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Selective Upregulation of Per1 mRNA Expression by ATP Through Activation of P2X7 Purinergic Receptors Expressed in Microglial Cells

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Abstract. Clock genes are believed to play a pivotal role in the generation and oscillation of circadian rhythm as a central clock in the hypothalamic suprachiasmatic nucleus in the mammalian brain. In this study, mRNA expression was for the first time demonstrated with clock genes in both cultured murine microglia and microglial cell line BV-2 cells. Exposure to ATP transiently increased Period-1 (Per1) mRNA expression without affecting that of other clock genes in BV-2 cells, while a similarly transient increase was shown in Per1 mRNA expression in a manner sensitive to P2X7 purinergic receptor antagonists in cultured microglia exposed to ATP. In BV-2 cells transfected with a Per1 promoter luciferase reporter plasmid, ATP significantly increased luciferase activity in a manner sensitive to a P2X7-receptor antagonist. In both microglia and BV-2 cells, a significant increase by ATP was seen in the immunocytochemical fluorescence intensity of cells expressing Per1 protein, with mRNA expression of different P2 receptors including P2X7. Per1 siRNA significantly decreased the number of cells with processes in BV-2 cells exposed to ATP. These results suggest that ATP selectively promotes Per1 expression through gene transactivation after stimulation of P2X7 purinergic receptors in microglial cells.

Keywords: clock gene, microglia, ATP, P2X7 subtype

Introduction

Circadian rhythmicity has been believed to originate in circadian core oscillators localized in the suprachiasmatic nucleus of the anterior hypothalamus as a central clock at the cellular level in mammals. However, recent studies showed that circadian rhythmicity also resides in a variety of cells from peripheral tissues, including heart (1), adipose tissue (2), pancreas (3), and liver (4). The suprachiasmatic nucleus would be not essential for driving peripheral oscillations but rather act as a synchronizer of peripheral oscillators, whereas the physiological rhythmicity may be under the direct control by their own local clock genes in different peripheral tissues (5). In mice,

the rhythmic transcription of two orthologs of the *Drosophila* Period (*per*) gene appears to be essential for the generation of circadian rhythms. Expression of mouse *per* (*mper*) genes is known to be positively regulated by other clock proteins belonging to the basic helix-loop-helix period/aryl hydrocarbon receptor nuclear translocator/single minded class, such as Clock and brain and muscle aryl hydrocarbon receptor nuclear translocator-like protein-1 (Bmal1). In addition, mPer proteins orchestrate multimeric protein complexes together with the products of cryptochrome (*cry*) genes such as *mery1* and *mery2*, which in turn negatively regulate the gene transcription mediated by Clock/Bmal1 protein complex (6–9). In the CNS, however, relatively little attention has been paid to the expression profiles of different clock genes and their protein products in residential cells other than neurons.

Microglia are one of glial cell types with different functions as an immunocyte in the CNS and known to be

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activated during infection, inflammation, trauma, ischemia, and other neurodegenerative disorders. Activated microglia are believed to elicit bidirectional functions, which may be destructive or protective in the CNS (10, 11), by releasing neurotoxic or neuroprotective factors. For example, large amounts of purines are leaked and/or released from damaged cells such as neurons and astroglia, as a result of brain disorders, which in turn activate adjacent microglia (12). Activated microglia then exhibit various phenotypes such as phagocytosis (13) and chemotaxis (14), in addition to releasing oxygen radicals (15), proinflammatory cytokines (16–18), and chemokines (19). Extracellular ATP is shown to induce these phenotypes through activation of purinergic receptors expressed by microglia. Purinergic receptors are classified into P1- and P2-receptor subclasses according to the agonist specificity: adenosine for the P1 subclass and ATP for the P2 subclass (20). P1 receptors are all metabotropic receptor isoforms grouped into A1, A2A, A2B, and A3 subtypes on the basis of their intracellular signals and molecular sequential homology, whereas P2 receptors are further classified into ionotropic P2X and metabotropic P2Y subtypes. In addition to endogenous ATP, moreover, the bacterial endotoxin lipopolysaccharide (LPS) is also shown to activate astroglia as well as microglia (21, 22).

