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Review

bHLH transcription factors in neural development, disease, and reprogramming

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ABSTRACT

The formation of functional neural circuits in the vertebrate central nervous system (CNS) requires that appropriate numbers of the correct types of neuronal and glial cells are generated in their proper places and times during development. In the embryonic CNS, multipotent progenitor cells first acquire regional identities, and then undergo precisely choreographed temporal identity transitions (i.e. time-dependent changes in their identity) that determine how many neuronal and glial cells of each type they will generate. Transcription factors of the basic-helix-loop-helix (bHLH) family have emerged as key determinants of neural cell fate specification and differentiation, ensuring that appropriate numbers of specific neuronal and glial cell types are produced. Recent studies have further revealed that the functions of these bHLH factors are strictly regulated. Given their essential developmental roles, it is not surprising that bHLH mutations and de-regulated expression are associated with various neurological diseases and cancers. Moreover, the powerful ability of bHLH factors to direct neuronal and glial cell fate specification and differentiation has been exploited in the relatively new field of cellular reprogramming, in which pluripotent stem cells or somatic stem cells are converted to neural lineages, often with a transcription factor-based lineage conversion strategy that includes one or more of the bHLH genes. These concepts are reviewed herein.

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1. Introduction

The vertebrate central nervous system (CNS) is comprised of a diverse array of neuronal and glial (astrocytes and oligodendrocytes) cell types that together make up functional brain regions that connect in a complex manner to allow for higher order cognitive functioning and sensory processing. Understanding how neural cells acquire their precise phenotypes is key to understanding brain function in normal and diseased states, and to deciphering new strategies to generate exogenous sources of neural cells for cellular repair. Members of the basic-helix-loop-helix (bHLH) family of transcription factors have emerged as essential regulators of neural cell fate specification and differentiation during embryonic development and in regions of the adult neural tube where neural stem cell (NSC) niches are maintained throughout life. Moreover, their differentiation properties have been exploited in the relatively new field of neural cell reprogramming. Indeed, one or more of these bHLH genes are included in many of the current transcription factor-based lineage neuronal conversion strategies. Here we focus on the roles of the bHLH genes in the developing CNS, describing their region-specific functions in the neural tube, and their transcriptional, post-transcriptional and post-translational regulation. We also cover associations between bHLH genes and disease, and finally, describe their central roles in neural cell reprogramming.

Due to space limitations, this review primarily focuses on murine bHLH genes, with studies in invertebrates, fish, frogs and humans only mentioned when comparisons are particularly insightful. Furthermore, due to length restrictions, we do not cover bHLH function in adult NSC niches, nor review the Hes/Hey genes, which are bHLH genes that are downstream effectors of Notch signaling.

2. A primer on bHLH transcription factors

2.1. bHLH structure and function

bHLH transcription factors are named based on their protein structure. The HLH domain is comprised of two alpha-helices connected by a non-conserved loop region and is used for dimerization, whereas the basic domain directs DNA binding (Fig. 1; reviewed in (Bertrand et al., 2002)). After dimerization, bHLH transcription factors bind E-box motifs with the consensus sequence CANNTG, with the central two nucleotides, as well as surrounding nucleotides, providing specificity of binding (reviewed in (Bertrand et al., 2002)).bHLH transcription factors can be broadly classified based on their patterns of expression as either class I or class II. Class I bHLH proteins are ubiquitously expressed, and are encoded by *Tcfe2a* (E12, E47), *Tcf4*, and *Tcf12* (Meredith and Johnson, 2000; Sun and Baltimore, 1991). Class II bHLH factors have tissue-specific expression profiles (reviewed in (Bertrand et al., 2002)) and are involved in a diverse array of developmental processes, including myogenesis (reviewed in (Berkes and Tapscott, 2005; Rudnicki et al., 2008)), fly sex determination (reviewed in (Garrell and Campuzano, 1991; Parkhurst and Meneely, 1994)), and haematopoiesis (reviewed in (Bloor et al., 2002; Curtis et al., 2012)). Herein, we focus on the class II bHLH transcription factors that are expressed and function in the developing nervous system.

Neural-specific bHLH genes are subdivided based on their homology to *Drosophila* genes that are expressed in the nervous system, including the *achaete-scute complex* (*AS-C*) (*achaete*, *scute*, *lethal of scute*) and *atonal* (*atonal*, *amos*, *cato*) gene families (Bertrand et al., 2002; Garcia-Bellido and Moscoso del Pradio, 1979; Jarman et al., 1994; Villares and Cabrera, 1987). There is a single murine *achaete-scute like 1* (*Ascl1/Mash1*) gene that is expressed in the nervous system, as well as the more distantly related *Nscl* family genes (*Nhlh1/Nscl1*, *Nhlh2/Nscl2*). In contrast, there are multiple *atonal*-related bHLH genes that are expressed in neural lineages, including members of the *Neurogenin* (*Neurog1*, *Neurog2*, *Neurog3*), *Neurod* (*Neurod1*, *Neurod2/Ndrf*, *Neurod6/Math2*, *Neurod4/Math3*), *Atonal* (*Atoh1/Math1*, *Atoh7/Math5*) and *Olig* (*Olig1*, *Olig2*, *Olig3*, *Bhlhe22/Bhlhb5*) families. While these subdivisions are based on sequence similarities, these genes can also be grouped based on functional properties into proneural and neural (neuronal or glial) differentiation genes.

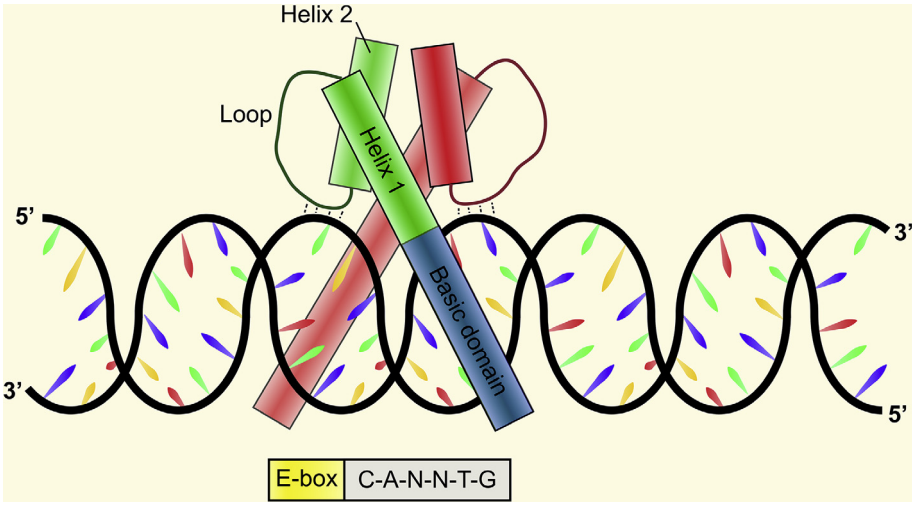

Fig. 1. Structure of bHLH transcription factors. bHLH transcription factors are comprised of two alpha helices, which mediate dimerization, and a basic domain, that binds E-box sequences in the DNA.

2.2. Proneural bHLH genes

Proneural bHLH transcription factors include a smaller subset of the neural-specific bHLH genes. In Drosophila, proneural genes in the *AS-C* and *atonal* families are expressed in uncommitted ectodermal cells, conferring a neural identity onto these cells, whereas in vertebrates, proneural genes are expressed after neural determination has occurred, in cells that have already acquired a neural identity. Despite these differences, proneural genes function similarly in flies and vertebrates, and in both species, they are necessary and sufficient to promote neuronal differentiation and to specify neuronal subtype identities (reviewed in (Bertrand et al., 2002; Guillemot and Hassan, 2017)). Moreover, in both invertebrates and vertebrates, proneural genes are characterized by their ability to induce Notch signaling, specifically the expression of Delta ligands, such as *Dll1* and *Dll3*. Finally, at a functional level, the loss of proneural gene function results in fewer neurons being born, while the gain of proneural gene function results in the generation of supernumerary neuronal cells.

Based on these strict definitions, the murine neural-specific bHLH genes with proneural activity include *Neurog1*, *Neurog2*, *Ascl1*, *Neurod4*, *Atoh1* and *Atoh7*. Both *Neurog1* (Ma et al., 1998) and *Neurog2* (Fode et al., 1998) were initially shown to have proneural activity in the peripheral nervous system (PNS), directing the differentiation of cranial ganglia neurons. Subsequent studies revealed that *Neurog2* also has proneural activity in the CNS, including in the neocortex (Fode et al., 2000), where it directs a neuronal fate while repressing a glial identity (Nieto et al., 2001), and in the spinal cord (Scardigli et al., 2001). *Ascl1* was first defined as a proneural gene in the PNS, including in the olfactory epithelium and autonomic nervous system (Guillemot et al., 1993), followed by the demonstration of its proneural properties in the ventral telencephalon (Casarosa et al., 1999; Horton et al., 1999). *Neurod4* was first characterized as a proneural gene in the tectum, where it acts in a redundant manner with *Ascl1* to promote neurogenesis while inhibiting gliogenesis (Tomita et al., 2000). Within the *Atonal* family, *Atoh1* was initially shown to function as a proneural gene in the cerebellum, where it directs the differentiation of granule neurons (Ben-Arie et al., 1997), and then later in the PNS, where it directs the differentiation of inner ear hair cells (Bermingham et al., 1999). Finally, *Atoh7* proneural activity was defined in the retina based on its requirement to direct the differentiation of retinal ganglion cells (Brown et al., 2001; Wang et al., 2001).

2.3. Differentiation bHLH genes

Several bHLH genes are expressed and function later in the development of neural lineages - either in committed neuronal/glial precursors or in postmitotic neuronal and glial cells. The genes that play important roles in neuronal and glial differentiation and maturation include members of the *NeuroD* (*Neurod1*, *Neurod2/Ndrf*, *Neurod6/Math2*), *Nscl* (*Nhlh1/Nscl1*, *Nhlh2/Nscl2*), and *Olig* (*Olig1*, *Olig2*, *Olig3*, *Bhlhe22*) families. These genes are widely expressed in distinct subsets of postmitotic neuronal and glial cells in different regions of the neural tube. For example, *Neurod1* is required for the differentiation of inner ear sensory neurons (Kim et al., 2001; Liu et al., 2000a) and granule cells in the cerebellum (Miyata et al., 1999) and hippocampus (Liu et al., 2000b; Schwab et al., 2000). *Neurod2* and *Neurod6* are together required for the formation of callosal connections in the cerebral cortex (Bormuth et al., 2013). In the retina, *Neurod1* is required for photoreceptor survival (Cho et al., 2007; Ochocinska et al., 2012), *Neurod2* is necessary for the differentiation of AII amacrine cells (Cherry et al., 2011), and *Neurod6* is required for the differentiation of non-GABAergic/non-glycinergic amacrine cells (Kay et al., 2011). *Neurod6* also cooperates with *Neurod1* for the differentiation of midbrain dopaminergic neurons (Khan et al., 2017), and for the terminal differentiation of hippocampal granule neurons (Schwab et al., 2000), whereas *Neurod2* is required to regulate the excitability of cortical pyramidal neurons (Chen et al., 2016). In the *Nsc1* bHLH family, *Nhlh1* and *Nhlh2* are required for the formation of pre-cerebellar neurons in the hindbrain (Schmid et al., 2007). Finally, of the Olig family bHLH genes, *Bhlhe22* is required for the differentiation of neurons in several CNS domains, including the dorsal horn of the spinal cord (Ross et al., 2010), dorsal cochlear nucleus in the brainstem (Cai et al., 2016) and retinal amacrine cells (Feng et al., 2006).

