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Transcriptional control of long-range cortical projections

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Long-range projection neurons of the neocortex form the major tracts of the mammalian brain and are crucial for sensory-motor, associative and executive functions. Development of such circuits involves neuronal proliferation, specification and migration, as well as axonal elongation, navigation and targeting, where growing axons encounter multiple guidance cues and integrate these signals to execute guidance decisions. The complexity of axon guidance mechanisms in the formation of long-range neuronal projections has suggested that they might be under control of transcription factors, which are DNA-binding proteins that regulate the expression of downstream genes. Here we discuss recent advances in our understanding of the control of axon guidance by transcriptional regulation, as well as future directions for the elucidation of the mechanisms and pathological relevance of this process.

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Introduction

The specificity of long-range cortical connections, such as callosal, corticospinal and corticothalamic projections, arises through a developmental sequence of events, from neuronal birth and specification, to circuit formation and refinement. The correct establishment of these connections during development is fundamental for healthy brain function[1]. Indeed, it has been suggested that aberrant brain connectivity may be involved in the aetiology of several neurodevelopmental conditions, such as schizophrenia [2], attention-deficit hyperactivity disorder [3], mirror movements [4] and autism [5]. The elongation, navigation and final establishment of long-range axonal projections are sequential processes that require coordinated expression of genes encoding multiple guidance receptors and ligands, both within the axon and the cellular environment [6]. The orchestration of such complex events is thought to be under the control of master regulatory proteins that influence the transcription of downstream genes in a stage and cell specific manner to guide long-range projections to their targets. Here, we outline the latest advances and current challenges in our understanding of the transcriptional regulation of axon guidance during the formation of corticocortical, subcortical and corticothalamic projections.

Transcription factors as master regulators of development

The correct formation of brain circuits involves multiple processes, including cell proliferation, neuronal differentiation, migration, identity specification and axonal elongation of long-range projection neurons [7]. These steps are under the regulation of transcription factors, which act as master regulators of development [7]. The terms 'master regulator gene' or 'transcriptional regulator' have been increasingly used in the literature to define proteins that, by binding DNA directly or indirectly through the formation of protein complexes, regulate the expression of multiple downstream genes, which specify cellular fate [8]. As a result, when the expression of certain master regulators is altered in the neocortex, long-range projection neurons undertake an alternative fate and extend their axons to different targets, a process known as cellular re-programming or re-specification [8]. Considering the wide variety of roles that transcription factors play during cortical development, axonal guidance defects can be directly or indirectly caused by their altered expression. For example, impaired migration may result in neurons that are located in the wrong region of the brain and/or layer of the cerebral cortex, causing them to be exposed to different guidance molecules specific to that area and produce an aberrant pattern of connectivity. Other effects of mis-expressed transcription factors can include altered neuronal morphology or impaired expression of membrane molecules, which are both crucial for interactions

with neighbouring cells, synaptic partners and, ultimately, projection patterns [9]. A combinatorial expression of transcriptional regulators that controls downstream regulatory proteins can thus often characterise different populations of long-range projection neurons with a specific pattern of connectivity [10].

Transcriptional regulators delineate three types of neocortical long-range projection neurons

Circuit mapping experiments employing retrograde tracers have demonstrated three major types of excitatory long-range projection neurons in the neocortex: first, corticocortical projection neurons, which are located primarily in layers (L) 2/3 and 5 of the cortex, and extend their axons to the contralateral hemisphere through the corpus callosum, as well as to other areas of the same hemisphere [11,12,13]; second, subcerebral projection neurons, which occupy L5 and project to subcortical targets, such as the midbrain, hindbrain and spinal cord [14•,15•]; and third, corticothalamic projection neurons, located in L6, which send connections to reciprocal regions of the sensory thalamus [16,17•]. These neuronal populations are born at different stages of cortical development [18,19] (with the exception of L5 corticocortical and subcerebral neurons) and express different combinations of regulatory genes that trigger specific developmental programs of neuronal fate [10] (Table 1). For example, the appropriate ratio of corticocortical and subcerebral projection neurons located in the deeper layers of the neocortex is regulated by the expression of specific transcription factors, such as CTIP1 [20,21]. In the following sections, each of the three subtypes of neocortical long-range projection neurons and our understanding of the regulation of their axonal elongation and guidance by transcription factors will be discussed.

