Microglia-derived purines modulate mossy fibre synaptic transmission and plasticity through P2X$_4$ and A$_1$ receptors

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Abstract

Recent data have provided evidence that microglia, the brain-resident macrophage-like cells, modulate neuronal activity in both physiological and pathophysiological conditions, and microglia are therefore now recognized as synaptic partners. Among different neuromodulators, purines, which are produced and released by microglia, have emerged as promising candidates to mediate interactions between microglia and synapses. The cellular effects of purines are mediated through a large family of receptors for adenosine and for ATP (P2 receptors). These receptors are present at brain synapses, but it is unknown whether they can respond to microglia-derived purines to modulate synaptic transmission and plasticity. Here, we used a simple model of adding immune-challenged microglia to mouse hippocampal slices to investigate their impact on synaptic transmission and plasticity at hippocampal mossy fibre (MF) synapses onto CA3 pyramidal neurons. MF -CA3 synapses show prominent forms of presynaptic plasticity that are involved in the encoding and retrieval of memory. We demonstrate that microglia-derived ATP differentially modulates synaptic transmission and short-term plasticity at MF-CA3 synapses by acting, respectively, on presynaptic P2X$_4$ receptors and on adenosine A$_1$ receptors after conversion of extracellular ATP to adenosine. We also report that P2X$_4$ receptors are densely located in the mossy fibre tract in the dentate gyrus-CA3 circuitry. In conclusion, this study reveals an interplay between microglia-derived purines and MF-CA3 synapses, and highlights microglia as potent modulators of presynaptic plasticity.

Introduction

Microglia are brain-resident macrophage-like cells of the central nervous system, and have been recently acknowledged as synaptic partners (Ben Achour & Pascual, 2010; Tremblay et al., 2010; Kettenmann et al., 2013). Microglia modulate neuronal activity in both physiological and pathophysiological conditions (Bessis et al., 2007; Roumier et al., 2008; Wake et al., 2009) through signalling mechanisms that are largely unknown. Purines are candidate signals, as microglia can release ATP (Pascual et al., 2012; George et al., 2015), which modulates synaptic transmission and plasticity (Neary & Zimmermann, 2009; Verkhratsky et al., 2009; Burnstock et al., 2011; Rodrigues et al., 2015).

ATP has pleiotropic effects in the brain, which depend on different types of purinergic receptors expressed by neurons and glial cells, but also on its degradation by extracellular ectonucleotidases to adenosine (Burnstock et al., 2011; Rodrigues et al., 2015). Purinergic receptors are divided into two families activated either by adenosine (P1 receptors) or by ATP (P2 receptors). P1 receptors are G-protein-coupled, and comprise four different subtypes: A$_1$, A$_2A$, A$_2B$, and A$_3$ (Fredholm et al., 2011). The P2 receptor family is subdivided in two subfamilies: the ionotropic P2X receptors (P2X receptors 1–7) family linked to increased cation permeability, and the metabotropic P2Y receptors (eight different subtypes) family coupled to G-proteins (Burnstock et al., 2011).

P1 and P2 receptors are widely distributed at brain synapses. In the hippocampus, A$_1$ receptors (A$_1$Rs) are located at both presynaptic and postsynaptic loci (Rebola et al., 2003), whereas A$_2A$ receptors (A$_2A$Rs) are mostly presynaptic (Rebola et al., 2005), but are also present in astrocytes (Matos et al., 2012) and microglia (Rebola et al., 2011). Adenosine decreases glutamatergic transmission via presynaptic A$_1$Rs (Dunwiddie & Masino, 2001), whereas it activates A$_2A$Rs to facilitate AMPA and N-methyl-D-aspartate receptor-mediated currents (Rebola et al., 2008; Dias et al., 2012) to modulate synaptic plasticity (Rebola et al., 2008; Costenla et al., 2011).

P2X receptors also modulate hippocampal synaptic transmission and plasticity (Pankratov et al., 2002). Presynaptic P2X$_2$ receptors facilitate CA3 excitatory transmission onto interneurons in the CA1 region (Khakh et al., 2003), whereas P2X$_4$ receptors (P2X$_4$Rs) tune the N-methyl-D-aspartate receptor-dependent component of long-term
plasticity at the Schaffer–collateral CA1 synapse (Sim et al., 2006). This is in accordance with the expression of P2X2, P2X3, P2X4 and P2X7 receptor subunits by hippocampal neurons (Rodrigues et al., 2005), whereas P2X7 receptors are mostly expressed by activated glial cells (Butt, 2011).

In this study, we investigated the role of microglia–purine interactions at hippocampal synapses between mossy fibres (MFs) and CA3 pyramidal cells. MF-CA3 synapses show prominent forms of presynaptic plasticity (Marchal & Mulle, 2004; Nicoll & Schmitz, 2005), and are instrumental in the encoding and retrieval of memories (Nakazawa et al., 2003; Bischofberger et al., 2006; Kesner, 2007). We report that ATP and adenosine, derived from lipopolysaccharide (LPS)-challenged microglia (George et al., 2015), do modulate synaptic transmission and short-term plasticity at MF-CA3 synapses by acting, respectively, on P2X4Rs and A1Rs. This study reveals an interplay between microglia and MF-CA3 synapses, and further supports microglia as true modulators of neuronal network through purinergic modulation of presynaptic function.

Materials and methods

Ethical approval

Experiments were carried out in accordance with the guidelines on the ethical use of animals from the European Communities Council Directive (86/609/EEC) and were evaluated by the Ethics committee n°50 of Bordeaux, affiliated with the CNREEA (Centre National de Réflexion Ethique sur l’Expérimentation Animale).

Microglial cell culture and pharmacological treatment

A murine microglial cell line, N9 (a gift from C. Verderio, CNR Institute of Neuroscience, Cellular and Molecular Pharmacology, Milan, Italy), was grown in RPMI-1640 (Sigma, St Quentin Fallavier, France) medium supplemented with 30 mM glucose (Sigma), 5% heat-inactivated fetal bovine serum (Gibco, Invitrogen, Merelbeke, Belgium), 100 μg/mL streptomycin, and 1 U/mL penicillin (Gibco, Invitrogen). Cells were kept at 37 °C under a humidified atmosphere with 95% O2 and 5% CO2. In order to trigger microglia actions at hippocampal synapses between mossy fibres (MFs) and CA3 pyramidal cells surrounded (10 μM) was added to the bath to inhibit GABA<sub>A</sub> receptors.

