

Involvement of P2X and P2Y receptors in microglial activation in vivo

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Abstract Microglial cells are the primary immune effector cells in the brain. Extracellular ATP, e.g., released after brain injury, may initiate microglial activation via stimulation of purinergic receptors. In the rat nucleus accumbens (NAc), the involvement of P2X and P2Y receptors in the generation of microglial reaction in vivo was investigated. A stab wound in the NAc increased immunoreactivity (IR) for P2X_{1,2,4,7} and P2Y_{1,2,4,6,12} receptors on microglial cells when visualized with confocal laser scanning microscopy. A prominent immunolabeling of P2X₇ receptors with antibodies directed against the ecto- or endodomain was found on *Griffonia simplicifolia* isolectin-B4-positive cells. Additionally, the P2X₇ receptor was colocalized with active caspase 3 but not with the anti-apoptotic marker pAkt. Four days after local application of the agonists α,β meATP, ADP β S, 2MeSATP, and BzATP, an increase in OX 42- and *G. simplicifolia* isolectin-IR was observed around the stab wound, quantified both densitometrically and by counting the number of ramified and activated microglial cells, whereas UTP γ S appeared to be ineffective. The P2 receptor antagonists PPADS and BBG decreased the injury-induced increase of these IRs when given alone and in addition inhibited the agonist effects. Further, the intra-accumbally applied P2X₇ receptor agonist BzATP induced an increase in the number of caspase-3-positive cells. These results indicate that ATP, acting via different P2X and P2Y receptors, is a signaling molecule in microglial cell activation after injury in vivo. The up-regulation of P2X₇-IR after injury suggests that this receptor is involved in apoptotic rather than proliferative effects.

Keywords CNS · Injury · Microglia · P2 receptors · Trauma

Abbreviations

ATP	adenosine 5'-triphosphate
α,β meATP	α,β methyleneATP
ACSF	artificial cerebrospinal fluid
ADP β S	adenosine 5'-(β -thio)diphosphate
BBG	brilliant blue G
BrdU	5-bromo-2'-deoxyuridine
BzATP	2',3'-O-(4-benzoyl-benzoyl)ATP
BSI-B4	lectin from <i>Bandeiraea simplicifolia</i> (<i>Griffonia simplicifolia</i>) isolectin B4 (peroxidase-labeled)
GFAP	glial fibrillary acidic protein
GSA-B4	lectin from <i>Bandeiraea simplicifolia</i> (<i>Griffonia simplicifolia</i>) isolectin B4 (biotin-conjugated)
2MeSATP	2-methylthioATP
IR	immunoreactivity
NAc	nucleus accumbens
pAkt	phosphorylated serine/threonine kinases
PPADS	pyridoxal-5'-phosphate-6-azophenyl-2',4'-disulphonic acid
RT-PCR	reverse transcription polymerase chain reaction
UTP γ S	uridine 5'-O-(γ -thio)triphosphate

Introduction

Microglial cells are the principal intrinsic immune competent cells of the brain. Resting microglial cells with a ramified morphology become activated under almost all pathological conditions, e.g., mechanical or chemical injury or inflammation. They change into ameboid-shaped motile phagocyt-

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otic and cytotoxic cells, which invade the site of injury and initiate repair or defense processes by secreting proteases, neurotrophic factors, and inflammatory mediators [1, 2]. This suggests that microglial cells are able to sense signal molecules entering the extracellular space by disturbances of brain homeostasis and promoting their transformation into the active state [3, 4]. Further, microglia express major histocompatibility complex antigen class II and may perform phagocytotic activity to clear cellular debris [4–7].

One of the most prominent compounds released by injury is the purine nucleotide ATP. Extracellularly applied ATP induces the activation of a cation conductance and a subsequent increase in intracellular Ca^{2+} [8]. ATP was shown to stimulate microglial cells to respond with rapid movements of their processes [9]. Further, ATP also induced the outgrowth of processes and membrane ruffling in cultured systems [10, 11]. Dependent on the concentration of ATP applied to in vitro systems, microglial cells secrete various biologically active substances, such as the cytokines tumor necrosis factor- α (TNF- α) [12], interleukin (IL)-1 β [13, 14], or IL-6 [15]. Boucsein and colleagues [16] have shown that in the presence of purinergic ligands, the lipopolysaccharide-induced release of TNF- α , IL-6, IL-12, and macrophage inflammatory protein-1 α was attenuated. In patch-clamp investigations with ATP-induced Ca^{2+} wave recordings of astrocytes, a current response in an adjacent microglial cell was observed, suggesting that microglia can sense the activity of astrocytes [17].

All these data suggest that a variety of ATP-sensitive ionotropic (P2X) and metabotropic (P2Y) receptors is expressed at resting and/or activated microglial cells [18–22]. The functional expression of ATP receptors on cultured microglia is well known [18, 23, 24]. The basic problem in studying this type of glial cells is that any manipulation *ex vivo* or *in vitro* will promote their transformation into the active form. We used an *in vivo* model of a stab wound injury in the rat nucleus accumbens (NAc) to observe the P2 receptor-mediated response of microglial cells to ATP released by injury. This technique enables local administration of receptor-specific purinergic ligands by microinfusion. Furthermore, our investigations focused especially on the P2X₇ receptor subtype on microglial cells *in vivo* and the functional consequences of injury-induced stimulation. The possible involvement of P2X₇ receptors in apoptotic and proliferative processes in microglia and astrocytes, respectively, was also investigated.

