulation of neuronal responses needs to be precisely coordinated during development, and is achieved by regulated expression of axon guidance receptors and intracellular effectors, which specify the responsiveness of the axons to extracellular cues. In this review we consider the evidence that transcription factors act as master regulators of this process, and propose that transcription factors specify connectivity by regulating the expression and function of genes that determine neuronal responses to axon guidance and synaptogenic molecules (BOX. 1). Although we focus on the regulation of connectivity in the vertebrate nervous system, there is also strong evidence that transcription factors contribute to neuronal connectivity in invertebrates, indicating that transcriptional control might be a general feature of nervous-system wiring. We begin by reviewing the recent progress made in the identification of the transcriptional mechanisms that control axon guidance decisions in various parts of the vertebrate nervous system, before considering the transcriptional control of synaptogenesis.

Development of retinal projections

Retinal ganglion cells (RGCs) project topographically to three main targets in the mammalian visual system: the dorsal part of the lateral geniculate nucleus (dLGN), the pretectum and the optic tectum (superior colliculus). Two features of RGC axon projections have been intensively studied. First, RGC axons leaving the retina have the choice of whether to cross the midline at the level of the optic chiasm. Second, axons that originate along the dorsoventral and nasotemporal axes of the retina project topographically along the anteroposterior and mediolateral axes, respectively, of their final targets. These two features of RGC axon projection underlie binocular vision and the retinotopic representation of visual space, respectively.

Control of midline crossing. Recent studies indicate that the axon guidance choice made by RGC axons at the optic chiasm is determined by the expression of transcription factors[4,5]. The zinc-finger transcription factor ZIC2 is specifically expressed in the ventrotemporal part of the retina that contains RGCs which project axons ipsilaterally, rather than crossing the midline[4,6] (FIG. 1a). Interestingly, ZIC2 is expressed by these RGCs before

LIM homeodomain transcription factor
A subclass of homeodomain transcription factors consisting of a zinc-binding motif that mediates protein–protein interaction.

Winged-helix transcription factor
Also called forkhead (or FOX). A class of transcription factors characterized by a 100-amino-acid, monomeric DNA-binding domain that folds into a variant of the helix-turn-helix motif and is made up of three α-helices and two characteristic large loops, or 'wings.'

their axons reach the midline but is downregulated soon after their axons cross the optic chiasm[4]. Mice with reduced expression of *Zic2* (*Zic2* knockdown or *Zic2*$^{KD/KD}$) have abnormal eye development[7] as well as malformations of the ventral diencephalon, including altered expression of axon guidance molecules that are known to control axon crossing at the optic chiasm, such as ephrin B2. Heterozygous *Zic2*$^{KD/+}$ mice were compared with *Zic2*$^{KD/KD}$ homozygous and wild-type littermates, revealing a direct correlation between the level of *Zic2* expression in RGCs and the proportion of axons that projected ipsilaterally. This suggests that the expression of *Zic2* might drive the decision to project ipsilaterally (FIG. 1c). Furthermore, overexpression of *Zic2* in dorsal RGCs was sufficient to make their axons sensitive to midline repulsive cues, an effect that required the presence of an intact DNA-binding domain in ZIC2 (REF. 4). These results show that ZIC2 has an instructive role in the responsiveness of RGC axons to midline repulsive cues at the optic chiasm.

One of the most likely candidate molecules to mediate the effects of ZIC2 at the optic chiasm is the ephrin B receptor EphB1, which is specifically expressed in the ventrotemporal retina where *Zic2* is expressed, and has been shown to help to prevent axon crossing at the optic chiasm[8] (FIG. 1d). However, whether ZIC2 controls *EphB1* expression directly or instead specifies ipsilateral RGC projections in an *EphB1*-independent manner remains to be determined.

The percentage of RGCs that project ipsilaterally at the optic chiasm correlates with the degree of binocularity in different species, which ranges from 40% binocularity in humans to 15% in ferrets and only 3–5% in rodents. There is also a strong correlation between the number of *Zic2*-expressing RGCs in the ventrotemporal retina and the increasing degree of binocularity in ferrets, mice, *Xenopus* and chicks[4]. What regulates the number of neurons that express *Zic2* in the ventrotemporal retina? A recent study shows that the LIM homeodomain transcription factor islet 2 (ISL2) is present in almost all RGCs except those in the ventrotemporal retina, and might inhibit *Zic2* expression[5] (FIG. 1b). Interestingly, some ISL2-positive RGCs are found in the ventrotemporal retina, but these are clearly distinct from RGCs that express *Zic2* and *EphB1*. *Isl2*-knockout mice have increased numbers of axons projecting ipsilaterally from RGCs located in or close to the ventrotemporal retina. The ventrotemporal retina of these mice contains more RGCs that co-express *Zic2* and *EphB1*. These results suggest that, in ventrotemporal RGCs, ISL2 represses the expression of *Zic2* and *EphB1* and therefore represses the genetic programme that underlies binocularity[5].

Specification of topography. Analysis of the projection of RGC axons to the optic tectum (retinotectal projections) has led to important insights into the establishment of topographic maps[9]. Importantly, the graded expression of EphA receptors in RGCs along the nasotemporal axis of the retina is crucial for the establishment of the topography of axon projection along the anteroposterior axis of the tectum, whereas graded EphB receptor expression along the dorsoventral axis of the retina gives RGC axons the ability to project topographically along the lateromedial axis of the developing tectum[10] (FIG. 2).

The graded expression of EphA and EphB receptors appears to be regulated by homeobox transcription factors. Ventral anterior homeobox 2 (*Vax2*) is expressed in a ventral-high to dorsal-low gradient in the retina, and both misexpression experiments in mice and chicks[11] and genetic loss-of-function studies in mice[12] point to a role for *Vax2* in patterning the expression of *EphA5*, *EphB2–B3* and ephrin B1–B2 along the dorsoventral axis of the retina (FIG. 2b). Retinotectal axons are misrouted in *Vax2*-knockout mice along the mediolateral axis of the tectum, supporting the idea that *Vax2* helps to specify the projection patterns of RGCs[13].

