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The bHLH genes in neural development

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ABSTRACT Groups of genes sharing similar motifs may be used at different steps of a same developmental process. In this review, we discuss the significance of this phenomenon in the case of the basic Helix-Loop-Helix (bHLH) proteins that are involved at different steps of the development of the peripheral nervous system (PNS) of *Drosophila*.

KEY WORDS: *peripheral nervous system, proneural genes, neuronal differentiation*

Introduction

Most insects are provided with large numbers of sensory bristles, many of which form at precise locations on their body and head. Each bristle is formed by several cells, all of which derive from a common, neurally competent, precursor cell (Bate, 1978). In flies it has been known for a long time that the formation of bristles requires the presence of two adjacent genes, *achaete (ac)* and *scute (sc)*. Mutations in either gene eliminate specific subsets of bristles, while the inactivation of both genes removes nearly all bristles in the adult. The genetic analysis of the *achaete* and *scute* genes has long concentrated on the organization of this locus, in relation to the then essential question of the divisibility of the gene, and the developmental role of these genes was largely left untouched (reviewed in Ghysen and Dambly-Chaudière, 1987).

In his classical paper, entitled "Two or three bristles", Curt Stern (1954) pointed out that the ability to form a bristle is a local property that is manifested not by one, but by a group of cells, and he suggested that this competence results from an underlying "prepattern". He also showed that the actual formation of the precursor cell in response to this prepattern shows a cell-autonomous requirement for *achaete*.

Antonio García-Bellido took up the genetic and developmental analysis of *ac* and *sc* in 1975. The genetic analysis (García-Bellido, 1979) confirmed the existence of a third gene, *lethal of scute (l'sc)*, adjacent to *ac* and *sc*, as already inferred by Raffel and Muller (1940), and suggested that the three genes belong to a complex locus, the *achaete-scute* complex (ASC). The developmental analysis (García-Bellido and Santamaria, 1978) revealed that *ac* and *sc* are required for the formation of nearly all external sense organs of the adult fly, and suggested that *l'sc* is required for the formation of the central nervous system (CNS). Furthermore, the analysis of loss-of-function (LOF) and gain-of-function (GOF) mutations in the *ac-sc* region (García-Bellido, 1979, García-Alonso and García-Bellido, 1986) led to the conclusion that *ac* and *sc* act as selector genes (García-Bellido, 1975) which control the decision to become a sensory precursor cell.

At the same time, Alain Ghysen proposed that the local competence to form a bristle is the consequence of a local expression of the *ac* and *sc* genes (Ghysen and Richelle, 1979; Richelle and Ghysen, 1979), and showed that the formation of ectopic bristles in flies carrying the GOF *Hairy-wing* mutation depends on the dosage of the wild-type *sc* gene, suggesting that the competence to form a bristle reflects the amount of Sc product present. This view has been largely confirmed by the subsequent analysis of the spatial pattern of expression of the *ac* and *sc* genes: both genes are expressed in clusters of undifferentiated ectodermal cells, and confer to these cells the competence to form sense organs (= proneural genes; Cabrera *et al.,* 1987; Romani *et al.,* 1989; Cubas *et al.*, 1991; Skeath and Carroll, 1991; Ruiz-Gomez and Ghysen, 1993).

The elucidation of the patterns of expression of *ac* and *sc* led to a re-appraisal of Stern's prepattern, now envisioned as the distribution of the products of "prepattern genes" that would act as activators and repressors of the *ac* and *sc* genes (Ghysen and Dambly-Chaudière, 1989; for a recent review, see Vervoort *et al.,* 1997).

Proneural genes and competence

The *ac* and *sc* genes perform a proneural function not only for the development of the adult sense organs, but also for the development of the larval sense organs, during embryogenesis

Abbreviations used in this paper: ASC, achaete-scute complex; ASH, achaete-scute homologs; bHLH, basic-helix-loop-helix; E (spl)C, Enhancer-of-split complex; GOF, Gain-of-Function; LOF Loss-of-Functio; PNS, peripheral nervous system; CNS, central nervous system.

