PRONEURAL GENES AND THE SPECIFICATION OF NEURAL CELL TYPES

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Certain morphological, physiological and molecular characteristics are shared by all neurons. However, despite these similarities, neurons constitute the most diverse cell population of any organism. Recently, considerable attention has been focused on identifying the molecular mechanisms that underlie this cellular diversity. Parallel studies in *Drosophila* and vertebrates have revealed that proneural genes are key regulators of neurogenesis, coordinating the acquisition of a generic neuronal fate and of specific subtype identities that are appropriate for the location and time of neuronal generation. These studies reveal that, in spite of differences between invertebrate and vertebrate neural lineages, *Drosophila* and vertebrate proneural genes have remarkably similar roles.

BASIC HELIX–LOOP–HELIX
A structural motif that is present in many transcription factors, which is characterized by two α-helices separated by a loop. The helices mediate dimerization, and the adjacent basic region is required for DNA binding.

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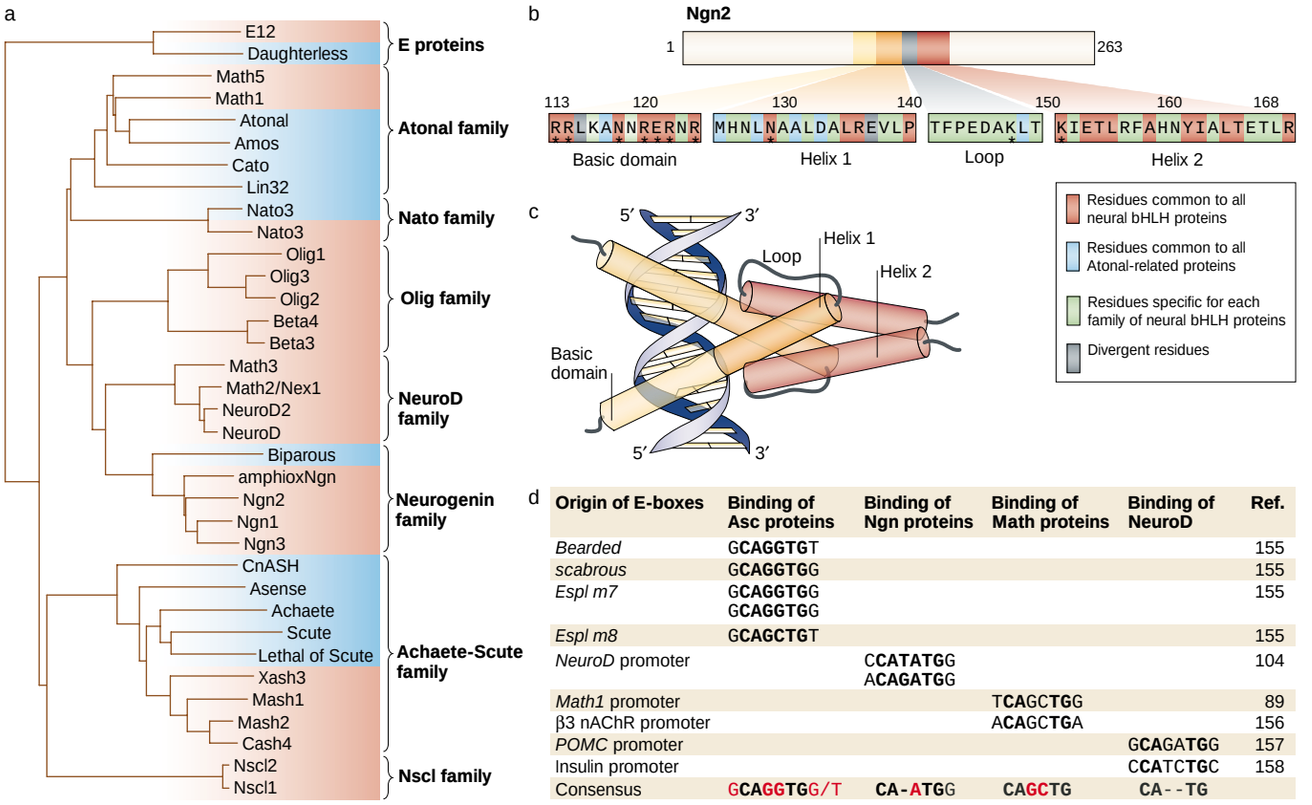
doi:10.1038/nrn874

Building a nervous system involves the production of a vast array of neuronal and glial cell types that must be produced in the correct numbers and at appropriate positions. The uniform epithelial sheath that constitutes the primordium of the nervous system in invertebrate and vertebrate embryos consists of cells that have the potential to generate both neurons and glia. However, multipotent progenitors that are located at different positions generate different neuronal and glial cell types. For example, progenitor cells in the ventral neural tube in vertebrate embryos initially produce motor neurons and later produce oligodendrocytes, whereas more dorsal progenitor cells produce interneurons and astrocytes. In *Drosophila*, neural development involves the transformation of ectodermal cells into progenitor cells, which undergo a limited number of divisions through fixed cell lineages before differentiating into neurons and glia. In vertebrate embryos, neuroepithelial cells have the self-renewing properties of stem cells. They produce intermediate progenitors that are restricted to a neuronal or glial fate, and proliferate to some extent before differentiating.

Genetic studies in *Drosophila* and vertebrate models have provided evidence that a small number of 'proneural genes', which encode transcription factors of the BASIC HELIX–LOOP–HELIX (bHLH) class, are both necessary and sufficient, in the context of the ectoderm, to initiate the development of neuronal lineages and to promote the generation of progenitors that are committed to differentiation. Importantly, proneural genes have recently been shown to integrate positional information into the neurogenesis process and to contribute to the specification of progenitor-cell identity. Current studies focus on understanding the mechanisms that underlie the multiple functions of proneural genes in neural development.

Structure and diversity of neural bHLH proteins

Identification of proneural genes. The study of proneural genes dates back to the second decade of the twentieth century, when mutant flies were found that lacked subsets of external sense organs or bristles[1]. By the late 1970s, a complex of genes that are involved in regulating the early steps of neural development in *Drosophila* had been identified[2]. Molecular analysis led to the isolation of the four genes of this complex, namely *achaete* (*ac*), *scute* (*sc*), *lethal of scute* (*lsc*) and *asense* (*ase*)[3,4], and to the discovery that the products of



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Origin of E-boxes	Binding of Asc proteins	Binding of Ngn proteins	Binding of Math proteins	Binding of NeuroD	Ref.
Bearded	G**CAGGTG**T				155
scabrous	G**CAGGTG**G				155
Espl m7	G**CAGGTG**G G**CAGGTG**G				155
Espl m8	G**CAGCTG**T				155
NeuroD promoter		C**CATATG**G A**CAGATG**G			104
Math1 promoter			T**CAGCTG**G		89
β3 nAChR promoter			A**CAGCTG**A		156
POMC promoter				G**CA**GA**TG**G	157
Insulin promoter				C**CA**TC**TG**C	158
Consensus	G**CAGGTG**G/T	**CA-ATG**G	**CAGCTG**	**CA--TG**	

Figure 1 | **Structure and properties of neural bHLH proteins. a** | Dendrogram of the sequence of the basic helix–loop–helix (bHLH) domain of invertebrate (blue) and vertebrate (red) neural bHLH proteins. Proteins have been grouped in distinct families on the basis of closer sequence similarities in the bHLH domain. **b** | Sequence of the bHLH domain of the mouse proneural protein neurogenin 2 (Ngn2). A colour code indicates the degree of amino-acid conservation between neural bHLH proteins at each position. Asterisks mark residues that make direct contact with DNA, on the basis of the crystal structure of other bHLH proteins[24–26]. **c** | Schematic representation of the structure of a bHLH dimer that is complexed to DNA (adapted, with permission, from *Nature* (REF. 25) © 1993 Macmillan Magazines Ltd). The basic region fits in the main groove of the DNA, and many residues in this region make direct contact with the E-box sequence. The two α-helices of both partners together form a four-helix bundle. **d** | Sequences of E-boxes that are present in the promoters of target genes and are specifically recognized by different families of neural bHLH proteins. Although neural bHLH proteins from different families recognize the common hexamer CANNTG, they must recognize different bases in the two central positions, as well as in adjacent positions.

these genes share sequence similarity with each other, as well as with the oncogene *myc*, the sex-determination gene *daughterless* (*da*) and the muscle-determination gene *MyoD*[4,5]. This work paved the way for the identification of the bHLH domain, a structural motif that is shared by these proteins and is responsible for their DNA-binding and dimerization properties[6].