TBX5 is a T-box transcription factor that is expressed in a dorsal-high to caudal-low gradient in the retina, and has been shown to repress expression of paired box gene 2 (*Pax2*) and *Vax2* in the dorsal half of the retina[14] (FIG. 2b). Misexpression of *Tbx5* is sufficient to induce the expression of ephrin B1 and ephrin B2 in the ventral half of the retina, leading to misprojection of RGC axons in the tectum. This supports a model in which patterning of *Tbx5*, *Pax2* and *Vax2* along the dorsoventral axis of the retina, which is possibly regulated by a combination of bone morphogenetic protein (BMP) and retinoic acid signalling[15–19], leads to the patterning of ephrin B and *EphB* expression that specifies RGC axon projections along the lateromedial axis of the tectum[20].

In chicks, the winged-helix transcription factors chick brain factor 1 (CBF1) and CBF2 (orthologues of forkhead box G1 (FOXG1) and FOXD1, respectively, in both mice and humans) are enriched in the nasal and temporal

Box 1 | **Diversity and specificity of transcription factors in the developing nervous system**

Transcription factors constitute a large family of proteins, with approximately 1,400 transcription factors making up approximately 7% of the mouse genome[88]. Transcription factors can be divided into several families based on the structure of their DNA-binding motif, which is usually composed of a conserved stretch of positively charged amino-acid residues.

Recent studies have started to comprehensively map the expression pattern of transcription factors expressed in the mouse brain[88] (see the Allen Brain Atlas, brain gene expression map and GenePaint). There is an extraordinary level of specificity in the pattern of expression of transcription factors, such that they are often expressed in cell-type-specific or layer-specific patterns. These databases provide a unique opportunity for the investigation of the role of combinatorial expression of transcription factors in the specification of neuronal phenotypes, including neuronal connectivity[89,90]. The emerging concept of molecular taxonomy raises the possibility that specific functional ensembles of neurons can be defined by the expression of a limited subset of genes that govern their morphology, connectivity and electrophysiological and neurochemical phenotype[91].

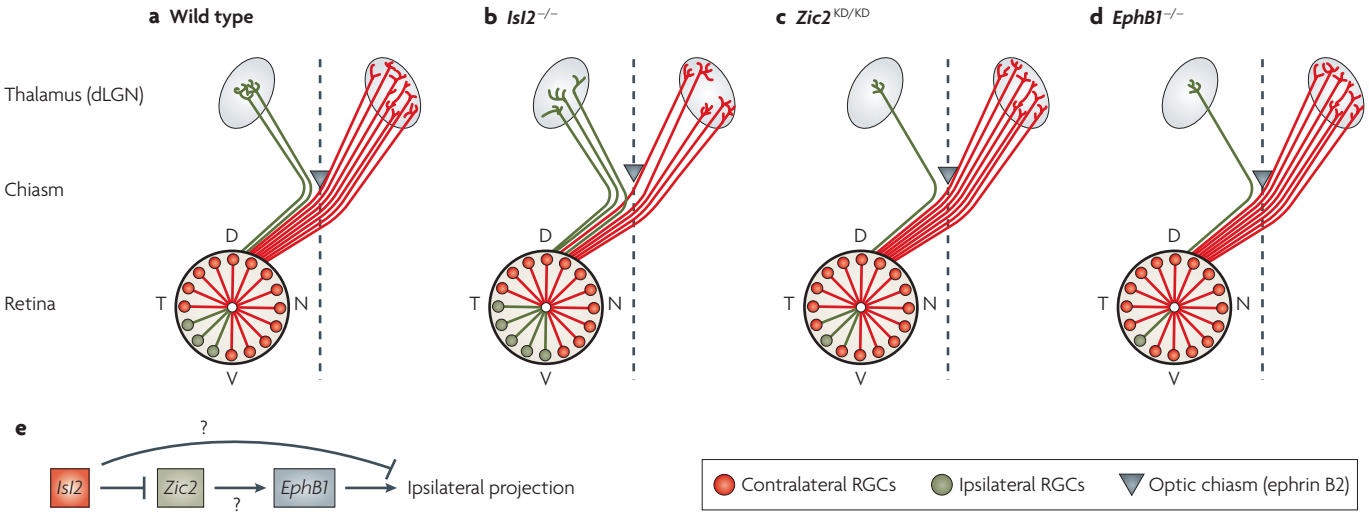

Figure 1 | **Transcriptional control of midline crossing by retinal axons.** Diagrammatic representation of retinal ganglion cell (RGC) axon crossing in wild type (**a**), and islet 1 (*Isl1*), *Zic2* and *EphB1* mutant mice (**b–d**). In wild-type mice, *Zic2*-expressing RGCs in the ventrotemporal crescent of the retina (green) do not cross the midline at the optic chiasm and therefore project ipsilaterally. In *Isl2*-knockout mice, a larger proportion of RGCs project ipsilaterally, suggesting that ISL2 normally represses *Zic2* expression in the ventrotemporal retina. By contrast, *Zic2*-deficient ($Zic2^{KD/KD}$) and *EphB1*-knockout mice present a decreased number of RGC axons projecting ipsilaterally, suggesting that both genes are specifying the repulsion of RGC axons to midline repellent present in the optic chiasm. A proposed relationship between *Isl2*, *Zic2* and *EphB1* is also shown (**e**), and explains how transcription factors can influence the axon guidance decisions defining binocularity in RGCs. D, dorsal; N, nasal; T, temporal; V, ventral.

retina respectively, and misexpression of *CBF1* in the temporal retina results in misprojection of axons along the anteroposterior axis of the tectum[21] (FIG. 2a). Recent misexpression studies have shown that CBF1 represses *CBF2* and *EphA3* expression and induces the expression of ephrin A2–A5 (REF. 22).

