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From proneural genes to the regulation of ciliogenesis

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Much of developmental biology is concerned with the processes by which cells become committed to particular fates in a regulated fashion, whereas cell biology addresses, among other things, the variety of differentiated forms and functions that cells can acquire. One open question is how the regulators of the former process lead to attainment of the latter. "High-level" regulators of cell fate specification include the proneural factors, which drive cells to commit as precursors in the sensory nervous system. Recent research has concentrated on the gene expression events downstream of proneural factor function. Here we summarize this research and describe our own research that has provided clear links between a proneural factor, *atonal* and the cell biological program of ciliogenesis, which is a central aspect of sensory neuron differentiation.

Over many years, Drosophila sensory neuronal development has been the scene for many fundamental discoveries, including the fields of patterning, transcriptional control of neural fate determination, Notch signaling, asymmetric cell division, structure and physiology of sensory cells (reviewed in ref. 1 and 2). Not least of these is the discovery and characterization of proneural genes. These basic-helix-loop-helix (bHLH) transcription factors are necessary and sufficient to induce neural fate commitment in progenitor cells in metazoans.[3] In Drosophila, *atonal* is a key proneural gene that specifies the precursors of several specialized sensory neuron types. These include mechano-sensory chordotonal (Ch) neurons that

are present in internal sense organs that mediate proprioception (Ch organs typically located to respond to joint or body movement), and hearing and gravitaxis (the large Ch array of Johnston's Organ in the antenna).[4] As with other proneural genes, transient *atonal* expression in ectodermal proneural clusters (PNCs) leads to sense organ precursor (SOP) specification via interaction with Notch signaling (lateral inhibition). After commitment, each SOP divides several times asymmetrically to give the 4–5 cells of an individual Ch organ. Typically one of these cells differentiates to form a bipolar Ch neuron while the remaining cells differentiate as support cells. One of these forms the characteristic scolopale 'basket' that houses the distal part of the Ch neuron dendrite (**Fig. 1A and B**).

