Neuroscience 277 (2014) 55–71

IMMUNOLOCALIZATION OF THE P2X4 RECEPTOR ON NEURONS AND GLIA IN THE MAMMALIAN RETINA

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Abstract—Extracellular adenosine 5′-triphosphate (eATP) acts as a neurotransmitter within the retina and brain, activating a range of ionotropic P2X and metabotropic P2Y receptors. In this study, the specific localization of the P2X4 receptor (P2X4-R) subunit was evaluated in the retina using fluorescence immunohistochemistry and pre-embedding immuno-electron microscopy. Punctate P2X4-R labeling was largely localized to the inner and outer plexiform layers of mouse, rat and cat retinae. In the mouse outer retina, double-labeling of P2X4-R with the horizontal cell marker, calbindin, revealed P2X4-R immunoreactivity (P2X4-R-IR) on horizontal cell somata and processes. In the inner retina, P2X4-R expression was found closely associated with rod and cone bipolar cell terminals, and the punctate labeling was observed on calretinin-positive amacrine cells. Using immuno-electron microscopy, P2X4-Rs were observed on processes post-synaptic to photoreceptor and bipolar cell terminals, likely representing horizontal, amacrine and ganglion cells, respectively. Furthermore, P2X4-R expression was also observed on Müller cells, astrocytes and microglia. These data suggest a role for P2X4-Rs in the lateral inhibitory pathways of the retina, modulating neuronal function of photoreceptors and bipolar cells. The expression on macro- and microglial cells implicates a role for P2X4-Rs in glial signaling, tissue homeostasis and immunosurveillance within the mammalian retina.

Key words: purinergic, extracellular ATP, synapse, retinal neurons, Müller cell, microglia.

INTRODUCTION

Extracellular adenosine 5′-triphosphate (eATP) acts as a neurotransmitter in the central and peripheral nervous systems and exerts its effects on two families of membrane-bound receptors: ligand-gated P2X cation channels (Khakh, 2001) and G-protein-coupled P2Y receptors (Lazarowski et al., 2003). Both P2X and P2Y receptors are further classified into different subtypes; seven P2X (P2X1-7) and eight P2Y (P2Y1, 2, 4, 6, 11–14) receptors have been characterized in mammals by molecular means (North and Barnard, 1997; Ralevic and Burnstock, 1998). eATP elicits a fast excitatory response via P2X receptors, resulting in non-selective cation permeability of Ca^{2+}, Na^{+} and K^{+} (North and Surprenant, 2000). Additionally, each P2X receptor subtype has unique gating and pharmacological properties that are defined by the trimeric arrangement of subunits assembled as either homomeric or heteromeric complexes (Nicke et al., 1998; Torres et al., 1999; Aschrafi et al., 2004; Nicke, 2008; Kawate et al., 2009).

P2X4 receptor (P2X4-R) expression has been localized to neurons throughout the CNS. It has been implicated in physiological functions including the modulation of neurotransmission (Le et al., 1998; Rubio and Soto, 2001), contributing to synaptic strengthening (Baxter et al., 2011). In addition, immunohistochemical studies have identified abundant P2X4-R immunoreactivity (P2X4-R-IR) on microglia in the brain and spinal cord (Cavaliere et al., 2003; Tsuda et al., 2003; Ulmann et al., 2008), and a role in mediating neuroinflammatory events post-injury has been suggested (de Rivero Vaccari et al., 2012). As a result, activation of glial P2X4-Rs has been extensively studied in microglial responses to injuries and degeneration in the CNS (Guo and Schluesener, 2005; Ulmann et al., 2008; Domercq et al., 2013).

The localization of P2X4-Rs in the mammalian retina has been identified with a variety of techniques. Reverse transcriptase polymerase chain reaction (RT-PCR) detected P2X 2, 3, 4, 5 and 7 gene expression in the rat retina (Brändle et al., 1998a,b; Wheeler-Schilling et al., 2000, 2001). At the protein level, P2X4-R expression has been identified in the rodent (Wheeler-Schilling et al., 2001; Kaneda et al., 2004) and macaque retinae (Ishii et al., 2003; Gu et al., 2013), where it was found to be localized to neuronal cell somata and microglia. However, a detailed analysis of P2X4-R expression on specific neuronal and glial cell populations in the retina has not been undertaken. Furthermore,

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Abbreviations: BSA, bovine serum albumin; DAB, 3,3′-diaminobenzidine; eATP, extracellular adenosine 5′-triphosphate; GCL, ganglion cell layer; GFAP, anti-glial fibrillary acid protein; GFP, green fluorescent protein; GS, glutamine synthetase; HEPES, 2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid; IPL, inner plexiform layer; INL, inner nuclear layer; NGS, normal goat serum; OPL, outer plexiform layer; P2X4-R, P2X4 receptor; P2X4-R-IR, P2X4-R immunoreactivity; PB, phosphate buffer; RT-PCR, reverse transcriptase polymerase chain reaction; PKC-α, protein kinase C-α; VGLUT1, vesicular glutamate transporter 1.

http://dx.doi.org/10.1016/j.neuroscience.2014.06.055
0306-4522/

although it has been implicated in synaptic signaling in other parts of the CNS, the synaptic characterization of P2X4-R in specific circuits of the retina remains to be determined. The aim of this study was to characterize P2X4-R expression in the mouse, rat and cat retinas, and to identify the specific retinal cell types expressing P2X4-R in the mouse retina so as to gain further insight into the potential physiological function of the purinergic system in retinal signaling.

