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REVIEW

All in the family: proneural bHLH genes and neuronal diversity

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ABSTRACT

Proneural basic Helix-Loop-Helix (bHLH) proteins are required for neuronal determination and the differentiation of most neural precursor cells. These transcription factors are expressed in vastly divergent organisms, ranging from sponges to primates. Here, we review proneural bHLH gene evolution and function in the *Drosophila* and vertebrate nervous systems, arguing that the *Drosophila* gene *atonal* provides a useful platform for understanding proneural gene structure and regulation. We also discuss how functional equivalency experiments using distinct proneural genes can reveal how proneural gene duplication and divergence are interwoven with neuronal complexity.

KEY WORDS: bHLH gene, Neural development, Neurogenesis, Neuronal diversity, Proneural gene

Introduction

The function of the nervous system relies on a large number of neurons with diverse functions. Even the simple nervous system of the nematode *C. elegans* has more than 100 classes of neurons, and this number is vastly larger in more complex metazoans (White et al., 1986). The specification and differentiation of most neurons rely on a class of transcription factors known as proneural basic Helix-Loop-Helix (bHLH) transcription factors (Huang et al., 2014), which are also important in attempts to achieve neuronal regeneration (Guillemot and Hassan, 2017). There are ∼125 bHLH genes in the human genome, as compared with 59 in *Drosophila* (Ledent et al., 2002; Simionato et al., 2007). Vertebrate bHLH factors are further categorized into subfamilies, including the Atoh (Atonal homolog), Ascl (Achaete-Scute complex-like), Neurogenin, Neurod and Olig factors (Fig. 1), whose members act during neurogenesis, neuronal differentiation and/or gliogenesis. These related genes probably arose from common ancestors by gene or genome duplication during evolution. This raises the question of whether genetic complexity might contribute to neuronal diversity. Here, we discuss the evolution and function of complex proneural networks. We highlight how gene replacement studies, some made feasible by new genome editing technologies, can help evaluate evolutionary changes in proneural bHLH gene functions and clarify the extent to which neuronal diversity depends on increasing genetic complexity or on other factors.

The Achaete-Scute gene complex and the proneural gene concept

The proneural concept was developed through the discovery and characterization of four *Drosophila* genes – *achaete* (*ac*), *scute* (*sc*), *lethal of scute* [*lsc*, or *l(1)sc*] and *asense* (*ase*) – that are responsible for development of much of the *Drosophila* CNS and PNS (Cubas et al., 1991; Garcia-Bellido and de Celis, 2009). Expression of these proneural genes defines regions of ectoderm with neurogenic competence, such that their default fate will be that of neural precursors unless diverted to another fate, for example by Notch signaling (Knust and Campos-Ortega, 1989; Simpson, 1990). *ac*, *sc* and *lsc* are proneural genes, conferring proneural competence that may or may not lead to neuronal determination in every cell, whereas *ase* is a neural precursor gene, expressed after the neural fate decision has been made. It has been suggested that the vertebrate homologs of these genes are expressed in ectoderm with previously acquired neural character, and therefore are not true proneural genes (Bertrand et al., 2002). However, we see this as a minor distinction because the same could be said for some proneural regions in *Drosophila*, and because it may not apply to some vertebrate tissues (Jarman and Groves, 2013). Like vertebrate proneural genes, *ac*, *sc* and *lsc* show a variety of overlapping and distinct expression patterns, reflecting both separate and redundant functions in the specification of different neural precursor cells, and exhibit cross-regulatory interactions whereby one gene functions to regulate the expression of another. Unlike most vertebrate proneural genes, however, *ac*, *sc*, *lsc* and *ase* are closely linked within a 100 kb segment of the X-chromosome, constituting the Achaete-Scute gene complex (AS-C) (Garcia-Bellido and de Celis, 2009).

The structure of the AS-C suggested that multiple proneural genes might be necessary to encode neuronal diversity. However, this idea has to be revisited, as it is now believed that the multigene AS-C of *D. melanogaster* is a recent evolutionary invention. Indeed, although proneural bHLH genes are found as far back as coelenterates, an AS-C of four linked genes is not found even in other dipteran insects. For example, the basal mosquito species *Anopheles gambiae* has only a single proneural gene of the AS-C class as well as a single neural progenitor gene related to *ase*. This simple arrangement seems to be the ancestral condition for insects and is also seen in the red flour beetle *Tribolium castaneum*, the honey bee *Apis mellifera* and the wasp *Nasonia vitripennis* (Negre and Simpson, 2009). Since the nervous systems of mosquitoes, honey bees or wasps does not appear to be significantly less complex than that of *D. melanogaster*, it can be argued that the multiple bHLH proteins in the AS-C are not really necessary to make complex neural structures. In addition, the potential contributions of multiple genes in other proneural gene families, such as the *ato*, *cato* and *amos* genes that diverged earlier in the lineage leading to *Drosophila*, or the Atoh genes that diverged in vertebrates (Fig. 1), merit consideration in light of this finding.

