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Beyond proneural: emerging functions and regulations of proneural proteins

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Proneural proteins, which include Ascl1, Atoh1 and Neurogenins épinière in vertebrates and Achaete-Scute proteins and Atonal in *Drosophila*, are expressed in the developing nervous system throughout the animal kingdom and have an essential and well-characterised role in specifying the neural identity of progenitors. New properties and additional roles of these factors have emerged in recent years, including the regulation of stem cell proliferation and the capacity to reprogram many types of cells into neurons. This review will focus on these recent findings. The review will also discuss the mechanisms that allow proneural proteins to induce the transcription of their target genes in different chromatin contexts and the phosphorylation events and other post-transcriptional mechanisms that regulate the proneural proteins themselves.

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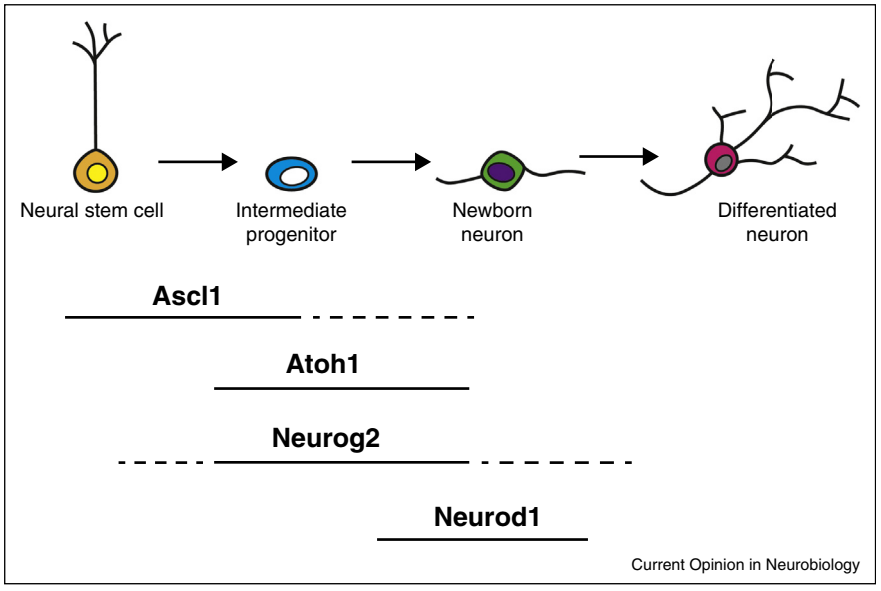
Introduction

Proneural proteins, first identified in *Drosophila* in the 1980s and later in vertebrates and other invertebrate species, are a small group of transcription factors of the basic-loop-helix class, which are necessary and sufficient to confer a neural (i.e. neuronal + glial) or neuronal fate to progenitor cells in the developing nervous system. This is an ancient function conserved through evolution from cnidarians to mammals, although variations exist between phyla regarding the exact stage in development at which these factors are expressed and function. Early expression in multipotent cells and a role in fate specification distinguish proneural proteins from neuronal differentiation factors such as members of the mammalian Neurod family, which are first expressed in cells already committed to a neuronal fate and promote their differentiation [1]. In addition to committing progenitor cells to a neuronal fate, proneural proteins also specify their identity, for example, sense organ identity in flies and the neurotransmission phenotype of mammalian neurons [1]. Although proneural proteins primarily act in progenitor cells, they sometimes remain expressed transiently in postmitotic neurons and regulate their migration and axonal and dendritic growth [2–4]. In this review, we will not discuss these well-established modes of expression and functions. We will focus instead on more recent findings, and specifically on a newly identified function of the proneural protein Ascl1 in neural stem cells, on a role for Ascl1 as pioneer factor, and on phosphorylation events that have recently been shown to greatly contribute to the regulation of the activity of proneural proteins.