In the present study, therefore, we have determined expression profiles of particular clock gene mRNA by microglial cells exposed to the stimulants, such as ATP and LPS, in order to elucidate their possible pathological significance using primary cultured mouse brain microglia and mouse microglial cell line BV-2 cells.

Materials and Methods

Materials

Pregnant ddY mice were supplied by Sankyo Labo Service (Tokyo). Mouse microglial BV-2 cells are a generous gift from Dr. Eui-Ju Choi (Korea University, Seoul, Korea). Dulbecco's modified Eagle medium (DMEM), penicillin-streptomycin, and ethidium bromide solution were obtained from Gibco BRL (Gaithersburg, MD, USA). LPS from *Salmonella enteritidis*, Poly-L-lysine, isolectin B4, ATP, adenosine, bis-benzimidazole trihydrochloride (Hoechst33342), oxidized ATP (oxATP), and 3'-O-(4-benzoyl)benzoyl ATP (BzATP) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) was provided by JRH Biosciences, Inc. (Lenexa, KS, USA). 5xFirst strand buffer, M-MLV Reverse transcriptase, Ultra Pure Agarose, Plus Reagent, Lipofectamine 2000, LipofectamineRNAiMAX, and Alexa Fluor 594 goat anti-rabbit IgG were supplied by Invitrogen (San Diego, CA, USA). The dual luciferase

reporter assay system and pL-SV40 were purchased from Promega (Madison, WI, USA). ISOGEN, trypsin, paraformaldehyde (PA), EDTA, 3,3'-diaminobenzidine (DAB), dimethyl sulfoxide, and anti-Iba1 antibody were purchased from Wako (Osaka). dNTP mix and 10× buffer were purchased from Takara Bio Inc. (Otsu). Anti-Per1 antibody was obtained from Abcam, Inc. (Cambridge, MA, USA). (1*R**,2*S**)-4-[2-Iodo-6-(methylamino)-9*H*-purin-9-yl]-2-(phosphonooxy)bicyclo[3.1.0]hexane-1-methanol dihydrogen phosphate ester tetraammonium (MRS2500), 4-([4-formyl-5-hydroxy-6-methyl-3-[(phosphonooxy)methyl]-2-pyridinyl]azo)-1,3-benzenedisulfonic acid (PPADS), and isoPPADS were purchased from Tocris (Ballwin, MO, USA). Other chemicals used were all of the highest purity commercially available.

Cell cultures

Mouse microglial cell line BV-2 cells were plated at a density of 2×10^4 cells/cm² in DMEM containing 10% FBS in appropriate dishes and then cultured at 37°C for 1 day under 5% CO₂.

Primary microglia

This study was carried out in compliance with the Guideline for Animal Experimentation at Kanazawa University, with an effort to minimize the number of animals used and their suffering. Microglia were isolated from neonatal mouse brains by the previously described method (23), with minor modifications (24). In brief, two to three whole brains of 1–2-day-old neonatal mice were briefly triturated by using a blue-tip-mount pipette with 2 ml DMEM and then incubated with 2.5 ml of 0.25% trypsin in Ca²⁺, Mg²⁺-free phosphate-buffered saline (PBS) containing 5.5 mM glucose at 37°C for 15 min. Trypsinization was stopped by the addition of 2.5 ml horse serum supplemented with 0.1 mg/ml of DNase I, followed by centrifugation at $350 \times g$ for 5 min and subsequent trituration of the precipitates by using a blue-tip-mount pipette with DMEM containing 10% FBS for plating on poly-L-lysine-coated plastic dishes. Cultures were maintained in a 5% CO₂ / 95% air humidified incubator at 37°C for 2 weeks, with medium change every week. After 2 weeks, cultures were incubated for 20–30 min with trypsin–EDTA solution containing 0.25% trypsin and 1 mM EDTA that was diluted to 1:4 in DMEM. Cells attached to the bottom of plastic dishes were collected as microglial preparations.

Isolectin B4 staining

Cultured microglia and BV-2 cells were washed with PBS, followed by fixation with 4% PA for 30 min at 4°C and subsequent blocking with PBS containing 4% normal

horse serum, 0.03% H₂O₂, and 10% Triton X-100. Cells were washed with PBS and then incubated with isolectin B4 overnight at 4°C. Cells were finally reacted with DAB for observation under a microscope.