Unlike the neuronal differentiation genes, *Olig1* and *Olig2* are best characterized for their roles in promoting glial, and more specifically, oligodendrocyte differentiation. *Olig1* was first shown to be necessary and sufficient to promote an oligodendrocyte fate in the embryonic neocortex (Lu et al., 2001a), whereas *Olig2* was first shown to be necessary (Takebayashi et al., 2002) and sufficient (Zhou et al., 2001) to promote oligodendrocyte differentiation in the spinal cord. In contrast, *Olig3* has been best characterized as a neuronal differentiation gene, controlling the differentiation of pre-cerebellar neurons (Liu et al., 2008), brainstem nuclei (Storm et al., 2009), and dorsal spinal cord neurons (Muller et al., 2005).

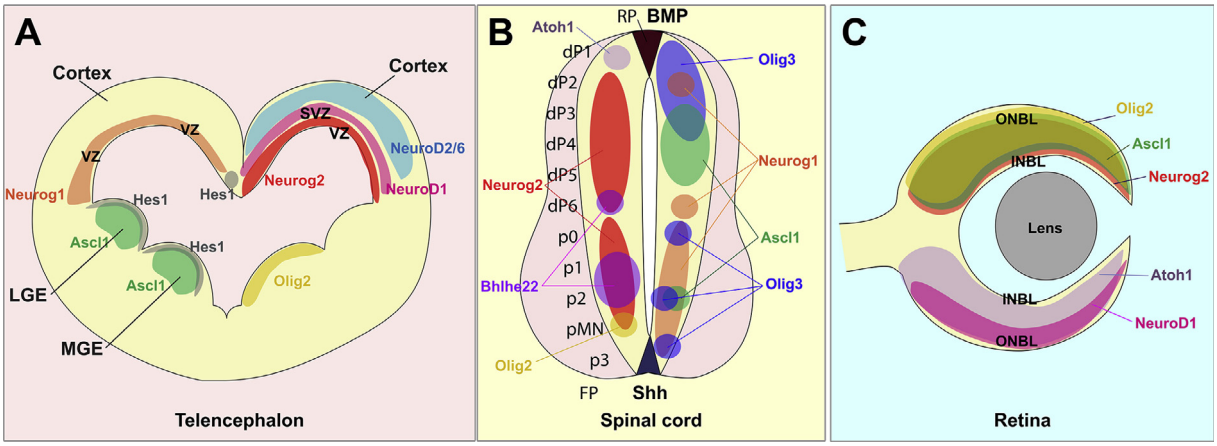

Fig. 2. Schematic representation of bHLH expression in different regions of the neural tube. (A) The telencephalon is divided into dorsal and ventral domains. In the dorsal telencephalon, which gives rise to the neocortex, *Neurog1*, *Neurog2* and *Ascl1* are expressed in cortical progenitors. In addition, *NeuroD* genes are expressed in postmitotic neurons. In the ventral telencephalon, *Ascl1* is the only proneural gene, but *Olig1/2* are also expressed in glial progenitors and OPCs. (B) The embryonic spinal cord is also divided into dorsal and ventral domains, with BMP and Wnt signals secreted from the roof plate (RP) and Shh secreted from the floor plate (FP) patterning the dorsal (dP1-dP6) and ventral (p0-p2, pMN, p3) progenitor domains. Multiple bHLH genes are expressed and function in the various progenitor domains, as highlighted in the text. (C) The embryonic retina is comprised of an outer neuroblast layer (ONBL) of dividing progenitors, and an inner neuroblast layer (INBL) of postmitotic, differentiated neurons. Various bHLH genes are expressed in these domains as highlighted in the text.

3. bHLH function in the embryonic neural tube

3.1. Overview

A major question for neurodevelopmental biologists is how distinct types of neuronal and glial cells are generated in different regions of the neural tube, especially when the same bHLH factors are often re-used or 're-purposed' in multiple CNS domains. We provide here an overview of how neuronal fates are specified by bHLH genes in various CNS domains, including the dorsal and ventral telencephalon, spinal cord, retina and hindbrain, with the goal of highlighting similarities and differences in proneural gene function in these domains.

3.2. bHLH gene function in the dorsal telencephalon

The dorsal telencephalon is the embryonic anlage of the neocortex, which, at maturity, is a six-layered neuronal structure. During embryogenesis, neocortical neurons are derived from cortical progenitor cells in the ventricular zone (VZ) and subventricular zone (SVZ) of the dorsal telencephalon (Fig. 2A). These progenitors give rise to glutamatergic pyramidal neurons between embryonic day (E) 10.5 and E17.0 in mouse (Takahashi et al., 1999), followed by the differentiation of astrocytes in late embryogenesis, and oligodendrocytes in the early postnatal period (Kessaris et al., 2006). The earliest-born neurons are the Cajal-Retzius neurons, which arise between E10.5 and E12.5 from progenitor domains in the pallial margins, including the ventral pallium, cortical hem and pallial septum (Bielle et al., 2005). Cajal-Retzius neurons form the marginal zone, which is layer I of the neocortex. Subplate neurons, which form a transient layer VII in most species, are generated next, at E11.5, and settle beneath the marginal zone (Takahashi et al., 1999). Together the marginal zone and subplate merge to form a preplate layer of neurons. As layer VI neurons differentiate, at ~E12.5, they migrate into the center of the preplate to separate it into an overlying marginal zone and underlying subplate (Takahashi et al., 1999). The remaining neuronal layers are generated in a sequential, inside-out fashion, with layer V neurons differentiating next and migrating past layer VI neurons to settle in a more superficial position, followed by the differentiation and migration of layer IV and finally, layer II/III (fused in mouse) neurons (Caviness et al., 1995).

3.2.1. Neurog1/Neurog2

Neurog1. and *Neurog2* are expressed in cortical (or pallial) progenitors in the VZ, and to a lesser extent in the SVZ, of the dorsal telencephalon beginning at the onset of neurogenesis, with *Neurog2* expressed throughout neurogenesis (E10.5-E17) and beyond, while *Neurog1* expression declines after E15.5 (Dixit et al., 2014b; Fode et al., 2000; Schuurmans et al., 2004). Spatially, *Neurog1* transcripts are more abundant in lateral domains, whereas *Neurog2* transcripts are distributed throughout the VZ. In loss-of-function studies, *Neurog2* is required for the genesis of a subset of the earliest-born Cajal-Retzius neurons (Dixit et al., 2014b). Interestingly, while fewer Cajal-Retzius neurons are generated in *Neurog2*$^{-/-}$ cortices, more of these early-born neurons are generated in *Neurog1*$^{-/-}$ cortices (Dixit et al., 2014b; Fode et al., 2000; Mattar et al., 2004). A possible reason is that *Neurog1* is required to limit the proneural activity of *Neurog2*, such that in the absence of *Neurog1*, *Neurog2* has enhanced proneural activity, and can promote the differentiation of more Cajal-Retzius neurons (CS, DD, data not shown; (Dixit et al., 2014b)). In contrast, in the lateral piriform cortex, these two genes are required together to generate lot1^{+} Cajal-Retzius neurons, which are not present in *Neurog1*$^{-/-}$; *Neurog2*$^{-/-}$ cortices (Dixit et al., 2014b).

The next cells to differentiate in the neocortex are the subplate neurons, which are reduced in number and disrupted in organization in the absence of *Neurog2* function (Mattar et al., 2004). The subplate is disrupted in *Neurog2*$^{-/-}$ cortices in part because *Ascl1* is upregulated, which leads to the misspecification of early-born (birth before E14.5) neurons to an aberrant GABAergic identity (Fode et al., 2000; Schuurmans et al., 2004). Notably, *Neurog2* forms a genetic switch with *Ascl1*, such that when *Neurog2* is turned off, *Ascl1* expression is turned on (Fode et al., 2000; Schuurmans et al., 2004). The upregulation of *Ascl1* results in the generation of ectopic GABAergic interneurons (Fode et al., 2000; Schuurmans et al., 2004), as well as oligodendrocyte precursor cells (OPCs) (Parras et al., 2007).

Not only are *Neurog1* and *Neurog2* both required for the expression of markers of a dorsal regional identity and glutamatergic

neurotransmitter phenotype in the embryonic neocortex, they are also sufficient to induce this phenotype (Fode et al., 2000; Mattar et al., 2008; Parras et al., 2002; Schuurmans et al., 2004), at least when misexpressed before E14.5 (Dennis et al., 2017; Li et al., 2012). Before E14.5, *Neurog2* can induce the expression of most *NeuroD* and *Nscl1* family genes in cortical and subcortical progenitors, all of which are expressed in subsets of glutamatergic neurons in the embryonic neocortex (Mattar et al., 2008). After E14.5, *Neurog2* activity is tightly regulated, as discussed further below.

Neurog1 can similarly promote neurogenesis in early cortical progenitors, but these gain-of-function studies have only been performed at E10.5 (Dixit et al., 2014b), so it is not clear whether there are similar temporal restrictions on *Neurog1* activity. In addition to promoting neurogenesis, *Neurog1* represses glial fates in two ways. First, Neurog1 protein sequesters CBP-Smad1-Stat3 transcriptional complexes away from the regulatory regions of genes involved in astrogliogenesis (Sun et al., 2001). Second, *Neurog1* activates the expression of *miR-9*, a micro RNA that targets components of the Jak-Stat pathway, including *Lifr-beta*, *Gp130*, and *Jak1* (Zhao et al., 2015). While structurally similar to Neurog1, a role in gliogenic repression by Neurog2 has not yet been shown.

In addition to regulating neuronal differentiation and subtype specification, *Neurog1* and *Neurog2* are also required for the proper migration of neocortical neurons, especially early-born deep-layer neurons (Schuurmans et al., 2004). An important Neurog2 residue that regulates the polarity of the leading process during neuronal migration is Y241, the phosphorylation of which is required to inhibit RhoA activity (Ge et al., 2006; Hand et al., 2005). To mediate the inhibition of RhoA, Neurog2 induces the transcription of *Rnd2*, a small GTP-binding protein (Heng et al., 2008).

There is also evidence that *Neurog2* regulates axonal projection patterns in the neocortex, as thalamocortical axons (Seibt et al., 2003), which target the subplate, corticofugal axons (Dennis et al., 2017; Hand and Polleux, 2011; Schuurmans et al., 2004), which project subcortically from layers VI and V, and callosal axons, which target the midline (Hand and Polleux, 2011), are all disrupted in *Neurog2*$^{-/-}$ cortices. Moreover, misexpression of *Neurog2* can confer a subcortical projection pattern onto newborn neurons even when expressed outside of the normal window of their birth (i.e., after E14.5), even though these ectopic neurons do not express normal deep-layer markers (Dennis et al., 2017).

3.2.2. Ascl1

Ascl1. is expressed in a limited number of cortical progenitors in the VZ and SVZ compared to its robust expression in the ventral telencephalon (as discussed below) (Britz et al., 2006). In the developing neocortex, similar to *Neurog2*, *Ascl1* is required for the differentiation of a subset of early-born, glutamatergic Cajal-Retzius neurons (Dixit et al., 2011). At later stages, when cortical plate neurons are differentiating, *Ascl1* controls radial migration by regulating the expression of Rnd3, which similar to Rnd2, is a small GTP-binding protein that inhibits RhoA (Pacary et al., 2011).

