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Epigenetic and transcriptional regulation of neuron phenotype

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ABSTRACT Understanding the structure and function of cells is central to cell biology and physiology. The ability to control cell function may benefit biomedicine, such as cell-replacement therapy or regeneration. If structure defines function and cells are composed of water, lipids, small metabolites, nucleic acids, and proteins, of which the latter are largely encoded by the DNA present in the same cell, then one may assume that the cell types and variation in cellular phenotypes are shaped by differential gene expression. Cells of the same cell type maintain a similar composition. In this review, I will discuss the epigenetic and transcription regulation mechanisms guiding cell fate- specific gene expression in developing neural cells. Differentiation involves processes of cell-fate selection, commitment and maturation, which are not necessarily coupled.

KEYWORDS: phenotypic convergence, differentiation, cellular competence, epigenetics, cell fate decision

Regulation of developmental fate transitions

Cell type-specific genome and gene-expression state is largely established by sequence-specific transcription factors (TFs) and local epigenetic modifications. Germ layer identity may be constituted by global editing. For example, preventing methylation at H3K27 via knockout of polycomb group genes *Eed* and *Suz12* shifts the fate of mouse embryonic stem cells (mESC) towards mesendoderm at the expense of ectodermal fate (Yu *et al.*, 2023). However, further diversification into cell types and differentiation is clearly regulated by highly specific TF-DNA interactions.

Cell fate selection is closely associated with specific TFs called "pioneer factors". Pioneer TFs bind DNA irrespective of its pre-existing conformational state or associated histone modifications and prime chromatin decondensation (Soufi *et al*., 2012). As such, the pioneer TFs reshape the chromatin landscape, opening new chromatin regions and enabling a new transcriptional state. The ability to bind heterochromatin and the preference for open or closed chromatin varies between TFs. Perhaps only a subset of target enhancers may mediate the pioneer function, while others require chromatin decondensation by prior activators or a topological shift. Little is known about the regulation of topological shifts. Euchromatin and heterochromatin are localized in distinct nuclear compartments, and developmental stage-specific gene enhancers can move between the nuclear compartment cell type specifically (Norrie *et al.*, 2019). Interestingly, generation of induced pluripotent cells can be greatly facilitated by deforming the nucleus (Song *et al.*, 2022).

Together with TFs, the chromatin state at enhancers and promoter CpG islands represent the other side of the coin in successful expression of required genes. Unlike differentiated cells, where a stable state of chromatin and histone modifications are maintained, the epigenetic state is dynamic during development. Developmental gene enhancers are associated with a poised state: a bivalent modification of histones (reviewed in Macrae *et al*., 2023) (Rada-Iglesias *et al.*, 2011, 2012). Stabilization of the current chromatin state may signify cell-fate commitment.

Gene expression programs and chromatin state in neurons

The nervous system in mammals and vertebrates is remarkably complex in terms of cell type number. Consistent with the higher number of distinct cell types, regulation of gene expression may

Abbreviations used in this paper: bHLH, basic helix-loop-helix; C. elegans, Caenorhabditis elegans; ChIP-seq, chromatin immunoprecipitation and sequencing; CNS, central nervous system; COE, Collier/Olf1/EBF family of transcription factors; DHS, DNase I hypersensitivity sites; Dll1, Delta-like 1 gene; DNA, deoxyribonucleic acid; E-box sequence, here, 5'-CANNTG-3', the bHLH TF binding sequence in DNA; ETS, E26 transformation-specific; G2/M, Gap 2 to mitosis transition in cell cycle; HD, homeodomain; HD-CUT, CUT-homeobox protein; HLH, helix-loop-helix protein domain; HMG, high-mobility group; iN, in vitro generated neurons; iPSC, induced pluripotent stem cells; kDa, kilodalton; MEFs, mouse embryonic fibroblasts; mESC, mouse embryonic stem cell; NFI, nuclear factor I; NPC, neural progenitor cells; NR, nuclear receptors; PNS, peripheral nervous system; RE, regulatory element; SOX, Sox family high-mobility group proteins; TF, transcription factor; ZF, zinc-finger domain.

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be more multivariate in the nervous system compared with other tissues. The intergenic regions flanking neural genes have longer intergenic regions (2 times longer than the intergenic regions of tissue-specific genes of other tissues) that also contain more regulatory elements (REs, also known as enhancers and silencers) (Jaura *et al.*, 2022). This feature has emerged in vertebrates, as neural genes in *Drosophila* and *Caenorhabditis elegans* are not associated with longer intergenic regions. Neural gene enhancers also seem to be used in a highly cell type-specific manner; in any region of the cortex, the number of region-specific REs vastly exceeds the number of REs active in all regions. For example, different genomic elements are accessible near commonly expressed genes *Sst* and *Negr1* in the cortex, hippocampus, and motor neurons in mice (Jaura *et al.*, 2022).

Interestingly, the neuronal gene expression program in its common (or pan-neuronal) units can be induced by several distinct TF combinations convergently. Mouse embryonic fibroblasts (MEFs) can be reprogrammed *in vitro* to neurons (iN) using a combination of the TFs Pou3f2 (Brn2), Ascl1, and Myt1l (BAM), or, less efficiently, using Ascl1 alone (Pang *et al.*, 2011). Similarly, expression of the basic helix-loop-helix transcription factor (bHLH TF) Neurog2 can be used to generate iN of glutamatergic identity (Thoma *et al.*, 2012). Ascl1 and Neurog2 function as pioneer factors; however, MEF-derived iN can also be created independent of Ascl1 by co-expression of Sox8 and Mytl1 or Dlx3 and Mytl1 (Wapinski *et al.*, 2013, 2017). The differentiation process is possibly relayed along the regulatory network and may also be activated by downstream layer regulators. Consistent with the hypothesis of feed-forward relay, in *Xenopus* embryo and mouse P19 cells, a similar glutamatergic neuron differentiation program can be initiated by either Neurog2 or NeuroD4, a direct transcriptional target of Neurog2 (Seo *et al.*, 2007).

