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Role of the purinergic P2Y2 receptor in hippocampal function in mice

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Abstract. – OBJECTIVE: The aim of this study is to investigate the role of the purinergic P2Y2 receptor in learning and memory processes.

MATERIALS AND METHODS: Behavioral, electrophysiological, and biochemical tests of memory function were conducted in P2Y2 receptor knockout (P2Y2R-KO) mice, and the findings were compared to those of wild-type mice with the help of unpaired Student's *t*-test.

RESULTS: The findings of the behavioral Y-maze test showed that the P2Y2R-KO mice had impaired memory and cognitive function. Electrophysiological studies on paired-pulse facilitation showed that glutamate release was higher in the P2Y2R-KO mice than in the WT mice. Furthermore, PCR and Western blot analysis revealed that the mRNA and protein expression of acetylcholinesterase E (AChE) and alpha-7 nicotinic acetylcholine receptors (α7 nAChRs) were increased in the hippocampus of P2Y2R-KO mice.

CONCLUSIONS: The findings of this study indicate that P2Y2 receptors are important regulators of both glutamatergic and cholinergic systems in the hippocampus.

Key Words:
P2Y2 receptor, Acetylcholinesterase, Nicotine, Cholinergic, Glutamatergic.

Introduction

P2 receptors are purinergic ATP receptors that are essential for regulating memory function[1]. ATP is an important regulator in the central nervous system[2], and once ATP is released in the extracellular space, it activates both excitatory and inhibitory P2 receptors[1]. P2 receptors are classified into several subtypes of ligand-gated cationic channels (P2X) and G-protein-coupled receptors (P2Y)[3]. P2Y purinoceptors (P2YRs), which are activated by extracellular nucleotides, belong to the superfamily of G-protein-coupled receptors and are composed of eight subtypes (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, and P2Y14). P2Y2 receptors, which are coupled with Gq proteins, stimulate phospholipase C and result in increased levels of inositol phosphates and mobilization of Ca^{2+} from intracellular stores, which in turn, activate downstream signaling pathways[4]. In addition, P2Y2 receptors are widely distributed throughout the body, including the brain. It has shown that P2Y2 receptors have functions in various cell types, such as astrocytes, glial cells, epithelial cells, and coronary artery smooth muscle cells.

A few researches have demonstrated that P2Y2 receptors are expressed in the brain. However, most of these studies illustrate the importance of P2Y2 receptors in regulating neurotransmitter release, enzyme activity, and pro-inflammatory cytokines in the brain[5], and reports describing the functional effects of P2Y2 receptors in learning and memory processes are rare. In this study, we contribute to the literature by examining the role of P2Y2 receptors in learning and memory function in P2Y2 receptor-knockout (P2Y2R KO) mice *via* behavioral, electrophysiological, and molecular methodologies. Here, we show that P2Y2 receptors are required for regulating learning and memory function and that knockout of the P2Y2 receptor results in memory deficits.

Materials and Methods

P2Y2R Knockout Mice

Wild-type (C57BL/6) mice and P2Y2R knockout (P2Y2R-KO or $P2Y2R^{-/-}$) mice on a C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and bred at

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the animal facility of Auburn University. DNA was extracted from mouse tail snip samples, and PCR genotyping was routinely performed according the instructions of the animal supplier. Mice were housed in the Biological Research Facility of Auburn University (AL, USA) in a controlled and pathogen-free environment (temperature, 25°C; 12:12-h light-dark cycle) with free access to water and a standard chow diet.