Ascl1 also functions in a redundant manner with *Neurog2* to control laminar fate specification via input into the cortical derepression circuit (Dennis et al., 2017). *Neurog2* and *Ascl1* are required together to turn off the expression of Tbr1, a layer VI marker, in upper layer neurons, which should not express this gene. *Neurog2* and *Ascl1* are also both required and sufficient for the generation of Ctip2^{+} layer V neurons, which are lost in *Neurog2*$^{-/-}$; *Ascl1*$^{-/-}$ cortices (Dennis et al., 2017). Finally, *Neurog2* and *Ascl1* are both required to repress the expression of Satb2, a marker of late-born upper layer neurons that are generated precociously in *Neurog2*$^{-/-}$; *Ascl1*$^{-/-}$ cortices (Dennis et al., 2017). Notably, there is also precocious gliogenesis in *Neurog2*$^{-/-}$; *Ascl1*$^{-/-}$ cortices, suggesting that these two proneural genes are required together to control the timing of neural cell differentiation (Nieto et al., 2001). In addition, *Neurog2* and *Ascl1* also cooperate in the guidance of callosal axons to the midline, and while the corpus callosum does not form in *Ascl1*$^{-/-}$ mutants, likely due to defects in the formation of midline glia that serve as guideposts for these axons (Casarosa et al., 1999; Dennis et al., 2017), axonal targeting to the midline is much more disrupted in *Neurog2*$^{-/-}$; *Ascl1*$^{-/-}$ cortices (Dennis et al., 2017).

Finally, at early postnatal stages, *Ascl1* is involved in the specification of an OPC fate in cortical progenitors (Nakatani et al., 2013), akin to its function in embryonic progenitors in the ventral telencephalon (see below).

3.3. bHLH gene function in the ventral telencephalon

At early embryonic stages (~E12.5), the ventral telencephalon is divided into the lateral (LGE), medial (MGE) and caudal (CGE) ganglionic eminences (Fig. 2A), all of which give rise to distinct populations of GABAergic neurons (reviewed in (Laclef and Metin, 2017)). These progenitor domains give rise to projection neurons that remain in the ventral telencephalon, populating the globus pallidus, amygdala and septum (MGE, CGE), and striatum (LGE, CGE) (Laclef and Metin, 2017). In addition, ventrally derived neurons migrate tangentially to dorsal domains of the telencephalon, with the MGE and CGE contributing to neocortical and hippocampal GABAergic interneurons, and the LGE giving rise to interneurons that populate the olfactory bulb (Laclef and Metin, 2017). Neuronal differentiation in this region of the brain occurs earlier than in dorsal domains, with the MGE giving rise to most neurons between E10.5 and E14.5, and migration beginning at ~E12.5 (Laclef and Metin, 2017).

The only proneural bHLH gene expressed in the ventral telencephalon is *Ascl1*, transcripts for which are found in subcortical (or subpallial) progenitors in the VZ and SVZ of the LGE, MGE, and CGE. *Ascl1* is required for the generation of GABAergic neurons, most notably in the MGE (Casarosa et al., 1999; Horton et al., 1999). The reduction in size of the MGE in *Ascl1*$^{-/-}$ brains is due to a reduction in proliferation, and lower expression of *Dll1* and downstream Notch effectors such as *Hes5* (Casarosa et al., 1999), highlighting the key connection between proneural genes and Notch signaling. *Ascl1* is also required to specify an OPC fate in the embryonic ventral telencephalon (Parras et al., 2007), raising the question of how it selects between GABAergic neuronal and oligodendroglial target genes. To preferentially promote an oligodendrocyte fate, *Ascl1* represses the generation of Dlx^{+} progenitors and a GABAergic neuronal fate (Petryniak et al., 2007). In addition, in the context of high RAS/ERK signaling, which is initiated downstream of several receptor tyrosine kinases (RTKs) and other signaling pathways, *Ascl1* promotes a glioblast rather than a neuronal identity (Li et al., 2014).

Ascl1 also has a third function in the ventral telencephalon, which is to promote the proliferation of subpallial progenitors, a function that was uncovered through genome wide profiling of target genes, many of which are positive cell cycle regulators (Castro et al., 2011). This activity of *Ascl1* was unexpected, as proneural genes are classically considered to promote cell cycle exit and neuronal differentiation, but misexpression of *Ascl1* can promote proliferation when misexpressed in certain cellular contexts (Castro et al., 2011; Li et al., 2014). A recent report suggests that *Neurog2* may similarly promote proliferation when misexpressed in the neocortex in a limited fashion, at least for the first 16 h post-overexpression (Hagey and Muhr, 2014).

During embryogenesis, OPCs are generated in the ventral telencephalon and migrate tangentially into the dorsal telencephalon, similar to GABAergic interneurons (Laclef and Metin, 2017). Both *Olig1* and *Olig2* are expressed in OPCs in the ventral telencephalon

and their expression is sustained during migration (Lu et al., 2000; Tekki-Kessaris et al., 2001; Zhou et al., 2000). Furthermore, *Olig1/2* are required for oligodendrocyte fate specification in the ventral telencephalon (Zhou and Anderson, 2002).

3.4. bHLH gene function in the spinal cord

In the spinal cord, progenitors are distributed in zones based on their position along the dorsoventral axis (reviewed in (Lu et al., 2015)). Dorsal progenitor zones range from dP1 (closest to the roof plate) to dP6 (more medial), while ventral zones range from p0 (adjacent to dP6) to p3 (closest to the floor plate), with a motor neuron progenitor zone (pMN) located between p2 and p3 (Fig. 2B; reviewed in (Ulloa and Briscoe, 2007)). As progenitors differentiate into interneurons the dorsal domains change from being classified as dorsal progenitor (dP) to dorsal interneuron (dI) domains. The specification of dorsoventral progenitor identity occurs in two steps, beginning with the establishment of fuzzy borders between progenitor domains in response to morphogen gradients; BMPs and Wnts secreted from the dorsal roof plate, and Shh from the ventral floor plate (Fig. 2B; (Ulloa and Briscoe, 2007)). These morphogens act in a concentration-dependent manner to regulate the expression of transcription factors that confer a regional identity. For example, Shh represses Class I target genes (e.g., *Pax6*, *Dbx2*) while inducing Class II target genes (eg., *Nkx2.2*, *Nkx6.1*) at specific morphogen concentrations. These Class I and Class II genes, which encode homeodomain transcription factors, are cross-repressive, leading to the sharpening of progenitor boundaries.

Many bHLH genes are also expressed in the developing spinal cord, including *Neurog1* (dP2, dP6, p0-p3), *Neurog2* (dP2-dP6, p0-p2, pMN), *Ascl1* (dP3-dP5, p2), *Atoh1* (dP1), *Olig2* (pMN), *Olig3* (dP1-dP3, p0, p2, p3) (reviewed in (Lai et al., 2016)) and *Bhlhe22* (dP6, p1, p2) (Skaggs et al., 2011). All of these genes are required for the differentiation of different populations of spinal cord neurons and glia. *Ascl1* is required for neurogenesis in the spinal cord (Battiste et al., 2007), with a specific reduction in dI3 and dI5 interneurons observed in *Ascl1*$^{-/-}$ spinal cords dorsally (Helms et al., 2005; Mizuguchi et al., 2006), while v2 interneurons are reduced ventrally (Parras et al., 2002). Moreover, overexpression of *Ascl1* promotes the formation of ectopic dI3 and dI5 interneurons (Muller et al., 2005). Finally, *Ascl1* is required for the generation of spinal OPCs, and their subsequent differentiation into myelinating oligodendrocytes (Sugimori et al., 2007; Sugimori et al., 2008; Vue et al., 2014).

In contrast, *Neurog2* functions in the ventral spinal cord, with a reduction in motor neurons and v1-v3 interneurons seen in *Neurog2*$^{-/-}$ mice (Parras et al., 2002; Scardigli et al., 2001). In the embryonic spinal cord, Neurog2 binds retinoic acid receptors (RAR), recruiting them to motor neuron differentiation genes so as to integrate RA motor neuron differentiation signals (Lee et al., 2009; Lee and Pfaff, 2003; Lee et al., 2004). *Olig2* turns on *Neurog2* expression in the pMN, and when these two genes are co-expressed, they induce motor neurons to differentiate (Mizuguchi et al., 2001; Zhou et al., 2001). Conversely, the down-regulation of *Neurog2* expression and upregulation of *Nkx2.2* converts *Olig2* to a pro-oligodendrocyte protein (Zhou et al., 2001). In contrast, *Olig3* is required for the differentiation of dI1-dI3 interneurons, acting through the inhibition of genes that are normally expressed in dI4-dI6 (Muller et al., 2005).

Atoh1 is required for the specification of dI1 dorsal interneurons, and has cross-repressive interactions with *Neurog1*, which is required for the differentiation of a distinct set of dorsal interneurons (Bermingham et al., 2001; Gowan et al., 2001). *Neurog1* is further involved in the ventral spinal cord, where, along with *Neurog2*, it is required for the differentiation of the full complement of motor neurons and ventral interneurons (Scardigli et al., 2001). Finally, *Bhlhe22* is required for the differentiation of v1 interneurons (Skaggs et al., 2011).

3.5. bHLH gene function in the hindbrain

The dorsal hindbrain, which surrounds the fourth ventricle and is also known as the rhombic lip, gives rise to neurons that populate hindbrain nuclei (e.g., cochlear nucleus, pre-cerebellar and deep-cerebellar nuclei, etc.). The hindbrain forms on the caudal side of the isthmus, also known as the mid-hindbrain boundary (MHB), with Fgfs acting as the main inductive pathway (reviewed in (Butts et al., 2014a)).

At maturity, the cerebellum has a very precise folding pattern, and is divided into 10 lobules and 4 transverse domains. It is comprised of two main cell types; Purkinje cells, which form a monolayer in the center, and mature granule cells, which are found in an underlying internal granular layer (IGL). During development, an external granular layer (EGL) containing granule cell precursors overlies the Purkinje cell layer.

In the embryonic and early postnatal period, granule cell precursors in the outer EGL express *Atoh1*, and proliferate in response to Shh, which is secreted by Purkinje cells (Ben-Arie et al., 1997). The EGL persists until P21, and during this time, *Atoh1* is required for the proliferation and amplification of granule cell precursors (Flora et al., 2009), and it prevents these cells from differentiating (Klisch et al., 2011). In order for granule cell precursors to differentiate, they must downregulate *Atoh1* expression and begin to express *Neurod1*, which inhibits transit amplification, at least in amphibians (Butts et al., 2014b).

Ascl1 is also expressed in VZ progenitors in the hindbrain, where it is required for the differentiation of cerebellar interneurons (Sudarov et al., 2011). *Neurog2*$^{+}$ progenitors also give rise to Purkinje cells, and in the absence of *Neurog2*, there is an overall reduction in cerebellar size, and Purkinje cells have poorly developed dendritic arbors (Florio et al., 2012).

There are also other hindbrain nuclei, and *Atoh1* is involved in their generation, including the respiratory rhythm generators, such that *Atoh1*$^{-/-}$ mice die perinatally from severe apnea (Rose et al., 2009). In addition, *Nhlh1* and *Nhlh2* are together required for the migration of neurons that form pre-cerebellar nuclei in the pons and medulla oblongota (Schmid et al., 2007). Finally, *Ascl1* is required for the differentiation of a subset of serotonergic neurons in the hindbrain (Pattyn et al., 2004).

3.6. bHLH gene function in the retina

The optic vesicles first arise from an evagination of the diencephalon at E8.5 in mouse (Wawersik and Maas, 2000). The optic vesicles then invaginate at E10 to form a bilayered optic cup that is the anlage of the neural retina and retinal pigment epithelium (RPE) (Fig. 2C). The first neurons to differentiate are the retinal ganglion cells (RGCs), which first appear at ~E11.5, followed closely by cone photoreceptors, and horizontal and amacrine cell interneurons, which are generated in an overlapping fashion during the embryonic period (Young, 1985). The remaining retinal cell types, including rod photoreceptors, bipolar cells and Müller glia, begin to differentiate in late embryogenesis, with their genesis peaking in the early postnatal period, and ending by postnatal day (P) 12.

