

## P2X7 receptor mediated phosphorylation of p38MAP kinase in the hippocampus

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### Abstract

This study was designed to explore the effect of P2X7 receptor (P2X7R) activation on the expression of p38 MAP kinase (p38 MAPK) enzyme in hippocampal slices of wild-type (WT) and P2X7R<sup>-/-</sup> mice using the Western blot technique and to clarify its role in P2X7 receptor mediated [<sup>3</sup>H]glutamate release. ATP (1 mM) and the P2X7R agonist BzATP (100 μM) significantly increased p38 MAPK phosphorylation in WT mice, and these effects were absent in the hippocampal slices of P2X7R<sup>-/-</sup> mice. Both ATP- and BzATP-induced p38 MAPK phosphorylations were sensitive to the p38 MAP kinase inhibitor, SB203580 (1 μM). ATP elicited [<sup>3</sup>H]glutamate release from hippocampal slices, which was significantly attenuated by SB203580 (1 μM) but not by the extracellular signal-regulated kinase (ERK1/2) inhibitor, PD098095 (10 μM). Consequently, we suggest that P2X7Rs and p38 MAPK are involved in the stimulatory effect of ATP on glutamate release in the hippocampal slices of WT mice.

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The family of mitogen-activated protein kinases (MAPK) is divided into three different subfamilies, including the extracellular signal-regulated kinases (ERK1/2), the c-Jun amino-terminal kinases (JNK) and the p38 MAPKs [1,2]. It is now well established that neuronal MAPK cascades play an important role in synaptic plasticity and memory formation. P38 MAPK is activated during different kinds of learning paradigms [3,4], and may play a role in the transcriptional events that lead to memory consolidation [5,6]. The p38 kinases are widely expressed in many tissues, including the brain, and are activated by dual phosphorylation of threonine 180 and tyrosine 182 within the Thr-Gly-Tyr site located in subdomain VIII [7]. In the hippocampus, p38 MAPK is heavily expressed in pyramidal neurons in the CA1 and CA3 regions [8], and is abundant

around the terminals of mossy fiber synapses [9], but also appears in astrocytes and in activated microglia following ischemic insult [10].

P38 MAPK could be activated in response to various extracellular signals, including ATP, which is an important extracellular messenger in the hippocampus. The action of ATP is conveyed by different subtypes of ionotropic P2X and metabotropic P2Y receptors, and among them, the functional role of homomeric P2X7 receptor (P2X7R) [11] in the nervous system has received particular attention recently [12–14]. Compelling evidence has shown that stimulation of P2X7R releases neurotransmitters and activates neurons, either directly or indirectly, eliciting changes in their excitability [15–18]. The activation of P2X7R promotes the release of glutamate and GABA in the hippocampus [16,18], an effect that is absent in the hippocampal slices of P2X7R<sup>-/-</sup> mice [19]. Moreover, increasing body of evidence support the role of P2X7R mediated excitatory amino acid release in physiological and pathological changes of neuronal activity in the CNS, e.g., in ischemia and in the following repair pro-

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cess [12–14]. Nevertheless, the participation of intracellular signalling pathways in this effect was unclear until now. Although the P2X7R agonist BzATP has been reported to produce synaptic depression at mossy fiber-CA3 synapses, which is sensitive to the inhibition of p38 MAPK [20], the involvement of P2X7R in this effect has been disproved more recently [21]. Nevertheless, there is still the open possibility that p38 MAPK is regulated upon P2X7R activation in the hippocampus. P2X7R has been shown to mediate the activation of p38 MAPK by ATP in non-neuronal cells [22–24], but there is no clear indication whether such an activation also plays a role in the information processing of the adult brain. This study was designed in order to explore whether the activation of P2X7R promotes p38 MAPK signaling in the hippocampus and, if so, whether this signaling event mediates the facilitative effect of ATP on glutamate release in mouse hippocampal slices.