Proneural proteins and stem cell proliferation

Although the primary function of proneural proteins is to endow progenitors with a neuronal fate, they also often drive progenitors out of the cell cycle and initiate their differentiation [1]. A few exceptions exist however, including mouse Atoh1 promoting granule cell proliferation during cerebellar development and in medulloblastoma [5,6] and *Drosophila* Asense, which contributes to the self-renewal of embryonic neuroblasts [7]. Ascl1/Mash1 has also been shown to promote the proliferation of neural stem cells and/or progenitor cells in the ganglionic eminences of the embryonic telencephalon and in the neurogenic regions of the adult mouse brain (dentate gyrus and ventricular-subventricular zone) through direct induction of cell cycle regulators such as *Cyclin D* genes [8,9,10••]. In the adult dentate gyrus, Ascl1 expression is restricted to stem cells and early intermediate progenitors and the onset of differentiation and cell cycle exit are induced by other factors including Tbr2 and Neurod1 (Figure 1). Possibly reflecting this proliferation-promoting function, Ascl1 has been implicated in the tumorigenicity of glioblastoma and other tumours [11,12]. Moreover, Ascl1 is expressed in, and might promote the proliferation of, neuronal progenitors derived from parenchymal astrocytes following ischemia, neurotoxic injury or viral-mediated transduction of Sox2 [13,14•,15•,16], suggesting a broader role of Ascl1 in activation of neural stem cells in response to a variety of physiological and pathological stimuli.

Figure 1



Our current understanding of the timing of expression of proneural genes in different lineages. A generic neuronal lineage is represented on the top of the figure. Continuous lines indicate the portion of the lineage when the proneural protein indicated above the line is consistently expressed, while dashed lines indicate expression in some lineages but not others (e.g. Ascl1 is expressed in the embryonic ventral telencephalon in neural stem cells in the ventricular zone, in all intermediate progenitors in the subventricular zone, and transiently in a subset of neurons in the mantle zone, but only in neural stem cells and early intermediate progenitors in the adult dentate gyrus [1,9•]).

Proneural proteins and neuronal reprogramming

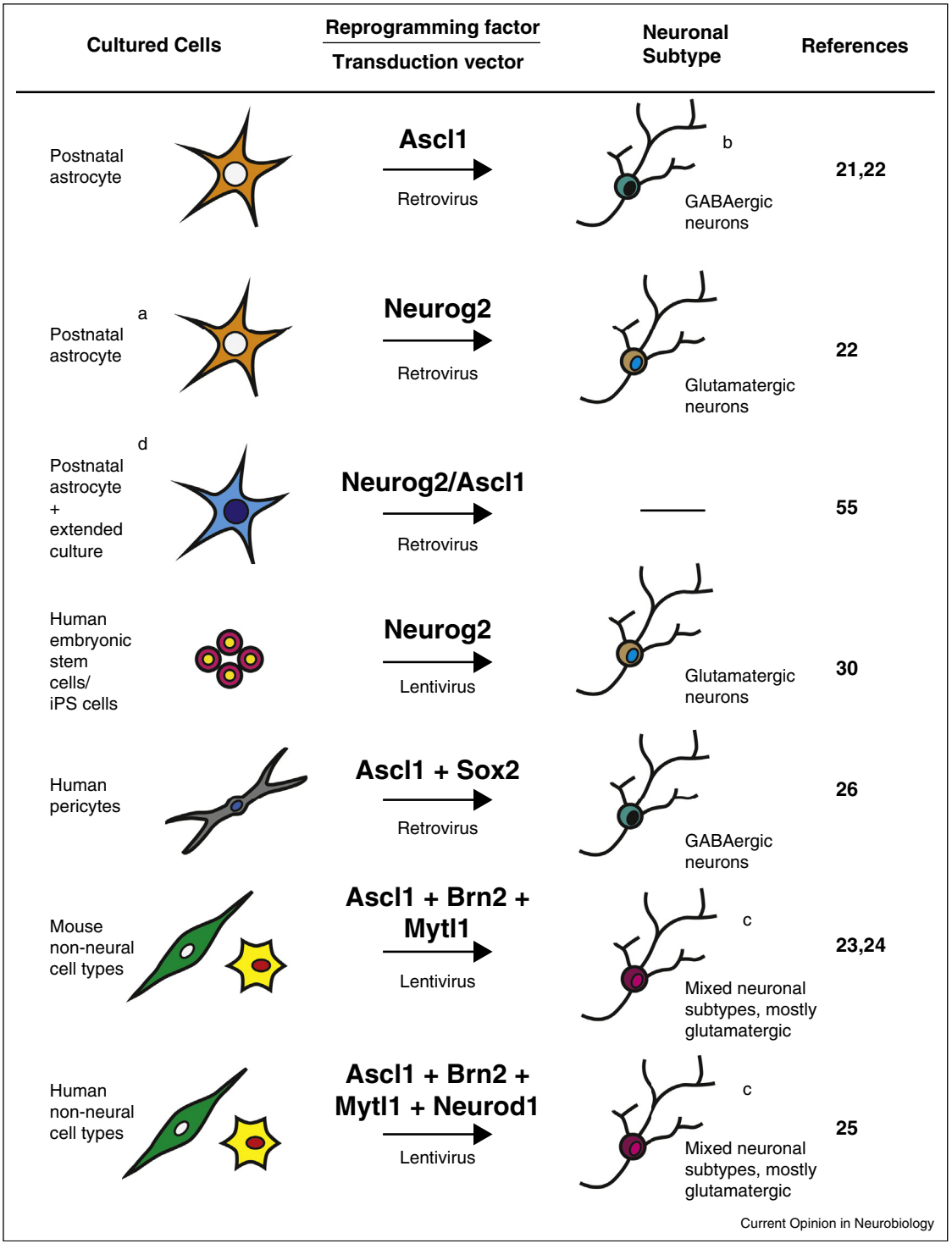
The pioneering work of Harold Weintraub established over 25 years ago that forced expression of a single transcription factor can be sufficient to convert a differentiated cell into another cell type [17,18]. Proneural proteins (e.g. Neurog1) and neuronal differentiation factor (e.g. Neurod1) were subsequently shown to have the capacity, when ectopically expressed in embryos, to convert non-neural ectoderm into neurons [19,20]. Magdalena Götz and colleagues extended this finding by showing that forced expression of Ascl1 and Neurog2 converts astrocytes in culture into fully differentiated and functionally mature neurons ([21,22]; Figure 2), and Marius Wernig and colleagues and others showed that Ascl1 in combination with other factors could directly reprogram (i.e. without an intermediate proliferative progenitor state) a variety of cultured cell types originating from mice, humans and other primates, into induced neuronal cells (iNs) [23–28]. Uniquely among proneural factors, Ascl1 alone can reprogram fibroblasts into iNs [29•]. Proneural proteins can also convert pluripotent cells into iNs, and expression of Neurog2 in human ESCs or iPSCs is currently the most efficient strategy to generate homogeneous populations of human neurons with a cortical-like identity [30•].