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(Dambly-Chaudière and Ghysen, 1987). However, while the formation of all adult external sense organs depends on *ac* and *sc*, some of the embryonic external sense organs were shown to depend on a previously unidentified member of the ASC, *asense (ase)* (Dambly-Chaudière and Ghysen, 1987). This requirement can be partially rescued by an exogenous *ase* genomic fragment (Jarman *et al.,* 1993a), indicating a proper proneural function for *ase*. In contrast to the other ASC genes which are expressed in clusters of cells before the appearance of the precursors, and turned off before the precursor divides, *ase* expression has never been reported in clusters of cells but only in precursors, and is maintained in their daughters (Brand *et al.,* 1993). Since very few larval organs depend strictly on *ase*, a proneural expression of this gene may have been overlooked, however. More surprisingly, it was observed that in the absence of the ASC, cells which may correspond to the *ase*-dependent precursors delaminate and express a precursor-specific gene (Jarman *et al.,* 1993a). It may be, therefore, that *ase* is not required for the formation of precursors but rather for the maintenance of their precursor fate. The analysis of *l'sc* function during central neurogenesis has revealed a proneural function similar to that of *ac* and *sc* for the sense organs, including an expression in proneural clusters (Jimenez and Campos-Ortega, 1990; Martin-Bermudo *et al.,* 1991).

Altogether, it appears that *ac*, *sc* and *l'sc* function as proneural genes, each promoting the formation of a set of precursors. Gain-of-function analyses have shown that all four ASC genes can act as proneural genes, in that the generalized expression of any of them in flies doubly mutant for *ac* and *sc* can rescue the formation of bristles, often in a nearly wild-type pattern (Rodriguez *et al.,* 1990; Dominguez and Campuzano, 1993; Brand *et al.,* 1993; Hinz et al., 1994). Thus the ASC genes can functionally substitute for each other in their proneural capability. The same conclusion had been drawn previously from the analysis of the LOF phenotypes: the LOF phenotype of one gene is increased when additional ASC genes are simultaneously inactivated, and reduced in the presence of extra doses of other ASC genes (García-Bellido, 1979).

In contrast to the external sense organs (es organs), the internal chordotonal organs (ch organs) are not under the control of ASC genes (Dambly-Chaudière and Ghysen, 1987). The ch organs are specified by another bHLH gene, *atonal*, whose protein only shows 50% identity with those encoded by the ASC (Jarman *et al.,* 1993b). Ectopic expression of *ato* promotes the formation of ectopic chordonal organs (in regions where chordotonal organs do form in wild-type), as well as of ectopic bristles (in some regions of the notum; Jarman *et al.,* 1993b). The latter effect is independent of the *ac/sc* products, as it is observed in flies lacking functional *ac* and *sc* genes (Jarman *et al.,* 1993b). Although this effect could result from the activation of *ase* or of *l'sc* gene by the *ato* product, another possibility is that Ato has the capability to promote the formation of either type of sense organ, depending on the region where it is acting.

The specificity of Ato resides in the b region of its bHLH motif, as revealed by domain swapping experiments (Chien *et al.,* 1996). Interestingly, the b regions of Ato and of the ASC proteins differ by only seven residues which, in the Ato protein, seem to project away from DNA, suggesting that the ch vs. es specificity of the bHLH factors depend on an interaction of the b domain with cofactor(s) (Chien *et al.,* 1996).

Antagonizing genes and precursor selection

Other bHLH genes have an antagonistic effect on the earliest steps of precursor formation. They are of two types: the HLH protein encoded by *extramacrochaete* (*emc*; Botas *et al.,* 1982; Ellis *et al.,* 1990, Garrell and Modolell, 1990), which lacks a basic domain altogether, and the bHLH "Hairy family" including the proteins encoded by *hairy* (Rushlow *et al.,* 1989), by at least seven genes of the *E(spl)-C (Enhancer of Split* Complex; Delidakis and Artavanis-Tsakonas, 1992; Knust *et al.,* 1992) and by *deadpan (dpn*; Bier *et al.,* 1992; see below).

In vitro experiments have shown that Emc is able to prevent Ac and Sc from binding to DNA by sequestering them into inactive heterodimers (Van Doren *et al.,* 1991). The pattern of expression of *emc* suggests that it acts as a prepattern gene, by making the regions where it is expressed refractory to the formation of sense organs (Cubas and Modolell, 1992).

The second group of genes may be more directly involved in the singling out of a precursor from among the proneural cells (reviewed in Ghysen *et al.,* 1993). All the proteins of this group have in common the presence of a particular type of b domain which does not bind to the canonical E-box but to a slightly different consensus sequence called N-box (Tietze *et al.,* 1992). Furthermore, they all contain another domain that allows them to interact with the product of the *groucho* gene (Hartley *et al.,* 1988), and thereby to act as transcriptional repressors (Paroush *et al.,* 1994). It is believed that these proteins antagonize the proneural bHLH genes (reviewed in Campos-Ortega, 1993; Jan and Jan, 1993). Indeed, genetic analyses have shown that both *hairy* and *E(spl)-C* genes act through the repression of the production and/or activity of the ASC proteins. Similarly, the analysis of *ac/sc* expression in *dpn* mutants suggests that Dpn may be required to switch off *ac/sc* expression in the precursors before their division (Bier *et al.,* 1992).

