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Review

From skin to nerve: flies, vertebrates and the first helix

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Abstract. Vertebrate and invertebrate nervous tissue is derived from early embryonic ectoderm, which also gives rise to epidermal derivatives such as skin. Proneural basic helix-loop-helix (bHLH) transcription factors are the key players in the formation of peripheral nervous system (PNS) and central nervous system (CNS) from naïve ectoderm to differentiated postmitotic neurons. The comparative approach and the use of a wide range of animal models have led to increasingly comprehensive investigations of this issue in the last decade. This review will focus on current studies of neural development in vertebrate and invertebrate PNS and on understanding how the bHLH domain structure encodes multiple functions required for neural specification.

Key words. *Drosophila*; vertebrates; bHLH; proneural gene; neural development; evolution.

Introduction

Epitheliogenesis

Both the adult skin and nervous system develop from the ectoderm on the surface of the post-gastrulation embryo. Although basic helix-loop-helix (bHLH) proteins are more widely known for their roles in nervous system development, recent evidence shows they also play important roles in epidermal development. The epidermis is constantly renewed, with a fine equilibrium between proliferation and differentiation. Skin is a dynamic structure with proliferation in the basal layer in contact with a basement membrane. As cells enter terminal differentiation, they cease proliferation, lose contact with the basement membrane and migrate upwards. On top of spinous layers and granular layers, terminal differentiation results in the assembly of the protective stratum corneum. A key feature of this protective outer layer is that it is continually shed and replenished by underlying keratinocytes to maintain the barrier. The stem cells necessary for maintenance of this cycling structure are located in the basal stratum of the epidermis and in specialized compartments of skin appendages (reviewed in [1–3]). Skin appendages develop from embryonic skin resulting from sequential epithelial-mesenchymal interactions and contribute to epidermal repair and regeneration (reviewed in [4]). Accumulating evidence suggests that stem cells from each location have the potential to interconvert between lineages [5–8].

Despite intensive effort, little is known about the precise steps and regulators that lead to mature epidermal cells. Among the transcription factors and signalling pathways that are important in controlling stem cell fate [9–13], recent studies have shown that a few bHLH proteins may play a role in epithelial cell differentiation and cell lineage induction [14, 15]. For example, Sosic and colleagues have shown that like *twist* in *Drosophila*, *Twist-1* and *Twist-2*/*Dermo-1*-in vertebrate are induced by a cytokine signalling pathway and regulate other genes. *Twist-2*/*Dermo-1*-null mice or *Twist-1* and *-2* heterozygous alleles show elevated expression of proinflammatory cytokines, resulting in perinatal death from cachexia. They further reveal that *Twist* represses κB-dependent transcription by inhibiting p65 trans-activation [14]. More recently, *cDermo-1* has been shown to express in developing

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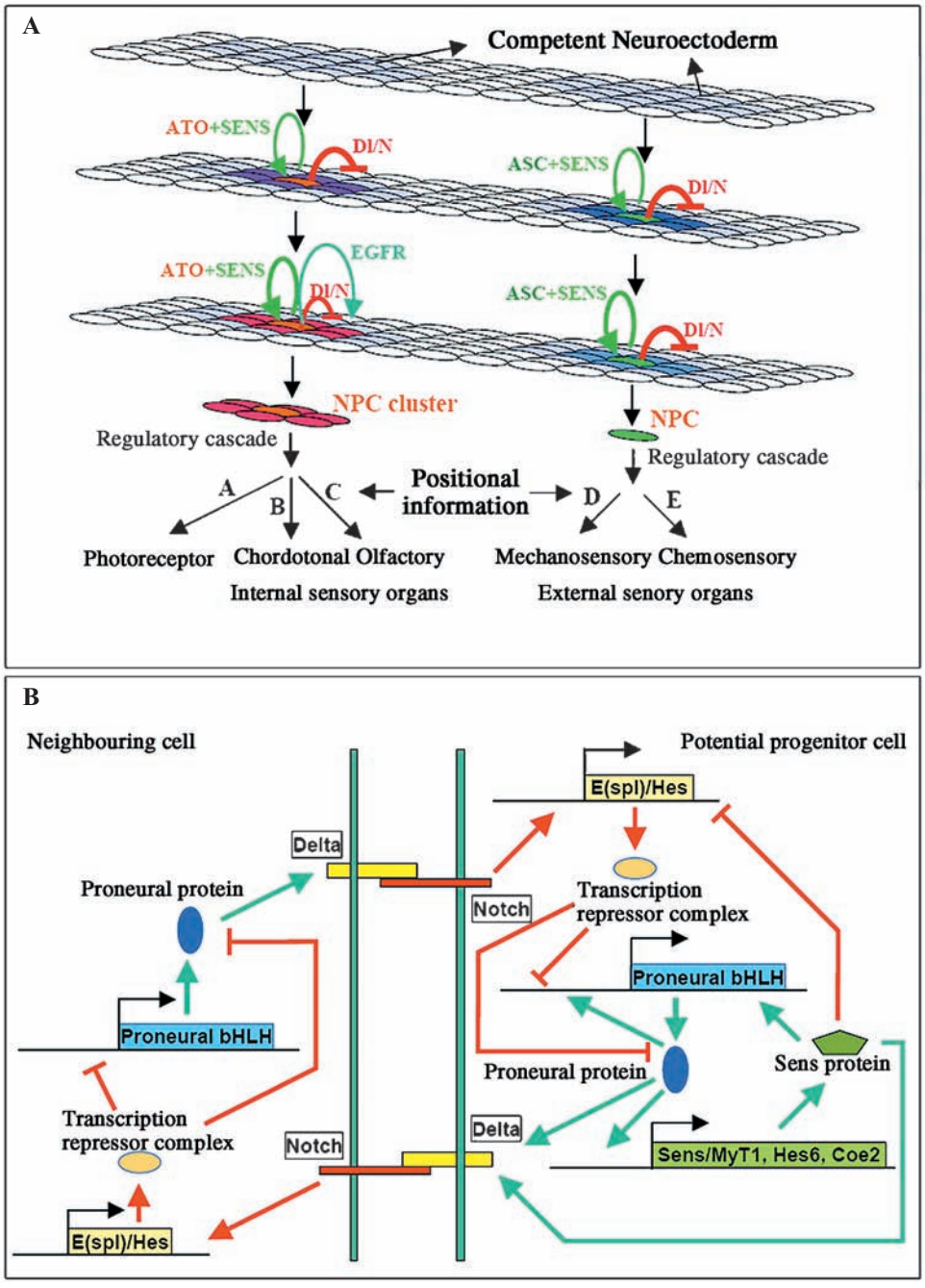