Determination of mRNA expression

Cultured cells were superficially washed with PBS twice, followed by extraction of total RNA using ISOGEN according to the manufacturer's instructions and subsequent synthesis of cDNA with 25 ng/ml oligo (dT)18 primer, 0.5 mM dNTP mix, and M-MLV Reverse Transcriptase (25). Reverse transcription polymerase chain reaction (RT-PCR) was conducted using upstream and downstream primers specific for each molecule (Table 1). In preliminary experiments, a clearly linear correlation was optimized with each primer set. Quantitative analysis was done at the cycle number with high linearity between mRNA expression and cDNA produc-

tion using primers for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). PCR reaction products were separated on 1.5% agarose gels with ethidium bromide for visualization. The relative abundance of each PCR product was determined by quantitative analysis of digital photographs of gels using Scion Image β 4.02 software (Scion Co., Frederick, MD, USA), followed by calculation of ratios of expression of mRNA for each gene over that for GAPDH (26).

Immunocytochemistry

Primary microglia and BV-2 cells were washed with PBS, followed by fixation with 4% PA for 20 min at 4°C and subsequent blocking with 10% normal goat serum in PBS containing 0.1% Triton X (27). Cells were then reacted with an antibody adequately diluted against Iba1 or Per1 protein overnight at 4°C. Finally, cells were reacted with the corresponding secondary antibody, anti-rabbit

Table 1. Primers used for RT-PCR in this study

	Sense primer (5'-3')	Antisense primer (3'-5')	Product size (bp)	Annealing temperature (°C)
Per1	CCAGGCCCGGAGAACCTTTTT	CGAAGTTTGAGCTCCCGAAGT	402	64
Per2	CTACCTGG CAAGGTGCAACAG	GGTTTGAATCTTGCCACTGG	696	64
Per3	TCCTGATGGTAAGACATTCCAG	GCGTGAACAATCACACTCACTT	500	64
Bmal1	TCAAGAATGCAAGGGAGGCC	AACAGGTAGAGGCGAAGTCC	599	64
Cry1	CGTCTGTTTGTGATTGCGGG	ATTACGCCACAGGAGTTGC	669	64
Cry2	AGAAGGTGAAGAGGAACAGCAC	TAGATGTATCGAGAGGGGAAGC	513	64
Dec1	CAGACAAGATACCGACTC	AACCACTACCGAGACATGCC	697	64
Dec2	ACAGGACAGAAACCTCCAAATCG	TGTTTCAGCTGAGCAATGCATTC	703	64
DBP	GGCCATGAGACTTTTGACCC	ACGTTCTTCGGGCACCTAGC	432	64
NPAS2	CCCTCTGTAAATGGCTTTGA	CTTTGCCAAACTGCATCAGA	319	60
Clock	CTATGCTTCTGTGTAACGCG	GCCTATTATTGGTGGTGGCC	719	64
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA	452	64
P2X1	CAGAAAGGAAAGGCCAAGGTATTC	TGACGACGGTTTGTCCCATTC	508	60
P2X2	CGTCTTCATCGTGCAGAAAA	CTGATCAAACGTGCAGTGCT	514	64
P2X3	TGGCGTTCTGGGTATTAAGATCGG	CAGTGGCCTGGTCACTGGCGA	435	60
P2X4	GCGTCTGTGAAGACCTGTGA	TAGCCAGGAGACACGTTGTG	416	64
P2X6	AGAGTAGTGCTGTGCCAGGAAAC	CCTCAAAGTCCCCTCCAGTCATAG	228	60
P2X7	TGTGAAGTCTCTGCCTGGTG	GTCCGCTTTTCCACATTGTT	440	60
P2Y1	TCCTCTTCATTCCGATGTGCC	TCTTCTTCTTGAGCCTGCCCAG	391	65
P2Y2	GGGACGAAGTGGGTACAAATGTC	GGTGTGGCAACTGAGGTCAAGTG	785	65
P2Y4	CAACCAATGCCAATGGAAGTACC	ACTTGTCCTCCGTGAAGAGATAG	414	60
P2Y6	TGCTTGGGTAGTGTGTGGAG	CCGGAAGTCTGTTGTGTGA	499	60
P2Y12	CCTGGGGTTGATAACCATTG	GTGGAAGGGAACAAAGCAAA	418	60
P2Y13	CGTCTGGTCCCTGATGTTCT	ACATGGCACCTTTTGTGTGA	487	57
P2Y14	GGCAGGTGAATGTGTTTGTG	CGAGCACGATGCTGAAGATA	462	60