Several bHLH genes are expressed in the embryonic retina, including the proneural genes *Neurog2*, *Ascl1*, *Neurod4*, and *Atoh7*, and the differentiation genes *Neurod1*, *Neurod2*, *Neurod6*, *Bhlhbe22* and *Olig2* (Cherry et al., 2011). In general, developmental defects in the retina are rather subtle when individual bHLH genes are knocked out. One exception is the proneural gene *Atoh7*, which is required for the generation of most RGCs in mouse (Brown et al.,

2001; Wang et al., 2001) and zebrafish (Kay et al., 2001). The timing of RGC neurogenesis is also controlled by *Neurog2*, with a delay in early RGC genesis observed in *Neurog2*$^{-/-}$ retinas (Hufnagel et al., 2010). The ectopic expression of *Ascl1*, which appears at E12.5 in the *Neurog2*$^{-/-}$ retina, as opposed to its normal onset after E14.5, has been suggested to rescue early RGC differentiation defects (Hufnagel et al., 2010). However, *Ascl1* is not required for the differentiation of RGCs (Tomita et al., 1996), and lineage tracing suggests that *Ascl1*$^{+}$ progenitors do not give rise to RGCs (Brzezinski et al., 2011), consistent with the lack of *Ascl1* expression until after E14.5 (Dixit et al., 2014a), when RGC genesis is almost complete. *Ascl1* is also not required for the genesis of other early-born retinal cell types, although there is a reduction in normally late-born bipolar cells in *Ascl1*$^{-/-}$ retinal explants grown to the equivalent of postnatal stages (Tomita et al., 1996). In Xenopus retina, *Ascl1* is required and sufficient to promote the differentiation of GABAergic neurons (Mazurier et al., 2014), but this function does not appear to be conserved in the murine retina, possibly because of compensatory effects from other bHLH genes.

Of the retinal differentiation bHLH genes, the loss of *Neurod1* results in the degeneration of photoreceptors (Cho et al., 2007; Ochocinska et al., 2012), while there is a reduction in AII amacrine cells in *Neurod2* mutants (Cherry et al., 2011). *Bhlhbe22* is required for the differentiation of GABAergic amacrine cells and cone bipolar cells (Feng et al., 2006; Huang et al., 2014). Finally, while *Olig2* function has not been assessed, based on lineage tracing, *Olig2*$^{+}$ retinal progenitor cells are biased towards terminal divisions and preferentially gave rise to rods and amacrine cells (Hafler et al., 2012).

More severe defects in retinal development are only observed in triple bHLH gene mutants (Akagi et al., 2004), highlighting the ability of these genes to compensate for one another. In *Ascl1*$^{-/-}$; *Neurog2*$^{-/-}$; *Neurod4*$^{-/-}$ retinas, fewer amacrine, horizontal and bipolar cells are generated, while more Müller glia and RGCs differentiate (Akagi et al., 2004). In *Neurog2*$^{-/-}$; *Neurod4*$^{-/-}$; *Neurod1*$^{-/-}$ retinas, there are only two cell layers, with the outer layer containing photoreceptors, a few amacrine cells and an expanded pool of Müller glia, whereas there are virtually no bipolar or horizontal cells. In the second layer there are a reduced number of RGCs (Akagi et al., 2004). Finally, in *Ascl1*$^{-/-}$; *Neurod4*$^{-/-}$; *Neurod1*$^{-/-}$ retinas, there are fewer photoreceptors and bipolar cells, whereas RGCs and Müller glia are increased in number (Akagi et al., 2004). The increase in Müller glia, which are the last born cell type, in all three triple mutants, suggests that the proneural genes are required to regulate the timing of the switch from neurogenesis to gliogenesis (Akagi et al., 2004). Another important finding is that in the absence of the different bHLH genes, other bHLH genes are upregulated (e.g. *Ascl1*, *Neurog2* and *Neurod4* are upregulated in *Neurod1* mutant retinas) (Cho et al., 2007), similar to the upregulation in *Ascl1* expression in *Neurog2* mutants in the neocortex (Fode et al., 2000). There are thus multiple cross regulatory interactions between bHLH transcription factors that appear to be compensatory.

4. Conclusions and Future directions

When comparing the functions of bHLH transcription factors across the different subdivisions of the neural tube, one of the most striking features is that individual factors can specify distinct cell fates in different domains. A major question is how the same transcription factor can have such diverse roles. One reason is that these bHLH transcription factors are tightly regulated through a host of different post-transcriptional and post-translational events, as highlighted further below. Another interesting feature of these genes is the extent of their regulatory interactions. Most notably, *Neurog2* is required to repress the expression of *Ascl1* in both the retina and forebrain, and other regulatory interactions between bHLH factors have been uncovered in the retina and other regions of the neural tube. These repressive functions of the proneural genes were not expected, as they were considered to be transcriptional activators. Indeed, the ability of Neurog2 to repress a set of genes is an indirect process, involving the transactivation of downstream repressors (Kovach et al., 2013). Unravelling the regulatory interactions between bHLH genes, and the extent to which they are redundant or have distinct functions, still remains a challenge.

5. Regulation of bHLH transcription factors

5.1. Regulation of bHLH transcription factor expression by signaling pathways

5.1.1. Notch signaling

bHLH transcription factors, especially those with proneural function, have a known association with the Notch signaling pathway (Fig. 3A). Proneural bHLH transcription factors transactivate Notch ligands (eg., Delta1 (Dll1)/Dll3), which then bind Notch receptors on neighboring cells to repress proneural expression through a process known as lateral inhibition (Castro et al., 2006). Upon Dll1/3 ligand binding, the Notch receptor is cleaved, resulting in nuclear translocation of the Notch intracellular domain (NICD), which interacts with Rbpj (CSL) to activate the transcription of *Hes* genes (Honjo, 1996; Ohtsuka et al., 1999; Selkoe and Kopan, 2003). Hes transcription factors recruit Groucho/TLE corepressor complexes to N-boxes (CACNAG) in promoter and enhancer elements to repress gene transcription (Bai et al., 2007; Kageyama et al., 2008; Ohsako et al., 1994; Ross et al., 2003). When Hes transcription factors become highly expressed, proneural genes are repressed (Bertrand et al., 2002; Chen et al., 1997; Ross et al., 2003). Hes transcription factors can further interfere with proneural transcription factors by binding and sequestering E-proteins that would normally dimerize with them (Alifragis et al., 1997; Bertrand et al., 2002; Kageyama et al., 2008; Ross et al., 2003).

While proneural activity in one cell may lead to proneural repression in neighboring cells, this repressive state is not stable. Hes proteins are autoregulatory, meaning that they are able to repress their own promoters, and can initiate oscillatory promoter activity (Shimojo et al., 2008; Takebayashi et al., 1994). Within a given cell, *Hes* oscillations create a state whereby proneural genes and *Hes* genes oscillate inversely with each other in two to three hour cycles (Imayoshi et al., 2013; Shimojo et al., 2008). Proneural genes thus create intercellular inhibition while *Hes* genes create intracellular oscillations. Similar oscillations have been observed with *Ascl1*, also under the repressive control of *Hes1* (Chen et al., 1997; Imayoshi et al., 2013). However, this mechanism is not common to all bHLH transcription factors, as *Olig2* has a distinct oscillatory pattern that is independent of *Hes1* (Imayoshi et al., 2013). Finally, *NeuroD* regulates Notch activity in the developing retina, but whether it acts in an oscillatory manner is unknown (Taylor et al., 2015).

5.1.2. Wnt signaling

Wnt signaling is an important regulator of bHLH gene transcription (Fig. 3B). Wnt signaling is able to induce expression of *Neurog2* and is a direct regulator of *Atoh1* (Gunhaga et al., 2003; Machon et al., 2005; Shi et al., 2010; Watanabe et al., 2005). Wnt signaling is also likely a direct regulator of *Neurog1*, as Lef binding sites reside within the *Neurog1* regulatory region (Hirabayashi et al., 2009). Indeed, Wnt signaling components are sufficient to induce ectopic expression of both *Neurog1* and *Neurog2* in the ventral

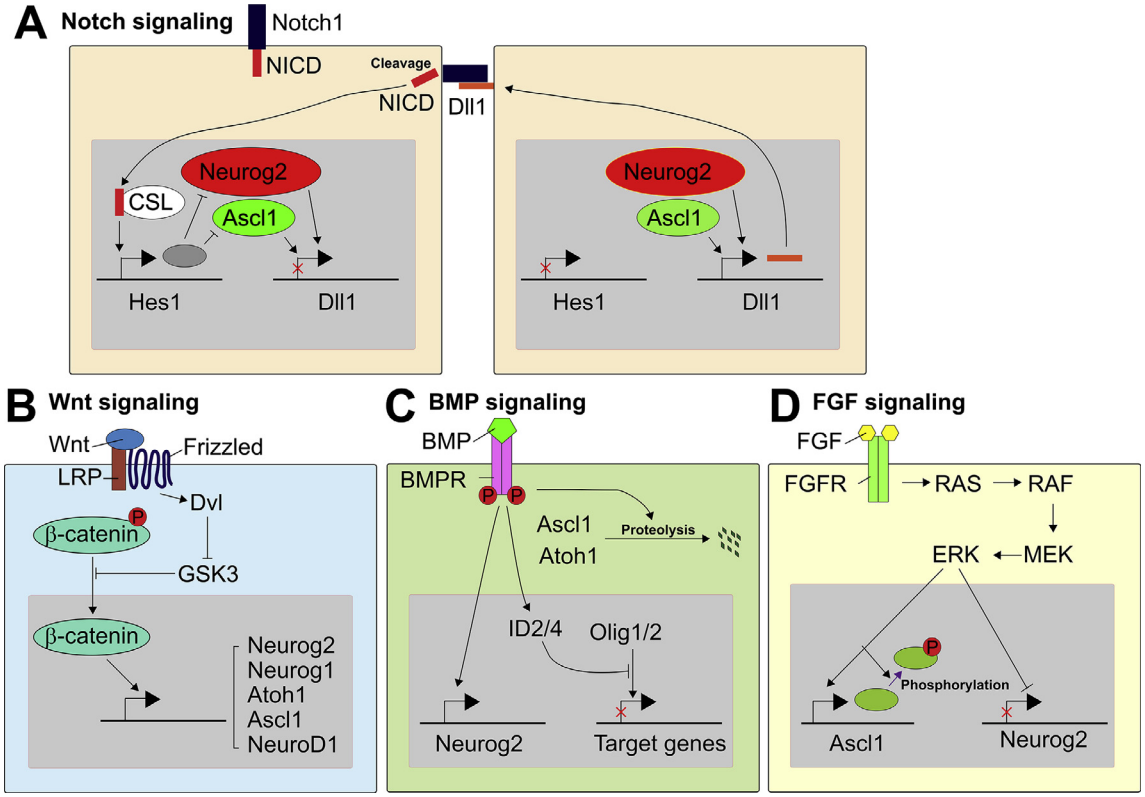

Fig. 3. Regulation of bHLH factors by Notch, Wnt, BMP and FGF signaling. (A) Notch signaling pathway, showing proneural transcription factors initiating Dll1 ligand expression in the emitting cell (to the right). Dll1 binds to the Notch receptor on the receiving cell (to the left), leading to the cleavage of the NICD, which translocates to the nucleus to interact with CSL and initiate the transcription of *Hes1*. Hes1 inhibits proneural gene expression. (B) Wnt signaling, showing Wnt ligand binding to the LRP/Frizzled receptor pair, leading to the stabilization of b-catenin, which translocates to the nucleus to initiate the expression of several bHLH genes. (C) BMP signaling, showing binding of BMPs to transmembrane serine/threonine kinase receptor dimers (type I and type II), which leads to the initiation of *Neurog2* expression, and the inhibition of *Olig1/2* expression. Ascl1 and Atoh1 are also degraded in response to BMP signaling. (D) FGF signaling, showing the binding of FGFs to transmembrane receptor tyrosine kinase (RTK) dimers, which activates RAS/RAF/MEK/ERK signaling, leading to the repression of *Neurog2* expression and the activation of *Ascl1* expression.

telencephalon (Gunhaga et al., 2003; Israsena et al., 2004; Machon et al., 2005; Watanabe et al., 2005). In the case of *Neurog2*, regulation by Wnt signaling is temporally restricted, as disruption of Wnt signaling reduces *Neurog2* expression during early but not late cortical neurogenesis (Bluske et al., 2012; Machon et al., 2005). Wnt is also able to increase *Ascl1* expression in cultured cells, and regulates *NeuroD1* expression in the hypothalamus and adult hippocampus (Kuwabara et al., 2009; Lee et al., 2016; Tang et al., 2002).