Drosophila atonal as a platform for understanding proneural gene structure and regulation

Ideally, a discussion of how proneural gene families arose by gene duplication and diversification would begin with the original progenitor genes. We currently lack access to those ancient genes, however, and therefore propose here that the *Drosophila* gene

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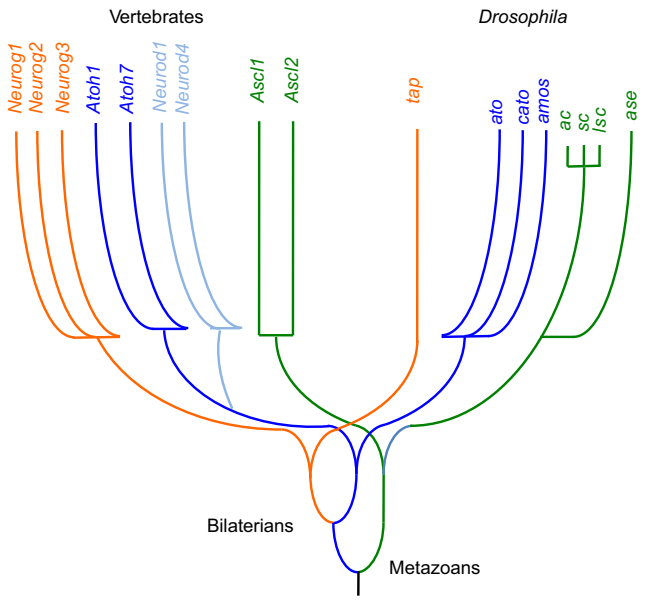

Fig. 1. Schematic phylogeny of proneural bHLH genes. The relationships and ancestry of extant proneural bHLH genes in vertebrates and *Drosophila* (Simionato et al., 2007). Each color represents a related subfamily. The most ancient divergence is thought to be between Achaete/Scute-like genes and Atonal/Neurogenin/Neurod-like genes. The divergence of Atonal/Neurod-like and Neurog-like genes predates the vertebrate-insect divide, but the Atonal/Neurod family diverged within the chordate lineage. Achaete/Scute-like genes diversified within insects, including a recent triplication in the lineage leading to *Drosophila* (Negre and Simpson, 2009).

atonal (*ato*) provides a useful model because it is not a member of a gene complex and often acts alone, i.e. without coexpression of other proneural genes. Accordingly, a description and analysis of *ato* regulation and function in *Drosophila* provides a useful basis for comparison with other proneural genes, and a starting point for understanding proneural gene evolution.

ato is a homolog of vertebrate Atoh genes (Jarman and Groves, 2013). In *Drosophila*, *ato* functions to specify the retina, the chordotonal organs (stretch receptors of the PNS) and some of the olfactory sense organs, all without assistance from the AS-C. The two other Ato family genes in *Drosophila* are unlinked and show limited overlap in function with one another; *amos* is required for specification of the remaining olfactory sensillae and for specifying some dendritic neurons, while *cato* acts as a neural precursor gene in the chordotonal sensory lineage (Maung and Jarman, 2007; zur Lage and Jarman, 2010).

Within the *Drosophila* eye, the role of *ato* is to specify the fate of R8 class photoreceptor neurons (Jarman et al., 1994). These are the founders of ommatidia – the individual units of the insect compound eye. Each R8 cell coordinates the induction of additional photoreceptor neurons from neighboring cells by a mechanism dependent on receptor tyrosine kinase signaling (Treisman, 2013). In the absence of *ato* no retinal neurogenesis takes place owing to the absence of the crucial R8 founder cell. The photoreceptor neurons of the eye differ markedly in structure and physiology from the other *ato*-dependent neurons, and are surrounded by very different support cells (Jarman and Groves, 2013). Clearly, distinct neural structures do not require distinct proneural genes, as *ato* exemplifies a single gene that defines multiple classes of neuron, albeit in combination with other genes that provide specific/unique contexts for *ato* function (Kiefer et al., 2005). This is also abundantly clear in vertebrates, where *Atoh1* has diverse roles in the CNS, PNS and gut (reviewed by Huang et al., 2014).

An early step in neural fate determination is the transcription of *ato*. Detailed studies show that *ato* expression can best be understood in terms of distinct mechanisms of initiation and then of maintenance (Fig. 2, Box 1). There is no R8-specific transcription factor capable of initiating *ato* transcription in only these cells. Instead, *ato* transcription initiates in all undifferentiated retinal cells at a particular stage. Because the *Drosophila* eye develops progressively, with a wave of differentiation initiating at the posterior eye margin, a corresponding wave of *ato* transcription crosses the eye primordium just in advance of neurogenesis (Fig. 2) (Jarman et al., 1994). This spatiotemporal pattern is induced by the morphogens Hedgehog (Hh) and Decapentaplegic (Dpp). This eye-specific response to these morphogens requires a particular code of pre-existing transcription factors that includes *eyeless* (*Drosophila Pax6*) and *sine oculis* (a *Drosophila* homolog of the vertebrate Six genes), which are already expressed in the eye primordium (Zhang et al., 2006; Baker and Firth, 2011).

Once initiated, the pattern of *ato* transcription (and protein) evolves rapidly, narrowing down first to proneural clusters of ∼10 cells and then to single cells that maintain expression for several hours and thereby acquire R8 photoreceptor fate (Fig. 2) (Jarman et al., 1994). In common with other proneural genes, this narrowing of *ato* expression is regulated by Notch, which establishes the final pattern of neuronal precursor cells. This refinement serves as a model for the Notch-dependent lateral inhibition that fine-tunes neural fate specification in numerous vertebrate and invertebrate developmental contexts (Fortini, 2009). During lateral inhibition, *ato* transcription no longer responds to Hh and Dpp. Instead, *ato* transcription is maintained by autoregulation, and the refinement of its expression by Notch reflects interruption of the autoregulatory loop in progressively more and more cells (Baker et al., 1996). Notch acts through transcriptional repressor proteins [E(spl) in *Drosophila*], which are sequence-specific DNA-binding proteins that can also be recruited to autoregulatory enhancers by protein-protein interactions with proneural proteins (Giagtzoglou et al., 2003). Blocking autoregulation in this way is an effective means of permanently extinguishing gene expression and might be exploited by Notch signaling in many situations (Baker, 2004). In addition, there is increasing evidence that Notch signaling affects proneural protein stability, which would also negatively impact autoregulation (Kiparaki et al., 2015; Weinberger et al., 2017).