## Materials and methods

**Animals.** All studies were performed according to the NIH Guide for the Care and Use of Laboratory Animals. Experiments were conducted on 2- to 5-months old C57BL/6J wild-type (WT) (P2X7R<sup>+/+</sup>) and C57BL/6J WT based P2X7R<sup>-/-</sup> (KO) male mice weighing 20–25 g. The original breeding pairs of P2X7R<sup>-/-</sup> mice were kindly supplied by Christopher Gabel from Pfizer Inc. (Groton CT, USA). The animals contained the

DNA construct previously shown to produce genetic deletion of P2X7R [25]. Genomic DNA was isolated from the tails of WT and P2X7R<sup>-/-</sup> animals, and the genotypes were confirmed by PCR analysis using gene-specific primers as described by Solle et al. [25].

**Western blot experiments from mouse hippocampal slices.** Hippocampal slices (400  $\mu$ m) were prepared from C57BL/6J WT based P2X7R<sup>+/+</sup> and P2X7R<sup>-/-</sup> (KO) mice as previously described [19]. Slices were dissected in ice-cold Ca<sup>2+</sup>-free artificial cerebrospinal fluid (ACSF; in mM: NaCl 125; KCl 2.4; MgCl<sub>2</sub> 0.83; CaCl<sub>2</sub> 1.1; KH<sub>2</sub>PO<sub>4</sub> 0.5; Na<sub>2</sub>SO<sub>4</sub> 0.5; NaHCO<sub>3</sub> 27; glucose 10; Hepes 110, pH 7.4; Ca<sup>2+</sup>-free ACSF was of the same composition except for 1.93 mM MgCl<sub>2</sub>) and placed for 10 min in polypropylene tubes containing Ca<sup>2+</sup>-free ACSF at 35 °C, equilibrated at pH 7.4 in O<sub>2</sub>/CO<sub>2</sub> (95:5, v/v), and then incubated at 35 °C in 900  $\mu$ L ACSF containing 1.1 mM Ca<sup>2+</sup> and 1  $\mu$ M tetrodotoxin (TTX, Alomone labs, Israel) for 50 min before pharmacological treatment. TTX was used to prevent indirect effects caused by neuronal firing. Adenosine 5'-triphosphate (ATP; 1 mM, Sigma), 2'(3')-O-(4-benzoylbenzoyl) adenosine 5'-triphosphate (BzATP; 100  $\mu$ M, Sigma), and anandamide (1  $\mu$ M, Tocris) were added for 5 min, whereas the p38 MAPK inhibitor SB203580 (1  $\mu$ M, Sigma) was allowed to react for 30 min after the incubation. The ACSF solution was then aspirated and the slices were lysed by sonication in homogenization buffer containing 1% sodium dodecyl sulphate (SDS; Sigma (v/v)), 1 mM sodium orthovanadate (Sigma) and 1  $\mu$ L of protease inhibitor cocktail (Sigma), with sterile water added to a total volume of 100  $\mu$ L. After centrifugation (4 °C, 10,000 rpm, 15 min), the protein concentration of the upper solution of each sample was measured using Folin reagent. The samples were prepared by boiling in sample buffer, and equal amounts of protein (70  $\mu$ g) were separated by tris-glycine-SDS-polyacrylamide gel electrophoresis (10%) and transferred onto a nitrocellulose membrane (Hybond-ECL, Amersham) using a MiniProtean-3 (Bio-Rad) apparatus.