Material and methods

Materials

Ketamine hydrochloride (Ketanest; Ratiopharm, Ulm, Germany), xylazine hydrochloride (Rompun; Bayer, Leverkusen, Germany), and thiopental natrium (Trapanal, Altana Pharma,

Konstanz, Germany) were used for anesthesia. For intracumbal microinjection, 2-methylthioATP (2MeSATP), α,β -methyleneATP (α,β meATP, both RBI; Natick, MA, USA); adenosine 5'-(β -thio)diphosphate (ADP β S), 2',3'-O-(4-benzoyl-benzoyl)ATP (BzATP), brilliant blue G (BBG), and 5-bromo-2'-deoxyuridine (BrdU) (all from Sigma, Deisenhofen, Germany); uridine 5'-O-(γ -thio)triphosphate (UTP γ S, Inspire Pharmaceuticals Inc., Durham, NC, USA); pyridoxal-5'-phosphate-6-azophenyl-2',4'-disulphonic acid (PPADS; Biotrend, Köln, Germany); and artificial cerebrospinal fluid [ACSF (in mM): 126 NaCl; 2.5 KCl; 1.2 NaH_2PO_4 ; 1.3 MgCl_2 , and 2.4 CaCl_2 ; pH 7.4] were used.

Antibodies directed against glial fibrillary acidic protein (GFAP; mouse anti-GFAP, Sigma, Deisenhofen, Germany; rabbit anti-cow GFAP, DakoCytomation, Hamburg, Germany); BrdU (mouse anti-BrdU, Clone Bu20a; DakoCytomation), active caspase 3 (rabbit, Promega, Madison, WI, USA); OX 42 (CD 11b, mouse anti-rat, complement receptor type 3; Serotec, Oxford, UK); (a) lectin from *Bandeiraea simplicifolia* (*Griffonia simplicifolia*) isolectin B4 (BSI-B4, peroxidase labeled) and (b) lectin from *B. simplicifolia* (*G. simplicifolia*) isolectin B4 (GSA-B4, biotin conjugated; Sigma, Deisenhofen, Germany, each); phosphorylated serine/threonine kinases (pAkt1/2/3; rabbit, Ser 473, raised against the short amino acid sequence containing phosphorylated Ser 473 of Akt 1, Akt 2, and Akt 3 of mouse origin, recommended for the detection of Ser 473 phosphorylated Akt 1 and corresponding phosphorylated Akt 2 and Akt 3 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); and the following P2 receptor antisera were used: rabbit anti-P2X₁, anti-P2X₂, and anti-P2X_{4,5,6} (Alomone Labs, Jerusalem, Israel) as well as rabbit anti-P2X₇ receptor-subtype (intracellular C-terminus binding, Alomone Labs), anti-P2X₃ (guinea pig; Neuromics Inc., Northfield, MN, USA). Furthermore, a goat anti-rat ecto-P2X₇ receptor antibody (a kind gift of Dr. M. Kim; [25]) was used for immunofluorescence double-labeling studies. Additionally, anti-P2Y₁, anti-P2Y₂, anti-P2Y₄, anti-P2Y₁₂ (rabbit; Alomone Labs, Jerusalem, Israel), anti-P2Y₆ (rabbit; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-P2Y₁ (rabbit, GlaxoSmithKline, Brentford, Middlesex, UK) receptor antibodies were tested. The secondary carbocyanine (Cy)2- and Cy3-conjugated IgGs as well as Cy2- and Cy3-conjugated streptavidin (Jackson ImmunoResearch, West Grove, PA, USA) were used. For histochemical detection, 3,3'-diaminobenzidine hydrochloride (DAB; Sigma Chemical Co., St. Louis, USA) was applied.

Animals

Male Wistar rats (280–320 g) were housed under a 12-h light, 12 h-dark cycle and allowed access to lab food and water *ad libitum*. All procedures using animals were approved by the committee of Animal Care and Use of the relevant local

governmental body in accordance with the law of experimental animal protection.

Surgery/microinjection

Anesthetized rats were fixed in a stereotaxic frame. At the coordinates AP=1.7 mm (rostral to bregma), L=1.5 mm (lateral to the sagittal suture), and V=6.5 mm (below the surface of the hemisphere), the injection cannulae connected to a syringe pump via an FEP tubing was inserted into the NAc [26]. By microinfusion, rats received ACSF, which was used as control as well as vehicle for the following P2 receptor agonists: 2MeSATP (nonselective), α, β meATP (P2X_{1,3}), ADP β S (P2Y_{1,11,12,13}), UTP γ S (P2Y_{4,6}), given as 0.1 nmol each, and BzATP (preferential P2X₇) 0.3 nmol. As antagonists, PPADS (30 pmol; nonselective) and BBG (1 pmol, P2X₇) were applied. When the effects of the P2 receptor agonists were pharmacologically proven, the microinfusion of the respective antagonists preceded infusion of the agonist and antagonist mixture. As a proliferation marker, BrdU (0.1 nmol) was applied together with the antagonist, the agonist, or ACSF alone. Test substances were injected in a volume of 1 μ l at a rate of 12 μ l/h.

Immunocytochemical studies and double-immunofluorescence studies

Immunocytochemical and double-immunofluorescence studies were done as previously described [26, 27]. After a postinjection time of 2 h and 4 days, the rats were transcardially perfused under thiopental sodium anesthesia with paraformaldehyde (2%) in sodium acetate buffer (pH 6.5; solution A) followed by paraformaldehyde (2%)/glutaraldehyde (0.1%) in sodium borate buffer (pH 8.5; solution B). The brains were immediately removed and stored overnight in solution B without glutaraldehyde. Serial coronal sections (50- μ m thick) from the NAc were obtained using a vibratome (Leica, Typ VT 1000S, Nussloch, Germany) and collected as free-floating slices (0.1 M Tris; pH 7.6).

Immunocytochemical studies

BSI immunoreactivity

Free-floating sections were rinsed with 0.05 M Tris-buffered saline (TBS, pH 7.6) and were treated with 1% hydrogen peroxide for 30 min to inactivate endogenous peroxidase activity. Immunolabeling was performed with lectin from BSI-B4 (1:200) in TBS containing 2% bovine serum albumin overnight at 4°C, followed by washing in 0.05 M Tris buffer (pH 8.0). Peroxidase activity was visualized with DAB (0.07%) containing nickel ammonium sulphate (1%) and hydrogen peroxide, which renders a black reaction product.