IgG antibody conjugated with Alexa 594, and observed under a fluorescence microscope for monitoring on a computer using Image J software for subsequent data analysis. The fluorescence intensity was quantified in five different visual fields selected at random in a well.

Luciferase assay

The Per1 promoter (−1803 to +40) cloned into luciferase reporter vector was a generous gift from Dr. Sassone-Corsi (Institute de Genetique et de Biologie Moleculaire et Cellulaire, France). BV2 cells were plated at 2×10^6 cells/cm² and transfected with 4 μ g Per1 promoter by using an electroporation device (Nucleofector™) according to the manufacturer's instructions. Cells were then cultured at 37°C in a humidified 5% CO₂ incubator for 24 h, followed by exposure to 1 mM ATP for 24 h in either the presence or absence of 100 μ M oxATP. Two days after transfection with the reporter plasmid, cells were lysed, and luciferase activity was determined using specific substrates in a luminometer according to the manufacturer's protocol (28).

Transfection with small interfering RNA (siRNA)

BV2 cells were transfected with Per1 siRNA at 10 nM by LipofectamineRNAiMAX for 24 h, followed by exposure to 1 mM ATP for 1 h for determination of Per1 mRNA expression. Cells were also exposed to 1 mM ATP for different periods up to 9 h for counting the

number of cells with at least one process under a microscope (BZ-8000; Keyence, Osaka). The number of cells was individually counted for calculation of the ratio of cells with at least one process over total cells in each visual field selected at random on a microscope.

Data analyses

Results are all expressed as the mean $\pm$ S.E.M. and the statistical significance was determined by the two-tailed and unpaired Student's *t*-test or one-way analysis of variance ANOVA with Bonferroni/Dunnett post hoc test as described previously.

Results

Expression of clock genes

For validation of the appropriateness of primary microglia and BV-2 cells used in this study, cells were stained for microglial markers such as isolectin B4 and Iba1. More than 90% of cells were positive to staining for isolectin B4 and Iba1 in murine microglia cultured for 1 day (Fig. 1A), while BV-2 cells were all positive to staining for isolectin B4 and Iba1 when cultured for 1 day (Fig. 1B). In addition, marked upregulation was seen in mRNA expression of inducible nitric oxide synthase in BV-2 cells exposed to 100 ng/ml LPS for 24 h (data not shown).

For evaluation of possible expression of clock genes in

Fig. 1. Validation of microglial cells. Primary cultured microglia derived from neonatal mice (A) and BV-2 cells (B) were fixed with 4% PA, followed by staining for isolectin B4 or Iba1 and subsequent staining with Hoechst33342 for DNA. More than 90% of cells were positive to staining for isolectin B4 and Iba1 in both primary microglia and BV-2 cells.

microglial cells, total RNA was extracted from cultured mouse microglia and BV-2 cells, in addition to mouse whole brain as a positive control, followed by determination of mRNA expression on RT-PCR analysis. The expression of mRNA was seen for all clock genes examined in cultured mouse microglia (Fig. 2A), while Clock mRNA was absent from BV-2 cells with high expression of mRNA for neuronal PAS domain protein 2 (NPAS2) (Fig. 2B), which is shown to form a heterodimer with Bmal1 protein as a transcriptional factor (29). Therefore, most clock genes would be expressed by cells derived from a microglial lineage.