The activity of reprogramming factors is guided by the chromatin landscape, but also shapes it. In endogenous neural progenitor cells (NPCs) and MEFs alike, the Ascl1-binding "E-box" motifs are associated with histone marks H3K4me1, H3K27ac, H3K9me3, forming a closed but permissive chromatin state (Wapinski *et al.*, 2013, 2017), while Pou3f2 binding occurs at already open chromatin (Wapinski *et al.*, 2013). In NS5 cell-derived iN, Ascl1 binding to DNA is globally activating in function and binding was preferentially detected in enhancers associated with H3K27ac and H3K4me1, with the proportion of open-chromatin binding increasing during differentiation; 80% of Ascl1-bound sites overlap with DNase I hypersensitivity sites (DHS) in early and 91% in the late differentiation stage. In the genomic loci of *NeuroD4*, *Ap3b2*, *Mcf2l*, and *Nrxn3*, Ascl1 binding was detected in closed chromatin in the early differentiation stage and precedes the appearance of DHS at binding site (Raposo *et al.*, 2015). In mESC-derived motor neurons, Neurog2 acts as a pioneer factor and the accessible REs are later bound by Pou3f2, Ebf2, Onecut2, and Isl1 (Rhee *et al.*, 2016), where Isl1 is a selector gene in motor neurons. Interestingly, Isl1 first binds DNA broadly, followed by a shift in its genome occupancy, mediated by Onecut2. Onecut2 recruits Isl1, displacing it from "transient" enhancers by protein-protein interaction (Rhee *et al.*, 2016). The function of very short-lived transient enhancers is still obscure. Such binding events might simply immobilize and accumulate TFs.

Altogether, neuronal fate acquisition and differentiation can be envisioned as a two-step process as follows: 1, broad marking and opening of the common neuronal program by pioneer type TFs, and 2, the restriction of a genetic program by cell type-specific factors (selector TFs) that maintain a subset of open chromatin elements while others are silenced.

As several TF combinations can convert mouse and human fibroblasts to iN, it would be interesting to show how the neurogenic programming factors guide cells. This guidance involves both activation of new chromatin regions and chromatin silencing. In iN, Myt1l functions as a lineage-specific repressor. Myt1l is also expressed exclusively in neural tissues *in vivo* and targets myogenic, cartilage, heart, and lung development-associated genes while neuronal gene promoters are depleted of Myt1l-binding motifs (Mall *et al.*, 2017). The activating neurogenic factor function seems to converge on a "core neuron transcriptome" that includes transcriptional repressors such as RE1-silencing transcription factor *Rest* and common and specific activators, including the common activator *Mecp2*, methyl-CpG binding protein 2 (Tsunemoto *et al.*, 2018). Pan-neuronal genes such as *Mapt*, *Tubb3*, *Map3*, and *Snap25* are reliably induced in iN generated using different TF combinations (Tsunemoto *et al.*, 2018). However, the genes governing neurotransmitter identity are often not expressed at endogenous levels or are not segregated in iN. For example, BAM-iN cells express both *Gad1/2* and *Slc17a6* and are excitatory interneurons by function (Pang *et al.*, 2011). Induced pluripotent stem cells (iPSC)-derived Neurog2-iN cultures contain a mixture of molecularly distinct central nervous system (CNS) and peripheral nervous system (PNS) neurons, including motor neurons and forebrain cholinergic and glutamatergic neurons (Chen *et al.*, 2020; Lin *et al.*, 2021).

Obtaining the mature neuron phenotype involves several developmental transitions *in vivo.* Consistently, mimicking endogenous developmental gene expression *in vitro* can increase the differentiation efficiency and the homogeneity of the induced neurons. Such guided differentiation assays have been developed for several neurodegenerative disease-associated cell types, including dopaminergic and serotonergic neurons, motor neurons, and various types of GABAergic and glutamatergic neurons (reviewed in Limone *et al.*, 2022). Using this strategy, sequential expression of Sox2 and Foxg1 followed by Ascl1, Dlx5, and Lhx6 has been used to produce cortical interneuron-like cells from mouse and human fibroblasts and human iPSC (Colasante *et al.*, 2015). The *in vitro* generated cortical GABAergic neurons expressed *Arx*, *Dlx1*, *Dlx2*, *Sox6*, *Satb1*, and *ErbB4*, acquired GABAergic phenotype and morphology, and integrated to mouse hippocampus upon transplantation, forming GABAergic inhibitory synapses in the host tissue (Colasante *et al.*, 2015). The purity and cell-type composition of the iN cultures may need further investigation. Ectopic expression of Dlx5, Lhx6, and phosphorylation-resistant Ascl1 in human pluripotent stem cells can convert 70-90% of cells to *Gad1*-positive GABAergic neurons. Adding micro-RNAs miR-9 and miR-124 further supported neuronal differentiation (Sun *et al.*, 2016). Molecular marker analysis showed that the protocol used in Sun *et al.,* rather induces a lineage identity and not a cell type, as a mixture of medial ganglionic eminence-derived interneuron subtypes including the SST-, calbindin-, calretinin-, and NPY-expressing interneurons were found. Nevertheless, the transplanted neurons successfully integrated in the mouse cortex, maintaining their subtype identity (Sun *et al.*, 2016). Guided differentiation assays for other neuron types exist. A combination of ectopic *Neurog2* with regional cues (retinoic acid and Smoothened inhibitor) and growth factors (GDNF, BDNF, and