Active caspase 3 immunoreactivity

Free-floating sections were rinsed with 0.1 M TBS (pH 7.4) and treated with 1% hydrogen peroxide for 25 min to inactivate endogenous peroxidase activity. Immunoreactivity (IR) was studied with rabbit anti-active caspase 3 (1:500) in TBS containing 10% normal horse serum (NHS) and 0.1% Triton X-100 overnight at 4°C, followed by biotinylated horse anti-rabbit IgG (1:100, Vector Labs, Burlingame, CA, USA) and preformed streptavidin/biotin-peroxidase complex (1:125, StreptABCComplex; DakoCytomation) for 2 h. DAB (0.05%) served as chromogen.

GFAP/BrdU immunoreactivity

The procedure was applied to free-floating slices as previously described [26]. Briefly, the GFAP staining procedure was carried out with rabbit anti-cow GFAP antiserum (1:600) and biotinylated protein A (1:400; Calbiochem, La Jolla, CA, USA). To detect the astroglial marker, the streptavidin/biotin technique based on a StreptABCComplex (1:125) and DAB (0.05%) as chromogen were used. Mitotic astrocytes were identified by immunostaining of the incorporated BrdU. After DNA denaturation (2 N HCl) and neutralization (borate buffer; 0.15 M; pH 8.5), the slices were incubated with a mouse monoclonal antibody against BrdU (1:75), followed by incubation with horse biotinylated anti-mouse immunoglobulins (1:100; Vector Labs., Burlingame, CA, USA) and with ABC Elite Kit (1:50; Vectastain; Vector). Peroxidase activity was visualized with DAB (0.07%) containing nickel ammonium sulphate (1%) plus cobalt chloride (1%) (DAB-Ni/Co) and hydrogen peroxide, which renders a black reaction product. For GFAP/BrdU double-staining experiments to characterize mitogenic changes, slices were first processed for anti-GFAP labeling followed by BrdU immunolabeling.

Double-immunofluorescence studies

After washing with TBS (0.05 M; pH 7.6) and blocking with normal goat serum (1% NGS) in TBS, slices were incubated in an antibody mixture of mouse anti-OX 42 (CD11b; 1:100) and rabbit anti-P2X receptor antibodies (P2X₁ 1:400; P2X₂ 1:400; P2X₄ 1:400; P2X₅ 1:400; P2X₆ 1:400; P2X₇ 1:1,000) or P2Y receptor antibodies [P2Y₁ 1:400; P2Y₂ 1:1,000; P2Y₄ 1:1,000; P2Y₁₂ 1:400 (Alomone Labs); P2Y₁: 1:1,500 (GlaxoSmithKline); P2Y₆ 1:200 (Santa Cruz Biotechnology, Inc.)] with 0.1% Triton X-100 in 1% NGS in TBS for 48 h at 4°C. The secondary antibodies employed for simultaneous localization of the two primary antisera were Cy2-conjugated goat anti-mouse IgG (1:500) and Cy3-conjugated goat anti-rabbit IgG (1:800), respectively. Cy2-conjugated goat anti-guinea pig IgG (1:400) and Cy3-conjugated goat anti-mouse

IgG (1:1,000) were used for visualization of guinea pig anti-P2X₃ (1:1,000). Sections were washed three times for 5 min each in 1% NGS [or 5% fetal calf serum (FCS) for guinea pig anti-P2X₃] in TBS and then incubated for 2 h in a solution containing a mixture of the secondary antibodies with 1% NGS (5% FCS) in TBS.

GSA-B4 and P2X or P2Y double labeling was performed in two steps: first the incubation of the slices with GSA-B4 (1:300), followed by treatment with Cy2-conjugated streptavidin (1:350); and second, by incubation with rabbit P2X/Y antibodies followed by Cy3-conjugated goat anti-rabbit IgG (1:800) as described above. The ecto-P2X₇ receptor antibody (goat anti-rat ecto-P2X₇ receptor, 0.12 µg/ml [25]) was combined with rabbit anti-caspase 3 (1:500), rabbit anti-pAkt (1:600), or rabbit anti-P2X₇ (C-terminal, 1:1,000) and subsequent detection with Cy2-conjugated donkey anti-goat IgG (1:1,000) and Cy3-donkey anti-rabbit IgG (1:1,000). Incubation of slices with goat anti-rat ecto-P2X₇ (1:600), rabbit anti-active caspase 3 (1:500), or rabbit anti-pAkt (1:600) and GSA-B4 (1:300) was followed by treatment with Cy3-conjugated donkey anti-goat (1:1,000) or Cy3-conjugated goat anti-rabbit IgG (1:400) and Cy2-conjugated streptavidin (1:350). Control experiments were carried out without the primary P2 receptor antibody or by preadsorption of the antibody with the peptides used for their generation. When slices were incubated with PBS instead of the primary antibody or with primary antibody serum, which had been preabsorbed with peptide antigen for 1 h before use, no immunofluorescence with either of the control procedures was observed. After intensive washing and mounting on glass slides, all stained sections were dehydrated in a series of graded ethanol, processed through n-butylacetate, coverslipped with entellan (Merck, Darmstadt, Germany), and analyzed by light microscopy or confocal laser scanning microscopy.

Confocal microscopy

Double immunofluorescence was investigated by a scanning confocal microscope (LSM 510, Zeiss, Oberkochen, Germany) equipped with an argon laser emitting at 488 nm (yellow-green Cy2-immunofluorescence) and a helium/neon laser emitting at 543 nm (red Cy3-immunofluorescence).

Quantification and statistical analysis

Changes in BSI-B4-IR were determined by densitometry (in regions 1 and 2, Fig. 1a) using a digital image analyzer (Diana II) in combination with an advanced image data analyzer (AIDA 2.0). Results are expressed as percentage of the BSI-B4-IR of the ACSF-treated sides in the same regions.