EXPERIMENTAL PROCEDURES

Animals

All experimental procedures using animals were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) and The University of Melbourne Animal Ethics Committee (Ethics #: 1112260). All efforts were made to minimize the number of animals used and their suffering. Adult C57BL/6J, wildtype mice and dark agouti rats were obtained from the Animal Resource Centre (WA, Australia), and were used at 6–8 weeks of age. All mice and rats were housed at the University of Melbourne and maintained in plastic cages with *ad libitum* access to food and water under a 12-h:12-h light–dark cycle. Fixed, adult cat ocular tissue was generously provided by Professor Robert Shepherd (The Bionics Institute, VIC, Australia). For assessment of P2X4-R expression on retinal microglia, heterozygous CX3CR1$^{+/GFP}$ mice on the C57BL/6J background that express enhanced green fluorescent protein (EGFP) under the control of the microglia-specific *cx3cr1* gene were used (B6.129P(Cg)-*Ptprc*^{a}*Cx3cr1tm1Lit/LittJ*, originally from the Jackson Laboratories, ME, USA; live breeders, kindly provided by Prof. Paul McMenamin, Monash University, VIC, Australia). For assessment of retinal ganglion cells, C57BL/6J mice transfected with yellow fluorescence protein driven by a Thy-1 promoter were used (B6.Cg-Tg(*Thy1-YFPH)2Jrs/J*, originally from the Jackson Laboratories, ME, USA; live breeders, kindly donated by A/Prof Anthony Hannan, Howard Florey Institute, VIC, Australia).

For all procedures involving collection of retinal tissue, mice were anesthetized by intraperitoneal injection of ketamine (67 mg/kg; Provet, VIC, Australia) and xylazine (13 mg/kg; Troy Laboratories, NSW, Australia) and sacrificed by cervical dislocation. Rats were anesthetized with an intramuscular injection of ketamine (50 mg/kg) and xylazine (5 mg/kg), and then sacrificed with a lethal intracardial overdose of sodium pentobarbital (60 mg/kg; Nembutal; Merial Australia, NSW, Australia).

Western blot analysis

The specificity of the P2X4-R antibody was confirmed by Western blot analysis using C57BL/6J mouse retinal and cortical tissues. Fresh cortex and retinae were sonicated in homogenizing buffer (40 mM HEPES; 320 mM Sucrose; pH, 7.5) containing a cocktail of protease inhibitors (1 tablet cOmplete®; Roche, VIC, Australia). Homogenates were centrifuged at 13,000*g* for 1 min at room temperature and the supernatants spun for 30 min to isolate membrane in pellet. Samples were diluted to 1:2 with sample buffer (0.5 M Tris–HCl, pH 6.8; 10% sodium dodecyl sulfate (SDS); 25% glycerol; 0.5% bromophenol blue; 5% beta-mercaptoethanol). Samples were boiled for 4 min then centrifuged at 13,000*g* for 2 min. Denatured protein was loaded onto 12% Acrylamide/Bis–Tris gels with a molecular weight marker (BenchMark™Prestained Protein Ladder, Invitrogen, VIC, Australia). Protein was separated on the gel by electrophoresis and transferred onto nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA). The membrane was briefly stained with Ponceau S to ensure protein transfer and then rinsed with dH_2O. Membrane-bound proteins were blocked with 0.5% skim milk powder in Tris-buffered saline with 0.05% Tween-20 (TBS-T) in the SNAP i.d. protein detection system (Millipore, North Ryde, NSW, Australia), incubated with the primary antibody directed against P2X4-R (rabbit anti-P2RX4; 1:300; Alomone Labs, Kensington, SA, Australia) diluted in 0.5% skim milk powder in TBS-T for 10 min. Membranes were washed three times with TBS-T, incubated with the secondary antibody (goat anti-rabbit 680; 1:3000; Li-COR Biosciences, Lincoln, NE, USA) diluted in 0.5% skim milk powder in TBS-T for 10 min and washed again in TBS-T. The membrane was imaged using an Odyssey CLx Infrared imaging system (Li-COR Biosciences, Lincoln, NE, USA).

Immunohistochemistry and confocal microscopy

For immunohistochemical localization of P2X4-R on retinal sections, the eyes were enucleated and the anterior segment and vitreous were removed. The posterior eyecups were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4) for 30 min and cryoprotected in graded sucrose (10%, 20%, 30%) in 0.1 M PB overnight. The tissue was then embedded in optimal cutting temperature (OCT) compound (Tissue-Tek, Sakura, Torrance, CA, USA), frozen at −20 °C, and sectioned transversely at 14 μm on a Microm HM550 cryostat (Thermo Scientific, Walldorf, Germany). Retinal sections were collected onto polylysine-coated slides (Thermo Scientific, VIC, Australia) and stored at −20 °C. For labeling of retinal sections, thawed slides were washed in 0.1 M PB and blocked with 10% normal goat serum (NGS), 1% bovine serum albumin (BSA) and 0.5% Triton-X-100 in 0.1 M PB (pH, 7.4) for 1 h. The sections were then incubated with primary antibodies (Table 1) overnight at room temperature. After washing with 0.1 M PB, sections were incubated with secondary antibodies (Table 1) for 1 h at room temperature in the dark. The sections were washed in 0.1 M PB then mounted with Mowiol/glycerol based mounting medium and covered with a glass coverslip. Negative controls were obtained by overnight pre-incubation of the P2X4-R primary antibody with five times the concentration of peptide antigen (KKYKYVEDYEQGLSGEMNQ) before processing for