CNTF) enabled 95% purity of induced lower motor neurons, and the neurons formed neuromuscular connections *in vitro* (Limone *et al.*, 2023). Arguably, the requirement for transfections or viral transductions is a risk. Production of midbrain dopaminergic neurons from human iPSC requires application of Shh activators, FGF8, and BMP inhibitors, followed by sorting of Corin+ floor plate-like cells and propagation under growth factors (Doi *et al.*, 2014). *In vitro* differentiated dopaminergic cells have therapeutic potential, as transplantation of these cells into the striatum results in improvement of movement in animal models of Parkinson's disease (Liu *et al.*, 2012; Kikuchi *et al.*, 2017).

Regulation of the common target genes of basic helix-loop-helix transcription factors

The transcription factors that induce neurogenesis *in vivo* are called "proneural". Proneural TFs are expressed in neuronal progenitors and induce differentiation by repressing re-entry to the cell cycle and promoting cell cycle exit. In mouse neuronal precursors, the main proneural factors are the bHLH TFs *Ascl1*, *Neurog2*, and *Neurog1*. bHLH TFs have pioneer TF function (Zhu *et al.*, 2018) and, as discussed earlier, Ascl1 and Neurog2 also promote neuronal fate *in vitro*.

Looking at the global chromatin landscape and the DNA binding activity of Ascl1 or Neurog2 during induced neurogenesis, Ascl1 and Neurog2 were shown to preferentially bind distinct genomic locations. A smaller number of REs could be bound by both TF. These REs contain either multiple E-box sequences or a single binding site, whose sequence is an average of Ascl1-only and Neurog2-only motifs and is probably recognized by both TFs (Fig. 1A) (Aydin *et al.*, 2019). Despite differences in binding locations, Ascl1 and Neurog2 activate somewhat overlapping sets of target genes. The target-gene activation seems to be remarkably cell type dependent, as approximately 80% of common targets were reported in *in vitro* differentiated mESCs (Aydin *et al.*, 2019) but only 3.1% in reprogrammed astrocytes (Masserdotti *et al.*, 2015). The high number of shared targets would not be surprising as Ascl1 and Neurog2 proteins are structurally and functionally similar. The same binding sites could be used without competition or loss of affinity, as Ascl1 and Neurog2 are rarely coexpressed in developing embryos. However, the situation seems to be more complex. When specific target genes are considered, there are examples of both shared and distinct enhancers mediating proneural function (Fig. 1).

An important feature of the neurogenic gene expression program induced by proneural factors is the expression of *Delta-like* genes. *Dll1 (Delta-like1)* is a common target of Ascl1 and Neurog2, which are thought to act via distinct enhancers (DeltaM and DeltaN, as described in mice and zebrafish) (Castro *et al*., 2006). The DeltaM enhancer is conserved in vertebrates and contains 2 E-boxes and a POU-HD binding octamer sequence (Fig. 1A). Binding at DeltaM, Ascl1 can activate *Dll1* expression alone or synergistically with Pou3f2 (Brn2) (Fig. 1B) (Castro *et al.*, 2006). POU TFs are common cofactors of Ascl1 in CNS. A conserved POU/E-box sequence combination similar to the *Dll1* enhancer is found in the proximity of several neurogenesis-associated genes, which could be interpreted as a coregulation unit. These genes include Notch signalling pathway proteins, TFs, neuronal migration regulators, and cell-cycle regulators (Castro *et al.*, 2006). A recent study (Aydin *et al.*, 2019) confirms the preferential binding of Ascl1 to DeltaM and Neurog2 to DeltaN in iN (Fig. 1A). Distinct and shared enhancers for Ascl1 and Neurog2 are also a feature of the mouse *Dll3* promoter. The *Dll3* promoter contains 7 E-box motifs (E0-E6) that mediate Dll3 gene activation, binding either Ascl1 or Neurog2 alone or synergistically with Tcfe2a and Nhlh1. Interestingly, the E0 motif is critical for Dll3 expression, but the TFs interacting with E0 are unknown and do not include Ascl1, Neurog2, Nhlh1, and Tcfe2a-E12 (Henke *et al.*, 2009).

Another common target of Ascl1 and Neurog2 is Gadd45γ (Seo *et al.*, 2007; Huang *et al.*, 2010). Gadd45 proteins are small 18-kDa cytoplasmic and nuclear proteins expressed in postmitotic neuronal cells (Tamura *et al.*, 2012). Gadd45γ function is important for G2/M checkpoint, cell cycle withdrawal, and induction of differentiation. Similar to Ascl1 overexpression, Gadd45γ overexpression in P19 cells leads to neuronal morphology, physiological characteristics of neurons, and expression of neuronal genes *Slc17a6*, *Syt4*, *Npy*, and *Tuj*. The Gadd45γ promoter contains 4 E-box motifs conserved in mouse, zebrafish, and *Xenopus*. Its regulation is independent of Pou3f2 function, as HD octamer is not present. Gadd45γ overexpression does not induce *Ascl1* or *Neurog2* expression (Huang *et al.*, 2010). As proneural proteins are downregulated soon after cell cycle exit, Gadd45γ may function as a relay protein in the neurogenic program (Table 1).