Additionally, the number of BSI-B4-labeled ramified and activated microglial cells as well as GFAP and GFAP/BrdU double-labeled cells were counted under a light microscope

(Axioskop; Zeiss) with a 20x objective within a square (0.5 × 0.5 mm) in region 1 (Fig. 1a). Each value represents five replications for each condition. The results were expressed as a percentage of ACSF-treated sides of the same regions. The individual groups were compared with one-way analysis of variance (ANOVA) using Bonferroni *t* test. A probability level of 0.05 or less was considered to be statistically significant.

Results

Immunocytochemistry

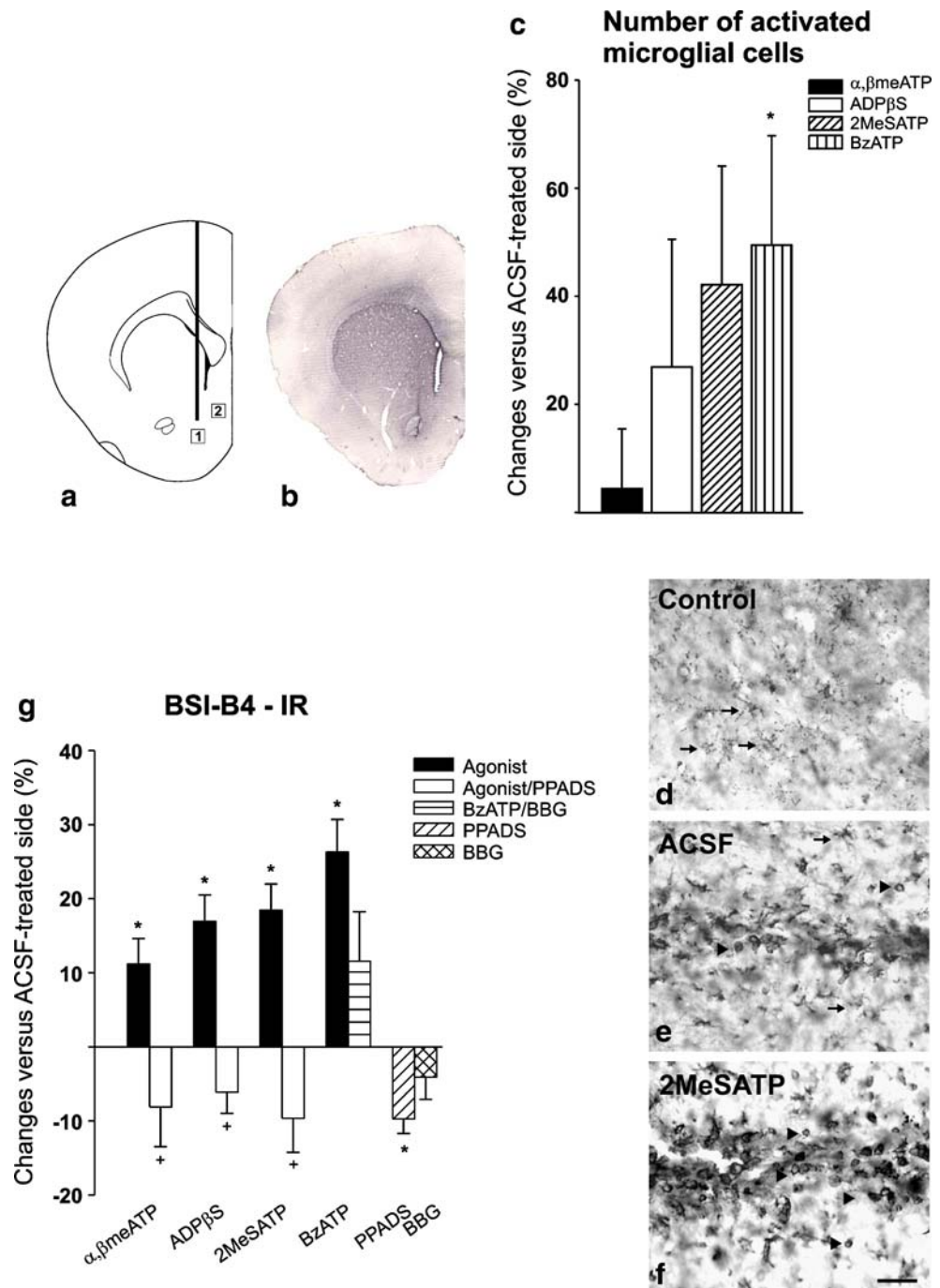
After mechanical injury (Fig. 1a,b), a characteristic BSI-B4 immunolabeling around the stab wound was observed in comparison with untreated controls, decreasing in relation to the distance from the lesion. In untreated rats, a great number of resting (process-bearing) microglial cells was present (Fig. 1d, arrows), whereas after stab-wound injury, changes in the number of resting microglial cells with retracted processes (Fig. 1e, arrow) and activated microglial cells (Fig. 1f, arrowhead) were observed. Quantification of BSI-B4-immunolabeling in the NAc (region 1; Fig. 1c) of rats 4 days after stab-wound injury indicated a variable influence of P2 receptor agonists on the number of activated cells. Whereas α,β meATP had no effect, 2MeSATP was more potent in tendency than was ADP β S in increasing the number of activated microglial cells. BzATP, which was injected into the NAc in a higher dose than were the previous ligands [28], also induced a marked enhancement of activated microglial cells (Fig. 1c). UTP γ S was without effect, both on the number of activated microglia and on resting microglia (not shown).

Quantification of BSI-B4-IR in the NAc (region 1 plus 2) by densitometry showed a significant increase for all agonists used with the exception of UTP γ S (not shown) when compared with ACSF-treated animals. The effects of α,β meATP, ADP β S, and 2MeSATP could be inhibited by pretreatment with PPADS and those of BzATP by pretreatment with BBG (Fig. 1g). PPADS and BBG given alone inhibited and failed to alter BSI-B4-IR, respectively.