5.1.3. BMP signaling

A similar temporally-dependent regulation of *Neurog2* is observed with Bmp signaling (Fig. 3C), as Bmp7, which is secreted from the hem, meninges and choroid plexus, is required after E14.5 to maintain *Neurog2* expression in SVZ progenitors in the cortex (Segklia et al., 2012). BMP signaling also limits oligodendrogenesis through an indirect action on Olig1/2 proteins (Samanta and Kessler, 2004). In the cerebellum, BMP signaling can prevent Shh-induction of proliferation of granule cell precursors by down-regulating Atoh1 expression through proteasome-mediated degradation (Zhao et al., 2008). Interestingly, BMP2 also induces the degradation of Ascl1 protein (Vinals et al., 2004).

5.1.4. Fgf signaling

When dissociated cortical cells are exposed to Fgf or downstream effectors in this pathway (eg., RAS/ERK signaling) *in vitro* or *in vivo*, there is a switch from Neurog2 to Ascl1 expression (Fig. 3D) (Abematsu et al., 2006; Gabay et al., 2003; Hack et al., 2004; Li et al., 2014). This signaling pathway thus controls the selection of cell fate by deciding which proneural gene is expressed, with Neurog2 promoting the differentiation of glutamatergic neurons in the cortex, while ectopic *Ascl1* specifies a GABAergic neuronal or OPC fate (Li et al., 2014).

5.2. Regulation of bHLH transcription factor function by phosphorylation

The proneural activity of bHLH proteins is also regulated by phosphorylation (Fig. 4; reviewed in (Guillemot and Hassan, 2017)). Neurog2 harbours multiple conserved SP and TP sites that are phosphorylated by proline-directed serine-threonine kinases, such as Cdk1, GSK3 and ERK. In Xenopus, phosphorylation of Neurog2 by Cdk1 has an additive effect, with DNA binding reduced with each additional phosphorylation event, functioning as a rheostat-like control mechanism to limit neurogenesis (Ali et al., 2011). Cdk1 also phosphorylates Ascl1 (Ali et al., 2014) and Neurod4 (Hardwick and Philpott, 2015) in Xenopus, both of which have six SP/TP sites, reducing the ability of these proneural proteins to promote primary neurogenesis.

A similar mode of regulation by proline-directed serine-threonine kinases has been observed in the murine neocortex, where low Wnt signaling at late stages (>E14.5) leads to elevated GSK3 activity and an increase in Neurog2 phosphorylation on SP sites (Li et al., 2012). Similarly, Ascl1 is phosphorylated by ERK, another proline-directed serine-threonine kinase (Li et al., 2014). Interestingly, when Neurog2 becomes phosphorylated, it changes its choice of dimerization partners, preferentially forming Neurog2-E47 heterodimers rather than Neurog2-Neurog2

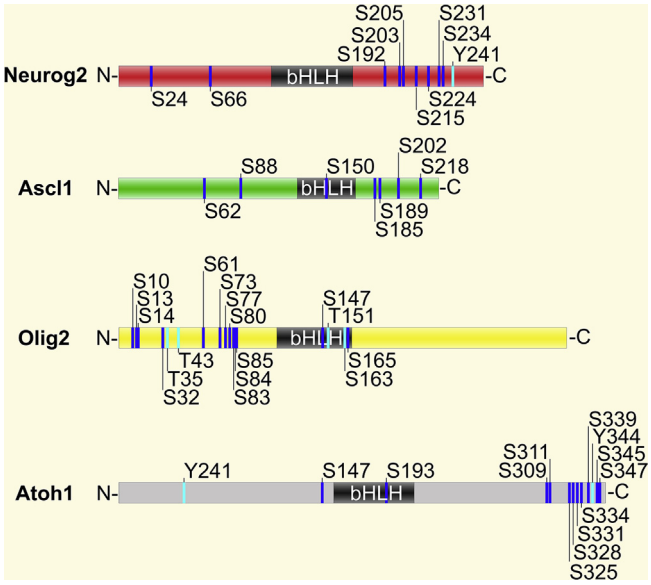

Fig. 4. Serine/threonine phosphoacceptor sites in bHLH proteins regulated by phosphorylation. All of the serine/threonine residues that could potentially be phosphorylated are depicted in murine protein sequences for Neurog2, Ascl1, Olig2 and Atoh1, all of which are regulated by phosphorylation.

homodimers, and these Neurog2-E47 heterodimers have a reduced ability to induce neuronal differentiation (Li et al., 2012). Neurog2 is also phosphorylated by GSK3 in the murine spinal cord, but here it promotes the association of Neurog2 with Nli1, an adaptor protein that recruits Lhx3 and Isl1 to target genes to induce motor neuron differentiation (Ma et al., 2008). Phosphorylation of proneural proteins by proline-directed serine threonine kinases may thus be a conserved mechanism, not only reducing proneural activity at different stages of development, but also directing neuronal subtype specification.

Interestingly, the inhibitory effects of SP/TP phosphorylation of Neurog2 are observed on neuronal differentiation genes, such as *xNeurod1*, but not on *xDelta* (Hindley et al., 2012). Similarly, at low levels of ERK activation, presumably correlating with reduced Ascl1 phosphorylation, Ascl1 can activate neuronal differentiation genes, whereas at higher levels of ERK activation, Ascl1 preferentially transactivates glial genes (Li et al., 2014). Ascl1 is directly phosphorylated by ERK, with serine to alanine mutations of the six putative ERK phosphoacceptor sites (SP sites) sites in Ascl1 enhancing the ability of Ascl1-SA6 to induce neurogenesis, and reducing its ability to promote gliogenesis (Li et al., 2014). In this context, Ascl1 phosphorylation is used to select downstream targets.

During spinal cord development, phosphorylation of Olig2 determines whether motor neurons or OPCs are specified, with the phosphorylated form of Olig2 specifying a motor neuron fate (Li et al., 2011). Olig2 phosphorylation changes Olig2 dimerization from Olig2-Neurog2 heterodimers (unphosphorylated) to Olig2-Olig2 homodimers (phosphorylated) (Li et al., 2011). The formation of unphosphorylated Olig2-Neurog2 heterodimers prevents Neurog2 from binding downstream targets required for motor neuron specification, thus leading instead to the selection of oligodendrocyte target genes and an oligodendrocyte fate.

Additional residues are phosphorylated in bHLH proteins, including a conserved S/T phosphoacceptor site in the second helix that is found in Neurog, Ascl and Atonal homologs from fly to humans (Quan et al., 2016). Phosphorylation of this site inactivates proneural activity to control the activities of these proteins in a precise temporal-spatial fashion (Quan et al., 2016). Neurog2 is also phosphorylated on Y241, leading to the transactivation of downstream targets associated with neuronal migration (Hand et al., 2005). For Atoh1, phosphorylation at S193 is required for proper maintenance of mechanosensory hair cells in the inner ear (Xie et al., 2017).

5.3. Epigenetic regulation of bHLH transcription factors

5.3.1. Ascl1

Transcription factor binding to nucleosomal DNA is considered to be pioneer activity (Fig. 5A). In the ventral telencephalon, over half of the sites that Ascl1 binds to in the enhancer regions of its target genes are found in closed chromatin (Raposo et al., 2015). More strikingly, upon binding, Ascl1 opens the chromatin so that other transcription factors can also bind, leading to its designation as a pioneer factor (Raposo et al., 2015). This pioneer activity of Ascl1 has also been observed during neuronal reprogramming of fibroblasts (Wapinski et al., 2013). Similarly, in glioblastoma cell lines, Ascl1 binds to nucleosome rich, closed chromatin, inducing a reorganization or opening that allows *Ascl1* to promote neurogenesis (Park et al., 2017). How Ascl1 induces chromatin opening remains to be deciphered.

5.3.2. Neurog1

Neurog1 expression is repressed by Polycomb group (PcG) proteins at later stages of development to allow gliogenesis to initiate (Fig. 5B) (Hirabayashi et al., 2009). It has also been found that a 4 kb enhancer region that controls *Neurog1* expression generates a non-coding RNA (utNeurog1) that is required for efficient *Neurog1* transcription (Fig. 5C) (Onoguchi et al., 2012). The tight regulation of *Neurog1* expression in the neocortex is thus regulated at the epigenetic level.

5.3.3. Neurog2

A major question is how proneural genes can activate their downstream targets in somatic cells in which the chromatin surrounding neuronal differentiation genes is closed. Neurog2 accomplishes this feat in part by binding to histone acetyltransferases, such as CBP/p300 (Lee et al., 2009). In Xenopus, Neurog2 interacts with an H3K9 demethylase known as KDM3A during the formation of primary neurons (Lin et al., 2017). This interaction facilitates Neurog2 binding to the regulatory regions of neuronal differentiation genes (*Neurod1*, *Tubb3*) by removing repressive H3K9me2 marks, and facilitating the addition of open chromatin marks H3K27ac and H3K4me3 (Lin et al., 2017). Xenopus Neurog2 has also been shown to interact with Brg1, a chromatin remodelling protein, as does Neurod1, and these interactions are essential for neuronal differentiation (Seo et al., 2005).