The initiation and maintenance of *ato* gene expression segregate to distinct enhancers (Sun et al., 1998) (Fig. 2). Transgenes containing eye initiation enhancers drive reporter gene expression only in the initial uniform expression domain, just overlapping the first hints of spatially restricted expression. By contrast, transgenes containing the eye autoregulatory enhancer drive reporter gene expression only in the periodic clusters of cells and the single R8 precursors that derive from them. It makes sense that input from the initiation enhancer ceases before lateral inhibition, otherwise inhibiting autoregulation would not extinguish gene expression. Conversely, the autoregulatory enhancer must become active when initiation ceases, or Ato protein would decay before gene expression could be maintained. The basis of temporal coordination between these two regulatory programs is only partially known (Chen and Chien, 1999; Lim and Choi, 2004; Baker et al., 2009).

After Ato expression is stable and Ato protein is active as a transcription factor, R8 photoreceptor fate can be specified. The eye-specific targets of Ato might be defined combinatorially with other, eye-specific factors. Some individual eye targets are known,

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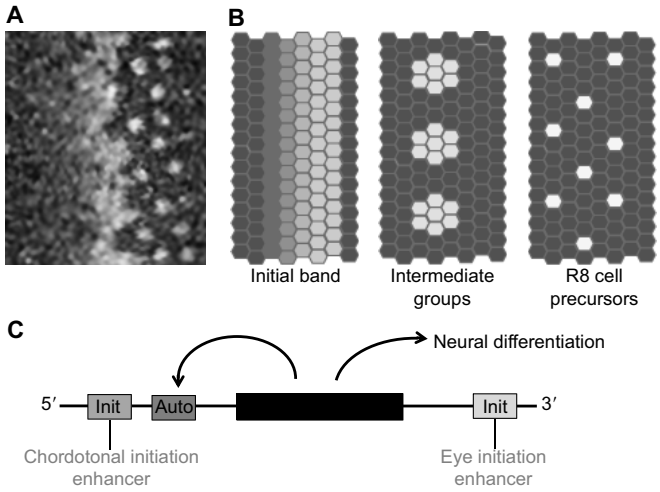

Fig. 2. Regulation of Atonal expression and transcription in the *Drosophila* eye. (A) Confocal image of Atonal (Ato) protein expression at the leading edge of the *Drosophila* retina differentiation wave. Towards the anterior (left), Ato expression progressively accumulates in all the cells, then rapidly evolves through a pattern of intermediate groups of cells that transiently maintain Ato protein while the surrounding cells lose expression, resolving to only isolated single cells that maintain Ato expression posteriorly (right). These are now the committed and postmitotic R8 photoreceptor precursors. The wave of differentiation advances anteriorly at a rate of one column every 90-100 min. (B) The complete Ato expression pattern can be dissected into at least three temporally overlapping phases (Jarman et al., 1995). First, Ato expression accumulates uniformly, reaching higher and higher levels more posteriorly until this uniform expression abruptly ceases. Replacing the uniform expression, and just overlapping with it in time, is a transient phase of expression in 'intermediate groups' of up to ten cells. The intermediate groups are proneural preclusters that will all develop as R8 photoreceptor neurons unless Notch signaling is activated (Baker et al., 1996; Dokucu et al., 1996). Then, each intermediate group is refined to a single Ato-expressing cell that maintains expression for three to four ommatidial columns (~6 h). (C) Uniform initiation of Ato expression depends on an eye initiation enhancer that is downstream of the coding region of the *ato* gene (black box), but is independent of functional Ato protein and of the 5′ autoregulatory enhancer. By contrast, expression in the intermediate groups of up to ~10 cells is autoregulatory and depends on the 5′ autoregulatory enhancer because the 3′ initiation enhancer becomes inactive at this stage. Single cells escape Notch activity to maintain autoregulatory Ato expression and become determined as R8 photoreceptor precursor cells. They maintain Ato expression for ~6 h, then Ato protein undergoes inhibitory phosphorylation, leading to the loss of expression. This loss is permanent because initiation is no longer active. Other neural regions that express Ato (e.g. chordotonal organs) rely on distinct enhancers (Baker et al., 1996; Sun et al., 1998; Quan et al., 2016).

such as the zinc-finger transcription factor Senseless (*sens*, a homolog of *Gfi1*) and Fasciclin 2 (Fas2), an adhesion molecule that is elevated transiently by Ato function (Frankfort et al., 2001; Acar et al., 2006; Quan et al., 2016). Studies of transcription in a variety of systems indicate that proneural genes activate many transcriptional targets including other transcription factors, structural proteins, receptors and channel proteins (Portman and Emmons, 2004; Reeves and Posakony, 2005; Cachero et al., 2011). In addition, it has been shown that both the levels and duration of Ato expression in R8 cells are important for proper development (White and Jarman, 2000). If Ato levels are too high, the subsequent recruitment of other photoreceptors by receptor tyrosine kinase signaling becomes overactive (White and Jarman, 2000). Conversely, failure to maintain full expression levels results in inadequate recruitment of other ommatidial cells (White and Jarman, 2000).

Ato expression is ultimately downregulated in the R8 neurons during their terminal differentiation (Jarman and Groves, 2013), a feature that is generally shared by vertebrate proneural bHLHs. In normal development, Ato is active in R8 precursors during only a short time window of 6-8 h, before transcription fades. Recent studies show that Ato protein itself persists for longer but becomes inactivated by phosphorylation so that autoregulatory *ato* transcription is not maintained, and the protein subsequently decays. Mutation of the phosphorylation site leads to reduced recruitment of other photoreceptors, apparently owing to persistently high levels of the cell adhesion protein Fas2 (Quan et al., 2016). Thus, a second, Notch-independent process ultimately interrupts autoregulation to terminate Ato expression.