A further proneural gene, *atonal* (*ato*), was isolated more recently in a PCR (polymerase chain reaction) screen to identify bHLH sequences related to that found in *achaete-scute* complex (*asc*) genes[7]. The *ato* gene belongs to a distinct bHLH family, sharing with *asc* genes approximately 45% identity in the bHLH domain, compared with 70% identity among *asc* family members (FIG. 1a,b). Two *ato*-related genes, *amos* (*absent MD neurons and olfactory sensilla*) and *cato* (*cousin of atonal*), were later isolated[8–10], defining a second family of proneural genes (FIG. 1a).

Proteins of the *asc* and *ato* families share several features that define them as proneural. First, most *asc* and *ato* family members (although not all, see below) are expressed in the ectoderm, before any sign of neural differentiation becomes apparent. In *Drosophila*, proneural genes are initially expressed by groups of ectodermal cells called 'proneural clusters', which are distributed in patterns that foreshadow the distribution of neural progenitor cells in the peripheral and central nervous systems (the PNS and CNS, respectively)[11]. Second, genetic analysis has revealed that genes of the *asc* and *ato* families are both required and sufficient to promote the generation of neural progenitor cells from the ectoderm[12,13]. This activity involves the activation of the Notch signalling pathway[14]. Last, all known proneural genes belong to the same class of bHLH transcription factors, indicating that they have similar biochemical properties. Interestingly, members of the *asc* and *ato* families account for all proneural activity in the PNS, but not in the CNS, where the generation of some neuroblasts does not require any of the known proneural genes[15]. Complete sequencing of the

Drosophila genome has revealed the existence of new bHLH genes, but none of them shows the expression pattern that would be expected of a proneural factor[16,17]. So, if further *Drosophila* genes with proneural activity exist, they might diverge in structure from those already identified.

Many genes that are related to *asc* and *ato* have been found in vertebrates[18,19] (FIG. 1a). Some of these genes have been isolated by RT-PCR on the basis of sequence conservation with their *Drosophila* counterparts or other vertebrate genes. Others have been isolated in YEAST TWO-HYBRID SCREENS through their ability to dimerize with other bHLH proteins. The vertebrate *asc* family includes *ash1*, which is present in all species analysed (for example, *Mash1* in mouse, *Cash1* in chick, *Zash1* in zebrafish and *Xash1* in *Xenopus*), and three other genes that, curiously, have each been found in only one class of vertebrates (*Mash2* in mammals, *Xash3* in *Xenopus* and *Cash4* in chick) (FIG. 1a). The number of vertebrate genes that are related to *Drosophila ato* is larger, but only two of them (*Math1* and *Math5* in the mouse) have a bHLH domain similar enough to that of *ato* to be considered as orthologues. Other vertebrate *ato*-related genes can be grouped into distinct families (for example, the neurogenin (Ngn) family, the NeuroD family and the Olig family) that are characterized by the presence of family-specific residues in their bHLH domain[18,20] (FIG. 1b), indicating that different members in each family share biochemical properties that distinguish them from other neural bHLH proteins. Most vertebrate proneural-related genes are expressed exclusively or principally in the developing nervous system, indicating some conservation of function. However, only a relatively small subset of the vertebrate *ato*-related genes has been shown to have a role in the selection of neural progenitor cells, as discussed below.

Biochemical properties of proneural proteins. Like other tissue-specific bHLH proteins, proneural proteins bind DNA as heterodimeric complexes that are formed with ubiquitously expressed bHLH proteins, or E proteins, encoded by the *Drosophila* gene *da*, or one of three mammalian genes: *E2A* (with its two alternative products E12 and E47), *HEB* and *E2-2* (REFS 21–23). The crystal structures of the bHLH domain of MyoD, Max and E47 show that bHLH dimers are formed by interactions between the two helices of each partner to form a four-helix bundle[24–26] (FIG. 1c). Because heterodimerization is a prerequisite for DNA binding, factors that interfere with dimerization effectively act as passive repressors of proneural gene activity. Products of the *Drosophila emc* (*extra macrochaetae*) and the vertebrate Id (inhibitor of differentiation) genes have a HLH domain, but lack an adjacent basic motif for DNA binding. These proteins have a high affinity for E proteins, so they can compete with proneural proteins, forming heterodimers that cannot bind DNA[23,27,28]. The vertebrate Hes/Her/Esr proteins constitute, with their *Drosophila* counterparts, the hairy and enhancer of split (Espl) factors, another family of proneural gene inhibitors[29,30]. These proteins have been shown to act as classical DNA-binding repressors of proneural gene transcription[31–33], but they are also thought to inhibit the activity of proneural proteins by interfering with proneural–E-protein complex formation[29,30].

Like other bHLH proteins, proneural proteins specifically bind DNA sequences that contain a core hexanucleotide motif, CANNTG, known as an E-box. Residues in the bHLH domain that directly contact DNA have been identified by modelling the structure of an Atonal–Daughterless bHLH heterodimer in a complex with DNA[34]. The basic region and helix 1 of the bHLH domain form a long α-helix that is connected by the loop region to helix 2 (FIG. 1c). The basic region fits in the main groove of the DNA, and seven of the ten bHLH residues that make direct contact with DNA are located in this region, whereas the other three are scattered throughout the HLH region. Interestingly, nine of the ten DNA-contacting residues are completely conserved in the different families of neural bHLH proteins, and they bind the conserved bases of the E-box or phosphate residues. These direct contacts are responsible for the common ability of neural bHLH proteins to bind to the core E-box sequence, but are unlikely to account for the divergence in DNA-binding specificity and biological activities between different neural bHLH-protein families. Indeed, a comparison of E-box sequences in the promoters of various target genes reveals a sequence specificity that goes beyond the four conserved bases of the core E-box, CA and TG (FIG. 1d). The basis for this DNA-binding specificity is unknown at present, but it might involve interactions between family-specific residues in the bHLH domain and cofactors[34] (FIGS 1b; see below).

Proneural proteins and most other neural bHLH factors act as transcriptional activators, and only a few, including Olig2, have been shown to act as repressors[21,22,35,36]. Ngns have been shown to induce transcription through recruitment of cofactors such as p300/CBP (CREB-binding protein) and PCAF (p300/CBP-associated factor)[37]. Further characterization of the cofactors that interact with proneural proteins will be important to further our understanding of the mechanisms that underlie their activities.

Genetic analysis of proneural functions

Proneural genes in Drosophila. Loss-of-function (LOF) and gain-of-function (GOF) studies have been crucial for the identification of neural bHLH genes that have proneural activity in *Drosophila* and vertebrates, and to precisely define their diverse contributions to neural development. Proneural genes were initially identified through naturally occurring LOF mutations in the *asc* complex of *Drosophila*, including deletions of several *asc* genes that resulted in the loss of defined subsets of neural progenitors (BOX 1). LOF analysis has shown that the *ac* and *sc* genes are required in a redundant manner for the generation of most embryonic and adult external sense organs (mechanosensory and chemosensory organs), as well as for a subset of CNS progenitors (neuroblasts). The expression of a third *asc* gene, *lsc*, is

RT-PCR
Reverse transcriptase–polymerase chain reaction (PCR) — a reaction in which messenger RNA is converted into DNA (reverse transcription), which is then amplified by PCR.

YEAST TWO-HYBRID SCREEN
A system used to determine the existence of direct interactions between proteins. It involves the use of plasmids that encode two hybrid proteins; one of them is fused to the GAL4 DNA-binding domain and the other one is fused to the GAL4 activation domain. The two proteins are expressed together in yeast; if they interact, then the resulting complex will drive the expression of a reporter gene, commonly β-galactosidase.

CHORDOTONAL ORGAN
A sense organ in insects that detects mechanical and sound vibrations.