Given the efficiency of neuronal reprogramming of cultured cells, transcription factors have also been transduced into the mouse brain *in vivo* to circumvent the notoriously limited ability of the mammalian brain to replace lost neurons, by reprogramming glial cells, including parenchymal astrocytes, NG2 glia and retinal Müller glia, into neurons ([31–33]; Figure 3). The cocktail of three transcription factors including Ascl1 that was originally shown to reprogram fibroblasts *in vitro* [23] can also convert resident parenchymal astrocytes or transplanted human astrocytes into neurons [34•]. However, the extent to which Ascl1 alone is able to convert astrocytes in the brain into neurons is disputed, with one study reporting neuronal reprogramming of astrocytes in the midbrain, striatum and cerebral cortex [35], while other studies reported very little or no neuronal reprogramming by transduction of Ascl1 alone into glial cells of the spinal cord or cerebral cortex [13,36,37]. Neurog2 has the capacity to induce the conversion of glial cells into neurons only when cells are both activated by injury and exposed to exogenous growth factors, suggesting that the *in vivo* environment of glial cells limits their lineage plasticity [38•]. In contrast, Neurod1, a neuronal differentiation factor acting downstream of Neurog2 during neurogenesis, can efficiently reprogram on its own reactive astrocytes and NG2 glia into mature neurons [39].