The possible origins of proneural gene complexes by gene duplication

If the single AS-C progenitor gene resembled *ato* in its regulation, then the complexity of the AS-C in *Drosophila* might have arisen from duplications of this ancestral gene (Fig. 3) (Taylor and Raes, 2004). Gene duplicates are expected to begin life identical in sequence, and it is easy to see that natural selection need not maintain both identical copies. Mutational loss or inactivation of one copy is thought to be a frequent outcome. When the duplicated gene has multiple functional elements that may be lost independently, a process of subfunctionalization can occur. For example, when an important feature of the expression pattern is lost from one copy, its retention by the other copy becomes essential. If both copies lose distinct subsets of the original gene's function, selection must maintain both partially defective copies to retain the full complement of functions. For this reason, subfunctionalization is thought to be a widespread feature of duplicated gene families and the underlying cause for the retention of both copies during evolution (Force et al., 1999; Taylor and Raes, 2004; Conant and Wolfe, 2008).

Considering duplication of a gene such as *ato*, with separate initiation and maintenance enhancers, one imagines that both descendant genes should have been capable not only of autoregulation but also of cross-regulation (Fig. 3). This situation would not have to be retained and many scenarios are possible as the enhancer and protein sequences drift during evolution. It is easy to imagine a variety of complex network topologies that could arise via subfunctionalization, with each network retaining the full function

Box 1. Gene initiation and maintenance programs

The existence of both initiation and maintenance programs for gene expression reflects fundamental features of cell fate determination. Most developmental fates are initially induced by extrinsic signals and only remain stable through subsequent development by becoming independent of them. In fact, classical embryology defined cell determination as 'acquired independence from inducing signals', for example as revealed after explanting from the embryo or after experimental transplantation to a new embryonic location (Slack, 1983). Maintenance of key transcription factors by autoregulatory mechanisms is one way that this independence is achieved (Baker, 2004). The cell-cell signals that induce cell fates during development usually belong to a small number of conserved developmental signaling pathways (including the TGFβ, Hedgehog, Notch, Receptor tyrosine kinase and Wnt pathways), each of which has many functions in different tissues and developmental stages (Gerhart, 1999; Pires-daSilva and Sommer, 2003). Reuse of these signaling pathways is made possible because they initiate cell fates but are not generally required to maintain them.

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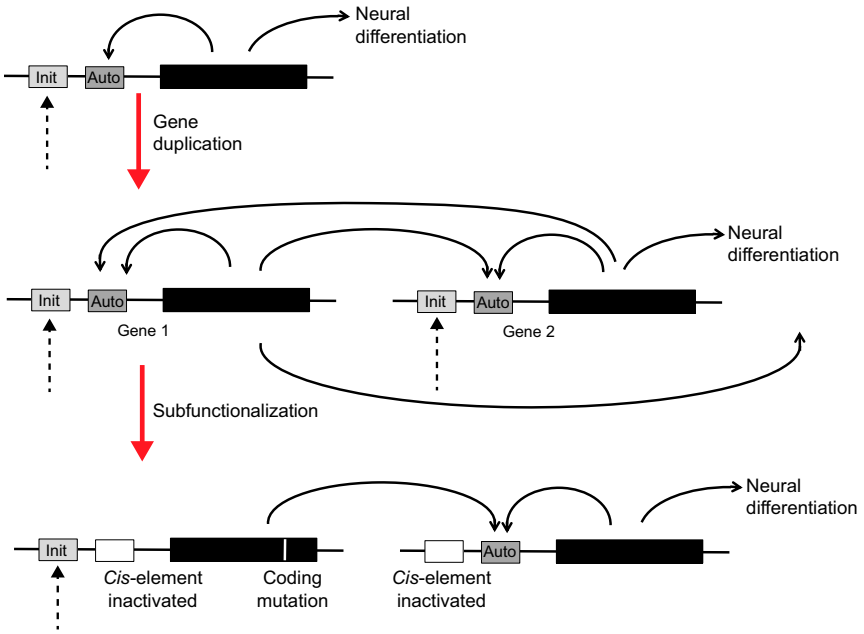

Fig. 3. Illustration of subfunctionalization. A hypothetical proneural gene with separate enhancers controlling initiation versus autoregulatory transcriptional activities is shown (top). The complete duplication of this gene (middle) produces two functionally redundant, identical genes (genes 1 and 2) that are expected to positively regulate one another through inheritance of the autoregulatory machinery. However, natural selection does not have to maintain redundant functions and can give rise to subfunctionalization (bottom). In the example shown, protein 1 has lost the capacity to specify neural differentiation because of a missense mutation in the coding region and has also lost the ability to autoregulate through divergence of its regulatory sequences, but remains functional as long as protein 1 cross-activates gene 2 and protein 2 then specifies neuronal differentiation. In this example, it would not be necessary for gene 2 to retain regulatory sequences for initiation, since this subfunction could be performed by gene 1. Duplicated genes 1 and 2 would now appear to have temporally distinct roles, although together they fulfill the function of a single ancestral gene. In principle, many other interdependent network topologies can arise through other patterns of subfunctionalization (Taylor and Raes, 2004; Conant and Wolfe, 2008).

of the original progenitor gene (Fig. 3) (Taylor and Raes, 2004; Conant and Wolfe, 2008). In many respects, the regulation of the AS-C looks like one possible outcome of this process. A variety of 'prepattern' pathways activate one or more AS-C genes in different proneural regions. Thereafter, the autoregulation and cross-regulation of AS-C genes play important roles in neural fate determination (Gómez-Skarmeta et al., 1995). These features of gene regulation could have been inherited from an autoactivating progenitor (Culi and Modolell, 1998).