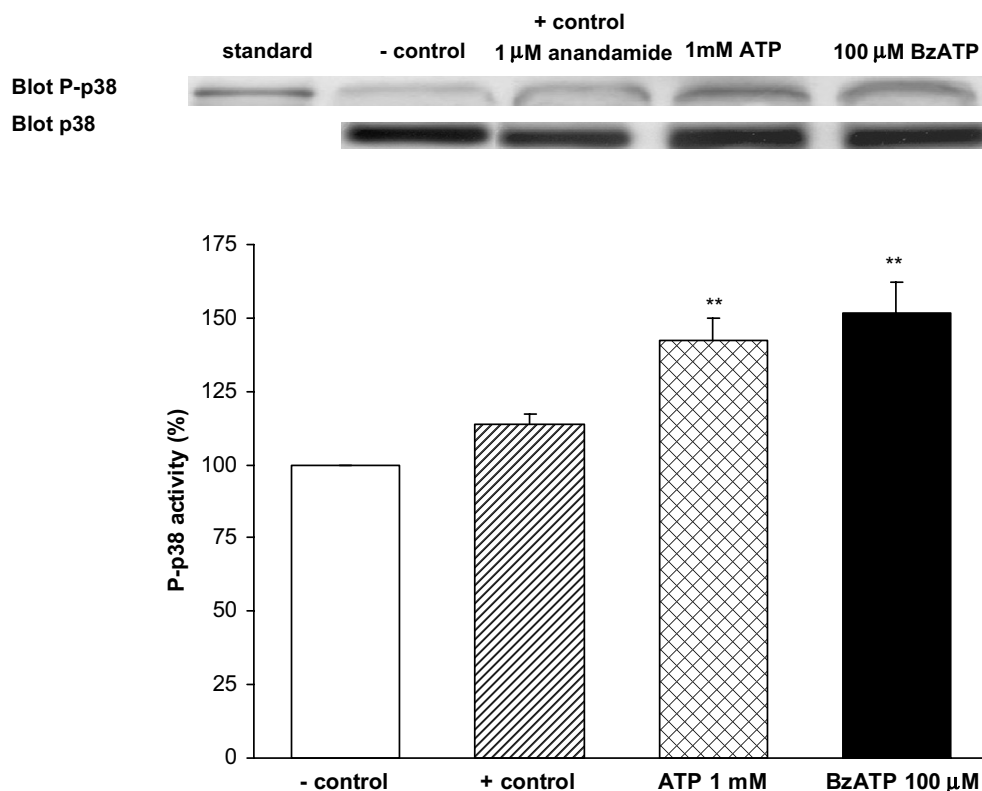


Fig. 1. The effect of P2 receptor agonists on p38 MAPK phosphorylation in the hippocampal slices of WT mice. ATP (1 mM) and BzATP (100  $\mu$ M) were applied for 5 min after incubation in ACSF at 35 °C for 50 min in the presence of 1  $\mu$ M TTX. Anandamide (1  $\mu$ M) was used as a positive control (+ control), whereas negative controls (– control) were evaluated in the absence of any drugs. The data represent three-independent experiments. The statistical analysis was done by one-way ANOVA and Dunnett's multiple comparison test (\*\* $P < 0.01$  vs. – control).

The membranes were then blocked with 5% dry milk in tris-buffered saline Tween 20 (TBST) buffer for 1 h at room temperature, incubated with p38C-20 primary antibody (1:500, Santa Cruz) for 1 h and washed in TBST buffer for 10 min three times. Before applying the P-p38 antibody (1:1000, Promega) for 2 h at room temperature, the membranes were blocked with 1% BSA in TBST buffer overnight at 4 °C, then washed with TBST buffer for 10 min three times. The membranes were incubated with secondary antibody (donkey anti-rabbit IgG-HRP, Santa Cruz, 1:3000 relative to the p38 C-20 primary antibody and 1:5000 relative to the P-p38 primary antibody) for 1 h at room temperature. The specific bands were detected and visualized by chemiluminescence (Amersham). The quantification of the immunoreactive bands was performed using the SynGene program by densitometer analysis.

**[<sup>3</sup>H]Glutamate release experiments from mouse hippocampal slices.** [<sup>3</sup>H]Glutamate ([<sup>3</sup>H]GLU) release experiments were carried out according to the method described in our previous papers (e.g., [16,19]). Briefly, male C57BL6 mice (20–25 g, Charles-River Ltd., Budapest, Hungary) were anesthetized under light CO<sub>2</sub> inhalation, and then decapitated. The hippocampi were dissected in ice-cold Krebs' solution and 400 μm thick slices were prepared using a McIlwain tissue chopper and incubated in 1 mL of 95% O<sub>2</sub>- and 5% CO<sub>2</sub>-saturated, modified Krebs solution (mM: NaCl 113, KCl 4.7, CaCl<sub>2</sub> 2.5, KH<sub>2</sub>PO<sub>4</sub> 1.2, MgSO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 25.0, and glucose 11.5, pH 7.4, 32 °C) containing 5 μCi/mL L-[G-<sup>3</sup>H]glutamate ([<sup>3</sup>H]GLU, specific activity 1.74 TBq/mmol; Amersham) for 45 min. After loading, the slices were superfused continuously with carbogenated modified Krebs' solution (32 °C, flow rate: 0.7 mL/min). Subsequently, perfusate samples were collected over a 3-min period and assayed for [<sup>3</sup>H]GLU. Preparations were subjected to 6-min perfusion of agonist (ATP) in the absence or presence of drugs (SB203580 and PD098095, Sigma), which

