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Nr4a2 is essential for the differentiation of dopaminergic neurons during zebrafish embryogenesis

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ABSTRACT

Nr4a2 is a member of the orphan nuclear receptor gene superfamily, which has been found to be critical for the development and maintenance of mesencephalic dopaminergic (DA) neurons. To uncover the molecular mechanisms by which Nr4a2 contributes to the development of DA neurons, we have applied zebrafish to study the topographic distribution of *nr4a2b* transcripts, as well as its correlation with neuronal progenitor marker (*neurogenin 1*) and DA neuron markers (*tyrosine hydroxylase*, *TH* and *DA transporter*, *DAT*) during neurogenesis. Our studies showed that although *nr4a2b* transcripts did not co-localize with *TH* and *DAT* transcripts in the posterior tuberculum (PT area), knockdown of Nr4a2 resulted in a significant decrease of TH^+ and DAT^+ DA neurons in the PT area, accompanied by a reduction of DA transmitter, which were partially rescued by the injection of mouse *Nr4a2* mRNA. Surprisingly, the number of $nr4a2b^+$ cells in Nr4a2-deficient embryos was increased by 1.6 fold. These results suggest that Nr4a2 may play an important role in the differentiation and maturation rather than the survival of DA progenitors in the PT area during zebrafish early embryogenesis.

Introduction

The catecholamine neurotransmitter dopamine (DA) plays a central role in the control of voluntary movement, cognition, and emotive behaviors in mammals (Lerner et al., 1994). DA neurons arising from substantia nigra pars compacta (SNc) and part of ventral tegmental area (VTA) project to the striatum to regulate locomotion (Goridis and Rohrer, 2002). While the physiological significance and clinical relevance (e.g., Parkinson's disease, PD) of midbrain DA neurons are well-recognized, the molecular mechanisms that regulate DA neuron development and homeostasis are poorly understood.

The development of mouse midbrain DA neurons is initiated at embryonic day 10 (E10) in the ventral–lateral neural tube adjacent to the floor plate and is regulated by the floor plate-derived morphogenic signal, sonic hedgehog (Shh) (Hynes et al., 1995; Wang et al., 1995). DA progenitors migrate to the ventral midbrain along the dorsal–ventral axis and express different genes such as Mash1, Neurogenin 2 (Ngn2) and Nr4a2 during different stages of development (Kele et al., 2006; Lo et al., 1998; Zetterstrom et al., 1997). These ventralized cells further differentiate to adopt specific cell fate along the anterior–posterior axis (Hynes et al., 1997). At the level of midbrain, this differentiation leads ultimately to the expression of DA synthetic enzymes including tyrosine hydroxylase (TH, a rate-limiting enzyme in DA synthesis) and DA transporter (DAT, a protein involved in DA reuptake). The expression of these genes in the ventral midbrain at E11.5 marks the establishment of a DA phenotype in progenitor cells (Solberg et al., 1993).

In vertebrate, a number of regulators have been identified to play an important role in either early midbrain DA progenitor commitment (Fezl, Engrail1/2, Ngn2, Lmx1a) (Andersson et al., 2006; Ang, 2006; Kele et al., 2006; Levkowitz et al., 2003), or specification (FoxA1, FoxA2, Nr4a2, Wnt-1, Wnt-5a, Pitx3) (Ang, 2006; Castelo-Branco et al., 2006; Ferri et al., 2007; Smidt et al., 2000). However, the mechanisms responsible for the differentiation from DA progenitors to mature DA linage remain elusive.

Nr4a2 is predominantly expressed in the central nervous system, especially in the SNc, VTA, and limbic areas (Zetterstrom et al., 1996). Numerous studies have demonstrated that Nr4a2 is essential for both development and terminal differentiation of ventral mesencephalic DA progenitors into a complete DA phenotype (Joseph et al., 2003; Saucedo-Cardenas et al., 1998). Defects in *NR4A2* gene or altered expression of this gene in SNc have been found in association with PD and certain psychiatric disorders (Le et al., 2003). Mice with a targeted deletion of the *Nr4a2* gene developed poor motor function and die

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1 day after birth, due to the lack of DA neurons in SNc and VTA, but not in other parts of the central neural system such as diencephalon, hypothalamus and olfactory bulb (OB) (Le et al., 1999; Zetterstrom et al., 1997). However, the molecular mechanism and signal pathway of agenesis of DA neurons are still unclear.

Multiple members of the *Neurogenin* family including *Neurogenin 1* and *Neurogenin 2* have been shown to play an overlapping role in regulating neuronal specification during mice DA neuron differentiation (Kele et al., 2006; Ma et al., 1996; Sommer et al., 1996). In zebrafish, *neurogenin 1* (*neurog1*) has been found to be expressed in the neuronal progenitors to regulate the development of spinal, cranial sensory, epiphysial neurons and DA neurons (Andermann et al., 2002; Blader et al., 2004; Cau and Wilson, 2003; Cornell and Eisen, 2002; Jeong et al., 2006).

The DA neurons are first detected in the basal forebrain of zebrafish embryos at ~24 hour post fertilization (hpf), as demonstrated by the expression of TH and DAT (Guo et al., 1999; Holzschuh et al., 2001; McLean and Fetcho, 2004). These DA neurons subsequently protrude ascending and descending projections, and are believed to be homologous to mammalian DA neurons in the basal forebrain and midbrain (Rink and Wullimann, 2001).