In addition to their neural commitment role, genetic analyses have shown that proneural factors influence a neuron's subtype identity, so that different proneural factors create neuronal diversity.[3] Thus, *atonal* commits cells to differentiate as Ch neurons, while another proneural gene, *scute*, performs this function for the closely related External Sensory (ES) neurons (**Fig. 1B**).[5] Like Ch neurons, ES neurons are derived from single SOPs that divide to give a bipolar sensory neuron and support cells. Typically, the support cells form a sensory bristle on the surface of the fly for reception of external stimuli such as touch. The Ch and ES developmental programs are very similar but terminal neuronal and support cell differentiation are modified according to the specialized structures and functions required for the two sensory subtypes.
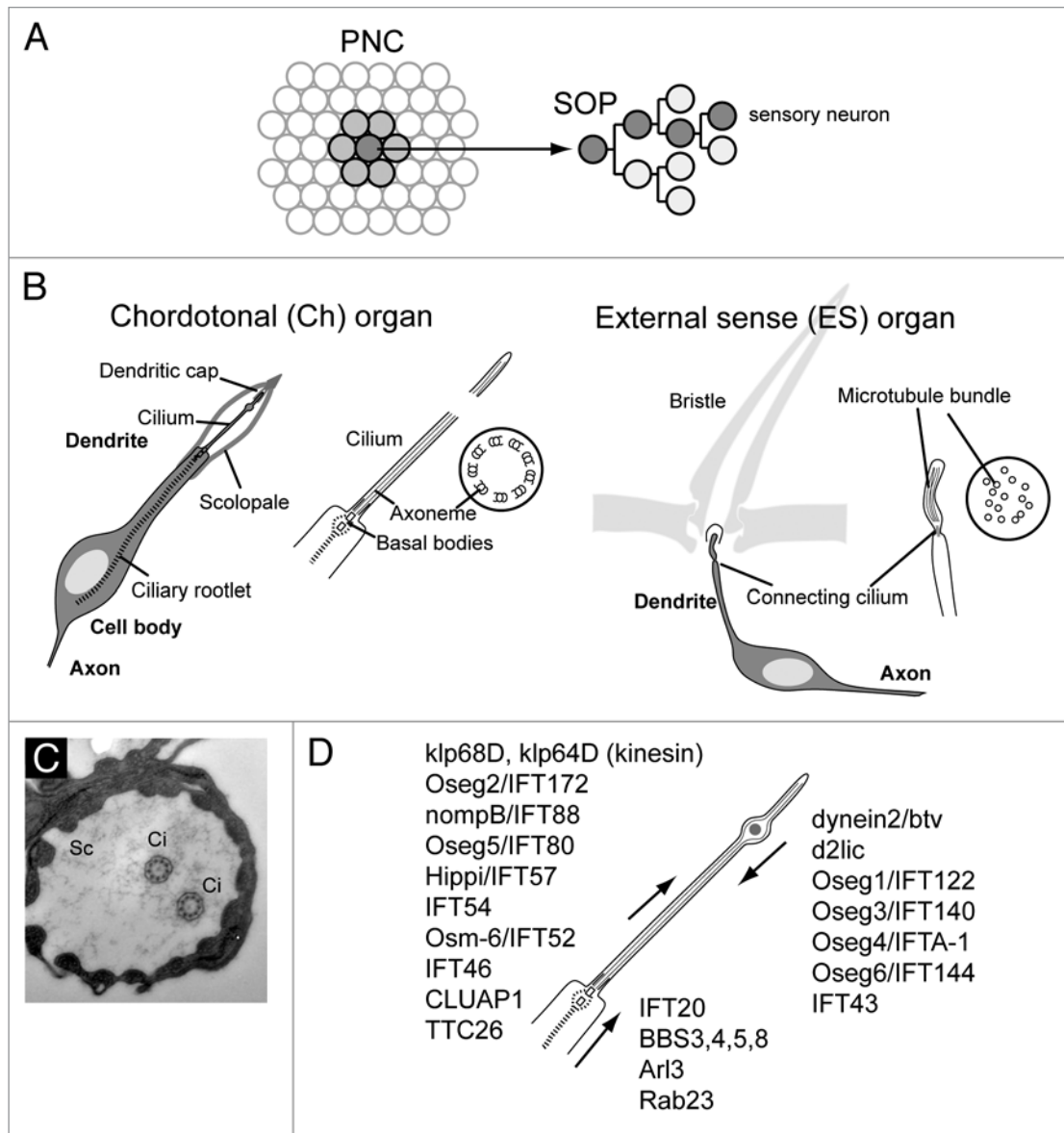
Figure 1. (A) Proneural genes are expressed in proneural clusters (PNC) from which single sense organ precursors (SOPs) are committed following interaction with Notch signaling. The SOP divides several times to give the sensory neuron (or sometimes 2–3 neurons) and support cells of a sense organ. (B) The sensory neurons of Ch and ES organs are similar but have differences in their ciliary dendrites (in addition to support cell differences). In Ch neurons the cilium at the dendrite tip is housed in a scolopale. It is anchored to a basal body and has a regular axoneme of nine microtubule doublets. In ES neurons the cilium is reduced to short segment connecting the dendrite to a sensory structure containing a bundle of disorganized microtubules. (C) The electron micrograph shows a transverse section through a single Ch scolopale of Johnston's Organ in the antenna. In this case the scolopale (Sc) houses two Ch ciliary dendrites (Ci), which are seen in cross section. (D) Some of the conserved ciliogenesis genes present in the early Ch neuron transcriptome. The genes are associated with anterograde IFT (left), retrograde IFT (right) and transport from the Golgi to the base of the cilium (below).

Therefore, proneural genes coordinate the shared developmental programs that lead to neuronal differentiation with distinct processes that govern the acquisition of specialized neuronal subtype properties.[6] While much research has focused on the developmental decision to commit as an SOP, the link between these high-level regulators of neural cell fate and the cellular pathways that ultimately must be activated to construct specialized cells is more obscure.

