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Extracellular nucleotide signaling in adult neural stem cells: synergism with growth factor-mediated cellular proliferation

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We have previously shown that the extracellular nucleoside triphosphate-hydrolyzing enzyme NTPDase2 is highly expressed in situ by stem/progenitor cells of the two neurogenic regions of the adult murine brain: the subventricular zone (type B cells) and the dentate gyrus of the hippocampus (residual radial glia). We explored the possibility that adult multipotent neural stem cells express nucleotide receptors and investigated their functional properties in vitro. Neurospheres cultured from the adult mouse SVZ in the presence of epidermal growth factor and fibroblast growth factor 2 expressed the ecto-nucleotidases NTPDase2 and the tissue non-specific isoform of alkaline phosphatase, hydrolyzing extracellular ATP to adenosine. ATP, ADP and, to a lesser extent, UTP evoked rapid Ca^{2+} transients in neurospheres that were exclusively mediated by the metabotropic $P2Y_1$ and $P2Y_2$ nucleotide receptors. In addition, agonists of these receptors and low concentrations of adenosine augmented cell proliferation in the presence of growth factors. Neurosphere cell proliferation was attenuated after application of the $P2Y_1$-receptor antagonist MRS2179 and in neurospheres from $P2Y_1$-receptor knockout mice. In situ hybridization identified $P2Y_1$-receptor mRNA in clusters of SVZ cells. Our results infer nucleotide receptor-mediated synergism that augments growth factor-mediated cell proliferation. Together with the in situ data, this supports the notion that extracellular nucleotides contribute to the control of adult neurogenesis.

KEY WORDS: Adult neurogenesis, ATP, Ecto-Nucleotidase, Neurosphere, P2 receptor, Subventricular zone

INTRODUCTION

In the adult rodent brain, neurogenesis persists in two restricted regions: the subventricular zone (SVZ) of the lateral ventricles and the dentate gyrus of the hippocampus (Altman and Das, 1965; Gage et al., 1998; Alavarez-Buylla and García-Verdugo, 2002). Within the SVZ, three principal closely adjacent cell types are distinguished morphologically and functionally: Type B and A cells, representing protoplasmic astrocyte-like stem cells and migrating neuroblasts, respectively; and the transit-amplifying type C cells (Doetsch et al., 1999; Peretto et al., 1999; Garcia et al., 2004). Neuronal precursors migrating towards the olfactory bulb (OB) are ensheathed by tubes of slowly proliferating type B cells (Menezes et al., 1995; Lois et al., 1996). In the OB, the neuroblasts differentiate into granular or periglomerular interneurons (Lois et al., 1996). Multipotent precursors with the characteristics of neural stem cells can be propagated from the SVZ as neurospheres in the presence of epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF2). They retain the ability to generate neurons, astrocytes and oligodendrocytes (Reynolds and Weiss, 1992; Reynolds and Weiss, 1996; Gritti et al., 1996; Hack et al., 2004). The extracellular signaling mechanisms controlling the various transition steps involved in adult neurogenesis are poorly understood. These steps include the initiation of the asymmetric division of type B cells to form type C cells, the high proliferation rate of type C cells, the generation of migrating neuroblasts, the suppression of neuroblast differentiation during migration or the induction of differentiation in the OB. Presumably, these steps are controlled in a step-wise manner by a multifactorial process. Polypeptide growth factors and cytokines, and additional secretory factors have been implicated in SVZ-derived cell proliferation and differentiation (Doetsch, 2003; Alvarez-Buylla and Lim, 2004; Abrous et al., 2005).

We have previously identified functional ATP receptors in the adult hippocampal progenitors in situ (Shukla et al., 2005). Furthermore, we have shown that, in the adult rodent brain, the nucleoside triphosphate-hydrolyzing ecto-enzyme ecto-nucleoside triphosphate diphosphohydrolase 2 (NTPDase2), is expressed at a high level by type B cells of the SVZ (Braun et al., 2003) and by hippocampal progenitors (Shukla et al., 2005). As ecto-nucleotidases modulate nucleotide receptor-mediated cell communication (Zimmermann, 2001), this raises the possibility that purinergic signaling is involved in the control of adult neurogenesis. Extracellular nucleotides act via ionotropic receptors (seven subtypes, $P2X_{1-7}$, permeable to Na^+, K^+ and Ca^{2+}) or G-protein-coupled receptors (eight subtypes, $P2Y_{1,2,4,6,11,12,13,14}$). P2X receptors are stimulated by ATP, whereas P2Y receptors are activated by ATP, ADP, UTP, UDP or nucleotide sugars, depending on the subtype (Ralevic and Burnstock, 1998).

In this study, we explored the possibility that adult neural stem cells express functional nucleotide receptors. We demonstrate that neurosphere cells from the adult SVZ express distinct nucleotide-hydrolyzing ecto-enzymes as well as defined P2 receptors whose activation elevates cytosolic Ca^{2+} concentrations and augments cell proliferation in a synergistic manner with mitogenic growth factors. Together with our in situ data, this supports the notion that extracellular nucleotides contribute to the control of adult neurogenesis in the SVZ.

