Journal of Chemical Neuroanatomy 78 (2016) 25–33

Contents lists available at ScienceDirect

Journal of Chemical Neuroanatomy

journal homepage: www.elsevier.com/locate/jchemneu

Validation of antibodies for neuroanatomical localization of the $P2Y_{11}$ receptor in macaque brain

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ARTICLE INFO

Article history:
Received 10 May 2016
Received in revised form 5 August 2016
Accepted 6 August 2016
Available online 8 August 2016

Keywords:
P2RY11
$P2Y_1$
$P2Y_2$
Lymphocyte
Primate
Rat

ABSTRACT

Focus on the purinergic receptor $P2Y_{11}$ has increased following the finding of an association between the sleep disorder narcolepsy and a genetic variant in *P2RY11* causing decreased gene expression. Narcolepsy is believed to arise from an autoimmune destruction of the hypothalamic neurons that produce the neuropeptide hypocretin/orexin. It is unknown how a decrease in expression of $P2Y_{11}$ might contribute to an autoimmune reaction towards the hypocretin neurons and the development of narcolepsy. To advance narcolepsy research it is therefore extremely important to determine the neuroanatomical localization of $P2Y_{11}$ in the brain with particular emphasis on the hypocretin neurons. In this article we used western blot, staining of blood smears, and flow cytometry to select two antibodies for immunohistochemical staining of macaque monkey brain. Staining was seen in neuron-like structures in cortical and hypothalamic regions. Rats do not have a gene orthologue to the $P2Y_{11}$ receptor and therefore rat brain was used as negative control tissue. The chromogenic signal observed in macaque monkey brain in neurons was not considered reliable, because the antibodies stained rat brain in a similar distribution pattern. Hence, the neuroanatomical localization of the $P2Y_{11}$ receptor remains undetermined due to the lack of specific $P2Y_{11}$ antibodies for brain immunohistochemistry.

1. Introduction

Patients suffering from the sleep disorder narcolepsy experience excessive daytime sleepiness with spontaneous sleep attacks and fragmented sleep during the night. Other symptoms include cataplexy which is characterized by a sudden loss of muscle tone often triggered by laughter (Lammers et al., 2000). Current research focuses on the hypothesis that narcolepsy is caused by a specific autoimmune destruction of the hypocretin/orexin-producing neurons in the lateral hypothalamus. The mechanism by which the immune system recognizes and destroys hypocretin neurons is still unknown.

A single nucleotide polymorphism (SNP) in the *P2RY11* gene (rs2305795) is associated with narcolepsy (Kornum et al., 2011). The genetic study of rs2305795 in narcolepsy was carried out across three ethnic groups, European, Asian and African Americans, with a total of 5689 individuals. Data shows a combined odds ratio (OR) of 1.28 with a *p*-value of $6.1*10^{-10}$ (Kornum et al., 2011). The narcolepsy-associated *P2RY11* SNP is located in the 3′-untranslated region (UTR) of the gene transcript. This region is often involved in gene expression regulation and concordantly, the disease-associated allele decreases the mRNA expression of *P2RY11* in natural killer (NK) cells and $CD8^+$ T lymphocytes (Kornum et al., 2011). Hence, a lower *P2RY11* gene expression in these cells correlates with an increased risk for developing narcolepsy.

The *P2RY11* gene encodes a G-protein coupled receptor termed $P2Y_{11}$, belonging to the family of purinergic receptors that senses extracellular nucleotides. The primary ligand of $P2Y_{11}$ is adenosine 5′-triphosphate (ATP), and stimulation of the receptor results in activation of adenylyl cyclase and phosphatidylinositol pathways (Communi et al., 1999). Not much is known about the normal function of this receptor, one reason being that no gene orthologue exists in murine species (as reviewed by (Dreisig and Kornum, 2016)). It is therefore not possible to produce knockout strains for this receptor, which have been very helpful in the research of other purinergic receptors. Most of the research conducted on $P2Y_{11}$

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http://dx.doi.org/10.1016/j.jchemneu.2016.08.002
0891-0618/

centres around its role in the human immune system. Here it is found to regulate various effects such as cytokine release in keratinocytes (Ishimaru et al., 2013), increase chemotaxis in granulocytes (Moreschi et al., 2008, 2006), delay apoptosis in neutrophils (Vaughan et al., 2007), and increase lymphocyte viability (Kornum et al., 2011).

Beside a relatively high expression in immune cells, *P2RY11* is found highly expressed brain (Moore et al., 2001). The cellular localization and function of the receptor in the brain are unknown. It remains unclear how the rs2305795 SNP in *P2RY11* increases the risk of developing narcolepsy, but one possible explanation is that $P2Y_{11}$ might have a protective role in neurons analogue to what has been observed in T lymphocytes (Kornum et al., 2011). If indeed so, it might be relevant for the disease process resulting in narcolepsy, where the hypocretin-producing neurons in the lateral hypothalamus are supposedly destroyed by the immune system. It is unknown if the hypocretin neurons in the brain express the $P2Y_{11}$ receptor and whether this affects viability of these neurons. Thus, this study aimed at investigating the neuroanatomical distribution of $P2Y_{11}$ in the primate brain with focus on the hypothalamic hypocretin neurons.

