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Pax2 regulates neuronal–glial cell fate choice in the embryonic optic nerve

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Abstract

During development, neural cell fate in the vertebrate optic nerve is restricted to the astroglial lineage. However, when isolated from the embryo and explanted in vitro, optic nerve progenitors generate neurons instead of astrocytes, suggesting that neuronal potentialities exist and are repressed in progenitors in vivo. Here we have investigated the mechanisms controlling cell fate in the optic nerve. The optic nerve is characterized by expression of the homeodomain transcription factor Pax2 which is maintained in differentiated astrocytes. We have observed that Pax2 is rapidly down-regulated in explanted optic nerves that generate neurons, and that its overexpression by electroporation in the optic nerve, or ectopically in the neural tube, is sufficient to block neuronal differentiation and allow glial development, showing that Pax2 plays a major role in controlling cell fate in the optic nerve. In vitro and ex vivo experiments further show that a signaling cascade that involves successively Sonic hedgehog and FGF is required to maintain Pax2 expression in optic nerve precursors whereby inhibiting the neuronal fate and promoting astroglial differentiation.
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Keywords: Optic nerve; Cell fate determination; Sonic hedgehog; FGF; Pax2; Neural tube; Glia

Introduction

The large variety of neuronal and macroglial cell types that populate the vertebrate central nervous system (CNS) develop from initially multipotent stem cells and/or progenitor cells of the neuroepithelium (Temple, 2001). In most CNS areas, the neural cell types locally generated are quite diverse and comprise several classes of neurons and glial cells (for reviews, see (Bertrand et al., 2002; Pearson and Doe, 2004). In a few CNS areas, however, neural cell production is restricted to a single cell type. Such is the case for a major component of the forebrain, the optic nerve, a purely glial territory. In the adult, the optic nerve entirely lacks neuronal cell bodies and comprises astrocytes and oligodendrocytes that wrap retinal ganglion cell axons on their way to the tectum. During development, however, oligodendrocytes are not generated locally but arise from extrinsic sources and migrate into the nerve in a proximo-distal direction (Small et al., 1987; Ono et al., 1997). Thus, in contrast to the neighboring retina, where neural progenitors generate a diverse progeny, including six types of neurons and one type of glia, optic nerve progenitors only produce astrocytes. This has lead to the assumption that cell fate in this region could be, from the onset, restricted to this particular glial cell type (Small et al., 1987). However, it has been shown that optic nerve precursors are not initially restricted to the astroglial fate. In particular, when isolated from influences of the surrounding tissues, optic nerve cells have the ability to generate neurons (Omlin and Waldmeyer, 1989; Giess et al., 1990), indicating that their developmental repertoire is broader than their normal fate in vivo and that neuronal potentialities must be repressed in this territory to allow only astrocytes to develop.

The optic nerve arises from the optic stalk, a constriction of the neuroepithelium connecting the optic vesicle to the diencephalon. This neuroepithelial domain is characterized from early stages by the specific expression of two homeodomain transcription factors, Pax2 and Vax1 (Nornes et al., 1990; Hallonet et al., 1998; Bertuzzi et al., 1999). Initial expression of both factors is under the control of the Shh signaling pathway (Chiang et al., 1996; Take-uchi et al., 2003). Overexpression of Shh at early stages of eye formation expands

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the Pax2 and Vax1 expression domains, and reduces that of Pax6 in the retina, resulting in severe malformations of the eye (Ekker et al., 1995; Macdonald et al., 1995; Zhang and Yang, 2001; Take-uchi et al., 2003). Mutually repressive influences between Pax2 and Pax6 have been well established, which progressively contribute to the definition of sharp boundaries between the retinal and optic nerve fields (Schwarz et al., 2000).

FGF signaling is another essential component in optic system morphogenesis (de Iongh and McAvoy, 1993; Pittack et al., 1997). FGF8, expressed in the retina (Vogel-Hopker et al., 2000) and in the optic stalk (Crossley et al., 2001), has been shown to direct retinal neuron specification (Martinez-Morales et al., 2005). FGF signaling can also promote Vax gene expression in the optic system (Take-uchi et al., 2003). However, the role of FGF signaling in the development of the optic stalk has not been investigated.

Pax2 and Vax1 play critical roles in eye morphogenesis, by defining the identity of the optic stalk field. In mutant mice for either factor, the retina–optic nerve boundaries are lost, leading to the expansion the retinal field at the expense of the optic stalk territory. In addition, the optic fissure does not close properly, resulting in optic nerve coloboma (Favor et al., 1996; Torres et al., 1996; Otteson et al., 1998; Bertuzzi et al., 1999; Hallonet et al., 1999). In the Pax2 mutant, most optic stalk cells degenerate, while a few differentiate into pigment cells (Schwarz et al., 2000), whereas in the Vax1 mutant optic nerve glia seem to develop normally, but fail to associate with axons, leading to defects in retinal axon trajectories (Bertuzzi et al., 1999; Hallonet et al., 1999).