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MATERIALS AND METHODS

Animals

Handling of animals was under veterinary supervision according to European regulations (University of Frankfurt). Mice (C57BL/6N or C57BL/6J) were obtained from Charles River Wiga (Sulzfeld, Germany). Unless stated otherwise, C57BL/6N mice were used. The generation of $P2Y_1$-deficient mice (C57BL/6J) has been previously described (Léon et al., 1999). Eight- to nine-week-old animals were anaesthetized with CO_2 and brains were excised for preparation of neurospheres.

Preparation of neurospheres

Neurospheres from wild-type or knockout animals were prepared as described previously (Johansson et al., 1999). In brief, the SVZ was excised with microblades from tissue surrounding the lateral wall of the lateral ventricles, using 400 μm thick coronal sections kept in ice-cold PBS buffer [in mM: 137 NaCl, 3 KCl, 15 Na^+/K^+-phosphate buffer (pH 7.4)]. The tissue was enzymatically dissociated with 0.5 mg/ml papain (14 U/mg) dissolved in DMEM/F12 media (Invitrogen, Karlsruhe, Germany) containing 0.1 mM EDTA, for 20-30 minutes at 37°C. The suspension was transferred into the same volume of trypsin inhibitor (0.7 mg/ml in DMEM/F12) with 1000 U/ml of DNase I (Sigma, Taufkirchen, Germany) and mechanically dissociated by triturating with a micropipette. Cells were centrifuged at 260 $\boldsymbol{g}_{av}$ for 6 minutes, resuspended in DMEM/F12, transferred into uncoated culture dishes and cultured with growth media [DMEM/F12, B27 as supplement, 10 mM HEPES buffer (pH 7.2) (all from Invitrogen), 100 U/ml penicillin and 10 μg/ml streptomycin (both Sigma)], containing 20 ng/ml of human recombinant EGF and 10 ng/ml FGF-2 (both from PeproTech, London, UK). After 7 days in vitro, neurospheres were collected in 15 ml falcon tubes, centrifuged at 260 $\boldsymbol{g}_{av}$ for 6 minutes, dissociated with accutase II (PAA Laboratories, Cölbe, Germany) at 37°C for 45 minutes, and seeded into growth media as described above. The resulting neurospheres were analyzed 7-8 days after subplating, unless stated otherwise.

Measurement of intracellular Ca^{2+} transients with fura-2 AM

For the analysis of the intracellular Ca^{2+} concentration, neurospheres were fixed for a short period (up to 60 minutes) onto poly-L-ornithine-coated (250 μg/ml) glass coverslips (12 mm). Neurospheres were maintained in culture medium (DMEM/F12 with supplement B27) and loaded for 15 minutes at 37°C with 4 μM fura-2 acetoxymethylester (fura-2 AM, Molecular Probes, Leiden, Netherlands). Before analysis, coverslips were washed twice with buffer A [in mM: 140 NaCl, 5 KCl, 2 $MgCl_2$, 5 $CaCl_2$, 10 glucose, 10 HEPES (pH 7.2)], transferred to a perfusion chamber on the heatable stage of an inverted microscope (Axiovert 100; Zeiss, Oberkochen, Germany) and analyzed as previously described (Schomerus et al., 1995). ATP, ADP, UTP, UDP, 2-MeSATP, 2-MeSADP, 2-ClATP, ADPβS, adenosine, UDP-glucose, α,β-meATP, β,γ-meATP, BzATP, suramin, PPADS, thapsigargin (all from Sigma) and MRS2179 (from Sigma or Tocris, Bristol, UK) were dissolved in prewarmed (37°C) Ca^{2+}-containing or Ca^{2+}-free buffer A and applied at the concentrations indicated. Inhibitors were applied 8-10 minutes before agonist application. Data are presented as 340 to 380 emission ratios or as approximate Ca^{2+} concentrations (nM). In the case of emission ratios, sequential measurements taken for several nucleotides from the identical neurosphere were normalized (normally to ATP=100%).

Nucleotide hydrolysis

Neurospheres were washed twice with phosphate-free physiological saline [in mM: 140 NaCl, 5 KCl, 10 HEPES, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose (pH 7.25)]. Ecto-nucleotidase activity was determined by measuring the formation of P_i liberated from nucleotides, as previously described for cultured CHO cells (Heine et al., 1999). Viable neurospheres were incubated at 37°C in phosphate-free physiological saline containing 1 mM ATP, ADP, AMP, *p*-nitrophenyl phosphate (PNPP, substrate of alkaline phosphatase) or *p*-nitrophenyl thymidine monophosphate (PNP-TMP, substrate of ecto-nucleotide pyrophosphatase/phosphodiesterases) (Bollen et al., 2000). Hydrolysis rates were constant for each experimental condition. At the end of the enzyme reaction, it was ensured that less than 10% of the initial substrate was hydrolyzed. To block alkaline phosphatase activity, neurospheres were preincubated for 15 minutes with 1 mM levamisole (Sigma) in phosphate-free physiological saline before incubation with the substrate solution containing the same concentration of levamisole.