Corticocortical projection neurons

Corticocortical projection neurons extend their axons to the contralateral hemisphere through the corpus callosum and/or to other cortical regions within the same hemisphere [11,12,13]. Relatively little is known about the molecular specification of neurons that project exclusively within the same hemisphere, so here we describe the current understanding of transcription factor expression in interhemispheric callosal projection neurons. Callosal neurons located in both L2/3 and L5 are largely characterised by the expression of the transcription factor SATB2. When *Satb2* is knocked out in mouse, corticocortical axons fail to form the corpus callosum and instead reach subcortical targets via the internal capsule and the contralateral hemisphere through the anterior commissure [33••]. From early stages of development, L2/3 and L5 callosal neurons also express the transcription factor LMO4, which is crucial for neuronal differentiation [30], as well as CITED2, which acts in progenitor cells to establish callosal identity [22••,40]. In addition to this, L2/3 and L5 callosal neurons are characterised by the expression of LHX2, which specifies a callosal fate, especially during deeper layer neurogenesis [26], as well as TLE3, the precise actions of which are currently unknown [27••]. Some transcriptional regulators are also exclusively expressed in neurons located in L2/3, such as the transcription factors CUX1 and CUX2, which regulate callosal projections [37], dendritic branching, spine formation and synaptogenesis [38], as well as POU3F3 and POU3F2 (BRN1 and BRN2), which are crucial for neuronal positioning [41] and formation of callosal projections [39].

Subcerebral projection neurons

Subcerebral projection neurons form the main descending tracts of the neocortex, including corticotectal, corticobulbar and corticospinal projections, and are characterised by the expression of the transcription factors BCL6, CTIP2 (BCL11B), FEZF2 (FEZL), and SOX5 [14•,23,31•,34•,36]. These transcription factors are essential for corticofugal projection formation, such that knockout mice for these genes fail to form cortical

Table 1

Transcriptional regulators differentially expressed in long-range projection neurons, based on protein and/or mRNA detection. L, neocortical layer

Corticocortical projection neurons	Subcerebral projection neurons	Corticothalamic projection neurons
L2/3 and L5	**L5**	**L6**
Cited2[22••]	*Bcl6* [14•,23]	*Cxxc5*[24•,25]
Lhx2[26,27••]	*Bhlhe22* (*Bhlhb5*)[27••,28]	*Foxp2*[24•,27••,29]
Lmo4[22••,30]	*Ctip2* (*Bcl11b*)[14•,31•]	*Gse1*[24•]
Satb2[32•,33••]	*Fezf2* (*Fezl*)[17•,34•]	*Nfe2l3* (*Nrf3*)[27••]
Ski[35]	*Sox5*[14•,36]	*Tbr1*[17•]
Tle3[27••]	*Tcerg1l*[27••]	*Tle4*[24•]
	Zfp703[27••]	*Zfpm2* (*Fog2*)[24•]
L2/3		
Cux1[37,38]		
Cux2[22••,38]		
Pou3f2 (*Brn2*)[22••,39]		
Pou3f3 (*Brn1*)[22••,39]		

connections to the spinal cord, mainly as a result of impaired differentiation of precursor cells into subcortical projection neurons [14•,23,31•,34•,36]. Subcortical projection neurons are also characterised by the expression of the transcription factor BHLHE22 (BHLHB5), which is a postmitotic regulator of area identity; *Bhlhe22*-knockout mice show disorganisation of the barrels in the somatosensory cortex, as well as altered differentiation of corticospinal neurons in the motor cortex [28]. Some transcription factors, such as ZFP703 and TCERG1L, have been shown to be differentially expressed in subcortical projection neurons, but further experiments are required to fully elucidate their effect on projection fate [27••].

Corticothalamic projection neurons

Corticothalamic projection neurons are characterised by the expression of TBR1 and its downstream effector TLE4, with knockout mice for these genes lacking both the corticothalamic and the reciprocal thalamocortical projections [17•,24•,42]. Corticothalamic projection neurons also express FOXP2 [24•,29], and ZFPM2 (FOG2), which controls corticothalamic neuronal identity and axonal targeting by downregulating the expression of CTIP2 [24•]. Additional transcription factors, such as CXXC5, which is a known activator of myelin genes [25], GSE1 and NFE2L3 [24•,27••], are also expressed in corticothalamic projection neurons, and are interesting candidates for future studies investigating their role in corticothalamic tract formation.

