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Analysis of transcriptional codes for zebrafish dopaminergic neurons reveals essential functions of Arx and Isl1 in prethalamic dopaminergic neuron development

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ABSTRACT

Distinct groups of dopaminergic neurons develop at defined anatomical sites in the brain to modulate function of a large diversity of local and far-ranging circuits. However, the molecular identity as judged from transcription factor expression has not been determined for all dopaminergic groups. Here, we analyze regional expression of transcription factors in the larval zebrafish brain to determine co-expression with the Tyrosine hydroxylase marker in dopaminergic neurons. We define sets of transcription factors that clearly identify each dopaminergic group. These data confirm postulated relations to dopaminergic groups defined for mammalian systems. We focus our functional analysis on prethalamic dopaminergic neurons, which co-express the transcription factors Arx and Isl1. Morpholino-based knockdown reveals that both Arx and Isl1 are strictly required for prethalamic dopaminergic neuron development and appear to act in parallel. We further show that Arx contributes to patterning in the prethalamic region, while Isl1 is required for differentiation of prethalamic dopaminergic neurons.

Introduction

Dopaminergic (DA) neurons modulate circuits controlling a wide array of behaviors as well as important aspects of physiology (Bjorklund and Dunnett, 2007; Iversen and Iversen, 2007). Most research focuses on the development of DA neurons of the ventral midbrain in mammals, because the degeneration of substantia nigra neurons causes Parkinson's disease (Ang, 2006; Smidt and Burbach, 2007). However, DA neurons develop in mammals, like in other vertebrates, in several distinct clusters at specific anatomical locations in mes-, di- and telencephalon (Smeets et al., 2000; Bjorklund and Dunnett, 2007). While signaling and transcription factors regulating dopaminergic neurons of substantia nigra and ventral tegmental area have been intensively studied (Prakash and Wurst, 2006), much less is known about the developmental control of other DA groups in the forebrain. In mammalian fore- and midbrain, a total of 10 major populations of DA neurons develop in the olfactory bulb (group A16), in the inner nuclear layer of the retina (A17), in the diencephalon (A11-15), and in the mesencephalon (A8-A10) (Hökfelt et al., 1984). The diencephalic DA groups are distributed in nuclei of the dorsal thalamus (A11), tuberal hypothalamus (A12), prethalamic zona incerta (A13), and preoptic area/rostral hypothalamus (A14, A15). More detailed studies will be required to identify not only anatomical features but also molecular characteristics of these DA groups. Several modes of DA transcriptional specification may be envisioned. First, complex regional transcriptional codes, different for each DA group, may independently converge towards the specification of the DA neurotransmitter phenotype, for example, through multiple independent enhancers in DA differentiation genes. Alternatively, DA group-specific sets of distinct members of defined families of transcription factors may converge on regulatory elements. Evolutionary conservation of some factors specifying DA neurons from *Caenorhabidtis elegans* to human (Flames and Hobert, 2009) has nurtured the quest to identify regulatory code elements common to DA or, more generally, monoaminergic differentiation (Flames and Hobert, 2011). Further studies of DA development in different brain regions and throughout vertebrate evolution are needed to uncover common mechanisms, which may also help to discover group specific features controlling individual projection behaviors and physiological traits.

Some DA groups are evolutionary conserved, e.g. those in the olfactory bulb and the amacrine cells in the retina are present in zebrafish and mouse. Other prominent systems have undergone anatomically significant changes in the course of evolution, e.g.

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the mesdiencephalic system of mammals, which is absent in zebrafish, or the zebrafish pretectal system, which is not formed in the mature mouse brain. The zebrafish has emerged as a widely used teleost model to study catecholaminergic and specifically DA systems organization and development, because it is highly amenable to genetic, experimental embryological and pharmacological analysis (Rink and Wullimann, 2002; Panula et al., 2010; Schweitzer et al., 2011; Yamamoto and Vernier, 2011). Using marker proteins for the detection of catecholaminergic (Tyrosine hydroxylase TH - two paralogous genes, *th* and *th2*), dopaminergic (Dopamine transporter *dat/slc6a3*), and noradrenergic neurons (Dopamine beta hydroxylase *dbh*), all catecholaminergic groups have been mapped anatomically from embryonic through larval to adult stages (Holzschuh et al., 2001; Candy and Collet, 2005; Chen et al., 2009; Filippi et al., 2010; Yamamoto et al., 2010). Furthermore, projection patterns of all these dopaminergic groups have been characterized in larval zebrafish (Tay et al., 2011). Zebrafish DA groups corresponding to most mammalian groups can be identified: a cluster of groups termed DC2, 4, 5, 6 in the ventral diencephalic posterior tubercular and hypothalamic areas, all of which depend on the Otp transcription factor activity (corresponding to A11; (Ryu et al., 2007); a prethalamic group (termed DC0/1—potentially corresponding to A13); medial (DC3) and caudal (DC7) hypothalamic groups (corresponding to A14 and A12); preoptic DA neurons (A15); an olfactory bulb (A16) and amacrine retinal group. In addition, zebrafish have a pretectal DA group, which has no corresponding structure in adult mammals, but a pretectal DA group has been reported in human embryogenesis (Puelles and Verney, 1998). Finally, zebrafish have an endogenous subpallial dopamine system, which may correlate with a small number of DA neurons reported in the primate striatum (Betarbet et al., 1997). While zebrafish lack a direct anatomical counterpart of mammalian A8–A10 groups and have no DA neurons in the mesencephalon, it has been discussed whether the teleostean diencephalic ascending system (Rink and Wullimann, 2001), or the endosubpallial system (Tay et al., 2011), may substitute some of the mesostriatal circuit function.

