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# Context dependence of proneural bHLH proteins

Lynn M Powell and Andrew P Jarman

A key point in neural development is the commitment of progenitor cells to a specific neural fate. In all animals studied, proneural proteins – transcription factors of the basic helix-loop-helix (bHLH) family – are central to this process. The function of these factors is strongly influenced by the spatial and temporal context in which they are expressed. It is important to understand the molecular mechanisms by which developmental context interacts with and modifies the intrinsic functions and properties of the proneural proteins. Recent insights have been obtained in *Drosophila* and vertebrates from analysis of how bHLH proteins interact with other transcription factors to regulate target genes.

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## Introduction

Neuronal diversity is acquired by various spatial and temporal mechanisms that pattern the events of neural development. The defining event of neurogenesis itself is the switch from uncommitted, cycling progenitor cell to committed neural precursor cell, which has a relatively restricted cell division potential before it or its daughter cells differentiate as neurons or glia. The details of this step differ in different organisms, but a consistent feature is the involvement of basic helix-loop-helix (bHLH) transcription factors. In *Drosophila*, proneural bHLH factors are initially expressed in ectodermal cells, giving the cells competence to undergo neural commitment, and are then upregulated upon neural commitment, at which point they also trigger Notch-mediated lateral inhibition to suppress the competence of surrounding cells. In vertebrates, the proneural function is also closely associated with committing cycling progenitor cells to a neuronal fate, which involves activation of Notch signalling, inducing cell-cycle exit, migration and terminal differentiation [1–4].

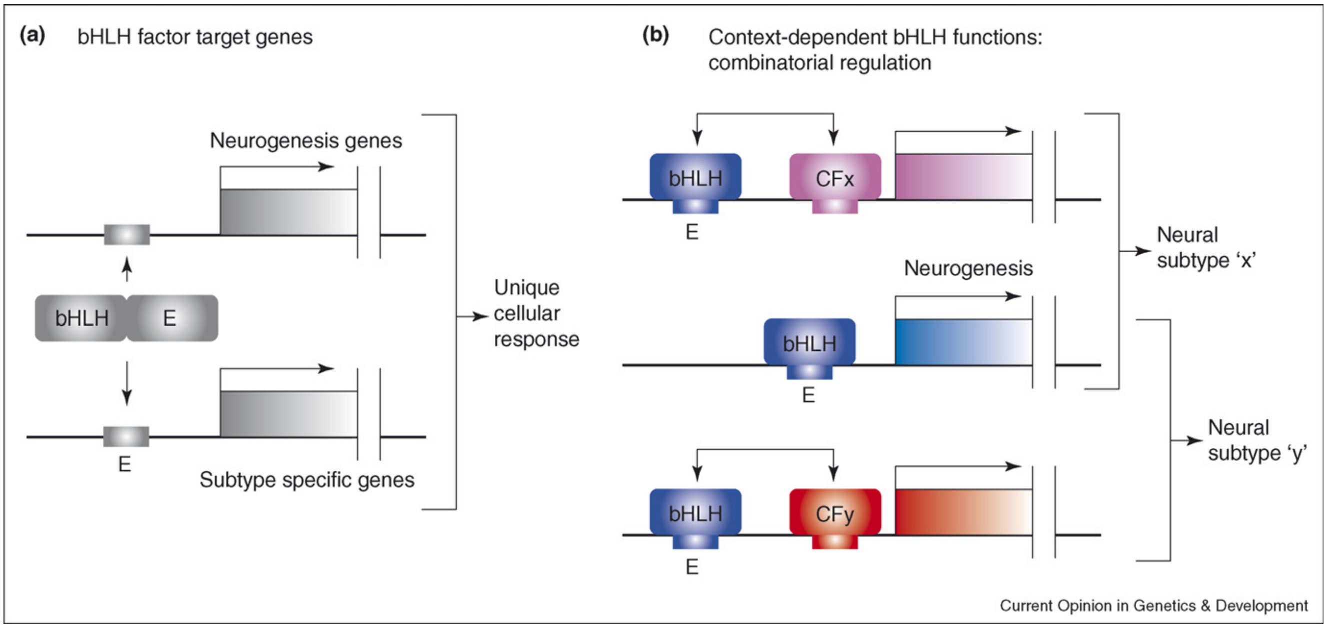
Proneural proteins are transcriptional activators that function as heterodimers with E proteins (Daughterless in *Drosophila* and typically E12 or E47 in vertebrates), which are generally more widely expressed and dimerise with multiple proneural factors. These bHLH heterodimers bind E box motifs (CANNTG) in the regulatory regions of their target genes (Figure 1a). A number of observations are of major importance with regard to proneural bHLH proteins, their regulation of specific target genes and how this relates to acquisition of neural diversity. Firstly, proneural proteins generally trigger neurogenesis, but specific proneural proteins are required for different neuronal and/or glial cell types, which implies that each bHLH protein regulates both common (shared) target genes for neurogenesis and unique target genes for neuronal subtype characteristics (Figure 1a). Thus, subtype specificity of bHLH factors underpins some of the cellular diversity in the nervous system [5–7]. Among other things, this has important implications for coaxing the production of specific neuronal subtypes from stem cells [8,9].