Interestingly, mice lacking *Foxd1* and *Foxg1* show deficits earlier in the visual projection pathway, at the level of the optic chiasm[23,24]. In *Foxd1*-knockout mice, RGCs in the ventrotemporal retina, which normally project axons ipsilaterally, end up projecting axons contralaterally, probably because they do not respond to chemorepulsive cues at the chiasm[23]. *Foxg1* is expressed predominantly in the nasal part of the retina and in the optic chiasm. *Foxg1*-knockout mice have an eightfold increase in the numbers of RGC axons projecting ipsilaterally, a defect that might be due to a mispatterning of axon guidance cues at the level of the midline in the optic chiasm[24] (FIG. 2a).

Overall, these results indicate that the combinatorial expression of transcription factors along the dorsoventral and nasotemporal axes of the retina specifies the pattern of expression of ephrins and Eph receptors that underlies the topographic projections of RGC axons to the visual centres. However, many questions remain to be addressed. For example, what are the patterning mechanisms that specify the graded expression of transcription factors along the axes of the developing retina? How are the transcriptional mechanisms that specify binocularity and topography coordinated in RGCs? And are any of these transcriptional mechanisms influenced by activity-dependent mechanisms in the retina, such as the retinal waves that have been shown to influence RGC connectivity? Further investigations will be required to answer these important questions.

Regulation of thalamocortical patterning

The thalamocortical pathway constitutes one of the most important and complex topographically organized projection pathways in mammals. Each thalamic nucleus projects to a given cortical area (inter-areal topography) to transmit information regarding a particular sensory modality. Once thalamic axons reach their target cortical areas, they establish topographic maps within these areas (intra-areal topography)[25–28]. There is evidence that the inter-areal topography of thalamic axonal projections is regulated by axon guidance cues in the main intermediate target of these axons, the ventral telencephalon[25,26,29]. The importance of ventral telencephalic cues in the initiation of the topography of thalamocortical projections was demonstrated recently in a study of the role of the transcription factor neurogenin2 (NGN2) in the specification of neuronal connectivity[29].

NGN2 is a basic helix-loop-helix transcription factor that is expressed in the rostral part of the developing thalamus[29,30], and specifies projections from ventrolateral and anterior thalamic nuclei to the frontal cortex[31–33]. *Ngn2*-knockout mice are characterized by a shift in the projection of thalamocortical axons that occurs first in the ventral telencephalon[29]. A new *in vitro* assay has been developed in which the projections of identified regions of the developing thalamus expressing green fluorescent protein could easily be assessed in a whole-mount preparation; using this 'whole-mount telencephalon' assay, it has been found that axons emerging from the rostromedial dorsal thalamus are preferentially targeted

Retinal waves
Waves of spontaneous neural activity generated partly by the bursting of retinal ganglion cells (RGC) and propagating throughout the retina. This correlated, spontaneous activity is conveyed to the lateral geniculate nucleus (LGN) and the visual cortex and is necessary for the segregation of eye-specific layers within the LGN, and the refinement of retinotopy.

Inter-areal topography
The topography of projection of axons originating from a thalamic nucleus to a specific set of cortical areas.

Intra-areal topography
The topography of projection of axons from one thalamic nucleus inside a given cortical area.

Basic helix-loop-helix
(bHLH). Approximately 15 charged residues (basic domain) conferring specificity of DNA binding as well as a helix-loop-helix motif mostly involved in dimerization.

Barrel cortex
The part of the rodent somatosensory cortex that receives sensory input from contralateral whisker follicles. The barrel cortex has been a useful model system to study cortical connections because of the ease with which anatomical representations of thalamocortical axon terminals can be visualized.

Transcriptional co-activator
A protein factor which on recruitment to a specific promoter activates gene transcription. Co-activators do not directly bind to DNA but are associated with transcription-factor complexes.

to the anterior ventral telencephalon, ultimately invading anterior cortical territories. Conversely, axons that emerge from progressively more caudolateral parts of the thalamus grow preferentially towards more caudal parts of the ventral telencephalon. These results show that *Ngn2* controls the topography of thalamocortical projections by controlling the responsiveness of thalamocortical axons to intermediate target cues in the ventral telencephalon[29].

A complementary study showed that some of these intermediate cues belong to the ephrin A family and that the responsiveness of thalamic axons to ephrin A5 is regulated by differential expression of EphA receptors along the rostromedial to caudolateral axis of the dorsal thalamus[34]. It is unclear, however, which effectors mediate the effects of NGN2 on the specification of anterior thalamic axon guidance. Recent experiments have ruled out the possibility that EphA receptor expression is controlled by NGN2 (J. Seibt, S. Dufour, P. Vanderhaeghen and F.P., unpublished observations). Further studies will be required to determine which axon guidance receptors mediate the functions of NGN2 in regulating the responsiveness of thalamocortical axons to axon guidance cues in the ventral telencephalon, and specifying their final targets[27,28].