Assessment of Spatial Memory

The Y-maze test assesses the ability of an animal to recognize places already explored and its propensity to explore a new place[6]. Therefore, the Y-maze was used to assess working memory and spatial memory functions in P2Y2R-KO mice and wild-type (WT) mice, as described previously[7]. The apparatus for the Y-maze test consists of three plastic arms separated by an angle of 120°. The apparatus was placed on a floor with a light at the top of each arm and a camera placed above the apparatus to record all the test sessions. The Y-maze tests were carried out when the mice were 12 weeks old. The training sessions were 15 min long, and animals were allowed to explore only two of the arms: the arm they were initially placed in (entry arm) and one (known arm) of the two other arms located to the left and right of the entry arm. During the second session, the mice were allowed to explore all three arms of the maze, including the newly available arm (novel arm), during a session lasting approximately 6 min. The first and second sessions were separated by a period of 3 h. The second session was video recorded to score the number and order of arm entries. An arm entry was registered when more than half of the mouse's body was within any of the three arms. The dwell time in each arm was also recorded for the all mice. The number of entries and time spent in the novel arm were scored and analyzed.

Preparations of Acute Hippocampal Slices

The animals were euthanized with CO_2, and then, decapitated for removal of the brain. The resected brains were washed with oxygenated cutting solution to remove the blood. Then, brain sections (thickness, 350 μm) were created with a vibratome Series 1000 tissue sectioning system (Technical Products International Inc., St. Louis, MO, USA) and washed with an oxygenated cutting solution (NaCl, 85 mM; KCl, 2.5 mM; $MgSO_4$, 4.0 mM; $CaCl_2$, 0.5 mM; NaH_2PO_4, 1.25 mM; $NaHCO_3$, 25 mM; glucose, 25 mM; sucrose, 75 mM; kynurenic acid, 2.0 mM; ascorbate, 0.5 mM). The slices were submerged in oxygenated artificial cerebral spinal fluid (ACSF) in a holding chamber for 2 h at 30°C, and following this, long-term potentiation (LTP) recording was started.

Extracellular Field Recordings

Sections were transferred into a submerge-type recording chamber, which was held between two nylon nets and viewed under a microscope. This submersion chamber was continuously perfused with oxygenated ACSF (32°C) at a flow rate of 2-3 ml/min. A platinum bipolar electrode was placed on the CA3 region of the hippocampus. A glass microelectrode (outer diameter, 1.5 mm) was filled with ACSF with a micropipette (Narishigie Scientific Instruments Lab., Tokyo), and then, placed on the stratum radiatum in the CA1 region of the hippocampus to record field excitatory postsynaptic potentials (fEPSPs) from the Schaffer collateral pathway. Two electrodes were inserted in the middle of the stratum radiatum with a Model 4D Digital Stimulus Isolation Amplifier to stimulate the CA3 region. Field potentials were recorded using the LTP Recording software with Axoclamp 2B (Axon Instruments, Foster City, CA, USA) and analyzed using the WinLTP software[8].

General and Real-Time RT-PCR Analysis

Tissue samples were obtained from isolated hippocampi of WT and $P2Y2R^{-/-}$ mice. Total RNA and DNA were extracted from tissue samples using the TRIzol reagent (Sigma-Aldrich, St. Louis, MO, USA), and the RNeasy and DNeasy kits, respectively (Qiagen, Hilden, Germany). Subsequently, total RNA was treated with RNase-free DNase (Ambion, Carlsbad, CA, USA) to eliminate possible traces of genomic DNA. For the synthesis of first-strand complimentary DNA (cDNA), 500 ng of total RNA was reverse transcribed using a cDNA synthesis kit (Applied Biosystems, Foster City, CA, USA). The cDNA samples were then amplified by PCR using 2.5 units of TaqDNA polymerase (Qiagen, Shanghai, China). Real Time-PCR was performed on an iCycler iQ5 detection system (Bio-Rad, Hercules, CA, USA) with SYBR Green reagents (Bio-tool). The sequences of the primers used were as follows: mouse nicotinic receptor (alpha polypeptide 7) mRNA, 5′-ACATGTCTGAGTACCCCGGA-3′ (forward) and 5′-AGGACCACCCTCCATAGGAC-3′ (reverse); mouse acetylcholinesterase (AChE) mRNA, 5′-ATCG-

GTGTACCCCAAGCAAG-3′ (forward) and 5′-TGCAGTTAGAGCCACGGAAG-3′ (reverse); mouse β-actin, 5′-ATGGATGACGATATCGCTGCG-3′ (forward) and 5′- CTAGAAGCACTTGCGGTGCAC-3′ (reverse). All the kits and reagents were used according to the manufacturers' instructions.

