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From *Trans* to *Cis*: Transcriptional Regulatory Networks in Neocortical Development

Mikihito Shibata¹, Forrest O. Gulden¹, and Nenad Sestan^{1,2}

¹Department of Neurobiology and Kavli Institute for Neuroscience, Yale School of Medicine, New Haven, Connecticut 06510, USA

²Department of Psychiatry and Program in Cellular Neuroscience, Neurodegeneration and Repair, Yale School of Medicine, New Haven, Connecticut 06510, USA

Abstract

Transcriptional mechanisms mediated by the binding of transcription factors to *cis*-acting regulatory DNA elements play critical roles in directing gene expression. While transcription factors have been extensively studied, less effort has gone towards the identification and functional characterization of *cis*-regulatory elements and associated epigenetic modulation. However, due to methodological and analytical advances, more comprehensive studies of regulatory elements and mechanisms are now possible. Here we summarize recent progress in integrative analyses of these regulatory components in the development of the cerebral neocortex, the part of the brain involved in cognition and complex behavior. These studies are uncovering not only the underlying transcriptional regulatory networks, but also how these networks are altered across species and in neurological and psychiatric disorders.

Keywords

transcription factor; *cis*-regulatory element; chromatin; epigenetics; gene expression; neuron; disease

cis-regulatory elements are integral components of transcriptional regulatory networks

A fundamental issue in developmental biology is how the multitude of structures necessary for a functioning organism arise from the shared genomic heritage of a single zygote [1]. The transformation from a zygote to an adult organism is composed of a series of developmental events that each rely on the precise spatio-temporal regulation of gene expression [1–3]. These programs are, in large part, mediated by *trans*-acting factors [i.e.,

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Contact information: Nenad Sestan, MD, PhD, 333 Cedar Street, SHM C323-C, New Haven, CT 06510, Office: (203) 737-2190; Lab: (203) 737-1435, nenad.sestan@yale.edu, Lab website: www.sestanlab.org.

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transcription factors (TFs)] that bind specific genomic sequences [4, 5] in *cis*-regulatory elements (CREs). According to their structural and functional features, TFs can be categorized into many distinct families [4]. Depending on their function, CREs can be divided into functionally different classes, with the proximal promoters, enhancers, insulators and silencers being the most widely studied [6–10]. While other regulatory elements operating in *trans*- or in *cis*-, including but not limited to chromatin remodelers, RNA-binding proteins, regulatory RNAs, and *cis*-regulatory RNA elements also exist and function at the transcriptional or post-transcriptional level [11], the complex sets of interactions among TFs and CREs form the central core of transcriptional regulatory networks (TRNs) (BOX 1) [1].

Box 1

Core classes of *cis*-regulatory elements in DNA

Cis-regulatory elements (CREs) in genomic DNA can be divided into functional classes, with promoters, enhancers, insulators, and silencers being the core components that have historically been the most widely studied. Promoters may be **proximal promoters**, located near the transcription start site and occupied by the primary transcriptional machinery, or **distal promoters**, bound by TFs but having a weaker regulatory influence. In either case, the promoter serves as a scaffold where general TFs such as TAF1 bind to initiate transcription. Besides RNA polymerase II, active promoters may be associated with histone H3 trimethylated lysine 4 (H3K4me3) or other markers of promoter activity [114].

Enhancers, silencers, and insulators can influence target gene transcription more globally. **Enhancers** may facilitate gene expression by serving as scaffolds to recruit both TFs and proteins with histone acetyltransferase activity. This allows the decondensation of chromatin, permitting the general transcription machinery greater access to promoters. In contrast to enhancers, **silencers** and **insulators** act as negative regulators of gene expression. These regulatory elements may prevent the spread of heterochromatin or prevent the activation of promoters unrelated to nearby enhancers. The effects of these CREs are orientation independent and may be temporally or tissue specific.

As with promoters, there are a number of markers for active enhancers, silencers, and insulators. For example, active enhancers may be associated with H3.3 [115], H2A.Z [7], H3K27ac [115], H3K4me1, or other markers. Among the markers for active silencers and insulators are REST [116], SUZ12 [117] and CCCTC-binding factor (CTCF) [118].

Super enhancers consist of clusters of enhancers spanning up to 50 kb, are densely occupied by master TFs and Mediator complexes, and play crucial roles in defining cell identity in many differentiated cell types including mouse progenitor B cells, T helper cells, and macrophages [112, 119]. Super enhancers may also activate the expression of master TFs and other genes that play important roles in cell type specification, potentially making the expression of genes regulated by super enhancers sensitive to perturbations impacting master TFs or Mediator complex.