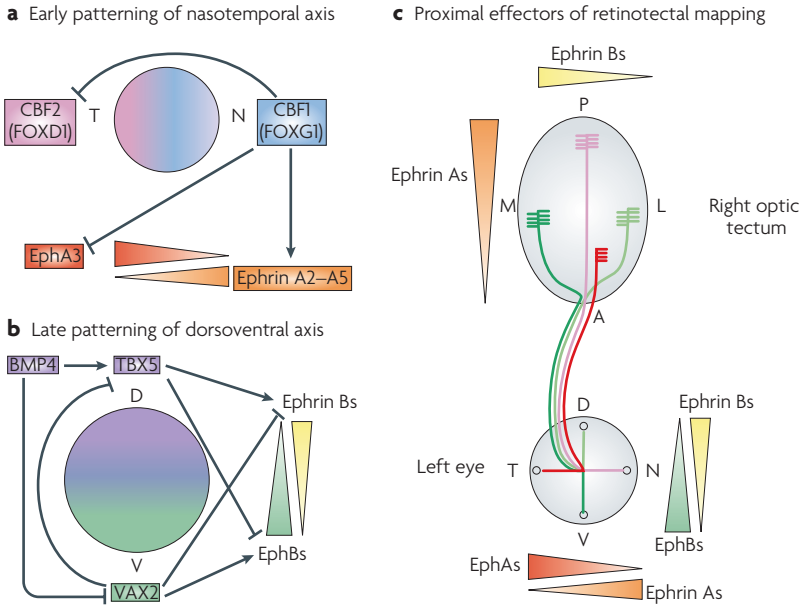

Figure 2 | **Transcriptional regulation of the topography of retinotectal projections. a** | Early patterning of the nasotemporal axis of the retina. Two forkhead transcription factors called *Foxg1* (chick brain factor (*CBF1*) in chicks) and *Foxd1* (*CBF2* in chicks) are expressed in complementary patterns in the nasal (N) and temporal (T) retina respectively. Based on the results of misexpression studies, CBF1 has been suggested to repress CBF2 and EphA3 receptor expression but induce the expression of ephrin A2–A5 in the nasal retina. **b** | Late patterning of the dorsoventral axis of the retina. Two transcription factors (T-box 5 (TBX5) and ventral anterior homeobox 2 (VAX2)) are present in complementary patterns in the dorsal (D) and ventral (V) part of the retina. Bone morphogenetic protein 4 (BMP4) has been proposed to induce *Tbx5* expression in the dorsal retina. VAX2 represses TBX5 expression. TBX5 represses the expression of EphB receptors but induces expression of ephrin B ligands in the dorsal retina. Conversely, VAX2 induces EphB receptor expression but represses ephrin Bs expression in the ventral retina. **c** | Ephrin–Eph signalling in the patterning of retinotectal projections. The topography of retinotectal projections is largely determined by EphA receptor expression, which determines the projections along the low-anterior and high-posterior gradient of ephrin A2–A5 across the tectum, and by EphB receptors, which determine the projection along the high-medial to low-lateral gradient of Eph along the body wall. A, anterior; L, lateral; M, medial; N, nasal; P, posterior; T, temporal.

Activity-dependent transcription. The refinement of thalamocortical axonal projections within their target regions requires neuronal activity and contributes to the establishment of functional cortical circuits. In several cortical areas, axons from the thalamus are initially intermixed and segregate into distinct domains during the first few postnatal weeks. The patterning of the thalamocortical axons in the barrel cortex is regulated by the transcription factor neurogenic differentiation D2 (NeuroD2) and the transcriptional co-activator LIM domain only 4 (LMO4)[35,36] (FIG. 3). *NeuroD2* and *Lmo4* were cloned in a screen for activity-regulated transcription factors and induce gene expression in response to calcium influx through voltage-sensitive calcium channels[37]. Functional analysis of NeuroD2 and LMO4 has implicated them in regulating connectivity in the somatosensory cortex.

The barrel cortex of rodents receives sensory input from the whisker follicles via subcortical relay stations in the brainstem and sensory thalamus. Thalamocortical axons project topographically to cortical layer 4, where the terminals of axons representing individual whiskers form dense arborizations surrounded by cortical neurons in discrete territories called barrels[38,39]. *NeuroD2* and *Lmo4* are expressed by cortical neurons. In both *NeuroD2*- and *Lmo4*-knockout mice, thalamocortical axons can project to layer 4, but cannot refine their territories into cortical barrels[35,36]. Moreover, the subcortical relay stations develop normally in both knockout lines. These results indicate that postsynaptic NeuroD2- and LMO4-mediated transcription is essential for the proper refinement of presynaptic axon terminals. It remains to be determined whether this process depends on a retrograde message that is expressed in cortical neurons by NeuroD2- or LMO4-mediated transcription. Although past studies have established a role for neurotransmitter receptors and their downstream signalling pathways in barrel cortex development, the discovery of NeuroD2 and LMO4 as essential regulators of axon terminal refinement highlights the importance of transcriptional programmes in the development of cortical circuitry[40–42]. Important goals of future studies will be to identify the mechanisms by which calcium signals regulate NeuroD2- and LMO4-mediated transcription, and to identify the effectors that link transcriptional activation to the reorganization of thalamocortical afferents.

Patterning cortical efferent projections

A defining feature of the cerebral cortex is that neurons in a given layer share specific patterns of axon projections. For example, pyramidal neurons in layer 6a project primarily to the thalamus, whereas pyramidal neurons in layer 5 project to subcortical targets such as the striatum, colliculus, pons and spinal cord. Neurons that project to the contralateral hemisphere (callosal projections) are found mainly in layers 2 and 3, but also

Fluorescence-activated cell sorting
(FACS). A technique that can rapidly separate cells in a suspension on the basis of their size and their fluorescence.

Transcription profiling
Genome-wide analysis of mRNA transcript expression in groups of cells using DNA microarray hybridization technology.

Small hairpin RNA
A sequence of RNA that makes a tight hairpin turn and can be used to silence gene expression in mammalian cells.

in layer 5 in the adult. In rodents, these layer-specific patterns of projections are also area-specific: for example, neurons that project to the superior colliculus are found only in layer 5 of primary and secondary visual areas, whereas neurons that project to the spinal cord are found only in layer 5 of the primary and secondary somatosensory cortex[43]). This areal specificity emerges during development, starting from an initial, more widespread and less specific pattern of projections. Elegant studies in rodents using retrograde tracing at different stages of postnatal development have shown that layer 5 neurons of virtually all cortical areas project to both the spinal cord and the colliculus at birth[44]. The projections are gradually refined by target-specific pruning of axon collaterals.

The molecular mechanisms that specify the layer- and area-specific projections of pyramidal neurons are only now beginning to be identified. Retrograde tracing of cortical pyramidal neurons from different target areas was used to purify neuronal subpopulations by fluorescence-activated cell sorting (FACS), followed by transcription profiling to identify genes that are expressed by specific subtypes of neurons[45]. One of these factors, CTIP2 (a zinc finger DNA-binding protein; also called BCL11B) is involved in the specification of corticospinal projections from layer 5 neurons[45]. In *Ctip2*-knockout mice, the axons of layer 5 neurons show axon path-finding defects, including a failure to extend past the pons into the spinal cord[45]. These results indicate that CTIP2 probably initiates a transcriptional programme that specifies the ability of a subset of layer 5 neurons to project to the spinal cord.

