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$P2X_2$ Receptor Deficiency in Mouse Vestibular End Organs Attenuates Vestibular Function

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Abstract—$P2X_2$ receptors are ligand-gated cation channels activated by extracellular ATP that modulate neural transmission in various neuronal systems. Although the function and distribution of $P2X_2$ receptors in the cochlea portion of the inner ear are well established, their physiological role in the vestibular portion is still not understood. Therefore, we investigated $P2X_2$ receptor localization in the peripheral vestibular portion, and assessed their physiological function *in vivo* using $P2X_2$ receptor knock out ($P2X_2$-KO) mice. Histological analysis revealed that $P2X_2$ receptors were localized on the epithelial surface of supporting and transitional cells of the vestibular end organs. To examine vestibular function in $P2X_2$-KO mice, we conducted behavioral tests and tested the vestibulo-ocular reflex (VOR) during sinusoidal rotations. $P2X_2$-KO mice exhibited significant motor balance impairment in the balance beam test. VOR gain in $P2X_2$-KO mice was significantly reduced, with no decrease in the optokinetic response. In conclusion, we showed that $P2X_2$ receptors are mainly localized in the supporting cells of the vestibular inner ear, and the loss of $P2X_2$ receptors causes mild vestibular dysfunction. Taken together, our findings suggest that the $P2X_2$ receptor plays a modulatory role in vestibular function.

Key words: inner ear, supporting cell, vestibular dysfunction, $P2X_2$-KO, VOR, ATP.

INTRODUCTION

$P2X_2$ receptors are ligand-gated nonselective cation channels that are activated by extracellular adenosine triphosphate (ATP) to act as neurotransmitters/neuromodulators in various neuronal systems (Brake et al., 1994; North, 2002; Housley et al., 2009). A number of studies have shown that $P2X_2$ receptors regulate auditory neurotransmission in the inner ear (Housley et al., 1999; Jarlebark et al., 2000; Lee and Marcus, 2008; Ito and Dulon, 2010). Within the vestibular portion of the inner ear, $P2X_2$ receptor gene expression has been detected in vestibular end organs by reverse transcriptase–polymerase chain reaction (RT–PCR) (Brandle et al., 1999; Kreindler et al., 2001) and *in situ* hybridization (ISH) (Housley et al., 1998). Previous studies have demonstrated the presence of $P2X_2$ receptor mRNA in vestibular epithelia, but no study has evaluated $P2X_2$ receptor subcellular localization. Therefore, we investigated $P2X_2$ receptor localization in the mouse peripheral vestibular nervous system using immunohistochemistry (IHC) and ISH. A previous electrophysiological study reported that ATP-sensitive cation channels (UTP, ADP and, UDP-insensitive) were expressed in vestibular transitional cells (VTC) on the endolymph (apical) side. These channels were identified as $P2X_2$ receptors by analyzing their sensitivity to ATP. Accordingly, $P2X_2$ receptors on non-sensory cells might regulate the endolymphatic ion concentration during vestibular transduction (Lee et al., 2001). However, $P2X_2$ receptors in the cochlea portion were expressed in outer hair cell stereocilia, supporting cells, Reissner's membrane, and spiral ganglion neurons (Jarlebark et al., 2000; Lee and Marcus, 2008). Moreover, a recent study using $P2X_2$ receptor knock out ($P2X_2$-KO) mice found that $P2X_2$ receptors are required to prevent noise-induced hearing loss (Housley et al., 2013; Yan et al., 2013). Although the function and distribution of $P2X_2$ receptors in the cochlear portion of the inner ear is

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Abbreviations: ATP, adenosine triphosphate; EDTA, ethylenediaminetetraacetic acid; ISH, *in situ* hybridization; OKN, optokinetic nystagmus; $P2X_2$-KO, $P2X_2$ receptor knock out; PB, phosphate buffer.

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well established, their physiological function in the vestibular portion remains unclear (Lee and Marcus, 2008; Ito and Dulon, 2010). Thus, the $P2X_2$-KO mouse model will be utilized to investigate the importance of the $P2X_2$ receptor in vestibular function by examining behavior using open-field, rotarod, balance beam, and vestibulo-ocular reflex tests.

EXPERIMENTAL PROCEDURES

Animals

Adult, male C57BL/6J mice (9–11 weeks of age and weighing 20–26 g) used in this study were purchased from Japan SLC Inc. (Hamamatsu, Japan). Every effort was made to minimize animal suffering and reduce the number of animals used. Using a breeding pair (strain name: B6.129-P2rx2^{tm1Ckn}/J, stock number: 004603) obtained from Jackson Laboratories, and back-crossing to an inbred strain (C57BL/6J) for ten generations, $P2X_2$-KO mice and WT littermates with defined genetic hybrid backgrounds were generated. A lack of $P2X_2$ gene expression in $P2X_2$-KO mice was verified by sequencing. All animal experimental protocols were approved by the Animal Care and Use Committee of the Osaka University Graduate School of Medicine. The methods were performed in accordance with the approved guidelines.