Immunocytochemistry

Neurospheres were washed with PBS, fixed with 2% paraformaldehyde (PFA) and washed in PBS containing 0.1% Triton-X100. Non-specific binding was blocked with 5% bovine serum albumin (BSA) in PBS. Primary antibodies were diluted in PBS containing 1% BSA. NTPDase2 and tissue non-specific alkaline phosphatase (TNAP) were detected using polyclonal antibodies against murine NTPDase2 (Braun et al., 2003; Shukla et al., 2005) and rat TNAP (Miki et al., 1986), respectively. Monoclonal antibodies were used for the detection of GFAP, S100β (both from Sigma), βIII tubulin (BabCo, Berkeley, USA), nestin and O4 (both Chemicon, Hampshire, UK). Appropriate secondary antibodies were applied for immunodetection. For TNAP immunolabeling, neurospheres were fixed with 4% PFA, washed in PBS followed by application of a blocking buffer consisting of 1% donkey serum, 50 mM glycine, 0.1% gelatine, 0.1% BSA in PBS. Neurospheres were examined using a Zeiss Axiophot microscope. Images were processed using Adobe Photoshop.

RT-PCR analysis

Total RNA of 7-day-old secondary neurospheres was isolated using a Potter homogenizer and a Qiashredder (Qiagen, Hilden, Germany) for homogenization of the cells and the RNeasy Mini Kit (Qiagen) for RNA isolation as previously described (Vollmayer et al., 2001). To avoid DNA contamination, a digestion with RNase-free DNase I (Qiagen) was performed during the purification protocol. Total RNA and genomic DNA were isolated with Trizol LS reagent (Invitrogen) according to the manufacturer's instructions from mouse brains excised from animals anaesthetized with CO_2. The cDNA species were synthesized with Omniscript (Qiagen) with a random octamer primer. PCR was performed employing the primers given in Table 1 and Taq DNA polymerase (Eppendorf, Hamburg, Germany). A touch-down PCR protocol was performed starting with an annealing temperature that was increased by 4°C and reduced by 0.4°C per cycle for the first 10 cycles until the annealing temperature indicated in Table 1 was obtained. Thirty-five further cycles were performed with the final annealing temperature. The PCR reaction was analyzed on agarose gels. A primer pair for the house keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a positive control. To exclude contamination with genomic DNA, a primer pair for the immunoglobulin heavy chain binding protein (BiP) was used. The binding sites of these primers were separated by an intron in the BiP gene. In all cases, the identity of the amplified PCR fragments was confirmed by DNA sequencing.

In situ hybridization

A mouse $P2Y_1$ cRNA probe was prepared by cloning a 319 bp fragment of the $P2Y_1$ PCR product (Table 1) into *Eco*RV-digested pBluescript SKII (+) (Stratagene, La Jolla, California, USA) by blunt-end ligation. Antisense and sense RNA probes were prepared using T7 and T3-RNA-Polymerase (MBI Fermentas, St Leon-Rot, Germany). Digoxigenin (DIG) labeling was carried out with DIG RNA labeling mix (Roche, Mannheim, Germany) according to the manufacturer's protocol. Hybridization was carried out on snap-frozen tissue sections deposited on 2% 3-aminopropyl triethoxysilane-coated slides. Sections were fixed with 4% paraformaldehyde and acetylated with 0.1 M triethanloamine (TEA) buffer (pH 8.0), containing 0.25% (v/v) acetic anhydride. Hybridization was carried out following prehybridization of sections for 60 minutes at 42°C in 100 μl prehybridization buffer containing 40% deionized formamide (Boehringer Mannheim, Germany), 1× Denhardt's solution, 4× SSC, 10 mM DTT (Sigma), 1 mg/ml yeast t-RNA (Sigma) and 1 mg/ml denatured and sheared salmon sperm DNA (Invitrogen). Each slide was covered with 50 μl of hybridization buffer containing prehybridization buffer with 10% dextran sulfate (Sigma) and 20-200 ng/ml of DIG-labeled cRNA probe and incubated for 16 hours at 42°C. Following a series of high stringency washes, hybridization was visualized by immunohistochemistry using an alkaline phosphatase-conjugated anti-digoxigenin antibody and precipitating alkaline phosphatase reaction product (naphthol-AS-BI-phosphate, Roche) according to the manufacturer's instructions.

Table 1. Details of primers used in RT-PCR

Protein	Primers (5′→3′)	Product length (bp)	T_m (°C)	GenBank Accession Number
$P2Y_1$	CCTGCGAAGTTATTTCATCTA GTTGAGACTTGCTAGACCTCT	319	55	AK036611
$P2Y_2$	TCTGCTTCCTGCCTTTCCAC TCCGTCTTGAGTCGTCACTG	309	56	NM_008773
$P2Y_4$	AGCCCAAGTTCTGGAGATGGTG GGTGGTTCCATTGGCATTGG	492	56	NM_020621
$P2Y_6$	ATCAGCTTCCTGCCTTTCC CTGTGAGCCTCTGTAAGAGATCG	214	56	NM_183168
BiP	ACACTTGGTATTGAAACTG GTGGCTTTCCAGCCATTC	560	52	NM_022310
GAPDH	AACGACCCCTTCATTGACC GTAGACTCCACGACATACTCAGC	197	55	NM_008084
NTPDase2	AAGACAGATATGCCAGCACTCC GATATTGAAGAGCCCAGAGACG	635	62	NM_009849
TNAP	AAGGGCCAGCTACACCACAACACG GCCCACGGACTTCCCAGCATC	267	54	NM_007431

Immunoblotting

For western blot analysis, neurospheres were suspended in 50 mM HEPES (pH 7.4) containing a mixture of protease inhibitors (in μg/ml: 2 chymostatin, 2 aprotinin, 1 pepstatin, 150 benzamidine, 2 antipain, and 2 leupeptin) and 0.5% Triton X-100, and homogenized using an Ultrathurax homogenizer (IKA-Werk, Staufen, Germany). Concentrated sample buffer with (TNAP) or without (NTPDase2) reducing agent was added and polyacrylamide gel electrophoresis was carried out on minigels (10% acrylamide). Immunoblotting using the polyclonal anti-NTPDase2 antibody (dilution 1:2000) and the anti-TNAP antibody (dilution 1:500) was performed using an enhanced chemiluminescence system (Amersham Biosciences, Freiburg, Germany).