Figure 1. Proneural proteins regulate neural commitment via Notch signalling-mediated 'lateral inhibition' and a regulatory cascade. (*A*) In the *Drosophila* PNS, proneural proteins are first expressed in competent neuroectoderm. A higher expression level of proneural genes in potential progenitor cells activates Notch/Delta-mediated lateral inhibition, resulting in the selection of progenitors. A regulatory cascade is activated in NPCs followed by proneural protein downregulation, resulting in neuronal differentiation. Different proneural proteins cooperate with co-factors and give rise to distinct types of neurons. External sensory neurons are derived from single AS expressing NPC. Internal sensory neurons are derived from Ato-expressing NPC clusters, where secondary NPCs are recruited by the Atonal-induced EGF signalling pathway. (*B*) Proneural bHLH proteins induce the Notch ligand Delta. The Notch intracellular domain induces the expression of downstream targets E(spl)/Hes, which in turn repress the expression of proneural genes in neighbouring cell. This process is called 'lateral inhibition'. Accumulation of proneural proteins in the potential NPCs is set up by upregulation of Sens/MyT1, Hes6 and Coe2, which inhibit E(spl)/Hes expression and upregulate proneural protein and Delta. High levels of proneural proteins in the NPCs initiate a program resulting in neuronal differentiation and cell fate commitment.

dermis. Overexpression in those cells induces the developmental program leading to skin appendage formation. Results from misexpression of *cDermo-1* in different places indicate that *cDermo-1* may initiate feather induction in a local skin identity manner [15].

Neurogenesis

The molecular mechanisms underlying vertebrate and invertebrate neurogenesis – the process by which cells in the ectoderm adopt a neural fate and cells within the neuroectoderm become neurons – are remarkably similar. In vertebrates ectodermal cells give rise to epidermal progenitors on the ventral side and neural progenitors on the dorsal side of the gastrulating embryo. Embryonic ectoderm can be induced to form neural tissue in the presence of a source of inducing signals secreted by the dorsal mesoderm [16, 17]. Several factors, such as Noggin, Chordin and Follistatin, which inhibit bone morphogenetic proteins (BMPs), are required for this process of neural induction [18]. In addition, more recent findings in chick and mouse demonstrate that the Wnt, fibroblast growth factor and insulin-like growth factor families also play an essential role in neural induction [19–22]. As a result, ectodermal cells segregate into the surface ectoderm, neural crest and neural tube [23]. In *Drosophila*, *short gastrulation* (*sog*), an orthologue of *Chordin*, blocks the activity of *Decapentaplegic* (*Dpp*), a homologue of *BMP4*, and defines the domain of the ectoderm that will become the neuroectoderm [24].

Similarities have also been seen in the mechanisms whereby neuroectodermal cells are selected to become neurons. Genetic studies in *Drosophila* and vertebrate models have provided evidence that a subset of the bHLH transcription factors – the so called proneural proteins – act cell autonomously to initiate development and differentiation of neural lineages (reviewed in [25–27]). In addition, the antagonistic relationship between these proneural proteins and the cell-cell communication process mediated by the Notch signalling pathway, called lateral inhibition, plays an essential role in preventing certain cells from becoming neurons [28–31]. It has been well documented that the bHLH transcription factors activate the Delta-Notch (Dl-N) signalling system by activating Delta. Proneural gene expression in the signal-receiving cells is repressed by activated Notch, resulting in the selection of the signal-sending cell as the neural or neuronal precursor cell (NPC) (reviewed in [25, 32–36]). The high level of proneural proteins in the selected NPCs initiates a regulatory cascade to define the distinct neural lineages. Figure 1A summarize these sequential steps in *Drosophila*.

The bHLH proneural proteins

The bHLH proteins form a particularly large and complex superfamily. They have been shown to be involved in haematopoiesis [37], neurogenesis [38], cardiac muscle development [39], mesodermal cell determination [40], dermal cell differentiation [41] and skeletal development [42], among other processes. Neural bHLH proteins are the subset of this superfamily involved in neurogenesis (reviewed in [33]).