Numerous other regulatory components exist. Other cis-elements affecting gene expression, if not always transcription, are 3' untranslated regions, splicing regulatory elements, and epigenetic factors including DNA methylation, histone modifications, chromosomal conformation, and chromatin state. Additional trans-regulatory elements include regulatory RNAs (i.e. miRNAs, lincRNAs and so on), RNA-binding proteins, splicing factors, and chromatin modifiers/remodelers.

Interactions between TFs and CREs are complex, dynamic, and modulated by various epigenetic processes [12]. While individual CREs may be regulated by the competitive or cooperative binding of many TFs, multiple CREs may also confer transcriptional control over the expression of any single gene [12]. Furthermore, TFs and CREs regulate transcription together with their cognate transcriptional co-factors, chromatin regulators, epigenetic modifications, RNA binding proteins, and non-coding RNAs [2, 13]. This combinatorial and multi-level transcriptional regulation greatly increases the complexity of gene expression regulation, facilitating the unique spatio-temporal patterns necessary for development and function [3].

Researchers have long recognized the importance of TFs in development, evolution, function and pathogenesis. Although the contributions of CREs have also been explicitly recognized, our understanding of their key biological roles and associated TRNs has been more elusive due to the difficulties associated with their systematic identification and functional characterization. Fortunately, thanks to a number of recent methodological and analytical advances, more comprehensive and functionally relevant studies of CREs are now possible in various complex tissues and physiological contexts (BOX 2).

Box 2

Advances in the study of transcription factors and *cis*-regulatory elements

Improving global scientific infrastructure has facilitated the attainment, preservation, and dispersal of organisms and tissues, including human and non-human primate brains, previously not easily available to the scientific community. This has allowed comparative genomics to flourish, leading to the identification of potential CREs through the recognition of sequence conservation across species [120, 121]. Similarly, improvements in tissue and cell processing methods have allowed the isolation of cells and discrete morphological areas on a level previously impossible [105, 122–126]. This has aided the creation of high-quality databases profiling the genome, transcriptome, epigenome, and regulome of multiple neural cell types, tissues, developmental periods, species, and diseases [10, 96, 125, 127] [97, 105] (see www.genome.gov/encode/; www.1000genomes.org; www.roadmapepigenomics.org; www.gtexportal.org/; www.brain-map.org; www.genepaint.org; enhancer.lbl.gov; www.brainspan.org and www.commonmind.org).

Technological advances have also facilitated the discovery and characterization of CREs. While the increasingly long lists of TFs annotated for their involvement in any given development process has provided progressively more “bait” for chromatin immunoprecipitation (ChIP) assays [23, 128], the ability to couple ChIP with next

generation sequencing, as in ChIP-seq, has allowed global assessments of CRE activity. The use of ChIP-seq using more general markers associated with specific CREs, such as EP300 (also known as p300), RNAP II (Pol II), and histone H3 acetyl Lys27 (H3K27ac), has also allowed these global searches for CREs to be performed independently of knowledge of (or antibodies against) TFs [10, 12, 96, 129] [130]. Recently developed techniques for mapping chromosomal interactions over long distances, such as ChIA-PET (chromatin-interaction analysis with paired-end tag sequencing), couple ChIP-seq with assays that identify both the distal and proximal gene regulatory elements of promoters by tethering conformationally related chromosomal regions [131–133]. Taken together, these advances allow both the identification of hundreds of thousands of CREs and novel opportunities to correlate promoter and enhancer occupancy with gene expression.

With the identification of so many regulatory elements has come an increased ability to validate and functionally characterize those elements. The Massively Parallel Reporter Assay (MPRA) and other similar approaches allow the rapid validation of hundreds of putative CREs in a single experiment [134]. Once validated, CREs can be functionally characterized through the use of BAC transgenesis [69], site-directed mutagenesis by CRISPR/Cas technology [135, 136], or other techniques drawn from an expanding molecular toolkit.

Among the most complex biological tissues, with a myriad of cell types and a very protracted development, is the cerebral neocortex. The neocortex is a portion of the brain unique to mammals, expanded in humans, and critical for cognition, perception and complex behavior [14–16]. The development of the neocortex, and consequently its function, depends on the highly orchestrated regulation of gene expression. Examples of the contributions of TFs to the developing neocortex, and to neurological and psychiatric disorders, are abundant [17–20], but, as is true for many complex biological systems, knowledge concerning CREs and their integration with TFs and other regulatory components into TRNs has lagged far behind.

Here we summarize recent progress in the study of selected key TFs, CREs, and associated regulatory processes in the control of gene expression underlying the development, evolution, and pathogenesis of the neocortex (BOX 3).