Cell proliferation

Neurospheres were dissociated with accutase II at 37°C for 45 minutes after 7 days in culture. Equal numbers of cells (5000 cells/well) were seeded into uncoated 24-well plates and cultured with growth medium (1 ml) containing 5 ng/ml of human recombinant EGF and 2.5 ng/ml FGF-2, unless otherwise stated. The P2 receptor agonists ADPβS, UTP, UDP, α,β-meATP, BzATP (50 μM each, all from Sigma) and adenosine (1-50 μM), and the $P2Y_1$ receptor antagonist MRS2179 (50 μM, Sigma) were added daily. Carrier solution was added as a control. After 4 days in culture, neurospheres were centrifuged at 260 g_{av} for 6 minutes, treated with accutase II at 37°C for 45 minutes and mechanically dissociated. The cell number was counted using a hemocytometer. All experiments were performed in triplicate.

Cell differentiation

To study their differentiation potential, secondary neurospheres were dissociated with accutase after 7 days. Cells were seeded (5000 cells/coverslip) onto poly L-ornithine-coated (0.25 mg/ml) glass coverslips in growth factor-free culture medium containing fetal bovine serum (2%). After 8 days, cells were rinsed with PBS (pH 7.4) and fixed for 30 minutes with 2% paraformaldehyde in PBS. Immunocytochemistry was performed as described above with primary antibodies against GFAP, βIII tubulin or O4 (Chemicon), to detect astrocytes, early neurons and oligodendrocytes, respectively. DAPI (1 μg/ml) was added to the appropriate secondary FITC-coupled antibodies. On each coverslip, the total number of DAPI-labeled nuclei was counted and related to the total number of cells immunostained for the respective antibody.

RESULTS

Neurospheres express ecto-nucleotidases

Neurospheres derived from adult tissue microdissected from the lateral wall of the lateral ventricles were cultured in the presence of DMEM/F12 media and EGF (20 ng/ml) and FGF2 (10 ng/ml). They were dissociated after 7 days and expanded under the same experimental conditions for another 7 days to form secondary neurospheres. After 7 days, all secondary neurospheres abundantly expressed the type B cell proteins nestin, GFAP and S100β (Fig. 1A-C). In addition, individual neurospheres contained few cells with extended processes that were immunopositive for the early neuron marker βIII tubulin (Fig. 1D), suggesting low onset of cell differentiation. We also verified the potential of neurospheres cells to differentiate. Seven-day-old secondary neurospheres were dissociated and cultured for 8 days in the absence of growth factors in fetal bovine serum-containing medium. When compared with the total of DAPI stained cells, 42% of the cells were GFAP-expressing protoplasmic astrocytes, 22% were βIII tubulin-positive early neurons with multiple processes and 21% were O4-expressing oligodendrocytes (8715 cells analyzed), revealing their multipotent potential (Reynolds and Weiss, 1992).

As type B cells in situ highly express the ecto-nucleotidase NTPDase2 (Braun et al., 2003), we first analyzed the ecto-nucleotidase activity of viable neurospheres. Neurospheres hydrolyzed externally added ATP, but also ADP and AMP (Fig. 1G). As NTPDase2 has a high preference for the hydrolysis of ATP (Zimmermann, 2001), this suggested that either NTPDase2 was not present in neurospheres or that another ecto-nucleotidase predominated in the nucleotide hydrolysis. As embryonic stem cells express alkaline phosphatase (Annerén et al., 2004), an enzyme that can dephosphorylate all three nucleotides, we probed for the presence of this enzyme in the adult neurospheres. Neurospheres hydrolyzed the alkaline phosphatase-specific substrate *p*-nitrophenyl phosphate (PNPP). Of the four isoforms of alkaline phosphatase, only the tissue non-specific form (TNAP) is effectively inhibited by 1 mM levamisole (Kozlenkov et al., 2004). Levamisole inhibited ATP hydrolysis by 82%. This suggests that NTPDase2 would contribute less than 20% to the total ATP hydrolysis. No hydrolysis was observed of *p*-nitrophenyl thymidine monophosphate (PNP-TMP), a substrate of members of the ecto-nucleotide pyrophosphatase/phosphodiesterase family (NPP1 to NPP3) (Bollen et al., 2000).