The defining feature of bHLH proteins is the presence of a basic helix-loop-helix domain. It comprises two α-helices separated by a variable loop. The first 8–13 amino acids of helix1 are highly basic and are required for DNA binding. Biochemical and structural studies have shown that the HLH domain mediates protein homo- or heterodimerization, while the basic region in the first helix makes contact with DNA in a sequence-specific manner. Different classes of neural bHLH proteins act either as positive or as negative regulators of transcription [31, 43, 44].

The concept of a 'proneural' gene was first defined in *Drosophila* and refers to genes that are responsible for endowing naïve ectodermal cells with neural fate [45–47]. Null mutants of these genes lose neural structure and convert NPCs to epidermal cells. There is strong evidence to suggest that, in vivo, proneural bHLH proteins form heterodimers with the widely expressed bHLH E proteins [called Daughterless (Da) in *Drosophila*]. These heterodimers bind to variations on a common hexamer CANNTG, or E-box [43, 44, 48–50], and regulate the transcription of target genes [44, 51].

Two proneural bHLH protein families have been identified so far (table 1): Achaete-Scute (AS) family and Atonal (Ato)-related protein family. They can be further divided into subgroups (reviewed in [33, 52]). Both families are found in vertebrates and invertebrates. Four genes in *Drosophila*, namely *achaete* (*ac*), *scute* (*sc*), *lethal of scute* (*lsc*) and *asense* (*ase*) belong to the *as* family [53, 54]. Their vertebrate homologues are called achaete-scute homologue (*Ash*). There are four genes which belong to the Ato-related family in *Drosophila*, named *atonal* (*ato*), *target of poxn* (*tap*), *absent MD neurons and olfactory sensilla* (*amos*), and *cousin of atonal* (*cato*) [55–59]. Vertebrate homologues of *ato* are called *Ath* and *neurogenin* (*Ngn*). Two other subgroups of *ato*-related genes in

Table 1. Proneural proteins in *Drosophila* and vertebrates.

Proneural protein family	*Drosophila*	Vertebrates
Ato	Ato, Amos, Cato Tap	Math1, Math5 Ngn1, Ngn2 NeuroD, Math2 Oligo
AS	Ac, Sc, LSc, Ase	Ash1, Ash2

Table 2. Expression of proneural proteins in embryos.

Gene	Expression pattern
ato	P cell; chordotonal organ; central brain; external sensory organ
amos	abdominal segment; antenno-maxillary complex; cellular blastoderm; sensillum precursor
ac	ectoderm; midline; neuroblast; procephalon
sc	ectoderm; neurectoderm; neuroblast; stomodeum
Math1	cranial ganglia; dorsal wall of the neural tube; spinal cord; midbrain; hindbrain; cerebellum; metencephalon; inner ear sensory epithelium; cochlear duct; vestibular component; primordium
Math5	hypothalamus; thalamus; cerebral cortex; corpus striatum; telencephalon; olfactory lobe; hindbrain; midbrain; ventricular layer; cranial; spinal cord; dorsal root ganglion; neural retina; nuclear layer; outer nuclear layer
Ngn1	olfactory pit; midbrain ; dorsal root sensory ganglia ; subset of cranial ganglia ; otic placode; epithelium; telencephalon; spinal cord; neural tube; pancreas
Ngn2	telencephalon; basal palte and dorsal of spinal cord; neural tube; ventral hindbrain; dorsal thalamus; optic vesicle; geniculate and petrosal ganglion; subset of dorsal root ganglion; cranial; lateral wall; pretectum; hypothalamus; cerebral cortex; retina
Mash1	olfactory pit; prosencephalon; midbrain; rhombencephalon; neural tube; forelimb bud; ventral spinal cord; nasal placode; ventral telencephalon; dorsal aorta; lateral wall; metencephalon; sympathetic ganglion; sympathetic and enteric neural crest; dorsal cerebral cortex; cerebellum; thalamus; hippocampus; ventral hypothalamus; mantle layer; corpus striatum; olfactory lobe; hindbrain; ventral zone; cranial; dorsal root ganglion; nasal cavity; dorsal telencephalon; autonomic nervous system; ear; retina; pancreas; nuclear layer

vertebrates are the *NeuroD* and *Olig* groups. They are more distinct from other *Drosophila ato* family proteins, but are characterized as *ato* family members owing to the presence of family-specific residues in their bHLH domain [33, 60]. The expression patterns of vertebrate and invertebrate proneural proteins in embryo are summarized in table 2.

Proneural bHLH proteins in *Drosophila* PNS neurogenesis

Function of proneural proteins

In the *Drosophila* PNS, different proneural genes are initially expressed in different groups of ectodermal cells, called proneural clusters, defined by patterning genes. The cells in these clusters have the potential to develop into NPCs [61]. The expression of proneural genes is necessary and sufficient to promote the generation of NPCs.

Developmental analysis of loss-of-function (LOF) mutations in the *as* complex locus revealed that the *ac* and *sc* genes are required for the formation of external sensory organs of the adult fly, which include mechanosensory and chemosensory bristles [62, 63]. Gain-of-function (GOF) analysis shows that *ac* and *sc* are also sufficient for external sensory organ NPC specification [47, 64–67].