In the simplest case, subfunctionalization can maintain multiple members of a gene family even when they have not acquired new functions compared with their common progenitor. However, it is also possible that when multiple functions divide among separate descendant genes, selection then optimizes each now-independent function to a greater degree, leading to enhanced, more specialized functions of the descendant family members. Finally, new functions sometimes arise in duplicated genes, although this is thought to occur less frequently (Taylor and Raes, 2004; Conant and Wolfe, 2008). Functional distinctions have been demonstrated between individual AS-C genes in transgenic assays (Chien et al., 1996), consistent with differences that contribute to neuronal diversity (Huang et al., 2014). Could individual genes have thus evolved new functions, or has each retained subsets of the ancestral functions?

Gene replacement to compare proneural gene function

In principle, the origins of functional distinctions between AS-C genes could be addressed in an experiment that tests whether the single homolog of the proneural AS-C genes from honey bees, wasps or mosquitoes could rescue any or all of the *Drosophila* AS-C genes. The AS-C structure would make this difficult, but a recent study has made use of gene knock-in methods to replace the *ato* coding region at the endogenous *Drosophila* locus with sequences encoding other proneural proteins, an assay that should test the functional attributes of various proteins precisely (Table 1) (Weinberger et al., 2017). These studies showed that Amos is almost indistinguishable from its paralog Ato in terms of function when expressed from the *ato* locus, whereas previous transgenic assays had suggested distinctions between these proteins (Maung and Jarman, 2007; Weinberger et al., 2017). Thus, the Ato family paralogs in *Drosophila* might have diverged primarily in expression pattern not protein function, although further studies are required to confirm this, including studies of the olfactory sensilla subtypes that depend on either *ato* or *amos*.

By contrast, neither *sc* nor the *Drosophila* Neurogenin-like gene *tap* could replace *ato*, reflecting the much earlier evolutionary divergence of the Achaete/Scute-like, Neurog and Ato families (Fig. 1). On the other hand, a sponge proneural gene equally resembling the Neurog and Ato gene families could partially substitute for *ato*, consistent with the idea that once-shared features of the Neurog and Ato genes might have been lost from at least the Neurog family gene *tap*. Interestingly, this study also suggested that the degrees of partial rescue by diverged family members, including mouse *Atoh1* and *Atoh7* and Atoh genes from cephalochordates and annelids, might largely reflect quantitative differences in protein stability, in particular destabilization by Notch signaling, rather than qualitative functional differences (Table 1) (Weinberger et al., 2017).

The expression, regulation and function of proneural genes in the vertebrate nervous system

To what extent are these studies of *Drosophila* proneural genes, and of the Ato gene family in particular, relevant to the more complex network of vertebrate proneural genes (Huang et al., 2014)? Based

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Table 1. Functional complementation studies within the proneural gene family

Donor gene	Target gene	Method	Functional complementation	Tissues tested	Reference
Dm *sc*	Dm *ato*	UAS-GAL4	Partial	Retina	Sun et al. (2000)
Dm *sc*	Dm *lsc*	UAS-GAL4	Partial	Retina	Chien et al. (1996)
Dm *ato*	Dm *amos*	UAS-GAL4	No	Olfactory	Maung and Jarman (2007)
Dm *amos*	Dm *ato*	UAS-GAL4	Yes	Retina	Maung and Jarman (2007)
Mm *Atoh1*	Dm *ato*	UAS-GAL4	Yes (both)	Chor/retina	Wang et al. (2002)
Dm *ato*	Dm *ato*	UAS-GAL4	Yes	Retina	Sun et al. (2003)
Mm *Atoh7*	Dm *ato*	UAS-GAL4	No	Retina	Sun et al. (2003)
Xl *Atoh7*	Dm *ato*	UAS-GAL4	Yes	Retina	Sun et al. (2003)
Xl *Neurod*	Dm *ato*	UAS-GAL4	Yes	Retina	Sun et al. (2003)
Dm *sc*	Dm *ato*	UAS-GAL4	No	Retina	Sun et al. (2003)
Bm *Ato*	Dm *ato*	UAS-GAL4	Yes	Retina	Yu et al. (2012)
Dm *ato*	Dm *ato*	GR	Yes (all)	Retina/chor/hear	Weinberger et al. (2017)
Dm *tap*	Dm *ato*	GR	No	Viability	Weinberger et al. (2017)
Dm *amos*	Dm *ato*	GR	Yes (all)	Retina/chor/hear	Weinberger et al. (2017)
Mm *Atoh1*	Dm *ato*	GR	Yes (all)	Retina/chor/hear	Weinberger et al. (2017)
Mm *Atoh7*	Dm *ato*	GR	Weak/no/no	Retina/chor/hear	Weinberger et al. (2017)
Bf *Ath*	Dm *ato*	GR	Partial (all)	Retina/chor/hear	Weinberger et al. (2017)
Pd *Ath2*	Dm *ato*	GR	No/weak/weak	Retina/chor/hear	Weinberger et al. (2017)
Aq *bHLH1*	Dm *ato*	GR	Partial/weak/weak	Retina/chor/hear	Weinberger et al. (2017)
Dm *sc*	Dm *ato*	GR	No	Viability	Weinberger et al. (2017)
Dm *ato*	Mm *Atoh1*	GR	Yes (all)	Brain/intestine/ear	Wang et al. (2002)
Mm *Ascl1*	Mm *Neurog2*	GR	Partial (both)	Brain/spinal cord	Fode et al. (2000)
Mm *Neurog2*	Mm *Ascl1*	GR	No (both)	Brain/spinal cord	Parras et al. (2002)
Mm *Ascl1*	Mm *Atoh7*	GR	No	Retina	Hufnagel et al. (2013)
Mm *Neurod1*	Mm *Atoh7*	GR	Yes	Retina	Mao et al. (2008)
Mm *Neurod4*	Mm *Atoh7*	GR	Partial	Retina	Mao et al. (2008)
Mm *Atoh7*	Mm *Neurod1*	GR	No	Retina	Mao et al. (2013)
Mm *Neurog1*	Mm *Atoh1*	GR	Partial	Ear	Jahan et al. (2015)
Mm *Atoh7*	Xl *Atoh7*	OE	No	Retina	Brown et al. (1998)
Dm *ato*	Xl *Atoh7*	OE	Yes	Retina	Sun et al. (2003)
Xl *Ascl1*	Xl *Atoh7*	OE	No	Retina	Moore et al. (2002)
Mm *Ascl1*	Xl *Atoh7*	OE	No	Retina	Brown et al. (1998)