were preperfused for 18 min before the second agonist application. The radioactivity released from the preparations was measured with a Packard 1900 Tricarb liquid scintillation spectrometer (Packard, Canberra, Australia). Release of [<sup>3</sup>H]GLU was expressed in Bq/g and as a percentage of the amount of radioactivity in the tissue at the sample collection time (fractional release). Basal outflow was calculated as the fractional release measured in a 3-min sample in the absence and presence of drugs, respectively. Agonist-induced [<sup>3</sup>H]GLU outflow (S<sub>1</sub> and S<sub>2</sub>) was expressed by calculating the net release in response to agonist application by the area-under-the-curve method, i.e. by subtracting the release before the agonist application from the values measured after agonist application. The effects of drugs on the agonist-evoked release of [<sup>3</sup>H]GLU were expressed as S<sub>2</sub>/S<sub>1</sub> ratios measured in the absence and presence of the drugs, respectively.

**Statistical analysis.** All data were expressed as means ± SEM of *n* observations. Statistical analyses were made by one-way ANOVA followed by Dunnett's post hoc test (multiple comparisons) or Student's *t*-test (pairwise comparisons). *P* < 0.05 was considered statistically significant.

## Results

### Western blotting experiments

Five minutes application of the P2 receptor agonists ATP (1 mM) and BzATP (100 μM), enhanced significantly the active form of p38 MAPK, whereas the total amount of