The proneural gene *ato* is involved in the selection of precursors of chordotonal organs (internal stretch receptors), the auditory organ (or Johnston's organ), a subset of olfactory sense organs and the founder photoreceptor cells, or R8 [57, 68, 69]. The *ato*-related gene *amos* is the proneural gene for a subtype of multidendritic neuron and most olfactory sensilla [55, 70]. Since there is no *tap* mutant available, its physiological function is not known. Expression studies suggest that *tap* is involved in neurogenesis as well, and further studies will help elucidate its role in *Drosophila* neural development.

The regulatory cascade

The expression of proneural genes in NPCs is downregulated before they divide. The ability of proneural genes to promote neural lineage development relies on the induction of downstream regulatory genes that control neuronal differentiation. These genes are expressed in selected precursors and are therefore called neuronal precursor genes. Some of them are neural bHLH genes, like *ase* [60]. It is generally not expressed in clusters of ectodermal cells, but instead in most or all selected progenitors. It is required for the correct differentiation of sensory neurons [71, 72]. Genetic analysis has shown that *ase* mutations alter the differentiation of sensory organs [72, 73]. Similar to *ase*, the third *ato* family gene, *cato*, is also expressed in NPCs and is involved in neuronal differentiation [56].

Regulation of NPC selection is both positive and negative. On the one hand, proneural genes inhibit their own expression in adjacent cells through activation of the Notch signalling pathway [74, 75]. This results in the expression of repressors, such as the Enhancer of split [E(spl)] bHLH proteins, which restrict proneural gene expression into single cells [76, 77]. On the other hand, positive feedback regulation is required to increase and/or maintain the levels of proneural gene expression in the selected neural progenitors. Proneural proteins can either positively auto-regulate or induce the Zn-finger protein Senseless

(Sens), which in turn represses E(spl) function [78, 79]. Recently, another Zn-finger protein, Charlatan (Chn), was identified. It specifically activates a certain enhancer of *ac/sc*, stimulating Ac/Sc expression. Absence of *chn* leads to loss of embryonic peripheral neurons and causes aberrant development of chordotonal organs, but does not seems to affect Ato function in the eye [80].

Another aspect of NPC selection is regulated by anti-neuronal bHLH/HLH genes. Two mechanisms have been found for anti-proneural function. One is to inhibit the formation of functional heterodimers between proneural proteins and Da by competitive binding. This mechanism has been shown for the HLH protein Extramacrochaetae (Emc) and the bHLH proteins Hairy (H) and E(spl) [81–86]. Another is to repress transcription of neural bHLH genes by binding site-dependent transcriptional repression, as has been shown for H and E(spl), which bind to a CACNAG sequence called N box in the promoter region of neural bHLH genes and recruit the transcriptional repressor Groucho through the WRPW motif in their carboxy-terminal regions [76, 84, 87–89]. More recently, it was shown that transcriptional activation of the C-terminal domain of Sc is required for E(spl) recruitment in an enhancer context-dependent manner [90]. Beside using the anti-neural bHLH/HLH genes to regulate timing of differentiation, other elements and signalling pathways which control cell cycle exit, cell proliferation and asymmetric/symmetric cell division also play important roles in proper neural lineage development (reviewed in [26, 91–93]). The cell autonomous proneural regulatory network is shown in figure 1B.

Functional diversity of Drosophila proneural genes

The two sets of proneural proteins, Ato-related and AS, share the common characteristics of selecting NPCs by activating the Notch signalling pathway and interacting with Zn-finger transcription factors [79]. However, external sensory organs are formed from single precursors that send only inhibitory signals to their neighbours (fig. 1A), while secondary precursors within the chordotonal precursor cluster (fig. 1A) and the non-R8 photoreceptors are recruited by EGFR signalling activated by Ato-expressing precursors [94, 95]. LOF and GOF studies have shown that different proneural genes are involved in the development of different types of sense organs. This indicates that proneural genes have a role in the specification of neuronal identity.

One interesting question is whether proneural bHLH proteins alone are sufficient to specify distinct NPCs. Jarman et al. showed that ectopic expression of Ato promotes the formation of ectopic chordotonal organs as well as ectopic bristles depending on levels of Ato expression and the context in which it is expressed [57]. Bristle formation does not depend on ectopic activation of *ac/sc*. Another example comes from the observation that *sc* can partially rescue the eye phenotype of *ato* null mutants, inducing formation of ommatidia apparently without first inducing R8 cells [96]. However, this study did not exclude that R8 photoreceptors are formed initially but undergo apoptosis later. It would be interesting to test this more carefully. It is important to note that no evidence exists to suggest that *ac* or *sc* can induce cordotonal organs. However, to fully exclude the possibility that both sets of proneural proteins have a certain capability to promote NPCs of either type of sensory organ in the correct context, reciprocal rescue experiments are needed. The overexpression of a homeodomain transcription factor, Cut, which is normally induced by AS and expresses only in the external sensory organ NPCs, is able to transform chordotonal organs to external sensory organs. In contrast, mutations in *cut* transform external sensory organs into chordotonal organs [97, 98]. Genetic data indicate that Ac/Sc induce *cut* expression, whereas Ato represses the activation of *cut* [97, 99]. The differential abilities in regulating *cut* may in part underlay the functional diversity of these two types of proneural genes.