In the present study, we have cloned one of the zebrafish homologues of human *NR4A2* and analyzed the topographic distribution of the gene in the forebrain during zebrafish embryogenesis. This homologue of human *NR4A2* is the same gene as *nr4a2b*, which was reported by Filippi et al. (2007). Our results showed that although *nr4a2b* transcripts did not co-localize with *TH* and *DAT* in the posterior tuberculum (PT area), it is partially co-expressed with *neurog1*. Knockdown of Nr4a2 caused a decrease in the number of TH^+ and DAT^+ neurons, accompanied by a simultaneous increase of $nr4a2b^+$ cells. These data suggest that Nr4a2 may play an important role in regulating differentiation and maturation rather than survival of DA progenitors in the PT area during early zebrafish embryogenesis.

Results

Cloning and structural characterization of zebrafish nr4a2b

To clone the zebrafish homologues of mammalian *Nr4a2*, we used the BLAST program to search the zebrafish genome database (http://www.ensembl.org/Danio_rerio/index.html). We identified two homologues of mammalian *Nr4a2* as reported recently (Filippi et al., 2007), named *nr4a2b* on chromosome 6, whose genomic organization is similar to that of human *NR4A2* (Fig. S1A), and *nr4a2a*, whose genomic position is so far unavailable. The zebrafish Nr4a2b protein (586 amino acids) shares approximately 82% similarities with its mammalian counterpart (Fig. S1B), and has a conserved phylogenetic relationship among species (Fig. S1C). In human, there are three genes in the vicinity of the *NR4A2* locus: *KCNJ3*, *NR4A2* and *GPD2*, which together define a 2 Mb genomic region on chromosome 2q22–q23 (Fig. S1D, left). By using the "reciprocal best hit" method (Barbazuk et al., 2000; Liu et al., 2002), we identified zebrafish expressed sequence tags (ESTs) that are the respective homologues of these three human genes: *gpd2*, *nr4a2b* and *kcnj3*. Each of the homologues is located within the same region of zebrafish chromosome 6 (Fig S1D, right). The observation that genomic synteny is conserved across this group of genes indicated that *nr4a2b* is one of the zebrafish homologues of human *NR4A2*.

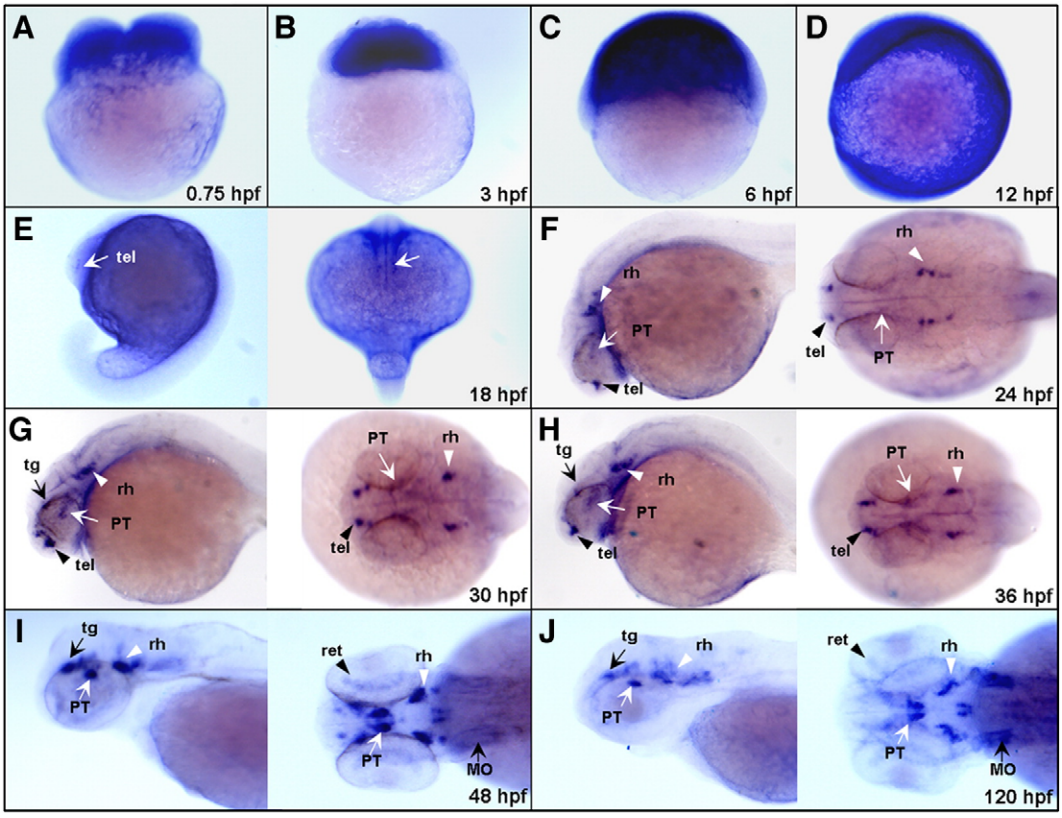

Fig. 1. Distribution of *nr4a2b* transcripts throughout the early developmental stages of zebrafish embryos. Anterior is to the left and dorsal is up. (A–D) *nr4a2b* is a maternal expressed gene and is universally expressed from 0.75 hpf to 12 hpf stage. (E) The first specific transcripts are found in brain at about the stage of 18 hpf (white arrows). (F) Two groups of $nr4a2b^+$ cells appear at 24 hpf. One is near OB in tel (black heads), and the other is around the rh (white arrowheads). A group of $nr4a2b^+$ cells emerges in PT (white arrows). (G–J) The $nr4a2b^+$ cells in PT area (white arrows) and in tg (black arrows in the lateral view) are expanded from 30 hpf to 120 hpf. At the stage of 48–120 hpf, *nr4a2b* transcripts display a complex pattern (I and J). It is expressed at MO (black arrows in the dorsal review) and ret (black arrowheads). MO, medulla oblongata; OB, olfactory bulb; PT, posterior tuberculum; rh, rhombencephalon; ret, retina; tel, telencephalon; tg, tegmentum.