Secondly, and somewhat paradoxically, an individual bHLH protein is required for several different neural cell types at different times or locations in development (see Table 1 for recent findings). Additionally, some neural bHLH proteins even have important non-neural roles. Therefore, target gene specificity of a bHLH factor must be modulated by developmental context. A striking example is *Drosophila* Atonal, which is required for precursors of chordotonal proprioceptors in most of the body, olfactory receptors in the antenna and R8 photoreceptors in the eye [5,10]. In each location, Atonal function cannot be substituted by other proneural bHLH genes [7]. bHLH proteins must therefore have different intrinsic properties that endow them with different target gene specificities, but these properties are modified strongly according to the context. This review summarises recent research that begins to illuminate the molecular mechanisms by which context modifies the intrinsic functions and properties of neural bHLH factors.

## Combinatorial regulation—cofactors provide spatial/temporal context

One explanation of context dependence of bHLH function is that certain subtype-specific target genes also require input from other regionally expressed transcription factors (here referred to as cofactors). Thus, context-dependent specificity results from different combinations

**Figure 1**



**(a)** bHLH proteins regulate target gene sets by binding to E box motifs as heterodimers with an E protein (the latter generally not shown in subsequent figures). **(b)** A bHLH factor may regulate different subtype-specific targets in different places (or at different times) through combinatorial regulation with coexpressed transcription cofactors (CF). Solid arrows indicate protein interactions leading to synergistic regulation of the target.

of bHLH factors and cofactors whose expression overlap in different places or times [11,12•]. A long-standing theme is combinatorial control by bHLH and homeodomain (HD) proteins, and the mouse and *Xenopus* retinas provide good examples of this [13–15]. For instance, a combination of Mash1 and the HD factor Chx10 is required for bipolar cell fate [14]. Another well-studied example, recently extended by Sugimori *et al.* [16••], comes from the mouse ventral spinal cord [17,18]. Here a combinatorial code of patterning factors (Olig2, Pax6 and Nkx2.2) and proneural bHLH factors (Neurogenin (Ngn) and Mash1) is postulated to produce neurons, astrocytes and oligodendrocytes in different locations or times [2,16••].

In the *Drosophila* eye, genes of the Retinal Determination Gene Network (RDGN) (particularly *sine oculis* and the PAX6 homologues, *eyeless* and *twin of eyeless*) are prime candidates for providing the context for neurogenesis by Atonal [19]. It is not difficult to envisage that the RDGN not only activates Atonal expression for retinal neurogenesis [20,21] but also modulates Atonal target gene regulation in an eye-specific manner [22]. However, whilst interactions within the RDGN are well characterised, much less is known about how these patterning factors interact with Atonal. Analysis of newly identified eye-specific targets of these RDGN factors may be fruitful [23,24].