Voltage-clamp recordings were performed on CA3 pyramidal neurons visualized with a differential interference contrast microscope (Eclipse FN-1; Nikon, Champligny sur Marne, France) equipped with an infrared camera (VX44; Till Photonics, Gräfelfing, Germany), by use of an Axopatch-200B amplifier (Axon Instruments, Sunnyvale, CA, USA). Signals were filtered at 2 kHz and digitized at 5 kHz via a DigiData 1322A interface (Axon Instruments). Series resistance (10–20 MΩ) was monitored during the recording with a −10 mV hyperpolarizing voltage step (length, 50 ms) delivered at the beginning of each recording. Neurons were rejected if the series resistance changed by >20% during the experiment. Neurons with a holding current of >300 pA at a holding potential of −70 mV were rejected. Data were collected and analysed with pCLAMP software 9.2 (Axon Instruments). N9 microglia were challenged with LPS (100 ng/mL) for 1 h or not challenged, before being added to the slices. The cells were trypsinized and collected, and then added to a hippocampal slice at a density of ~100 000 cells per slice. N9 cells were allowed to adhere to the slices for 15–20 min, and the unattached cells were washed away, leaving approximately 400–500 cells stuck at the slice surface. Voltage-clamp recordings were obtained from CA3 pyramidal cells surrounded (10–100 μm) by three to five N9 cells in close vicinity. Excitatory postsynaptic currents (EPSCs) from MF–CA3 synapses were evoked by minimal-intensity stimulation of MFs (Marchal & Mulle, 2004; Sachidhanandam et al., 2009). A glass electrode, with a tip diameter of ~1 μm, was placed on the hilus of the dentate gyrus to stimulate MFs. The baseline stimulation frequency for all experiments was 0.1 Hz. EPSCs were identified according to the following criteria: robust low-frequency facilitation, low release probability at low stimulation (0.1 Hz), rapid rise time of individual EPSCs, and being free of secondary peaks during decay. LCCG-1 (10 μM), a metabotropic glutamate receptor 2 agonist, was added routinely to confirm the selective stimulation of MFs, and cells were excluded if the inhibition was <80%. Pyridoxal phosphoehosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS), ivermectin, 5-(3-bromophenyl)-1,3-dihydro-2H-benzofuro[3,2-e]-1,4-diazepin-2-one (BDBD), apyrase, ARLE7156 and SCH58261 were obtained from Tocris (Bristol, UK), and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) and 2-chloro-N6-cyclopentyladenosine (CPA) were obtained from Abcam (Cambridge, UK). The antibody used for staining P2X7Rs (Ref. HPA039494) was obtained from Atlas Antibodies (Stockholm, Sweden).

Immunohistochemistry

Mice (P19–P21) were anaesthetized by intraperitoneal administration of pentobarbitol (50 mg/kg body weight), and were fixed by transcardial perfusion with 50 mL of 4% paraformaldehyde. Brains were removed and postfixed in 4% paraformaldehyde overnight, and then saturated in a solution with 30% sucrose for 24 h. They were then frozen in cold heptane, and stored at −80 °C. Brains were cut on a cryostat at −20 °C into 40-μm-thick coronal sections. Sections were...
collected into antifreeze medium and stored at −20 °C. After a thorough wash in 0.1 M phosphate-buffered saline (PBS) with 0.3% Triton X-100 (PBST), free-floating sections were incubated with 3% normal donkey serum (NDS) with 0.3% Triton X-100 for 20 min at room temperature. Sections were then incubated overnight at 4 °C with the primary antibody anti-P2X4R (1 : 400) in PBST–NDS solution. Slices were washed three times with 1× PBS to rinse off the excess primary antibody before incubation for 2 h at room temperature with Alexa 488 goat anti-rabbit (Synaptic Systems, Göttingen, Germany) at 1 : 400 in PBST–NDS solution. After being washed three times with 1× PBS, slices were fixed with formaldehyde medium containing 4',6-diamidino-2-phenylindole. Fluorescent images were acquired with a Leica DM6000 TCS SP8X microscope, with a diode laser (excitation – blue, 405 nm; white, 488 nm; and emission – green, 500–560 nm). Images were obtained by mosaic acquisition of the whole hippocampus at ×20.

Statistical analysis

Values are presented as mean ± standard error of the mean (SEM), and n indicates the number of different cells. For statistical analysis, non-parametric tests were used. Student’s t-test was used for comparison between two groups, and one-way ANOVA followed by a Newman–Keuls post hoc test for comparison between more than two groups. Statistical differences were considered to be significant at P < 0.05. Note that, although the impacts of several drugs and modulators are presented as percentage values for the sake of clarity, statistical comparisons were always carried out with the absolute values. Statistical analysis was performed with PRISM 5.0 (GraphPad Software, La Jolla, CA, USA).

Results

LPS-treated microglia (LPS-microglia) increase basal synaptic transmission at MF–CA3 synapses

We first studied the effect of activated microglia on basal synaptic transmission. For this purpose, N9 microglia were first activated by pretreatment with LPS (100 ng/mL for 1 h) (Gomes et al., 2013). Activated microglia were then added to acute hippocampal slices with previously described methodology (Centonze et al., 2009). In our experimental condition, microglia covered the whole hippocampus and surrounded almost every CA3 pyramidal cell (Fig. 1A and B). Although microglia do not penetrate deeply into the slice, we recorded CA3 pyramidal cells surrounded (10–100 μm) by an average of three to five microglia in close vicinity, i.e. similar to the estimated density of microglia in the CA3 area (Jinno et al., 2007). Consistent with previous observations (Centonze et al., 2009), no noticeable differences in morphology between non-treated microglia (Fig. 1A) and LPS-microglia (Fig. 1B) were observed. However, we have not carried out detailed morphological analysis to determine whether the physical interaction between LPS-microglia and MF–CA3 synapses is similar to the interaction between endogenous microglia and MF–CA3 synapses.