Profiles of clock gene expression in activated BV-2 cells

To evaluate the functionality and/or responsiveness of clock genes expressed, BV-2 cells were exposed to stimulants of microglial cells for 1–24 h for determination of mRNA expression. Both ATP (30) and LPS (21, 22) are shown to stimulate different microglial activities. In BV-2 cells exposed to 1 mM ATP for 1 to 24 h, mRNA expression of both Per1 and Per2 was more than doubled only when determined 1 h after the addition (Fig. 3). The increase was returned to the control level within 3 h after the addition of ATP. However, ATP failed to significantly affect mRNA expression of other clock genes in BV-2 cells exposed to 1 mM ATP for 1 to 24 h. These included Per3, Dec1, Dec2, Cry1, Cry2, Dbp, Bmal1, and NPAS2. Similarly, BV-2 cells were exposed to 100

ng/ml LPS for 1–24 h, followed by determination of mRNA levels of different clock genes. In contrast to the action by ATP, however, LPS did not significantly affect mRNA expression of all clock genes examined during the exposure to BV-2 cells for 1–24 h (Fig. 4).

To confirm the upregulation by ATP of mRNA expression of both Per1 and Per2, primary microglia were also exposed to 1 mM ATP for 1–24 h. A significant increase was transiently seen in Per1 and Per2 mRNA expression in primary microglia exposed to 1 mM ATP for 3 h with a decline to the level before exposure within 6 h (Fig. 5). In contrast to BV-2 cells, however, Dbp mRNA levels were significantly decreased 6 h after the addition of 1 mM ATP in primary cultured microglia. Other clock gene mRNA levels were not significantly affected during the exposure to ATP for 1 to 6 h. Accordingly, ATP would selectively and transiently increase Per1 and Per2 mRNA expression amongst different clock genes in both microglial BV-2 cells and murine primary microglia.

Possible involvement of P2X7 purinergic receptors

The increase by ATP was significantly prevented by the addition of different antagonists for P2 purinergic receptors in primary microglia exposed to 1 mM ATP for 3 h (Fig. 6A). These included PPADS as a P2-receptor antagonist, isoPPADS as a P2X-receptor antagonist, and oxATP as a P2X7-receptor antagonist. The P2Y-receptor antagonist MRS2500 did not significantly prevent the increase by ATP in microglia, by contrast, whereas the

Fig. 2. RT-PCR of clock genes in microglial cells. Total RNA was extracted from cultured mouse microglia (A) and BV-2 cells (B), in addition to adult mouse whole brain, followed by determination of mRNA expression using primers specific for each clock gene. The expression of mRNA was seen for all clock genes examined in cultured mouse microglia, while Clock mRNA was absent from BV-2 cells with high expression of NPAS2 mRNA.

Fig. 3. Effects of ATP on clock gene mRNA expression in BV-2 cells. BV-2 cells were exposed to 1 mM ATP for different periods up to 24 h, followed by extraction of total RNA and subsequent determination of mRNA expression using primers specific for each clock gene. Values are the mean $\pm$ S.E.M. in 3 different experiments. * $P < 0.05$, ** $P < 0.01$: significantly different from each control value obtained in cells not exposed to ATP. Per1 mRNA expression was more than doubled only when determined 1 h after the addition of ATP amongst the different clock genes examined.

P1-receptor agonist adenosine failed to significantly increase Per1 mRNA expression at 1 mM in microglia. In primary microglia, indeed, a significant increase was seen in Per1 mRNA levels after exposure to the selective P2X7-receptor agonist BzATP at 300 μ M (Fig. 6B). In the presence of the P2X7-receptor antagonist oxATP, moreover, ATP failed to significantly increase Per1 mRNA expression in BV-2 cells (Fig. 6C). In BV-2 cells transfected with a Per1 promoter reporter plasmid, ATP induced a significant increase in luciferase activity in a manner sensitive to oxATP (Fig. 6D). Thus, ATP would predominantly promote transactivation of the Per1 gene through a mechanism relevant to activation of the P2X7 purinergic receptor subtype in microglial cells.

Expression of Per1 protein

We next attempted to evaluate whether Per1 protein is similarly induced in cells exposed to ATP as seen with mRNA expression. Under our experimental conditions, however, western blotting analysis was rather difficult due to several technical reasons including the limited availability of sufficient proteins required for the detec-

tion by the anti-Per1 antibody from cultured microglial cells. Accordingly, cells were cultured for 12 h in either the presence or absence of 1 mM ATP, followed by immunocytochemical staining for Per1 protein in place of western blotting and subsequent staining with Hoechst33342 for nuclear DNA. In primary microglia (Fig. 7A) and BV-2 cells (Fig. 7B), immunoreactive Per1 protein was markedly detected in a number of cells stained with Hoechst33342. Repetition of these experiments for quantification clearly revealed a significant increase in the fluorescence intensity due to immunoreactive Per1 protein in cultured microglia and BV-2 cells. These results suggest that ATP would induce expression of both the mRNA and corresponding protein for Per1 in both primal microglia and BV-2 cells.