Double-immunofluorescence studies at P2X and P2Y receptor subtypes

Multiple immunofluorescence labeling in combination with laser scanning microscopy was used to characterize the P2X/Y receptor subtype expression after injury in comparison with untreated controls. Under control conditions, no P2X or P2Y (with the exception of P2Y₁) receptor-IR was coexpressed on single GSA-B4-positive cells. The mechanical injury associated with the introduction of the injection cannula resulted in the expression of P2X_{1,2,4,7} and P2Y_{1,2,4,6,12}

Fig. 1 **a** A rat brain slice including needle tracts and regions of interest within the nucleus accumbens (NAc) in which immunoreactivity (IR) and cells were evaluated (1 core 1; 2 core 2; according to [29]). **b** BSI-B4-marked microglial cells after stab-wound injury (overview). **c** Quantification of the number of activated microglial cells after injury and microinjection of P2-receptor-agonists 2MeSATP, ADP β S, α , β meATP (0.1 nmol each), and BzATP (0.3 nmol) after a post-injection time of 4 days. Values are expressed as percentage of ACSF-treated controls and represent mean \pm SEM of six animals per group. **d–f** BSI-B4-IR of microglial cells in the NAc of rats, illustrating changes in the glial morphology: **d** A great number of resting (process-bearing) microglial cells (arrows) under control conditions; **e, f** changes in the number of resting microglial cells (arrow) and activated microglial cells (arrowhead) after different treatment conditions (scale bar=200 μ m). **g** Quantification of the effects of agonists 2MeSATP, ADP β S, α , β meATP (0.1 nmol each), and BzATP (0.3 nmol) alone and in combination with PPADS (0.1 nmol/0.03 nmol each) or BBG (1 pmol) on BSI-B4-IR of microglial cells in brain slices of the NAc of the rat after a postinjection time of 4 days. Data are expressed as percentage of the ACSF-treated side and represent the mean \pm SEM of six animals per group (* p <0.05, vs. ACSF group; + p <0.05, agonist vs. antagonist/agonist group)

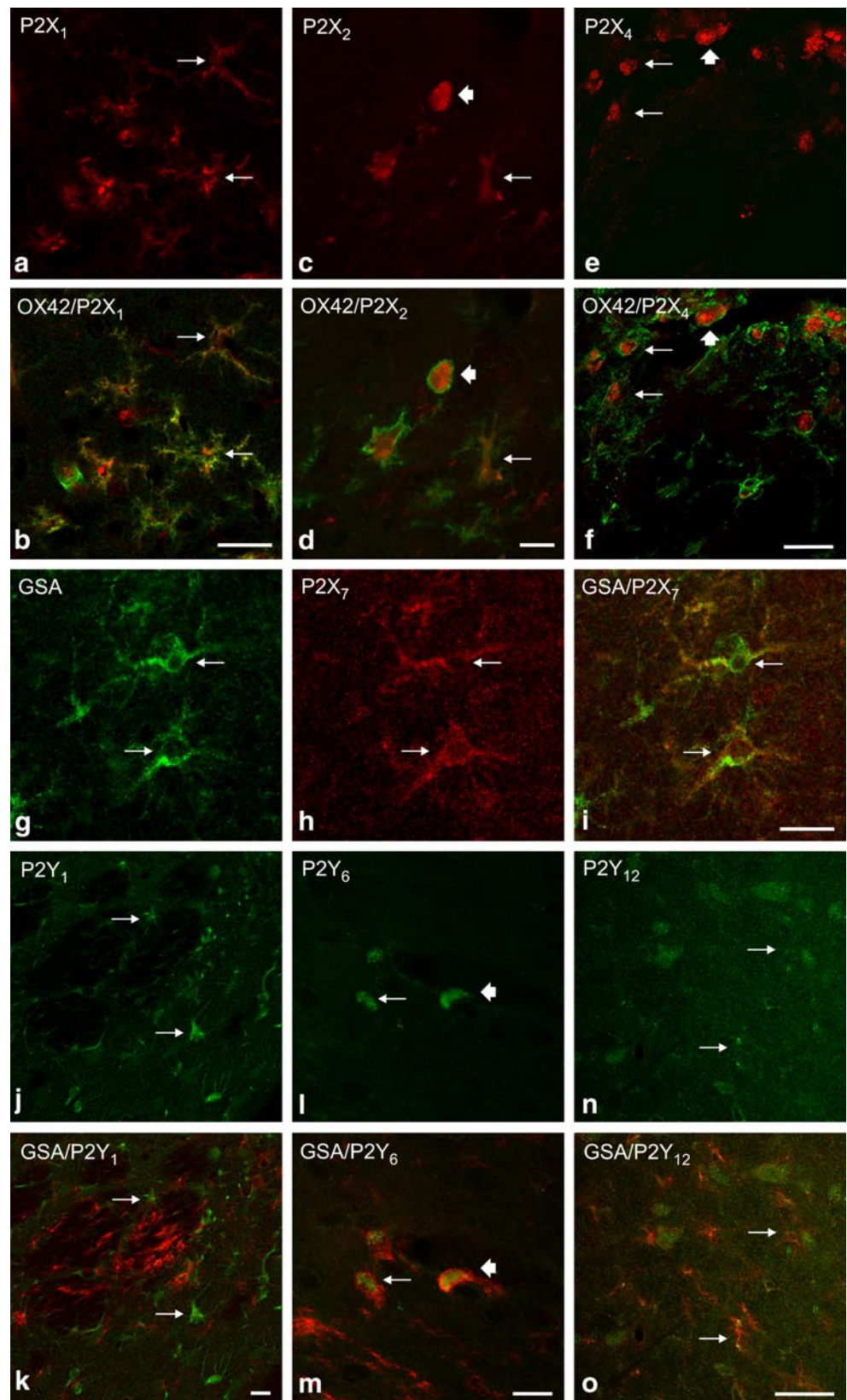


receptors in the lesioned area, observed earliest 2 h until 4 days after injury. An up-regulation of immunolabeling of P2X_{1,2,4,7} receptor subtypes was unequivocal; examples are shown in Fig. 2 (a–i). In the affected area, P2Y_{1,2} and P2Y₁₂ fluorescence labeling was observed on GSA-B4-positive cells with low intensity. P2Y₄ labeling appeared preferentially on process-bearing microglial cells (not shown). P2X₂-IR (Fig. 2c,d), P2X₄ (Fig. 2e,f), and P2Y₆-IR (Fig. 2l,m) were found on both resting and especially on activated microglial cells, the latter being identified by the

lack of processes. No immunofluorescence was observed for P2X_{3,5,6} receptor subtypes on either type of microglial cells before and after stab wounding.