In conclusion, Ascl1 and Neurog2 both function as pioneer TFs, modifying the chromatin accessibility landscape and as cell fate determinants that regulate the terminal differentiation genes. The neuronal differentiation program is somewhat convergent, while the potential to execute the full target program is context-dependent and likely shaped by epigenetic modifications or preset nuclear environment. Proneural TFs can act via shared or unique REs, while the convergently regulated, common target genes have multiple REs.

Phenotypic convergence in invertebrates

In this section, I highlight some interesting recent studies that have addressed the generalizable regulatory logic of cell fate determination. There are exceptional cases where cell fate is defined by a single TF. In the ventral nerve cord motor neurons of the nematode *C. elegans*, synaptic acetylcholine production as well as acetylcholine neurotransmission genes are activated by COE-type TF unc-3 (Kratsios *et al.*, 2012). In mouse serotonergic neurons, the capacity of serotonin neurotransmission is induced and maintained by the ETS (E26 transformation-specific) family TF Fev (Liu *et al.*, 2010; Wyler *et al.*, 2016). Such critical fate-determining TFs have been called "selector genes". In most cases, however, the regulatory landscape is complex (Kutejova *et al.*, 2016) and is perhaps better exemplified by the regulation of *eat-4/Vglut* gene expression in *C. elegans* glutamatergic neurons, where *eat-4* is controlled by a modular enhancer responsive to multiple alternative TFs of different TF classes (Serrano-Saiz *et al.*, 2013). Similar regulatory logic seems to apply for pan-neuronal genes *Rab-3*, *ric-4/Snap25*, and *snb-1/Vamp*, where any partial deletion of an enhancer does not eliminate the expression in all neurons (Stefanakis, Carrera and Hobert, 2015).

A recent study of selector gene-based regulation of cellular phenotype used annotated regulatory links between the known selector genes and terminal differentiation genes in *C. elegans* for regulatory link modelling (Mora-Martinez, 2021). The model assigned different numbers of selector-type TFs per target gene,