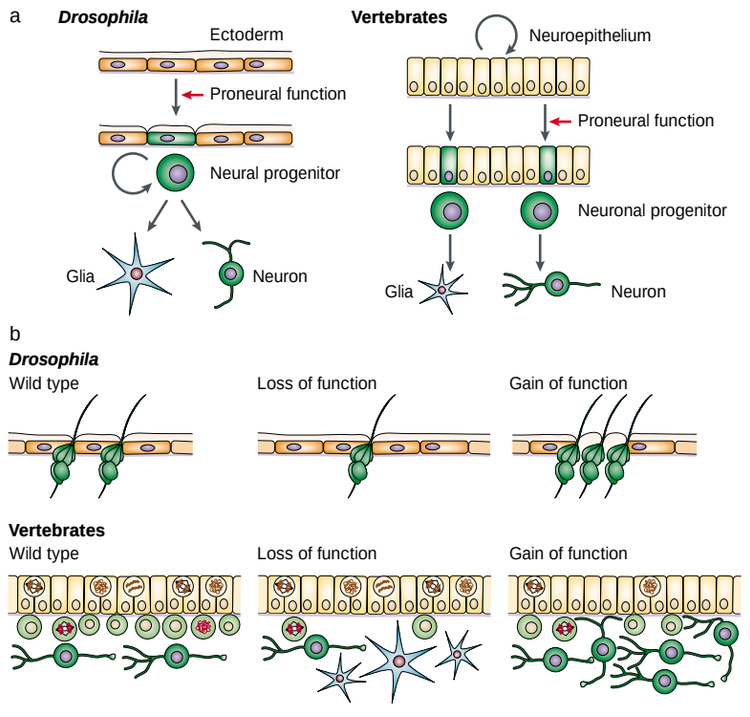
restricted to the CNS primordium, and this is the most important *asc* gene for the generation of neuroblasts[15]. In contrast to the other *asc* genes, the fourth gene of the complex, *ase*, is not expressed in clusters of ectodermal cells, but instead in all progenitors of the PNS and CNS after they have been produced[38]. It is required for the correct differentiation of sensory organs rather than for the selection of their progenitors, so it meets the definition of a neuronal-precursor gene[12,39]. In spite of the divergence of their functions, GOF analyses have shown that all *asc* genes have the same intrinsic activity. When ectopically expressed, the four *asc* genes induce the development of ectopic external sense organs at the expense of epidermis[39,40] (BOX 1). So, GOF and LOF mutations have opposite phenotypes, indicating that *asc* genes have characteristics of selector genes for neural development[2].

In contrast to external sense organs, internal CHORDOTONAL ORGANS (proprioceptors) are specified by *ato* and not by *asc* genes[7]. LOF analysis has revealed that *ato* also has a proneural role for the specification of founder photoreceptors of the retina, a subset of olfactory organs and some multidendritic neurons in the PNS[9,41]. In the CNS, *ato* expression is restricted to a small population of embryonic precursors after their selection, and to the neurons that they generate in the larval and adult brain[42]. Loss of *ato* function does not lead to a proneural phenotype in the embryonic brain, but causes defects in axonal branching and arborization, indicating that *ato* functions later in the CNS than in the PNS[42]. Although *asc* genes and *ato* together account for the origin of much of the *Drosophila* PNS, a subtype of multidendritic neuron, and most olfactory sensilla, still develop in *asc*/*ato* double mutants[7]. The *ato*-related gene *amos* is the proneural gene for these remaining sensory organs[9,10]. By contrast, the third *ato* family member, *cato*, is expressed in the PNS after neural-precursor selection, similar to *ase* in the *asc* family; and LOF analysis has shown that *cato* is not involved in the generation of sense organs, but in their differentiation, as shown by the widespread defects in neuronal morphology that are observed in mutants[8]. Interestingly, GOF studies have revealed important differences between *ato* and *asc* genes, and among *ato*-family members, in their capacity to induce particular types of sense organ when ectopically expressed[7,9,10,34]. This provides strong evidence that proneural genes of the *asc* and *ato* families are involved not only in promoting the selection of neural progenitors, but also in specifying their identity.

Proneural genes in vertebrates. Genetic analysis has revealed that vertebrate neural bHLH genes are functionally highly heterogeneous. Genes of the *asc* and Ngn families, and possibly members of the family of *ato* homologues, have a similar proneural function to that of their *Drosophila* counterparts, whereas other neural bHLH genes are involved in specifying neuronal fates or in neuronal differentiation, but have no proneural role. Mice that carry a null mutation in *Mash1* have severe defects in neurogenesis in the ventral telencephalon and the olfactory sensory epithelium[43–46], whereas *Ngn1* or *Ngn2* single-mutant mice lack complementary sets of cranial sensory ganglia, and *Ngn1/2* double mutants lack, in addition, spinal sensory ganglia and a large fraction of ventral spinal cord neurons[47–50]. These neurogenesis

Box 1 | Are proneural functions similar in invertebrates and vertebrates?

Proneural genes in organisms as different as fly and mouse have remarkably similar functions. In both species, proneural genes are needed for the selection of progenitors and for their commitment to differentiate along a particular lineage, an activity that is carried out, in part, by activating Notch signalling. However, there are important differences, both in the cellular context in which *Drosophila* and vertebrate proneural genes act, and in the types of decision that they make (see text for references). In *Drosophila*, proneural genes are first expressed in quiescent ectodermal cells that have both epidermal and neuronal potential. Proneural activity results in the selection of progenitors that are committed to a neural fate but remain multipotent, with sense organ progenitors giving rise to neurons, glia and other non-neuronal cell types, and some neuroblasts of the central nervous system also generating both neurons and glia. Progenitors of the peripheral and central nervous systems only begin to divide after proneural gene expression has subsided (see panel **a** of the figure). Loss of proneural gene function in *Drosophila* results in fate transformation of sense organs (green) into epidermal cells (orange), whereas ectopic expression of proneural genes results in the opposite phenotype (panel **b**). In vertebrates, by contrast, proneural genes are first expressed in neuroepithelial cells that are already specified for a neural fate and are self-renewing. Proneural activity results in the generation and delamination of progenitors that are restricted to the neuronal fate and have a limited mitotic potential. In some lineages at least, proneural genes are involved in the commitment of neural progenitors to the neuronal fate at the expense of a glial fate (panel **a**). In the mouse, loss of proneural activity results in a failure of neuroepithelial stem cells (yellow) to generate, by division, committed neuronal progenitors (green), and in the precocious generation of glial progenitors (blue). In the chick, the precocious expression of proneural genes leads to premature cell-division arrest of neuroepithelial cells and to their neuronal differentiation (panel **b**). So, the actual fates that are specified depend on the cellular context in which proneural proteins act, which differs between different organisms, as well as among different lineages of the same organism.



ROOF PLATE
The point of fusion of the neural folds, forming the dorsal-most part of the neural tube.

defects are associated with a loss of progenitor populations, and a failure to express the Notch ligands Delta and Serrate/Jagged, and to initiate Notch signalling (see below), similar to proneural phenotypes in *Drosophila*[43,44,46–49,51]. Interestingly, the loss of neuronal progenitors that results from mutations in *Mash1* and *Ngn2* is correlated in certain lineages (for example, in the cerebral cortex) with a premature generation of astrocytic progenitors, implicating Ngn and Mash genes both in the commitment of multipotent progenitors to a neuronal lineage and in the inhibition of a glial fate (FIGS 2 and 3; BOX 1). However, the role of bHLH genes in lineage selection and inhibition of glial fate is not strictly correlated with a proneural function, because *NeuroD* and *Math3*/*NeuroM*, two *NeuroD*-family genes with characteristics of differentiation genes (see below), have also been implicated in the neuronal-versus-glial cell-fate decision in defined CNS regions[52,53]. GOF analysis of Ngn genes, by ectopic expression in the surface ectoderm of *Xenopus* and zebrafish embryos, or in the neural tube of chick embryos, has shown that these genes can promote the generation of supernumerary neural progenitors, activate Notch signalling ectopically, drive progenitor cells out of division, and promote neuronal differentiation through activation of the differentiation genes *NeuroD* and *ath3* (REFS 35,54,55). So, as in *Drosophila*, ectopic expression and null mutations of vertebrate proneural genes produce opposite phenotypes.

The generation of mouse mutants for *Math1* and *Math5* has shown that these two *ato*-family genes are essential for the development of a small number of neuronal lineages in the CNS. *Math1*-mutant mice completely lack cerebellar granule cells, as well as hair cells in the inner ear and other non-neural cell types[56–58]. Ablation of *Math1* also results in the loss of the dorsal-most population of spinal interneurons (D1 interneurons) and in an increase in the number of adjacent ROOF PLATE cells, whereas ectopic expression of *Math1* in the neural tube induces D1 interneurons at the expense of other interneuron types, indicating a role for *Math1* in the specification of interneuron identity[58,59]. Interestingly, many *Math1*-dependent cell types belong to the proprioceptive sensory pathway, which is important for hearing and balance, revealing a striking conservation between *Math1* and *ato* at the functional level[20,58]. Similarly, *ato* function in the *Drosophila* visual system seems to have a parallel in vertebrates with the other orthologue, *ath5*. LOF mutation of *ath5* in the mouse and zebrafish results in the loss of most retinal ganglion cells (RGCs), and in a concomitant increase in other retinal cell types, implicating *ath5* in the specification of the RGC fate in multipotent retinal progenitors[60–62]. Conversely, in *Xenopus*, overexpression of *Xath5* in retinal progenitors promotes the differentiation of RGCs[63], and the chicken *Cath5* gene directly regulates the promoter for the β3-subunit of the neuronal acetylcholine receptor (β3 nAChR), an early marker of RGC differentiation[64].