Listed are genes for which loss of target gene function has been replaced by donor gene function (including self-replacements), along with the method used, tissues (or overall viability) examined and a summary of the result. Dm, *Drosophila melanogaster*; Mm, *Mus musculus*; Xl, *Xenopus laevis*; Bm, *Bombyx mori*; Bf, *Branchiostoma floridae*; Pd, *Platynereis dumerilii*; Aq, *Amphimedon queenslandica*. GR, gene replacement; OE, overexpression; chor, chordotonal organ; hear, hearing.

on amino acid sequence conservation within their bHLH domains, it is thought that the vertebrate Atoh, Neurog and Neurod subfamilies evolved from a common ancestor with *ato*, *cato*, *amos* and *tap*, whereas *Drosophila* AS-C genes are most related to the vertebrate *Ascl1* and *Ascl2* genes (Fig. 1). However, the vertebrate genes differ functionally from one another, and from the *Drosophila* genes, in a variety of ways. *Ascl2* is not expressed in the nervous system and is not relevant here. Others are expressed in, and required for, the development of distinct neural cell populations.

The expression domains of vertebrate Ascl and Atoh gene families have been comprehensively described (see Huang et al., 2014). In the vertebrate retina, for example, *Atoh7* (also known as *Ath5*) function is crucial for retinal ganglion cell (RGC) formation (Kanekar et al., 1997; Brown et al., 2001; Kay et al., 2001; Wang et al., 2001). By contrast, mutations in mouse *Neurog2* delay the temporal progression of retinal neurogenesis (Hufnagel et al., 2010). *Neurod1* is required for photoreceptor and amacrine cell differentiation (Morrow et al., 1999), while *Ascl1* (also known as *Mash1*) maintains a progenitor pool for late fates and regulates rod photoreceptor and bipolar cell neurogenesis (Jasoni and Reh, 1996; Tomita et al., 1996b). Of the three members of the Neurogenin family, *Neurog1* and *Neurog2* are expressed in the developing forebrain, olfactory system, spinal cord, dorsal root ganglia, and cranial ganglia, whereas *Neurog3* is expressed in the developing spinal cord, hypothalamus, and pancreatic endocrine progenitor cells. In addition to being required in the retina, *Ascl1* is required in the ventral forebrain and dorsal spinal cord. Yet another distinction, exemplified by retinal bHLH factors, is temporally separable expression. *Neurog2* and *Ascl1* are present in actively mitotic progenitors, whereas *Atoh7* is expressed by progenitors completing terminal mitosis, and *Neurod1* and *Neurod4* are only present in postmitotic cells.

There are also differences between related sets of genes. For instance, *Atoh1* (also known as *Ath1*) and *Atoh7* appeared during whole-genome duplications in basal vertebrate species (Fig. 1) but have non-overlapping, distinct expression domains, as well as a subdivision of *ato* sensory functions. Whereas *Atoh7* is crucial for RGC neurogenesis, *Atoh1* is required by inner ear sensory hair cells, dorsal spinal cord interneurons, Merkel cells and cerebellar granule cells (Bermingham et al., 1999, 2001; Ben-Arie et al., 2000). These two genes also exhibit separate expression domains in zebrafish, frog, chick and mouse embryos, although their complete functional separation can only be tested by gene replacement. Remarkably, the genetic hierarchy of retinal neurogenesis exhibits similarities in flies and mammals: in mammals, Pax6 (a mouse homolog of *Drosophila* Eyeless, which activates *ato* expression) activates multiple bHLH factors, while Shh signaling and Notch signaling refine spatiotemporal expression. In line with this, it has been shown that *Atoh7* has a conserved Pax6 binding site in its 5′ primary enhancer, located ∼1.5 kb upstream from the ATG codon (Riesenberg et al., 2009; Willardsen et al., 2009). Although not formally tested, there are also consensus binding sites for Gli and Hes proteins, which are the transcriptional effectors of Hh and

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Notch signaling pathways. Together, these findings suggest that vertebrate *Atoh7* performs a similar function in the retina to that performed by *Drosophila ato* in the fly eye. However, even though *Atoh1* is not expressed in the vertebrate retina, it behaves more like the *ato* gene, since it positively autoregulates itself (via a 3′ enhancer) (Helms et al., 2000) and can partially substitute for *ato* in the fly eye (Table 1) (Wang et al., 2002; Weinberger et al., 2017). In addition, the mammalian *Atoh7* gene differs in its acquisition of a second 'shadow' enhancer, the deletion of which in humans causes the agenesis of RGCs (Ghiasvand et al., 2011). Although there is a partial *atoh7* shadow enhancer in bony fishes, it is located closer to the primary enhancer, and a comparison of mouse and human genomes shows that these two regulatory elements are increasingly farther apart (Ghiasvand et al., 2011). This is suggestive of an additional, mammalian-specific mode of regulation in the eye, whereby changing chromatin configurations or epigenetic factors might be needed to rapidly open or close the *Atoh7* locus.