Challenges to elucidating the mechanisms of transcriptional regulation of axon guidance

Despite the differential expression of transcriptional regulators in all three types of long-range projection neurons, as well as the altered axonal projection patterns in knockout mice for these genes, the mechanisms by which these proteins influence circuit formation are not fully understood. For example, most studies have identified a correlation between the expression of transcriptional regulators and axon guidance genes, without experimentally demonstrating their mechanisms of interaction [43]. This may be because transcriptional regulators often simultaneously control multiple processes during cortical development [44], and their mode of action is temporal, spatial, concentration and gradient dependent [45]. For example, the transcription factor SIP1 has been shown to play a role in several aspects of brain development, including the regulation of microtubule-depending axon guidance and branching in all three populations of long-range projecting neurons [46]. Similarly, CTIP1 is expressed in corticocortical and corticothalamic, but not subcerebral neurons [20], although it has broader roles in the cortex, such as the regulation identity of functional areas [21], and migration of upper layer neurons via negative regulation of Sema3c transcription [47]. Furthermore, the extent of differential expression and co-expression of transcription factors within neurons, such as CTIP2 and SATB2, changes throughout development [48], highlighting that a multifaceted network of gene interactions and mechanisms of feedforward activation and/or feedback repression can also regulate the temporal sequence of gene expression [49].

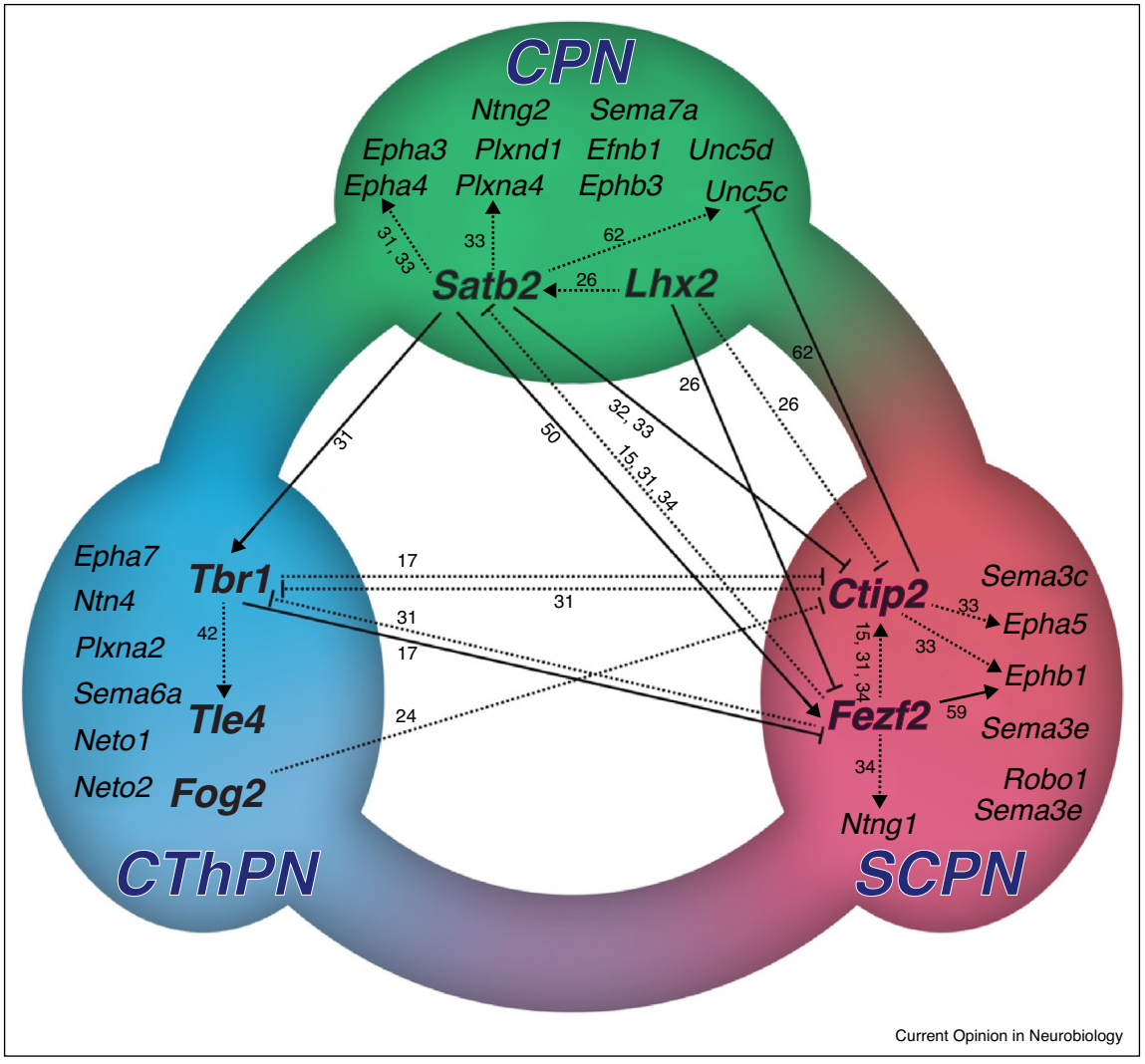
Interactions between transcriptional regulators

