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Evolution of Developmental Control Mechanisms

Msxb is a core component of the genetic circuitry specifying the dorsal and ventral neurogenic midlines in the ascidian embryo

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ABSTRACT

The tail ascidian larval peripheral nervous system is made up of epidermal sensory neurons distributed more or less regularly in ventral and dorsal midlines. Their formation occurs in two-steps: the ventral and dorsal midlines are induced as neurogenic territories by Fgf9/16/20 and Admp respectively. The Delta2/Notch interaction then controls the number of neurons that form. The genetic machinery acting between the inductive processes taking place before gastrulation and neuron specification at tailbud stages are largely unknown. The analysis of seven transcription factors expressed in the forming midlines revealed an unexpected complexity and dynamic of gene expression. Their systematic overexpression confirmed that these genes do not interact following a linear cascade of activation. However, the integration of our data revealed the distinct key roles of the two upstream factors *Msxb* and *Nkx-C* that are the earliest expressed genes and the only ones able to induce neurogenic midline and ESN formation. Our data suggest that *Msxb* would be the primary midline gene integrating inputs from the ventral and dorsal inducers and launching a pan-midline transcriptional program. *Nkx-C* would be involved in tail tip specification, in maintenance of the pan-midline network and in a posterior to anterior wave controlling differentiation.

1. Introduction

The peripheral nervous system (PNS) is defined as the part of the nervous system outside of the central nervous system (CNS), the brain and the spinal cord in vertebrates. It enables animals to receive a large part of their information from the environment and is thus fundamental to adjusting their behavior based on external cues. In vertebrates, the PNS arises during embryonic development from discrete dorsally located structures, the neural crest and the placodes (Baker and Bronner-Fraser, 2001, 1997). These structures are unique to vertebrates and their emergence is thought to have been instrumental in the switch from passive filter feeding behavior of invertebrates to active predation typical of vertebrates (Glenn Northcutt, 2005; Simoes-Costa and Bronner, 2013). Interestingly, in ascidians that belong to the sister group of vertebrates, rudimentary cranial neural crest and neurogenic placodes have recently been identified (Abitua et al., 2015, 2012). We have previously proposed that the ascidian tail dorsal midline ectoderm could be homologous to the vertebrate caudal neural plate border (Pasini et al., 2006). This midline is a neurogenic territory that gives rise to epidermal sensory neurons (ESNs) that are thought to be mechanoreceptors. It is specified together with CNS early during development (32-cell stage) through an inductive process involving a Fgf ligand. We have recently characterized part of the genetic interactions that lead to the transcription of *Msxb*, a transcription factor essential for dorsal midline fate (Imai et al., 2006; Roure et al., 2014). Following midline fate acquisition, the Notch pathway controls the number of sensory neurons within this neurogenic domain by launching a regulatory cascade involving several proneural genes and a microRNA miR-124 (Akanuma et al., 2002; Chen et al., 2011; Joyce Tang et al., 2013; Pasini et al., 2006).

Interestingly, in the ascidian *Ciona intestinalis*, tail ESNs are not limited to the dorsal midline. They are present along the ventral tail ectodermal midline. Similarly to the dorsal ESNs, they are specified in two steps: a neurogenic midline is induced, then ESNs are selected through the Notch pathway (Pasini et al., 2006). The primary inducer is however different: this is Admp, a Bmp ligand, which acts at gastrula stages. While a ventral neurogenic territory does not exist in vertebrates, it has recently been shown that amphioxus (cephalochordate) possess a similar region that is specified like *Ciona* ventral midline (Bmp signaling defines a ventral neurogenic territory, then Notch controls the number of ESNs) (Lu et al., 2012). While dorsal and ventral midlines have

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different spatial origins and are induced by distinct ligands, they appear to form a uniform tissue as revealed by cell organization and ESNs differentiation. In particular they are characterized by a set of genes, the midline genes, that are uniformly expressed in the midline tissue with no detectable dorso-ventral differences (Pasini et al., 2006). While the genetic circuitry controlling ESNs specification downstream of Notch starts being elucidated (Chen et al., 2011; Joyce Tang et al., 2013), little is known about midline genes with the exception of *Msxb* that has been shown to be essential solely for dorsal midline formation (Imai et al., 2006). In order to better understand neurogenic midline fate acquisition we have analyzed the function and regulation of seven midline transcription factors.

2. Material and methods

2.1. Embryo obtention and manipulation

C. intestinalis type B were provided by the Centre de Ressources Biologiques Marines in Roscoff (EMBRC-France). Embryo obtention, injection and electroporation were performed as described in (Pasini et al., 2006). Staging was described according to (Hotta et al., 2007). Standard control-MO (5′-CCTCTTACCTCAGTTACAATTTATA 3′) and *Msxb*-MO (5′-GGATTCGTTTACTGTCATTTTTAAT-3′) were purchased from GeneTools LLC and were injected at 0.25 to 0.50 mM.

2.2. Gene model identifiers

The genes described in this study are represented by the following gene models in the KH2012 *C. intestinalis* assembly: *Msxb* (KH.C2.957), *Nkx-C* (KH.C1.922), *Klf1/2/4* (KH.C5.154), *Achaete-scute a-like2* (KH.L9.13), *Cagf9/tox* (KH.C3.330), *Orphan bHLH-1* (KH.C7.269), *Dll-C* (KH.C7.770), *Fog* (KH.C10.574), *SoxB2* (KH.S164.12), *ETR* (KH.C6.128) and *KH.C8.111* (KH.C8.111).