Using immunocytochemistry, RT-PCR and western blotting, expression of both NTPDase2 and TNAP was demonstrated in neurospheres (Fig. 1E,F,H,I). The protein bands of 70 kDa and

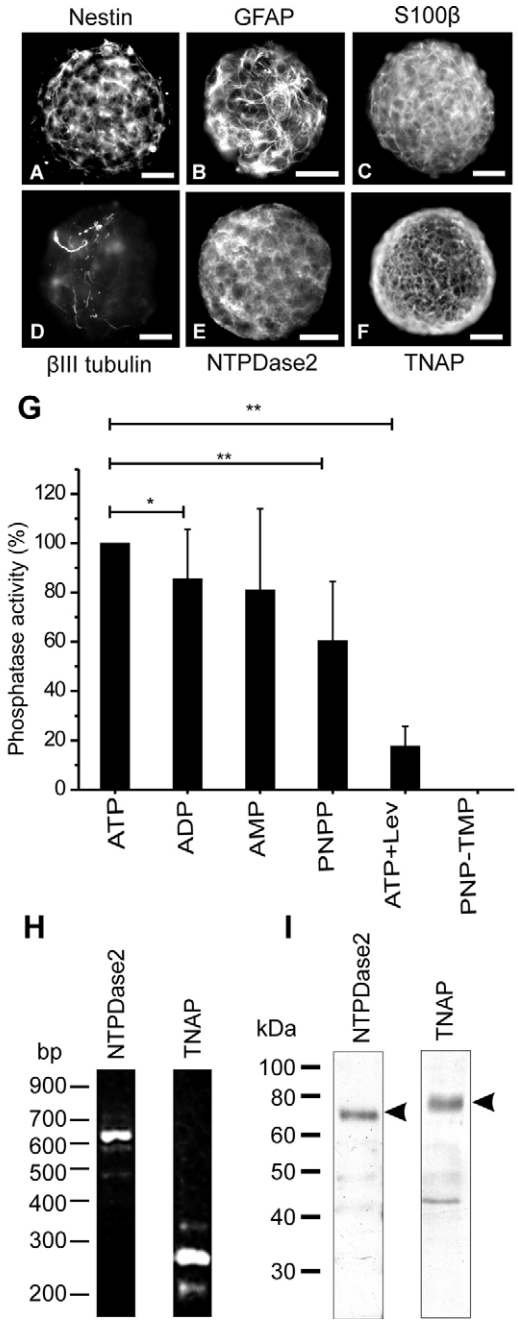

Fig. 1. Characterization of neurospheres.
(**A-C**) Immunocytochemistry identified nestin (A), GFAP (B), S-100β (C), βIII tubulin (**D**), and the ecto-nucleotidases NTPDase2 (**E**) and TNAP (**F**) in all neurospheres investigated (representative images). Scale bars: 50 μm. (**G**) Analysis of ecto-phosphatase activity. Viable neurospheres were incubated in the presence 1 mM ATP, ADP, AMP, PNPP or PNP-TMP. To determine the contribution of TNAP activity, ATP hydrolysis by neurospheres was analyzed in the presence of 1 mM levamisole. No phosphodiesterase activity (PNP-TMP hydrolysis) was observed. Phosphatase activities were normalized to the activity obtained with ATP as a substrate. The 100% value corresponds to 3.2±1.1 nmoles Pi/min/100 neurospheres (mean±s.d., n=9-12, *P<0.05, **P<0.01). (**H**) RT-PCR products revealing the presence of mRNAs in neurosphere extracts encoding NTPDase2 and TNAP. (**I**) Immunoblot using total neurosphere protein (5 μg/lane) detecting protein bands of 70 and 80 kDa (arrowheads) corresponding to NTPDase2 and TNAP, respectively.

80 kDa obtained in western blots for NTPDase2 and TNAP, respectively, corresponded to the values expected for the two enzymes (Zimmermann, 2001). No immunosignals were obtained in western blots using antibodies against the related ecto-nucleotidases NTPDase1 and NTPDase3 and against ecto-5´-nucleotidase (not shown). Taken together, these data demonstrate that neurospheres express surface-located ecto-nucleotidase activity whereby the predominant enzyme is TNAP.

Calcium transients evoked by identified nucleotide receptors

To probe for the presence of functional P2 receptors, we performed Ca^{2+} imaging of fura-2-loaded neurospheres. Neurospheres responded with rapid Ca^{2+} transients to the application of nucleotides (Fig. 2A). ATP and ADP evoked equal responses, UTP was less potent (28%) and UDP was essentially ineffective (Fig. 2B). After application of ATP, the absolute Ca^{2+} concentration amounted to 213 nM. The responses to ATP, ADP and UTP were dose dependent and maximal activation was obtained at a concentration of 50 μM (Fig. 2C). This nucleotide concentration was used in future experiments to activate neurospheres.

Of the murine phospholipase C-coupled P2 receptors, $P2Y_1$ receptors are activated by ATP and ADP (Vöhringer et al., 2000), $P2Y_2$ and $P2Y_4$ receptors by UTP and ATP, and $P2Y_6$ receptors by UDP (Wildman et al., 2003). $P2Y_{11}$ receptors are not apparent within the murine genome (Boeynaems et al., 2005). The response pattern obtained in Fig. 2 would thus be compatible with a contribution of P2X, $P2Y_1$, $P2Y_2$ or $P2Y_4$ receptors. In order to identify the receptor subtypes involved in Ca^{2+} signaling, we applied additional P2 receptor agonists (Fig. 3A). Comparable Ca^{2+} responses were evoked by the $P2Y_1$ receptors agonists 2-MeSATP, ADPβS, 2-ClATP and 2-MeSADP. By contrast, the P2X receptor agonists α,β-meATP ($P2X_1$, $P2X_3$), β,γ-meATP ($P2X_3$), BzATP ($P2X_7$) and the $P2Y_{14}$ receptor agonist UDP-glucose and adenosine yielded marginal responses. If P2X receptors were involved in raising cytosolic Ca^{2+}, omission of extracellular Ca^{2+} should reduce the amplitude of the Ca^{2+} signal. This was not the case (Fig. 3B). In accordance with the involvement of phospholipase C-coupled P2Y receptors, depletion of cytosolic Ca^{2+} stores by application of thapsigargin (5 μM), an inhibitor of the Ca^{2+} pump of the endoplasmic reticulum, strongly decreased the Ca^{2+} response to either ATP or ADP (Fig. 3C).

