available at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/brainres

BRAIN RESEARCH

Research Report

Six1 and Six4 promote survival of sensory neurons during early trigeminal gangliogenesis

Yoshiyuki Konishi[a], Keiko Ikeda[a], Yoichiro Iwakura[b], Kiyoshi Kawakami[a,*]

[a]Division of Biology Center for Molecular Medicine, Jichi Medical University, 3311-1, Yakushiji, Shimotsuke, Tochigi 329-0498, Japan
[b]Division of Cell Biology, Center for Experimental Medicine, Institute of Medical Science, University of Tokyo, Tokyo, Japan

ARTICLE INFO

Article history:
Accepted 28 July 2006
Available online 30 August 2006

Keywords:
Six1
Six4
Trigeminal ganglion
Cranial ganglia
Sensory neurons
Survival
Apoptosis
Bcl-x

ABSTRACT

Survival of sensory neurons is tightly regulated in cell-type and developmental-stage-specific manners. The transcriptional regulatory mechanisms underlying this regulation remain to be elucidated. In the present study, we investigated the role of Six1 and Six4 in the development of trigeminal ganglia. Abundant expression of Six1 and Six4 was noted in sensory neurons during early trigeminal gangliogenesis. Loss of both *Six1* and *Six4* in mice caused severe defects in the trigeminal ganglia, wherein massive apoptosis accompanied by activation of caspase-3 was observed at early but not late stages of gangliogenesis. In $Six1^{-/-}Six4^{-/-}$ mice, trigeminal sensory neurons were generated, but showed reduced expression of Bcl-x compared with the wild-type mice. Accordingly, neurons from the deficient mice could not survive in culture even in the presence of neurotrophins. Our results suggest a cell-intrinsic role of Six1 and Six4 in the survival of early-generated trigeminal sensory neurons.

1. Introduction

The role of neurotrophins in the developmental stage-specific survival of sensory neurons has been studied extensively (reviewed by Davies, 1997). In contrast, the cell-intrinsic mechanisms that regulate neuron survival temporally remain to be elucidated. In trigeminal (V) ganglia, neurons require neurotrophin-3 (NT-3) and brain-derived neurotrophic factor (BDNF) as survival factors around embryonic day 10 (E10) to E13, whereas they require nerve growth factor (NGF) after E12 when they send axons to innervate the target tissues (Buchman and Davies, 1993; Davies et al., 1993; Huang et al., 1999a; Wilkinson et al., 1996).

Some of the transcription factors have been implicated in the survival of trigeminal neurons, although none has been so far linked directly to early gangliogenesis. For example, in *Brn3a*-deficient mice, trigeminal sensory neurons survive normally until E13.5, but undergo apoptosis in later development (Huang et al., 1999b; McEvilly et al., 1996). Cyclic AMP-responsive element binding protein (CREB) is another transcription factor that promotes survival of peripheral neurons, as well as trigeminal sensory neurons. CREB is phosphorylated and becomes transcriptionally active in the presence of neurotrophins, hence has been suggested to mediate survival signals (Bonni et al., 1995, 1999; Finkbeiner et al., 1997; Ginty et al., 1994). Thus, the reported association of CREB with sensory neuron survival appears to involve late rather than early stages of development (Lonze et al., 2002). The existence of additional transcriptional factors that regulate survival early in the generation of sensory neurons remains to be indicated.

Six1 belongs to the *Six*-class homeobox gene family homologous to *Drosophila so*. It plays critical roles in the

* *Corresponding author*. Fax: +81 285 44 5476.
E-mail address: kkawakami@jichi.ac.jp (K. Kawakami).

0006-8993/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.brainres.2006.07.103

development of sensory organs as well as muscle and kidney in vertebrates (Kawakami et al., 2000; Laclef et al., 2003; Oliver et al., 1995; Ozaki et al., 2004; Xu et al., 2003; Zheng et al., 2003). *Six1* is highly expressed in cranial ganglia during embryogenesis in *Xenopus* and in mice (Laclef et al., 2003; Pandur and Moody, 2000; K. Kawakami, unpublished observations), and mice deficient in *Six1* lose sensory neurons in a subset of cranial ganglia (Zou et al., 2004). Another member of the *Six* family, *Six4*, is expressed in overlapping regions with *Six1*, but deletion of *Six4* produces no remarkable defect during development (Esteve and Bovolenta, 1999; Kobayashi et al., 2000; Ghanbari et al., 2001; Ozaki et al., 2001), and it is thought that this gene might partially compensate for *Six1* function.

The aim of the present study was to determine the roles of Six1 and Six4 in the survival of trigeminal neurons. The results showed that expression of Six1 and Six4 in the sensory neurons of developing trigeminal ganglia appeared at around E10.5. Deletion of both *Six1* and *Six4* resulted in defective development of trigeminal ganglia. Increased neuronal apoptosis was observed in trigeminal ganglia of the $Six1^{-/-}Six4^{-/-}$ mice at E10.5, but not at E13.5. Moreover, neurons prepared from $Six1^{-/-}Six4^{-/-}$ mice could not survive in culture even in the presence of neurotrophins. These findings indicate a novel role for Six1 and Six4 in the regulation of survival of early-generated sensory neurons during development.