Precursor genes and neural fates

In precursor cells, the expression of the proneural genes subsides before the precursor divides. A new panel of genes are then expressed in the precursors, the so-called neuronal precursor genes or pan-neural genes (Jan and Jan, 1993). These genes are expressed in all precursors whatever their origin, and are believed to control neural properties common to all precursors and to some of their progeny. Some neuronal precursor genes encode transcriptional regulators with bHLH domains, like *ase*, which is expressed in all sensory precursors and in all neuroblasts (Brand *et al.,* 1993; Jarman *et al.,* 1993a). Genetic analysis has shown that *ase* mutations alter the differentiation of some sensory organs, e.g., the bristles of the anterior wing margin (Dominguez and Campuzano, 1993). In *ase* mutants, most of the stout mechanosensory bristles of the wing margin are malformed (socketless, shatfless or duplicated bristles). The chemosensory bristles of the wing margin are also abnormal. It has been shown that the corresponding precursors do not divide properly in *ase*⁻ flies (no division or aberrant divisions).

Thus, *ase* seems to fulfil two roles in the PNS: an early *ac/sc* independent role to allow the development of a subset of embryonic external sense organs, and a late role in the differentiation of the precursors of the wing margin bristles, or their progeny. Even

for this second role, the ASC genes can functionally substitute for each other since the wing margin bristle phenotype of *ase* mutants can be rescued by extra doses of the *ac* and *sc* genes (Dominguez and Campuzano, 1993)

The adult *ase* phenotype is restricted to a small subset of sense organs, indicating that, although *ase* is expressed in all precursors, in most of them the absence of *ase* function has no phenotypic consequence (Brand *et al.,* 1993; Jarman *et al.*, 1993a). The same is true for the other known bHLH pan-neural gene, *dpn* (Bier *et al.,* 1992). Much like *ase*, *dpn* is expressed in all precursors but its inactivation produces no obvious morphological defects.

Neuronal differentiation genes

Downstream in the genetic cascade, a new member of the neural bHLH family has recently been identified which is expressed much later, at the time the neuron will differentiate (Bush *et al.,* 1996; Gautier *et al.,* 1997). In the PNS, its expression is transient and restricted to one neuron of each adult and embryonic chemosensory organs (Gautier *et al.,* 1997). We have shown that this specific expression is under the control of *poxn* (Gautier *et al.,* 1997), which specifies the chemosensory lineage (Dambly-Chaudière *et al.,* 1992), and have called this gene *target of poxn (tap)*. The gene has been cloned independently by Bush *et al.* (1996) who called it *biparous (bps)*, as it is expressed in the CNS in specific subsets of both neuronal and glial cells at the time they differentiate. Although no functional analysis has been reported so far, the expression pattern of *tap* suggests that it could control the specific differentiation of the neuronal and glial cells in which it is expressed.

Besides its late expression in the chemosensory neurons, *tap* is also expressed in the antennal disc at the time when the precursors to the olfactory organs form (Ledent *et al.*, 1998). This suggests that *tap* may have, in addition to its potential role in controlling the specific differentiation of one chemosensory neuron, a proneural function for olfactory organs.

Vertebrate bHLH genes

Structural homologs to the bHLH factors acting during Dr*osophila* neurogenesis have been found in vertebrates, where they are also involved in neurogenesis (see Lee, 1997, for a detailed review). The first to be isolated were the ASC genes homologs (ASH): two in rat, mouse and chicken (Johnson *et al.,* 1990; Jasoni *et al.,* 1994) and probably four in *Xenopus* (Zimmerman *et al.,* 1993; Ferreiro *et al.,* 1994).

In *Xenopus*, a gain-of-function analysis of these *ASH* genes revealed a proneural capability (Ferreiro *et al.*, 1994; Turner and Weintraub, 1994). Two of them (*XASH-3a* and *b*) are expressed at very early stages of neural plate formation in a spatially restricted pattern (Zimmerman *et al.,* 1993) and thus may have a function similar to that of their *Drosophila* homologs. The other pair of *XASH* gene is expressed much later (Ferreiro *et al.,* 1994) and is probably involved in other aspects of neural development.

A requirement at late steps of neurogenesis has also been demonstrated for one of the mammalian *ASH* gene, *MASH-1* (Johnson *et al.,* 1991), which is required for the differentiation of a subset of sensory neurons (Guillemot *et al.,* 1993; Sommer *et al.,* 1995). *MASH-2* appears to function only in the formation of extra-embryonic derivatives (Guillemot *et al.,* 1994). Thus neither of the *MASH* genes seems to play any role in the early steps of neurogenesis.