Transcription factors and associated proteins do not exert their effects in isolation, but rather are involved in an intricate regulatory network, with combinatorial expression ultimately specifying guidance factor expression and projection fate determination (Figure 1). For instance, the corticocortical transcription factor SATB2 represses the subcerebral gene *Ctip2*, thus inhibiting a subcortical fate in favour of a commissural one [33••]. TBR1 specifies corticothalamic projections, and can downregulate the subcerebral transcription factor *Fezf2* [17•], which in turn acts upstream of *Ctip2* and downregulates *Satb2* to inhibit a corticocortical fate [31•,34•]. In contrast, SATB2 has also been shown to upregulate the expression of *Fezf2* [50•]. This enables SATB2 to promote subcerebral as well as corticocortical projection fate in a cell context-dependent manner. Indeed, it has been shown that SATB2 plays a crucial role in the formation of subcortical projections when it is co-expressed with CTIP2 during early stages of cortical development [48,51•,52•]. Additional cofactors that may not directly bind to DNA, are also important regulators of gene transcription. For example, SKI is recruited by SATB2 to form the repressor complex that binds to regulatory regions of *Ctip2*, downregulating its expression and maintaining corticocortical identity [35]. Lack of SKI in SATB2-positive neurons causes these neurons to co-express CTIP2 and project to subcerebral targets [35]. This network is precisely regulated to ensure that a correct neuronal fate is attributed to neurons generated at specific stages of development [53].

Transcriptional regulation of axon guidance

Transcriptional regulators not only can affect axon guidance cell autonomously, such as for example by altering expression of axon guidance genes in a neuronal projection-specific manner [51•], but also in a cell non-autonomous way. Examples of the latter include controlling the development and positioning of guidepost cells, which guide axons to their appropriate targets by releasing cues in strategic positions [54,55], as well as the formation of tissue substrates for axonal growth [56]. Recent advances in understanding the cell autonomous transcriptional regulation of axon guidance were made by Molyneaux *et al.* (2015), who undertook a broad RNA-sequencing approach to molecularly characterise corticocortical, subcerebral and corticothalamic neurons, by sorting cells according to their exclusive expression of SATB2, CTIP2 and TLE4, respectively [27••]. Amongst the lists of genes involved in multiple processes, the authors discovered

Figure 1



Network of transcriptional regulators (bold) and axon guidance cue interactions. Continuous lines represent demonstrated gene interactions, while dashed lines represent suggested interaction based on knockout experiments. Flat-headed arrows: downregulation; normal arrows: upregulation. CPN: callosal projection neurons, CThPN: corticothalamic projection neurons, SCPN: subcerebral projection neurons. Reference numbers supporting each interaction are indicated.

axon guidance cues that are differentially expressed among these three populations (Figure 1, Table 2) [27••]. This approach can be combined with previous studies demonstrating interactions between transcriptional regulators and axon guidance cues to find new candidates that might be involved in the specification of cortical projections (Table 2).