Recent analysis has revealed that another zinc-finger-containing transcription factor, called Fez-like (FEZL; also called FEZ1 or ZFP312), is involved in specifying the projection patterns of layer 5 neurons that project to subcortical targets such as the spinal cord[46–49]. In *Fezl*-knockout mice, layer 5 neurons do not project to the spinal cord. In addition, they upregulate the T-box family transcription factor T-brain 1 (*Tbr1*), which specifies the projection patterns of layer 6 neurons[50], and downregulate the expression of several layer 5 markers, including the transcription factor *Ctip2* (REFS 46–49). Furthermore, forced expression of *Fezl* in cortical neurons induces *Ctip2* expression, and is sufficient to induce projection to subcortical targets such as the thalamus and the spinal cord[47,49]. These results show that FEZL regulates the pattern of subcortical axon projections in a subset of layer 5 neurons at least in part by controlling the expression of *Ctip2*. FEZL and CTIP2 do not seem to be involved in the general differentiation programme of layer 5 neurons, as the migration of these neurons is unaltered in *Fezl*- and *Ctip2*-knockout mice. However, subtle changes in the dendritic arborization of layer 5 neurons has been reported in neurons in which *Fezl* expression is downregulated using small hairpin RNA[47]. Further studies will be required to determine whether FEZL controls both axon projection and dendrite morphology or if the changes in dendrite morphology that result from FEZL knockdown are a secondary consequence of the change in axon projections.

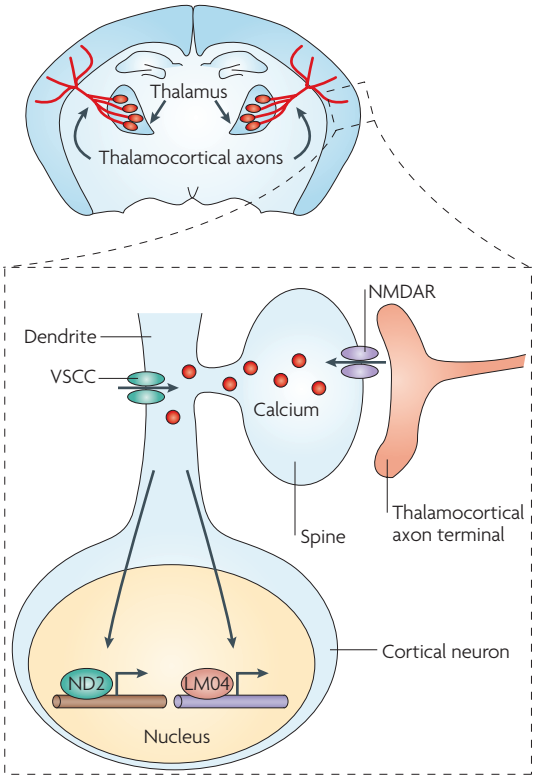

Figure 3 | **Regulation of thalamocortical patterning by NeuroD2 and LMO4.** Thalamocortical projections (red) are diagrammatically represented in the upper panel. In the lower panel an individual cortical neuron receiving thalamocortical input is shown. Calcium influx into cortical neurons activates neurogenic differentiation D2 (NeuroD2; ND2)-mediated and LIM domain only 4 (LMO4)-mediated transcription. In the absence of NeuroD2 or LMO4, thalamocortical axons can project from the thalamus to the cortex; however, they cannot form barrels, which are axon rich domains receiving sensory input from individual whiskers. NeuroD2 and LMO4 were the first two examples of transcription-factor control of barrel-cortex connectivity to be identified. VSCC, voltage-sensitive calcium channel; NMDAR, *N*-methyl-D-aspartate receptor.

An important area of future investigation will be to determine how FEZL and CTIP2 specify the projection of layer 5 axons to subcortical targets. It will be interesting to determine if, as in the case of RGCs, their transcriptional targets include axon guidance receptors or intracellular effectors that control the responsiveness of axons from layer 5 neurons to guidance cues produced by subcortical targets.

Patterning of motorneuron projections

Spinal motorneurons are perhaps the best-studied model of the transcriptional regulation of cell identity[51]. Motorneurons that project to specific muscle groups are clustered in specific pools[52]. Motorneurons that innervate axial muscles are located in the median motor column (MMC) and are present at all segmental levels of the spinal cord. By contrast, motorneurons that innervate limb muscles are located in the lateral motor column (LMC) and are generated only at levels of the

Hox transcription factors
A subgroup of homeobox genes that are found in a special gene cluster, the Hox cluster (also called Hox complex). There are four classes (A to D) of Hox genes that function in patterning the body axis, including the CNS.

neural tube that correspond to the limb fields[53–55] (FIG. 4). Classical experiments on the developing chick spinal cord have shown that the innervation of a specific muscle by motorneurons is initiated before target innervation by the sorting of axons into specific fascicles. These early studies demonstrated indirectly that the projection of distinct pools of motorneurons to a specific muscle is probably determined intrinsically[54,56].

The discovery that specific pools of motorneurons are organized by the expression of combinations of LIM homeobox transcription factors such as LIM1 and ISL1 represented a key step in our understanding of the molecular mechanisms that determine motorneuron projections[57–59]. More recently, a combinatorial code of Hox transcription factors expressed along the rostrocaudal axis of the spinal cord has been shown to have a key function in defining the identity of motorneuron pools, including the specification of particular target muscles[60].

