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Developmental expression of the three *iroquois* genes of amphioxus (*BfIrxA*, *BfIrxB*, and *BfIrxC*) with special attention to the gastrula organizer and anteroposterior boundaries in the central nervous system

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ABSTRACT

Here we describe the developmental expression of the three *iroquois* genes (*BfIrxA*, *BfIrxB*, and *BfIrxC*) of amphioxus. *BfIrxB* transcription is first detected at the gastrula stage in mesendoderm just within the dorsal lip of the blastopore (a probable homolog of Spemann's organizer) and in ectoderm. In early neurulae, expression begins in presumptive pharyngeal endoderm, somitic mesoderm, and neural plate. Mid-neurulae express *BfIrxB* throughout the hindbrain, posterior somites, pharyngeal endoderm, and notochord. In early larvae, expression is largely downregulated in the nerve cord, somites and notochord, but remains strong in the pharyngeal endoderm associated with the forming gill slits; also, a late expression domain appears in the ciliary tuft ectoderm. *BfIrxA* and *BfIrxC*, are not as widely expressed as *BfIrxB*. Both are first expressed in the presumptive hindbrain and presumptive pharyngeal endoderm at the early neurula stages. In the mid-neurula, additional expression domains appear in the extremities of the notochord. Neural expression is downregulated by late neurula. In the early larva, expression is chiefly limited to pharyngeal endoderm associated with the forming gill slits, excepting a small new domain of *BfIrxC* (not *BfIrxA*) expression in the ciliary tuft ectoderm. In comparison to developing vertebrates, embryos and larvae of amphioxus express *iroquois* genes in fewer tissues. Thus, *iroquois* genes of the proximate ancestor of the vertebrates evidently assumed numerous new roles during vertebrate evolution, including the division of the central nervous system into several sub-regions along its anteroposterior axis.

1. Results and discussion

In some *Drosophila* mutants, lateral sensory bristles fail to form on the thorax, leaving a dorsal ridge of bristles like a "Mohawk" haircut—this mutation was named "iroquois" after one of the tribes of Mohawks (Dambly-Chaudière and Leyns, 1992; Leyns et al., 1996). When *iroquois* genes were characterized (Gómez-Skarmeta et al., 1996), they were found to code for proteins with a distinctive 63-amino-acid homeodomain of the TALE (= three amino acid loop extension) superclass, which was subsequently demonstrated to be widespread in fungi, plants and animals. In comparison to other proteins encoded by TALE superclass genes, iroquois proteins are distinguished by an IRO box of about a dozen characteristic amino acids (Bürglin, 1997). By now, *iroquois* (*iro/irx*) genes are known from many multicellular animals, but not other organisms (Irimia et al., 2008; Larroux et al., 2008; Mukherjee and Bürglin, 2007; Perović et al., 2003). In general, basal animal phyla, like placozoans and cnidarians, have only a single *iroquois* gene, whereas many more derived invertebrates have three such genes (two exceptions are echinoderms and hemichordates, which have only one *iroquois* gene). The trio of *iroquois* genes in many invertebrates evidently resulted from two rounds of tandem gene duplication independently in each phylum (Irimia et al., 2008). Finally, during vertebrate evolution, whole genome duplications have produced multiple trios of these genes (Peters et al., 2000).

Triplicate *iroquois* genes are unusual in that, after duplication, the coding regions of the copies tend to be more persistent than would be predicted by the "birth-and-death" model proposed for

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multigene families by Nei and Rooney (2005). In contrast, the duplicated regulatory regions tend to be simplified and rearranged into a single control region that apparently directs transcription by all three structural genes. This unitary control may help keep the duplicated coding regions of *iroquois* genes tightly clustered (de la Calle-Mustienes et al., 2005; Irimia et al., 2008). Studies of *Drosophila* and vertebrate *iroquois* genes show that they typically help divide the body into large compartments with distinct borders early in development and later participate in subdividing these compartments by influencing the development of cell groups within them (Cavodeassi et al., 2001).

In the present study, we describe the developmental expression of the three *iroquois* genes in the invertebrate chordate, amphioxus, which is considered to be the most basal living representative of the phylum Chordata (Delsuc et al., 2006) and the best available stand-in for the proximate ancestor of the vertebrates. We find that amphioxus *iroquois* genes are transcribed in all three germ layers, but in only a relatively few tissues. In comparison, vertebrate *iroquois* genes are expressed in a strikingly broad spectrum of developing tissues (Houweling et al., 2001; Lecaudey et al., 2005), reflecting wide-spread cooption of this gene family to help direct the development of new structures arising during vertebrate evolution.