The involvement of P2 receptors in the nucleotide-mediated raise in cytosolic Ca^{2+} was further corroborated by the application of P2 receptor antagonists (100 μM) (Fig. 4). The $P2Y_1$-receptor-specific antagonist MRS2179 strongly reduced the response evoked by ATP or ADP (by 65% and 83%, respectively). Similarly, the non-specific P2 receptor antagonists PPADS and suramin inhibited the responses to ATP, ADP and UTP (between 75% and 95%). Application of the antagonists alone had no affect on cytosolic Ca^{2+} levels (data not shown). Furthermore, pre-application of the TNAP inhibitor levamisole (1 mM) had no effect on the rapid ATP-, ADP- or UTP-evoked Ca^{2+} signals (not shown). This suggests that this enzyme caused no significant agonist hydrolysis within the application period.

These results suggested that the $P2Y_1$ receptor had the highest impact on Ca^{2+} signaling. We therefore analyzed Ca^{2+} responses in $P2Y_1$-receptor-deficient mice. $P2Y_1$-deficient mice are viable with no apparent abnormalities affecting their development, survival or reproduction. They reveal, however, deficits in platelet aggregation (Léon et al., 1999). As the knockout mice had a different genetic background (C57BL/6J), we analyzed wild-type mice of this strain

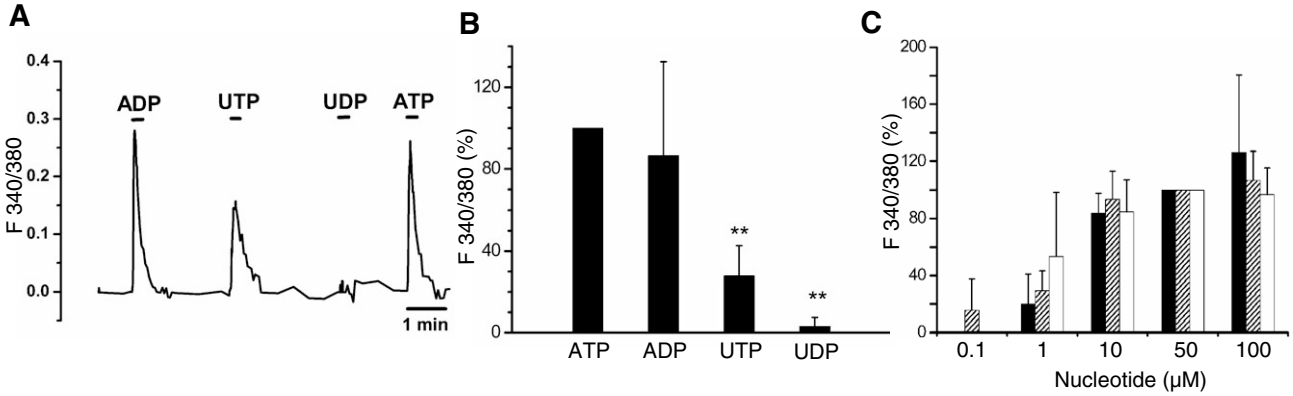

Fig. 2. Neurospheres express functional P2 receptors. (**A**) Neurospheres were loaded with the Ca^{2+} indicator fura-2 AM and stimulated with the P2 receptor agonists ATP, ADP, UTP and UDP (50 μM each). Representative plot of rapid and transient cytosolic Ca^{2+} elevations recorded from a single neurosphere, evoked by successive application (horizontal bars) of nucleotides. (**B**) Responses to ATP, ADP, UTP and UDP evoked in identical neurospheres, normalized to the ATP response (100%) (mean±s.d., n=14, $^{**}P$<0.01). (**C**) Dose dependence of the neurosphere response. Ca^{2+} responses to increasing concentrations of either ATP (black), ADP (hatched) or UTP (white) were successively recorded from single neurospheres. The response to 50 μM nucleotide was set to 100% (mean±s.d., n=3-9).

as controls (Fig. 5). Neurospheres isolated from C57BL/6J wild-type mice revealed the same pattern of evoked Ca^{2+} responses as neurospheres from the initially investigated C57BL/6N mice (ATP=ADP>UTP>>>UDP) (comp. Fig. 2B). The UTP- and ATP-activated $P2Y_2$/$P2Y_4$ receptor should have remained functional in the knockout mice. Accordingly, equal responses were obtained for ATP and UTP in neurospheres from $P2Y_1$-deficient animals. UDP and ADP yielded negligible responses, excluding a significant contribution of $P2Y_6$ receptors and demonstrating that ADP evoked its effects via the $P2Y_1$ receptor.