5.4. Conclusions and future directions

From these studies, it is evident that the regulation of bHLH gene expression and function is very tightly regulated. Furthermore, it is clear that we have only scratched the surface in this area, given for example, that it remains unclear how Ascl1 opens chromatin. The strict controls on bHLH expression highlights the importance of these genes in regulating cell fate decisions, and the very tight regional and temporal controls that are required to ensure that bHLH transcription factors are only active in the correct time and place. Future studies should be directed to examining whether some of the regulatory mechanisms described in this review apply to a larger number of bHLH factors, or whether they are unique attributes of the genes described. For example, it would be surprising if only *Neurog1* was subject to PcG-mediated repression, given the high relatedness to *Neurog2*. However, one could

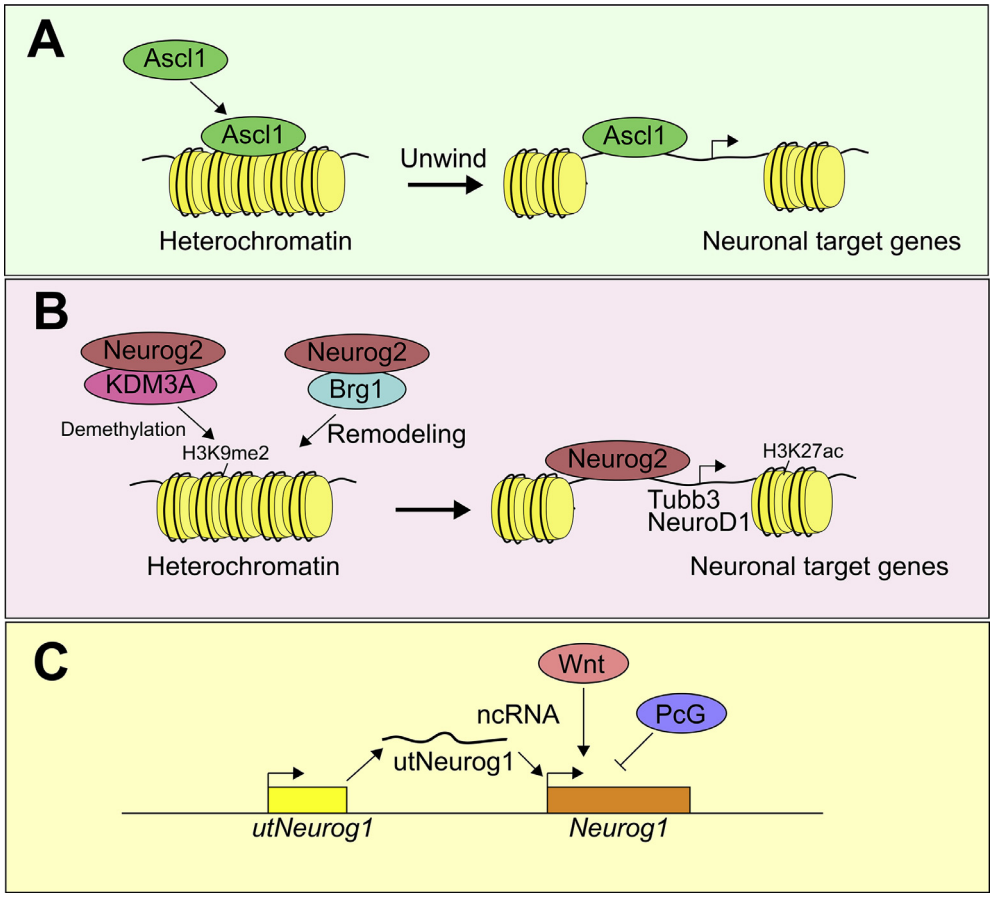

Fig. 5. Epigenetic regulation of bHLH transcription factors. Different epigenetic mechanisms have been shown to regulate, or be regulated by, the bHLH genes. (A) Ascl1 is a pioneer transcription factor because it can bind to closed chromatin and induce chromatin opening of neuronal target genes. The mechanisms are unknown. (B) Neurog2 can bind KDM3A to demethylate H3K9me2 repressive sites, allowing for new chromatin modifications that open the chromatin (eg. H3K37ac3). Neurog2 can also bind Brg1, a chromatin remodeler, to directly open the chromatin. (C) *Neurog1* transcription is repressed by Polycomb group (PcG) genes. In addition, an *utNeurog1* non coding transcript is generated from the enhancer region upstream of *Neurog1*, which is required to facilitate the transcription of *Neurog1*.

also argue that *Neurog1* is expressed in a much shorter time window in the neocortex, so PcG-repression may allow for the distinct expression patterns of these two genes.

6. bHLH transcription factors in health and disease

6.1. bHLH genes in cancer

6.1.1. Glioma

While the direct mutation of bHLH genes is not the underlying cause of most cancers, their dysregulation has been observed in various tumors. Gliomas are brain tumors that originate in glial cells, and they include both astrocytomas (e.g., glioblastoma multiforme, or GBM), which are the most aggressive gliomas, and oligodendrogliomas (ODG) (Fig. 6A). Gliomas represent approximately 80% of all malignant brain tumors, and are thus a major focus of health research. Strikingly, *ASCL1* is expressed in many gliomas, at low and high levels depending on tumor type (Phillips et al., 2006; Rheinbay et al., 2013; Rousseau et al., 2006; Somasundaram et al., 2005), and it is required for the proliferation and maintenance of these cells in an animal model of GBM (Rheinbay et al., 2013). *ASCL1* influences tumor growth through regulation of the Wnt pathway, as knockdown of *ASCL1* leads to decreased Wnt signaling (Rheinbay et al., 2013). There is also evidence that higher levels of *ASCL1* expression may make tumors more responsive to Notch inhibition, which allows for the latent proneural activity of *ASCL1* to be activated, leading to cell cycle exit and neuronal differentiation (Park et al., 2017). In contrast, glial tumors that express low levels of *ASCL1* are not responsive to Notch inhibitors, suggesting that *ASCL1* status should be considered as a prognostic of drug responsiveness. Finally, a more recent study has found that GBMs expressing a higher level of *ASCL1* have the potential to undergo neuronal differentiation and stop tumor spread in response to the inhibition of Notch signaling (Park et al., 2017). To induce neurogenesis, ASCL1 acts as a pioneer factor, binding to DNA and opening chromatin around its differentiation targets (Park et al., 2017).

ASCL1 is also expressed in pilocytic astrocytomas and gangliogliomas, which are less aggressive tumors. While these tumors share the same *bRAFV600E* mutation, the higher level of ERK activation in pilocytic astrocytomas results in more glial and less abnormal neuronal cells in the tumor (Li et al., 2014). This increase in abnormal gliogenesis is thought to be due in part to the hyperphosphorylation of ASCL1 by ERK, which promotes a glial fate (Li et al., 2014).

Astrocytomas are also characterized by the expression of *OLIG2* (Fig. 6B) (Bouvier et al., 2003; Ligon et al., 2004; Ligon et al., 2007; Lu et al., 2001b; Marie et al., 2001; Ohnishi et al., 2003), which is required for tumorigenicity (Ligon et al., 2007; Mehta et al., 2011; Suva et al., 2014). In mouse models of glioma, the loss of *Olig2* allows for enhanced survivability and a lack of symptoms (Ligon et al., 2007; Mehta et al., 2011; Suva et al., 2014). The phosphorylation state of Olig2 is also important as a phosophomimetic

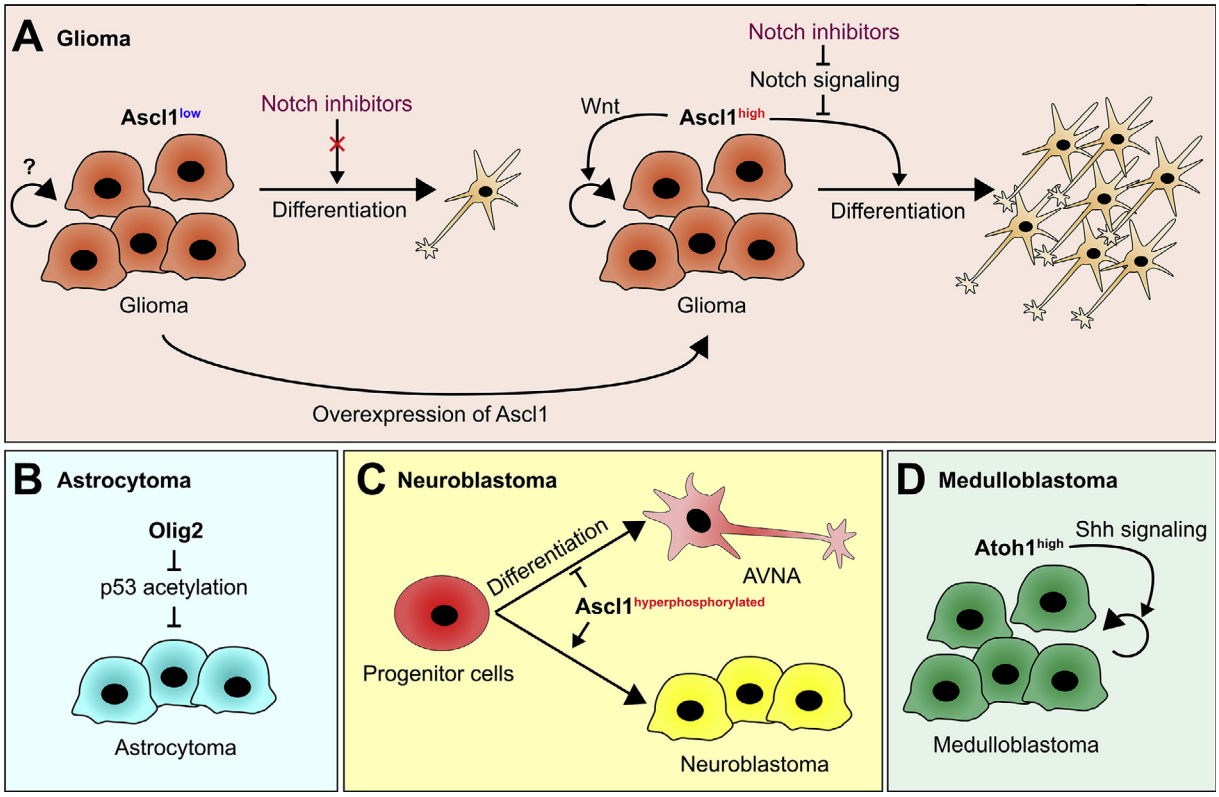

Fig. 6. Effects of bHLH transcription factors in tumorigenesis. While bHLH transcription factors are not normally mutated in cancers, they are deregulated in their expression or post-translational modifications. (A) *ASCL1* is expressed at high levels in some gliomas, which makes these tumors sensitive to Notch inhibitors. By increasing *ASCL1* expression levels in *ASCL1*^{lo} tumors, differentiation can ensue and reduce tumor growth. (B) *OLIG2* prevents p53 acetylation in astrocytomas. (C) Phosphorylation of ASCL1 prevents the differentiation of anteroventral noraderenergic cells (AVNA), which are thought to be the cells that give rise to neuroblastoma. (D) *ATOH1* is upregulated in medulloblastoma, promoting Shh signaling and proliferation.

form of Olig2 increases tumorigenicity in a glioma model (Sun et al., 2011). The role of *OLIG2* in tumorigenicity appears to be due, at least in part, to the OLIG2 protein suppressing acetylation of p53 in glioma cells (Mehta et al., 2011). While *OLIG2* expression may not directly induce astrocytomas, targeting *OLIG2* activity may represent a potential therapeutic strategy for managing tumor growth.

6.1.2. Neuroblastoma

Neuroblastoma is considered to be a developmental tumor arising from undifferentiated progenitors that normally give rise to the sympathetic nervous system (Fig. 6C; reviewed in (Maris et al., 2007)). The presentation of neuroblastoma is highly variable and can occur anywhere in the sympathetic nervous system. A recent report showed that ASCL1 is hyper-phosphorylated on SP sites in neuroblastoma cell lines, and that phosphorylated ASCL1 prevents the differentiation of anteroventral noraderenergic cells (AVNA), which are thought to be the cells that give rise to neuroblastoma (Wylie et al., 2015). A defect in differentiation may thus be the underlying cause of neuroblastoma, and the phosphorylation status of ASCL1 may contribute to this defect (Wylie et al., 2015).

6.1.3. Medulloblastoma

Medulloblastoma is the most common brain tumor found in children, and is a malignant tumor of the cerebellum (Fig. 6D). In a subset of medulloblastomas, *ATOH1* is upregulated (Salsano et al., 2004), and tumor formation is prevented in mouse models in which *Atoh1* is deleted (Flora et al., 2009). These data have suggested that the upregulation of genes associated with a granule cell precursor identity are associated with a poor prognosis (Schuller et al., 2008; Yang et al., 2008). The role of *ATOH1* in controlling medulloblastoma proliferation appears to occur through regulation of the Shh signaling pathway (Flora et al., 2009). *NHLH1* has also been shown to be a target of Shh signaling in the developing cerebellum and in medulloblastoma (De Smaele et al., 2008).