1.1. Developmental expression of *BfIrxB*

Expression of *BfIrxB* is first detected by in situ hybridization at mid-gastrula (Fig. 1A and B), strongly in the dorsal mesendoderm just within the dorsal blastopore lip (the likely homolog of Spemann's organizer according to Yu et al. (2007)) and more weakly in the ventroanterior ectoderm. Later in the gastrula stage (Fig. 1C), transcription in the mesendoderm is down-regulated middorsally leaving expression domains dorsolaterally on either side of the midline; in addition, strong expression begins dorsally in the ectoderm where the neural plate will soon form. By the early neurula stage (Fig. 1D–J), transcription continues in the neural plate, in the ventroanterior ectoderm and in the nascent somites; in addition, a new expression domain appears in the ventroanterior mesendoderm. In the hatching neurula (Fig. 1K and L), there are expression domains in the neural plate, in the ventroanterior endoderm, and in the mesoderm in association with the most posterior somites. By the early mid-neurula stage (Fig. 1M–Q), expression continues in the neural plate, the posterior somitic mesoderm, and the ventroanterior endoderm; in addition, there is expression in the newly formed notochord. Double in situ hybridization shows that the anterior and posterior limits of the neural expression domains of *BfIrxB* and *AmphiOtx*, respectively, overlap somewhat throughout the neurula stage (e.g., Fig. 1Q). In the late neurula (Fig. 1**R–V**), *BfIrxB* expression continues in the notochord, in the posterior somites, in the pharyngeal endoderm (strongest on the right side), and in the neural tube (where the neural expression is now limited to a few scattered cell groups). By the early larval stage (Fig. 1W–Y), conspicuous expression continues in the pharyngeal endoderm in regions where the gill slits will soon open. Notochordal expression is restricted to its posterior extremity, and neural expression is no longer detectable. A small new expression domain has appeared in the ectoderm that is associated with the ciliary tuft near the ventroanterior rim of the larval mouth.

1.2. Developmental expression of *BfIrxA* and *BfIrxC*

BfIrxA and *BfIrxC*, the two most closely related *iroquois* genes in amphioxus (according to the gene tree of Irimia et al. (2008)) are expressed in domains that are almost congruent with *BfIrxB*, although the last is also transcribed in three other domains (namely the gastrula organizer, the somitic mesoderm, and the ectoderm bearing the ciliary tuft). The earliest expression of *BfIrxA* is detectable by in situ hybridization in the neural plate at the early neurula stage (Fig. 2A). In the hatching neurula (Fig. 2B), neural plate transcription continues, and a second expression domain appears in the ventroanterior mesendoderm. By the late neurula (Fig. 2C), the neural expression is weakening in the neural tube and at either end of the notochord, but remains strong in the pharyngeal endoderm. In the early larva (Fig. 2D and E), weak expression remains at the anterior end of the notochord, and conspicuous expression continues in the pharyngeal endoderm in the region of the forming gill slits and in a group of ectodermal cells associated with the ciliary tuft; however, transcription is no longer detectable anywhere in the neural tube.

BfIrxC expression, which is not detectable in the mid-gastrula (Fig. 2F), first appears in two domains in the early neurula (Fig. 2G–I)—the neural plate and the ventroanterior mesendoderm. The same neural and mesendodermal expression domains are still present and are more prominent in the hatching neurula (Fig. 2J and K). By the early mid-neurula (Fig. 2L–N), expression is conspicuous in the pharyngeal endoderm, but is reduced to a relatively few cells in the nerve cord; moreover, the posterior region of the notochord has started to express the gene. In the late neurula (Fig. 2O), the pharyngeal and notochordal expression continue, but the neural expression has almost disappeared. By the early larval stage (Fig. 2P and Q), the only strong expression is in the pharyngeal endoderm associated with the forming gill slits, although there is moderate expression in some of the coelomic epithelial cells.

1.3. Discussion

This discussion compares *iroquois* gene expression between amphioxus and vertebrates. A broader comparison would be problematical, first because expression of *iroquois* genes has yet to be studied in most invertebrates and second because, in the most extensively studied invertebrate, *Drosophila*, these genes are involved only in ectoderm development, whereas they are expressed in all three germ layers of amphioxus and vertebrates. Even so, *iroquois* genes of amphioxus are involved in the development of markedly fewer tissues than are the homologous genes of vertebrates. For example, although the vertebrate pronephros, diencephalon and heart express *iroquois*, the corresponding organs of amphioxus do not. *Iroquois* genes are also expressed in several vertebrate-specific structures (limb buds, digits, neural crest, telencephalon, vibrissae, mammary gland, and lungs) (Houweling et al., 2001). Expression domains that amphioxus and vertebrates have in common are the organizer region of the gastrula, the somitic mesoderm, the notochord, the pharyngeal endoderm, and the hindbrain (Bosse et al., 1997, 2000; Cohen et al., 2000; de la Calle-Mustienes et al., 2005; Glavic et al., 2001; Lecaudey et al., 2005; Tan et al., 1999; Wang et al., 2001). Vertebrate *iroquois* genes have received the most attention for their developmental roles in the gastrula organizer and the developing central nervous system (CNS).

The present study adds *iroquois* to the suite of genes known to be expressed in the organizer of the amphioxus gastrula (Langeland et al., 2006; Yu et al., 2007). At the gastrula stage of both *Xenopus* (Glavic et al., 2001) and amphioxus the expression zones *of iroquois*, *chordin*, *goosecoid*, *lim-1* are virtually congruent. Although the function of amphioxus organizer genes has not been studied, their expression patterns are similar to those in vertebrates (Yu et al., 2007), suggesting that most, if not all, amphioxus organizer genes have similar functions as their vertebrate counterparts. Specifically, *Xenopus Xiro*-1 induces organizer-specific genes (*chordin*, *goosecoid* and *Xlim-1*) and acts as a context-dependent repressor of ventral marker genes (*bmp-4*, *Xwnt-8*, *Xvent-1*) (Glavic et al., 2001).