Box 3

Epochs of neocortical development

The vast majority of cells occupying the adult neocortex originate either from radial glia (RG) in the ventricular zone (VZ), intermediate progenitor cells (IPCs) in the subventricular zone (SVZ), or progenitor pools located within the ganglionic eminences. In these proliferative niches, multiple TFs act to maintain progenitor cells in a proliferative state. Other TFs promote the production of differentiated neurons, glia, and astrocytes. The mechanisms mediating the choices **between proliferation and differentiation and neurogenesis and gliogenesis** are tightly regulated.

As nascent neurons exit mitosis, they **migrate** from these proliferative niches to their ultimate destinations. Interneurons arising from the ventrally located ganglionic eminences tend to migrate tangentially along cortical layers. Cells arising in the VZ or SVZ of the dorsal telencephalon migrate radially along a radial glial scaffold and deposit themselves in a time-dependent manner. Preplate neurons, including Cajal-Retzius cells, are born first. These cells secrete Reelin, an instructive guidance molecule for the formation of neocortical layers. Newly born neurons migrate radially, first splitting the preplate and forming the cortical plate between the marginal zone and the subplate. Subsequently born neurons migrate into the cortical plate in an inside-out manner as progressively younger neurons migrate past their older siblings. At the end, later-born, superficial-layer neurons reside in layers 2 through 4 and earlier-born deep-layer neurons in L5–6. A schematic depicting this process can be seen in Figure 1.

Cellular diversification and differentiation generates the wide diversity of neuronal types present in the adult neocortex. While strong evidence for the prepatterning of cells within the ventricular zone exists, many of the TFs implicated in the development of specific cell fates only begin to be expressed as cells complete their terminal mitosis. At this time cells may express TFs essential for the attainment of multiple distinct cell fates. However, through a series of feedback mechanisms including mutual cross-repression, cells ultimately settle on a final identity. An example of a transcriptional regulatory network involved in cell type specification is shown in Figure 2.

Additionally the **assembly and function of neuronal circuits** is essential for normal function. Like differentiation, the genetic and molecular components essential for both synaptogenesis and connectivity may be expressed during or be influenced by earlier developmental epochs. Other factors, including extracellular stimuli, can influence the expression of TFs, the activity of CREs, and the function of TRNs.

Transcriptional regulation of neocortical neurogenesis and gliogenesis

The neocortex is mainly composed of a myriad of neuronal and glial cell types. Neurons are broadly classified into two major groups, glutamatergic excitatory projection neurons (also known as pyramidal neurons) and GABAergic inhibitory local circuit interneurons, which follow substantially different developmental programs [17–23]. Projection neurons, which account for approximately 80% of neocortical neurons, originate from stem/progenitor cells within the neocortical wall in the dorsal forebrain (Fig. 1), whereas interneurons arise primarily from progenitor cells in the ventral forebrain and migrate tangentially into the cortex. Glial cells, classified mainly as astrocytes, ependymal cells, and oligodendrocytes, also originate from the same lineage of neural progenitors [24].

Mediating the transitions between proliferation and differentiation of neural progenitor/stem cells in the ventricular zone (VZ) and subventricular zone (SVZ) is essential for determining neocortical size and other aspects of normal development [14]. A number of TFs have been implicated in controlling the onset, progression, and termination of neocortical neurogenesis. Among these, EMX1, EMX2, FOXG1, HES1, HES5, LHX2, and PAX6 are highly expressed by cortical progenitor cells throughout neurogenesis and have been shown to play

critical roles in promoting the maintenance of a progenitor state [25–27] [28, 29]. Other TFs, such as FEZF2 (FEZL or ZFP312), ID4, NGN1, NGN2, and NR2E1 (TLX) are enriched in early progenitor cells [30–32], whereas TFAP2C (AP2-gamma), CUX1, CUX2, POU3F3, POU3F2 (BRN1 and BRN2, respectively) and TBR2 are enriched in later progenitor cells [31, 33–36]. Each of these TFs may promote neurogenesis in some developmental contexts. For example, several TFs, such as INSM1, NGN2, TBR2, and TFAP2C, have been shown to promote the generation of basal progenitors in the SVZ from VZ progenitor cells [33, 36–38].

Knowledge of the CREs involved in these processes is essential for understanding how these suites of TFs are integrated to promote the appropriate developmental pathways.