Western Blot Analysis

Hippocampi from 12-week-old P2Y2R-KO and WT control mice were lysed using lysis buffer. The samples were sonicated with a Q-sonica homogenizer (Qsonica, Newtown, CT, USA) at a frequency of 30 Hz pulses for 20 s. Following this, the samples were centrifuged at 12,000′ g for 20 min. The supernatant was collected, divided into 100-µL aliquots, and stored at -80°C. The proteins were quantified using the BCA assay (Pierce, Oakland, CA, USA), combined at the appropriate ratio with 4′ Laemmli buffer (containing β-mercaptoethanol obtained from Sigma-Aldrich), and denatured by heating at 90°C for 5 min. The denatured proteins were separated using 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-p, Millipore, Billerica, MA, USA). The membranes were then blocked for 2 h in 5% non-fat dried milk dissolved in Tris-Buffered Saline containing 0.01% Tween 20 (TBST). They were then washed with TBST and incubated overnight at 4°C with one of the following primary antibodies: anti-AMPA-GluR1 (1:1,000, Cell Signaling Technology, Danvers, MA, USA), anti-PSD-95 (1:1,000, Cell Signaling Technology), and anti-GAPDH (1:1,000, Cell Signaling Technology, Danvers, MA, USA). The membranes were then probed for 4 h at room temperature with the corresponding secondary anti-rabbit antibodies (1:10,000) conjugated with fluorophore DyLight 550. Protein bands were visualized using a FLA-5100 imager with a 532-nM green laser and the LPG filter set (Fujifilm Inc., Tokyo, Japan). Fluorescent signals were visualized by excitation at λ532 nm and emission at λ570 nm. Finally, protein expression was determined based on the densities of these bands relative to that of GAPDH for both the P2Y2R-KO mice and WT control mice.

Data Analysis

Data are presented as mean ± standard error of the mean (SEM) values for each group, and they were analyzed using the GraphPad Prism 5 software. Two-tailed unpaired Student's *t*-tests were used to analyze the data obtained from the Y-maze, electrophysiological, neurochemistry, and Western blot analyses, as well as water intake, percentage fat mass, percentage lean mass, percentage body water, and blood glucose and plasma insulin concentrations. $p \leq 0.05$ was considered to indicate statistical significance.

Results

Behavioral Performance in Y-maze Tests

There was no significant difference in the total time spent in the novel arm between the WT and P2Y2R-KO groups (Figure 1A), or in the number of mice that chose the novel arm at the beginning of the test session (2/12, P2Y2R-KO group; 2/7, WT control group). However, significantly fewer entries into the novel arm were recorded for the P2Y2R-KO mice than for the WT mice ($p < 0.05$) (Figure 1B). The P2Y2R-KO mice also exhibited a tendency to spend more time in the entry and other arms than in the novel arm, but the difference in time spent in these two arms compared to the WT control group was not significant ($p > 0.05$). Similarly, although the WT mice showed a propensity to spend more time in the novel arm, the difference compared to the P2Y2R-KO mice was not significant ($p > 0.05$).

Basal Synaptic Transmission and LTP in P2Y2 KO Mice

Theta burst stimulation (TBS) of the presynaptic fibers in the hippocampal CA1 region induces LTP of the synaptic transmission (Figures 2A and 2B). Input-output relationship graphs were constructed to evaluate the strength and response of synaptic transmission in the WT and P2Y2R-KO mice (Figure 2C, $p > 0.05$; n = 5). There were no significant differences between the WT and P2Y2R-KO mice in terms of fEPSP amplitude across the different stimulus intensities (Figure 2D).