Ascl1 as a pioneer factor

The ability of Ascl1 to convert multiple cell types into neurons suggests that it is able to activate target genes when these genes are not expressed and are actively

Figure 2



Summary of *in vitro* reprogramming experiments performed by transducing proneural genes in various cell types. The cell types reprogrammed are indicated on the left of the figure, the proneural genes and the viral vectors used for their transduction are indicated in the second column, the types of induced neuronal cells produced are indicated in the 3rd column, and the reference to the articles reporting the experiments are indicated in the last column. Notes: a. cultured astrocytes originating from the postnatal cortex or the injured adult cortex; b. induced neuronal cells (iNs) forming synapses and displaying electric activity; c. forced expression of Ascl1 produces iNs of mostly glutamateric excitatory character while in the embryonic brain, Ascl1 induces predominantly GABAergic neurons; d. Neurog2 is unable to reprogram postnatal astrocytes that have been maintained in culture, due to the recruitment of RE1-Silencing Transcription factor (REST) which progressively reduces chromatin accessibility at Neurog2 target genes essential for reprogramming, including NeuroD4 [55•].

Figure 3

transduced cell type	reprogramming factor / transduction vector	time between gene transduction and analysis	neuronal features	references
astrocytes in adult striatum	**Ascl1 + Brn2 + Mytl1** lentivirus	6 weeks	morphology and molecular markers	28
NG2 glia in injured adult cortex	**Ascl1** retrovirus	N/A	no reprogramming	30
astrocytes in adult striatum	**Ascl1** lentivirus	N/A	no reprogramming	10
astrocytes in injured adult spinal cord	**Ascl1** lentivirus	N/A	no reprogramming	31
activated glial cells in injured striatum and cortex	**Ascl1** retrovirus	N/A	no reprogramming	32
astrocytes in postnatal and adult brain	**Ascl1** adeno-associated virus	between 10 and 45 days	morphology, markers, action potentials,synaptic currents	29
activated glial cells in injured striatum and cortex	**Neurog2 + FGF2 + EGF** retrovirus	7 and 14 days	molecular markers	32
astrocytes and NG2 glia in injured cortex	**NeuroD1** retrovirus	7 and 14 days	molecular markers, action potentials, synaptic currents	33
NG2 glia in injured adult cortex	**Sox2** retrovirus	12 and 24 days	morphology, markers, action potentials, synaptic currents	30
astrocytes in adult striatum	**Sox2** lentivirus	5 weeks	morphology, markers, action potentials, synaptic currents requires BDNF+Nogin or Valproic Acid for maturation	50

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Summary of *in vivo* reprogramming experiments performed by transducing proneural genes in glial cells of the adult mouse brain. The cell types reprogrammed are indicated on the left of the figure, the proneural genes and the viral vectors used for their transduction are indicated in the second column, the time between gene transduction and analysis is indicated in the third column, the neuronal features assessed in the reprogrammed cells are indicated in the fourth column, and the reference to the articles reporting the experiments are indicated in the last column. Note that a study showed that expression of Ascl1 alone could convert reactive astrocytes in the midbrain, striatum and cerebral cortex into mature neurons of both GABAergic and glutamatergic subtypes that integrate morphologically and functionally into existing circuits [35]. In contrast, other studies have reported very little or no neuronal reprogramming by transduction of Ascl1 alone into glial cells of the spinal cord or cerebral cortex [13,36,37,38•]. Although very similar, these different studies used different viral systems for Ascl1 delivery, resulting in different levels of expression or target cell specificity, which may explain these different results.

repressed. Indeed, an analysis of genomic occupancy of ectopically expressed Ascl1 in fibroblasts from the Wernig lab showed that Ascl1 is an 'on target' pioneer factor that is able to recognize the regulatory elements of its neuronal target genes even when they are nucleosome-bound in fibroblasts [40••]. This pioneering activity of Ascl1 has been related to the structure of its DNA binding domain, which is shorter than that of other bHLH proteins (i.e. Olig2, Neurod1, MyoD and Tal1) and therefore likely to contact fewer nucleotides in its consensus binding site, which might allow Ascl1 to bind to this site even when the remaining nucleotides are engaged in nucleosome interactions [41••]. Interestingly, Ascl1-occupied loci are enriched in a trivalent chromatin signature comprised of H3K4me1, H3K27ac and H3K9me3 in cell types that are efficiently reprogrammed by Ascl1, whereas enrichment is low in cell types that are refractory to Ascl1 reprogramming, suggesting a role for this trivalent chromatin state in Ascl1 recruitment [40••]. To convert human pericytes into neurons, Ascl1 cannot act alone but requires co-expression with Sox2, suggesting that Sox2 is required in these cells to induce a chromatin state permissive for Ascl1 binding, or alternatively that Sox2 guides Ascl1 to essential target sites [26•,42,43]. Importantly, Ascl1 appears to have a similar pioneering activity during normal development, since it is also bound to closed chromatin in neural stem cells and many of its target genes shift to an open configuration during differentiation [40••,44••,45]. In contrast to Ascl1, there is no evidence that Neurog2 acts as a pioneer factor, although it efficiently converts pluripotent stem cells and astrocytes into neurons, probably reflecting the accessibility of the regulatory elements of its target genes in these cell types [21,30•].