To study basal synaptic transmission, MF-CA3 EPSCs (MF-EPSCs) were evoked with a minimal stimulation protocol (Marchal & Mulle, 2004). The average amplitude of MF-EPSCs recorded at a low stimulation rate (0.1 Hz) (−73 ± 5 pA; n = 10) was consistent with previous observations (Marchal & Mulle, 2004). The addition of non-treated microglia had no effect on basal synaptic transmission (−76 ± 12 pA; P = 0.8, n = 9) whereas the addition of LPS-microglia increased basal synaptic transmission (−106 ± 12 pA; P < 0.05, n = 9; data not shown) (Table 1).

LPS-microglia decrease short-term plasticity at MF-CA3 synapses

MF-CA3 synapses are distinct from most other synapses in the central nervous system, because they show prominent forms of presynaptic short-term plasticity (Nicoll & Schmitz, 2005), including pronounced paired-pulse facilitation (PPF) (two stimulations separated by 10–200 ms), represented by variation of the paired-pulse ratio (PPR) (pulse 2/pulse 1), and frequency facilitation (FF) (tonic stimulation switching from 0.1 Hz to >1 Hz). Activity-dependent neurotransmitter release among central nervous system synapses depends largely on the basal release probability, which is linked to the presynaptic calcium concentration. Hence, PPF at MF-CA3 synapses has been reported to depend solely on the residual calcium concentration in the presynaptic terminal (Salin et al., 1996; Nicoll & Schmitz, 2005). MF-EPSCs recorded from slices with added LPS-microglia showed decreased PPF, estimated from the PPR, as compared with slices with no added microglia (LPS-microglia, 277 ± 31%; control, 392 ± 32%; P < 0.05, n = 9; Fig. 1C and E) and slices with non-treated microglia added (microglia, 357 ± 35%, n = 9; Fig. 1C and E). Added LPS-microglia also significantly decreased FF as compared with control (LPS-microglia, 281 ± 18%; control, 482 ± 39%; P < 0.001, n = 9; Fig. 1D and F) and slices with non-treated microglia added (microglia, 430 ± 18%; P < 0.05, n = 9; Fig. 1D and F). As both PPF and FF were similar between control slices and slices with added non-treated microglia, our results indicated that the decrease in short-term plasticity observed with added LPS-microglia was probably attributable to the immune challenging of microglia.

Inhibition of microglial A2ARs prevents changes in short-term plasticity

The immune challenging of microglia with LPS (100 ng/mL) is known to drive microglia to release ATP, and this is abolished by blocking adenosine A2ARs on microglia (Gomes et al., 2013; George et al., 2015). We thus hypothesized that ATP released from LPS-microglia was involved in the observed changes in short-term synaptic plasticity. Taking advantage of the fact that neuronal presynaptic A2ARs do not, as such, modulate basal synaptic transmission or either PPF or FF at MF synapses (Rebola et al., 2008), we antagonized A2ARs on microglia by pre-incubation with the A2AR antagonist SCH58261 (50 nM) for 20 min (Lopes et al., 2004) prior to activation of microglia by LPS and addition to acute slices.

The pre-incubation of LPS-microglia with SCH58261 significantly reduced the decrease in FF induced by the addition of LPS-microglia (LPS-microglia, 281 ± 18%; LPS-microglia + SCH58261, 373 ± 27%; P < 0.05, n = 9) (Fig. 1F) but had no significant effect on the decrease in PPF (LPS-microglia, 277 ± 31%; LPS-microglia + SCH58261, 324 ± 31%; P = 0.65, n = 9) (Fig. 1E; Table 2). Thus, the blockade of A2ARs in LPS-microglia alleviated some of the changes observed in MF-CA3 short-term synaptic plasticity. As we have previously shown that A2ARs control ATP release from microglia (George et al., 2015), this supports the hypothesis that ATP released from LPS-microglia might impact on MF-CA3 synaptic activity, leading to changes in short-term synaptic plasticity.
To further validate the putative role of ATP, released by LPS-microglia, in the observed changes in FF and PPF, we manipulated extracellular ATP by either promoting its degradation with apyrase or by blocking its extracellular degradation with ARL67156 (Frenguelli et al., 2007). Addition of apyrase (10 U/mL) decreased basal synaptic transmission at low frequency (0.1 Hz) within 5 min (control, 98 ± 1%; apyrase, 54 ± 1%; P < 0.001, n = 4) (Fig. 2A and B). In addition, apyrase abolished the decrease in FF induced by LPS-microglia (LPS-microglia, 281 ± 18%; LPS-microglia + apyrase, 393 ± 24%; P < 0.01, n = 4) (Fig. 2E). Apyrase also abolished the decrease in PPF induced by LPS-microglia (LPS-microglia, 277 ± 31%; LPS-microglia + apyrase, 466 ± 86%; P < 0.05, n = 4) (Fig. 2F). Conversely, ARL67156 was used in control slices.

For each experimental condition, values are normalized to control. †, increase; ‡, decrease; –, no effect.

**LPS-microglia regulate FF**

To further validate the putative role of ATP, released by LPS-microglia, in the observed changes in FF and PPF, we manipulated extracellular ATP by either promoting its degradation with apyrase or by blocking its extracellular degradation with ARL67156 (Frenguelli et al., 2007). Addition of apyrase (10 U/mL) decreased basal synaptic transmission at low frequency (0.1 Hz) within 5 min (control, 98 ± 1%; apyrase, 54 ± 1%; P < 0.001, n = 4) (Fig. 2A and B). In addition, apyrase abolished the decrease in FF induced by LPS-microglia (LPS-microglia, 281 ± 18%; LPS-microglia + apyrase, 393 ± 24%; P < 0.01, n = 4) (Fig. 2E). Apyrase also abolished the decrease in PPF induced by LPS-microglia (LPS-microglia, 277 ± 31%; LPS-microglia + apyrase, 466 ± 86%; P < 0.05, n = 4) (Fig. 2F). Conversely, ARL67156 was used in control slices.
For each experimental condition, values are normalised to LPS-microglia. ↑, increase; ↓, decrease; −, no effect.

Adenosine is involved in LPS-microglia-mediated regulation of PPF

Our data strongly suggest that ATP is the key molecule responsible for the microglia-induced changes in presynaptic forms of short-term plasticity. However, ATP is readily converted into adenosine by extracellular ectonucleotidases (Cunha et al., 1998), namely in synapses (Cunha, 2001), and presynaptic adenosine A1Rs are present at MF–CA3 synapses (Scanziani et al., 1992; Moore et al., 2003; Kukley et al., 2005; Rebola et al., 2008). Therefore, we investigated whether adenosine derived from the ATP released by LPS-microglia could mimic the effects of ATP or abolish the effects of extracellular ATP.