## Enhancers provide the molecular context for specific target gene regulation

Cellular context is achieved by the co-expression of bHLH factors and cofactors. Combinatorial control is widely expected to result from the co-occurrence of binding sites for the bHLH and cofactors in the enhancers of specific target genes, with different cofactors combining with a bHLH factor to regulate different targets (Figure 1b). Thus, enhancers act in their well-known role as integrators of diverse regulatory inputs. However, few examples are currently known in detail. One is the 'recruitment' enhancer of *atonal*, which is regulated by the combination of Atonal (for autoregulation) and the ETS factor, Pointed [25] (Figure 3a). The proteins bind cooperatively to adjacent binding sites in the enhancer. This combination of sites also occurs in the enhancer of another Atonal target—the *dacapo* gene, which encodes a cdk inhibitor [26]. Thus, a subset of Atonal targets only responds in the presence of Pointed, which itself is produced in response to receptor tyrosine kinase (RTK) signalling.

An analogous example in mouse is provided by the combinatorial regulation of *Delta1* by Mash1 and Brn factors, which act co-ordinately by binding cooperatively to adjacent E box and POU protein binding sites in the *DeltaM* enhancer [27••] (Figure 3b). The two binding sites form a characteristic motif that is also found in other likely

**Table 1**

**Some major functions of *Drosophila* and mouse proneural factors in the nervous system**

| | |
|---|---|
| *Drosophila* | |
| Atonal | Proprioceptors (chordotonal organs)<br>Olfactory receptors (sensilla coeloconica) [5]<br>Eye (R8 photoreceptor)<br>Subset of brain neurons (differentiation) |
| Achaete/Scute | External sense organs (tactile/chemosensory bristles)<br>CNS neuroblast subset |
| Amos | Olfactory receptors (sensilla basiconica and trichodea) [5]<br>Dbd neurons |
| Mouse | |
| Math1 (Atoh1) | Cerebellum (Granule Cells) [53]<br>Inner ear (sensory hair cells)<br>Mechanoreceptors (Merkel cells)<br>Dorsal spinal cord (commissural neurons) [28] |
| Ngn2 (Neurog2) | Ventral neural tube (motorneurons)<br>Forebrain (glutaminergic neurons)<br>Dentate gyrus [54]<br>Midbrain dopaminergic neurons [55]<br>Retinal ganglion cells [6] |
| Mash1 (Ascl1) | Dorsal neural tube (commissural neurons)<br>Neural crest (autonomic neurons)<br>Forebrain (GABAergic neurons) [56]<br>Forebrain (oligodendrocytes) [57,58]<br>Midbrain dopaminergic neurons [31] |

Only some of the most recent papers are shown here. Recent reviews on developmental roles of bHLH factors can be found in references [2,10].

downstream targets, including *Delta3*, *Insm1* (Zn finger factor involved in differentiation) and *Fbw7* (involved in cell-cycle arrest and Notch degradation). In both these examples, the cofactor interaction is required only for a subset of targets, supporting a model in which bHLH factors interact with different cofactors to activate different subprograms of neurogenesis (Figure 1b) [28].

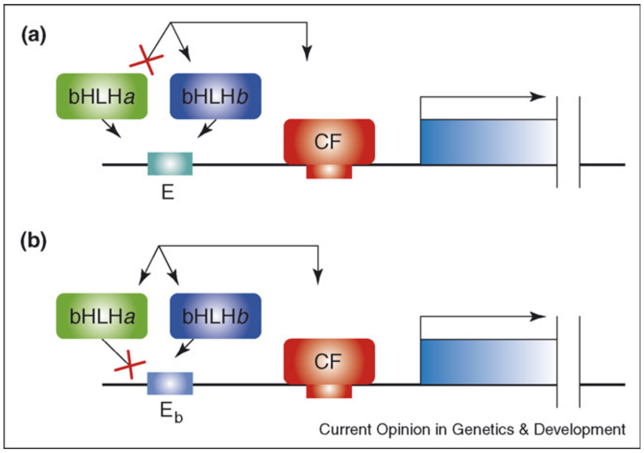
Another example of combinatorial regulation is the cooperation between Ngn2/NeuroM and the LIM-HD factors, Lhx3 and Isl1, to specify motor neurons in the chick neural tube. Part of their joint function is to activate the Hb9 motor neuron-specifying factor [18]. This is achieved via two E boxes and two LIM-HD sites in the *Hb9* motor neuron enhancer (Figure 3c). Interestingly, in this case the sites are not directly adjacent, and instead a bridging cofactor, NLI, mediates the interaction between the DNA binding factors. This arrangement mirrors a *Drosophila* interaction between Scute and the GATA factor, Pannier, which is bridged by the NLI homologue, Chip [29].