Post-transcriptional regulation of proneural proteins

Proneural genes are expressed in a transient manner in discrete progenitor populations, through tight regulation of their transcription [1]. In addition to this well-documented mode of regulation, proneural proteins have recently been shown to undergo a complex set of phosphorylations that profoundly influence their activities. The first evidence came from a pioneering study by Franck Polleux and colleagues, who showed that the phosphorylation of a mammalian-specific tyrosine residue in Neurog2 is required for its role in neuronal migration and dendritic morphology, but not for its proneural function [3]. Little else was reported on the subject afterwards but in the last 5 years, a number of studies from the groups

of Anna Philpott and Carol Schuurmans have reported extensive phosphorylation of all the major mammalian proneural proteins *in vivo*. Phosphorylation of multiple serine residues in Neurog2 and Ascl1 has been shown to act as a rheostat regulator of DNA binding, a clear departure from the classical all-or-nothing notion of proneural protein function [46,47•]. These phosphorylation events are regulated by cyclin-dependent kinases, suggesting a model in which neuronal progenitors gradually extinguish proneural protein activity by a succession of phosphorylation events as they exit the cell cycle. Further analysis showed that these serine phosphorylation events influence the choice of transcriptional targets by proneural proteins, suggesting a model whereby highly phosphorylated proteins (in cycling progenitors) are only able to bind and activate target genes with an open chromatin such as the Notch ligand gene *Dll1*, while un-phosphorylated proteins (in postmitotic neurons) are able to bind and activate targets with a less accessible chromatin, such as the differentiation genes *Neurod1* for Neurog2 and *Myt1* for Ascl1, through recruitment of chromatin-remodelling factors [46–48].

Wnt signalling has been proposed to control the time window of activity of proneural proteins through regulation of their phosphorylation during development of the mammalian cerebral cortex and of *Drosophila* sensory bristles. By reducing the activity of the GSK3β/Shaggy kinase, Wnt signalling promotes the de-phosphorylation and stimulates the activity of Neurog2 in the mouse cortex and of Drosophila Scute in sensory bristles. Phosphorylation by GSK3β modulates Neurog2 activity by influencing its choice of dimerization partner rather than its DNA binding affinity [49,50].