P2X₄Rs modulate short-term synaptic plasticity

To further characterize the role of ATP derived from LPS-microglia in the modulation of FF, we investigated which subtype of purinergic receptor was involved. ATP can bind to metabotropic (P2Y) and ionotropic (P2X) receptors. A role of P2X receptors has been reported in hippocampal synapses, mostly in the context of synaptic plasticity (Soto et al., 1996; Khakh et al., 1999; Pankratov et al., 2002; Priel & Silberberg, 2004; Sim et al., 2006; Pougnet et al., 2014). As shown in Fig. 4A, the effect of LPS-microglia on FF was prevented by blocking P2X receptors with the broad-spectrum antagonist PPADS (50 μM) (Lambrechts et al., 2002) (LPS-microglia, 281 ± 18%; LPS-microglia + PPADS, 427 ± 56%; P > 0.05, n = 9). In contrast, PPADS had no effect on the decrease in PPF induced by LPS-microglia (LPS-microglia, 277 ± 31%; LPS-microglia + PPADS, 281 ± 22%; P = 0.9, n = 9) (Fig. 4B). These results provide evidence that P2X receptors specifically modulated FF, and not PPF.

We next investigated which subtype of P2X receptor was responsible for the effects of LPS-microglia on FF. The presynaptic expression of P2X₄Rs at MFs remains controversial (Armstrong et al., 2002; Sperlagh et al., 2002; Kukley et al., 2004; Yu et al., 2008), whereas the involvement of P2X₄Rs in the modulation of synaptic strength (Baxter et al., 2011) and/or plasticity has been documented (Sim et al., 2006) at CA3–CA1 synapses. However, the presence of P2X₄Rs at MF synapses remains to be studied. We first investigated whether P2X₄Rs were involved in the modulation of basal synaptic transmission. Application of the selective P2X₄ antagonist 5-BDBD (10 μM) (Donnelly-Roberts et al., 2008) decreased basal synaptic transmission at low frequency (0.1 Hz) within 5 min of its addition (control, 98 ± 1%; 5-BDBD, 65 ± 4%; P < 0.01, n = 9) (Fig. 4C and D). Conversely, ivermectin (3 μM), a positive allosteric modulator of P2X₄Rs that potentiates P2X₄R currents (Khakh et al., 1999; Sim et al., 2006), increased basal synaptic transmission at low frequency (0.1 Hz) within 10 min of its addition (control, 98 ± 1%; ivermectin, 128 ± 7%; P < 0.05, n = 6) (Fig. 4E and F). Consistent with these results, basal synaptic transmission was also decreased in slices obtained from P2X₄R knockout (KO) mice (control, 98 ± 1%; P2X₄R KO, 85 ± 6%; P < 0.05, n = 9; data not shown). Therefore, ATP modulates basal synaptic transmission at MF–CA3 synapses through P2X₄Rs.

We then investigated the putative role of P2X₄Rs in the modulation of FF. Selective blockade of P2X₄Rs with 5-BDBD on its own had no effect on FF (control, 482 ± 39%; 5-BDBD, 438 ± 25%; P = 0.12, n = 9), whereas potentiation of P2X₄Rs by ivermectin significantly decreased FF (control, 482 ± 39%; ivermectin, 344 ± 35%; P < 0.05, n = 9) (Fig. 4G). Conversely, 5-BDBD on its own decreased PPF (control, 392 ± 32%; 5-BDBD, 227 ± 16%; P < 0.01, n = 9), whereas ivermectin had no effect (control, 392 ± 32%; ivermectin, 369 ± 40%; P = 0.66, n = 9) (Fig. 4H). These results provide good evidence that activation of P2X₄Rs modulates FF but not PPF, and suggest a presynaptic site of action for ATP. To further strengthen the hypothesis of presynaptic localization of P2X₄Rs, we used a new antibody raised against P2X₄Rs and checked its specificity in slices from P2X₄R KO mice. As

|    | LPS-microglia | SCH58261 Apyrase | DPCPX | CCPA | 5-BDBD KO |
|----|---------------|-----------------|-------|------|----------|
| BT | ↑             | ↓               | ↑     | ↑    | ↓        |
| PPF| ↓             | ↑               | ↑     | ↓    | ↓        |
| FF | ↑             | ↑               | ↑     | ↑    | ↑        |

Table 2. Comparison of basal transmission (BT), PPF and FF with respect to LPS-microglia (slice + LPS-microglia)
shown in Fig. 4I, we observed strong immunostaining for P2X4R (green) on the MF tract (upper panel) that was almost completely absent in slices from P2X4R KO mice (lower panel). It is worth noting that we did not observe clear P2X4R immunoreactivity in the CA1 area, in contrast to previous reports (Rubio & Soto, 2001; Sim et al., 2006), suggesting the possibility that different antibodies may

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recognize different known molecular structures of homomeric P2X4Rs (Zemkova et al., 2015), different known heteromeric receptor complexes containing P2X4R subunits (Antonio et al., 2014), or P2X4Rs with different subcellular localizations (Xu et al., 2014).

P2X4Rs mediate the LPS-microglia-induced decrease in FF

We then investigated the possible involvement of P2X4Rs in the modulation of FF by LPS-microglia. Blockade of P2X4Rs in hippocampal slices before the addition of LPS-microglia prevented the decrease in FF (LPS-microglia + 5-BDBD, 378 ± 28%; LPS-microglia, 281 ± 18%; P < 0.01, n = 9) (Fig. 5B) but did not alleviate the decrease in PPF (LPS-microglia + 5-BDBD, 242 ± 25%; LPS-microglia + CCPA, 250 ± 38%; P = 0.9, n = 9) (Fig. 5C). Because P2X4Rs are also dynamically involved in controlling endogenous microglia (Toulme & Khakh, 2012; Koizumi et al., 2013) as well as N9 microglia (Xiang & Burnstock, 2005), we investigated whether the effect of 5-BDBD was attributable to P2X4Rs expressed at MF-CA3 synapses or those expressed by N9 microglia added to the slice. For this purpose, LPS-microglia were added to hippocampal slices obtained from P2X4R KO mice.