Unfortunately, the known repertoire of CREs active in different types of cortical progenitor cells during neurogenesis is largely incomplete and ones that have been functionally characterized in this context are rare. One well-studied example of such a CRE concerns the seemingly paradoxical ability of PAX6 to promote both the expression of genes for progenitor self-renewal as well as those for neuronal differentiation. These conflicting abilities are resolved in part through a low-affinity enhancer, E1, of *NGN2* [39]. When concentrations of PAX6 are low, PAX6 is unable to bind E1 and does not induce the expression of the proneural gene *Ngn2*. Instead, PAX6 induces the expression of genes promoting self-renewal while HES1 directly represses several genes that promote differentiation [40]. However, when levels of PAX6 are higher, PAX6 is able to bind E1 and induce the expression of *NGN2*. Other TFs, including FEZF1 and FEZF2, are subsequently induced and directly repress *Hes1* and the related *Hes5* [41]. This shifting ratio between PAX6 and HES1 is directly tied to neurogenesis [40].

Other examples of regulatory elements mediating neurogenesis also exist. The BAF (mSWI/SNF) chromatin remodeling complex is involved in regulating the size and thickness of the neocortex [42] and the ability of PAX6 to bind to specific CREs and regulate downstream target genes is modulated by this complex during adult neurogenesis [43]. These studies suggest that epigenetic mechanisms such as chromatin remodeling [11] may play a critical role in the transcriptional regulation of neurogenesis throughout development. In addition, specific non-coding RNAs have been shown to regulate cortical neurogenesis by targeting multiple key TFs [44, 45], providing a regulatory feedback mechanism and another layer of complexity.

TFs and CREs also control the switch from neurogenesis to gliogenesis that generally occurs later in development. Here again, key examples highlight the importance of epigenetic regulation, such as DNA methylation, in controlling transcriptional regulatory interactions and gene expression. During neocortical neurogenesis, promoters of astrocyte-specific genes, such as *glial fibrillary acidic protein (Gfap)*, are heavily methylated and silenced [46]. Astrocyte fate is impeded by NGN1, which promotes neuronal fate by competing with STAT3 for EP300-SMAD activator complexes [47]. However, at the end of neurogenesis, the *Gfap* promoter is demethylated to facilitate both astrocyte differentiation and the transformation of the radial scaffold of progenitor cells into glial progenitors, differentiated astrocytes, and ependymal cells [46]. This is possible because the suppression of NGN1 expression, coupled with the demethylation of promoters of astrocyte-specific genes begun

by NFIA [48], allows the JAK-STAT3 and BMP pathways to initiate the expression of pro-astrocytic genes.

Another major class of glial cells, oligodendrocytes, develop from proliferating oligodendrocyte precursor cells that arise in proliferative zones in multiple regions of the ventral and dorsal forebrain, migrate throughout the developing white matter, and divide a limited number of times before they terminally differentiate. Among the TFs that promote oligodendrocyte specification and maturation are OLIG1, OLIG2, SOX10, YY1, MRF, and ZFP219 [49, 50]. Interestingly, DLX1 and DLX2, TFs necessary for interneuron fate specification, and OLIG1 and OLIG2 control neuronal versus oligodendroglial cell fate acquisition in the developing ventral forebrain [51]. DLX1/2 promote neurogenesis at the expense of the oligodendrocyte lineage through repression of *Olig2* expression [51, 52]. By contrast, OLIG1 directly binds to the *Dlx1/2* I12b intergenic enhancer to repress *Dlx1/2* expression, leading to the acquisition of an oligodendrocyte fate [52].

Transcriptional regulation of neuronal migration

Following neurogenesis, nascent projection neurons move toward the dorsal (pial) surface of the developing brain to ultimately form the six-layered architecture of the mature cerebral neocortex. Several TFs implicated in other epochs of neuronal development, including SOX5, TBR2, NGN1, POU3F3, and POU3F2, are also involved in the radial migration of nascent projection neurons in the dorsal telencephalon [53–55]. Presumably, these TFs regulate the expression of genes involved in cytoskeletal dynamics and other aspects of cell movement, but until recently the details of this regulation have remained unknown. Recent work concerning a CRE of *RND2* offers insight into these processes.

Rnd2 is a gene encoding a rhoA-like GTPase involved in regulating cytoskeletal dynamics and implicated in neuronal migration [56]. The expression of *RND2* is regulated by competitive binding of NGN2 and ZBTB18 (ZFP238 or RP58) to a CRE in the 3' region of *Rnd2*. In the absence of NGN2 binding, *RND2* is not expressed and the natively *RND2*-expressing neurons are incapable of migrating from the SVZ to the cortical plate [57]. In contrast to NGN2, ZBTB18 represses *Rnd2* [58]. Neurons depleted of *Zbtb18*, which show an upregulation of *Rnd2*, also cannot migrate from the SVZ due to the failure of the multipolar-bipolar transition [59]. Furthermore, NGN2 activates *Zbtb18* transcription by binding to its promoter [60], whereas ZBTB18 represses *Ngng2* [59, 61]. NGN2 and ZBTB18 therefore form a feedback loop operating through a shared CRE to fine-tune the expression of *Rnd2*, thus controlling the radial migration of projection neurons.