2. Materials and methods

2.1. Gene expression

P2RY11 gene expression was investigated in macaque brain from three different regions i.e. prefrontal cortex, hypothalamus and cerebellum. Tissue punches were collected bilaterally from 6 mm fresh frozen brain blocks of control animal and unilaterally from two animals that received intrathecal adeno-associated virus type 9 (AAV9)-GFP (1 mL, 2.0×10^{12} vector genomes), which was manufactured and purified as described previously by (Samaranch et al., 2014). Convection-enhanced delivery was monitored with real-time MRI by adding a MR-visible contrast agent (2 mM gadolinium, Prohance, Bracco Diagnostics, Monroe Township) to vector preparation as described in detail by (Fiandaca et al., 2008). All procedures were carried out in accordance with the UCSF Institutional Animal Care and Use Committee (San Francisco, CA) and Valley Biosystems (Sacramento, CA). Necropsy was performed by transcardial perfusion with ice-cold phosphate-buffered saline (PBS). Brains were sliced in 6 mm blocks and cut longitudinally in halves before flash freezing them in isopentane and stored at −80 °C.

Punches were quickly transferred to Qiazol lysis reagent (#79306, Qiagen) at 4 °C before homogenization using sonication and left at 4 °C until the following day. RNA was extracted using RNeasy Lipid Tissue Mini Kit (#74804, Qiagen) according to manufacturer's instructions. An on-column DNAse digestion (#79254, Qiagen) was included in the protocol. RNA was eluted in 60 μL RNAse-free water and the concentration measured on Nanodrop. cDNA synthesis was performed with iScript™ Reverse Transcription Supermix (#1708840, BioRad) according to manufacturer's instruction and product stored at −20 °C until further use. Gene expression was measured in MicroAmp® Fast Optical 96-Well Reaction Plate (#4346906, Applied Biosystems) after mixing cDNA samples with TaqMan Universal Master Mix (#4440043, Applied Biosystems) and specific primer/probes for cynomolgus macaque i.e. *P2RY11* (#mf02913762_g1, ThermoFisher Scientific) and *GAPDH* (#mf04392546_g1, ThermoFisher Scientific). The plate was run on ABIPrism 7000 Sequence detection system. Statistical difference was tested by calculating the ΔCt-values and comparing each group in a one-way ANOVA using GraphPad Prism 6.

2.2. Antibodies

This article reports application of three $P2Y_{11}$ antibodies applied in various immunoassays i.e. western blot, immunocytochemistry (ICC), flow cytometry, and immunohistochemistry (IHC). The three commercial antibodies that target three different human $P2Y_{11}$ receptor epitopes were tested (Table 1). For two of the three antibodies the exact immunogenic peptide sequence was considered proprietary and could not be disclosed by the suppliers.

2.3. Macaque monkey blood smear

Whole blood was drawn in heparinized tubes from macaque monkeys and diluted 1:1 with PBS. Lymphocytes were collected following density gradient centrifugation with Ficoll (#17-1440-02, GE healthcare Life Sciences) at 1,000*g* for 10 min. Cells were washed twice in PBS using a slow spin at 300 g for 10 min before resuspended in PBS. Cell suspension was smeared onto Fisherbrand Superfrost Plus microscope slides (#12-550-15, Fisher Scientific) and allowed to dry.

2.4. Immunocytochemical DAB staining

Blood smear slides were fixed either 1 min in 4 °C acetone or 15 min at room temperature (RT) in 4% paraformaldehyde (PFA). Blood smears were blocked for endogenous peroxidase activity in PeroxAbolish (#PXA969 M, Biocare Medical) for 20 min at RT. Cells were washed three times in PBS and blocked for unspecific binding in Background Sniper (#BS966MM, Biocare Medical) for 30 min at RT before incubation in 0.1 mg/mL primary antibody diluted in Da Vinci Green Diluent (#PD900 M, Biocare Medical) for 2.5 h at RT with rabbit polyclonal IgG (#I-1000, Vector Laboratories) as isotype control antibody. Cells were washed three times in PBS before incubation in secondary MACH horseradish peroxidase (HRP)-polymer antibody solution targeting rabbit IgG (#RHRP520L, Biocare Medical) for 1 h at RT. DAB development was performed in solutions prepared as 5 mL dH_2O + 2dr buffer + 4dr DAB + 2dr H_2O_2 provided in DAB Peroxidase Substrate kit (#SK-4100, Vector Laboratories) for 2:30 min and stopped in dH_2O. Smears were coversliped with Shandon-Mount mounting medium (#1900333, Thermo Scientific) and imaged at 20× magnification.

2.5. Immunofluorescent staining of macaque blood smears

Blood smears were fixed analogue to above, blocked in Background Sniper and incubated in 0.2 mg/mL primary antibody

Table 1
Antibodies targeting the human $P2Y_{11}$ receptor applied in western blot, immunocytochemistry (ICC), flow cytometry, and immunohistochemistry (IHC).

$P2Y_{11}$ antibodies			
Catalogue number, supplier	Species/type	Concentration	Epitope/immunogenic peptide sequence
LS-A866, LifeSpan Biosciences	Polyclonal rabbit IgG	1 mg/mL	17 amino acid sequence in 3rd cytoplasmic loop
ab183096, Abcam	Polyclonal rabbit IgG	1 mg/mL	C-terminal, APKPSEPQSRELSQ
ab140878, Abcam	Polyclonal rabbit IgG	1 mg/mL	16 amino acid sequence in 3rd extracellular loop