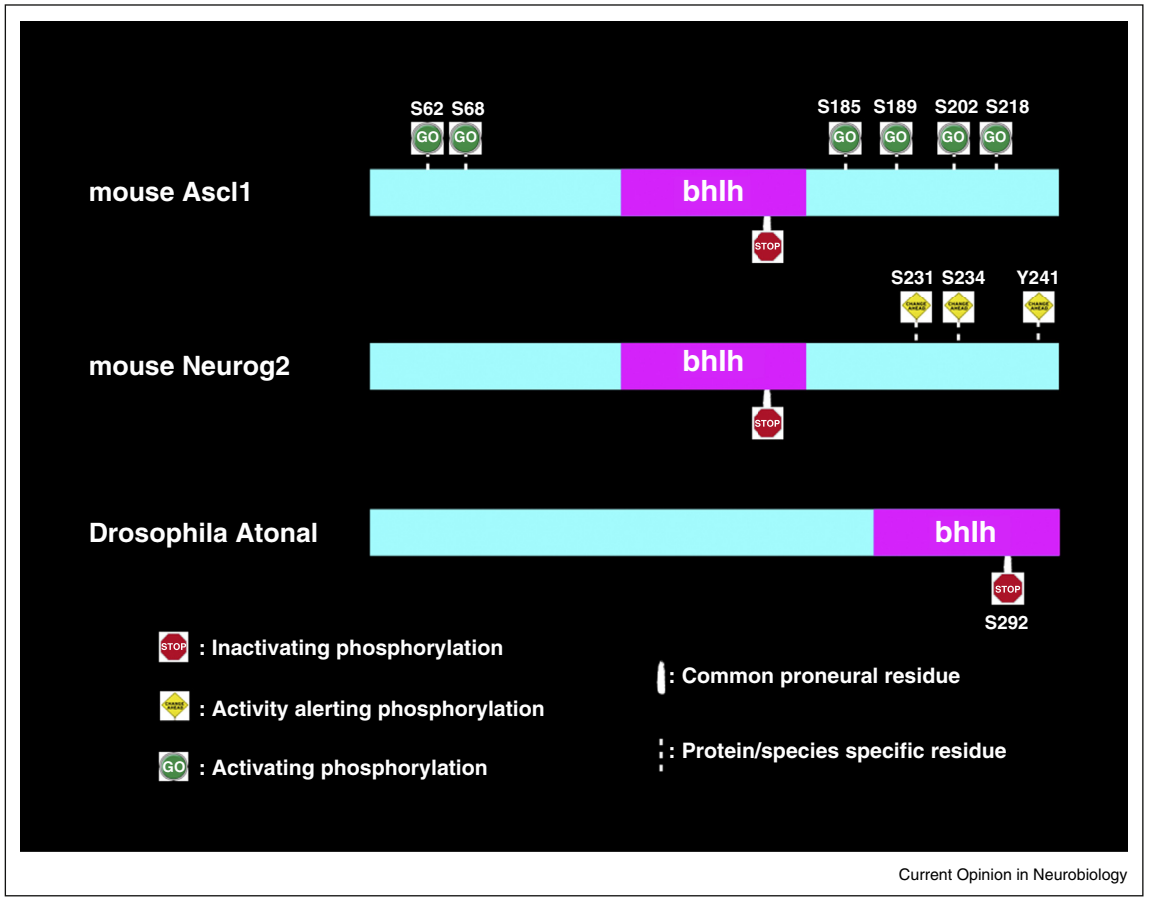
Most of the Cdk and GSK3β phosphorylation sites are located outside of the bHLH domain, which mediates DNA binding and heterodimerisation, and are not conserved across proneural homologues and paralogues, suggesting that each proneural protein has evolved its own sophisticated fine-tuning regulatory mechanisms. In contrast, phosphorylation of a residue present at the very same location in the bHLH domain of all *Drosophila* and vertebrate proneural proteins has recently been shown to act as an on/off switch for *in vivo* DNA binding and transcriptional activity of all proneural proteins, and to be essential for the rapid termination of their fate specification functions in early neural progenitors [51••]. Interestingly, when examined at endogenous levels using a knock-in approach for *Drosophila ato* the data indicate that the phosphorylated isoform — although lacking proneural activity — persist for several hours in non-dividing neurons. Whether it plays a role in early neuronal differentiation, akin to what has been shown for phosphorylated Neurog2, remains unclear. Thus, while a conserved post-translationally modified residue controls in a similar — potentially binary — way all proneural proteins, modifications of non-conserved residues may fine-tune the context-specific functions of individual proneural proteins (Figure 4). What this means to their comparative differential endogenous activities remains to be investigated, but the deep conservation of the inactivating residue across all proneural proteins indicates this may be a fundamental feature of neurogenesis across animal species.