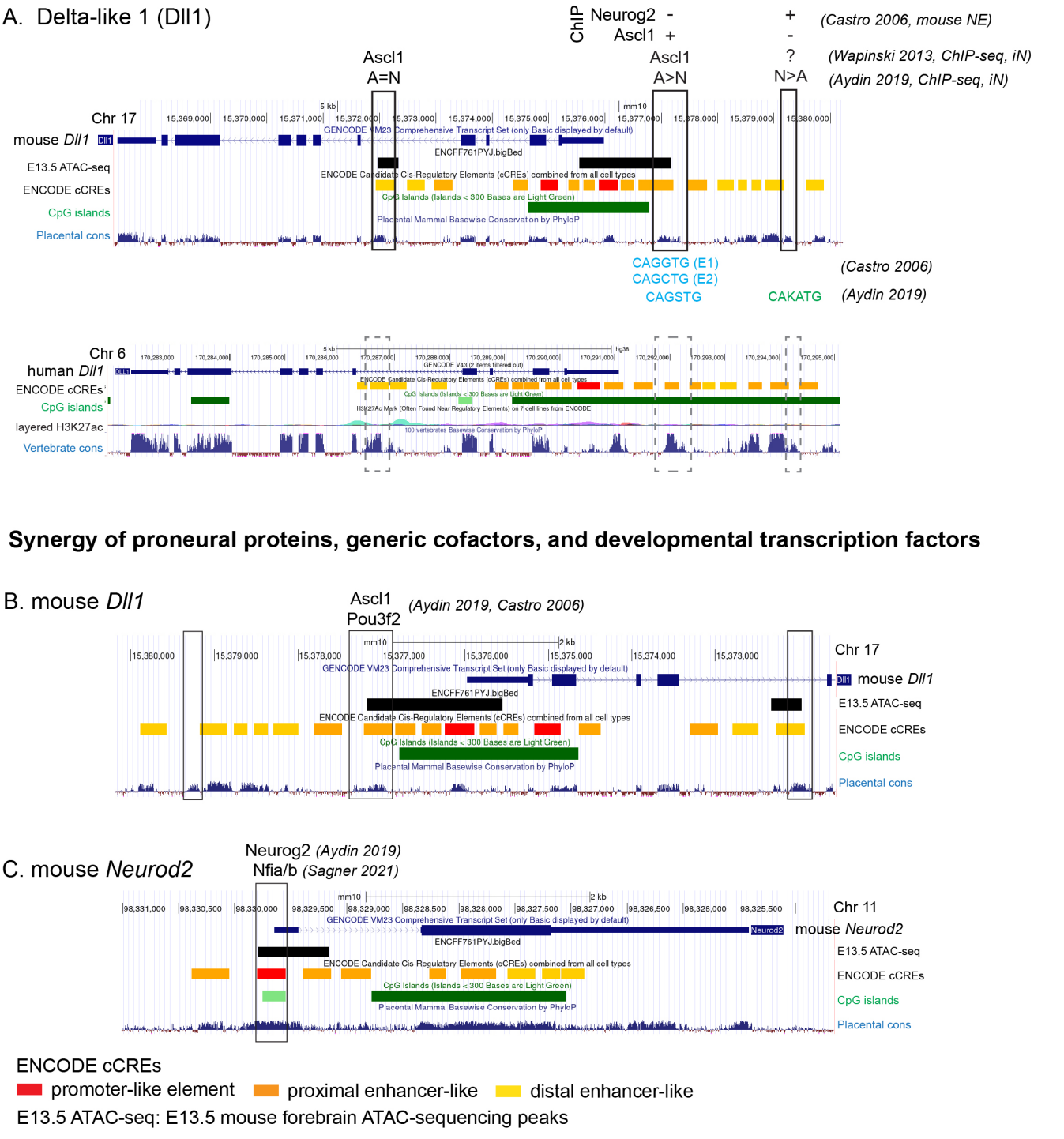

Fig. 1. Convergence of transcription factor activity on the regulatory elements upstream of the Dll1 and Neurod2 genes in developing neuronal precursors. (A) Genomic loci of the mouse and human Delta-like 1 (Dll1) genes. The conserved regulatory elements (RE) are outlined in boxes. The binding of Ascl1 and Neurog2 in each RE, according to different studies, is shown above the RE. The consensus sequence of the E-box in the RE is shown below the box, when known, according to Castro *et al.*,2006 and Aydin *et al.*,2019. A, Ascl1; N, Neurog2; The "=", "<" and ">" signs indicate whether the RE is preferentially bound by Ascl1, Neurog2 or both, according to Aydin *et al.*,2019). **(B,C)** Genomic loci of the mouse Delta-like 1 (Dll1) and Neurod2 genes, showing the synergistic activity of transcription factors in REs. The references are indicated. Mouse genome mm10 or human genome hg38, gene models, location of ENCODE CREs, CpG islands and the conservation in placental or vertebrate animals was fetched from the UCSC genome browser. The E13.5 mouse ATAC-seq features are from the E13.5 mouse forebrain ATAC-seq experiment (ENCODE accession number ENCFF761PYJ).

depending on the level of cell-type specificity of the target gene: the cell type-specific genes were strongly linked to only 2-5 TFs, while the broadly expressed genes could be equally linked to about 10 different TFs (Mora-Martinez, 2021). In another study, the cell-type markers and expressed TF combinations were correlated in 52 single-cell clusters and 17 cell types isolated from the developing eye of *Drosophila melanogaster,* using single-cell and bulk RNA-sequencing (Konstantinides *et al.*, 2018). There, 1/3 of the expressed genes correlated with a single TF expression and were expressed on average in 2.2 clusters. The other 2/3 of genes correlated with a combination of TFs and were expressed on average in 22 clusters (Konstantinides *et al.*, 2018). Testing various models of regulation, the expression pattern of neural differentiation genes was better explained by a model where a gene can be regulated by several TFs convergently.

Some common principles arise from these studies. First, only a minority of differentiation genes are expressed in a unique single-cell cluster (or cell type), while the expression of most genes is not highly restricted across cells. Second, different regulatory networks may explain the regulation of more restricted, cell subtype-specific genes and more broadly expressed, cell type- or cell class (pan-neuronal)-specific genes. The model of a redundant modular architecture seems to be common to enhancers regulating the pan-neuronal or neuron class-specific genes, and a master regulator type control (or selector gene model) to the subtype-specific gene expression (Hobert and Kratsios, 2019).

Phenotypic convergence in vertebrate nervous system

Similar to invertebrates, a shared cell lineage or embryonic field of origin does not always correlate with chromatin or gene expression state similarity in vertebrates. Similar molecular identity can be derived from distinct lineages, and these divergences and convergences are also apparent in single-cell RNA-seq using genetic lineage tracers in mice and zebrafish *in vivo* (Wagner *et al.*, 2018; Chan *et al*., 2019). However, the genetic regulatory mechanisms are much less studied in vertebrates.

As an example of phenotypic convergence at cell subtype level, inhibitory GABAergic interneuron fate is controlled by distinct selector genes in spatiotemporally and molecularly distinct neuroepithelial cell lineages, such as HD-TFs Dlx1, Dlx2, and Dlx5 in the telencephalon and anterior diencephalon; zinc-finger TF Gata2 and bHLH TF Tal2 in posterior diencephalon and midbrain; and Gata2, Gata3, and Tal1 in hindbrain (Achim, Salminen and Partanen, 2014). bHLH TF Ptf1a acts as a selector gene in GABAergic dorsal spinal cord interneurons, cerebellar granule neurons, and GABA- or glycinergic retinal amacrine cells (Jin and Xiang, 2019). All these TFs function as selector genes, promoting GABAergic over glutamatergic neurotransmitter phenotype. Interestingly, Ptf1a also promotes differentiation of the acinar cells of the pancreas (where it regulates amino acid biosynthesis and secretion) and the paracrine serotonergic neurons in the enteric nervous system (Hoang *et al.*, 2016; Jin and Xiang, 2019). In neurons, GABAergic neurotransmission is governed by the expression of glutamic acid decarboxylase genes *Gad1* and *Gad2* and the GABA vesicular transporter gene *Slc32a1*, which are expressed in all GABAergic neuron subtypes. Chromatin immunoprecipitation and sequencing (ChIP-seq) analyses confirmed the binding of Dlx1 and Dlx2 near *Gad1*, *Gad2*, and *Slc32a1* in forebrain GABAergic interneurons (Le *et al.*, 2017; Pla *et al.*, 2018; Lindtner *et al.*, 2019). Dlx TF target enhancers are also found near *Nrxn3* and *Arx*, which are important in GABAergic neuron maturation. Dlx1, Dlx2, and Dlx5 bind largely overlapping sites in the genome, and importantly, their binding can be associated with either an increase or a decrease in RE accessibility (Lindtner *et al.*, 2019). Interestingly, Dlx TF-binding enhancers are highly enriched in the Ascl1-binding variant of E-box (Lindtner *et al.*, 2019), consistent with a transcriptional relay. The availability of enhancers can also be modulated by Nkx2-2, Nkx2-1, and Lhx6 (Sandberg *et al.*, 2016; Kim *et al.*, 2021). It is not known where the other selector TFs bind.