Beyond initial morphogenesis, factors regulating cell fate in the optic system have been well studied in the retina. For example, Pax6 has been shown to control neuronal identity of retinal precursors, since in conditional Pax6 mutant mice, in which Pax6 is inactivated after initial retinal morphogenesis, only amacrine cells develop at the expense of the other neuronal types (Marquardt et al., 2001). In comparison, little is known about cell fate regulation in the optic nerve. Loss of Shh activity in the early chick and mouse optic system from its retinal source modifies cell fate in the optic disk and distal part of the optic nerve, leading either to neuronal development of optic stalk neural precursors (Zhang and Yang, 2001) or to cell death and pigment cell differentiation (Dakubo et al., 2003). Possible functions for Pax2 and Vax1 in cell specification in the optic nerve once morphogenesis is completed have not been characterized.

In the present study, we have defined some of the factors which repress neuronal differentiation in the embryonic chick and mouse optic nerve and lead to glial fate restriction of optic nerve precursors. We have focused on Pax2, and show that this transcription factor is able to regulate neuronal/glial cell choice in the optic nerve. Pax2 expression is lost in explanted optic nerves that generate neurons. It is maintained by Shh activity which in parallel totally blocks neuronal differentiation. However, Shh effects are indirect and are mediated by FGF signaling, suggesting that ongoing FGF activity is required to inhibit neuronal differentiation and promote astroglial development. Pax2 overexpression in optic nerve explants is sufficient to block neuronal differentiation and allow glial development. Furthermore, ectopic expression of Pax2 in the trunk neural tube also strongly inhibits neuronal development and induces glial cells. We propose that a regulatory cascade involving successively Shh, FGF and Pax2 regulates neuronal–glial cell fate choice in the optic nerve.

Materials and methods

Fertilized White Leghorn chick eggs obtained from commercial source were incubated at 38°C and staged according to the series of Hamburger and Hamilton (Hamburger and Hamilton, 1992). *Pax6*^{Neu} mutant mice (*Small Eye* allele) were a gift of Pr. Dr. P. Gruss.

Dissections, cultures and treatments

Optic nerves of embryos were dissected free of surrounding tissues at different stages (E4.5, E6 and E8) in sterile PBS. In all experiments, the whole optic nerve was excised, but without contamination by retinal or diencephalic tissues (see Results). At E4.5, optic nerves were grown as single explants. At later stages, they were subdivided into two to three explants. Cultures were grown on collagen gel-coated 12-mm plastic coverslips placed in 14-mm four-well dishes (Nunc). The culture medium was Neurobasal supplemented with 1% of B27 supplement (Gibco). The recombinant N-terminal fragment of the human Shh protein (Biogen) was used at concentrations of 4, 12, 24, and 50 nM. The recombinant mouse FGF8b and recombinant human FGF4 (R&D systems) were used at 100 ng/ml. The recombinant human BMP protein (R&D Systems) was used at a final concentration of 20 ng/ml. Cyclopamine, a gift of Dr. Frederic Rosa (Ecole Normale Supérieure, Paris, France), was used at a concentration of 4 μM. SU5402 (Calbiochem) was used at a concentration of 50 μM. Some experiments aiming to block the Shh signaling pathway were performed ex-vivo. The head was dissected at E5, opened along the dorsal midline, leaving the mesenchyme surrounding the optic nerve. These explants were then grown for 12 h with or without cyclopamine.

Staining procedures

For immunohistochemical analyses, heads of embryos were fixed in 3.7% formaldehyde (Sigma) in PBS overnight at 4°C. Tissues were then sectioned at 80 μm on a vibratome (Leica) before being processed. Optic nerve explants were fixed in the same fixative for 30 min at room temperature. O4 immunohistochemistry was performed as previously described (Giess et al., 1992). For the detection of intracellular antigens, fixed cultures and sections were first permeabilized using Triton-X-100 (0.5% in PBS). Then they were blocked using BSA (1% in PBS) and primary antibodies were applied at the appropriate dilution in 0.1% Triton-X-100/BSA 1% in PBS and incubated overnight at 4°C. After rinsing, they were incubated for 30 min with biotinylated secondary antibody directed against either mouse or rabbit IG (Amersham, 1:50), followed either by FITC or TRITC conjugates coupled to streptavidin (Amersham, 1:50). In some cases, they were revealed for 1 h using goat anti-mouse or goat anti-rabbit antibodies coupled to Alexa 488, Alexa 546, or Alexa 647 (Molecular Probes). Apoptosis was detected by TUNEL staining using Cell Death Detection Kit (Roche) as recommended by the manufacturer. Sections and optic nerve explants were analyzed with either a Zeiss LSM-410 or a Leica SP2 confocal microscope. Apoptosis was quantified by measuring mean pixel intensity in optic nerve explants using image-J software.

Antibodies

Neurons were identified either with Tuj1 monoclonal antibody (Babco), which recognizes neuron-specific β3-tubulin, used at 1/2000 or with NeuN antibody (Chemicon), used at 1/500 or with anti HuC/D (Molecular Probes) used at 1/500. Astroglial cells were evidenced by a rabbit antiserum directed against the glutamate–aspartate transporter (GLAST), a gift from Dr. Masahiko Watanabe used at 1/500. The anti-Pax2 antiserum (Babco) was used at 1/500 dilution. Mouse antibodies against Pax6, (used at 1:2) and HNK1 (used at 1:500)

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