Injury-induced prominent expression of the P2X₇ receptor was observed on microglial cells by double labeling with GSA-B4 (Fig. 2g–i) and also on astrocytes, as described earlier [29]. The two different P2X₇ receptor antibodies, one directed against the extracellular domain of the rat P2X₇ receptor [25] and the other against its C terminus (C-terminal P2X₇) (Alomone Labs) labeled the same cells (Fig. 3a,b; thin

Fig. 2 Confocal images of examples of double immunofluorescence labeling to characterize the colocalization of P2X_{1,2,4,7} and P2Y_{1,6,12} receptor subtypes on **a–f** OX42- or **g–o** GSA-B4-labeled microglial cells in the NAc of rats 4 days after stab-wound injury (*thin arrow* process-bearing microglial cell; *thick arrow* activated microglial cell) (scale bars: **a, b**=20 μm; **c, d**=100 μm; **e–i**=20 μm; **j, k**=100 μm; **l, m**=20 μm; **n, o**=50 μm)



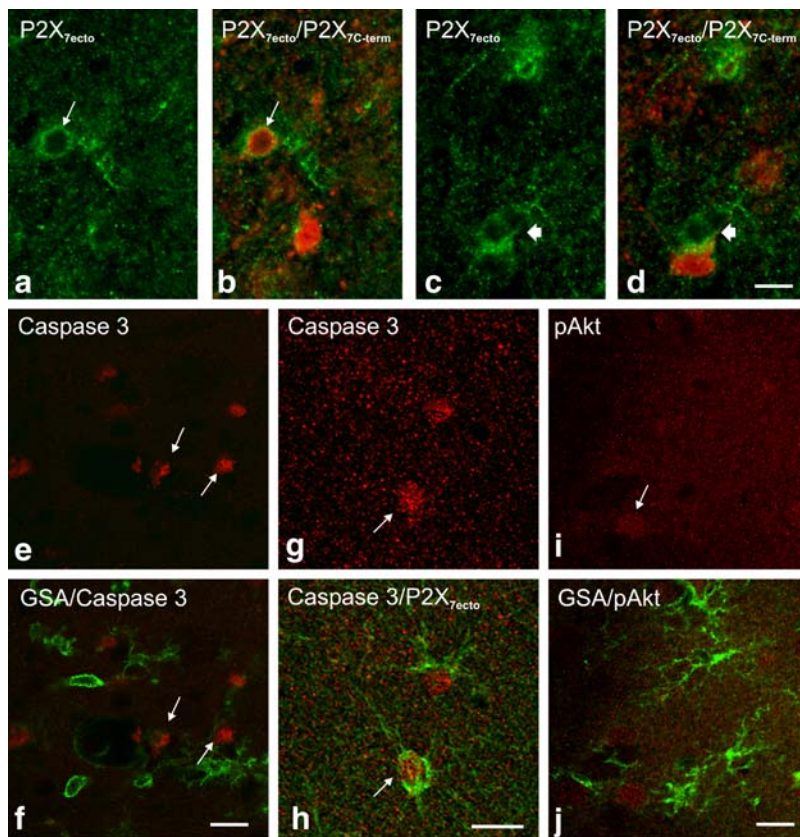


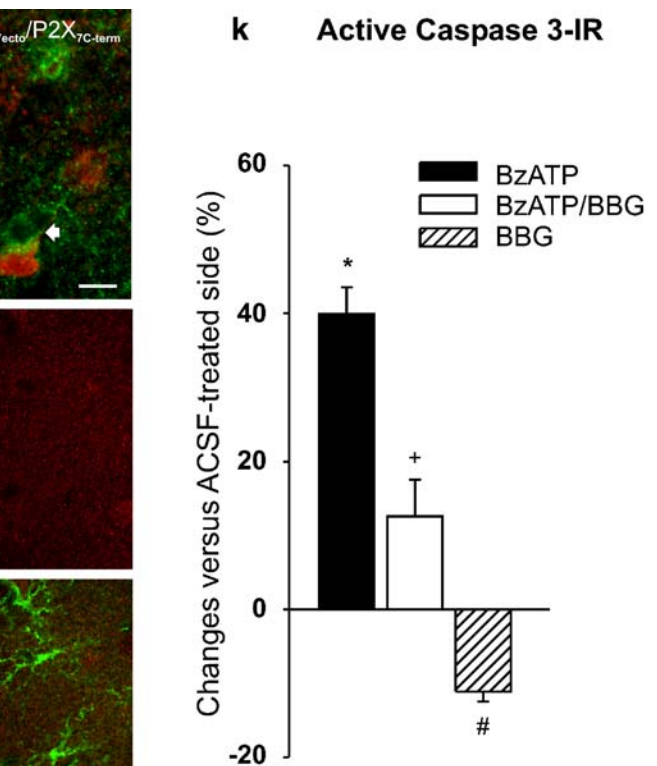
Fig. 3 Apoptosis or proliferation after P2X₇ receptor stimulation after stab-wound injury in the NAc of rats 4 days after injury. **a–d** Colocalization of P2X₇-IR detected by antibodies directed against the C-terminus (*C-term*) (**b, d**) and the ectodomain (**a–d**) of the rat P2X₇. **e–j** Confocal images of double-labeling studies: Coexpression of active caspase 3 on single GSA-B4-positive cells (**e, f**) and active caspase 3 and the ecto-P2X₇ receptor subtype (**g, h**). No double labeling of pAkt on GSA-B4-

arrow) but also different cells (Fig. 3c,d; thick arrow). Additionally, the ecto-P2X₇ antibody labeled GSA-positive cells (data not shown).