Expression of the P2Y2 Receptor in the Hippocampus of WT Mice

Previous studies[9,10] have demonstrated that the P2Y2 receptor is widely expressed in the nervous system. First, we demonstrated that the P2Y2 receptor was expressed at the mRNA and protein levels in the hippocampus of WT mice (Figures 3A and 3B). Next, we designed primers to amplify the nicotine receptor (α7) and acetylcholinesterase (AChE) mRNA. Interestingly, nicotine receptor expression was three-fold higher in the

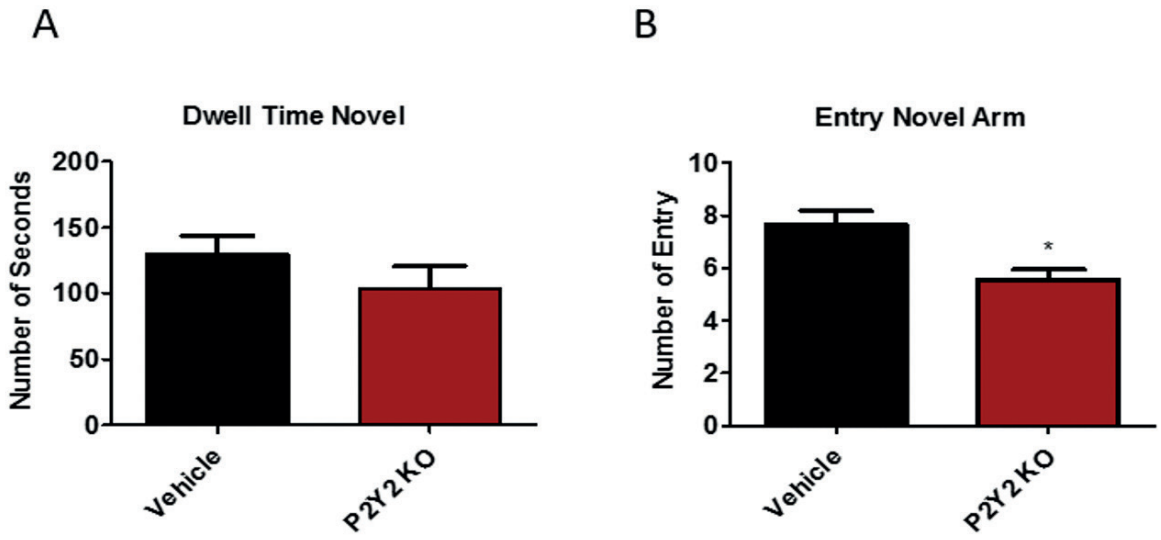

Figure 1. Comparison of number of entries and dwell time in the novel arm between the P2Y2R-KO mice and vehicle mice. The Y-maze results for 11 WT and 7 P2Y2R-KO mice are shown. **A**, Representative total dwell time spent in the novel arm in WT and P2Y2R-KO mice. **B**, Representative total number of entries into the novel arm in WT and P2Y2R-KO mice. Data represent the mean±SEM values, and *p*-values were calculated by two-tailed unpaired Student's *t*-tests.

hippocampus of P2Y2RKO mice than in the hippocampus of WT mice. In addition, increased acetylcholinesterase (AChE) mRNA levels were also detected in the hippocampus of P2Y2R-KO mice (Figures 4A, 4B and 4C). All these data indicate the potential functional significance of the P2Y2 receptor in the hippocampus.

Discussion

The findings of the present study demonstrate that the P2Y2 receptor is involved in modulating learning and memory processes in the hippocampus. Specifically, our researches in P2Y2R-KO mice showed that they had behavioral memory deficits and increase in LTP. Additionally, PPF analysis revealed that glutamate release was higher in the P2Y2R-KO mice than in the control mice. By combining the data obtained from the PCR, Western blot, and AChE assays, we found that the facilitation of glutamate release and increase in acetylcholine levels in the nerve terminals was caused by a decrease in AChE in the P2Y2R-KO mice.