The Cut data suggest that selecting neural precursors per se is genetically separable from specifying lineage identity, at least downstream of proneural activity. The ability of Ato to repress *cut* means that the proneural genes themselves control lineage specification. This raises the question whether different structural properties of proneural genes mediate the different aspects of their activities. Another interesting question is the extent to which lineage specification requires cooperation with other factors in the correct context. However, one thing is certain: in vivo, distinct proneural bHLH proteins are required to coordinate with context specific co-factors to promote the formation of distinct cell fates.

Proneural bHLH proteins in vertebrate PNS neurogenesis

Function of proneural proteins

After neural induction, vertebrate neurogenesis, does not occur homogeneously and simultaneously throughout the neural plate. Its pattern responds to precise, positionally distinct developmental cues within the neural plate, rather similar to *Drosophila* proneural clusters. Within each domain, neuronal precursors are defined by the lateral inhibition process of Delta-Notch signalling. Some transcription factors, including the AS- and Ato-related proneural bHLH proteins, the Zn-finger protein Gli/Zic, Winged helix (XBF) and Iroquois (Iro), act complementarily to define proneuronal clusters and are required for neuronal differentiation within the neural plate [61, 100, 101].

In general, unlike *Drosophila*, vertebrate early-acting bHLH proteins appear to control neuronal vs. glial cell fate rather than neural vs. epidermal cell fate decisions. However, a case was found in the chick caudal neural plate where it appears that some neural plate cells are committed to epidermis [102]. It is worth noting that an AS bHLH protein family gene is expressed in the most caudal part of the neural plate both in frog (*Xash3*) and in chick (*Cash4*) [103–105].

Homologues of the proneural bHLH proteins acting in *Drosophila* neurogenesis have been found in vertebrates (table 1). More than one AS homologue (Ash) exists in rat, mouse, chicken, *Xenopus* and zebrafish [104, 106–108]. Similarly, homologues of the Ato-related protein family also exist in all vertebrates. They can be subdivided into Aths (vertebrate homologue of Ato), Ngns/Ngnr (vertebrate homologue of Tap) and the other subgroups NeuroD and Olig [60].

If one defines proneural genes as those sensitive to lateral inhibition and expressed in neural or neuronal proliferative progenitors, then clearly several vertebrate bHLH proteins can be defined as proneural proteins, including X-Ngnr1 (Ato family) and Xash (AS family) in *Xenopus*, Mash1 (AS family), Ngn1, Ngn2 and Math1 (Ato family) in mouse [106, 109–114]. Others, such as NeuroD in *Xenopus* and Math2 in mouse are found in postmitotic cells and are regulated by proneural proteins, and therefore can be considered as neuronal differentiation regulators [115, 116]. In addition to the bHLH domain, in the NeuroD subgroup (NeuroD, Math2/Nex1 and mNDRF/KW8/NeuroD2) [117, 118], a 40-amino acid sequence was found that contains a leucine-proline-rich region and a junctional sequence (Met-His-Gly/Asp/Asn) important for internal E-box residue specification [119].

Genetic analysis of vertebrate proneural bHLH genes has shown that the general rules of how these genes regulate neurogenesis are similar to their *Drosophila* counterparts. LOF analysis suggests that progenitor populations are lost and Notch signalling is not activated in some null mutants. For example, *Mash1*null mutants show severe defects in neurogenesis in the olfactory sensory epithelium [38, 120]. *Ngn1* or *Ngn2* single mutant mice show complementary sets of cranial sensory ganglia defects [111, 121–123]. It is likely that in these cases, *Ngn1* and *Ngn2* act to specify neuronal (vs. glial) fate as opposed to early neural progenitors.

Vertebrate neural crest contains multipotent progenitors, and bHLH proteins appear to play a role in the cell fate specification of these progenitors. Sensory neurons and autonomic neurons are two major neuronal classes of vertebrate PNS neurons [124]. Ato-related proteins, such as Ngns, are required for sensory neuron specification, while AS proteins, such as Mash1, are required for autonomic neuron specification [111, 122, 125].

In the vertebrate retina, the seven basic neural and glial cell types form through position-dependence and cell-cell interaction. A large body of studies has revealed the role of proneural bHLH proteins in retinal development. It is known that retinal ganglion cells (RGCs) require *Ath5*, an orthologue of *ato* [126, 127], amacrine cells and photoreceptors require *NeuroD* [128], bipolar cells require *Ash1* and *Ath3* [129, 130], and bipolar cells and photoreceptors require *Ngn2* [131]. *Ath5* begins to express in the ventronasal optic cup and spreads dorsally and temporally. It regulates the expression of the *Brn3* subfamily of POU homeodomain transcription factors for RGC development and survival [132, 133]. However, the ability of Ath5 to activate target genes becomes inhibited in later progenitors [134]. *Mash1* is transiently expressed by differentiating bipolar cells, and *Mash1*-null mutations show a decrease in bipolar cell number while increasing Muller glial cells [135]. *Math3* is also expressed in bipolar cells, but *Math3*-null mutations do not affect bipolar cell development [135]. Double mutation studies indicate that Mash1 and Math3 cooperatively regulate neuronal vs. glial cell fate determination in the retina [135]. An excellent overview of bHLH factors involed in retinal cell fate determination is given in a recent review [136].