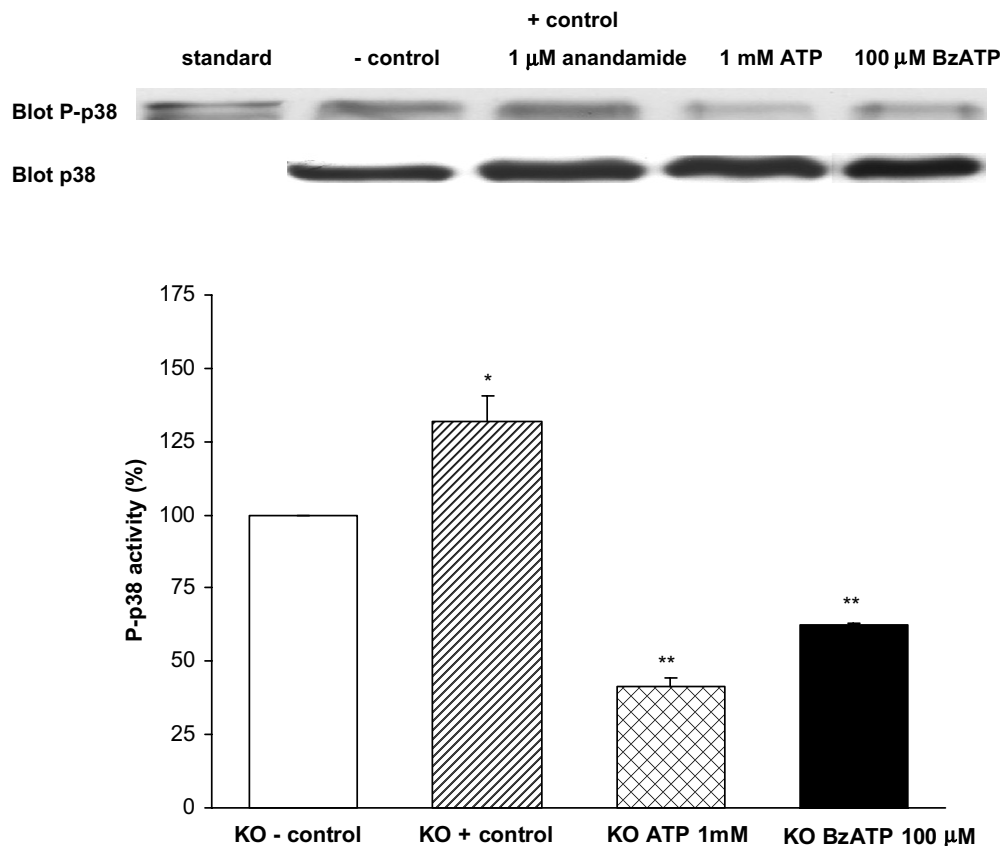


Fig. 2. The effect of P2 purinoceptor agonists on p38 MAPK phosphorylation in the hippocampal slices of P2X7R<sup>-/-</sup> mice. ATP (1 mM) and BzATP (100 μM) were added for 5 min after incubation in ACSF at 35 °C for 50 min, in the presence of 1 μM TTX. Anandamide (1 μM) was used as a positive control (+ control), whereas negative controls (– control) were evaluated in the absence of any drugs. The data represent three-independent experiments (\**P* < 0.05, \*\**P* < 0.01, vs. – control).

p38 MAPK was not modified in the hippocampal slices of the WT mice (Fig. 1). The CB1 receptor agonist anandamide (1  $\mu$ M), was used as a positive control [26]. Although the change did not reach the level of significance in WT mice, anandamide appreciably increased p38 MAPK phosphorylation (Fig. 1).

Using the same protocol as described above, the stimulatory effect of ATP (1 mM) and BzATP (100  $\mu$ M) on the active form of p38 MAPK was absent in the hippocampal slices of P2X7R<sup>-/-</sup> mice (Fig. 2). Moreover, we observed that the p38 MAPK phosphorylation declined notably in these animals. The CB1 receptor agonist anandamide (1  $\mu$ M) significantly increased the active form of p38 MAPK in the hippocampal slices of P2X7R<sup>-/-</sup> mice, whereas the total amount of inactive form was invariable by any treatment (Fig. 2).

In order to confirm the involvement of p38 MAPK in the effect of purinoceptor agonists in inducing the phosphorylation of p38, Western blot analysis using the specific p38 MAPK inhibitor, SB203580 (1  $\mu$ M), was applied to hippocampal slices of WT mice. SB203580 (1  $\mu$ M) was added for 30 min, after incubation in ACSF at 35 °C for 20 min and before the treatment with ATP (1 mM) and BzATP (100  $\mu$ M) for 5 min, and in this way prevented the effect of both P2 receptor agonists on the p38 MAPK phosphorylation (Fig. 3).

### [<sup>3</sup>H]Glutamate release experiments

After incubating the hippocampal slices with [<sup>3</sup>H]GLU, the tissue uptake of radioactivity was  $0.96 \pm 0.12 \times 10^5$  Bq/g ( $n = 11$ ) in the hippocampal slices. Similarly to that observed in our previous studies [16,19], when the slices were exposed to 10 mM ATP for 6 min, the tritium efflux promptly and reversibly increased; the total [<sup>3</sup>H]GLU efflux evoked by ATP (S<sub>1</sub>) was  $5.501 \pm 0.16\%$  (Fig. 4A). A subsequent identical stimulus (S<sub>2</sub>), applied 30 min later, elicited a comparable amount of tritium release, resulting in an S<sub>2</sub>/S<sub>1</sub> ratio of  $0.894 \pm 0.052\%$  ( $n = 8$ ). When the p38 MAPK inhibitor, SB203580 (1  $\mu$ M), was perfused 18 min before S<sub>2</sub>, the 10 mM ATP-evoked [<sup>3</sup>H]GLU release significantly decreased (Fig. 4A). SB203580 (1  $\mu$ M) did not affect the resting outflow of [<sup>3</sup>H]GLU ( $4.571 \pm 0.135\%$  and  $4.452 \pm 0.188\%$  in the absence and presence of SB203580,  $n = 4$ ,  $P > 0.05$ ).