Transcriptional regulation of neuronal diversification and differentiation

The organization of the neocortex into functionally distinct layers and areas is rooted in the diversification of developing projection neurons into distinct subtypes. The early progenitor pool gives rise to subplate (SP) and deep-layer (L5–6) neurons, whereas the late progenitor pool generates upper-layer (L2–4) neurons (Fig. 1). Further diversification into many subtypes occurs during the early postmitotic and postmigratory life of projection neurons (Fig. 1) [23, 54, 62–65].

Studies of laminar position and cortical projection neuron lineage specification illustrate both the importance of the spatial distribution of relevant TFs and the ability of CREs to integrate the effects of these TFs. These processes are also modulated by a number of other transcriptional and post-transcriptional regulatory processes. For example, FEZF2, which is enriched in L5 and, to a lesser extent, L6 projection neurons, is required for specifying subcerebral (corticofugal) projection neurons, including corticospinal neurons [66–70]. Acting along with FEZF2 in immature L5 corticospinal neurons are members of the SOX family of TFs (SOX4, SOX5, and SOX11) as well as BCL11B (CTIP2) (Fig. 3) [54, 69, 71, 72]. Similar suites of TFs exhibiting layer- and subtype- specific expression profiles are expressed by developing projection neurons in L6 (SOX5, TBR1, and NR2F1), L4 (NR2F1, LHX2, CUX1/2, and BHLHB5), and L2–4 (CUX1/2, BHLHB5, and SATB2).

Knowledge of the transcriptional interactions, CREs involved, and epigenetic machinery is necessary for understanding the cell-type specific expression of these TFs. SOX5 is required for L5/6 projection neuron migration and specification, but it also represses *Fezf2* in L6 corticothalamic projection neurons through direct repression mediated by the E4 enhancer near *Fezf2* [54, 71] (Fig. 3A,B). Conversely, SOX4 and SOX11 compete with SOX5 for the activation of the E4 enhancer [69] (Fig. 3A, B). The *Fezf2* locus is also bound by TBR1, further promoting the repression of *Fezf2* in L6 neurons [23, 73] (Fig. 3B). In turn, FEZF2, among other transcription factors including BCL11B, represses the *Tbr1* locus (Fig. 3A). SATB2, another DNA-binding protein expressed by immature neurons across several cortical layers whose expression is indirectly regulated by FEZF2, activates the expression of TBR1 as well as suppresses the expression of BCL11B (Fig. 3C,D) [70, 74].

A problem inherent to the “closed” TRN described above, however, is that it must in some way be broken to allow the transition from the production of deep layer neurons to the production of upper layer neurons. This is at least partially mediated by the TF FOXG1. FOXG1 is expressed in neocortical progenitors and upper layer neurons and directly suppresses TBR1 expression by binding the 5′ region of the *Tbr1* locus. This results in the upregulation of deep layer genes including *Fezf2* and *Bcl11b*. The deep layer neurons then themselves produce a cell-extrinsic signal that, coupled with the repression of *Tbr1* by FOXG1, makes progenitors competent to differentiate into upper layer neurons [75].

The cell-type-specific and sustained expression of TFs necessary for the specification, development, and later maintenance of neuronal cell types is mediated at least in part by epigenetic means [76]. This is also true for cortical projection neurons. For example, BCL11B, which is expressed by the subcerebral projection neurons in L5/6, is a recently identified subunit of the BAF chromatin remodeling complexes [77]. As such, it likely cooperates with other TFs to target distinct chromatin loci containing CREs involved in the cell-type specific activation or repression of gene expression. A different example involves the interaction of AF9 with DOT1L histone H3 methyltransferase, a protein that mediates the methylation of histone H3 lysine 79 (H3K79) at the transcription start site of *Tbr1* [78]. This epigenetic modification suppresses *Tbr1* expression in upper layer neurons and promotes the fate maintenance of the intracerebral projection neurons in those layers. Similarly, SATB2 is itself a chromatin modifier and binds directly to matrix attachment

regions. This allows SATB2 to recruit histone deacetylases and silence the expression from loci including *Bcl11b* [62, 63, 79].

OLIG1/2, discussed earlier as TFs necessary for gliogenesis, also play critical roles in oligodendrocyte differentiation. For example, OLIG2 transcriptionally pre-patterns the chromatin-remodeling factor SMARCA4 (BRG1) [80]. Other studies have also linked DNA methylation and chromatin modifications to glial differentiation and maintenance [81] and such findings will only increase in frequency as transcriptomes and epigenomes are compiled in parallel from diverse neuronal cell types.