Identification of P2Y receptors

Using RT-PCR we identified mRNA for $P2Y_1$, $P2Y_2$ and $P2Y_6$ receptors but not for $P2Y_4$ receptors (Fig. 6A). Positive PCR signals for $P2Y_4$ were obtained with genomic DNA from mouse brain, suggesting that the primers employed were functional. The PCR signal for the $P2Y_6$ receptor was unexpected as UDP evoked Ca^{2+} signals close to baseline. To exclude the possibility that the mRNA sample prepared from neurospheres contained genomic DNA, we applied primers for the immunoglobulin heavy chain binding protein (BiP). The binding sites of these primers were separated by

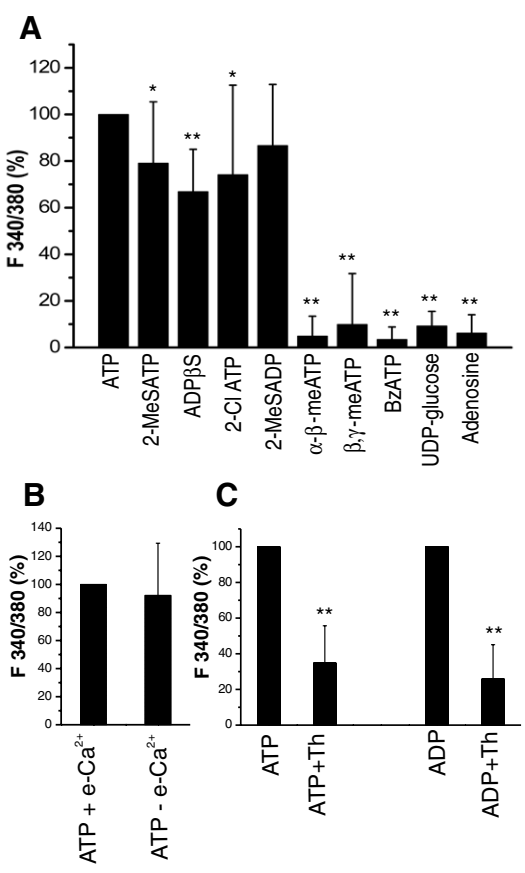

Fig. 3. Profiling of receptors responsible for cytosolic Ca^{2+} responses. (**A**) Ca^{2+} signals recorded from fura 2-loaded neurospheres evoked by a variety of P2-receptors agonists. Whereas all $P2Y_1$-receptor agonists (ATP, 2-MeSATP, ADPβS, 2-ClATP, 2-MeSADP) induced a comparable response, the P2X receptor agonists α,β-meATP, β,γ-meATP and BzATP, the $P2Y_{14}$ receptor agonist UDP-glucose, and the P1-receptor agonist adenosine were ineffective (50 μM each). Ca^{2+} signals were normalized to the ATP response (100%) obtained in the identical neurosphere. (**B**) Independence of the cytosolic Ca^{2+} response on extracellular Ca^{2+} (e-Ca^{2+}) following application of ATP (50 μM). (**C**) Dependence of the cytosolic Ca^{2+} response on intracellular Ca^{2+}. Pre-application for 10 minutes of the Ca^{2+} store blocker thapsigargin (Th, 5 μM) strongly diminished the Ca^{2+} elevations evoked by ATP or ADP (50 μM each). Responses were normalized to the ATP (or ADP) response (100%) obtained in the identical neurosphere (mean±s.d., n=5-19 (A), 27 (B) or 7-9 (C); $^{*}P$<0.05, $^{**}P$<0.01).

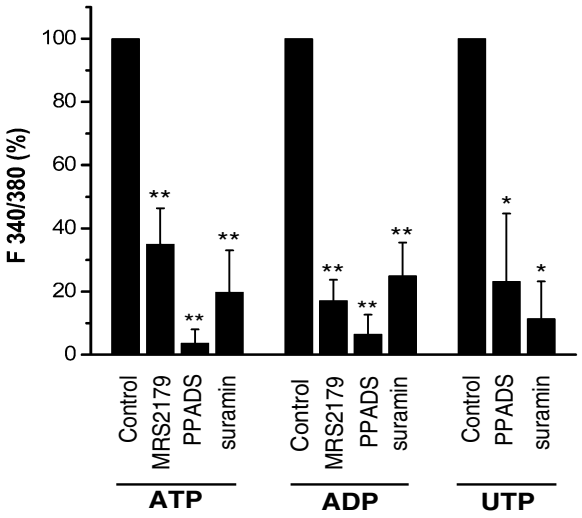

Fig. 4. P2-receptor antagonists reduce the cytosolic Ca^{2+} elevations. Inhibitors (100 μM each) were applied 8 minutes prior to agonist application. The $P2Y_1$-receptor-specific antagonist MRS2179 reduced the Ca^{2+} signal evoked by ATP or ADP (50 μM each). The non-selective P2-receptor antagonists suramin or PPADS reduced the Ca^{2+} signals evoked by either ATP, ADP or UTP (mean±s.d., n=5-8, *P<0.05, **P<0.01).