Transcriptome Analyses of Sensory Neuron Development

Several recent functional genomic and computational biology studies have made substantial progress in determining the genes activated downstream of proneural factors. Reeves and Posakony carried out a microarray analysis of wing disc cells expressing an *E(spl)-m4-GFP* reporter that were isolated by fluorescence-activated cell sorting (FACS).[7] These cells represent mostly *scute*-expressing proneural clusters. Two hundred four (204) genes were

found to be 2-fold or greater expressed in these cells compared with the rest of the wing disc. Twenty-seven (27) genes out of a sample of 43 were verified by in situ hybridization as being expressed in PNCs or SOPs, and several genes were shown to be direct proneural targets. More recently, Buffin and Gho reported preliminary results from a laser microdissection technique for isolating microchaete SOPs from fixed pupae.[8] Microarray analysis allowed the identification of 127 2-fold enriched genes. In both these studies, SOP genes detected included *mira*, *peb*, *neur*, *phyl*, *quail*, *insv*, *CG32150*. Nevertheless, overall there is relatively little overlap between the two gene lists. For instance, of the top 25 genes in the first study, only five were detected in the second. This presumably reflects the different stages targeted (PNC vs. SOP) as well as the different neural cell types (macrochaetae vs. microchaetae), although technical issues may also be involved given the laborious nature of the sample preparation required. A third recent study used transcriptome analysis of whole eye discs combined with computational analysis to discover genes directly downstream of *atonal*.[9] This study suggests that a major function of proneural factors is to manipulate signaling pathways during neurogenesis.

All these studies find new candidate SOP genes that are a rich source for future understanding of neurogenesis. However, these and prior genetic studies (reviewed in refs. 10–13) are largely concerned with regulation of SOP specification or fate maintenance. To provide insight into how subsequent neural development and subtype differentiation are regulated, we followed the *time course* of transcriptome changes downstream of *atonal* function in embryonic Ch cells.[14] GFP-tagged Ch SOPs were purified from staged embryos by FACS. Expression profiling was carried out at 1-hour time points covering the first three hours of Ch neuron development. The ease of obtaining large, synchronized populations resulted in highly robust expression data.[14] At the earliest time point, a large number of known or previously suspected neural genes are present among the 141 genes enriched in Ch cells, including many of the SOP genes found in one or more of the studies above

(such as *cpo*, *nrm*, *phyl*, *cato*, *sens*, *sca*, *nvy*, *spdo, CG32150*). Some caveats should be mentioned. Since Ch cells are compared to the rest of the embryo, the method may under-represent Ch expressed genes that are also expressed in other neural cells, particularly in the far more predominant CNS. Some pan-neural genes (CNS and PNS) are indeed poorly represented (*scrt, dpn*). Nevertheless, pan-sensory genes (PNS-wide) are readily detected in addition to Ch-specific genes (and these can be very highly enriched, such as *CG32150*). Perhaps not surprisingly, only 11% (16/141) Ch-expressed genes are in common with those from wing disc PNC cells,[7] and only 8% are in common with microchaete SOPs. A mere 3.5% of genes are shared between all three datasets.

Over the three time points, there is an increasing representation of genes known or suspected to be involved in the cell biological process of ciliogenesis. This is an important aspect of Ch neuron differentiation. Ch neurons develop a highly structured sensory dendrite based on a modified cilium (**Fig. 1B and C**).[15] Ciliogenesis itself is an important process that is the current focus of intensive cell biological research.[16] In vertebrates, ciliated cells are widespread, both in the PNS (e.g., photoreceptors, olfactory neurons), other adult tissues (e.g., kidney, lung) and embryonic cells, which bear a primary cilium required for paracrine signal transduction.[16] Ciliogenesis is a highly conserved process that begins with the docking of the centrosome at the cell membrane, where it becomes the basal body that nucleates the formation of the microtubule axoneme that forms the core of the cilium. Axoneme extension is coordinated with ciliary membrane expansion. These require the specialised transport process known as Intraflagellar Transport (IFT) [17] and homologues of genes disrupted in the human ciliopathy, Bardet-Biedl syndrome (BBS genes).[20] Many known or suspected ciliogenesis genes have been identified in Drosophila.[17-19] In contrast to vertebrates, the only ciliated cells in Drosophila are sensory neurons and sperm, and mutations in ciliary genes characteristically result in flies that are uncoordinated and male sterile. This restricted tissue distribution of cilia is also the reason why

ciliogenesis genes can be readily detected by Ch transcriptome analysis (**Fig. 1D**).