Although the role of *Math1* and *Math5* in specifying the identity of neuronal lineages has been clearly established, direct evidence is still lacking that these genes are also required as proneural genes to generate these lineages[20]. There is no doubt that *ath1* has an intrinsic proneural activity, as shown in GOF experiments. The *Xath1* gene of *Xenopus* has the capacity to induce ectopic neuronal differentiation in non-neural ectoderm, although without inducing early neural markers, and ectopic expression of *Math1* in the chick neural tube leads to precocious differentiation of neuroepithelial cells[59,65]. Moreover, *Math1* can induce ectopic chordotonal organs in *Drosophila*, and can partially rescue proneural defects in *ato*-null flies[66]. However, there is no

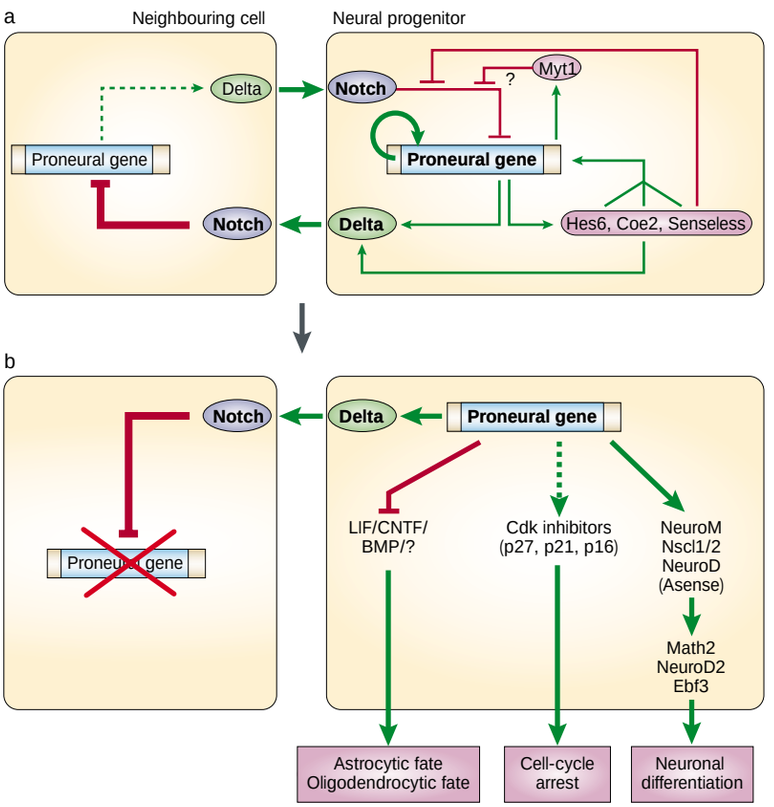


Figure 2 | **Regulatory pathways controlled by proneural genes in neuronal commitment. a** | Groups of ectodermal cells, called 'proneural clusters' in *Drosophila*, initially express a proneural gene and the Notch ligand Delta at similar levels. Through lateral inhibition, a regulatory loop takes place between the cells, involving the upregulation of *Delta* expression by the proneural gene and downregulation of proneural gene expression by the Notch signalling pathway. As a result, a slightly elevated level of proneural gene activity in one cell, the future neural progenitor, leads to the repression of proneural expression in neighbouring cells, and to a further increase in proneural gene expression in the same cell. Further regulatory loops that are controlled by proneural genes are required to accumulate high levels of proneural protein in the future progenitor (see text). **b** | Proneural gene expression is induced at a high level in the neural progenitor, where it initiates a programme that leads to neuronal differentiation. In neighbouring cells, Notch signalling both represses the expression and inhibits the activity of proneural genes, resulting in a block in differentiation. Proneural genes induce the expression of several helix–loop–helix genes that are implicated in differentiation steps, such as *asense* in *Drosophila*, and members of the *NeuroD* family and *Ebf3* (early B-cell factor 3) in vertebrates. In parallel, vertebrate proneural genes also inhibit glial differentiation by blocking gliogenic signals by different mechanisms from those that promote the neuronal fate[112]. In addition, vertebrate proneural genes promote cell-cycle exit by inducing the expression of cyclin-dependent kinase (Cdk) inhibitors, possibly by an indirect mechanism that involves the activation of a neuronal-differentiation gene such as *NeuroD*.

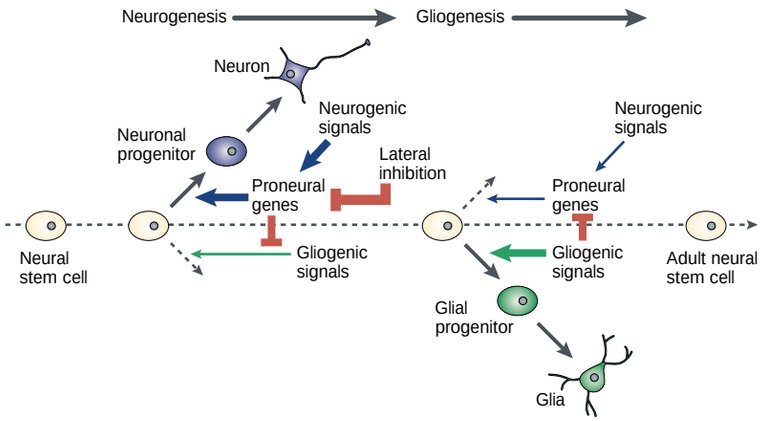


Figure 3 | **A model of the role of vertebrate proneural genes during the neurogenic and gliogenic phases of neural development.** Neural stem cells are multipotent and can generate all neural cell types; that is, neurons, astrocytes and oligodendrocytes. Generally, neural stem cells first generate neurons, and later produce glia, and the switch from neurogenesis to gliogenesis is the result of changes in stem-cell properties that are controlled by both extrinsic and intrinsic cues[106,146]. Proneural genes are intrinsic determinants that are likely to be part of the switch mechanism from neurogenesis to gliogenesis. The figure focuses on the interactions between these genes and extrinsic cues that promote either neuronal or glial fates. During the first period of differentiation, neurogenic signals induce proneural gene expression. Bone morphogenetic protein 2 (BMP2) and erythropoietin (Epo) have been shown to induce or upregulate the expression of *Mash1* in the embryonic peripheral nervous system and the adult central nervous system, respectively[147,148]. Proneural proteins accumulate at a high level in a subset of progenitor cells, resulting in the activation of a neuronal-differentiation pathway, the inhibition of glial differentiation, and cell-cycle arrest (FIG. 2). At the same time, through a process of lateral inhibition, Notch signalling downregulates and/or inhibits proneural genes in other cells that are thereby prevented from entering the neuronal pathway. So, the regulation of proneural genes is a key mechanism that maintains a balance between progenitors entering a neuronal-differentiation pathway, and progenitors remaining self-renewing and undifferentiated, and so available to produce other types of neuron or glial cell. Subsequently, gliogenesis is initiated by several developmentally regulated gliogenic signals, such as fibroblast growth factor 2 (FGF2), ciliary neurotrophic factor (CNTF) and BMPs[149]. These signals activate glial differentiation[113] and, in parallel, inhibit neurogenesis through various mechanisms, including activation of proneural inhibitors of the Id and Hes family[150], degradation of proneural protein[151], and possibly repression of proneural gene transcription. Notch signalling has been shown to have a gliogenic activity[152,153], and is likely to act, in part, by inhibiting proneural gene activity. Stem cells persist in the adult brain[106], but the potential role of proneural genes in their differentiation has not yet been addressed. So, evidence is accumulating that proneural proteins, which integrate both neurogenic and gliogenic signals, and regulate neurogenesis and gliogenesis, are essential components of an intrinsic function that computes the response of neural stem cells to a changing environment[154].

evidence, at present, that progenitors of the lineages specified by *Math1* or *Math5* are missing in LOF mutants, as is observed in Ngn or *Mash1* mutants. So, it remains to be seen whether *Math1* and *Math5* normally function as proneural genes to select progenitor cells from a pool of neuroepithelial stem cells and to drive their differentiation.

Mutational analysis in the mouse has so far established a clear proneural activity for only a few genes, namely *Mash1*, *Ngn1* and *Ngn2*, and possibly *Math1* and *Math5*. Are these genes sufficient to account for the selection of all neural progenitors? In the PNS, Ngns are involved in the generation of all cranial and spinal sensory progenitors[47–49], but *Mash1* seems to be dispensable for the generation of neuronal progenitors in sympathetic ganglia[67], implying that another, as yet unidentified gene with proneural activity might exist in this lineage. In the spinal cord, *Mash1*, *Ngn1* and *Ngn2* are expressed in most progenitors, except in two domains that are located at the ventral and dorsal ends of the neural tube, where *Ngn3* and *Math1* are expressed, respectively[59]. Although the role of proneural genes has not yet been systematically examined in most regions of the brain, *Mash1*, *Ngn1* and *Ngn2* are co-expressed in the dorsal telencephalon, and the three genes could together account for the generation of all progenitors of the cerebral cortex[51,68]. By contrast, *Mash1* is the only known proneural gene to be expressed in the ventral telencephalon, and although a large fraction of progenitors is missing in this region in *Mash1* mutants, other progenitors persist and differentiate normally[43,46], indicating that, in the CNS as well, other genes with proneural activity remain to be identified.