At the molecular level, a potential conservation of pathways involved in specification and differentiation of mammalian DA neurons has been investigated in zebrafish by analyses of expression and function of transcription factors previously shown to be essential for specific DA groups in mammals. With respect to Nurr1/Nr4a2, Pitx3 and Lmx1b, which are crucial in mammalian mesdiencephalic development (Smidt and Burbach, 2007), none of these transcription factors appeared to be expressed by zebrafish ventral diencephalic DA neurons establishing ascending projections (Filippi et al., 2007). Furthermore, morpholino knockdown analyses failed to reveal a function for Nr4a2, Pitx3 and Lmx1b in the development of zebrafish neurons with ascending projections (DC2,4–5 groups; Filippi et al., 2007; but see Blin et al., 2008; Luo et al., 2008). However, Nr4a2 is co-expressed in and required for differentiation of preoptic, pretectal, and amacrine DA neurons in zebrafish (Filippi et al., 2007). Other studies revealed that DC2,4–6 groups depend on the expression of the transcription factor Orthopedia (Otp) in regard to their specification and/or differentiation (Del Giacco et al., 2006; Ryu et al., 2007). The analysis of Otp mutant mouse embryos revealed that A11 group DA neurons do not differentiate in the absence of Otp function (Ryu et al., 2007), suggesting that zebrafish DC2,4–6 DA groups are homologous to the mammalian A11 group. This idea is also supported by the similar projection behaviors of Otp-dependent/A11 DA neurons in zebrafish and mouse, which in both species integrate ascending telencephalic and descending diencephalospinal projections (Bjorklund and Skagerberg, 1979; Takada et al., 1988; Takada, 1993; Tay et al., 2011).

Thus, while an anatomical framework has been established and some conserved molecular mechanisms have been identified that link teleostean and mammalian DA group specification, we are far away from knowing DA group specific transcriptional codes. The comparison of zebrafish and mammalian systems would enable identification of core transcriptional regulators, and help to elucidate the regulatory logic. It may be possible that different members of the same core classes or families of factors accommodate analogous functions in the distant brain regions in which DA neurons develop, or completely distinct codes may converge on regulation of transmitter, electrical and projection phenotypes. Even in the second case, evolutionary comparison should help to identify core mechanisms for each DA group. In this study, we used previous knowledge from mammalian systems, as well as gene expression data from public databases, to identify transcription factors co-expressed in each of the DA groups of the early larval zebrafish brain. As result, we present molecular identity profiles for each DA group, which support evolutionary relations of these groups to mammalian systems. We then focus our attention on the prethalamic group of DA neurons, which has not been previously studied in zebrafish. Using knockdown antisense technology, we demonstrate that the transcription factors Arx and Isl1, which are co-expressed in mature prethalamic DA neurons, are also required for differentiation of the DA phenotype.

Materials and methods

Zebrafish maintenance

Zebrafish were kept and bred according to standard procedures (http://zfin.org). Fertilized eggs were raised at 28.5 °C in medium containing 0.2 mM phenylthiourea in order to inhibit pigmentation. Embryos were staged according to (Kimmel et al., 1995).

Whole mount in situ hybridization and immunofluorescence

For whole mount in situ hybridization (WISH) expression analysis, digoxigenin-labeled antisense riboprobes were generated for each gene (Table 1). To characterize the phenotype of the morphant embryos, the following additional neuronal markers were used: *ascl1a* (Allende and Weinberg, 1994), *dat/slc6a3* (Holzschuh et al., 2001), *ddc* (probe based on RefSeqNM_213342.1 nucleotides 1063–2377), *elavl3/HuC* (Good, 1995), *gad1/gad67* and *gad2/gad65* (Martin et al., 1998), *vglut2a/slc17a6b* and *vglut2b/slc17a6a* (Higashijima et al., 2004). Standard colorimetric in situ hybridization was performed (Hauptmann and Gerster, 1994). Fluorescent in situ hybridization (FISH) was carried out as described (Filippi et al., 2007).

After completion of the whole mount FISH, embryos were equilibrated in PBTD (Phosphate buffered saline; 0.1% Tween20; 1% DMSO) for 10 min, and blocked for at least 1 h in blocking solution (PBTD; 10% goat serum; 1% protease-free Bovine Serum Albumin) at room temperature. They were then incubated in fresh blocking solution containing a rabbit polyclonal anti-TH primary antibody (1:500 dilution; Ryu et al., (2007)), overnight at 4 °C. This antibody has been shown to bind specifically to TH (TH1) and not to the paralogous TH2 (Filippi et al., 2010). The following day, the embryos were extensively washed with PBTD containing 1% goat serum, and subsequently incubated in blocking solution containing a goat anti-rabbit Alexa555-conjugated secondary antibody (1:1000 dilution; Molecular Probes), overnight at 4 °C. After washing out the secondary antibody with several changes of PBTD, the embryos were equilibrated in 80% glycerol, and mounted for imaging.