Beside the essential role of proneural bHLH proteins in neuronal differentiation, recent studies have shown that they also act through independent mechanisms to inhibit gliogenesis [35, 36, 135]. Studies on both *Mash1*/*Math3* double mutant [135] and Mash1/Ngn2 double mutant [35] deficient mice show increased gliogenesis in addition to reduced neurogenesis. Sun and colleagues proposed that Ngn1 inhibits gliogenesis by binding to the CBP/Smad1 or p300/Smad1 co-activators and competing them away from promoters of glial genes [36]. A recent study has further demonstrated that Mash1 is required for specification of both neurons and oligodendrocytes [137]. These findings suggest that proneural bHLH proteins integrate different contextual information and play multiple roles in nervous system development.

The regulatory cascade

Although the roles of proneural bHLH proteins in regulating neurogenesis are well established, their downstream targets genes remain poorly defined. A screen for downstream effectors of Ngn2 in the cortex using a subtractive hybridization method identified 16 misregulated genes in an Nng2 mutant [138]. These genes include transcription factors as well as genes involved in migration and axonal pathfinding, such as *Sema3C* and *EphA5*. Further analysis of these genes should provide a better understanding of the molecular mechanisms underlying neurogenesis. Xseb4R, an RNA-binding protein, has been isolated in *Xenopus* [139]. LOF and GOF studies suggest that *Xseb4R* strongly promotes neural differentiation and is

involved in retinogenesis. It is responsive to proneural transcription cascade-upregulated by the proneural gene *XNgnr1* and differentiation gene *XNeuroD* and is inhibited by Delta-Notch signalling [140]. A study of commissural neurons shows that a Bar-class homeobox gene, *Mbh1*, is necessary and sufficient for specification of commissural neurons, as a direct downstream target of Math1. Further Mbh1 enhancer analysis indicates that Math1 directly activates the expression of Mbh1 by binding to the E-box in its promoter region, probably in a collaboration with other factors [141].

GOF experiments in *Xenopus* [142] and LOF analysis in mouse [110, 111, 122] have shown that the bHLH *NeuroD* family genes are induced by proneural proteins and act downstream of vertebrate proneural genes in the later stage, similar to *ase* and *cato* in *Drosophila*. The activation of *NeuroD* subfamily genes is directly promoted by proneural proteins. Most structures of the PNS transiently express high levels of *neuroD* RNA during embryogenesis, as well as in differentiating adult neurons. Ectopic expression of *NeuroD* family genes promotes neuronal differentiation [116, 143]. LOF analysis shows that NeuroD family proteins are required for the proliferation, differentiation and survival of many types of neurons [144, 145]. This is clearly different from the loss of progenitor cells in mice which lack *Mash1* or *Ngn*. Therefore, proneural proteins act through a regulatory cascade of bHLH proteins to specify subsets of neuronal lineages.

Interactions among proneural genes and Notch signalling are remarkably conserved (fig. 1B). Neural progenitors activate Notch signalling to inhibit proneural protein expression in neighbouring cells, while simultaneously forming a positive regulatory loop within the NPC. This regulatory loop is maintained by inducing expression of other factors, such as *Hes6*, *Coe2* and the Zn-finger protein gene *MyT1*. These progenitors exit the cell cycle and activate neuronal bHLH genes, such as *Math2/NeuroD2/Ebf3,* for initiating neuronal differentiation. In parallel, they inhibit glial differentiation by blocking gliogenic signalling. Two groups of neural bHLH transcription repressor genes, *inhibitor of differentiation* (*Id*) [146, 147], which is a homologue of *emc*, and *Hes/Her/Esr*, which resemble *Drosophila hairy* and *E(spl)*, have also been identified in vertebrates [25, 148]. They are under the control of Notch signalling and appear to exert their inhibition using the same mechanisms as their *Drosophila* counterparts.

Functional diversity of proneural genes

Most animals have more than one paralogue of each bHLH gene. Most if not all of these express in complementary subtype progenitors and play distinct roles. Two mouse *ato* family genes, *Math1* and *Math5* are such examples. *Math1* mutant mice lack cerebellar granule cells in the central nervous system (CNS) and lose inner ear hair cells, which are essential for hearing and balance [112, 149]. GOF and LOF studies also indicate a role for *Math1* in the specification of interneuron identity [150, 151]. LOF mutation of *Ath5* in mouse and zebrafish results in the loss of most RGCs [126, 152]. Conversely, in *Xenopus* and chicken, overexpression of *Math5* or *Cath5* promotes differentiation of RGCs [127, 153].

Despite evidence showing that aspects of proneural function can be interchangeable, it is clear that different proneural genes play different roles in vivo. For instance, the phenotype of *Ngn2*-null mutant embryos is transient and is recovered after an delay in an *Ngn1*-dependent manner [125]. *Ngn2* is required for large-diameter sensory neuron (trkc+/trkB+) development, whereas *Ngn1* is required for small-diameter sensory neuron (trkA+) development. It is possible that both *Ngn1* and *Ngn2* can potentially promote the differentiation of both types of neurons, but they are normally expressed at different times and therefore at least encode the proper timing of neural differentiation and presumably act with different co-factors.