How does the expression of combinations of transcription factors specify the projection of motorneuron axons to specific muscles? At the brachial level of the developing mammalian spinal cord, two LIM homeodomain transcription factors (LIM1 and LMX1B) control the initial trajectory of motor axons in the developing limb[61]. The expression of *Lim1* by a lateral set of LMC neurons ensures that their axons select a dorsal trajectory in the limb (FIG. 4a). Furthermore, LMX1B functions within limb mesenchymal cells to control the dorsoventral axonal trajectory of both medial and lateral LMC neurons[61]. Studies in *Drosophila melanogaster* and *Caenorhabditis elegans* have indicated that LIM homeodomain proteins have an evolutionarily conserved role in the control of motor axon trajectories[62,63]. There is also evidence that EphA–ephrin A interactions control motor axon projections at the level of the ventral base of the limb[64–67]. It has been shown that LIM1 and ISL1 control the dorsoventral specificity of motor axon projections at least in part by controlling the expression of EphA4 in lateral LMC neurons, and thereby conferring responsiveness to ephrin A ligands that are induced by LMX1B in the ventral portion of the limb[68] (FIG. 4b,c). These results indicate that LIM1 delimits the dorsoventral specificity of axon projection by controlling *EphA4* expression, whereas the expression of *Lmx1b* by mesenchymal cells in the ventral half of the developing limb controls the expression of ephrin A5 and thereby defines a territory that is avoided by lateral LMC axons[68].

Another class of receptors, the neuropilins — which mediate repulsion of growth cones in response to class 3 semaphorins — has been implicated in the early dorsoventral segregation of LMC axons in the limb[69]. Semaphorin 3A (SEMA3A)–neuropilin 1 (NPN1)

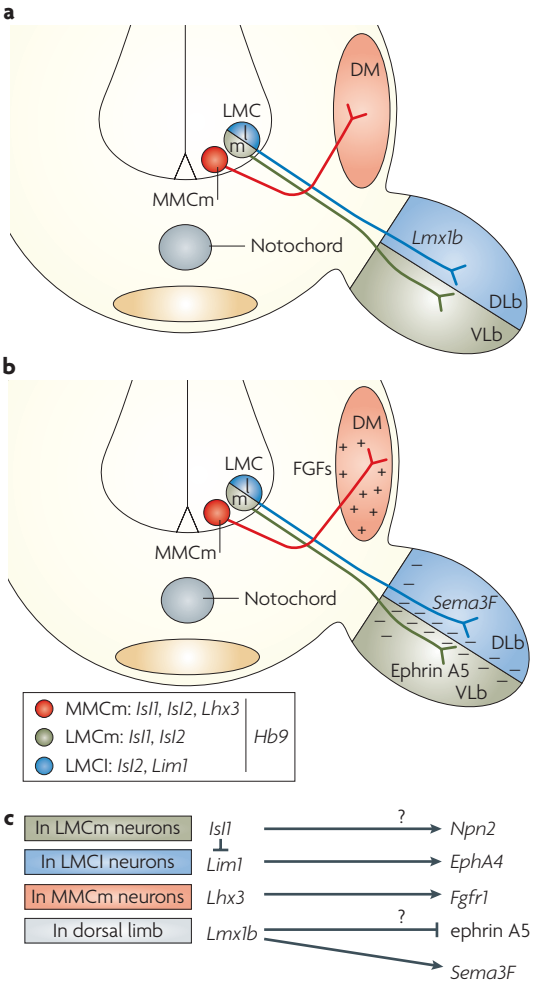

Figure 4 | **Specification of motorneuron projections.** Diagrammatic representation of transcription factors and axon guidance signals specifying motorneuron projections in the limb. **a** | Cross section through developing spinal cord and limb bud showing the distribution of transcription factors expressed by subsets of motorneurons and the targets they innervate. At the brachial level of the developing spinal cord, motorneurons located in the lateral motor column (LMC) can be divided in a medial (LMCm) and a lateral (LMCl) pool projecting to the ventral and the dorsal part of the limb, respectively (VLb and DLb). The medial part of the medial motor column (MMCm) projects to the dermomyotome (DM). All motorneurons express the transcription factor *Hb9*, but each pool can be defined by the expression of specific combinations of homeodomain transcription factors: MMCm neurons express islet 1 (*Isl1*), *Isl2* and LIM homeobox 3 (*Lhx3*), LMCm neurons express only *Isl1* and *Isl2* whereas LMCl neurons express *Isl2* and *Lim1*. Cells in the dorsal half of the limb express another transcription factor, *Lmx1b*. **b** | Distribution of axon guidance signals known to influence motorneuron projection patterns. In MMCm neurons, LHX3 controls the expression of fibroblast growth factor receptor 1 (*Fgfr1*), which confers responsiveness to dermomyotome-derived FGFs that act as strong attractants for MMCm axons. Expression of *Lim1* in LMCl neurons induces the expression of *EphA4*, which confers responsiveness to the chemorepellent cue ephrin A5 located in the ventral part of the limb. In LMCm neurons, neuropilin2 (*Npn2*) expression confers responsiveness to the chemorepellant cue semaphorin 3F (*Sema3F*) expressed in the dorsal part of the limb. **c** | The relationships between transcription factors expressed in motorneurons and the guidance molecules that they regulate. Question marks indicate suggested but not proven relationships between transcription factors and axon guidance cues.

Dermomyotome
A transitory epithelial sheet of cells formed during somite maturation. The dermomyotome is the source of most of the mesodermal tissues in the body, giving rise to cell types as diverse as muscle, connective tissue, endothelium and cartilage.

Plexus
A network of intersecting nerves.

signalling controls the timing of the ingrowth of motor axons into the limb, whereas SEMA3F–NPN2 signalling guides the axons of a medial subset of LMC neurons to the ventral limb[69].

Members of the fibroblast growth factor family — such as FGF8 — that are expressed in the dermomyotome also attract the axons of brachial motorneurons[70]. Interestingly, reprogramming limb-innervating motorneurons to become dermomyotome-innervating neurons using the LIM homeodomain transcriptional factor LHX3 is sufficient to induce FGF receptor 1 expression and as a consequence induce FGF-responsiveness in the motorneuron axons[70]. These results indicate that, in addition to its well-characterized function as a patterning cue early in development, FGF signalling might also be a novel effector pathway of the LIM homeodomain transcriptional code in the guidance of motorneuron axons to their targets.