A new family of related bHLH proteins (Neurogenin and NeuroD) has recently been identified in *Xenopus* and mouse (Lee *et al.,* 1995; Ma *et al.,* 1996; Sommer *et al.,* 1996; Lee, 1997). The localized expression of *neurogenin (ngn)* precedes the formation of neuronal precursors, and injection of *ngn* mRNA is sufficient to induce ectopic neurogenesis in *Xenopus* (Ma *et al.,* 1996). Taken together, these observations suggest that n*gn* is a proneural gene. In contrast, the structurally related *NeuroD* genes are expressed at a later step, at the onset of neuronal differentiation and in mature neurons, suggesting that *NeuroD* could control neuronal differentiation (Lee *et al.,* 1995). The only member of the *ngn/NeuroD* family identified in flies so far is *tap* (Gautier *et al.,* 1997).

A growing number of *ato* homologs (ATH) are also known in vertebrates and seem to be involved in early steps of neural development (Lee, 1997). However, there is as yet too little information about the function of these genes to make meaningful comparisons with the role of *ato* in fly neurogenesis. Finally, several *Hairy/E(spl)* genes have been cloned in mammalians and appear to fill repressive functions similar to that of their *Drosophila* counterparts (Ishibashi *et al.,* 1995).

Evolution of the neural bHLH gene function

According to the temporal expression of the bHLH genes in the formation of fly sense organs, it appears that bHLH genes may act at different steps of neurogenesis, from the determinative step where they are involved in the early decision to form neural derivatives, to the differentiative step where they may specifiy neuronal properties. Interestingly, a similar situation is observed in vertebrates, where some bHLH genes (e.g., *neurogenin*, *ngn*) act at early steps of neurogenesis, while others (e.g., *NeuroD)* act at the time the neurons undergo differentiation.

A comparison of the fly and vertebrate coding sequences also reveals the existence of two subfamilies of bHLH domains: the fly *ac-sc* type, which is present in the "ASH" (*achaete-scute* homologs) genes of all vertebrates examined so far, and the vertebrate *ngn - NeuroD* type, which is present in the *tap/bps* gene of *Drosophila*. The fly gene *atonal* is somewhat different from both, and might represent a third family of motifs. The ASH type seems evolutionarily oldest, as it is present in *Hydra* (Grens *et al.,* 1995). Given the relatively large divergence between the two types of bHLH motifs, however, we cannot exclude that *Hydra* also contains a *ngn*-like bHLH gene.

Within each subfamily, one may find early- and late-acting genes, e.g., *ngn* and *NeuroD*, or *ac-sc* and *ase*. Interestingly, the early- and late-acting bHLH genes belonging to one family tend to be expressed in similar regions: in frogs, the late expression of *NeuroD* occurs in regions where *ngn* had been expressed previously (Lee *et al.,* 1995; Ma *et al.,* 1996), and the expression of *ase* in flies follows the expression of *ac* and *sc* (Brand *et al.,* 1993; Jarman *et al.,* 1993a). In the case of *tap*, the situation seems different, since the same gene is expressed both at a very early and at a very late step, although in different organs - in the olfactory and in the gustatory organs respectively (Gautier *et al.,* 1997; Ledent *et al.*, 1998). It must be noted, however, that olfactory and gustatory organs belong to the general family of chemosensory receptors.

One possible interpretation of these results is that, much like in the myogenic bHLH family where early and late genes are sequentially activated in the same cells and progressively lead to the formation of myoblasts (Bate, 1993; Weintraub, 1993), each bHLH family would contain determinative and differentiative genes that would act sequentially.

We envision the evolution of this sequential action of bHLH genes as initiated by an ancestral bHLH gene controlling the various aspects of neuronal differentiation. Slightly impaired versions of this gene may have arisen by duplication and mutation, that set cells on a neural differentiative path but stop short of actually differentiating. This would provide cells with an indelible "proneural" character while still allowing them to proliferate. The activation of the fully active differentiation gene would then mark the end of proliferation and the completion of the differentiation program. Thus determination, or commitment, would be partial differentiation set on by a partially active differentiation gene. A similar evolution might be at the origin of the similar organization of the myogenic family of bHLH genes.

Such a system might be unique to heterodimerizing bHLH genes, because they can interact directly with each other, offering large possibilities of modulating target recognition, and of activating or inactivating each other, thereby imposing a sequential pattern of expression. Alternatively, it may be that a large similarity between the molecular nature of the determined and differentiated stages will be a regular feature of many histotypes.

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