Some of the axon guidance genes that were specifically expressed in one of the three groups of projection neurons have previously been shown to be regulated by the transcription factor used to isolate these populations [22••,33••] and/or have been shown to play an important role in the specification of projection fates (Figure 1, Table 2). For example, axon guidance genes that were highly enriched in SATB2-positive callosal neurons, such as *Unc5c* (or *Unc5h3)*, *Plxna4*, and *Epha4* [27••], were previously shown to be downregulated in *Satb2*-knockout animals, confirming that they are directly or indirectly regulated by SATB2 (Figure 1) [33••]. In addition to this, *Unc5c* and *Epha4* have also been shown to be necessary for callosal development and restoring their expression in *Satb2* mutants partially rescues the formation of cortico-cortical connections via the corpus callosum [31•,62•]. Other genes that were reported to be highly enriched in SATB2-positive neurons, such as *Efnb1* and *Ephb3*

Table 2

Summary of axon guidance genes differentially expressed by corticocortical (SATB2^{high}CTIP2^{low}TLE4^{low}), subcerebral (SATB2^{low}CTIP2^{high}TLE4^{low}) and corticothalamic (SATB2^{low}CTIP2^{med}TLE4^{high}) projection neurons (adapted from Molyneaux et al. 2015). Some of these genes have been shown to be directly or indirectly regulated by transcriptional regulators. In addition to this, some are crucial for the formation of the axonal tract corresponding to the neuronal population where they are highly enriched, while others control the formation of different long-range circuits. See text and references for further details

Gene	Corticocortical neurons	Subcerebral projection neurons	Corticothalamic projection neurons	Notes
Efnb1	High	Low	Low	Crucial for callosal formation [57]
Efnb2	High	Low	Low	Crucial for callosal formation, elevated expression in Satb2$^{-/-}$ [33••,57]
Efna4	High	Low	Low	Unknown role in callosal formation
Epha3	High	Low	Low	Unknown role in callosal formation
Epha4	High	Low	Low	Crucial for callosal and subcortical tract formation, reduced expression in Satb2$^{-/-}$ [33••,57]
Epha5	Low	Medium	Medium	Unknown role in subcortical projection formation, elevated expression in Satb2$^{-/-}$ [33••]
Epha7	Low	Medium	High	Crucial for corticothalamic projection formation [33••,58]
Ephb1	Low	High	High	Crucial for subcortical and corticothalamic projection, elevated expression in Satb2$^{-/-}$ [33••,59•]
Neto1	Low	Medium	High	Unknown role in corticothalamic projection formation
Neto2	Low	Low	High	Unknown role in corticothalamic projection formation
Ntn4	Low	Low	High	Unknown role in corticothalamic projection formation
Ntng1	Low	High	Low	Highly expressed in corticospinal neurons [14•,34•,60]
Ntng2	High	Low	Low	Unknown role in callosal formation
Plxna2	Low	Medium	High	Unknown role in corticothalamic projection formation
Plxna4	High	Low	Low	Unknown role in callosal formation, reduced expression in Satb2$^{-/-}$ [33••]
Plxnd1	High	Low	Low	Unknown role in callosal formation
Robo1	Low	High	Low	Involved in the specification of corticofugal, thalamocortical, and corticocortical projections [61]
Sema3c	Low	High	Low	Unknown role in subcortical projection formation
Sema3e	Low	High	Low	Unknown role in subcortical projection formation
Sema6a	Low	Medium	High	Unknown role in corticothalamic projection formation
Sema7a	High	Low	Low	Unknown role in callosal formation
Unc5c	High	Low	Low	Crucial for callosal and subcortical tract formation, reduced expression in Satb2$^{-/-}$ [33••,62•,63]
Unc5d	High	Low	Low	Unknown role in callosal formation

[27••], have not yet been investigated in *Satb2*-knockout animals, and their transcriptional regulation remains unknown. However, they represent excellent candidates for investigating their regulation by SATB2, as previous experiments have shown abnormal callosal projections in the absence of these guidance cues [57].

Interestingly, a few axon guidance cues shown to be upregulated in *Satb2*-knockout animals promote subcerebral projections, likely due to the increased expression of CTIP2 [33••] (Figure 1). Due to the feedback loop in the network that regulates the expression of transcription factors, it is unclear whether the expression of these specific axon guidance cues is a consequence of the loss of SATB2 or of the upregulation of CTIP2. Some genes, such as *Epha5* and *Ephb1*, were found to be highly enriched in CTIP2-positive neurons [27••], suggesting that they may be directly or indirectly regulated by CTIP2. Moreover, *Ephb1*, which regulates the formation of the corticospinal tract [59•], is under direct transcriptional control of FEZF2 [59•], which also controls, directly or indirectly, the expression of *Ntng1* [34•]. Although *Ntng1* is highly enriched in CTIP2-positive neurons [27••], as well as in retrogradely labelled corticospinal neurons [14•], little is known about its role in corticospinal tract formation, however it has been suggested to promote thalamocortical axonal outgrowth [60]. Relatively less is known about the transcriptional regulation of axon guidance genes involved in the formation of the corticothalamic tract. However, some of the genes highly enriched in TLE4-positive neurons, such as *Epha7*, play a critical role in the guidance of corticothalamic axons, with altered cortical expression of *Epha7* causing a disruption of their topographic organisation [58,64].