There are also examples of both positive autoregulation and cross-regulation among the vertebrate proneural genes. Mouse *Atoh1* positively autoregulates its own expression in the dorsal neural tube, cerebellum, vestibular and auditory systems (Helms et al., 2000). By contrast, neither *Atoh7* nor *Neurog2* autoregulates in the mouse eye. However, *Neurog2* appears first along the advancing retinal wavefront, and directly activates transcription through an evolutionarily conserved *Atoh7* retinal enhancer (Hufnagel et al., 2007; Skowronska-Krawczyk et al., 2009), so Neurog2 cross-regulation of *Atoh7* could be considered analogous to the Ato autoregulation, or AS-C cross-regulation, that is observed in the fly. Cross-regulation between *Neurog2* and *Atoh7* is potentially mammalian specific, however, because there is no *Neurog2* homolog in zebrafish (Furlong and Graham, 2005), in which initiation of *atoh7* expression depends at a minimum on Hh signaling (Neumann and Nuesslein-Volhard, 2000; Masai et al., 2005).

Could other mechanisms thus be used to replace autoregulation to maintain stable neural fate? Positive autoregulation has not been found in studies of some *Drosophila* neurons, indicating that although the segregation of initiation and autoregulation provides an important conceptual insight into mechanisms of fate specification, similar outcomes might also be achieved by other regulatory mechanisms (Holohan et al., 2006; Zhou et al., 2017). It is possible that transcription factors further downstream are responsible. The Ato-Sens hierarchy that functions in the fly eye is not found in the vertebrate retina, although the *Sens* homolog *Gfi1* works with *Atoh1* during vertebrate auditory and intestinal development (Shroyer et al., 2007; Kirjavainen et al., 2008). Instead, *Atoh7* transcriptionally activates both *Pou4f2* and *Isl1*, which act synergistically to lock in an RGC fate during vertebrate retinal neurogenesis (Wu et al., 2015). Immediately downstream of Pou4f2-Isl1 is a hierarchy of transcription factors [Onecut1 (Oc1), Oc2, Ebf1, 3, Irx2, 5, 8, Myt1] that control integral aspects of the RGC differentiation program (reviewed by Zagozewski et al., 2014). Each factor acts similarly in other parts of the CNS. Some of these genes have homologs that are also active in the fly eye, suggesting that there might be some deep conservation of mechanisms. Indeed, the autoregulatory expression of *ato* in the fly eye is transient, lasting only ∼8 h, and the neural fate of photoreceptor cells must thus be maintained by other genes thereafter.

Although proneural gene autoregulation may not be universal, mouse Atoh1 and Neurog2 proteins do exhibit the conserved phosphorylation event that terminates autoregulation of fly Ato. As mentioned above, the phosphorylation of Ato on a particular serine residue blocks protein-DNA binding, thereby halting stimulation of neurogenesis by all three bHLH proteins (Quan et al., 2016). Curiously, substitution of Ser with Ala in Atoh1 also appears to reduce function (Xie et al., 2017). The bHLH domain of mammalian Atoh7 also possesses the relevant serine, but the importance of this particular protein phosphorylation mechanism in the mouse retina remains unclear for both Neurog2 and Atoh7 (Brown et al., 1998). However, additional phosphorylation events that post-translationally regulate bHLH proneural activity have been described. In the frog retina, GSK3 phosphorylates a distinct C-terminal serine in *Xenopus* Neurod (Moore et al., 2002). This serine is not present in *Xenopus* Atoh7, thereby allowing GSK3 activity to behave as a toggle switch between amacrine and RGC fates. During motor neuron formation in mice, Neurog2 is phosphorylated at particular serine residues, enabling it to complex with the LIM-domain proteins Islet1 and Lhx3 and execute a motor neuron specification program in the ventral spinal cord (Ma et al., 2008). More generally among bHLH proteins, phosphorylation of multiple amino acid residues has increasingly been shown to control key aspects of differentiation (Hardwick and Philpott, 2015; Philpott, 2015; Wylie et al., 2015; Hardwick et al., 2016; Azzarelli et al., 2017; Krentz et al., 2017). Thus, post-translational regulation of multiple bHLH factors, rather than transcriptional cross-regulation, correlates with situations in which neurogenesis occurs rapidly and/or bHLH factors are co-expressed in the same progenitor cell.

Other types of transcriptional cross-regulation can also occur in vertebrates. For example, *Neurog2* and *Ascl1* cross-regulate one another in multiple contexts (Fode et al., 2000). In the developing forebrain, these factors are expressed in distinct dorsal (*Neurog*2) and ventral (*Ascl1*) domains and are thought to instruct separate forebrain identities via similar neural determination programs. Loss of *Neurog2* causes ectopic dorsal *Ascl1* expression, and dorsal misexpression of *Ascl1* is sufficient for induction of ventral-specific markers and fate. Cross-regulation is negative and non-autonomous; the molecular mechanism involves bHLH factor activation of a Notch ligand, which signals to an adjacent cell to downregulate the other bHLH factor, rather than to itself as would be typical for classical lateral inhibition (Kageyama et al., 2008; Shimojo et al., 2008). Because *Neurog2*-mediated suppression of *Ascl1* maintains dorsal identity, functional distinctions between *Neurog2* and *Ascl1* were expected, but surprisingly the ectopic expression of *Neurog2* in ventral forebrain progenitors is unable to induce progenitors to adopt a dorsal fate, although it does generally induce neuronal differentiation (Parras et al., 2002).