Interestingly, the comparison between the GOF phenotypes of *asc*-related and Ngn genes has pointed to divergences in the core proneural activities of these two gene families that were not apparent in LOF studies. Ectopic expression of Ngns leads to a rapid cell-cycle withdrawal and a highly efficient differentiation of progenitor cells. By contrast, the ectopic expression of *asc*-family genes in non-neural ectoderm is less efficient at promoting proliferation arrest and neuronal differentiation, although *Mash1* has the same capacity as Ngns to drive the differentiation of neuroepithelial cells[35,55,69–71]. This divergence in activities has been interpreted as reflecting a greater sensitivity to Notch-mediated inhibition of differentiation for *asc*-family genes than for Ngn genes[70,72]. It could underlie important differences in the way that the selection of progenitors is coupled with their proliferation and differentiation in different lineages, resulting, for example, in greater or lesser expansion of progenitor populations, depending on which proneural gene is active in a given lineage.

Mechanisms of proneural activity

In recent years, several regulatory genes and pathways that are controlled by proneural genes and involved in the progression of neural lineages have been identified, revealing some of the mechanisms that underlie proneural function.

Notch signalling. An essential role of proneural proteins is to restrict their own activity to single progenitor cells. Proneural genes inhibit their own expression in adjacent cells, thereby preventing these cells from differentiating. This is achieved through activation of the Notch signalling pathway, in a process termed 'lateral inhibition', which is initiated by the induction of a Notch ligand (FIG. 2a). Expression of the ligand in the future progenitors activates the Notch signalling cascade in neighbouring cells, resulting in the expression of repressors — Espl genes in *Drosophila*, and their homologues Hes/Her/Esr in vertebrates — that, in turn, directly downregulate proneural gene expression. In invertebrates and vertebrates, proneural genes are initially expressed in groups of equivalent neurectodermal cells[7,11,54,55,73]. Through lateral inhibition, this initial pattern is refined and

proneural gene expression is restricted to single cells that enter a neural-differentiation pathway[14,72,74]. This process must be reiterated, because Notch activity is transient, whereas the expression of proneural genes persists in the neurectoderm throughout the period of neurogenesis. This allows the transformation of a spatial pattern of proneural gene expression in groups of epithelial cells into a collection of individual progenitors that are generated over a protracted period of neurogenesis and, as a result, adopt distinct fates[74,75]. Key to the process of lateral inhibition in *Drosophila* is the direct and dose-dependent transcriptional activation of the Notch receptor ligand Delta by proneural genes[76–78], and a similar regulation of the ligands Delta and Serrate/Jagged by *Mash1* and Ngns takes place during vertebrate neurogenesis[43,44,47,48,72].

Positive-feedback loops. The control of progenitor fate commitment by proneural genes can be viewed as a two-step process: an initial, reversible phase of selection of progenitors, when proneural genes are only expressed and/or active at a low level, and progenitors are not yet committed to differentiation; and a later phase of irreversible commitment of progenitors to differentiation, when proneural genes have reached a high level of expression or activity[37,79–81] (FIG. 2). Notch signalling is involved in the initial upregulation of proneural gene expression, but other positive-feedback mechanisms are required to increase and/or maintain the levels of proneural gene expression in the selected neural progenitors. In particular, transcription factors such as the *Drosophila* ZINC FINGER protein Senseless[82], and the vertebrate HLH proteins Xcoe2 (REF. 83) and Hes6 (REFS 84,85), are induced by proneural genes and can, in turn, upregulate proneural gene expression, as shown in ectopic-expression experiments. *Senseless* acts, in part, by inhibiting the Notch signalling pathway in selected progenitors through the repression of Espl genes[82], whereas *Hes6* interferes at a post-transcriptional level with the inhibitory activity of the bHLH factor Hes1 on *Mash1* transcription and function[84]. The zinc-finger protein Myt1 (myelin transcription factor 1) is also induced by proneural genes, and it promotes neuronal differentiation when expressed ectopically in a *Xenopus* embryo assay[86]. Myt1 acts by an unknown mechanism to confer insensitivity to lateral inhibition to the selected progenitors.

Autoregulation of proneural genes in *Drosophila* also has a role in the accumulation of proneural proteins in neural progenitors[87,88]. In vertebrates, autoregulation has been shown for the *ato*-family gene *Math1* (REF. 89), but it does not seem to have a role in the regulation of *Mash1* or Ngn genes[43,68]. Accumulation of Scute protein in sensory precursors depends on the activity of a specific enhancer in the *sc* gene — the SMC enhancer — which contains functional E-boxes and mediates autoregulation[79]. Epidermal growth factor (EGF) signalling, which is activated by *asc* genes through the induction of *rhomboid* (*rho*), also contributes to the autoregulation of proneural genes, acting at the level of the SMC enhancer to promote Scute protein accumulation in sensory precursors[90].

Cascades of neuronal-differentiation genes. The expression of proneural genes in individual neural progenitors is transient. In the vertebrate neural tube, proneural genes are downregulated before progenitor cells exit the proliferative zone and begin to differentiate[55,91,92]; and in *Drosophila*, they are downregulated before progenitors begin to divide and generate sensory organs in the PNS and ganglion mother cells in the CNS[7,93,94]. So, the ability of proneural genes to promote full neuronal differentiation must rely on the induction of downstream regulatory genes that implement neuronal-differentiation programmes. Many of the neuronal-differentiation genes are structurally related to proneural genes, leading to the idea that, in the nervous system, as in muscles, distinct bHLH genes acting in cascades underlie the sequential steps of cell determination and differentiation[18,80,95,96]. bHLH genes that can drive neuronal differentiation when ectopically expressed, but are expressed later than proneural genes and are under their transcriptional control, have been identified in both *Drosophila* and vertebrates. One example is the *Drosophila asc* gene *ase*, a direct transcriptional target of *ac* and *sc* that is expressed in most neuronal precursors in the CNS and PNS[38]. This expression pattern indicates that *ase* has a generic role in neuronal differentiation, an idea that is supported by the differentiation defects observed in external sense organs in the absence of *ase* function[39].

In vertebrates, bHLH genes of the *NeuroD* family also have characteristics of differentiation genes, such as their expression in immature neurons rather than in neuroepithelial cells, their capacity to promote neuronal differentiation when ectopically expressed[97,98], and their LOF phenotypes. *NeuroD* is required for the proliferation, differentiation and survival of granule cells in the cerebellum and hippocampus[99,100], and mutation of *Math2*/*Nex1* or *NeuroD2* in the mouse also results in defects in the differentiation and survival of cerebellar and hippocampal neurons[101,102], which are clearly distinct from the loss of progenitors observed in mice that lack *Mash1* or Ngn. GOF experiments in *Xenopus* embryos and LOF analysis in mice have unravelled the EPISTATIC relationships that exist between differentiation genes and proneural genes. For example, *Ngnr1* (a *Xenopus* Ngn gene), *Xath3* and *NeuroD* are expressed sequentially, and the ectopic expression of *Ngnr1* induces the expression of both *Xath3* and *NeuroD*, whereas *Xath3* and *NeuroD* can cross-activate each other, but do not induce *Ngnr1* expression[55,103]. Similar results have been obtained in the mouse, where *Ngn1* or *Ngn2* is required for the expression of *Math3* and *NeuroD* in cranial sensory neurons[47,48], and *Mash1* acts upstream of *Ngn1* and *NeuroD* in the olfactory sensory epithelium[44]. So, *NeuroD*-family genes act downstream of vertebrate proneural genes in a manner very similar to *ase* and *cato* in *Drosophila* neurogenesis. Activation of *NeuroD* by Ngns is likely to be direct[104], as is the activation of *ase* by *ac* and *sc*[38].

ZINC FINGER
A protein module in which cysteine or cysteine–histidine residues coordinate a zinc ion. Zinc fingers are often used in DNA recognition and in protein–protein interactions.

EPISTASIS
When one gene masks the expression of another. If mutant *a* gives phenotype A and mutant *b* gives phenotype B, and if the double mutant *ab* gives phenotype A and not B, then gene *a* is epistatic to gene *b*.