The structure of target enhancers therefore provides the important *molecular* context for bHLH protein function: collocation of binding sites within enhancers of targets determines whether a particular target gene responds to bHLH factors in a particular cellular context.

## bHLH selectivity—DNA or protein interactions?

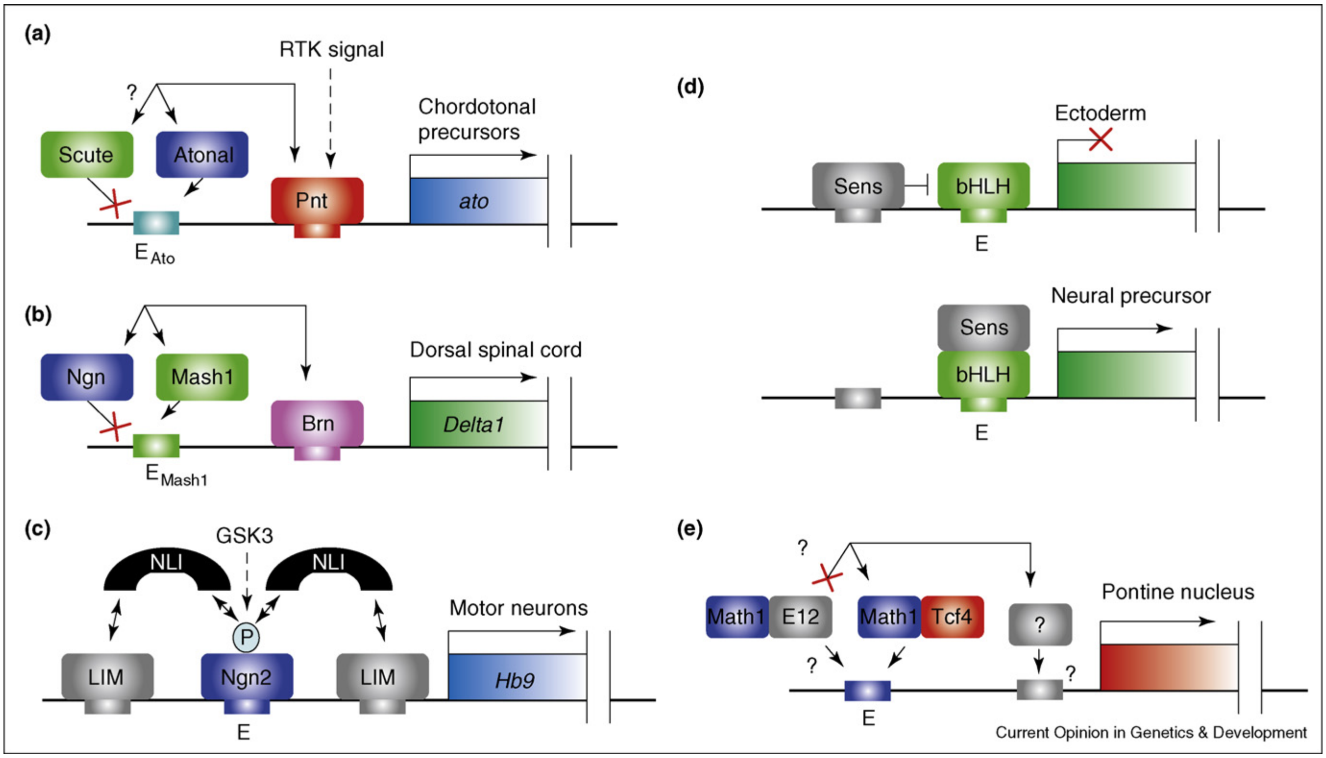
Whilst these are powerful examples of combinatorial control, an important but largely unanswered question is what provides the *specificity* of co-regulation by different bHLH proteins and different cofactors. Multiple bHLH factors and multiple potential co-regulators may be present in the same cells, but targets only respond to a specific combination. Is specificity for a particular bHLH factor driven by specificity of protein–protein interactions or DNA binding specificity (Figure 2)? *In vivo* E box occupancy by different bHLH factors can be measured by chromatin immunoprecipitation (ChIP), but this may not clearly answer the question since occupancy will depend on protein interactions as much as on DNA binding affinity. In structure–function experiments, functional differences between bHLH proteins have been mapped to non-DNA-contacting residues within the bHLH domain, whilst DNA-contacting residues tend to be highly conserved between different bHLH factors [7,10,30–33]. It is attractive to think therefore that these ‘specificity residues’ contact different cofactors thereby providing specificity of target gene regulation (Figure 2a). However, it is also clear that different proneural bHLH factors *do* have different E box binding site preferences *in vivo* if not *in vitro*. For instance, some Scute and Atonal-specific target genes have functionally distinct Scute- and Atonal-specific variant E box motifs [34]. Moreover, these E box motifs are differentially used by Scute and Atonal