It is known that *Ngn2* and *Mash1* express complementarily in most regions of the nervous system, and have distinct roles. In multipotent cortical progenitors the proneural proteins Mash1, Ngn1 and Ngn2 play key roles. *Ngns* are expressed in the dorsal telencephalon, which gives rise to glutamatergic neurons, wherease *Mash1* is predominantly expressed in the ventral telencephlon, which gives rise to GABAergic and cholinergic neurons [154, 155]. When the coding sequence of *Mash1* is knocked into the *Ngn2* locus, the cortical progenitors in the dorsal telencephalon are misdirected in their fate and become GABAergic neurons [123, 156]. When Ngn2 replaces Mash1, ventral telencephalon neurons differentiate normally and show no change in phenotype [157]. These data indicate that the ability of proneural bHLH factors to play a role in neural subtype specification relies strongly on the cellular context.

Increasing evidence suggests that different bHLH proteins, partially due to their spatial and temporal regulation, coordinate with different co-factors, and therefore integrate all regulatory information and specify neuronal differentiation. For example, bHLH proteins cooperate with ETS proteins, Paired homeodomain proteins and Lim homeodomain proteins [26, 92, 136, 158] as well as chromatin remodelling proteins [159]. A recent study showed that bHLH proteins Ngnr1 and NeuroD physically associate with chromatin remodelling complex SWI/SNF via its catalytic subunit Brg1. The transactivation of proneural bHLH proteins in neuronal differentiation may therefore be mediated by Brg1. Although the sequences of bHLH proteins are highly conserved and functions are occasionally interchangeable, their distinct functions

still cannot be solely explained by their differential expression and/or co-factors. The structural diversity of neural bHLH proteins itself plays an important role in adapting to developmentally and evolutionarily contextual specificities.

The bHLH domain: structural diversity encodes functional specificity

A large body of analysis in both vertebrates and invertebrates shows that proneural gene activity is highly dependent on cellular context. Perhaps a key function of proneural bHLH proteins is to allow neural cells to correctly interpret specific positional cues provided by local factors. An interesting question is how this integrative specificity is achieved. Increasing evidence supports the possibility that direct protein-protein interactions between proneural protein and co-factors are essential for the specific functions of proneural proteins. A recent study gives a direct example of a specific binding pocket for the ETS protein Pointed needed to mediate specific functions of Ato [160]. The Sc C-terminal containing a transcriptional activation domain has recently been found to be required for E(spl) recruitment [90]. It is noteworthy that E(spl) use different domains to contact Sc (the N-terminal region) [90] vs. Sens (the middle Orange region) [161], which probably allows it to repress a Sc/Sens complex more effectively. These findings suggest that protein-protein interactions may play a common role in both positive and negative regulation. Studies have shown that direct interactions between regionally expressed transcription factors and proneural proteins regulate the transcription of target genes in a region-specific manner [162, 163]. In one case, a bridging factor brings the *Drosophila* GATA factor Pannier to the AS-Da heterodimer, which binds to the E-box in the promoter region [162]. In another case, certain binding site combinations serve an architectural function to mediate or enable transcriptional synergy, which drives target gene activation in specific cells [163]. DNA-protein level regulation of proneural downstream target genes can be found as well [164–167]. A study from last year has shown that although neural bHLH proteins from different families recognize the common hexamer CANNTG, they recognize different bases in the two central positions, as well as in the adjacent positions [164]. Therefore, different proneural proteins interact with distinct co-factors regulating transcription of distinct downstream target genes via both protein-protein and protein-DNA interactions.

It is reasonable to assume that the functional diversity of neural bHLH proteins is mediated by their structural diversity. Indeed, some bHLH proteins lack certain subdomains and others contain additional domains [31, 76, 90]. Domain exchange experiments have shown that although the sequence of the bHLH domain is highly conserved between different proneural families and across different species, a few distinct amino acids alter functional specificity. For example, placing three amino acids from the basic region of MyoD into E12 creates myogenic specificity [119]. Swapping the basic region of Ato to Sc allows Sc to induces chordotonal organs [168], suggesting that Ato-specific function relies on a motif in the basic region, perhaps mediating protein-protein interactions. In vertebrates, the comparison between *Xash1* and *XNgnr1* proneural activities demonstrates that although overexpression of both these genes induces neuronal differentiation, they do induce distinct downstream targets, and these functional specificities rely on the first helix [169]. A single amino acid mutation which changes the three-dimensional structure of Mash1 by introducing an additional helical turn endows it with myogenic activity [170]. Another study compared the function of different domains of Mash1 and Math1 and showed that different motifs in different domains are required for distinct functions, probably reflecting the importance of unique protein-protein interactions [171]. A recent study comparing the mechanisms used for regulating the selection of NPCs in vertebrates and *Drosophila* by the proneural proteins Ngn1 and Ato has shown that divergence in proneural activity is encoded by three amino acids in the basic region. This motif in Ato and Ngn1 induces and interacts with the Zn-finger proteins Sens and MyT1, respectively. In addition, a five-amino acid motif in the second helix domain of Ngn1 is sufficient to induce neurogenesis in vertebrates [172].