The decrease in FF induced by LPS-microglia was reduced in slices obtained from P2X4R KO mice (LPS-microglia + P2X4R KO, 337 ± 23%; LPS-microglia, 281 ± 18%; P < 0.05, n = 6) (Fig. 5D), whereas the decrease in PPF induced by LPS-microglia was preserved in slices from P2X4R KO mice (LPS-microglia + P2X4R KO, 265 ± 41%; LPS-microglia, 277 ± 31%; P = 0.8, n = 9) (Fig. 5E).

The ectonucleotidase inhibitor ARL67156 (10 μM) mimicked the LPS-microglia-induced decrease in FF (control, 482 ± 39%, n = 9; Fig. 3).
Fig. 4. Extracellular ATP modulates basal synaptic transmission and FF via presynaptic P2X4Rs. (A) Graphical representation of FF. PPADS (50 μM) blocked the LPS-microglia-induced decrease in FF (control, 482 ± 39%; LPS-microglia, 281 ± 18%; LPS-microglia + PPADS, 427 ± 56%; n = 9 for all conditions). (B) Graphical representation of PPR. PPADS (50 μM) had no effect on the LPS-microglia-induced decrease in PPR (control, 392 ± 32%; LPS-microglia, 277 ± 31%; LPS-microglia + PPADS, 281 ± 22%; P = 0.9, n = 9 for all conditions). (C) Summary plot of the effect of 5-BDBD (10 μM) on basal synaptic transmission. 5-BDBD induced a decrease in basal synaptic transmission within 5 min of addition (control, black squares; 5-BDBD, open squares). (D) Graphical representation of the basal synaptic transmission shown in C (control, 98 ± 1%; 5-BDBD, 65 ± 4%; n = 9 for both conditions). (E) Summary plot of the effect of ivermectin (3 μM) on basal synaptic transmission. Ivermectin increased basal synaptic transmission within 10 min of addition (control, black squares; ivermectin, open squares). (F) Graphical representation of the basal synaptic transmission shown in E (control, 98 ± 1%, n = 9; ivermectin, 128 ± 7%, n = 6). The values in A, B, D and F are mean ± SEM; *P < 0.05 and ***P < 0.001, unpaired t-test. (G) Graphical representation of FF. Ivermectin (3 μM) decreased FF; whereas 5-BDBD (10 μM) had no effect (control, 482 ± 39%, n = 9; ivermectin, 344 ± 35%, n = 6; 5-BDBD, 438 ± 25%, n = 9). (H) Graphical representation of PPR. 5-BDBD (10 μM) decreased PPR, whereas ivermectin (3 μM) had no effect (control, 392 ± 32%, n = 9; ivermectin, 369 ± 40%, n = 6; 5-BDBD, 227 ± 16%, n = 9). The values in G and H are mean ± SEM; *P < 0.05 and **P < 0.01 from one-way ANOVA followed by a Newmann–Keuls multiple comparison test. (I) The hippocampal MFs showed strong immunostaining for P2X4R (green) in a wild-type (WT) mouse (upper panel), whereas it was virtually absent in a slice from a P2X4R KO mouse (lower panel). 4’,6-Diamidino-2-phenylindole staining for nuclei is shown in blue. Scale bar: 200 μm.
Fig. 5. LPS-microglia-derived ATP regulates FF via P2X4Rs. (A) Representative traces of EPSCs triggered at low frequency of stimulation (0.1 Hz). (B) Graphical representation of FF. Blockade of P2X4Rs by 5-BDBD (10 μM) inhibited the effect of LPS-microglia on FF (control, 482 ± 39%; 5-BDBD, 438 ± 25%; microglia, 430 ± 18%; microglia + 5-BDBD, 374 ± 35%; LPS-microglia, 281 ± 18%; LPS-microglia + 5-BDBD, 378 ± 28%; n = 9 for all conditions). (C) Graphical representation of the PPR. 5-BDBD (10 μM) had no effect on the decrease in the PPR induced by LPS-microglia, whereas it reduced it when applied alone (control, 392 ± 32%; 5-BDBD, 227 ± 16%; microglia, 357 ± 35%; microglia + 5-BDBD, 212 ± 18%; LPS-microglia, 277 ± 31%; LPS-microglia + 5-BDBD, 242 ± 25%; n = 9 for all conditions). (D) Bar representation of FF. Genetic deletion of P2X4R (P2X4R KO) abolished the LPS-microglia-induced decrease in FF (control, 482 ± 39%; P2X4R KO, 419 ± 43%; microglia, 340 ± 18%; microglia + P2X4R KO, 377 ± 31%; LPS-microglia, 281 ± 18%; LPS-microglia + P2X4R KO, 337 ± 23%; n = 9 for all conditions). (E) Graphical representation of the PPR. The LPS-microglia-induced decrease in the PPR was preserved in P2X4R KO slices (control, 392 ± 32%; P2X4R KO, 253 ± 32%; microglia, 357 ± 35%; microglia + P2X4R KO, 216 ± 57%; LPS-microglia, 277 ± 31%; LPS-microglia + P2X4R KO, 265 ± 41%; n = 9 for all conditions). (F) Graphical representation of FF. Addition of ARL67156 (10 μM) mimicked the LPS-microglia-induced decrease in FF, whereas prior blockade of P2X4Rs by 5-BDBD (10 μM) prevented this effect (control, 482 ± 39%, n = 9; ARL67156, 183 ± 29%, n = 3; LPS-microglia, 281 ± 18%, n = 9; ARL67156 + 5-BDBD, 442 ± 97%, n = 3). (G) Graphical representation of the PPR. Increasing the level of endogenous ATP with ARL67156 (10 μM) had no effect on the PPR (control, 392 ± 32%, n = 9; ARL67156, 324 ± 71%, n = 3; LPS-microglia, 277 ± 31%, n = 9; ARL67156 + 5-BDBD, 281 ± 78%, n = 3). Values in all graphs are mean ± SEM. *P < 0.05, **P < 0.01 and ***P < 0.001 from one-way ANOVA followed by a Newman–Keuls multiple comparison test.
LPS-microglia, 281 ± 18%; P < 0.001, n = 9; ARL67156, 183 ± 29%; P < 0.001, n = 3) (Fig. 5F), whereas prior blockade of P2X$_4$Rs by 5-BDBD (10 μM) prevented this effect (ARL67156 + 5-BDBD, 442 ± 97%; P < 0.02, n = 3) (Fig. 5F). In contrast, prior blockade of P2X$_4$Rs by 5-BDBD, before addition of ARL67156, had no further effect on PPF (Fig. 5G).