6.2. bHLH genes in neurodevelopmental, neuropsychiatric and neurodegenerative disorders

6.2.1. Eye disorders

The most notable pathologies linked to mutations in bHLH genes are found in the eye, mostly affecting *ATOH7*, which is required for RGC differentiation and early eye development in animal models (Huang et al., 2014). Patients with *ATOH7* mutations develop persistent hyperplasia of the primary vitreous and have bilateral optic nerve aplasia (Prasov et al., 2012). Moreover, in a genetic screen of consanguineous Pakistani families with Nonsyndromic Congenital Retinal Non-Attachment (NCRNA), a form of blindness caused by separation of the retina and posterior globe, three families contained mutations in a conserved region of *ATOH7* (Keser et al., 2017). A separate study found a 6.5 kb deletion in an enhancer element for *ATOH7* in a Kurdish population with NCRNA (Ghiasvand et al., 2011). Finally, a weaker link has been made between *ATOH7* and primary open angle glaucoma, a glaucoma subtype associated with an imbalance in the production and drainage of aqueous humor in the anterior chamber (Axenovich et al., 2011; Ramdas et al., 2011). Similarly, *ATOH7* is one of 8 genes with possible pathogenicity associated with microphthalmia, anophthalmia and coloboma (MAC) (Williamson and FitzPatrick, 2014). The severity of human ocular defects associated with *ATOH7* mutations are more severe than observed in mutant mice, but are consistent with an early role for *Atoh7* in retinal development (Huang

et al., 2014). Finally, visual impairments have also been detected in a patient who was homozygous for a null mutation in *NEUROD1*, which led to several visual defects, including nyctalopia, blurred vision, and constriction of the visual field (Orosz et al., 2015). These defects are consistent with the known role of *Neurod1* in photoreceptor survival in animal models (Cho et al., 2007; Ochocinska et al., 2012).

6.2.2. Neuropsychiatric disorders

The link between bHLH transcription factors and neuropsychiatric disorders is fairly weak, yet there are some connections. A polymorphism within the *NEUROG1* regulatory region has been linked to language deficits (Ho et al., 2008). A region linked to schizophrenia, Chr 5q31, contains the *NEUROG1* locus in humans. Within schizophrenia patients, those with a *NEUROG1* polymorphism have decreased verbal memory, language skills, and visuospatial abilities (Ho et al., 2008). Schizophrenia patients with a polymorphism in the *NEUROG1* regulatory region have decreased cerebral gray matter volume (Ho et al., 2008). While *NEUROG1* polymorphisms do not appear to have a causative role in schizophrenia, these polymorphisms do indicate more severe outcomes. The deficits observed in schizophrenia patients with *NEUROG1* polymorphisms likely highlight important developmental functions for *NEUROG1* in the developing human cortex.

Mutations in *NEUROD2* have been associated with schizophrenia and schizoaffective disorder (Spellmann et al., 2017). *NEUROD2* polymorphisms in schizophrenia patients were associated with diminished verbal memory and executive functions (Spellmann et al., 2017).

6.2.3. Neurodegenerative disorders

Given the variety of developmental functions of bHLH genes, some associations with neurodevelopmental disorders or diseases with aberrant cell cycling were expected. However, neurodegenerative disorders with an adult onset seemed less likely to be linked to bHLH mutations. Indeed, only one gene, *ASCL1*, has been implicated in a neurodegenerative disorder, with polymorphisms in this gene associated with Parkinson's disease, a neurodegenerative disorder that affects dopaminergic neurons in the nervous system (Ide et al., 2005). The role of *ASCL1* in Parkinson's disease appears to be mediated through interactions with *PHOX2b*. *ASCL1* may be involved in Parkinson's disease through proper developmental control of the locus coeruleus, a region believed to provide protection against the neurodegenerative effect of Parkinson's disease (Ide et al., 2005). *NEUROG2* has been investigated for a potential association with Parkinson's disease, but the results indicated that it is unlikely to be involved (Deng et al., 2010). Finally, *NEUROD6* is downregulated in Alzheimer's Disease, which is a common cause of dementia, and has been suggested to be a potential biomarker (Satoh et al., 2014).

7. Conclusions and future directions

Collectively, these studies indicate that mutations in bHLH genes are not a major feature of hereditary disease, probably because most of these genes are essential to embryonic development, neural cell differentiation and survival. Indeed, neuropsychiatric and neurodegenerative disorders have very little association with bHLH genes. The greatest exception is *ATOH7*, mutations of which are linked to visual impairments in more than one population. However, a better link needs to be established between *ATOH7* mutations and visual development outside of the consanguineous populations currently studied.

Furthermore, somatic mutations in bHLH genes are not commonly found in tumors, although the de-regulated expression of many of these genes is observed in many cancers. Targeting bHLH genes may thus still be meaningful in tumor treatment. For instance, given the progenitor-like behaviour of tumor cells, dysregulation of bHLH factors may also disrupt proliferation within the tumor, as has been shown for *Olig2* in mouse models (Ligon et al., 2007; Mehta et al., 2011; Suva et al., 2014). Indeed, the overexpression of *Neurog2* or *Neurod1* in GBM stem cells promotes cell death, with remaining live cells acquiring neuronal features (Guichet et al., 2013). Another study similarly found that the overexpression of *Ascl1*, *Brn2* and *Neurog2* can induce human glioma cells to undergo neuronal differentiation and stop proliferating (Zhao et al., 2012), while another study reported that the expression of *Sox11* and *Neurog2* together in human glioma cells *in vivo* blocks tumor growth (Su et al., 2014). The possibility that bHLH transcription factors may be exploited for the treatment of brain tumors is thus a real possibility.

8. bHLH transcription factors in reprogramming

8.1. A general overview of neuronal reprogramming

Developmental biologists have paved the way for the field of direct neural cell reprogramming by identifying both intrinsic and extrinsic cues that induce neurogenesis and gliogenesis and specify neural subtype identities (Masserdotti et al., 2016). The use of developmental genes in reprogramming strategies has led to the successful generation of a large variety of neural cell types resembling cells in the CNS and PNS. The further refinement of neural cell reprogramming strategies brings hope for the treatment of neurodegenerative disorders and for the repair of damage caused by stroke or other insults. In developing techniques for reprogramming cells, there has been a major focus on the use of bHLH genes, especially proneural transcription factors (Fig. 7).

8.2. Fibroblasts and other somatic cells

Stepwise lineage conversion strategies have been devised for the reprogramming of somatic cells, such as fibroblasts, to an induced neuronal (iN) identity. bHLH transcription factors have emerged as critical architects of neuronal reprogramming. They were first shown to induce neuronal differentiation in P19 cells, with *Ascl1*, *Atoh1*, *Neurod1*, *Neurod2*, *Neurod4*, *Neurod6*, *Neurog1*, *Neurog2*, or *Nhlh1* inducing neurogenesis in this embryonal carcinoma cell line (Farah et al., 2000). The first report of direct neuronal reprogramming in fibroblasts used three factors; *Ascl1*, *Brn2* and *Mytl1* (BAM) (Vierbuchen et al., 2010). Since then the field has exploded and multiple approaches have been devised. *Ascl1* has emerged as a potent reprogramming factor *in vitro*, acting in combination with other transcription factors to convert mouse and human fibroblasts (Caiazzo et al., 2011; Kim et al., 2011; Pang et al., 2011; Pfisterer et al., 2011; Son et al., 2011; Vierbuchen et al., 2010), hepatocytes (Marro et al., 2011), cardiomyocytes (Chuang et al., 2017) or astrocytes (Rivetti di Val Cervo et al., 2017) to neurons. While proneural genes can induce neuronal reprogramming, neuronal subtype identity is determined by the combination with other factors. For example, transfection of fibroblasts with *Ascl1* alone is sufficient to generate iNs (Chanda et al., 2014), but when *Ascl1* is cotransfected with *Pitx3*, the resulting iNs are dopaminergic (Kim et al., 2011), whereas when *Ascl1* is overexpressed with *Neurog1*, *Brn2*, *Myt1l*, *Isl1*, and *Klf7*, the result is nociceptor-like sensory neurons (Wainger et al., 2015). Notably, *Ascl1* is replaceable in sensory neuron programming, as *Neurog1* or *Neurog2* along with *Brn3a* can also induce nociceptor as well as mechanoreceptor and proprioceptor sensory neurons

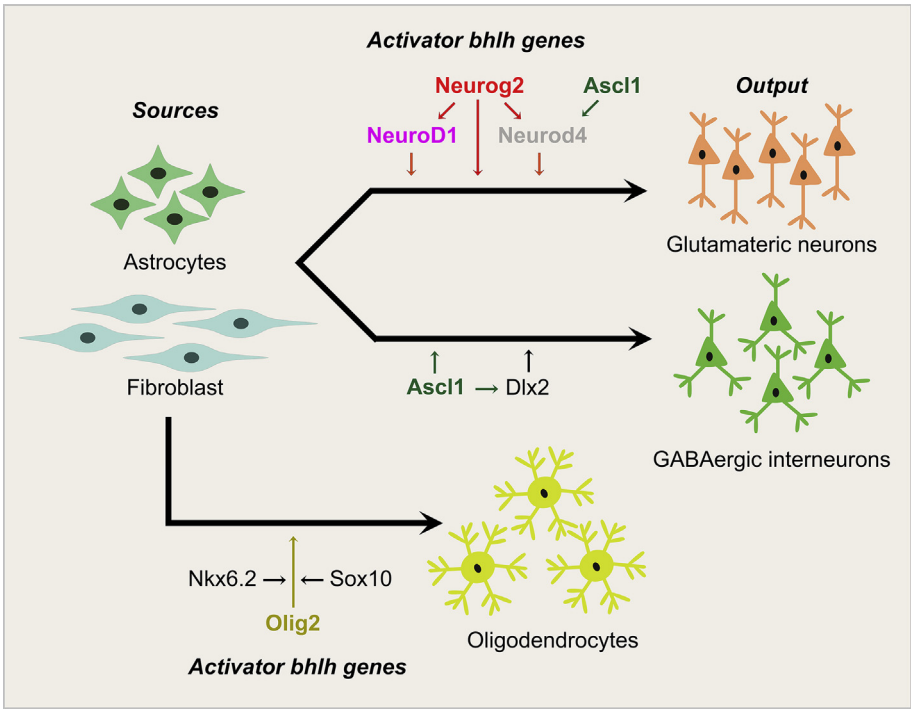

Fig. 7. Neural cell reprogramming using bHLH transcription factors. Three main sources of cells are used for reprogramming or directed differentiation; pluripotent cells (ES and iPS), astrocytes (usually *in vivo*) and fibroblasts. Multiple bHLH genes are efficient at neuronal induction, including *Neurog2*, *Ascl1*, *Neurod1* and *Neurod4*. These genes can produce multiple different types of neurons – schematized here are glutamatergic and GABAergic. *Olig2* can also be used in combination with other factors to generate oligodendrocytes.

(Blanchard et al., 2015). Proneural factors thus appear functionally redundant in inducing fibroblasts into sensory neurons.

Much like *Ascl1*, *Neurog2*'s ability to reprogram fibroblasts appears dependent on other factors. When cotransfected with *Sox11*, *Lhx3*, and *Isl1*, *Neurog2* is able to convert fibroblasts into motor neurons (Liu et al., 2016). When forskolin and dorsomorphin are added, *Neurog2* instead converts fibroblasts into cholinergic neurons (Liu et al., 2013). For retinal cell reprogramming strategies, overexpression of the bHLH transcription factor *NeuroD1* with *Crx*, *Rax*, and *Otx2*, induces fibroblasts to form photoreceptors (Seko et al., 2014). These induced photoreceptors appear functional as they exhibit electrophysiological responses to light stimuli.