The finding that proneural genes and neuronal-differentiation genes are structurally related raises the question of whether their distinct functions are solely the consequence of being expressed at different stages in neural lineages, or whether there are also differences in their intrinsic biochemical properties. Although this issue has not yet been directly addressed, several instances have been reported in which a proneural gene controls a differentiation step in a neuronal lineage, such as for *Mash1* in the sympathetic lineage[67], *Ngn1* in the olfactory epithelium[44], or *ato* in the brain[42], indicating that proneural genes have a dual capacity to promote the selection of progenitors and to regulate differentiation steps. In muscles, the specificity of myogenic bHLH genes for either the determination or the differentiation of myoblasts has been directly addressed by a gene-swapping experiment in mice, which showed that the differentiation gene myogenin (*Myog*) is less efficient than the determination gene *Myf5* (myogenic factor 5) at remodelling chromatin and activating transcription at previously silent loci[105]. So, it is likely that differences in both temporal expression patterns and intrinsic molecular properties account for the distinct developmental roles of early-expressed determination and late-expressed differentiation bHLH genes.

Inhibition of glial fates. Neurons and glia are generated from common multipotent progenitors in a temporally coordinated manner[106]. Vertebrate proneural genes have recently been shown to promote neuronal fates and to inhibit glial fates simultaneously, indicating that they have an important role in the switch mechanism from neurogenesis to gliogenesis[107] (FIG. 3). In mice that are doubly mutant for *Mash1* and *Math3*, or for *Mash1* and *Ngn2*, there is a block in neuronal differentiation and a compensatory premature differentiation of astrocytes in different brain regions[53,68]. Conversely, misexpression in the retina of different neural bHLH genes is sufficient to both induce neuronal differentiation and inhibit the differentiation of Müller glia[108,109]. The role of proneural genes in controlling the neuronal-versus-glial fate decision of multipotent progenitors appears to extend to the oligodendrocyte lineage, as *Ngn2* has been shown to repress oligodendrocyte specification in the spinal cord. The downregulation of *Ngn2* expression has been proposed to be a key step in the transition between the phases of neurogenesis and oligodendrogenesis in the ventral spinal cord[110,111].

Commitment to the neuronal fate by proneural genes is explained, at least in part, by the activation of the cascade of bHLH differentiation genes discussed above, but the mechanisms that are involved in the inhibition of glial fates are less clear. A recent study has proposed an unexpected mechanism to account for the inhibition by *Ngn1* of astrocytic differentiation in the cerebral cortex[112]. The gliogenic factors BMP (bone morphogenetic protein) and CNTF (ciliary neurotrophic factor) synergistically stimulate transcription of the astrocyte marker GFAP (glial fibrillary acidic protein), by inducing formation of a complex that includes the Stat1 (signal transducer and activator of transcription 1), Stat3 and

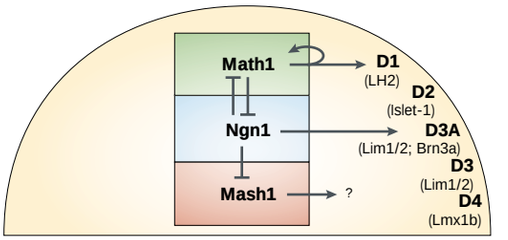


Figure 4 | **bHLH proteins in the dorsal spinal cord.** Regulatory relationships between basic helix–loop–helix (bHLH) proteins and neuronal populations in the dorsal spinal cord (adapted, with permission, from REF. 59 © 2001 Elsevier Science). Different neural bHLH proteins, expressed in distinct dorsoventral progenitor domains of the dorsal spinal cord, control the specification of different interneuron subtypes. The domains of neural bHLH gene expression are established and/or maintained by cross-repression[51,59].

Smad1 (mothers against decapentaplegic, homologue 1) transcription factors and the general co-activators p300/CBP[113]. Ngn1 has been shown to interfere with transduction of these glial-differentiation pathways, first by associating directly with p300/CBP–Smad1 complexes and interfering with the formation of complexes that include Stat1/3, and second by preventing the phosphorylation of Stat1/3 (REF. 112). Formation of Ngn1–p300/CBP complexes might be important not only to prevent glial gene transcription, but also to potentiate the transcription of *Ngn1*-responsive neuronal genes[37,112]. This indicates that, as in the haematopoietic system, lineage commitment of neural progenitors involves the suppression of alternative fates[114].

It is interesting to note that the expression of *Mash1* and *Ngn2* has been reported in precursors of the oligodendrocytic and astrocytic lineages, respectively[68,115,116]. These expression data indicate that, in the context of restricted glial progenitors, proneural genes might have functions that are distinct from their better-characterized role in lineage specification, perhaps in the differentiation of glial lineages.

Cell-cycle regulation. Differentiation in the nervous system is tightly coupled with cell-cycle withdrawal. The crucial step of cell-division arrest has been proposed as a mechanism to insulate already specified progenitor cells from the influence of extrinsic fate-determining cues[117]. Numerous lines of evidence indicate that proneural genes not only determine the neuronal fate of progenitors, but also promote the arrest of their division, and are therefore involved in coupling these two crucial processes[118]. For example, overexpression of *Ngn2* in the chick neural tube results both in premature cell-cycle exit and premature neuronal differentiation in neuroepithelial cells[35,36]. Similarly, transient expression of certain neural bHLH genes in the P19 cell line promotes cell-cycle exit as well as neuronal differentiation[97]. Regulation of the cell cycle by vertebrate proneural genes is likely to involve the activation of cyclin-dependent kinase (Cdk) inhibitors, such as p16, p21 and p27. Expression of neural bHLH genes in P19 cells induces p27 (REF. 97) (FIG. 2). Direct activation of Cdk-inhibitor genes might take place in some lineages at

the level of neuronal-differentiation genes, as *NeuroD* can activate the *p21* gene promoter in HeLa cells, and elevated *p21* expression and ectopic mitosis are observed in the enteric epithelium of *NeuroD*-knockout mice[119]. *Drosophila* proneural genes are expressed mainly in non-dividing cells, but there are instances in which *asc* genes have been shown to inhibit cell-cycle progression. In the developing wing, *ac* and *sc* downregulate the expression of the phosphatase *string* (Cdc25), which is involved in promoting the G2–M transition[120]. E-boxes have been found 5′ of the gene, so *asc* genes could directly regulate *string*[120]. In the optic lobes, *ase* participates in controlling the expression of *Dacapo*, a fly homologue of the vertebrate Cdk inhibitor p27 (REF. 121).

Neuronal-subtype specification

Functional studies of proneural proteins in *Drosophila* and vertebrates initially focused on their common proneural functions. However, the structural diversity of these proteins (FIG. 1b) has raised the possibility that they have further and divergent roles in neural development. Proneural genes are often expressed in restricted progenitor domains that correlate with the production of particular types of neuron, so they could be involved in the specification of neuronal-subtype characteristics. A clear example of such a correlation is provided by the dorsal embryonic spinal cord, in which *Math1*, *Ngn1* and *Mash1* are expressed in discrete dorsoventral progenitor domains that produce distinct types of interneuron[59] (FIG. 4). Indeed, mutational analysis in the mouse has shown that the activities of *Math1* and *Ngn1* are essential for the correct specification of these progenitor populations, thereby linking proneural genes with the acquisition of a specific neuronal fate[58,59].

Sense organ/neuronal-type specification in Drosophila. The first direct evidence that proneural genes have a role in the specification of neuronal identity was obtained in *Drosophila*, in which LOF studies had shown that different proneural genes are involved in the development of different types of sense organ[7,41,104]. Misexpression experiments showed that such phenotypes are not simply a consequence of distinct expression patterns, but also due to divergent activities of proneural genes[7,34,104,122]. Misexpression of *asc* genes in imaginal discs induces ectopic external sense organs, whereas *ato* and *amos* efficiently generate chordotonal organs, and *amos* also has a unique ability to generate multidendritic neurons. So, proneural genes have an active role in the induction of sense-organ-subtype characteristics. A similar activity has been shown in the CNS for the specification of MP2 neurons. The generation of these neurons depends on *ac* and *sc* activity, and although other neural bHLH genes (including *lsc*, *ase* and *ato*) can rescue the delamination of MP2 precursor cells in an *ac*/*sc*-null mutant background, the correct specification of MP2 neurons can only be obtained by the expression of *ac* or *sc*[123,124].

These results indicate that different proneural proteins must regulate different target genes that are involved in specifying the fate of neurons or sense organs. One such gene is *cut*, a HOMEOBOX gene that is specifically expressed in precursors of external sensory organs, where it controls the binary decision between external sense organ and chordotonal organ fates[125]. Genetic data indicate that *asc* genes induce *cut* expression, whereas *ato* represses the activation of *cut*[122,125]. So, the ability of *ato* and *asc* genes to convey different sense-organ-subtype information is, in part, a consequence of their differing abilities to regulate *cut* expression.