To test whether ERK1/2 MAPK plays a role in the 10 mM ATP-induced [<sup>3</sup>H]GLU release from mice hippocampal slices, we utilised the ERK1/2 MAPK inhibitor PD098095. In the presence of PD098095 (10  $\mu$ M), both the resting [<sup>3</sup>H]GLU efflux ( $4.425 \pm 0.085\%$ ,  $n = 8$ ,  $P > 0.05$ ) and the 10 mM ATP-evoked [<sup>3</sup>H]GLU release were similar to the values for the control (Fig. 4B).

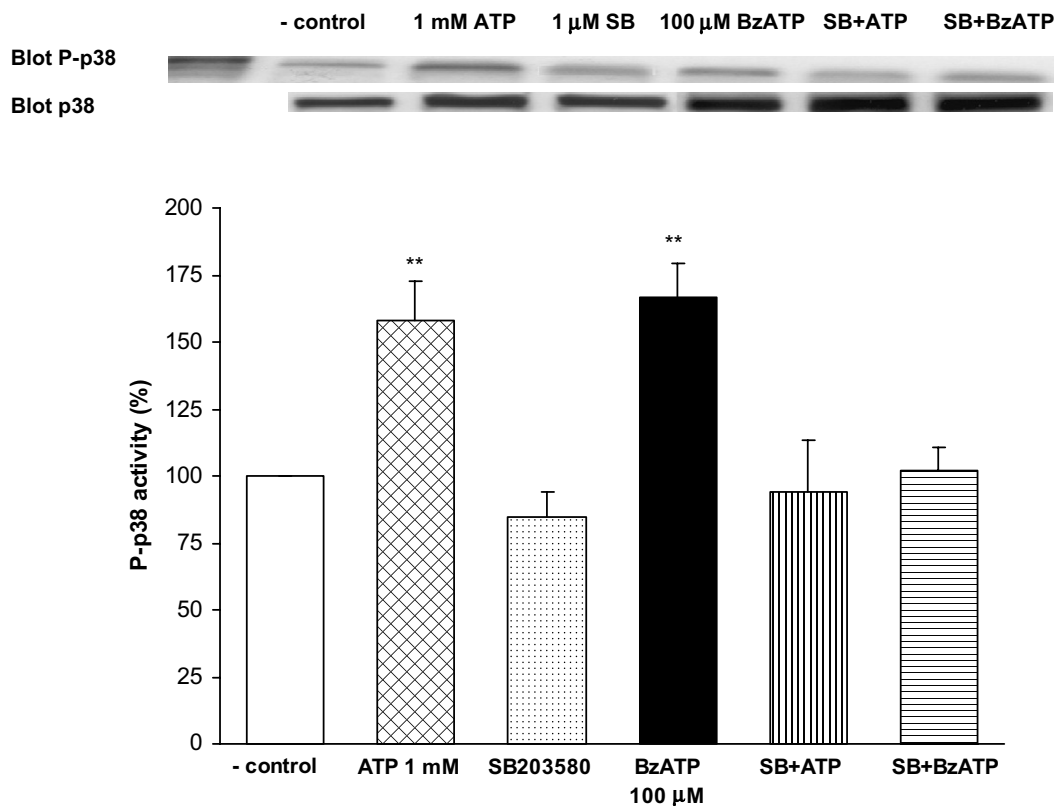


Fig. 3. The effect of SB203580 on the p38 MAPK phosphorylation in the hippocampal slices of WT mice. The p38 MAPK inhibitor SB203580 (1  $\mu$ M), applied for 30 min after incubation in ACSF at 35 °C for 20 min, in the presence of 1  $\mu$ M TTX, prevented the stimulatory effect of the P2 purinoceptor agonists ATP (1 mM) and BzATP (100  $\mu$ M) on p38 MAPK phosphorylation (%). The data represent three-independent experiments (\*\* $P < 0.01$  vs. – control).