It is not fully understood how the selector genes repress the alternative fate. In the mouse spinal cord, the neuromere-specific TFs Nkx2-2, Nkx6-1, and Olig2 negatively regulate each other's expression and possibly some of their targets by directly binding to distinct REs (Kutejova *et al.*, 2016). The DNA binding of Dlx TFs near genes that are involved in telencephalic neuroepithelium patterning (Otp, Gsh1, Ebf3, Gbx2, and Pax7) was associated with RE silencing (Lindtner *et al.*, 2019). Perhaps the selector gene can negatively regulate the nuclear factors defining alternative fates, one transitional decision layer up. Alternatively, the activator TFs may simply be sequestered and RE silenced by an independent mechanism.

It is not fully clear if proneural genes and selector genes function independently or synergistically. Rather, both may be possible depending on the target gene. Examples are scarce, but there seems to be some differences between vertebrates and invertebrates in this case. In *C. elegans*, the proneural TFs are very often indispensable for the selector gene expression. For example, the expression of bHLH TFs Atonal (lin-32) and Achaete-Scute (hlh-14) precedes the expression of selector TFs in several cell types, including a POU-HD TF *unc-86* in IL2 and URX neurons; DLX TF *ceh-43* and SIX family TF *ceh-32* in IL1 neurons, and an LHX TF *lin-11* in AVJ neurons - and both the bHLH TF and the selector TF functions are required for proper differentiation (Masoudi *et al.*, 2021). This is unlike the situation in mouse GABAergic neuron lineages. For instance, although Ascl1 can directly regulate *Dlx1/2* expression in mouse telencephalon, expression of *Dlx1/2* in telencephalon is delayed but not abolished in the absence of *Ascl1* (Horton *et al.*, 1999). The same is true for the expression of *Gata2*, *Tal2*, *Six3*, and *Lhx1* in the diencephalic and midbrain GABAergic neurons in the *Ascl1* mutant mouse (Peltopuro, Kala and Partanen, 2010; Virolainen *et al.*, 2012). Possibly, the vertebrate animals feature more redundancy in bHLH TF function. Notably, Ascl1 and Pou3f2 function is synergistic in early neural gene activation (Fig. 1C).

Building and maintaining a competent state

Generic cofactor waves

As discussed, pioneer factor and selector gene potential is dependent on cellular competence. Aside the epigenetic state of chromatin, another important factor in defining and establishing competence is the availability of generic transcriptional regulators or cofactors.

Neuronal progenitor, precursor and neuron state descriptors can be defined as genes co-expressed in neuronal cells at different time points during and after cell-cycle exit. Some general characteristics of such temporal code have been described recently (Sagner *et al.*, 2021) and are summarized in Table 1. In several

regions of the neural tube, *Onecut2(1/2/3)* expression was found in neuronal precursors early, *Pou2f2* and *Zfhx3(3/4)* in intermediate developmental stages, and *Nfia, Nfib,* and *Neurod2/6* during the later stages of development (Sagner *et al.*, 2021). Interestingly, CUT transcription factors are also expressed in all neuron types in *C. elegans* and control neuronal identity by regulating pan-neuronal gene expression in cooperation with the cell type-specific selector genes (Leyva-Díaz and Hobert, 2022). In mouse stem cell-derived neurons, the temporal code is subject to regulation by TGF-β signalling (Sagner *et al.*, 2021). Nuclear Factor I (NFI) TFs are generic regulators of differentiation in several cell types (Chen *et al.*, 2017). In neurons, Nfia and Nfib activate expression of *Neurod2* via the same REs (Fig. 1C) (Sagner *et al.*, 2021). NFI TFs may also function synergistically with other TFs to regulate early and late

TABLE 1

CELL CYCLE EXIT REGULATORS, CHROMATIN-, HISTONE-, DNA-MODIFYING ENZYMES AND TRANSCRIPTION FACTORS EXPRESSED IN NEURONS DURING DIFFERENTIATION

