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EXPRESSION OF *mKirre* IN THE DEVELOPING SENSORY PATHWAYS: ITS CLOSE APPOSITION TO NEPHRIN-EXPRESSING CELLS

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Abstract—*mKirre* is a novel member of the immunoglobulin superfamily, which is abundant in the developing and adult brain. In the present study, we showed *mKirre* gene expression in mouse sensory organs during development using *in situ* hybridization and immunohistochemistry. At embryonic day (E) 11.5, E15.5, and E17.5, we first detected signals for *mKirre* mRNA in the developing cochleae, retinae, and olfactory neuroepithelia, respectively. After birth, strong signals were observed in these sensory organs. In addition, at this stage, we found its expression in trigeminal ganglion neurons and neuronal populations forming sensory pathways in the olfactory bulb, midbrain, and pons. Furthermore, double-immunofluorescence staining revealed that nephrin-immunoreactivity was overlapping to *mKirre*-expressing cells in the developing sensory organs. These results suggest that *mKirre* may be involved in the establishment of the pathway from sensory organs to the brain not only in a homophilic manner but also with its heterophilic interaction to nephrin.

Key words: retinae, olfactory epithelia, cochleae, trigeminal ganglia, *in situ* hybridization, immunohistochemistry.

In mammalians, the transduction of external stimuli from environments, such as odor, light, and sound, first takes place in the specific sensory organs: these senses are initiated by specialized primary sensory cells of the sensory organs. The primary sensory cells activated by external stimuli relay the signals to various neuronal populations, which form the neuronal circuits specialized for each sense in the brain. The formation and establishment of the neuronal circuits for each sense require the precise projection of fate-committed neurons to their assigned targets by the recognition of guidance cues in the brain during the development. Although various classes of molecules have been identified as guidance cues, the immunoglobulin superfamily (IgSF) is one of the large families of axon guidance molecules (Rougon and Hobert, 2003).

In both invertebrates and vertebrates, the IgSF proteins are implicated in the control of various developmental processes, such as neuronal migration, axon guidance, and synaptic adhesion. In vertebrates, some members of IgSF proteins are expressed in the developing sensory organs (von Campenhausen et al., 1997; Kawauchi et al., 2003; Rougon and Hobert, 2003; Litwack et al., 2004; Weiner et al., 2004).

The *Drosophila* kin of *irre* (*kirre*) is a member of IgSF and also known as *dumbfounded* (*Duf*). The *kirre* protein is expressed in founder cells in muscle-forming mesoderm and contributes to myoblast aggregation (Ruiz-Gomez et al., 2000; Strünkelnberg et al., 2001). Its mammalian homologue, *mKirre*, identified in a bone marrow stromal cell line, plays an important role in the support of the hematopoiesis, and is expressed in bone stromal cells and the adult brain (Ueno et al., 2003). In addition, we have investigated the expression pattern of *mKirre* in the developing mouse brain using *in situ* hybridization technique, and have shown that *mKirre* mRNA is expressed in sensory-related regions of the CNS, such as the olfactory bulb, vestibular nucleus, and trigeminal nucleus, in perinatal mice (Tamura et al., 2005).

Recently, it has been reported that olfactory sensory neurons expressing the same type of odorant receptor fasciculate their axons by homophilic adhesive interaction of *mKirre* in mice (Serizawa et al., 2006). On the other hand, some reports have demonstrated that *mKirre* and its homologues interact with the other members of IgSF, nephrin in mice (Gerke et al., 2005) and *sticks-and-stones* (*SNS*) or *Hibris* (*Hbs*) in *Drosophila* (Galletta et al., 2004; Bao and Cagan, 2005). To further understand the role of *mKirre* in the development of the nervous system, we investigated the expression pattern of the *mKirre* gene in sensory organs, such as olfactory neuroepithelia, retinae, and cochleae, during mouse embryogenesis and in neonatal mice using *in situ* hybridization and immunohistochemistry.

EXPERIMENTAL PROCEDURES

Animals

Pregnant C57BL/6J mice (Nihon SLC, Hamamatsu, Japan) were used in the present study. These mice were kept under a 12-h

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Abbreviations: bc, basal cells; BSA, bovine serum albumin; CN, cochlear nucleus; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; *Duf*, *dumbfounded*; E, embryonic day; FSAG, facial-stato-acoustic ganglia; *Hbs*, *Hibris*; IC, inferior colliculus; IgSF, immunoglobulin superfamily; inbl, inner blastic layer; INL, inner nuclear layer; IPL, inner plexiform layer; *irreC*, irregular chiasm *C*; *kirre*, kin of *irre*; onbl, outer blastic layer; P, postnatal day; PB, phosphate buffer; PBS, phosphate-buffered saline; PFA, paraformaldehyde; rc, receptor neurons; RGC, retinal ganglion cell layer; *Rst*, Roughest; RT, room temperature; sc, supporting cells; SC, superior colliculus; *SNS*, *sticks-and-stones*.

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light/dark cycle with food and water *ad libitum*. All experiments were performed according to National Institutes of Health guidelines and were performed under the control of the Animal Research Control Committee in accordance with the Guidelines for Animal Experiments of Wakayama Medical University and Japanese Government Notification on Feeding and Safekeeping of Animals (No. 6). All efforts were made to minimize the number of animals used and their suffering.