Sorting long-range projection neurons based on their differential expression of markers or retrograde tracers has generated important lists of genes that may be directly or indirectly controlled by transcriptional regulators. These approaches alone, however, cannot completely shed light on our understanding of the transcriptional control of axon guidance. First, they exclude regulatory networks that are shared across neuronal populations. For instance, the transcription factor EMX1 is ubiquitously expressed in pyramidal cortical neurons and

affects midline crossing of callosal axons via upregulation of the guidance receptor *Nrp1* [65]. Similarly, the expression of other callosal axon guidance genes, such as *Slit1*, *Sema5b* and *Unc5a*, is under control of the transcription factor NEUROD [66]. Second, sorting cells by transcription factor expression may introduce artefacts, as these populations are often very heterogeneous. For example, most of the cells expressing CTIP2 in the upper layers and up to 40% of CTIP2-positive cells in the deeper layers are GABAergic interneurons [67]. Therefore, more studies that incorporate additional classification methods are required to fully elucidate the contribution of transcriptional regulators, including their precise mechanisms of spatially- and temporally-specific action, in the guidance of long-range projections.

Conclusion

The axons of long-range projection neurons that make up the major white-matter tracts of the brain are guided to their targets through a succession of choice points involving several axon guidance cues. Transcription factors are thought to play an important role in this process by controlling the expression of myriad guidance genes, but their mechanisms of action have only recently begun to be systematically investigated. For example, several studies have found differential enrichment of multiple axon guidance genes in corticocortical, subcerebral and corticothalamic neurons, suggesting that axonal projection phenotypes might be under tight transcriptional control [14•,22••,33••]. However, only a few axon guidance genes have been shown to be specific for a single cell-identity [27••], and most of those that are highly specific are known to play additional roles in different brain regions, demonstrating that the same signalling pathways can be involved in the formation of distinct axonal tracts [68] (Table 2). Therefore, further research is required to elucidate the genetic regulation of axon guidance during neocortical development.

Current, emerging approaches will enable further discovery in this field. Single-cell RNA sequencing experiments have provided important datasets of gene expression that define neuronal populations in the cortex of mice [69,70,71,72] and humans [73,74]. However, most cortical datasets currently available come from tissue at later developmental stages, after completion of neural circuit formation, and many contain only a few dozen cells per category. While this is a common limitation of the single-cell transcriptomic approach, a recent study sequenced around 690,000 individual cells from the adult mouse brain [72]. Such a large dataset would seem suitable for further gene discovery, however embryonic brain stages would be required to further examine the transcriptomic regulation of axon guidance. To further advance the field, conditional knockout mouse models or reporter mice that label specific cell types expressing (or mis-expressing) a particular transcription factor could be used, followed by cell sorting and transcriptome sequencing at different stages of development. Similarly, chromatin immunoprecipitation and sequencing (ChIP-seq) allows the identification of DNA-binding sites of specific transcription factors to explore their downstream effectors and elucidate their mechanisms of action [9,66]. Transgenic reporter mice have also been particularly effective in identifying regulatory elements required for gene expression that sub-delineate specific cell populations even further [75]. These methods, coupled with identification of downstream targets, will allow a more precise understanding of the regulation axon guidance molecules by transcription factors.

Finally, extracellular and epigenetic mechanisms, such as electrical activity [76], chromatin, histone and DNA modifications [77], cis-acting regulation via promoters, silencers and enhancers [78], as well as post-transcriptional [79] and post-translational [80] modifications, can also affect the expression of regulatory proteins at specific time points of cortical development. Each step in the genetic and epigenetic regulation of axon guidance is required for the correct establishment of brain circuits, and defects in any of these could lead to neurodevelopmental disorders. Understanding the precise network of interactions within spatial, temporal and functional contexts will substantially enhance our knowledge of the development of brain connectivity in health and disease.

Conflict of interest statement

Nothing declared.

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