2.3. In situ hybridization

Whole mount *in situ* hybridization were performed as previously described (Bertrand et al., 2003). Dig-labeled probes were synthesized from the following clones obtained from *C. intestinalis* cDNA libraries (Gilchrist et al., 2015; Satou et al., 2002): *Msxb* (cien92596), *Nkx-C* (citb089b03), *Klf1/2/4* (ciem823l14), *Achaete-scute a-like2* (cien82323), *Cagf9/tox* (long isoform: cidg851f11, short isoform: cima838k22), *Orphan bHLH-1* (ciem814e10), *Dll-C* (cilv080b23), *ETR* (citb028e11) and *KH.C8.111* (citb002h08). Effects of overexpression were analyzed for each marker on 50 electroporated embryos for at least 2 independent electroporation experiments. Embryos electroporated with pFog > Venus were used as controls (Roure et al., 2007).

2.4. Generation of electroporation constructs

All the genes that we have analyzed are predicted to possess a single transcript encoding a unique protein, with the exception of *Cagf9/tox* where two transcripts are predicted each with a unique transcription start site and first exon. The two transcripts encode a short and a long isoform, the short isoform corresponding to the long isoform deleted of the first 225 amino acids. Overexpression of each isoform was analyzed. Since the short isoform had no effect or an effect similar to overexpression of the long isoform (Fig. S1), only results for the more active long isoform are presented.

Electroporation constructs for overexpression were generated using the Gateway technology (Roure et al., 2007) with the promoter of *Fog* driving expression throughout ectoderm from the 16-cell stage (Pasini et al., 2006; Rothbacher et al., 2007) or with the promoter of *SoxB2* driving expression throughout b-line ectoderm except the b6.5 lineage from the 64-cell stage (Fig. S2). A 1363 bp fragment upstream of *SoxB2* was amplified by PCR from genomic DNA using the primers ataaagtaggctACTAATCTGGTGACACCCACGTTC and caaaagttgggtTGGGGTCGTCGAGTAGATTATACAG to generate the pSoxB2_upstream > nlsLacZ construct. pENTRY clones containing the full coding sequence were obtained from a full-ORF gene collection (Gilchrist et al., 2015) for *Msxb* (cien92596), *Klf1/2/4* (ciem823l14), *Achaete-scute a-like2* (cien82323), *Cagf9/tox* (long isoform: cidg851f11, short isoform: cima838k22) and *Orphan bHLH-1* (ciem814e10). For Nkx-C, the pENTRY clone was generated by amplification of the coding sequence from the clone citb089b03 using the primers aaaaagcaggctcagaaaaaATGGCGCATGCTTGCAATG and agaaagctgggtTTAACGTCGTAAGGTAGTGGG followed by a BP reaction. No cDNA clone was found to contain the entire coding sequence of Dll-C. Two overlapping clones (cilv069c15 and cilv008b23) were used to reconstitute a full ORF using two consecutive PCR amplifications. The first PCRs were done using the clone cilv069c15 and the primers (B1-DllC 5′-ggggacaagtttgtacaaaaaagcaggctcagaaaaaATGAGCGCCTACGGTTACAAC-3′ and DllC-rev 5′-ACGTCACTTCCCATCACGTG-3′) and the clone cilv008b23 and the primers (B2-DllC 5′-ggggaccactttgtacaagaaagctgggtTTATTTTAAGTAACCCGAGTCCC-3′ and DllC-fw 5′-GGCATCTCTGCATCCGTGG-3′). The final PCR was done on the first PCR products using the primers B1-DllC and B2-DllC.

3. Results and discussion

3.1. Dynamic expression of tail epidermis midline transcription factors

By searching the *Ciona* expression databases (Ghost and Aniseed) (Satou et al., 2005; Tassy et al., 2010), we identified seven transcription factors (TF) expressed in tail dorsal and ventral midlines at tailbud stages: the homeobox genes *Msxb*, *Nkx-C* and *Dll-C*, the bHLH factors *Achaete-scute a-like2* and *Orphan bHLH-1*, the HMG encoding gene *Cagf9/tox* and the Zn finger factor *Klf1/2/4*. None of these genes was exclusively expressed in tail midlines. For example, several of them were expressed in trunk epidermis, in regions that will also give rise to ESNs like the palps. *Msxb* was also expressed in the pigment cell forming region of the CNS (Abitua et al., 2012). *Orphan bHLH-1* was expressed in the notochord. Here we will solely describe their expression in forming midlines from gastrula to late tailbud stages when ESNs are post-mitotic and about to differentiate (Figs. 1, 2A, S3, and S4).

3.1.1. Initiation of expression

Careful analysis of the onset of expression of these seven genes led us to distinguish three different behaviors. The first category includes a single gene *Msxb* whose expression in the dorsal midline precursors was initiated very early, at the 64-cell stage (Roure et al., 2014). This expression occurred with no interruption during gastrulation and neurulation (Figs. 1, and S3). Expression in the ventral midline was initiated posteriorly at late gastrula/neurula stages and extended rapidly anteriorly (Figs. 1, and 2B–D). The second category includes *Achaete-scute a-like2*, *Klf1/2/4* and *Cagf9/tox*. Their dynamic of gene activation was similar to *Msxb* but started later: dorsal expression was initiated throughout the dorsal midline at late neurula stages and preceded the posterior to anterior initiation of expression in the ventral midline (Fig. 1). The sequence of gene activation is as follows: *Achaete-scute a-like2* and *Klf1/2/4*, followed by *Cagf9/tox*. The third category includes *Nkx-C*, *Dll-C* and *Orphan bHLH-1* that were sequentially activated at the tip of the tail epidermis at late gastrula/neurula stages. The