The findings in spinal cord development further support the notion that transcription factors influence axonal projections by regulating the expression of guidance receptors. It remains to be determined how specific combinations of transcription factors specify the expression of axon guidance receptors. It is also not known how interactions between different types of axon guidance receptors contribute to the guidance of motor axons in a local environment that contains multiple axon guidance cues.

Specification of sensorimotor circuits

One of the best examples of connectional specificity is the patterning of sensory and motorneuron projections in the spinal cord and limb. Sensory neuron cell bodies are located in dorsal root ganglia and extend processes that innervate the periphery (limb) and spinal cord. Different subclasses of sensory neurons innervate distinct muscle groups and have different termination zones in the spinal cord. Reciprocally, subpopulations of motorneurons extend dendrites to specific regions of the spinal cord and extend axons to different muscle groups. The pattern of innervation of motor- and sensory neurons is precisely specified in order to ensure proper control of movement.

There is considerable evidence that, in addition to influencing the trajectory of motor axons, transcriptional programmes also regulate the organization of motorneurons and sensory axons into functional circuits. The differentiation of specific pools of motorneurons is associated with the expression of E-twenty-six (ETS) transcription factors such as *Pea3* and *Er81* (REFS 71,72). Their initial expression coincides with the arrival of motorneuron axons at the plexus of the limb, and their expression depends on target-derived signals. In *Pea3* mutant mice, axons from specific motorneuron pools fail to branch normally within their target muscles and their cell bodies are mispositioned in the spinal cord[73]. Interestingly, glial cell-line-derived neurotrophic factor (GDNF) is present in the plexus of the developing forelimb and later in specific muscles innervated by *Pea3*-expressing motorneurons[74]. In the absence of GDNF signalling, as in the *Pea3* knockout, motorneurons that normally innervate these muscles are mispositioned in the spinal cord and their axons fail to arborize within the muscle. In GDNF signalling mutants (GDNF-knockout and GDNF receptor α1-knockout mice), *Pea3* fails to be induced in motorneurons[74]. Similarly, in dorsal root ganglion neurons, the expression of *Er81* requires the expression of the muscle-derived neurotrophin *Nt3* (REF. 75).

The idea that neuronal phenotype can be determined by target-dependent mechanisms is reinforced by a recent study that showed that patterns of dendritic arborization as well as the specificity of presynaptic inputs might depend on target-induced transcription programmes[76]. Two cervical motorneuron pools show specific patterns of dendritic arborization within the spinal cord and are also contacted by a distinct group (Ia) of proprioceptive afferents (IaPA). The induction of *Pea3* by GDNF is essential for cell-type-specific dendritic arborization and the selectivity of IaPA connectivity[76]. Together, these results indicate that the specification of functional neural circuits requires a tight interaction between target-derived trophic factors and the expression of neuron-subtype-specific transcription factors that regulate final cell-body position, axon arborization within targets, and dendritic arborization to specify functional circuits.

It will be of interest to determine if PEA3 influences dendritic patterning by regulating the expression of guidance receptors. The dendritic trajectory of cortical neurons is known to be influenced by SEMA3A–NPN1 interactions[77], and a similar mechanism might be involved in specifying motorneuron dendritic patterns.

Transcriptional control of synaptogenesis

Transcription factors also seem to exert an important influence on connectivity by regulating synapse formation (FIG. 5). Members of the MEF2 (myocyte enhancer factor 2) family are transcription factors that are highly expressed in neurons and have essential roles in various cellular process such as cell division, differentiation and apoptosis[78]. In neurons, expression of *Mef2a* and *Mef2d* in hippocampal neurons is necessary and sufficient to restrict the number of excitatory synapses on these neurons[79]. RNA interference-mediated knockdown of MEF2A/D in cultured hippocampal neurons results in a significant increase in excitatory synapses, as judged by the colocalization of pre- and postsynaptic markers. Another consequence of MEF2A/D knockdown is an increase in the frequency, but not the amplitude, of miniature excitatory postsynaptic currents (mEPSCs), suggesting that the number of excitatory synapses, rather than their strength, has increased. However, the overexpression of a constitutively active form of MEF2 is sufficient to reduce the number of excitatory synapses. These observations indicate that MEF2-mediated transcription might induce synapse elimination in developing neurons. The transcriptional activity of MEF2 is regulated by calcium signalling and by various signalling pathways, including