Possible role of the P2X₇ receptor after injury in vivo

To find out whether the P2X₇ receptor is related to apoptotic and/or proliferative processes after injury, we used the early apoptotic marker active caspase 3 and the proliferation marker BrdU. After BzATP microinjection, a significant increase in the number of active caspase-3-positive cells was found in comparison with ACSF-treated animals (Fig. 3k). This effect was reduced by pretreatment with the P2X₇ receptor antagonist BBG. Further, BBG given alone decreased the number of active caspase 3-immunopositive cells in comparison with ACSF-treated rats (Fig. 3k).

Double immunofluorescence-labeling showed colocalization of active caspase 3 at single GSA-B4-positive cells in the NAc of rats (Fig. 3e,f). Furthermore, colocalization of the ecto-P2X₇ receptor subtype on caspase-3-positive cells was also documented (Fig. 3g,h). Additionally, a possible co-



labeled microglial cells 4 days after injury (**i, j**) (scale bars: **b–g**=20 μ m). **k** Quantification of the number of active caspase 3-positive cells in region 1 (see Fig. 1 **a**). Values are expressed as percentage of controls and represent the mean \pm SEM of six animals per group. (* p <0.05, vs. ACSF group; + p <0.05, agonist vs. antagonist/agonist group; # p <0.05, antagonist vs. antagonist/agonist group)

expression of the anti-apoptotic and possibly proliferation-promoting pAkt on GSA-B4-positive cells was studied 4 days after injury. However, no colocalization of pAkt with GSA-B4-positive cells (Fig. 3i,j) was found by immunofluorescence labeling. Eventually, no colocalization of P2X₇ and pAkt was observed to this time point (data not shown).

A possible influence of P2X₇ receptor stimulation on the increase in cell number of activated and ramified microglial cells after BzATP stimulation was also studied. Changes in the number of activated microglia are shown in Fig. 1c. By quantification of ramified (resting) microglial cells in region 1 (Fig. 1a), an increase to $123.8 \pm 5.3\%$ in comparison with ACSF-treated rats was found, similar to that after treatment with 2MeSATP ($124.0 \pm 7.8\%$). For comparison, no characteristic changes were found after microinfusion of ADP β S ($108.3 \pm 11.6\%$) and α, β meATP ($101.7 \pm 6.9\%$). Besides the effects of BzATP on the number of active and resting microglial cells, no influence of BzATP treatment on the number of GFAP (specific marker for fibrous astrocytes)-positive cells (BzATP $1.7 \pm 7.3\%$; BBG $0.4 \pm 3.0\%$; BzATP/BBG $0.96 \pm 4.8\%$) was found in comparison with ACSF-treated rats. There was also no

effect on the number of GFAP/BrdU double-labeled cells (proliferating cells) (BzATP $14.8 \pm 4.5\%$; BBG $8.8 \pm 11.2\%$; BzATP/BBG $15.9 \pm 5.9\%$) in comparison with ACSF-treated rats.

Discussion

The results presented here demonstrate the involvement of various P2 receptors in microglial response to the placement of cannulas and microinjection, both resulting in tissue damage and thereby in high extracellular ATP concentrations [30]. Microglial cells stimulated by ATP may participate both in neurodegeneration (apoptosis and/or necrosis) and neuroprotection.

Cultured microglial cells from mouse or rat brain were shown to respond to ATP with the activation of a cationic conductance, accompanied by an increase in cytosolic Ca^{2+} [8], followed by changes of microglial morphology [31, 32]. The exposition of cultured microglial cells to ADP or ATP further induced membrane ruffling and markedly enhanced chemokinesis, a prerequisite to perform tissue surveillance in the brain [33].

In vivo, microglial cells are homogeneously distributed within the NAc characterized by rod-shaped somata with thin and highly ramified processes extended in all directions. Injury by stab wound caused microglial cell activation around the lesion track, characterized by swollen somata and thicker processes. The additional administration of P2X and P2Y receptor agonists enhanced the occurrence of activated microglial cells detected by their BSI-IR. The observed changes suggest that the stimulation of P2X_{1,3} receptors has only minor relevance in the process of microglia activation, whereas in agreement with the subsequent pharmacological investigations, involvement of P2X₇ and P2Y_{1,2,4,6,12} receptors in this process is highly probable.

Whereas P2X_{1–7} receptor-IR was absent in the non-affected NAc, the expression of P2X_{1,2,3,4,7} receptors but not of P2X_{5,6} receptors was detected after the stab-wound lesion. P2X_{2,4,6} receptors are widely expressed in central and peripheral neurones as well as other excitable cells [22, 34, 35], and the P2X₇ receptor is expressed in macrophage-type cells such as microglia but also mediates purinergic responses in astrocytes and neurons [36]. Changes in microglial expression of P2X receptors (P2X_{1–7}) during postnatal development of the rat have been described [37]. Obviously, microglial cells are involved in long-term trophic events such as cell proliferation, differentiation, movement, and death during development [37].

Upregulation of P2X₁-IR at microglial cells, as found in this study, was described at activated astrocytes after stab-wound injury in the adult rat NAc [29]. Further, the P2X₂ receptor was found to be situated at activated microglia in our study and previously at astroglial cells [29]. After is-

chemia in vivo, P2X₂ and P2X₄-receptor-IR in the hippocampus became up-regulated in different cellular phenotypes [38]. P2X₂ was expressed on neuronal cell bodies and fibers in hippocampal pyramidal cells, whereas intensive P2X₄-IR was localized at microglial cells. Confocal microscopic analysis with OX 42 [39] at organotypic cultures showed that P2X₄ and P2X₇ are expressed on microglia, whereas P2X₁ and P2Y_{1,2,12}, although present in the slices, do not colocalize at microglia, and the P2X₆ receptor is absent. P2X₄ receptors seem to be functionally relevant for microglial chemotaxis, which was suppressed by the knockdown of the P2X₄ receptor in cultured microglia by ribonucleic acid (RNA) interference through the lentivirus vector system [33].