The P2Y2 receptor and its role in memory function are not clear. In the present study, P2Y2R-KO mice were used as a model to investigate the role of the P2Y2 receptor in memory function. Compared to WT control mice, the P2Y2R-KO mice showed impaired working memory based on their performance in the Y-maze test, which is a hippocampal-dependent task (Figure 1). These cognitive deficits were manifested in P2Y2R-KO mice in the form of significantly fewer entries into the novel arm compared to the control mice. This means that the P2Y2R-KO mice preferred to avoid new environments. In addition, once the mice entered the novel arm, the P2Y2R-KO mice exhibited a shorter, but not statistically significant, dwell time than the WT mice. It has been reported that P2Y2 receptor activation results in a decrease in glutamate release from the presynaptic terminals of the hippocampus[11]. Accordingly, our electrophysiological study indicated that there was an increase in hippocampal glutamate release in P2Y2R-KO mice. Thus, our findings reveal the function of the P2Y2 receptor in glutamate release in the hippocampus, and therefore, its role in memory function.

Acetylcholinesterase, an enzyme that breaks down acetylcholine to choline and acetyl-co-enzyme A, is important for learning and memory processes[12]. AChE is found mainly in the synaptic cleft and plays an important role in reducing excessive acetylcholine in the synapse and regulating synaptic function. AChE receptors (AChRs) in the