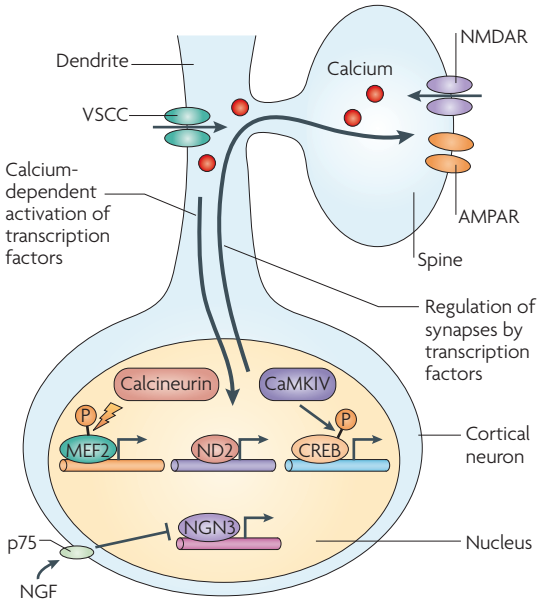

Figure 5 | **Transcription factors implicated in the regulation of synapse formation and synapse elimination.** Excitatory synapse numbers are suppressed by transcriptionally active myocyte enhancer factor 2A/D (MEF2A/D). Calcium influx through voltage-sensitive calcium channels (VSCC) or NMDARs (*N*-methyl-D-aspartate receptors) results in the activation of calcineurin, which dephosphorylates MEF2A/D at Ser408. Dephosphorylation activates MEF2A/D, which results in the suppression of excitatory synapse numbers. Neurogenic differentiation D2 (NeuroD2; ND2)-mediated transcription is activated by calcium influx into cortical neurons. In the absence of NeuroD2, the ratio of NMDAR: AMPAR (α-amino-3-hydroxy-5-methyl-4-isoazolepropionic acid receptor)-mediated glutamatergic neurotransmission remains high, suggesting a more immature state of the thalamocortical synapse. Calcium/calmodulin-dependent protein kinase IV (CaMKIV) activates cyclic AMP-response element binding protein (CREB)-mediated transcription, which then causes an increase in the numbers of silent synapses. Nerve growth factor (NGF) through binding to its receptor p75 causes a reduction in overall levels of neurogenin 3 (NGN3), which results in an increase in the total number of inhibitory synapses.

calcium/calmodulin-dependent kinases (CaMKs), extracellular signal-related kinase (ERK), p38 kinase and the phosphatase calcineurin[80]. Calcineurin-dependent dephosphorylation of MEF2 increases its binding to DNA and the expression of reporter genes. In neurons, MEF2A/D-dependent restriction of synapse numbers also requires calcineurin-dependent dephosphorylation of specific residues on MEF2A/D[79].

Which genes are regulated by MEF2A/D? A microarray screen that compared gene expression profiles in MEF2A/D-knockdown versus control neurons in response to membrane depolarization identified *Arc* (the gene that encodes activity-regulated cytoskeletal-associated protein) and *SynGAP* (synaptic RAS guanosine triphosphate) as MEF2 targets[79]. Interestingly, both of these genes have been implicated in the regulation of synaptic signalling. It remains to be seen whether *Arc* and/or *SynGAP* is required for MEF2A/D-dependent restriction of excitatory synapse numbers.

In cerebellar granule neurons, MEF2A is required for the differentiation of dendritic terminal structures called dendritic claws, but not for overall dendritic arborization[81]. As dendritic claws are sites of excitatory synaptic contacts, it will be worthwhile to investigate whether the regulation of excitatory synapse numbers by MEF2A/D and the morphological differentiation of synaptic terminals are causally linked or if these two processes are independently controlled by MEF2.

Another transcription factor that has been implicated in regulating synapse numbers is the basic helix–loop–helix protein NGN3 (REF. 82). The overexpression of *Ngn3* in cultured hippocampal neurons results in a reduction in the number of inhibitory synaptic terminals, and thereby increases the ratio of excitatory/inhibitory input. Moreover, *Ngn3* is downregulated by the binding of nerve growth factor (NGF) to its receptor p75, and correlative evidence indicates that the NGF–p75–NGN3 pathway might regulate the excitatory:inhibitory ratio in neurons.

Recently, the CaMKIV–cyclic AMP-response element binding protein (CREB) pathway, which regulates the development and plasticity of dendrites, has been shown to influence synapse formation[83,84]. The overexpression of a constitutively active form of CaMKIV or wild-type CREB in the hippocampus enhances long-term potentiation (LTP) at the CA1 synapse and increases the generation of new silent synapses. These synapses are dominated by NMDA (*N*-methyl-D-aspartate) receptor responses with little or no contribution from AMPA (α-amino-3-hydroxy-5-methyl-4-isoazolepropionic acid) receptors, and are considered to be potent substrates for LTP and experience-dependent modifications[85–87]. An important consequence of activation of the CaMKIV–CREB pathway might therefore be the generation of new synaptic contacts that can potentially serve as sites of learning and memory formation.

Finally, there is evidence that activity-dependent transcription is crucial for synaptic maturation. Most glutamatergic synapses start with a low AMPA receptor:NMDA receptor ratio and gradually acquire AMPA receptors during development. The increase in AMPA receptors depends on the NeuroD2 transcription factor[35]. NeuroD2-mediated transcription is calcium-regulated, and AMPA currents fail to develop in NeuroD2-null neurons. It remains to be determined whether this is mediated by a direct effect on the transcription of AMPA receptor subunits, or by an effect on a protein such as stargazin that regulates the synaptic trafficking of AMPA receptors.

These examples highlight the fact that transcription factors can exert a significant influence on synapse formation, stability and maturation. The evidence suggests that these factors act by regulating the responsiveness of neurons to neurotransmitters. It will be interesting to determine the mechanisms by which these factors control the distribution of neurotransmitter receptors, which is likely to be important both for synapse development and plasticity.

Silent synapses
Excitatory glutamatergic synapses that contain NMDA (*N*-methyl-D-aspartate)-type but no AMPA (α-amino-3-hydroxy-5-methyl-4-isoazolepropionic acid)-type glutamate receptors and as a result do not respond to stimulation at resting membrane potentials.

Conclusion

For much of the past decade, the investigation of molecular mechanisms of axon guidance and synapse formation was focused on proximal mediators, such as axon guidance molecules and synaptogenic factors. These molecules, however, cannot lead to the establishment of functional circuits without precise regulation of their spatial and temporal expression. Important advances in the last few years have revealed that a broad array of transcription factors serve as master regulators of genetic programmes that control axonal and dendritic patterning as well as synapse formation. It is likely that these transcription factors exert their influence by regulating the expression of genes that are directly involved in controlling the responses of neurons to guidance factors and neurotransmitters. Defining these relationships should be a major goal of the field and will greatly increase our understanding of the mechanisms by which transcriptional programmes regulate neuronal connectivity.

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Competing interests statement
The authors declare no competing financial interests.

DATABASES

The following terms in this article are linked online to:
Entrez Gene: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene
CTIP2 | EphB1 | ephrin B2 | GDNF | LMO4 | *Mef2a* | *Mef2d* | NGN2 | *Pea3* | TBX5 | *Vax2* | ZIC2

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