How is specificity in the regulation of target genes achieved? Different DNA-binding specificities alone might not account for the functional specificity of proneural factors, which might also depend on physical interactions with further factors, as indicated by experiments involving misexpression of chimeric

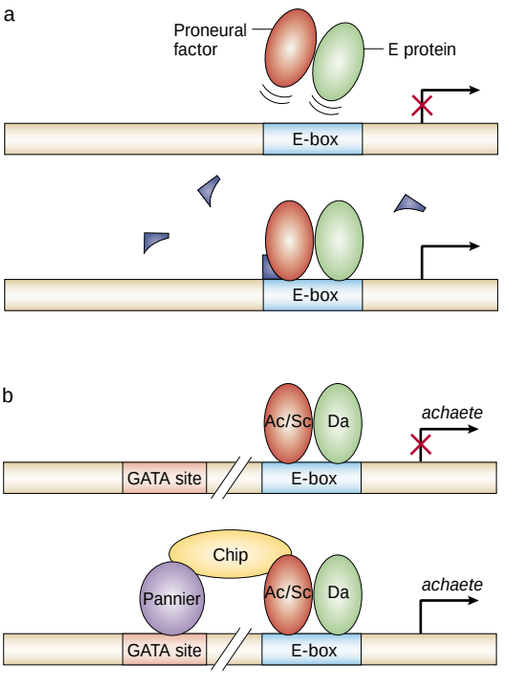


Figure 5 | **Models of interactions of proneural proteins with cofactors that confer functional specificity.** **a** | Functional specificities among proneural proteins. The functional specificities of the *Drosophila* proteins Scute (Sc) and Atonal, which are proneural factors for external sense organs and chordotonal organs, respectively, reside in residues that are located in the basic domain[34]. Residues that differ between the basic regions of Scute and Atonal are predicted not to directly contact the DNA, but to be involved in interactions with cofactors. In this model, a cofactor interacts with both the basic motif of the proneural protein and the DNA sequence, and provides the proneural protein with specificity for binding to a particular E-box sequence (FIG. 1d). Cofactors might also control the transcriptional activity of proneural proteins by interacting with the transcriptional machinery (not shown). **b** | Region-specific activity of a proneural protein. To activate the *achaete* promoter in a region-specific manner, the heterodimer that is formed by Achaete (Ac) or Scute and Daughterless (Da) requires an interaction with the GATA factor Pannier, which is bound to an upstream enhancer element by the bridging factor Chip[128]. Regional expression of Pannier thereby spatially restricts the activity of the Achaete/Scute–Daughterless heterodimer on this promoter.

HOMEOBOX
A sequence of about 180 base pairs that encodes a DNA-binding protein sequence known as the homeodomain. The 60-amino-acid homeodomain comprises three α-helices.

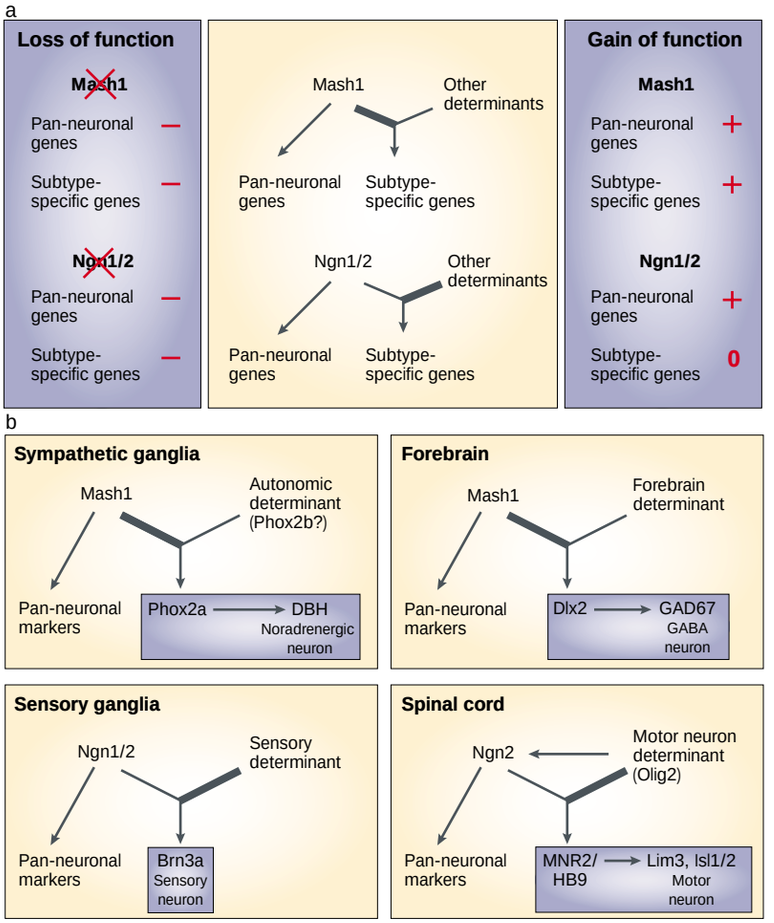


Figure 6 | **Context-dependent activity of Mash1 and the neurogenins. a** | Comparison of the loss-of-function (LOF) and gain-of-function (GOF) phenotypes of *Mash1* and neurogenins (Ngns). *Mash1* and *Ngn1/2* activate both generic and subtype-specific neuronal-differentiation programmes. LOF mutations in these genes result in a failure to activate both programmes. Reciprocally, ectopic expression of *Mash1* and *Ngn1/2* activates a generic neuronal-differentiation programme, but only *Mash1* has the capacity to override endogenous subtype-specification programmes and ectopically activate subtype-specific gene expression (see text for references). The greater dependence on cellular context of Ngn activity compared with *Mash1* might be due to a greater dependence on locally expressed cofactors. Alternatively, it might reflect interactions of *Ngn1/2* and *Mash1* with cofactors that have different distributions: cell-type-specific cofactors for *Ngn1/2* and regionally expressed cofactors for *Mash1*. **b** | Models of interactions of proneural proteins with different co-determinants. *Mash1* has an instructive role in specifying an autonomic fate in the peripheral nervous system (PNS) and a GABA (γ-aminobutyric acid) neuronal fate in the forebrain, probably through activation of the homeobox genes *Phox2a* and *Dlx1/2*, respectively. The regulation of different genes in different regions of the embryo implies that the target-gene specificity of *Mash1* is modulated by regional co-determinants. *Phox2b*, which is required together with *Mash1* to regulate *Phox2a* in sympathetic precursors, is a candidate autonomic cofactor. *Ngn2* contributes to the specification of a sensory neuron fate in the PNS, and to the specification of the motor neuron fate in the ventral spinal cord. *Ngn2* requires interactions with neuronal-subtype co-determinants to specify neuronal identities. For example *Olig2*, a determinant of somatic motor neuron fate, interacts with *Ngn2* to induce a motor neuron programme (see main text for references).

proneural proteins in the *Drosophila* PNS. A chimeric protein that is generated by replacing the basic domain of Scute with that of Atonal can induce ectopic chordotonal neurons and rescue *ato*-null mutants[34]. So, the basic domain of Atonal encodes important information for the specification of the chordotonal fate, reminiscent of the role of the basic region of the bHLH protein MyoD in conferring specificity for the transactivation of muscle-specific genes[126]. Importantly, the residues that differ between the basic domains of Atonal and Scute are located opposite the surface that contacts DNA, indicating that they form an interface for interactions with cofactors[34]. Such cofactors could affect the interaction of proneural proteins with their DNA-binding sites and/or modulate their transcriptional activity. Sequence comparisons of E-boxes in the promoters of target genes indicate that different neural bHLH proteins use different E-box sequences (FIG. 1d), confirming that functional specificity could be controlled, in part, at the level of DNA binding, and that cofactors might have an important role in regulating this property of proneural proteins (FIG. 5a). The structural basis for the specificity of proneural proteins is unlikely to reside solely in their basic domain; for example, Amos and Atonal differ only by a single amino acid in their basic DNA-binding domain, but have different specification properties when they are ectopically expressed[104].