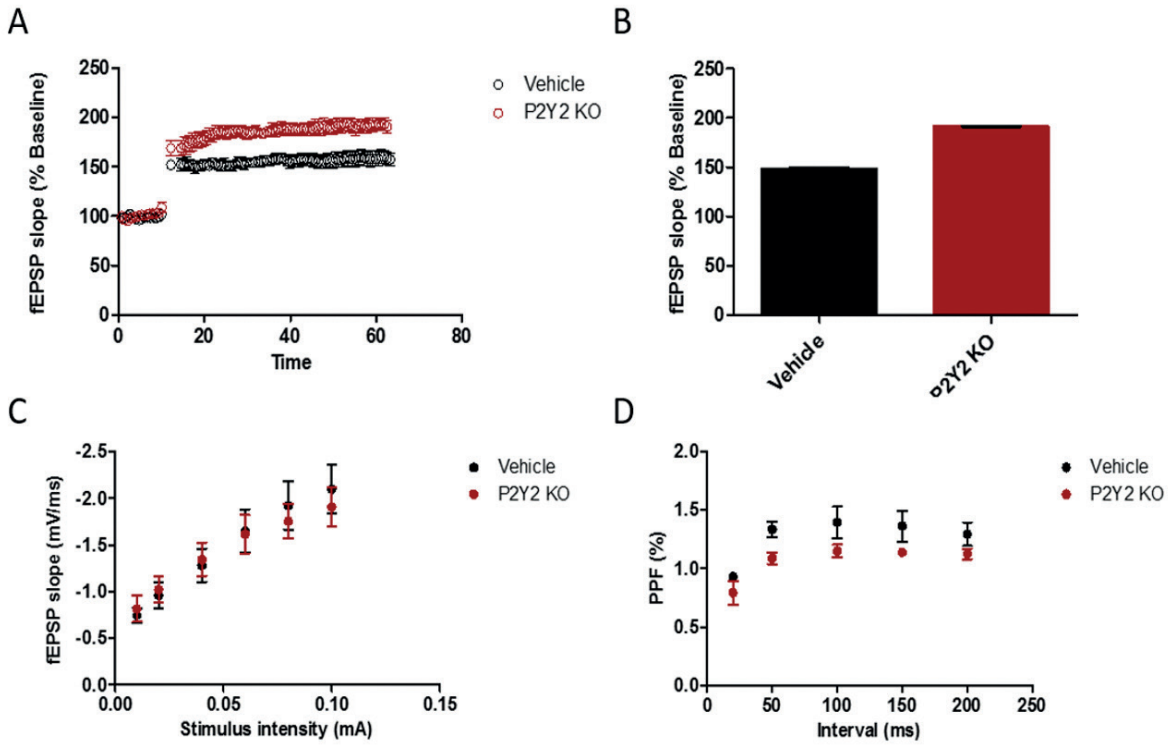

Figure 2. Long-term potentiation from the Schaffer collateral in the CA1 area of the hippocampus. Field excitatory postsynaptic potentials (fEPSPs) were measured in the hippocampus sections of P2Y2R-KO and WT mice. **A,** Long-term potentiation (LTP) was induced by theta burst stimulation (TBS) and measured at 55–60 min after TBS. Representative synaptic responses recorded indicate that LTP was higher and more persistent after TBS in the of P2Y2R-KO mice (Red) than in the WT mice (Black). **B,** Percentage changes in synaptic responses in the P2Y2R-KO and WT tissues ($p < 0.01$; n = 6). **C,** Basal synaptic transmission in the P2Y2R-KO and WT mice. **D,** Comparison of paired-pulse facilitation (PPF) in P2Y2R-KO (Red) and WT (Black) mice. PPF ratio (slope2/slope1) in P2Y2R-KO and WT mice measured at different inter-pulse intervals (50 ms, 100 ms, 150 ms, and 200 ms) ($p < 0.05$, n = 6).

brain play a central role in memory formation and cognition[13,14], and AChE is also recognized as the primary cholinesterase in the brain[15]. Previous studies[16,17] have shown that decreased P2Y2 receptor expression is associated with a decrease in AChE expression and activity. However, in our study, we found that both AChE expression and activity were higher in P2Y2R-KO mice than in WT mice. To validate our results, we assessed alpha-7 nicotinic acetylcholine receptor (α7 nAChR) expression. The results showed that there were higher levels of α7 nAChR in the hippocampus of P2Y2R-KO mice than in the hippocampus of WT mice. Accordingly, our gene expression analysis also showed that there were higher levels of AChE and α7 nAChR mRNA in the P2Y2R-KO mice than in the WT mice. With regard to the effects of nicotine on its receptors, it is well recognized that small concentrations of nicotine can enhance the function of nicotinic re-

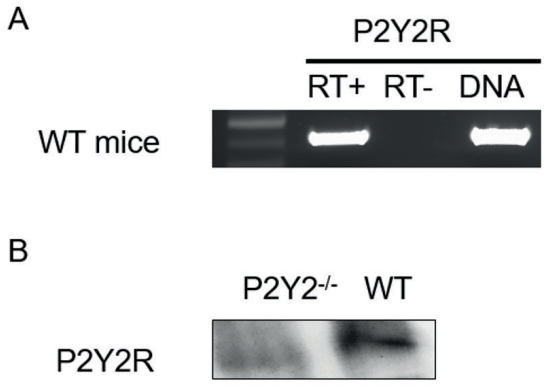

Figure 3. Expression of the P2Y2 receptor in the hippocampus of P2Y2R-KO and WT mice. **A**, PCR detection of P2Y2 receptor (P2Y2R) mRNA in WT mice. DNA extracted from the hippocampus was used as the positive control. **B**, Western blot analysis of P2Y2 receptor protein expression in P2Y2R-KO and WT mice.