Clearly, ciliogenesis is one of the key differentiation events that must ultimately be initiated by proneural factors. Moreover, an analysis of ciliogenesis gene regulation also provides potential insights into neural subtype specification. The ciliary dendrite of Ch neurons is anatomically and physiologically distinct from that of ES neurons (**Fig. 1B**).[15,21] This is an important aspect of their neuronal subtype distinction, and therefore subtype-specific variations in ciliogenesis must ultimately be regulated by the different neuronal subtype-determining activities of *atonal* and *scute*.

One might imagine that constructing the distinct Ch and ES ciliary dendrites requires subtype-restricted gene products. To some extent this is true: for instance the Ch cilium uniquely requires the TRPV channel subunits encoded by *nanchung* and *inactive* for sensory reception.[21,22] However, when we examined expression patterns of ciliogenesis genes detected in the transcriptome analysis, another possibility presented itself. Many core ciliogenesis genes (those required universally for cilium formation) are expressed differently in Ch and ES cells. Notably, these genes are transiently expressed in ES cells, but strongly and persistently expressed in Ch cells—the so-called Ch-enriched expression pattern.[14] This suggests that dendrite differences could depend on qualitative differences in the shared ciliogenesis program.

Regulating Ciliogenesis

These observations potentially link functional and structural differences between sensory neurons to differences in the regulation of ciliogenesis genes. This provides an opportunity to connect the developmental high-level regulators to a cell biological pathway required for subtype-specific differentiation. Ultimately, ciliogenesis must be under proneural factor control, and differences in ciliogenesis between sensory neuron subtypes must be due to differences in regulation by *atonal* and *scute*. The transcriptome analysis suggested two key transcription factors as candidates in the regulatory network linking *atonal* to Ch neuron ciliary differentiation. The
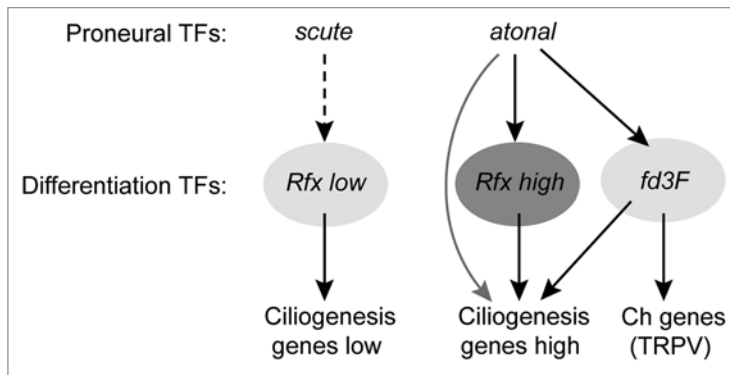
Figure 2. A provisional gene regulatory network linking proneural factors to ciliogenesis. The dashed arrow represents the fact that *Rfx* is not a direct target of *scute*. The grey arrow linking *atonal* to ciliogenesis represents the direct regulation of *dilatory*, but it is unknown whether other ciliogenesis genes may be directly regulated. "Low" and "high" refer to the transient/weak and persistent/strong expression in ES and Ch cells respectively—dubbed the Ch-enriched expression pattern.

first, *Rfx*, is highly expressed in Ch cells at all time points. This highly conserved transcription factor is well known as a regulator of ciliogenesis genes during sensory neuron differentiation.[19,23,24] At the later time point, the Ch transcriptome is highly enriched in known and predicted *Rfx* target genes, including the IFT-B genes. In turn, enhancer analysis showed that *Rfx* is directly activated by *atonal* in Ch cells. Unexpectedly, however, we found that in ES cells *Rfx* is only expressed later and it is unlikely to be directly activated by *scute*. That is, *Rfx* is expressed in a Ch-enriched pattern like many of its ciliogenesis target genes.[14] This suggests that differences in *Rfx* regulation and function may underlie subtype differences in sensory dendrite structure. Thus *Rfx* not only links *atonal* to ciliogenesis, but it also provides a potential link between different proneural factors and ciliary dendrite specialisation in different sensory neurons.

Proneural genes are normally suggested to regulate a combination of pan-sensory (shared ES/Ch) and subtype-specific targets.[3,5] Thus, the Ch-enriched expression pattern of *Rfx* marks it out as a different kind of target gene (in fact its regulation is remarkably similar to that of the *atonal*-related *cato* gene[25]). The pattern suggests that part of *atonal's* subtype function is to "tweak" ciliogenesis for ciliary specialisation. Compared to the well-formed Ch cilium, the ES cilium is strongly reduced, being just a short connecting cilium to the outer dendritic segment (**Fig. 1B**).