Expression of purinergic receptor subtypes

For evaluation of the possible expression of different purinergic receptor subtypes by microglial cells, RT-PCR analysis was done with primary microglia and BV-2 cells, in addition to mouse whole brain as a positive control, using primers specific for each purinergic recep-

Fig. 4. Effects of LPS on clock gene mRNA expression in BV-2 cells. BV-2 cells were exposed to 100 ng/ml LPS for different periods up to 24 h, followed by extraction of total RNA and subsequent determination of mRNA expression using primers specific for each clock gene. Values are the mean $\pm$ S.E.M. in 3 different experiments. No significant change was seen in *Per1* mRNA expression in cells exposed to LPS for 1 to 24 h.

tor subtype. Marked mRNA expression was seen for P2X1, P2X4, P2X7, P2Y1, P2Y2, P2Y4, P2Y6, P2Y12, P2Y13, and P2Y14, but not for P2X2, P2X3, or P2X6, subtypes in primary microglia (Fig. 8A), while BV-2 cells expressed mRNA for P2X1, P2X3, P2X4, P2X6, P2X7, P2Y1, P2Y2, P2Y4, P2Y6, P2Y12, P2Y13, and P2Y14, but not for P2X2 subtypes (Fig. 8B). It is thus likely that most P2 purinergic receptor subtypes are constitutively expressed by microglial cells.

Microglial cells with processes

In order to evaluate the possible functional significance of *Per1* in microglial functions, BV-2 cells were transiently transfected with siRNA for *Per1*, followed by further culture for an additional 24 h and subsequent exposure to 1 mM ATP for different periods to count the number of cells with at least one process under phase contrast microscopic observation as an index of the microglial activity. *Per1* siRNA significantly decreased *Per1* mRNA expression by more than 50% in cells not exposed to ATP, while exposure to ATP significantly increased *Per1* mRNA expression irrespective of *Per1* siRNA transfection (Fig. 9A). Although *Per1* siRNA did

not markedly affect the number of cells with processes, exposure to ATP markedly reduced the number of cells with at least one process in BV-2 cells transfected with *Per1* siRNA within 9 h after exposure (Fig. 9B). Repetition and quantification revealed that exposure to ATP significantly and transiently decreased the number of cells with a process when determined 3–6 h after exposure, while prior transfection with *Per1* siRNA led to a significant reduction of the number of cells with processes within 6 h after the exposure to 1 mM ATP (Fig. 9C). Taken together, the clock gene product *Per1* would play a role in the mechanism underlying the outgrowth of processes in microglial cells.

Discussion

The essential importance of the present findings is that clock genes were constitutively expressed by microglial cells such as mouse microglia and BV-2 cells. In addition, ATP predominantly promoted the expression of mRNA and corresponding protein for *Per1* in association with the activation of the P2X7 purinergic receptor subtype expressed by these microglial cells. To our knowl-

Fig. 5. Effects of ATP on clock gene mRNA expression in primary microglia. Primary microglia were exposed to 1 mM ATP for different periods up to 24 h, followed by extraction of total RNA and subsequent determination of mRNA expression using primers specific for each clock gene. Values are the mean $\pm$ S.E.M. in 3 to 7 different experiments. *Per1* and *Per2* mRNA expression was more than doubled only when determined 1 h after the addition of ATP amongst the different clock genes examined. * $P < 0.05$, ** $P < 0.01$, significantly different from each control value obtained in cells not exposed to ATP.