Interneurons are subdivided into several types depending on their origin and molecular markers. Parvalbumin (PVALB)- and somatostatin (SOM)-positive interneurons are generated from the MGE and calretinin (CALB2 or CR)-positive interneurons arise from the caudal ganglionic eminence (CGE). A number of TFs have been implicated in controlling the specification and differentiation of these interneurons, including NKX2.1 (TITF1), ASCL1 (MASH1), LHX6, SOX6, NR2F1 and NR2F2 (COUP-TF1 and COUP-TF2, respectively), and members of the *DLX* family [21, 82, 83].

Whereas NKX2.1, LHX6, SOX6, NR2F1, and NR2F2 play discrete roles in the specification of different subtypes of interneurons, *DLX1/2/5/6* and *ASCL1* are important for the broad specification and differentiation of interneurons. For example, three CREs of *DLX1*, called URE2, I12b, and I56i, are associated with different subtypes of interneurons [84]. URE2 is active in PVALB/CALB2/NPY/NOS1-positive interneurons, whereas I12b and I56i are specifically active in SOM/VIP/CALB2-positive interneurons. This suggests distinct combinations of *Dlx1* enhancers are used for the specification of interneurons. Taken together, these examples demonstrate that a combination of TFs, CREs, epigenetic modifications, and extrinsic signals function to first specify and then maintain diverse neural cell types.

Transcriptional regulation of the assembly and function of neural circuits

Developmentally regulated changes in TRNs continue long after neurogenesis has subsided and distinct cell fates have been determined. Sensory experience-driven synaptic activity during postnatal development initiates distinct transcriptional regulatory programs, allowing cellular functions and processes like synaptogenesis to be adapted to the environment [85, 86]. For example, BDNF is a member of the neurotrophin family and is implicated in neuronal survival, differentiation, neurite outgrowth, and synaptic plasticity. Some of the CREs of the *Bdnf* gene are bound and activated by CREB, ARNT2, NPAS4, CARF, and USF1, all proteins whose ability to induce gene expression is regulated by membrane activity [87].

Activity-dependent regulation is not unique to the *BDNF* gene. CREB-binding protein (CREBBP or CBP) is a DNA-binding transcriptional co-activator associated with enhancer activity that enables the general transcriptional machinery to access promoters, thus facilitating gene expression. Upon membrane depolarization, the number of CBP binding sites in the genome increases from approximately 1,000 to approximately 28,000 [88]. This includes, as assessed by the presence of the enhancer marker histone H3 lysine 4

monomethylation (H3K4me1), approximately 12,000 activity-regulated enhancers. Many of these enhancers are bound by FOS [85]. Greatly increased binding of CREB, NPAS4, and SRF to both enhancers and promoters is also observed. The activity of multiple TRNs of potentially thousands of genes may therefore be regulated by synaptic activity and, by extension, the cellular environment and experience. Additionally, here again knowledge of the CREs and regulatory mechanisms involved is necessary for understanding patterns of gene expression.

Evolution of neocortical *cis*-regulatory elements

Although the neocortex is present in all mammals, there are substantial variations in its development and organization that may underlie some species-specific aspects of cognition and behavior. Changes in coding sequences undoubtedly contribute to the phenotypes unique to any given species [89–91], but a significant portion of that uniqueness may be due to changes in TRNs including CREs and other regulatory components [92–94]. Moreover, changes in CRE sequence and activity have been linked with evolution across mammals [69, 92, 93, 95]. For example, only 58% of loci occupied by the enhancer-associated proteins EP300/CBP in mid-fetal human neocortex were similarly enriched for ChIP-seq reads at the orthologous site in the mouse genome [96].

While the functional consequences of such changes in CRE activity are largely unknown, these data suggest massive changes in the regulatory architecture governing the emergence and development of the neocortex. Recent studies demonstrate the reliance of this architecture on CREs, as the main regional domains of the forebrain can be further divided into several subdomains demarcated by enhancer activities [96, 97]. Additionally, these results suggest how the activities of a relatively small number of TFs with broad expression gradients may be integrated to produce comparatively fine distinctions within a tissue. CREs are therefore potentially powerful substrates for evolution to act upon in the creation of new subdomains and cell types within the telencephalon and elsewhere.