Embryos at embryonic day (E) 9.5, E11.5, E13.5, E15.5, and E17.5 were obtained by cesarean section under deep diethyl ether anesthesia. These embryos were immersed in ice-cold 0.1 M phosphate buffer (PB) containing 4% paraformaldehyde (PFA) at 4 °C overnight. Postnatal mice were deeply anesthetized with diethyl ether at postnatal day 0 (P0) and transcardially perfused with ice-cold 0.85% NaCl followed by ice-cold 4% PFA. For immunohistochemistry, the embryos and postnatal mice were fixed with Zamboni's fixative. The heads were quickly removed and postfixed with the same fixative at 4 °C overnight. Following the postfixation, they were immersed in 30% (w/v) sucrose in 0.1 M PB and embedded in O.C.T. medium (Miles, Elkhart, IN, USA).

In situ hybridization histochemistry

In situ hybridization histochemistry was performed as previously described (Tamura et al., 2005). Briefly, the *mKirre* cRNA probe for *in situ* hybridization histochemistry was generated from a 371-bp cDNA fragment of *mKirre* (Ueno et al., 2003). Frozen sections were cut on a cryostat at 6-μm thickness. The slides were hybridized with ^{35}S-labeled sense or antisense cRNA probes at 55 °C for 16 h. The high stringency washes were performed in 0.1× saline–sodium citrate buffer at 50 °C. After dehydration, the slides were submerged in NTB-2 liquid emulsion (Kodak, Rochester, NY, USA) and exposed at 4 °C for 10 days. After being developed in D-19 developer (Kodak), the slides were counterstained through the emulsion with Mayer's hematoxylin, and then examined under dark-field lateral illumination microscopy (XF-WFL; Nikon, Tokyo, Japan). The sense cRNA probe failed to hybridize in all the sections examined (data not shown). The nomenclature used follows that of Franklin and Paxinos (1997) and Jacobowitz and Abbott (1998).

Double-immunofluorescence staining

Slide-mounted 6-μm cryostat sections of the mouse heads were processed double-immunofluorescence staining, as described previously (Morikawa et al., 2004). Briefly, sections were preincubated in 0.1 M phosphate-buffered saline (PBS) containing 5% normal donkey serum at room temperature (RT) for 1 h, and then incubated with primary antibodies containing 1% bovine serum albumin (BSA) and 0.1% Triton X-100 (4 °C, overnight). Primary antibodies were used at the following dilutions: rabbit anti-*mKirre* 1:100 (Ueno et al., 2003); goat anti-nephrin 1:50 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The next day, sections were washed in 0.1 M PBS containing 0.1% Triton X-100, and were incubated in the Cy2-/Cy3-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA), containing 1% BSA and 0.1% Triton X-100 (RT, 1 h), diluted 1:800. All sections were counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI). Immunofluorescence images were acquired using an epifluorescence microscope (BX50; Olympus, Tokyo, Japan) equipped with a digital CCD camera (DP71; Olympus).

The following controls were performed: (i) incubation with protein A-purified goat or rabbit IgG instead of the primary antibody; (ii) incubation without the primary antibody or without primary and secondary antibodies; and (iii) incubation with the antigenic peptide (1:5; Santa Cruz Biotechnology) used to produce anti-nephrin antibody prior to staining with it. All controls revealed no labeling (data not shown).

RESULTS

mKirre gene expression during mouse retinogenesis

At E15.5, expression of *mKirre* mRNA was first detected in the developing retinae. Its signals were observed in the inner blastic layer (inbl; Fig. 1A, arrowheads), which is the origin of the retinal ganglion cell layer (RGC), the inner plexiform layer (IPL), and inner nuclear layer (INL) of the retinae (Young, 1985). Consistent with this result, *mKirre* mRNA was detected in the RGC, IPL, and INL at E17.5 (Fig. 1B, arrowheads), and its expression became stronger in the neonatal stage (Fig. 1C). In contrast, the signals for *mKirre* gene were not observed in the outer blastic layer (onbl) in any stages of the retinal development (Fig. 1A–C).

Developmental expression of *mKirre* mRNA in the olfactory epithelia

Until E15.5, we did not observe signals for *mKirre* mRNA in the developing olfactory epithelia (Fig. 1D). Its gene expression was first detected in the epithelia at E17.5 (Fig. 1E, arrowheads). After birth, strong signals for *mKirre* were observed in the olfactory epithelia (Fig. 1F). At this stage, its gene expression was observed in the receptor neurons (rc), but not in the basal (bc) and the supporting cells (sc) (Fig. 1F).

Expression of *mKirre* mRNA in the developing cochleae

At E9.5, no signals for *mKirre* were detected in the otic vesicles, which are primordium of the cochleae, while its gene expression was first observed in the facial-statoacoustic ganglia (FSAG) (data not shown), which are the origin of the vestibulocochlear ganglia (Sher, 1971). At E11.5, the expression of *mKirre* mRNA was observed in the cochlear epithelial cells (Fig. 1G, arrowheads) and the FSAG (Fig. 1G). In neonatal mice, *mKirre* mRNA was expressed in some cochlear epithelial cells (Fig. 1H, arrowheads) and spiral ganglia (Fig. 1H), but not in vestibular ganglia (Fig. 1H, inset).

Expression of *mKirre* mRNA in the developing trigeminal ganglia

Signals for *mKirre* mRNA were first detected in the trigeminal ganglia at E9.5 (data not shown). We found many cells expressing *mKirre* mRNA in the trigeminal ganglia at E15.5 (Fig. 2A). After birth, *mKirre* gene expression was observed in a subpopulation of primary sensory neurons in these ganglia (Fig. 2B).

Distribution of *mKirre* gene in the developing olfactory bulb, midbrain, and brainstem

Intriguingly, we observed strong signals for *mKirre* mRNA in the sensory organs during perinatal stages, as shown above. In addition, *mKirre* mRNA was highly expressed in the mitral cell layer of olfactory bulb in E17.5 embryos (Fig. 2C) and neonates (Tamura et al., 2005). Thus, we further investigated the expression pattern of *mKirre* mRNA in the