**ATP and adenosine modulate synaptic plasticity independently of each other**

Our data strongly suggest that extracellular ATP released from LPS-microglia acts on P2X$_4$Rs to modulate FF, and that adenosine catabolized from extracellular ATP acts on A1Rs to modulate PPF. Adenosine being the end-product of ATP, we had to disentangle any possible indirect effects of ATP on PPF or adenosine on FF. For this purpose, FF and PPF were investigated in the presence of DPCPX in slices obtained from P2X$_4$R KO mice. The LPS-microglia-induced decrease in FF (Fig. 6A and B) was reduced in P2X$_4$R KO mice (LPS-microglia, 281 ± 18%; LPS-microglia + P2X$_4$R KO, 337 ± 23%; P < 0.05, n = 9), but was preserved in the presence of DPCPX (LPS-microglia + P2X$_4$R KO + DPCPX, 402 ± 38%; P < 0.001, n = 5) (Fig. 6B). This indicates that A1Rs have no effect on the modulation of FF by P2X$_4$Rs. Conversely, genetic deletion of P2X$_4$Rs did not prevent the ability of DPCPX to block the LPS-microglia-induced decrease in PPF (Fig. 6C and D). Hence, ATP released by LPS-microglia and its end-product adenosine act independently on P2X$_4$Rs and A1Rs, respectively, to modulate short-term synaptic plasticity.

**Discussion**

Over the last 10 years, the notion of microglia as passive sentinels within the brain has been noticeably widened, and microglia are now acknowledged to constitute a new partner in synaptic function (Tremblay et al., 2010) [reviewed in Kettenmann et al. (2013)]. Indeed, microglia can secrete a full array of neuroactive molecules, including several proinflammatory cytokines (interleukin-1β, interleukin-6, and tumour necrosis factor-α), trophic factors such as brain-derived neurotrophic factor, and also neurotransmitters such as ATP, glycine, and glutamate [reviewed in Kettenmann et al. (2011)]. In the present study, we focused on the putative effect of microglia-derived purines on synaptic function at MF-CA3 synapses. In this experimental paradigm, N9 microglia were previously challenged with an immune stimulus (LPS) to induce them to release ATP (George et al., 2015), and were then added to hippocampal slices.

Combining pharmacological, electrophysiological and immunohistochemical approaches, our study demonstrated that ATP released

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**Fig. 6.** ATP and adenosine act independently on P2X$_4$Rs and A1Rs. (A) Representative traces of EPSCs recorded at 0.1 Hz and 1 Hz, illustrating FF in different conditions. (a) Control. (b) LPS-microglia. (c) LPS-microglia + P2X$_4$R KO. (d) LPS-microglia + P2X$_4$R KO + DPCPX. (B) Graphical representation of FF. Blockade of A1R by DPCPX (100 nM) had no effect on the inhibitory effect of P2X$_4$R KO on the LPS-microglia-induced change in FF (control, 482 ± 39%, n = 9; P2X$_4$R KO, 317 ± 23%; P < 0.001 from one-way ANOVA followed by a Neumann–Keuls multiple comparison test). (C) Representative traces of EPSCs recorded at 0.1 Hz, illustrating the PPR in different conditions. (a) Control. (b) LPS-microglia. (c) LPS-microglia + DPCPX. (d) LPS-microglia + P2X$_4$R KO + DPCPX. (D) Graphical representation of the PPR. Genetic deletion of P2X$_4$Rs had no effect on the DPCPX-induced inhibition of the LPS-microglia-induced decrease in the PPR (control, 392 ± 32%, n = 9; P2X$_4$R KO + DPCPX, 388 ± 66%, n = 5; LPS-microglia, 277 ± 31%, n = 9; LPS-microglia + DPCPX, 383 ± 35%, n = 9; LPS-microglia + P2X$_4$R KO + DPCPX, 417 ± 36%, n = 5). Values in all graphs are mean ± SEM. *P < 0.05, **P < 0.01 and ***P < 0.001 from one-way ANOVA followed by a Neumann–Keuls multiple comparison test.

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by LPS-microglia modulates MF-CA3 synaptic transmission both directly via presynaptic P2X_{4}Rs and through its metabolite adenosine, by acting on presynaptic A_{1}Rs. Each pathway selectively modulates a different type of short-term presynaptic plasticity at MF-CA3 synapses, namely FF for P2X_{4}Rs and PPF for A_{1}Rs. This study reveals complex crosstalk between microglial release of ATP, purinergic receptor activation, and hippocampal synaptic function.

**P2X_{4}Rs modulate basal synaptic transmission**

Basal synaptic transmission at MF-CA3 synapses is governed by presynaptic intracellular calcium homeostasis and AMP levels (Nicoll & Schmitz, 2005). Within the ionotropic P2X receptor family, P2X_{4}Rs show the highest permeability for calcium (Soto et al., 1996; Burnstock et al., 2011), and, through this property, P2X_{4}Rs can modulate basal synaptic transmission. In good agreement with this, the blockade of P2X_{4}Rs by 5-BDBD or their genetic deletion in P2X_{4}R KO mice decreased basal synaptic transmission, whereas ivermectin, which selectively potentiates ATP-induced currents at P2X_{4}Rs (Khakh et al., 1999; Priel & Silberberg, 2004), increased it. As expected, the degradation of extracellular ATP by apyrase also decreased basal synaptic transmission. All together, these results suggest that extracellular ATP tonically modulates basal synaptic transmission, most likely by increasing the presynaptic calcium concentration via its influx through P2X_{4}Rs.

Interestingly, the end-product of ATP catabolism, i.e. adenosine, by acting on presynaptic A_{1}Rs, also tonically decreases basal synaptic transmission (Moore et al., 2003; Kukley et al., 2008; Rebola et al., 2008), probably through the direct A_{1}R inhibition of calcium influx via presynaptic P/Q-type and N-type calcium channels (Gundlunger et al., 2007). Our results suggest that purines modulate basal synaptic transmission via a complex interplay between ATP and adenosine signalling, which produce opposite effects.