**Figure 2**



Mechanisms for specificity *between* bHLH factors. **(a)** Multiple bHLH factors can utilise an E box, but specific cofactor interactions determine that only bHLH factor ‘*b*’ results in target gene activation. **(b)** Multiple bHLH factors can interact with the cofactor, but specific DNA–protein interactions determine that only bHLH factor ‘*b*’ utilises that particular E box motif and results in target gene activation.

**Figure 3**



Examples of specific target gene regulation by bHLH factors. **(a)** In *Drosophila* Atonal and Pointed bind cooperatively to adjacent sites in an *atonal* autoregulatory enhancer. Scute apparently cannot utilise the E box motif; it is not clear whether it can interact with Pnt. **(b)** Mouse Mash1 and Brn proteins bind cooperatively to adjacent sites in the *DeltaM* enhancer. Ngn2 apparently cannot utilise the E box motif even though it can interact with Brn proteins. **(c)** Phosphorylation of Ngn2 is required for its interaction with NLI, thereby switching its ability to activate a subset of targets with Lim-HD factors. NeuroM also binds to this E box. **(d)** *Drosophila* proneural factor activity is regulated by Sens acting as a binary switch. Sens can act as DNA-binding repressor or a non-DNA binding coactivator. **(e)** Alternative E protein dimerisation partners may regulate Math1 target gene specificity in the Pontine nucleus, putatively via specific interaction with an unknown cofactor.

even in a non-neural cell culture system, suggesting that specialised cofactors may not always be required for bHLH specificity [35]. How can these observations be reconciled? Non-DNA-contacting 'specificity' residues in the bHLH domain may well cause conformation effects on DNA-contacting residues, thereby changing DNA interaction properties, perhaps in a manner induced or modified by cofactor interactions.

An elegant example of specifity in bHLH-cofactor interaction is provided by the *DeltaM* enhancer mentioned above. Whilst bHLH proteins Mash1 and Ngn2 are both generally capable of synergising with the Brn proteins, it appears that selective E box utilisation determines that only Mash1 synergises at the *DeltaM* enhancer [27••] (Figure 3b). A different explanation has been suggested for the mouse *Hb9* promoter. Whilst NeuroM and Mash1 can both bind an E box in the *Hb9* promoter (as measured by ChIP), only NeuroM can synergise with LIM-HD cofactors bound to the same promoter (via the NLI adaptor protein). Although not proven, it is suggested that this selective protein interaction provides the *Hb9* promoter's specificity [18] (Figure 3c). Conceivably, expression of different adaptor proteins may be an important mechanism for determining target gene specificity in other contexts [36•]. In the chick retina, ChIP studies show that bHLH factors regulating the Ath5 gene may interact with different E boxes at different developmental stages [37]. Perhaps temporal changes in the co-expression of different cofactors and/or adaptor proteins may influence this.