Specific examples of critical new insights into the functional relevance of species differences in the sequence and activity of CRE sequences on neocortical development and evolution have started to emerge. For example, the acquisition by mammals (and possibly birds) of the E4 enhancer sequence of *Fezf2* may have contributed to the emergence of the corticospinal projection system, thus enabling the highly skilled motor control typical of these species [69] (Fig. 2C, D). Such examples are not limited to TF binding sites, as DNA sequence changes in other regulatory sites, including sites bound by RNA-binding proteins in neocortically expressed transcript have also undergone evolutionary changes. One example of this is the human *NOS1* mRNA and FMRP [98].

Neocortical *cis*-regulatory elements in neurological and psychiatric disorders

Alterations to TRNs have been linked to many disorders, including neurological and psychiatric disorders. Such alterations can arise from trans-regulatory changes that modify the expression or function of TFs or from *cis*-regulatory changes that affect the binding of

TFs, RNA-binding proteins, and non-coding RNAs. For example, many copy number variants or mutations in TFs previously implicated in neocortical circuitry development, including ARX2, FEZF2, TBR1, SATB2, and SOX5 [65, 99–104], have been linked to neurological and psychiatric disorders.

A growing number of studies, in particular genome-wide association studies, have also identified genetic polymorphisms or expression quantitative trait loci (eQTL) in putative CREs and regulatory non-coding RNAs that can lead to variation in gene expression levels between neural cell types, brain regions, timepoints or individuals [105–107]. Other brain disease-associated SNPs occur in non-coding regions that overlap with putative CREs [12, 108] [109–111]. Some of these, such as SNPs associated with Alzheimer's disease, have also been reported to be enriched in super enhancers, which mediate the establishment and maintenance of cell type identity, as compared to more typical enhancers [112]. However, the respective functional contributions of these *cis*- and *trans*-regulatory changes, as well as the vast majority of eQTLs, to molecular and cellular processes have remained largely unexplored.

Importantly, CREs allow the data from studies on evolution and brain disorders to synergize. A comparison of the distribution of H3K4me3, a promoter-specific histone modification associated with active transcription, in the prefrontal cortices of human, chimpanzee, and macaque brains found 410 regions enriched for H3K4me3 that were unique, and 61 regions that were uniquely lost, in humans [113]. Among the genes potentially affected were *DPP10*, *CNTN4*, *CHL*, and other genes previously linked to brain disorders. Analyses of TRNs involving these genes may provide insights into how the human brain may have become increasingly and possibly uniquely susceptible to certain neurological and psychiatric disorders as compared to non-human primates.

Future directions

Our focus here on TFs and the CREs that mediate, either directly or indirectly through changes in chromatin structure and epigenetic modifications, the binding of TFs has led us to generally exclude from discussion many other regulatory modalities, including regulatory RNAs and chromatin remodelers, utilized by TRNs. Furthermore, we limited the scope to examples where molecular interactions between TFs and CREs were well-defined, and many studies that have assessed CREs, including eQTL, on a global scale were therefore not discussed. Despite these omissions, recent advances make clear that CREs do not function merely as passive conduits carrying developmental cues linearly along from TFs. Rather, they integrate diverse sets of developmental programs promoted by the cellular environment, external stimuli, and TFs themselves. This is seen in the ability of CREs to mediate competition or synergism between multiple TFs [54, 69, 71, 96] and the ability of CREs to make large swaths of the genome accessible, as in the new availability of the thousands of CBP binding sites present following membrane depolarization [85, 88]. Such integration and regulation is bi-directional; super enhancers may recruit some master TFs and regulate the expression of others while at the same time DNA-binding proteins modify CREs to regulate the activity of super enhancers [112].

As the necessity for incorporating CREs into TRNs is becoming all the more clear, so too is the need for the systematic discovery and characterization of CREs active in different neural cells, regions, timepoints, species, individuals and disease states. Combining these data with transcriptomic and other data sets, including interactomes and epigenomes, will further aid not only the identification of target genes but also the development of new insights into how changes in TRNs contribute to changes in gene expression patterns. Importantly, developing in parallel with new techniques for the identification of putative CREs are new methods for the large-scale validation of these CREs. The reconstruction of TRNs, in particular through the rapid introduction or manipulation of CREs in the mouse genome by CRISPR/CAS editing, further allows functional assessments of CREs and their roles in TRNs. Taken together, these advances will allow scientists to characterize TRNs in neocortical development, evolution, function and dysfunction as never before.