**Microglia-derived adenosine but not ATP impacts on PPF**

The decrease in PPF induced by LPS-microglia, which was preserved in the presence of PPADS or 5-BDBD but also in P2X_{4}R KO slices, strongly suggests that adenosine but not ATP was involved in this effect. Activation of A_{1}Rs at MF-CA3 synapses is known to inhibit synaptic transmission (Scanziani et al., 1992; Moore et al., 2003; Kukley et al., 2005), and its presynaptic localization has been confirmed by electron microscopy (Rebola et al., 2008). The blockade by DPCPX of the decrease in PPF induced by LPS-microglia and its mimicking by CCPA are good evidence that adenosine resulting from the degradation of microglia-derived ATP modulates PPF. Unexpectedly, the blockade of P2X_{4}Rs by 5-BDBD on its own also reduced PPF. This could indicate that endogenous ATP in the presence of receptors blocked by 5-BDBD would be more readily available to be converted into adenosine, activating A_{1}R and leading to a decrease in PPF. Hence, PPF was not affected in P2X_{4}R KO mice, supporting our contention that the effect of 5-BDBD involves greater availability of adenosine acting on A_{1}Rs rather than an effect of P2X_{4}R.

Owing to a very low initial release probability, the PPF at MF-CA3 synapses is assumed to be directly linked to the probability of glutamate release, which is dependent on the residual calcium concentration in the presynaptic terminal (Salin et al., 1996; Nicoll & Schmitz, 2005). Therefore, one could expect that microglia-derived adenosine acting on A_{1}Rs by reducing calcium influx into MF terminals (Gundlunger et al., 2007) would decrease PPF instead of increasing it. However, conflicting results have been reported for the impact of A_{1}R activation on PPF at MF-CA3 synapses, ranging from a reduction by half of PPF by DPCPX (Moore et al., 2003) to no effect at all (Kukley et al., 2005). These opposite results were attributed to differences in the ambient levels of adenosine in slices. Our results are consistent with a moderate level of ambient adenosine, as shown by the weak increase in basal synaptic transmission caused by DPCPX, similarly to what was previously reported (Kukley et al., 2005; Rebola et al., 2008), and by the lack of a significant effect on PPF.

**Pathophysiological relevance of microglia-derived purine modulation of MF-CA3 synaptic plasticity**

There is compelling evidence that the purinergic system is instrumental in neuroinflammation (Di Virgilio et al., 2009), which can impact on synaptic function (Ben Achour & Pascual, 2010; Pascual et al., 2012). ATP is a major trigger of microglia activation through P2X_{4}Rs and P2X_{7} receptors (Di Virgilio et al., 2009; Koizumi et al., 2013), and adenosine also regulates microglia responses through the activation of A_{3}ARs in vivo (Rebola et al., 2011), by controlling the microglial production of inflammatory cytokines, and growth factors such as BDNF and ATP (Gomes et al., 2013; George et al., 2015). By taking advantage of a simple paradigm based on the addition of activated N9 microglia releasing ATP, the present study provides evidence that microglia-derived purines in a neuroinflammatory paradigm modulate, via presynaptic P2X_{4}Rs and A_{1}Rs, different forms of presynaptic short-term plasticity at MF-CA3 synapses. However, the simplicity of the paradigm used also introduced some limitations in the mechanistic process underlying this control of short-term plasticity by microglia-derived ATP. Indeed, our experimental approach does not allow us to rule out the participation of endogenous microglia, astrocytes and/or neurons in possible amplification of the N9 microglia-derived ATP to control presynaptic short-term plasticity at MF-CA3 synapses. In this

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respect, a recent study showed that the production of tumour necrosis factor-α and ATP by microglia was driving nearby astrocytes to release ATP, promoting, in turn, astrogial release of glutamate and a subsequent increase in CA1 synaptic transmission in acute hippocampal slices (Pascual et al., 2012). However, this documented ATP-induced ATP release from astrocytes occurs only for ATP concentrations in the high micromolar range (Anderson et al., 2004), whereas we have previously estimated that N9 microglia release nanomolar concentrations of ATP (George et al., 2015). Certainly, future studies will be required to unravel the intricate interplay between different cell types in the purinergic control of presynaptic short-term plasticity at MF-CA3 synapses triggered by a neuroinflammation-associated increase in the concentration of microglia-derived ATP.

The CA3 subregion of the hippocampus has been reported to be involved in the encoding, storage and subsequent retrieval of memories (Nakazawa et al., 2003; Bischofberger et al., 2006; Kesner, 2007). Owing to the sparse connectivity between dentate gyrus granule cells and CA3 pyramidal neurons and the prominent ability of MF-CA3 synapses to facilitate markedly the MF pathway has been particularly involved in the pattern separation process (Bischofberger et al., 2006; Gilbert & Kesner, 2006; Leutgeb et al., 2007; Neves et al., 2008). Therefore, a decrease in short-term plasticity induced by neuroinflammation could impact on the pattern separation process. Relevant to this, it is well known that murine models of Alzheimer’s disease, which show prominent neuroinflammation, have impaired spatial memory, as is also found in Alzheimer’s patients. It would be interesting to use behavioural paradigms to test the pattern separation process in mice with conditional deletion of P2X3Rs and/or A1Rs in granule cells, in order to test the possible involvement of this subnetwork.

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Abbreviations

5-BBD, 5-(3-bromophenyl)-1,3-dihydro-2H-benzofuro[3,2-e]-1,4-diazepin-2-one; A1R, A2AR, A3AR, A4AR receptor; CCPA, 2-chloro-N'-cyclopentyladenosine; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; EPSC, excitatory postsynaptic current; FF, frequency facilitation; KO, knockout; LPS, lipopolysaccharide; LPS-microglia, lipopolysaccharide-treated microglia; MF, mossy fibre; MF-EPSC, mossy fibre-CA3 excitatory postsynaptic current; NDS, normal donkey serum; P, postnatal day; PBS, phosphate-buffered saline; PBST, 0.1 M phosphate-buffered saline with 0.3% Triton X-100; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid; PPF, paired-pulse facilitation; PPR, paired-pulse ratio; P2X3R, P2X4R receptor; SEM, standard error of the mean.