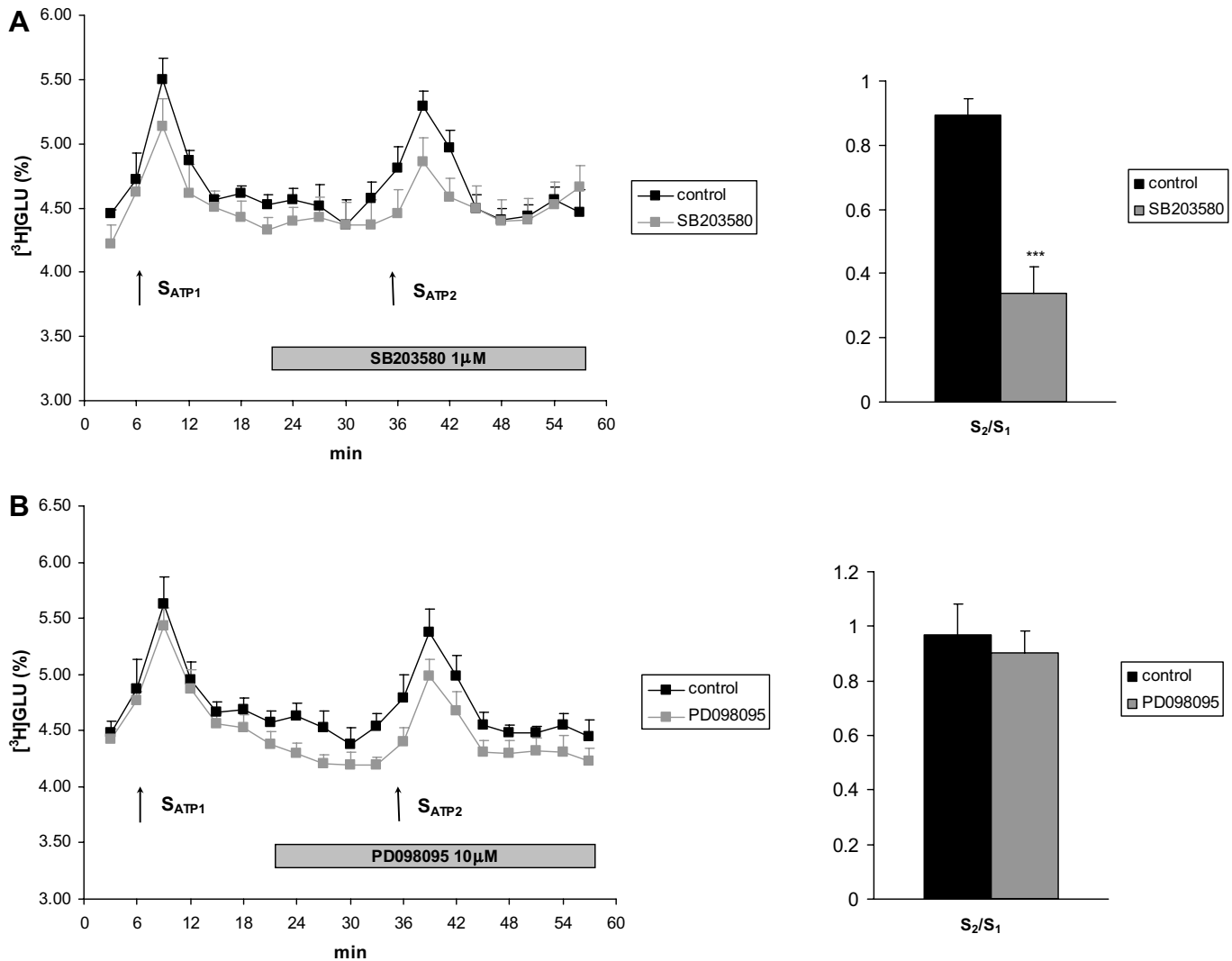


Fig. 4. The effect of the p38 MAPK inhibitor, SB203580 (1  $\mu$ M) (A), and the ERK1/2 MAPK inhibitor, PD098095 (10  $\mu$ M) (B), on [ $^3$ H]GLU release from the hippocampal slices of WT mice. After 60 min preperfusion, ATP (10 mM) was perfused for 6 min twice, as indicated by the arrows ( $S_{ATP1}$  and  $S_{ATP2}$ ). SB203580 (1  $\mu$ M) (A) and PD098095 (10  $\mu$ M) (B) were perfused 18 min before the second ATP application, as indicated by the horizontal bars. [ $^3$ H]GLU release was expressed as fractional release (for calculation see Materials and methods) (left panels). The effect of SB203580/PD098095 was quantified as  $S_2/S_1$  ratios in the absence (black bar) and presence (grey bar) of SB203580/PD098095 (right panels). The data represent the means  $\pm$  SEM of 4–8 (A) and 8–11 (B) identical experiments. The statistical analysis was made by Student's *t*-test (\*\*\* $P$  < 0.001).

## Discussion

The principal new finding of our study is the demonstration of increased phosphorylation of p38 MAPK in brain slices in response to the activation of P2X7 receptors. Thus, consistent with the activation of P2X7R, the endogenous ligand ATP and the P2X7 receptor agonist BzATP elicited an increase in the active form of p38 MAPK, BzATP being the more potent agent, and all these changes were sensitive to inhibition by SB203580, a specific inhibitor of p38 MAPK. However, both BzATP and ATP could be metabolized extracellularly in the hippocampus, giving rise to the formation of Bz-adenosine and adenosine, respectively [21,27]. A recent study revealed that  $A_1$ -adenosine receptors also activate p38 MAPK in the hippocampus [28], an effect that likely

underlies  $A_1$ -receptor-mediated synaptic depression. Yet, the involvement of  $A_1$ -adenosine receptors in the stimulation of p38 MAPK by ATP and BzATP is not very likely, as these effects were completely absent in mice deficient in P2X7Rs and adenosine does not activate P2X7Rs. Interestingly, ATP and BzATP significantly attenuated p38 MAPK phosphorylation in P2X7 $^{-/-}$  mice: these effects are most likely due to the activation of other P2X receptors by BzATP and ATP, as these agonists are also active at other P2X receptor subtypes, such