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Highlights

- Recent advances have facilitated the study of neural *cis*-regulatory elements
- This new work is reshaping our understanding of transcriptional regulatory networks
- Brain-specific transcriptional regulatory networks are altered in evolution and disease

Figure 1. Schematic illustration of neurogenesis in the mammalian neocortex

Neuroepithelial cells (NPCs) undergo symmetric cell division to produce an initial pool of cortical progenitors that later transform into apical radial glia cells (aRGCs). Neocortical aRGCs line the ventricular zone from where they extend a long radial process stretching to the basal surface. aRGCs begin asymmetric cell division to generate another aRGC and a nascent projection neuron. The neuron then migrates radially from the ventricular zone (VZ) along the basal process of a RGC into the cortical plate (CP). The earliest born neurons migrate to form the preplate. Later migrating neurons split the preplate into the marginal zone (MZ) and subplate (SP). The MZ also consists of Cajal-Retzius cells originating from multiple sites in the forebrain including the cortical hem. As neurogenesis proceeds, diverse subtypes of neurons are generated through the successive asymmetric division of RGCs. Early-born nascent projection neurons settle in the deep layers (Layers 5 and 6; red layers) and later-born projection neurons settle in towards mid neurogenesis stage. Additionally, some populations of RGC daughter cells become intermediate progenitor cells (IPC) or basal radial glial cells (bRGC) in the subventricular zone (SVZ). After the neurogenic stages, the radial scaffold detaches from the apical surface and aRGCs become gliogenic, generating astrocytes, or transform into ependymal cells. Tangential migration of interneurons is observed in the MZ, intermediate zone (IZ) and SVZ. Neocortical projection neurons mature into cortical projection neurons (CPNs), which show layer- and subtype-specific morphology and axonal projection patterns. Subcerebral projection neurons, which extend axons to the striatum, thalamus, brainstem, and spinal cord, are located in the deep layers. Upper layer projection neurons project axons within the telencephalon (i.e., cerebrum). Adapted from Kwan et al, 2012.

Figure 2. Transcriptional regulatory networks controlling specification and differentiation of cortical projection neurons

(A) Key transcription factors (TFs) involved in neurogenesis and cell type specification. Solid lines indicate direct activation or repression of a downstream target by the upstream TF indicated. Dashed arrows indicate regulation by unknown mechanisms. Orange arrows indicate indirect results of the effects of the TFs indicated. (B–D) Models for the regulation of the transcription of *Fezf2*, *Bcl11b* (aka *Ctip2*), and *Tbr1* during cell type specification. (B) Regulation of *Fezf2* transcription. SOX4 and SOX11 activate *Fezf2* transcription while SOX5 suppresses this expression by competing for the E4 enhancer. The physical interaction was tested by EMSA and ChIP [69]. TBR1 suppresses *Fezf2* expression by binding to another CRE. This physical interaction was tested by ChIP [23]. (C) SATB2 represses *Bcl11b* expression by binding to MAR (matrix attachment regions) sequences (orange boxes) [62]. (D) SATB2 induces the expression of *Tbr1* by binding to three MAR sequences [70].

Figure 3. The identification and characterization of the E4 enhancer of *Fezf2*

In this example of *cis*-regulatory element (CRE) discovery and functional characterization, transcriptome analysis and comparative genetics were coupled with BAC transgenesis to identify and characterize an enhancer of the *Fezf2* gene. (A) *Fezf2* is a transcription factor (TF) identified in a genome-wide search for genes enriched among corticospinal motor neurons and their predecessors as compared to other cortical projection neurons. Within the *Fezf2* locus are four conserved non-exonic regions, labeled E1, E2, E3, and E4, with high levels of homology across species. The *Fezf2* coding region is also indicated. (B) Schematic of a BAC reporter where a reporter gene, *Gfp*, is driven by the native regulatory environment governing *Fezf2*. (C) GFP expression, driven by the unmanipulated *Fezf2*-Gfp BAC, is present throughout the dorsal telencephalon and the corticospinal tract (arrow). This is also true when the putative enhancers E1, E2, and E3 are deleted (). However, deletion of the E4 enhancer (right panel) results in a loss of GFP expression from the dorsal telencephalon and the apparent absence of the corticospinal tract. (D) Sequence-level homology of the E4 enhancer of *Fezf2* in several species. Putative binding sites for SOX proteins are shaded. Note the generally high levels of homology except for the lack of homology in zebrafish at the second shaded SOX binding site. (E) SOX11 stimulates expression of a construct containing the E4 enhancer of *Fezf2*. The construct containing the zebrafish E4 sequence, which lacks the second consensus SOX binding site, is not induced by SOX11. (F) The putative SOX binding sites were mutagenized in mouse and zebrafish, effectively swapping mouse sequence into zebrafish and vice versa. (G) Swapping the sequences of the second SOX binding site between zebrafish and mouse reduces the ability

of SOX11 to induce transcription from mouse E4 while increasing the ability of SOX11 to induce transcription from zebrafish E4. No change is observed when the sequences of the first and third putative SOX binding sites are swapped.

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