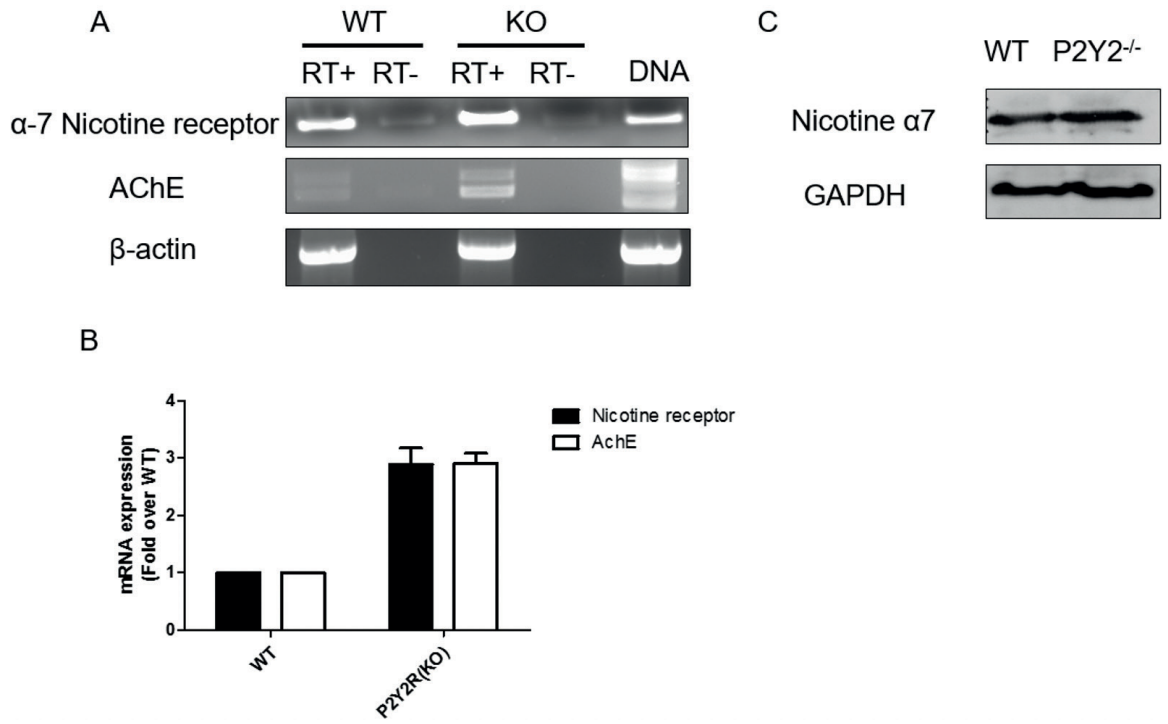

Figure 4. Upregulation of the nicotine receptor in P2Y2R-KO mice. Two pairs of primers were designed to quantify the mouse alpha-7 nicotinic acetylcholine receptor (α7 nicotine receptor) and acetylcholinesterase E (AChE) mRNA in hippocampus tissues from WT and P2Y2R-KO mice by real-time RT-PCR (n = 3). **A**, Agarose gel images of PCR products. **B**, Relative expression of α7 nicotinic acetylcholine receptor and AChE mRNA in WT and P2Y2R-KO mice. **C**, Western blot showing P2Y2 receptor protein expression (relative to expression of the internal control GAPDH) in hippocampus tissues from WT and P2Y2R-KO mice.

ceptors, while high concentrations are inhibitory and stimulate endocytosis. Therefore, nicotine can either stimulate or desensitize nicotinic receptors; hence, the concentration of acetylcholine, the endogenous ligand of nicotinic receptors, is vital for memory regulation and cognitive function. Thus, based on nicotinic acetylcholine receptor kinetics, the present results indicate that in mice that lack P2Y2 receptors, increased levels of AChE result in a decrease in acetylcholine levels in the synapse and an increase in α7 nAChR expression, and this effect leads to a higher LTP response. Thus, P2Y2 receptors might play a role in cognitive function through *via* glutamatergic and cholinergic mechanisms.

Conclusions

In summary, the present study provides important insight into the functional and molecular mechanisms of the hippocampal nerve terminals and, therefore, further clarifies the relationship between P2Y2 receptor expression and learning and memory processes in the brain.

Conflict of Interest

The Authors declare that they have no conflict of interest.