Proneural protein oscillations

The finding that Ascl1 promotes both cell proliferation and cell differentiation, sometime sequentially in the same lineage (e.g. in the embryonic telencephalon and adult ventricular-subventricular zone), raises the interesting question of how the switch between different activities of proneural proteins is controlled. Besides the change in level of protein phosphorylation between progenitors and postmitotic neurons discussed above, other mechanisms might also modulate the function of proneural proteins by regulating their interactions with target genes. The dynamics of proneural protein expression in particular has been proposed to play an important role in regulating their activity. The levels of Ascl1 and Neurog2 transcripts and proteins oscillate in neural progenitors with periods of 2–3 hours, as a consequence of repression by oscillating Hes proteins downstream of Notch signalling [52,53••]. Proneural protein expression becomes stabilised when Notch signalling is down-regulated and progenitors exit the cell cycle and differentiate. Ascl1 oscillations have been shown to be required for its proliferation-promoting activity while its sustained expression has been shown to promote neuronal differentiation [53••]. Finally, while much recent attention has been paid to phosphorylation as a mechanism of both hard and soft tuning of proneural activity, other modes of regulation, including the regulation of protein stability, are only beginning to receive attention. Ascl1 is actively degraded by the E3 ubiquitin ligase HUWE1/UREB1/MULE, and the rapid elimination of Ascl1 from proliferating stem cells in the adult hippocampus by HUWE1 has been shown to be essential for their return to quiescence and the long-term maintenance of hippocampal neurogenesis [10••]. HUWE1 similarly destabilises the proneural protein Atoh1 in cerebellar granule neuron progenitors, and the signalling molecule Sonic hedgehog has been shown to enhance Atoh1 expression by preventing its phospho-dependent degradation by HUWE1 [54•].

How the stabilisation of its expression results in Ascl1 switching from progenitor-specific targets to neuron-specific targets, is still not understood. Other mechanisms, such as changes in expression level, interactions with temporally regulated co-factors and regulation of nuclear entry and exit, might also contribute to the switch between the different activities of proneural protein. Whether these different mechanisms operate independently or act in concert to regulate proneural protein

Figure 4



Schematic representation of phosphorylation events modulating proneural protein function. While a conserved modification on a Serine residue within the bHLH domain (solid line, red STOP sign) appears to act as a binary activity switch, several protein-specific, class-specific and species-specific residues (dotted lines; green GO sign, orange CHANGE sign) act as rheostat-like modulators for context-dependent proneural protein functions.

activity (e.g. oscillations or phosphorylation regulating interaction with co-factors) remains to be studied.

Conclusion

Proneural proteins have long been known for their central role in the regulation of neurogenesis, during which they coordinate the acquisition of a neural cell identity with the regulation of cell proliferation and the initiation of differentiation. To fulfil these complex functions, proneural proteins have evolved sophisticated modes of regulation of their expression and activity that are only beginning to be understood.

Conflict of interest statement

Nothing declared.

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This study compares the genomic binding of Ascl1 when expressed in mouse fibroblasts and in neural stem cells and concludes that Ascl1 is an 'on-target' pioneer factor that recognises the same sites in different cell types even when they are not in an open chromatin configuration. Recruitment of Ascl1 is shown to correlate with a trivalent chromatin signature in fibroblasts and other cell types permissive to Ascl1 reprogramming activity, whereas this signature is less enriched in cell types that are refractory to Ascl1 activity.

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This article characterises the genomic sites bound by Ascl1 in proliferating neural stem cells and differentiating neurons and correlates binding to the open or close configuration of the chromatin at these sites. In proliferating cells, Ascl1 binds locations with both an open and a close chromatin, and some of the closed sites open during differentiation, supporting the idea that Ascl1 normally acts as a pioneer factor during neurogenesis.

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This study shows that three bHLH proteins, Ascl1, Olig2 and Hes1, are expressed in an oscillatory manner in proliferating neural progenitors, and that Ascl1 expression is stabilised and the two other factors are down regulated when cells differentiate into neurons, while Olig2 expression is stabilised when cells differentiate into oligodendrocytes and Hes1 is stabilised when cells differentiate into astrocytes. Moreover, optogenetic manipulation of Ascl1 dynamics shows that oscillatory expression of Ascl1 promotes proliferation while its sustained expression promotes neuronal differentiation.

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The authors of this paper show that the E3 ubiquitin ligase HUWE1 destabilises the proneural protein Atoh1 in cerebellar granule neuron progenitors when it is phosphorylated. The signalling molecule Sonic hedgehog stimulates Atoh1 expression by promoting its dephosphorylation and protecting it from HUWE1-mediated degradation.

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In this paper, Neurog2 is shown to efficiently reprogram immature astrocytes originating from the postnatal mouse brain into neurons. However, Neurog2 becomes inactive when transduced in astrocytes that have been maintained in culture, due in part to the repressor REST preventing Neurog2 from accessing the promoter of *Neurod4*, a key target gene during reprogramming.