Tissue preparation

Animals were deeply anesthetized by intraperitoneal injection of sodium pentobarbital (Nembutal; 200 mg/kg body weight) and decapitated after cessation of spontaneous respiration. Temporal bones [including the cochlea, semicircular canals, otoliths, vestibular ganglion (VG), and facial nerve trunk] were immediately dissected with small scissors and fine forceps under an upright light microscope (Leica, Wetzlar, Germany), and placed in chilled phosphate-buffered saline (PBS, pH 7.4). Temporal bones were stored overnight in 4% paraformaldehyde before decalcification in EDTA solution (10% in PBS, pH 7.2) for a total of 7 days. Next, the bones were cryoprotected in 0.1 M phosphate buffer (PB) containing 30% sucrose for 24 h before freezing for cryostat sectioning into 10-μm-thin sections. Each sample was processed for IHC and ISH. Whole brains were collected with small scissors and fine forceps under an upright light microscope. Brains were stored overnight in PB containing 4% paraformaldehyde, cryoprotected in 0.1 M PB containing 30% sucrose for 24 h at 4 °C, and then frozen for cryostat sectioning into 14-μm-thin sections. Each sample was processed for IHC.

Immunohistochemistry

Procedures were essentially identical to those previously described (Ishida et al., 2009). Rabbit antibody against the carboxyl terminus of rat $P2X_2$ (APR-003, lot No. AN-04) was purchased from Alomone Labs (Jerusalem, Israel). As previously reported, rabbit anti-$P2X_2$ receptor antibody does not stain $P2X_2$-KO mouse tissue, but does stain WT tissue (Finger et al., 2005). For frozen inner ear section analysis, each section was incubated overnight at 4 °C with primary antibodies diluted in blocking solution (1:500 for rabbit $P2X_2$ antibody). After rinsing with PBS for 30 min at room temperature, sections were incubated with appropriate secondary antibodies, specifically, Alexa® Fluor 488 (Thermo Fisher Scientific, Waltham, MA, USA), in PBS containing 0.3% Triton™ X-100 for 90 min at room temperature. After rinsing with PBS for 30 min at room temperature, some sections were incubated with rabbit anti-myosin VIIA antibody conjugated to Alexa Fluor 647 (Bioss Inc., Woburn, MA, USA) or Alexa Fluor® 594 phalloidin (Thermo Fisher Scientific, Waltham, MA, USA). Nuclei were counterstained with Hoechst® 33,342 (Thermo Fisher Scientific, Waltham, MA, USA). After staining, sections were examined using a fluorescence microscope (Olympus, Tokyo, Japan). For frozen brain section analysis, each section was incubated overnight at 4 °C with primary antibodies ($P2X_2$ antibody) diluted in blocking solution. After rinsing with PBS for 30 min at room temperature, sections were incubated with an alkaline phosphatase-conjugated goat anti-rabbit IgG secondary antibody (Thermo Fisher Scientific, Waltham, MA, USA) for 90 min at room temperature. The signal was developed using BCIP/NBT (Roche, Mannheim, Germany) as a chromogenic substrate. For whole-mount analysis, the temporal bone was opened above the otoliths (utricle and saccule). The sensory portion of the otoliths was dissected, and the otoconial membrane was detached from the surface of the sensory epithelium. Each tissue was stained with anti-$P2X_2$ receptor antibody and then was incubated with Rhodamine-conjugated phalloidin (Cytoskeleton, Denver, USA) (as described above). Images were captured by a LSM710 confocal microscope (Zeiss Microimaging, Jena, Germany).

In situ hybridization

Procedures were essentially identical to those previously described (Hondoh et al., 2010; Takimoto et al., 2014). Samples were embedded in Optimal Cutting Temperature (O.C.T.) compound (Sakura Finetek, Tokyo, Japan), frozen immediately, and then cut on a cryostat (10-μm-thick sections). Digoxigenin (DIG)-labeled cRNA probes were generated by *in vitro* transcription using T7 or SP6 RNA polymerase and the following nucleotide sequence: $P2X_2$ receptor, nucleotides 460–1477 (GenBank/EBI Data Bank accession NM_153400.4). After hybridization, slides were incubated with a sheep anti-DIG Fab fragment conjugated to alkaline phosphatase (1:3000; Roche, Mannheim, Germany). Signal was developed using BCIP/NBT (Roche, Mannheim, Germany) as chromogenic substrates. Hybridization signal specificity was confirmed using appropriate sense cRNA probes.

Quantification of VOR and optokinetic nystagmus (OKN)

Surgical preparation for head implant surgery and the experimental setup have been described previously (Imai et al., 2016; Takimoto et al., 2016). The VOR test was performed in a dark room. Mice were placed on a