2. Results

2.1. *Six1 is highly expressed in trigeminal sensory neurons during development*

To determine the role of Six1 in the development of trigeminal ganglia, we first investigated the expression of Six1 by using the rat polyclonal antibody against Six1 (Figs. 1A–C). Six1 was localized within the regions corresponding to the trigeminal ganglia at E10.5 (Fig. 1B). E11.5 trigeminal ganglia showed reduced but persistent Six1 expression (Fig. 1C). Previous studies showed expression of Brn3a and Islet1/2 in the developing ophthalmic trigeminal sensory neurons (Artinger et al., 1998; Fedtsova et al., 2003), and high expression levels of Pax3 in ophthalmic trigeminal neuronal precursors, but low expression levels in postmitotic trigeminal neurons (Baker and Bronner-Fraser, 2000; Baker et al., 2002; Fedtsova et al., 2003). To address whether Six1 is expressed in the sensory neuronal population, we performed double immunostaining with the rabbit polyclonal anti-Six1 antibody and mouse monoclonal antibodies against these trigeminal neuron molecular markers. While expression of Six1 was observed within and outside the ganglion, the strongest staining was observed distal to the neural tube, an area rich in sensory neurons (Fig. 1D). Trigeminal neurons immunopositive for Brn3a or Islet1/2 tended to show also strong Six1 immunoreactivity (Figs. 1G–L) at E10.5, whereas only a small population of cells revealed strong double staining of both Pax3 and Six1 (Figs. 1D–F) at E10.5. These findings suggest that Six1 is abundantly expressed in many of the developing sensory neurons in trigeminal ganglia.

2.2. *Six1/Six4-deficient mice show defective development of trigeminal ganglia*

To understand the function of *Six1* in the developing trigeminal ganglia, we analyzed the trigeminal ganglia of *Six1*-deficient mice by histological examination. Cresyl violet staining showed no apparent defect in the size of trigeminal ganglia due to ablation of *Six1* or *Six4* (Figs. 2A–C; Ozaki et al., 2001), but a significant reduction in size following deletion of both *Six1* and *Six4* (Fig. 2D). The number of neurons was significantly reduced in the trigeminal ganglion of $Six1^{-/-}Six4^{-/-}$ compared with those of wild type, $Six1^{-/-}$, $Six4^{-/-}$ and $Six1^{+/-}Six4^{+/-}$ (Fig. 3A). These observations suggest that Six1 and Six4 are critical but functionally redundant proteins for the development of trigeminal ganglia. Consistent with this conclusion, Six1 and Six4 were coexpressed in a considerable population of cells in developing trigeminal ganglia at E10.5 (Figs. 1M–P). We also noticed more pyknotic nuclei in the trigeminal ganglia of $Six1^{-/-}Six4^{-/-}$ mice than in wild-type mice at E10.5, but not at E13.5 (Figs. 2E–H, 3B), consistent with a previous report of wild-type trigeminal ganglia (Huang et al., 1999b). Increased apoptosis in early gangliogenesis might therefore play at least a partial role in the observed defect of the double-deficient mice.

2.3. *Increased apoptosis of trigeminal neurons in Six1/Six4-deficient mice during early gangliogenesis*

In apoptotic cells, endonucleases cleave DNA and produce ssDNA at terminals. Thus, an antibody against ssDNA has been used as a molecular tool to detect apoptotic nuclei

Fig. 1 – Six1 is expressed in trigeminal sensory neurons during development. A–C: Transverse sections at the trigeminal level prepared from mouse embryos at the indicated developmental stages. Immunohistochemistry was performed by using a rat polyclonal antibody for Six1. Six1 was highly expressed in trigeminal ganglia at E10.5 (B). D–L: Paraffin-embedded sections of trigeminal ganglia were subjected to double immunostaining with a rabbit polyclonal antibody for Six1 together with the mouse monoclonal antibody for Pax3 (D–F), Brn3a (G–I) or Islet1/2 (J–L). Dispersed spots appeared by Brn3a and Islet1/2 antibodies were presumably nonspecific signals coming from blood cells. Magnification for E–L similar to that shown on D. M–P: Sections from E10.5 mouse embryo prepared as described in B were subjected to immunohistochemistry using the rat polyclonal antibody for Six1 together with the rabbit polyclonal antibody for Six4. The region indicated by the rectangle in M is shown at the lower right corner of each panel at high power magnification. Magnification for N–P similar to that shown on M. Q–S: Sections of the trigeminal ganglia were subjected to immunohistochemistry as described for M–P except using no primary antibody. Magnification for R–S similar to that shown on Q. Vg, trigeminal ganglia; nt, neural tube; Vr, trigeminal nerve root; Vd, Vp, trigeminal ganglia regions distal and proximal to the neural tube.