In contrast to the forebrain, *Ascl1* and *Neurog2* act sequentially in spinal cord progenitor cells (Scardigli et al., 2001; Parras et al., 2002; Helms et al., 2005). *Ascl1* is expressed by proliferative dorsal interneuron progenitor cells [(dI) 3 and 5 regions], where it is required for specification. *Neurog2* expression follows that of *Ascl1*, and modulates the size of each cell population. Finally, in the ventral midbrain, *Neurog2* and *Ascl1* are co-expressed in dopaminergic neuronal precursors. The specification of these cells requires *Neurog2*, but not *Ascl1* (Andersson et al., 2006), but when *Ascl1* is used to replace *Neurog2* in a gene replacement knock-in mouse model, midbrain dopaminergic neurogenesis is partially rescued (Table 1) (Parras et al., 2002). The overlapping *Neurog2* and *Ascl1* function in the ventral midbrain questions how distinct their functions are in the forebrain and spinal cord, where the similarities are as apparent as the differences.

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How functionally distinct are vertebrate proneural genes?

If the multiplicity of vertebrate proneural genes contributes to the complexity of neural development then this may be revealed by functional differences between proteins. This indeed is sometimes evident. For example, bHLH domains from the AS-C and Ato families prefer to bind to slightly different DNA sequences (Powell et al., 2008). However, even though the Ascl, Atoh and Neurog families diverged long ago and are likely to have the most distinct properties, there is still evidence for interchangeable, and hence shared, proneural functions of *ato* and *sc* in the development of *Drosophila* chordotonal organs (zur Lage and Jarman, 2010). The best test of shared function is the capacity of one protein to substitute for another in knock-in experiments (Table 1). In the most dramatic example of such experiments, *ato* from *Drosophila* substitutes for *Atoh1* in mouse, even in the mouse intestine despite the fact that the fly intestine does not express *ato* (Wang et al., 2002). This is a powerful demonstration that genes long separated by evolution can retain ancestral functions at the protein level, while their regulatory sequences can diverge to allow species-specific expression patterns. It also highlights that distinct expression patterns do not always indicate divergent protein function at the molecular level.

In contrast to *Atoh1*, mouse *Atoh7* shows less rescue of *ato* eye phenotypes in *Drosophila* (Table 1) (Sun et al., 2003; Weinberger et al., 2017). This might reflect loss of ancestral functions by *Atoh7* that are retained by *Atoh1*. However, mouse *Atoh7* cannot even induce RGCs in the frog retina, whereas both *Drosophila ato* and *Xenopus Atoh7* can (Brown et al., 1998; Sun et al., 2003). Because *Xenopus Atoh7* does rescue the *Drosophila ato* eye phenotype partially (Brown et al., 1998; Sun et al., 2003) (Table 1), it appears that the mammalian *Atoh7* gene has lost ancestral functions at some point after the initial duplication and divergence of the *Atoh1* and *Atoh7* genes. A potential test of whether Atoh7 protein has acquired novel functions would be to examine whether fly *ato* can rescue the mouse *Atoh7* retinal defects, as it does for *Atoh1*.

Is it possible that other bHLH genes fulfill shared ancestral functions? Only *Ascl1;Neurod4* double mutants completely lack bipolar neurons, suggesting that both genes contribute overlapping but distinct functions to define these cells. Since *Ascl1* and *Neurod4* are expressed successively, in principle it could be that their expression dynamics are the only reason neither alone is fully sufficient. It should be noted, however, that defining specificities for proneural genes by replacement studies may lead to complicated results. Substituting *Atoh7* for *Neurod1* reprograms retinal cells to adopt an erroneous RGC fate, even though *Neurod1*, together with *Neurod4*, rescues RGC genesis in the absence of *Atoh7* (Mao et al., 2008, 2013) (Table 1). It is unclear whether it is protein expression levels and stability that normally distinguish the functions of Atoh7 and Neurod1, or local environmental cues or additional intrinsic factors modify the outcome of *Neurod1* expression.

In summary, studies that have examined functional differences between vertebrate proneural genes provide evidence for both functional distinctions and for conserved, shared functions, but it is not yet clear to what extent proneural gene number itself contributes to neural diversity.

Conclusions

As we have highlighted (and as summarized in Table 1), functional complementation studies have revealed many differences between proneural bHLH genes but also provide examples of functional similarity. Particularly striking is the functional replacement of *Drosophila ato* and mouse *Atoh1*, despite their evolutionary separation (Ben-Arie et al., 2000; Wang et al. 2002), and the comparative evidence that the complexity of the AS-C might not contribute much to neuronal complexity in insects (Negre and Simpson, 2009). The complex expression and function of the AS-C could have evolved by duplication of progenitor genes similar to *ato*, as could vertebrate proneural gene networks that show some regulatory similarities. It should be noted, however, that whereas a positive outcome in gene replacement argues powerfully for shared functions between proneural proteins, negative outcomes can have multiple explanations. Some distinctions between proneural bHLH genes must be due to their interactions with other regulatory factors, which explains, for example, how genes such as *ato* and *Atoh1* can be responsible for different neuronal cell types in distinct tissues (Kiefer et al., 2005; Huang et al., 2014). There is also increasing evidence that, in addition to transcriptional regulation, the regulation of bHLH protein stability is important (Qu et al., 2013; Kiparaki et al., 2015; Weinberger et al., 2017; Li and Baker, 2018). Also, genes under constraint by multiple mechanisms may behave less effectively in gene replacement experiments.

Although new functions may have been acquired during evolution to enable neuronal diversity, it is also likely that functional attributes that were already present in an evolutionary precursor have been selectively lost. For example, whereas a *Drosophila* Neurog protein lacks Ato function, a sponge gene possibly resembling the Neurog/Ato common ancestor exhibits partial rescue, suggesting that an ancestral function has been lost from the *Drosophila* Neurog protein (Weinberger et al., 2017) (Table 1). At present, only a small minority of possible replacements have been tested in endogenous gene replacement studies, which provide the most reliable results (Table 1), but the availability of new gene editing methods promises more such studies in the future. These studies might clarify the extent to which neuronal complexity relies on a multiplicity of proneural bHLH genes, and how much complexity could already have been encoded by ancient progenitor genes.

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Competing interests

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