Regulation of IFT activity is known to control flagella length in Chlamydomas[26] and we propose that regulation of IFT gene expression is a method by which IFT activity could be regulated in sensory neurons. We also speculate that *Rfx* and its ciliogenesis targets were highly expressed in ancestral multimodal ciliated sensory cells, and then their expression became less prominent in ES neurons when they emerged as a subtype in evolution.

This hypothesis of *Rfx* function remains to be proven, and indeed *Rfx* is not the whole story. The second transcription factor we identified is *fd3F*, a novel specific Forkhead protein that ranks very highly in the Ch transcriptome and is likely to be an *atonal* target (although this is not yet proven). Unlike *Rfx* (and even *atonal*), *fd3F* is completely Ch-neuron specific.[14] Like *atonal* mutants, *fd3F* mutant flies are uncoordinated, but their Ch neurons differentiate relatively normally. However, the TRPV subunits *nan* and *iav* are not expressed, making the Ch neurons nonfunctional. We have now identified several direct target genes of *fd3F* (including *nan* and *iav*) and all appear to be involved in ciliogenesis or ciliary function (Newton F.G. and Jarman A.P, in prep.). It seems that downstream of *atonal*, *fd3F* combines with *Rfx* to tweak ciliogenesis in Ch cells (**Fig. 2**). Interestingly, Forkhead family genes have been linked to ciliary modification in a range of animals (e.g., *Foxj1*).[16,27]

The finding of intermediate transcription factors linking *atonal* to ciliogenesis conforms to the common expectation that proneural factors stand aloof from regulating differentiation itself. Unexpectedly, however, several ciliogenesis genes show very early onset of expression, even beginning in SOPs and definitely preceding overt neural differentiation. We found that *dilatory*, which encodes a component of the ciliary base that is required for ciliary transport,[28] is directly regulated by *atonal* in newly formed Ch SOPs (although later maintenance of *dilatory* expression depends on *Rfx*). This raises the questions: what are ciliogenesis proteins like *dilatory* doing in still-dividing SOPs, and how many other early-expressed differentiation genes are directly regulated by *atonal*? Perhaps direct regulation of differentiation genes reflects the likely ancestral role of proneural factors in directly driving cycling progenitor cells to become postmitotic and differentiate as neurons, a role closer to their function in vertebrates. Concerning the identity of the differentiation targets, there may be a direct relevance to *Atoh1* function in the vertebrate inner ear, where it acts not as a proneural gene but a pro-hair cell gene, the hair cell being a mechanosensory cell that forms a kinocilium during differentiation.[29,30]

Finding Direct Targets

Even with this wealth of expression data, discerning which genes are direct proneural targets is a challenging task. In our case, we took the standard route of reporter gene analysis combined with site-directed mutagenesis of potential E box binding sites. Otherwise, computational identification of proneural protein binding sites is not trivial. For instance, the ES-neuron enhancer of *Rfx* contains two well-conserved Scute-specific E box sequences, but they are likely to be nonfunctional since mutation of both has no effect on enhancer activity (unpublished data). Recently, however, sophisticated computational analyses have been successfully employed toi dentify potential target genes. Rouault et al. used a training set of enhancers from known SOP-expressed genes to identify candidate binding sites and then used these to detect other possible SOP-expressed genes genome-wide.[31]

This resulted in the identification of 11 new SOP enhancers, with many more candidates, and it identified some SOP-associated DNA motifs including E boxes. This promising approach has yet to be combined with transcriptome data. Aerts et al. developed a computational tool, cisTargetX, which they used to discover over-represented sequence motifs associated with genes found to be downstream of *atonal* in the eye disc, which resulted in many new candidate *atonal* target genes.[9] When cisTargetX is employed to find over-represented motifs in our transcriptome data, it returns E boxes at the early time point and X boxes (Rfx binding sites) in the later time point, suggesting many new candidate targets for future analysis (unpublished observations). Whilst computational methods of target detection are improving all the time, these studies would greatly benefit from genome location data, but this is technically challenging due to the small cell populations involved.

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