as the P2X1 and P2X3 receptors [29], and the hippocampus is known to express all cloned P2X receptor subunits [30]. Consistently with previous results [31,32], in our experiments hippocampal slices exhibited a high constitutive expression of p38 MAPK, and this activity might be under the regulatory influence of other P2 receptors as well.

Within the central nervous system, P2X7R expression has been reported on activated microglia [33], astrocytes [34,35], and presynaptic nerve terminals, including the mossy fiber terminals of the hippocampus [16,34,36]. Therefore, the p38 MAP kinase activity that increased in response to P2X7R activation could represent such an activity either by neurons, astrocytes or microglia, and further studies are needed to delineate the cell-type specificity of this effect. Moreover, the effect of P2X7R activation could be either direct or indirect: since the long cytoplasmic C-terminus of the P2X7R subunit contains several protein–protein interaction motifs [37], a direct interaction with the p38 MAPK enzyme is a likely option, but we cannot exclude the possibility, either, that the increase in p38 activity is the secondary consequence of events downstream from P2X7R activation.

Our study also showed that one (but probably not the only) functional readout of P2X7R activation, subject to the regulatory influence of p38 MAPK, is increased glutamate release. Supporting this idea, SB203580 (the p38MAPK inhibitor) significantly, although not completely, decreased ATP-evoked [<sup>3</sup>H]GLU release, an effect previously proven to be P2X7R-mediated [16,19]. In contrast, PD098095 (the ERK1/2 inhibitor) was without effect, indicating the specificity of the involvement of p38 MAPK among MAPKs in this effect.

In conclusion, we report here for the first time the increased expression of p38 MAPK enzyme following P2X7R activation in the mouse hippocampus. This interaction might play a critical role in increased glutamate release, as well as in other downstream effects upon P2X7R activation, in various physiological and pathological states (e.g., synaptic plasticity, neurodegeneration, and neuroinflammation) where P2X7Rs and p38MAPKs are known to be both activated.

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