After spinal-nerve injury, P2X₄ receptor expression strikingly increased at the ipsilateral site in hyperactive microglia but not in neurons or astrocytes [40]. Intraspinal administration of P2X₄ receptor antisense oligodeoxynucleotide decreased induction of P2X₄ receptors and suppressed tactile allodynia after nerve injury.

The major P2X receptor expressed at microglial cells is the P2X₇ receptor, characterized in several reports in vitro [13, 16, 41]. This receptor subtype appeared to be strongly coexpressed with the microglial marker GSA-B4 after the mechanical lesion used in our study, suggesting an outstanding role of this receptor in injury-induced microglial responses. It is thought to be closely related to the immune functions of microglia, such as production of inflammatory cytokines, superoxide, and nitric oxide [42]. The P2X₇ receptor is reported to be little susceptible to desensitization in the presence of ATP, which makes it quite different from other purinergic receptors, including the P2X₄ receptor, which can be desensitized within a few minutes [23, 43]. Up-regulation of microglial P2X₇ receptors has been observed in several pathological models, such as in vivo ischemia [41, 44, 45], as well as multiple sclerosis and amyotrophic lateral sclerosis of the human spinal cord [46]. The P2X₇ receptors were also specifically up-regulated around β -amyloid plaques in a mouse model of Alzheimer's disease (Tg2576) [47] and in human proliferative vitreoretinopathy on Müller glial cells [48].

Within the P2Y receptor family, in our study, the P2Y₁ receptor was markedly expressed on microglial cells of untreated rats and was up-regulated in concert with P2Y_{2,4,6,12} receptors after injury. In the rabbit retina, microglial cells express functional P2Y₁ receptors [49]. Activation of these receptors stimulates phenotypic alterations characteristically for microglial activation.

Our data also demonstrate a strong colocalization of the P2Y₆ receptor with GSA-IR. In addition to the microglial function to build a barrier between injured and healthy tissue, these cells are able to clear dead cells or dangerous debris from brain tissue. This phagocytotic process was shown to be triggered by activation of P2Y₆ receptors by the endogenous agonist UDP [50].

Several authors report on ATP-induced migration of microglial cells to the affected sites of the brain [9, 10, 51–53]. Expression of the P2Y₁₂ receptor on microglial cells after injury seems to be strongly related to the motility of this cell type. In primary cultured microglial cells, exposition to ADP or ATP induced membrane ruffling and markedly enhanced chemokinesis, which could be blocked by the P2Y₁₂ receptor antagonist cangrelor (AR-C69931MX), suggesting that G_{i/o}-coupled P2Y receptors are involved [10]. The high dynamic of intact microglial processes as well as their chemotactic response to injury or local injection of ATP was shown in mouse cortex using time-lapse, two-photon laser imaging [9]. Investigations in P2Y₁₂ receptor-deficient mice gave further support that P2Y₁₂ receptors are required for the nucleotide-evoked chemotaxis [33, 54]. Eventually, expression of P2Y₁₂ receptors was observed in native and axotomized facial nuclei, and the number of P2Y₁₂-expressing cells increased following facial nerve axotomy, considered to be a crucial component in the regeneration cascades of motor neurons [55].

The published data confirm that not all known P2 receptors can be detected by their immunoreactivity. However, the mRNA of the P2X_{1–7} [29] and P2Y_{1,2,4,6,12} subtypes [27] was detectable in the NAc of untreated rats. The P2 receptors present on microglia may be differently involved in the processes of their activation, as shown by the inhibition of BSI-B4-IR using PPADS and BBG. It can be suggested by pharmacological studies that P2Y₁ and P2X₇ receptors may play a predominant role in the response of microglia to the consequences of a stab wound in the NAc.

Retinal detachment and application of ADPβS onto control retinas induced phenotype alterations of microglial cells (decrease of soma size, retraction of cell processes) and had no influence on microglial cell density [49]. In the stab-wound model, stimulation of P2 receptors with agonists preferring P2Y₁ and P2X₇ receptor subtypes promoted the morphological changes of resting to activated microglial cells.

P2X₇ receptors are ATP-gated ion channels; their sustained activation leads to cytolysis in an apoptotic fashion in the microglia [56]. P2X₇ receptor-mediated apoptosis involves activation of the proteolytic pathway of caspase activation, which leads to nuclear DNA damage [57]. In our study, immunocytochemistry showed expression of P2X₇ and active caspase 3 at GSA-positive cells after injury without agonist application. The active caspase 3 has been defined as the main executioner of programmed cell death and as an early apoptotic marker [58]. The microinfusion of BzATP increased the number of caspase-3-positive cells, suggesting an involvement of P2X₇ receptors in apoptotic changes after traumatic events. The BzATP-induced increase in ramified and activated microglia (and proliferating astrocytes) was only marginal.

The phosphoinositide-3-kinase/serine-threonine-kinase Akt (PI3-K/Akt) pathway is associated with cell proliferation, promoting cell survival and inhibiting apoptosis [59]. Because in the present study performed at 4 days after injury no coexpression of pAkt with GSA and P2X₇ was found, the evidence for a preferential apoptotic pathway triggered by P2X₇ receptors is unlikely.

Up-regulation of P2 receptors after mechanical injury might be both the cause and the result of the induction of apoptotic/necrotic cell death activating microglial cells with harmful or beneficial effects. The present results from in vivo investigations implicate that ATP via both P2X and P2Y receptor-stimulation acts as a signaling molecule in microglial cells in vivo. An up-regulation of P2X₇-IR after injury suggests involvement of this receptor subtype in apoptotic rather than in proliferative events.

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