A further level of complexity results from the fact that proneural factors elicit different biological responses when expressed in different cellular contexts. For example, *ato* can promote the formation of chordotonal organs, photoreceptors or olfactory sense organs, depending on the IMAGINAL DISC in which it is expressed. Its role in the specification of olfactory organs does not require the regulation of *cut*, so there is regional specificity in the regulation of target genes by *ato*[127]. A recent study has provided evidence that direct interaction with a regionally expressed transcription factor can underlie the region-specific activation of a target gene by a proneural protein[128]. Transcription from the *ac* promoter in a particular region of the thorax was shown to require the formation of a complex that comprised an Achaete/Scute–Daughterless heterodimer bound to an E-box in the proximal promoter, along with the GATA factor Pannier bound to an upstream enhancer element, and the bridging factor Chip associated with the proneural bHLH domain (FIG. 5b). So, functional interaction between a promoter that contains E-boxes and enhancers that bind regional cofactors such as Pannier is a potential mechanism to confer regional specificity on the transcriptional activity of proneural proteins.

IMAGINAL DISC
A single-cell-layer epithelial structure of the *Drosophila* larva that gives rise to wings, legs and other appendages.

Box 2 | Subtype specification by Olig genes

Among the large family of neural basic helix–loop–helix (bHLH) factors, many proteins that are devoid of proneural function have, nevertheless, been implicated in the specification of various neural cell types. Work carried out on the recently identified Olig genes[139–142] (FIG. 1a) has led to exciting findings on how bHLH factors act in a combinatorial manner with other cell-type determinants to specify diverse subtype identities. Initial observations indicated that *Olig1* and *Olig2* genes, which are both expressed in oligodendrocyte progenitors, have a role in the specification of this glial lineage. This hypothesis was later supported by the observation that ectopic expression of *Olig2* induces oligodendrocyte markers[111,143], and by recent loss-of-function studies that show that *Olig1* and *Olig2* are required for the generation and maturation of oligodendrocytes in the brain and the spinal cord, respectively[144,145]. However, *Olig1/2* expression is not restricted to oligodendrocyte progenitors, and in the ventral spinal cord, for example, these genes are expressed in a progenitor population that first generates motor neurons, and only later produces oligodendrocytes. Moreover, recent gain- and loss-of-function data have shown that *Olig2* serves as a key determinant of somatic motor neuron identity[35,36,144,145], in cooperation with *Ngn2* (REFS 35,36). The switch from a programme of motor neuron differentiation to one of oligodendrocyte differentiation in *Olig2*-expressing progenitor cells seems to involve the downregulation of *Ngn2* (REF. 111). So, these data indicate that Olig genes act in a combinatorial manner with proneural genes to specify both neuronal and glial cell identities.

Neuronal-subtype specification in vertebrates. Vertebrate proneural genes have also been implicated in the specification of neuronal subtypes, coupling the selection of progenitor cells with the specification of their identity. The role of *Mash1* in the specification of noradrenergic neurons is probably the best-studied case[129]. In the PNS, *Mash1* expression is restricted to precursors of sympathetic, parasympathetic and enteric neurons, which all share a noradrenergic neurotransmitter phenotype, and LOF and GOF analysis has shown that Mash1 acts in sympathetic ganglia in a combinatorial manner with a determinant of the noradrenergic phenotype, the homeodomain protein Phox2b, to induce the expression of the related homeobox gene *Phox2a* and of the noradrenaline-synthesizing enzyme dopamine β-hydroxylase (DBH)[130–133]. *Mash1* is also the main noradrenergic determinant in noradrenergic centres of the brain, such as the locus coeruleus, where it induces the expression of both *Phox2a* and *Phox2b*[129,134] (FIG. 6b).

GOF studies have strengthened the case for *Mash1* being an instructive determinant of neuronal-subtype identity, by showing that it can override endogenous differentiation programmes and re-specify progenitor identity when ectopically expressed in the CNS (FIG. 6a). Interestingly, these experiments have implicated *Mash1* in the specification of further neuronal subtypes. For example, it is expressed in ventral domains of the forebrain that produce mainly neurons that use GABA (γ-aminobutyric acid) as a neurotransmitter; and forced expression in the dorsal-forebrain domains induces ectopic expression of markers of GABA neurons, revealing a role for *Mash1* in the specification of this neuronal phenotype[51,135]. Significantly, in the differentiation pathways of both noradrenergic and GABA neurons, *Mash1* seems to act by inducing the expression of a homeobox gene, indicating that transcriptional cascades in which a proneural factor regulates a cell-fate determinant with a homeodomain motif might be a common strategy in the generation of different subtype identities (FIG. 6b). The implication of *Mash1* in the specification of multiple neuronal subtypes indicates that it must cooperate with regionally expressed determinants that modify its specificity, similar to the interaction of Achaete/Scute with Pannier (FIGS 5b and 6). The identification of these factors will be of crucial importance for understanding the contribution of proneural genes to the generation of cell diversity in the nervous system.

Recent reports have also attributed subtype-specification functions to Ngn genes. In the PNS, Ngns are likely to have a role in the specification of sensory neurons, as misexpression of *Ngn1* in chick embryos results in the induction of several sensory markers[136], and expression of *Ngn1* or *Ngn2* in dissociated neural tube cultures induces sensory neurons more efficiently than it promotes neuronal differentiation[70]. The capacity of Ngns to induce sensory neurons is, however, strictly constrained by the local concentration of the extrinsic signal BMP2, and ectopic-expression experiments in the CNS have provided further evidence that the cellular context dictates the specificity of Ngns for particular subtypes. In particular, ectopic expression of *Ngn2* alone is not sufficient to override endogenous differentiation programmes and re-specify progenitors, in clear contrast to results obtained with *Mash1* (REFS 35,135) (FIG. 6a). The context dependence of *Ngn2* is also clearly illustrated by the fact that *Ngn2* needs to cooperate with a determinant of motor neuron fate, *Olig2*, to induce motor neurons at ectopic positions in the spinal cord[35,36]. Nevertheless, *Ngn2* has a specific role in motor neuron induction, as shown by the fact that *Mash1* cannot replace *Ngn2* for the ectopic induction of motor neurons with *Olig2* (REF. 35). Altogether, these experiments indicate that, more than *Mash1*, Ngns are dependent on interactions with other neuronal-fate determinants to specify particular neuronal subtypes (FIG. 6). Interestingly, the expression of several homeodomain proteins in different types of spinal cord neuron is affected in *Ngn2*-null mutants, without overt defects in neuronal production[50]. This supports the idea that the regulation of homeodomain proteins that determine cell-fate functions is an important part of the role of proneural proteins in the specification of neuronal-subtype characteristics.

It is important to mention that bHLH genes that have no proneural function have also been implicated in the specification of neuronal identity (BOX 2). Detailed studies of the retina have, in particular, shown a role for differentiation genes of the *NeuroD* family[137]. For example, *NeuroD* and *Math3* have been shown to be both necessary and sufficient for the generation of amacrine interneurons[52,109], whereas *Math3* is involved with *Mash1* in the specification of the bipolar neuron fate[138]. Interestingly, bHLH genes need to work together with homeobox genes to specify retinal neuron fates. The induction of amacrine cells requires the co-expression of *NeuroD* or *Math3* with the homeobox gene *Chx10*, whereas bipolar cells are induced when *NeuroD* or *Math3* is co-expressed with *Pax6* (REFS 109,138). So, the specification of neuronal fates can be carried out by

non-proneural bHLH proteins, and is, in some cells, uncoupled from the selection of progenitors.

Conclusion

There is growing evidence that proneural genes have a vital role in specifying various aspects of the neuronal phenotype in vertebrates and *Drosophila*. Perhaps because proneural genes can interact with locally expressed determinants, they have a pivotal role in integrating positional information and the progression of neural lineages. It is possible that the structure of proneural proteins, in particular their bHLH domain, contributes to this central role in neurogenesis, as it might facilitate the establishment of functional interactions with multiple factors. Divergences in the core proneural activity of bHLH proteins could also contribute to the dedication of different proneural factors to different lineages. *Mash1* and Ngns are thought to differ in their sensitivity to Notch signalling, and thereby in their efficiency at initiating neuronal differentiation. Other differences in their proneural activities might also exist; for example, in cell-cycle regulation or in their capacity to suppress glial-differentiation programmes. Such differences might result in proneural genes coordinating not only the selection of neuronal progenitors, but also the expansion of the pool of progenitors or the timing of their differentiation, with the acquisition of a lineage identity. Future studies might reveal new roles for proneural genes, beyond their proneural function, which will help us to understand fully the logic behind the coupling of proneural and subtype-differentiation programmes.

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Acknowledgements
We are grateful to C. Goridis, P. Ramain, C. Schuurmans and U. Strahle for their critical comments on the manuscript, and to G. Gradwohl for his help with figure 1a.

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DATABASES
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ac | *amos* | *asc* | *ase* | *ato* | *cato* | Chip | *cut* | *da* | *Dacapo* | Delta | *emc* | *lsc* | *myc* | *MyoD* | Notch | Pannier | *rho* | *sc* | Senseless | *string*
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