It is worth noting that the class-specific residues in the bHLH domain are located on the outer surface of the protein dimer, and that all the specific functions discussed above are encoded by these residues (fig. 2). Taken together these data suggest that physical interactions with context-specific factors may play an important role in proneual bHLH protein function. Such co-factors could affect the interaction of proneural proteins with their DNA binding sites and regulate their transcriptional activity or the choice of target genes. All these data support the idea that the structural specificity of proneural genes integrates spatial and temporal information to specify neural lineage development. Therefore, identifying new co-factors, possible binding sites and fishing out distinct downstream targets are now the crucial requirements for better understanding the mechanisms involved in regulating neural development by bHLH proteins.

From a developmental point of view, the diversity of proneural proteins could allow them to regulate different processes to give rise to different cell lineages in a positionally and temporally specific manner. From an evolutionary point of view, this variety could allow organisms to diversify developmental processes using the same conserved machinery.

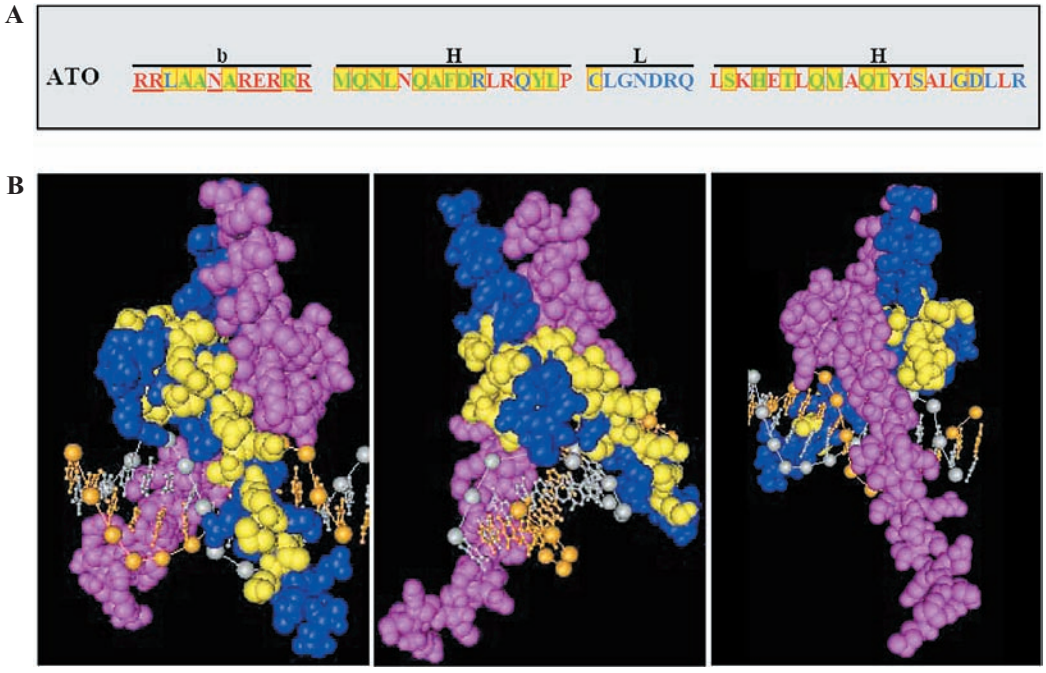

Figure 2. Structural specificity in the bHLH domain encodes functional specificity. (*A*) The amino acid sequence of the bHLH domain, using Atonal as an example. Red indicates residues common to all neural bHLH proteins. Green residues are specific for each subfamily of proneural bHLH proteins. Blue marks the divergent residues. Underlined residues are required for DNA binding. Sequences involved in functional specificity are labelled by yellow boxes. (*B*) Three views of a model of the 3D structure of a proneural protein heterodimer using the crystal structure of MyoD as a model. The proneural protein is marked in blue. The partner E protein is in purple. The side chains of the amino acids shown to encode functional specificity are marked in yellow. Note that these residues protrude to the surface of the dimer complex, providing the possibility of additional protein-protein interactions.

Conclusion

Growing evidence has revealed that proneural genes play crucial roles in specifying different aspects of neural development in vertebrates and *Drosophila*. Proneural proteins integrate temporal and positional information by interacting with co-factors and regulating the development of distinct neural lineages. The structure of proneural proteins, especially their bHLH domain, mediates their vital role in neurogenesis. The structural divergence of proneural proteins seems to underlie the developmental and evolutionary dedication of different proneural factors to different neural lineages.

Compared with the knowledge we have for the proneural genes themselves, little is known about their target genes, in particular the ones required for cell fate specification. It would be also interesting to find specific co-factors for distinct lineages and investigate how proneural bHLH proteins interact with these distinct pathways.

Finally, bHLH families arose from ancestral sequences by duplication in the protostomian and deuterostomian lineages. Currently, there are two alternative models to explain the evolutionary divergence of bHLH families. A classic model proposes that duplications gave rise to fully redundant copies, allowing accumulation of random mutations on which natural selection could act. Out of billions of pseudogenes, a benefitial mutation occurs that gives rise to a novel function in a process called neofunctionalization. An alternative model suggests that gene duplications derived from partial regulatory sequence deletions result in novel expression patterns of an otherwise identical copy. In the long run, each copy evolves new functions in a process called duplication-degeneration-complementation or subfunctionalization [173]. These two models are not necessarily mutually exclusive. Both scenarios may have ocurred, giving rise to the diverse array of modern, highly specialized bHLH genes.

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