edge, this is the first direct demonstration of the selective upregulation by ATP of *Per1* mRNA expression in a manner associated with activation of P2X7 purinergic receptors in microglial cells. Although previous studies have already shown the functional expression of molecular clock genes in different cells other than neurons to date (31), there is no direct evidence for the modulation by ATP of the expression of mRNA and corresponding protein for *Per1* in microglia cells in the literature. Evidence that clock genes are functionally expressed by macrophages, which are derived from a cellular lineage identical to microglia, with a role in inflammatory immune responses is indeed accumulating (32, 33). The constitutive expression of clock genes gives support for the possible circadian rhythmicity of a variety of functions in microglia as seen in macrophages, whereas the present findings give support to the promotion of a negative feedback regulation after upregulation of *Per1* mRNA amongst different clock genes expressed by microglia as shown in suprachiasmatic neurons (6–9). From this point of view, it should be noted that sustained exposure to ATP led to a rapid but transient increase in

Per1 mRNA levels throughout whole exposure periods in primary microglia and BV-2 cells. Overexpressed *Per1* would negatively regulate the transactivation mediated by the *Bmal1*/*Clock* complex of *Per1* promoter at upstream E-box elements as a negative feedback loop in microglial cells as seen in the suprachiasmatic neurons (5–9).

The underlying mechanism for selective and transient upregulation by ATP of *Per1* mRNA expression genes in microglial cells has not been clarified so far. Parathyroid hormone was shown to up-regulate mRNA expression of both *Per1* and *Per2*, but not *Per3*, through activation of the cAMP/protein kinase A pathway in cultured rat costal chondrocytes (34), whereas adenosine has been well known for years to promote the formation of intracellular cAMP through activation of P1 purinergic receptors in different eukaryotic cells (35). Moreover, intracellular cAMP formation is believed to be facilitated by activation of particular metabotropic P2Y purinergic receptor subtypes with seven transmembrane domains (20). By taking into consideration the present negative data with MRS2500 and adenosine, however, it is likely that ATP

Fig. 6. Effects of purinergic ligands on Per1 mRNA expression. A) Cultured primary microglia were exposed to 1 mM ATP for 3 h in either the presence or absence of different antagonists for determination of Per1 mRNA expression. A transient increase was seen in Per1 mRNA expression 3 h after the addition of ATP, while the increase was prevented by the addition of different compounds endowed to antagonize P2 purinergic receptors. B) Primary microglia were exposed to the P2X7 agonist BzATP at 300 μ M for 3 h for determination of Per1 mRNA expression. C) BV-2 cells were exposed to 1 mM ATP for 1 h in either the presence or absence of the P2X7 antagonist oxATP for determination of Per1 mRNA expression. D) BV-2 cells were transfected with luciferase reporter plasmid linked to Per1 promoter fragment, followed by exposure to 1 mM ATP for 24 h in either the presence or absence of 100 μ M oxATP. In the presence of oxATP, ATP failed to significantly increase Per1 mRNA expression in BV-2 cells, while ATP induced a significant increase in luciferase activity in a manner sensitive to oxATP. Values are the mean $\pm$ S.E.M. in 3 to 7 separate experiments. * P < 0.05, ** P < 0.01: significantly different from each control value in cells not exposed to ATP. # P < 0.05: significantly different from the value in cells exposed to ATP alone.

Fig. 7. Immunocytochemistry of Per1 protein in microglial cells. Primary microglia (A) and BV-2 cells (B) were exposed to 1 mM ATP for 12 h, followed by immunocytochemical detection of Per1 protein and subsequent quantification of the fluorescence intensity. A significant increase was seen in the fluorescence intensity due to immunoreactive Per1 protein in cultured microglia and BV-2 cells exposed to 1 mM ATP. Values are the mean $\pm$ S.E.M. in 58 to 62 visual fields selected at random. ** P < 0.01: significantly different from each control value in cells not exposed to ATP.

Fig. 8. RT-PCR of purinergic receptor subtypes in microglial cells. Total RNA was extracted from cultured mouse microglia (A) and BV-2 cells (B), in addition to adult mouse whole brain, followed by determination of mRNA expression using primers specific for each receptor subtype. The expression of mRNA was seen for most all purinergic receptor subtypes examined in both cultured mouse microglia and BV-2 cells.