Notably, neural lineage conversion of fibroblasts with bHLH transcription factors has not been limited to neurons. OPCs have also been successfully generated from fibroblasts using *Olig2* and *Sox10* with either *Zfp536* or *Nkx6.2* (Najm et al., 2013; Yang et al., 2013). The ability to induce OPCs is of considerable importance in finding ways to treat demyelinating diseases such as optic neuritis and multiple sclerosis.

8.3. Pluripotent stem cells – Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs)

The generation of iNs from pluripotent stem cells, whether it be from embryonic stem (ES) or induced pluripotent stem (iPS) cells, is considered a differentiation process, rather than a lineage conversion event. Neural induction is initiated in pluripotent cells in a stepwise manner by first inhibiting BMP and Activin/TGFβ signaling to generate induced NSCs, and then initiating neuronal differentiation by the addition of various small molecules and signaling molecules (Chambers et al., 2009). Several other procedures have been developed based on signaling molecules that can generate neurons with precise phenotypes, including striatal (Aubry et al., 2008), dopaminergic (for the treatment of Parkinson's Disease), and spinal cord-like neurons (reviewed in (Wang and Zhang, 2017)). However, such procedures are generally time-consuming and relatively inefficient. The induction of neuronal differentiation can be greatly improved by the overexpression of transcription factors, notably including several bHLH genes. For instance, *Neurog1* and *Neurog2* have been overexpressed in human iPS cells to induce neuronal differentiation (Busskamp et al., 2014). By combining *Neurog2* with *Islet1* and *Lhx3*, human ES cells can be converted into motor neurons (Hester et al., 2011). Finally, *Neurog2* alone is also sufficient to convert human ES or iPS cells into neurons (Zhang et al., 2013). The efficiency of neuronal differentiation from a source of human pluripotent cells has also been dramatically improved by the use of synthetic mRNAs for the bHLH factors *Neurog1*, *Neurog2*, *Neurog3*, *Neurod1* and *Neurod2*, as opposed to the plasmid-based or viral expression constructs used by others (Goparaju et al., 2017). Such an approach has the advantage of not altering the genome, as occurs with viral expression constructs, and mRNAs can be quickly translated into protein product.

Aside from the more commonly used bHLH transcription factors, *Atoh1* has been used along with *Gfi1* and *Pou4f3* to reprogram mouse ES cells into hair cells of the inner ear (Costa et al., 2015). *Neurog1* transfection of mesenchymal stem cells was also able to induce some aspects of a cochlear neuron identity (Schack et al., 2016). The inability of *Neurog1* to fully specify cochlear neurons may be a limitation of *Neurog1* in inducing cochlear neurons or a limitation of using mesenchymal stem cells for reprogramming to a neuronal fate.

8.4. Glia and other neural cells in the brain

While some promising work has been done using fibroblast reprogramming, many labs have turned their focus to *in vivo* strategies for repairing the brain, namely the reprogramming of astrocytes into neurons. Several bHLH transcription factors have

been used to successfully convert astrocytes into neurons. *Neurog2*, *Ascl1*, and *NeuroD1* are each sufficient to convert astrocytes into neurons (Berninger et al., 2007; Brulet et al., 2017; Guo et al., 2014). In wound injury models, the expression of *Olig2* inhibits the transformation of glial cells into neurons, as *Olig2* knockdown, using *miR-Olig2*, is enough to reprogram glial cells into neurons (Buffo et al., 2005). Much like its developmental role, *Ascl1* is promiscuous in reprogramming specific subtypes of neurons. When transfected into astrocytes of the dorsal midbrain, *Ascl1* converts astrocytes into neurons with glutamatergic and GABAergic identities (Liu et al., 2015). In contrast, *Ascl1* does not induce neurogenesis when misexpressed in the adult neocortex (Grande et al., 2013), and in the hippocampus and spinal cord, instead promoting oligodendrogliogenesis (Jessberger et al., 2008; Ohori et al., 2006).

Perhaps the most successful bHLH factor for reprogramming astrocytes is *NeuroD4*. Expression of *NeuroD4* was sufficient to reprogram both mouse and human astrocytes into neuronal fates (Masserdotti et al., 2015). While *Neurog2* can regulate *NeuroD4* expression, the ability to bind the *NeuroD4* promoter is limited by REST repression (Masserdotti et al., 2015). The restrictions imposed by REST are circumvented by reprogramming using *NeuroD4* instead of *Neurog2* in astrocytes.

In using astrocytes an important consideration in reprogramming strategies is whether the location of the astrocytes within the CNS restricts the neuronal subtypes that may be generated from them. Previous attempts to reprogram astrocytes have had variable outcomes that appeared to be in accordance with the source of astrocytes (Liu et al., 2015; Masserdotti et al., 2015). However, it is difficult to compare outcomes when there is variability in the techniques used. Chouchane et al. (2017) have nicely shown how regional identity influences reprogramming using astrocytes transfected with *Neurog2*. Astrocytes from both the neocortex and the cerebellum were isolated and transfected with *Neurog2*. *Neurog2* transfected astrocytes were subsequently transplanted back into the neocortex, with only the cortically isolated astrocytes successfully generating glutamatergic neurons (Chouchane et al., 2017). The source of astrocytes thus influences the ability of a transfected agent to reprogram specific neuronal subtypes.

8.5. Müller glia in the retina

While regeneration occurs not at all, or in a very limited manner, in warm-blooded vertebrates, it is a very efficient process in cold-blooded vertebrates, such as fish and frogs (reviewed in (Gemberling et al., 2013)). One of the best studied regenerative systems is the neural retina, where retinal cell types are replaced in a very specific fashion in fish and frogs (i.e., if photoreceptors die, then they are the cells that are replaced) (reviewed in (Gemberling et al., 2013)). The origin of new cells in a degenerative model are the Müller glia, which de-differentiate and proliferate to become retinal progenitor cells that can then re-differentiate into lost cell types, whatever they might be (Fig. 8; reviewed in (Gemberling et al., 2013)). One of the key factors that determines whether a proliferative Müller glial cell will undergo regeneration is the proneural gene *ascl1a* (Fausett et al., 2008), which is upregulated in the subset of proliferative Müller glia that will undergo neurogenesis upon damage. Interestingly, *Ascl1* is not upregulated in mammalian Müller glia in an injury model, but in a conditional *Ascl1* gain-of-function transgenic line, the increase in *Ascl1* expression can induce new neurogenesis in mice (Pollak et al., 2013; Ueki et al., 2015). However, this process is only efficient in young and not old animals (Jorstad et al., 2017). One reason may be that *Ascl1* target genes are in condensed chromatin and are not accessible, and indeed, the overexpression of *Ascl1* along with exposure to an HDAC inhibitor, which de-condenses chromatin, can induce new neurogenesis in older animals (Jorstad et al., 2017). However, this new neurogenesis does not include the derivation of new photoreceptors, which are often the cells that degenerate in retinal degenerative diseases, suggesting that additional studies are required to identify the conditions that can allow new photoreceptor genesis in older mammals. When retinal damage is induced by neurotoxic injury, transfection of bHLH transcription factors promotes the differentiation of Müller glia into progenitors, which

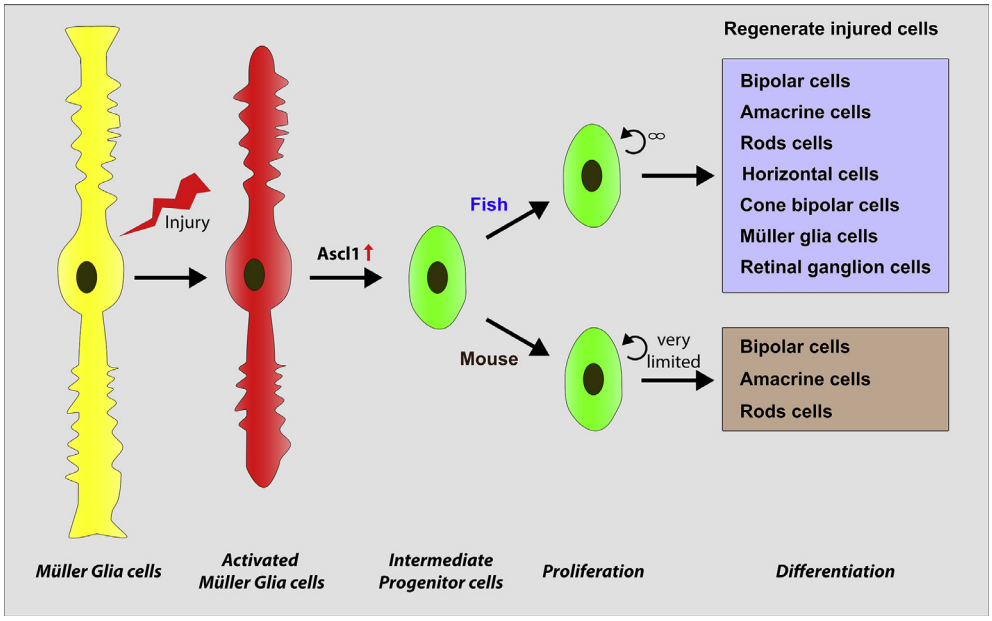

Fig. 8. Regeneration of retinal cells from Müller glia, comparing fish and mouse. In cold-blooded vertebrates, Müller glial cells de-differentiate in response to injury and become stem cell-like, giving rise to all seven populations of retinal cells. In contrast, in mammals there is a very limited proliferative response by Müller glia, and only a limited set of new neurons are generated.

then differentiate into other retinal cells types to replace those that are lost. In this model, co-expression of *Neurod1* with *Pax6* promotes differentiation into amacrine cells, while co-expression of *Neurod4* and *Pax6* promotes differentiation into both amacrine cells and horizontal cells (Ooto et al., 2004).

8.6. Summary and future perspectives

While developmental biologists may have paved the way for the field of direct neuronal reprogramming, the attempts to induce neurons using developmental approaches have highlighted developmental questions that still need to be resolved. Proneural genes have emerged as critical architects of neuronal reprogramming (Wilkinson et al., 2013). However, they are not active in all contexts; *Ascl1* is a potent neuronal reprogramming factor in fibroblasts (Caiazzo et al., 2011; Kim et al., 2011; Pang et al., 2011; Pfisterer et al., 2011; Son et al., 2011; Vierbuchen et al., 2010), hepatocytes(Marro et al., 2011), cardiomyocytes (Chuang et al., 2017), astrocytes (Rivetti di Val Cervo et al., 2017) and pluripotent cells (Yang et al., 2017), and not in the adult neocortex (Grande et al., 2013), hippocampus and spinal cord (Jessberger et al., 2008; Ohori et al., 2006). Similarly, *Neurog2* is used less often for neuronal reprogramming as it must be combined with other signals to become a potent lineage converter (Gascon et al., 2016). Thus, by better understanding how proneural genes are regulated, they can be more efficiently used in regenerative medicine.

The transcription factors that are co-expressed with bHLH factors such as *Ascl1* and *Neurog2* alter the resulting subtype of the induced neuron. These changes are likely a result of an altered epigenetic landscape within the cell. A better understanding of how factors upstream of proneural genes regulate epigenetic status will allow for a better refinement of the strategies used for inducing specific neuronal subtypes. Developmental biologists thus still have important questions to answer before specific neuronal subtypes can be consistently and efficiently produced. The reprogramming of astrocytes is a promising approach, as it holds the benefit of mobilizing cells *in vivo* to repair damage to the brain. However, astrocyte reprogramming still faces the same ethical hurdles of introducing genetic material into human patients and the potential for tumorigenic effects.

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