References

Anderson, C.M., Bergher, J.P. & Swanson, R.A. (2004) ATP-induced ATP release from astrocytes. J. Neurochem., 88, 246–256.

Antonino, I.S., Stewart, A.P., Varanda, W.A. & Edwardson, J.M. (2014) Identification of P2X2/P2X3/P2X6 heterotrimeric receptors using atomic force microscopy (AFM) imaging. FEBS Lett., 588, 2125–2128.

Armstrong, J., Brust, T., Lewis, R. & MacVicar, B. (2002) Activation of presynaptic P2X7-like receptors depletes mossy fiber–CA3 synaptic transmission through p38 mitogen-activated protein kinase. J. Neurosci., 22, 5579–5585.

Baxter, A.W., Choi, S.J., Sim, J.A. & North, R.A. (2011) Role of P2X4 receptors in synaptic strengthening in mouse CA1 hippocampal neurons. Eur. J. Neurosci., 34, 213–220.

Ben Achour, S. & Pascual, O. (2010) Glia: the many ways to modulate synaptic plasticity. Neurochem. Int., 57, 440–445.

Bessis, A., Béchade, C., Bernard, D. & Roumier, A. (2007) Microglial control of neuronal death and synaptic properties. Glia, 55, 233–238.

Bischofberger, J., Engel, D., Frotscher, M. & Jonas, P. (2006) Timing and efficacy of transmitter release at mossy fibre synapses in the hippocampal network. Eur. J. Physiol., 453, 361–372.

Burstrom, G., Fredholm, B. & Verkruthska, A. (2011) Adenosine and ATP receptors in the brain. Curr. Top. Med. Chem., 11, 973–1011.

Butt, A.M. (2011) ATP: a ubiquitous gliotransmitter integrating neuron–glial networks. Semin. Cell Dev. Biol., 22, 205–213.

Centonze, D., Muzio, L., Rossi, S., Cavasini, F., De Chiara, V., Bergami, A., Musella, A., D’Amelio, M., Cavallucci, V., Martorana, A., Bergamaschi, A., Cencioni, M.T., Diamantini, A., Butti, E., Coni, G., Bernardi, G., Cecconi, F., Battistini, L., Furlan, R. & Martino, G. (2009) Inflammation triggers synaptic alterations and degeneration in experimental autoimmune encephalomyelitis. J. Neurosci., 29, 3442–3452.

Contractor, A., Swanson, G. & Heinemann, S. (2001) Kainate receptors are involved in short- and long-term plasticity at mossy fibre synapses in the hippocampus. Neuron, 29, 209–216.

Costenla, A.R., Diógenes, M.J., Canas, P.M., Rodrigues, R.J., Nogueira, C., Maroco, J., Agostinho, P.M., Ribeiro, J.A., Cunha, R.A. & de Mendonça, A. (2011) Enhanced role of adenosine A2A receptors in the modulation of LTP in the rat hippocampus upon aging. Eur. J. Neurosci., 34, 12–21.

Cunha, R.A. (2001) Regulation of the ecto-nucleotidase pathway in rat hippocampal nerve terminals. Neurochem. Res., 26, 979–991.

Cunha, R.A., Sebastião, A.M. & Ribeiro, J.A. (1998) Inhibition by ATP of hippocampal synaptic transmission requires localized extracellular catabo- lism by ecto-nucleotidases into adenosine and channeling to adenosine A1 receptors. J. Neurosci., 18, 1987–1995.

Di Virgilio, F., Ceruti, S., Bramanti, P. & Abbraccchio, M.P. (2009) Purinergic signalling in inflammation of the central nervous system. Trends Neu- rosci., 32, 79–87.

Dias, R., Ribeiro, J. & Sebastião, A. (2012) Enhancement of AMPA currents and GluR1 membrane expression through PKA-coupled adenosine A2A receptors. Hippocampus, 22, 276–291.

Donnelly-Roberts, D., McGarthy, S., Shihe, C., Honore, P. & Jarvis, M.F. (2008) Painful purinergic receptors. J. Pharmacol. Exp. Ther., 324, 409–415.

Dunwiddie, T.V. & Masino, S.A. (2001) The role and regulation of adenosine in the central nervous system. Annu. Rev. Neurosci., 24, 31–55.

Fredholm, B., Izerman, A., Jacobson, K., Linden, J. & Müller, C. (2011) International Union of Basic and Clinical Pharmacology. Nomenclature and classification of adenosine receptors – an update. Pharmacol. Rev., 63, 1–34.

Frenguelli, B.G., Wigmore, G., Llaudet, E. & Dale, N. (2007) Temporal and mechanistic dissociation of ATP and adenosine release during ischaemia in the mammalian hippocampus. J. Neurochem., 101, 1400–1413.

George, J., Gonçalves, F.Q., Cristóvão, G., Rodrigues, L., Meyer Fernandes, J.R., Gonçalves, T., Cunha, R.A. & Gomes, C.A. (2015) Different danger signals differently impact on microglial proliferation through alterations of ATP release and extracellular metabolism. Glia, 63, 1636–1645.

Gilbert, P. & Kesner, R. (2006) The role of the dorsal CA3 hippocampal subregion in spatial working memory and pattern separation. Behav. Brain Res., 169, 142–149.

Gomes, C., Ferreira, R., George, J., Sanches, R., Rodrigues, D.L., Gonçalves, N. & Cunha, R.A. (2013) Activation of microglial cells triggers a release of brain-derived neurotrophic factor (BDNF) inducing their proliferation in an adenosine A2A receptor-dependent manner: A2A receptor blockade prevents BDNF release and proliferation of microglia. J. Neuroinflamm., 10, 16.

Guntheringer, A., Bischofberger, J., Johenning, F.W., Turvinen, M., Schmitz, D. & Breustedt, J. (2007) Adenosine mediates transmission at the hippocampal mossy fibre synapse via direct inhibition of presynaptic calcium channels. J. Physiol., 582, 263–277.

Jinno, S., Fleischer, F., Eckel, S., Schmidt, V. & Kosaka, T. (2007) Spatial arrangement of microglia in the mouse hippocampus: a stereological study in comparison with astrocytes. Glia, 55, 1334–1347.