A simple model for bHLH function suggests that common (shared) neurogenesis targets are regulated via non-specific E boxes (Figure 1b). Indirect evidence suggests that this is true for the *senseless* gene, which has a single E box responding both to Scute and Atonal [38], and Prokineticin 2, with an E box that binds both Ngn1 and Mash1 [39]. However, other shared targets respond to different bHLH factors via separate subtype-specific enhancers. This includes *Brd* (regulated by two enhancers containing Atonal- and Scute-specific E boxes, respectively)[34] and mouse *Delta1* (two enhancers for Mash1 and Ngn1/2, respectively) [27••]. Why should some shared targets be regulated in this way? Possibly it allows such targets to be regulated with different dynamics in different neural subtypes.

## Mechanisms providing signalling context

Signalling context (the signals a cell is receiving) is clearly important for modulating bHLH activity in both space and time. The Atonal/Pointed interaction mentioned above provides an obvious mechanism: Atonal can only regulate some targets when Pointed is present, which itself is produced when the cell responds to RTK signalling [25]. Recently, the motor neuron *Hb9* paradigm has been extended to show how bHLH specificity may be affected by signalling context via direct post-translational modification. Phosphorylation of Ngn2 has previously been shown to have several roles, including regulation of its stability [40]. Ma *et al.* [41••] have now shown that serine phosphorylation by GSK3 is required for the subtype determination function of Ngn2, but apparently not its neurogenesis function. For the *Hb9* motor neuron enhancer, this phosphorylation is necessary for Ngn2's interaction with the NLI adaptor (Figure 3c). Thus, a post-translational modification modulates the target gene specificity of a bHLH factor via controlling its capacity for protein–protein interactions. Tyrosine phosphorylation was previously shown to be required for Ngn2 function in migration and dendrite morphology of cortical pyramidal neurons [42], and it is possible that this modification also allows regulation of a specific subset of targets.

Expression of a coactivator protein may also underlie a temporal switch in *activity* rather than *specificity*. In *Drosophila* neurogenesis, proneural factors are thought to regulate only a few targets in neurally competent ectodermal cells (notably concerned with lateral inhibition) but many more upon commitment of the neural precursors. What promotes this switch in activity? A compelling series of papers shows that the Zn finger GPS factor, Senseless, is part of this switch: it can act as both a positive and negative modulator of proneural protein activity (Figure 3d) [38,43,44••]. There is a strong possibility that some aspects of this interaction are conserved [45,46]. It is not clearly known what switches the activity of Senseless, although a concentration-dependent switch is suggested [38]. Senseless provides temporal context for proneural bHLH activity rather than subtype specificity. However, recent evidence suggests that Senseless interaction may enhance the E box selectivity of different proneural factors, perhaps by strengthening bHLH recognition of different E box motifs [35].

Finally, the role of bHLH dimerisation partners should not be overlooked. In vertebrates there are several E protein partners, opening the possibility that choice of E protein may also provide a way of modulating proneural bHLH function. Flora *et al.* [47•] found a specific requirement for Math1/Tcf4 heterodimers (as opposed to heterodimers of Math1 with E47, E12 or HEB) in the generation of the pontine nucleus neuronal structure (derived from the rhombic lip of the hindbrain [48]). They propose that this may reflect a unique ability of Math1/Tcf4 heterodimers to interact with tissue-specific cofactors [47•] (Figure 3e), although choice of dimerisation partner might also affect DNA binding site preferences [49].

## Conclusions

Although exciting progress is being made, we still have a very patchy understanding of the molecular mechanisms of bHLH specificity in relation to the context. One message is that enhancers provide the all-important molecular context that enable different protein interactions to occur at different target genes even within the same cell [11]. A major bottleneck in progressing is a dearth of subtype-specific enhancers to be analysed in structure–function studies. Genome-wide target gene identification, bioinformatic and gene network approaches promise to add much more in future [50•,51,52•].

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