			Developmental stage			DNA-binding domain	Target sequence motif	Human neurological conditions associated with gene mutation	Functional study of human gene mutation in human [HS] or mouse [MM]
			"early"	"mid"	"late"				
Diff. state	**Cell-cycle exit regulators**		Lin28a Dnajc2 Dll1 Dll3 Fbxw7 Cdc25b	Gadd45g				Brain malformations (*DLL1*) (Fischer-Zirnsak *et al.,* 2019)	
Chromatin state: generic cofactors	**Chromatin modifiers**		Hmga1 Hmga2 Hmgb1 Mecp2	Hmga1 Hmgb1 Dnmt3b		HMG-box	structure (HMGA, HMGB) me-CpG (Mecp2)	Rett syndrome (*MECP2*) (Collins and Neul, 2022; Good *et al.,* 2021)	
	Transcription factors	**SOX**	Sox11	Sox2 Sox6 Sox4	Sox9	HMG-box	CTTTGT	Coffin-Siris syndrome (*SOX11*) (Wang *et al*., 2023; Al-Jawahiri *et al*., 2022) Eye malformation (*SOX2*) (Kelberman *et al*., 2008; Zenteno *et al*., 2006) Intellectual disability (*SOX4*) (Angelozzi *et al*., 2022; Grosse *et al*., 2023)	
		HLH (inhibitory)	Id3	Id4	Id4	-	-	NA	
		bHLH	Tcf12	Tcf4	Tcf4 Neurod2 Neurod6	basic domain	CANNTG	Pitt-Hopkins syndrome (*TCF4*) (Popp *et al*., 2022) Craniosynostosis, neurodevelopmental delay (*TCF12*) (Kennedy-Williams *et al*., 2021; Sharma *et al*., 2013) Neurodevelopmental delay, epileptic encephalopathy (*NEUROD2*) (Sega *et al*., 2019) Downregulated in Alzheimer's disease brain (*NEUROD6*) (Fowler *et al*., 2015; Satoh *et al*., 2014)	TCF4 mutations affect neuron type and differentiation in human cerebral organoids [HS] (Papes *et al*., 2022) Exonic polymorphisms in *NEUROD2* associated with differential response to psychoactive drugs [HS] (Spellmann *et al*., 2017)
		NFI		Nfia Nfib	Nfia Nfib Nfix	CAAT-box	(T)GCCA(A)	Brain malformations (*NFIA*) (Negishi *et al*., 2015; Labonne *et al*., 2016) Intellectual disability and macrocephaly (*NFIB*) (Schanze *et al*., 2018) Marshall-Smith syndrome (*NFIX*) (Uzman *et al*., 2023)	
		NR	Nr6a1 (Gcnf)		Nr1a1 (Thra)	cysteine-rich domain (Zn-finger)	TF-specific sequences	Broad range of neurodevelopmental defects (*MYT1L*) (Coursimault *et al*., 2022) Pain insensitivity (*ZFXH2*) (Habib *et al*., 2018) Intellectual disabilities (*NR1A1*) (Krieger *et al*., 2019) Primrose syndrome (*ZBTB20*) (Melis *et al*., 2020) Motor discoordination and apraxia of speech (*BCL11A*) (Bruce *et al*., 2022)	NR6A1 regulates hypocretin transcription in hypothalamus [HS] (Tanaka *et al*., 2010) NR1A1 regulates neurogenesis [HS] [MM] (Krieger *et al*., 2019) Bcl11a regulates development of cortical projection neurons and midbrain dopaminergic neurons [MM] (Dias *et al*., 2016; Woodworth *et al*., 2016; Tolve *et al*., 2021)
		ZF	Myt1l	Zfhx2 Zfhx3 Zfhx4 Zfp422	Sall3 Zbtb20 Bcl11a				
		HD	Pou3f2		Pou2f2	helix-turn-helix	ATGCAAAT	Neurodevelopmental delay, autism (*POU3F2*) (Schönauer *et al*., 2023)	POU3F2 regulates proliferation and differentiation of human NPCs [HS] (Chen *et al*., 2018)
		HD-CUT	Onecut2	Onecut3		homeo-domain	ATC[A/G]ATA	NA	ONECUT2 regulates MITF, microphthalmia gene [MM][HS] (Jacquemin *et al*., 2001)

Transcription factors are shown by class: high-mobility group (SOX), helix-loop-helix (HLH), basic helix-loop-helix (bHLH), nuclear factor I (NFI), nuclear receptors (NR), zinc-finger (ZF), homeodomain (HD), and CUT-homeobox transcription factors (HD-CUT). Hmga and Hmgb are high-mobility group (HMG) proteins that bind DNA conformation specifically, facilitating unwinding and histone displacement. Mecp2 is methyl-CpG binding protein 2. Compiled information from Sagner *et al.,* (2021), Huang *et al.,* (2010), Mall *et al.,* (2017) and Tsunemoto *et al.,* (2018). Developmental stages are grouped as in Sagner *et al.,* (2021). DNA-binding sequence motifs of transcription factors are from JASPAR (https://jaspar.genereg.net/). Human neurological conditions associated with a gene are shown where causal variants have been identified. The list may not be comprehensive and excludes characterized phenotypes with no neurological component.

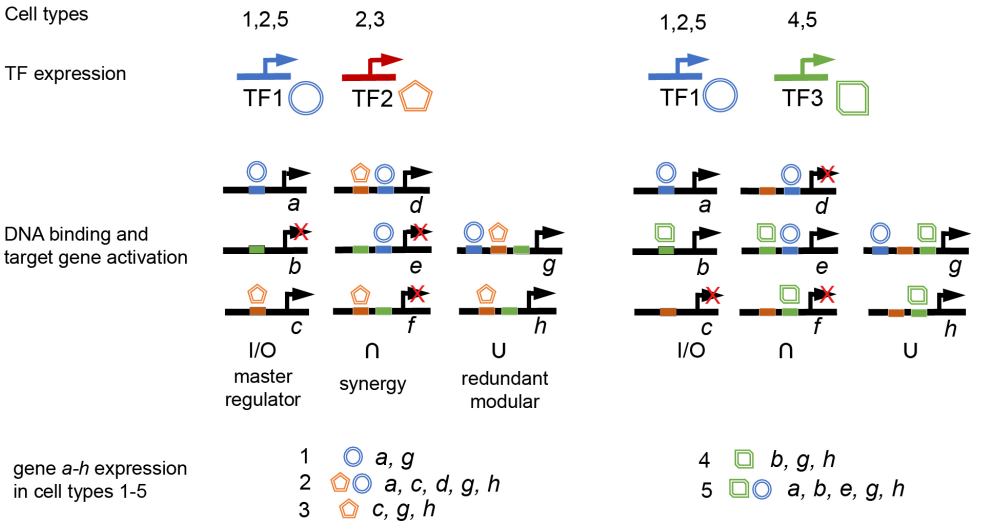

Fig. 2. Gene regulatory logic. The combination of gene expression for 8 genes (a-h) in five cell types (1-5) expressing different combinations of transcription factors (TF1-TF3). All TFs are considered activating, and regulatory elements are considered available. Repressive interactions are not shown for simplicity. TF binding sites are indicated in matching color. Three enhancer types are shown. Master regulator: RE, where TF binding will lead to gene expression; Synergy: binding at all sites required for gene expression; Redundant modular: binding at any RE is sufficient for gene expression.