References

1) Guzman SJ, Gerevich Z. P2Y receptors in synaptic transmission and plasticity: therapeutic potential in cognitive dysfunction. Neural Plast 2016; 2016: 1207393.
2) Pedata F, Melani A, Pugliese AM, Coppi E, Cipriani S, Traini C. The role of ATP and adenosine in the brain under normoxic and ischemic conditions. Purinergic Signal 2007; 3: 299-310.
3) Puchałowicz K, Tarnowski M, Baranowska-Bosiacka I, Chlubek D, Dziedziejko V. P2X and P2Y receptors—role in the pathophysiology of the nervous system. Int J Mol Sci 2014; 15: 23672-23704.
4) von Kugelgen I. Excitatory P2-receptors at sympathetic axon terminals: role in temperature control of cutaneous blood flow. Br J Pharmacol 2006; 148: 561-562.
5) Sperlágh B, Heinrich A, Csölle C. P2 receptor-mediated modulation of neurotransmitter release—an update. Purinergic Signalling 2007; 3: 269-284.
6) Alhowail AH, Chigurupati S, Sajid S, Mani V. Ameliorative effect of metformin on cyclophospha-

mide-induced memory impairment in mice. Eur Rev Med Pharmacol Sci 2019; 23: 9660-9666.
7) Valentim AM, Ribeiro PO, Olsson IA, and Antunes LM. The memory stages of a spatial Y-maze task are not affected by a low dose of ketamine/midazolam. Eur J Pharmacol 2013; 712; 39-47.
8) Anderson WW, Collingridge GL. The LTP Program: a data acquisition program for on-line analysis of long-term potentiation and other synaptic events. J Neurosci Methods 2001; 108: 71-83.
9) Weisman GA, Ajit D, Garrad R, Peterson TS, Woods LT, Thebeau C, Camden JM, Erb L. Neuroprotective roles of the P2Y(2) receptor. Purinergic Signal 2012; 8: 559-578.
10) Weisman GA, Camden JM, Peterson TS, Ajit D, Woods LT, Erb L. P2 receptors for extracellular nucleotides in the central nervous system: role of P2X7 and $P2Y_2$ receptor interactions in neuroinflammation. Mol Neurobiol 2012; 46: 96-113.
11) Rodrigues RJ, Almeida T, Richardson PJ, Oliveira CR, Cunha RA. Dual presynaptic control by ATP of glutamate release via facilitatory P2X1, P2X2/3, and P2X3 and inhibitory P2Y1, P2Y2, and/or P2Y4 receptors in the rat hippocampus. J Neurosci 2005; 25: 6286-6295.
12) Pepeu G, Giovannini MG. Cholinesterase inhibitors and memory. Chem Biol Interact 2010; 187: 403-408.
13) Placzek AN, Zhang TA, Dani JA. Nicotinic mechanisms influencing synaptic plasticity in the hippocampus. Acta Pharmacol Sin 2009; 30: 752-760.
14) Sacco KA, Bannon KL, George TP. Nicotinic receptor mechanisms and cognition in normal states and neuropsychiatric disorders. J Psychopharmacol 2004; 18: 457-474.
15) Campanari ML, Navarrete F, Ginsberg SD, Manzanares J, Sáez-Valero J, García-Ayllón MS. Increased expression of readthrough acetylcholinesterase variants in the brains of alzheimer's disease patients. J Alzheimers Dis 2016; 53: 831-841.
16) Tung EK, Choi RC, Siow NL, Jiang JX, Ling KK, Simon J, Barnard EA, Tsim KW. P2Y2 receptor activation regulates the expression of acetylcholinesterase and acetylcholine receptor genes at vertebrate neuromuscular junctions. Mol Pharmacol 2004; 66: 794-806.
17) Xu ML, Bi CW, Cheng LK, Mak S, Yao P, Luk WK, Lau KK, Cheng AW, Tsim KW. Reduced expression of P2Y2 receptor and acetylcholinesterase at neuromuscular junction of P2Y1 receptor knock-out mice. J Mol Neurosci 2015; 57: 446-451.