Fig. 9. Effects of Per1 siRNA on ATP-induced process retraction in BV-2 cells. BV-2 cells were transfected with either si-control or Per1 siRNA for 24 h and subsequent exposure to 1 mM ATP for different periods up to 9 h and subsequent counting of the number of cells with at least one process under phase contrast microscopy. Per1 mRNA expression 25 h after the exposure is shown in panel A, whereas typical phase contrast micrographic observations are shown in the panel B with quantitative data in the panel C. In panel B, processes are marked by black arrows. Per1 siRNA transfection significantly decreased the number of cells with at least one process after exposure to ATP for 9 h. Values are the mean $\pm$ S.E.M. in 3 separate experiments. * $P < 0.05$, ** $P < 0.01$: significantly different from each control value in si-control cells not exposed to ATP. # $P < 0.05$, ## $P < 0.01$: significantly different from the value in si-control cells exposed to ATP in parallel experiments.

selectively promotes Per1 mRNA expression through a mechanism relevant to activation of the ionotropic P2X purinergic receptor subtype, but not of either the P1- or P2Y-receptor subtype, in microglial cells. The prevention by different antagonists argues in favor of the possible involvement of the P2X7 purinergic receptor subtype in selective upregulation of Per1 mRNA expression in microglial cells exposed to ATP at a high concentration. Although the P2X7 subtype is thought to orchestrate an ion channel permeable to Ca^{2+} rather than Na^{+} ions (36), the exact intracellular signaling mechanism after Ca^{2+} influx remains to be elucidated in future studies. Promoter analysis would give us a clue for signal flows from Ca^{2+} rather than cAMP in the cytoplasm to Per1 promoter in the nucleus in microglial cells. In our preliminary experiments using culture medium deficient of Ca^{2+} ions, however, we failed to demonstrate the dependence of the increased mRNA level as well as promoter activity for Per1 due to the high cytotoxicity after the removal of Ca^{2+} ions from culture medium for primary microglia and BV-2 cells so far. Therefore, the final conclusion on the involvement of a P2X7 purinergic receptor subtype should await the use of a variety of P2X7-receptor antagonists other than oxATP.

Under the normal physiological conditions in the brain, extracellular ATP concentrations are kept much lower than intracellular levels. Under pathological conditions, however, ATP is leaked and/or released from adjacent damaged cells, such as neurons and glia, thereby increasing extracellular ATP to levels sufficient to activate microglia through the P2X7 purinergic receptor subtype, which has very low affinity for ATP compared to other purinergic receptor subtypes (37). In recent studies, clock genes are shown to play a role in the pathogenesis and etiology of particular diseases including implantation failure (38), bipolar disorder (39), and tumor formation (40), in addition to a role as a central clock to generate and oscillate circadian rhythmicity. Alternatively, Per1 would play a particular role as a transcription modulator responsive to extracellular signal inputs mediated by P2X7 purinergic receptors in microglia, in addition to participating in the generation of circadian rhythmicity in neurons. The present findings thus give rise to an idea that Per1 upregulation may be at least in part responsible for a variety of functional alterations induced by extracellular ATP accumulated around microglial cells under pathological conditions in the brain. Although both ATP and LPS markedly reduce the number of processes in microglia on automated morphological image analysis as a result of cellular activation, the morphological transformation is accompanied by secretion of the inflammatory cytokine tumor necrosis factor- α from microglia exposed to LPS, but not to ATP (41), in contrast to Per1 mRNA

expression shown in this study. As glutamate is also shown to promote the release of ATP from cultured spinal cord microglia through activation of a particular ionotropic glutamate-receptor subtype (42), extracellular ATP could in turn activate microglia to lead to transient retraction of processes after accumulation in response to different neurocrine and autocrine signals. Elucidation of the physiological and pathological significance of upregulation of Per1 mRNA by ATP in microglia, accordingly, is a prerequisite for future analyses of possible circadian rhythmicity in a variety of microglial functions.

It thus appears that clock genes are constitutively expressed by microglia with responsiveness to extracellular ATP at high concentrations in a manner related to activation of P2X7 purinergic receptor subtype. Molecular clock signaling is thus a potential target for the discovery and development of an innovative drug useful for the therapy and treatment of a variety of neurodegenerative and neuropsychiatric disorders relevant to activation of microglia in the brain.

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