developmental genes. In cerebellar granule neurons, NFI proteins are recruited by Etv1 to the promoters of late differentiation genes, regulating synapse formation and maturation (Ding *et al.*, 2016). *Nfia* mutant mice show severe defects in neurodevelopment and misregulation of genes associated with brain maturation (Wong *et al.*, 2007). NeuroD genes are involved in regulation of neuronal migration. In E13.5 and E14.5 cortical subventricular zone neuronal precursors, Neurod1 is coexpressed with *Tcf12* and *Tcf4*, and the Tcf12-Neurod1 interaction is important for cortical interneuron migration (Singh *et al.*, 2022).

In the differentiation process, generic wave factors complement fate determinant function, may stabilize gene expression programs, and therefore can be seen as commitment factors. It is not known whether the wave is reversible or how it is tuned by external signalling. Mutations in the generic cofactor genes are often associated with brain malformations, neurodevelopmental delay linked to number of other developmental defects outside the nervous system (such as craniofacial development), or both (Table 1). This is expected given the generic cofactor and early developmental functions of the genes. The regulation of later-expressed cell type-specific genes in neurons may affect finer aspects of neurodevelopment, psychology, or behaviour. However, it is currently poorly understood how individual aspects of complex neuropsychiatric disorders or even features of normal behaviours are linked to the cell types in the brain.

Stepwise activation of terminal differentiation gene enhancers

As the chromatin state can be copied and maintained over cell divisions, the activity of temporally differentially expressed TFs can coregulate gene expression. For example, ASEL/ASER neurons, a morphologically symmetric neuron pair in *C. elegans*, arises from early separating progenitor lineages in development. At lineage separation, the TF tbx37/38 primes the *lsy-6* promoter in ASEL progenitors for activation by che-1, a TF expressed later in the lineage, resulting in molecular and functional asymmetry (Charest *et al.*, 2020). *lsy-6* encodes a micro-RNA that targets an NKX homeobox TF cog-1 that, in turn, negatively regulates the expression of multiple genes, including the chemosensory receptor gcy-7 expressed in ASEL and the TF lim-6 that regulates the chemosensory receptor gcy-5 specific in ASER (Johnston and Hobert, 2003). che-1 and tbx37/38 are never coexpressed in ASER/ASEL lineages and priming of the *lsy-6* promoter in ASEL is not affected by loss of any other TBX TF in *C. elegans* (Charest *et al.*, 2020).

It would be interesting to further explore how early expressed TFs establish lineage-specific accessible states in gene loci or enhancers. One possible mechanism is the bivalent state via recruitment of histone modifiers. Early priming may explain how spatiotemporal code factors such as HOX, FOX, POU, LHX, and ETS TFs cooperate with the later-expressed selector TFs in vertebrates.

When continuous target expression is required, a feed-forward mechanism may be preferred. In developing neurons, once cell type-specific gene loci are activated, the maintenance of gene expression and accessible chromatin state is often relayed over from the initial selector genes to the same transcription factor family members. In the midbrain and diencephalic GABAergic neurons, the initial selector genes Gata2 and Tal2 are downregulated soon after the cell fate commitment, concomitant with the upregulation of Gata3 and Tal1, which could occupy the same enhancers in neuronal maturation stages (Kala *et al.*, 2009; Achim *et al.*, 2013). In forebrain GABAergic neurons, Dlx1/2 are expressed first, followed by Dlx5/6 expression (MacDonald *et al.*, 2013). Relay to a different TF family member would require adaptation in RE sequences. Stage- or context-specific elements may cluster and form super-enhancers as seen in the retina of mice, where transcription of the *Vsx2* gene requires activation of different stage-specific elements within a *Vsx2* super-enhancer (Bian *et al.*, 2022). Vsx2 is a selector-type TF that regulates both early and late-expressed genes in retinal bipolar neurons. Variation in RE sequences would allow maintenance of *Vsx2* expression in subsequent stages of differentiation and its co-regulation with a temporally distinct set of target genes.

The compendium of TF-RE interactions across cell types and states is not yet fully explored. In addition to the number and sequence of REs and transcription factor availability, RE availability and the type of TF cooperation must be considered (Fig. 2). Single-cell multi-omics may have the potential to map the shift in active enhancers concomitant with the expression of transcriptional regulators and target gene expression, discovering regulatory interactions that can be tested in high-throughput assays such as Perturb-Seq (Peidli *et al.*, 2024; Kim *et al.*, 2024). The studies in the context of whole developing organisms or adult functions are yet to follow.

Remarks

Phenotypic convergence, similar to the concept of convergent evolution, refers to the phenomenon of similar cell types arising from molecularly distinct precursors. As such, phenotypic convergence has been discussed by studying the cell types derived from complex samples, such as whole embryos or the nervous system, using single-cell sequencing. However, perhaps the term should rather be used in reference to the cellular characteristics and not about the cell type as the unit.

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