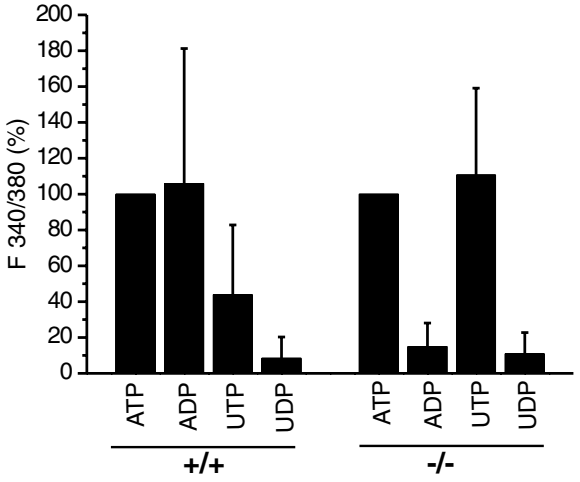

Fig. 5. P2-receptor-evoked cytosolic Ca^{2+} signals in neurospheres derived from $P2Y_1$-receptor knockout mice (C57BL/6J). The relative agonist potency in neurospheres from C57BL/6J wild-type mice corresponds to that of C57BL/6N mice (compare with Fig. 2B). In neurospheres derived from $P2Y_1$-knockout mice, the ATP and UTP responses were equal and ADP and UDP were essentially inactive (mean±s.d., n=20-38).

an intron in the BiP gene. Only the nucleotide corresponding in size to the spliced mRNA was amplified from neurosphere-isolated mRNA. By contrast, the fragment corresponding in size to the unspliced sequence was obtained from the genomic DNA sample. Taken together, these results suggest that $P2Y_1$ and $P2Y_2$ receptors were mainly responsible for the ATP, ADP and UTP-evoked Ca^{2+} transients, that the $P2Y_4$ receptor was absent, and that functional protein levels for the $P2Y_6$ receptor were negligible. In situ hybridization further demonstrated that the $P2Y_1$ receptor was expressed in the SVZ (Fig. 6B). As previously reported for the $P2Y_1$ receptor in the human brain (Moore et al., 2000), the receptor is also widely distributed in the murine brain. In addition, we identify select clusters of subependymal cells containing $P2Y_1$ receptor mRNA.

Synergistic activation of neurosphere cell proliferation by growth factors and nucleotides

We further investigated the possibility that activation of $P2Y_1$ and $P2Y_2$ receptors affects neurosphere cell proliferation. Neurospheres were dissociated after 7 days in culture and agonists or antagonists were added daily. Unless indicated otherwise, the cell number was determined after 4 days. Preliminary experiments revealed that addition of $P2Y_1$- and $P2Y_2$-receptor agonists did not significantly alter neurosphere cell proliferation at the high growth factor concentrations generally used in the culture system. We therefore reduced the concentrations of EGF and FGF2 to 5 ng/ml and 2.5 ng/ml, respectively, when replating cells previously grown at elevated concentrations. This did not significantly alter the formation of neurospheres and cell proliferation (high $3.3\pm1.2\times10^6$, reduced $2.7\pm1.1\times10^6$±s.d., cells/well, n=4). We also showed that culturing neurospheres at reduced growth factor concentrations did not change cell fate and the expression of cell markers (Fig. 7A,B). Under either experimental condition, all neurospheres analyzed after 4 days expressed nestin. No expression of βIII tubulin was observed at 4 days, indicating lack of cell differentiation during this short culture period. The daily addition of adenosine, of the $P2Y_1$-receptor agonist ADPβS, of the $P2Y_2$ receptor agonist UTP (50 μM each), or of ADPβS and UTP had no effect on the general appearance of neurospheres and the expression of nestin (Fig. 7C-F). It did not induce expression of βIII tubulin (not shown), suggesting that addition of these agonists did not change cell type.

A significant stimulatory effect on cell number was obtained on application of ADPβS or UTP (50 μM, each) at reduced growth factor concentration (Fig. 7G). Cell density was increased by 48% and 54%, respectively. This was further augmented by simultaneous addition of the two agonists (to 91%). By comparison, UDP, α,β-meATP and BzATP had no effect on cell proliferation. When applied in the absence of growth factors, ADPβS or UTP did not support survival of expanded cells and no neurospheres were formed (not shown). As extracellular ATP can be completely dephosphorylated by viable neurospheres, we also investigated the effect of adenosine on cell proliferation. At a concentration of 50 μM, adenosine essentially abrogated neurosphere development. Cell number was still decreased to 56% at 10 μM adenosine, but 1 μM adenosine increased cell number by 60%.

Evidence for endogenous receptor-mediated stimulation of cell proliferation

These results raised the possibility that nucleotides may be constitutively released from neurosphere cells and exert a synergistic P2 receptor-mediated effect on growth factor-mediated cell proliferation. We therefore compared cell proliferation in neurospheres from $P2Y_1$-receptor knockout and wild-type mice 4 days after replating. The cell number in $P2Y_1$ knockout neurospheres was significantly decreased by 53% when compared with the respective controls (Fig. 7H). In addition, we analyzed the effect of the selective and non-hydrolysable $P2Y_1$-receptor antagonist MRS 2179 (50 μM) on wild-type neurosphere cell proliferation. When applied daily within the first 4 days of replating, the cell number was reduced by 27%. In an additional series of experiments, replated cells were first grown for 4 days in medium containing 20 ng/ml EGF and 10 ng/ml FGF2 and then cultured for

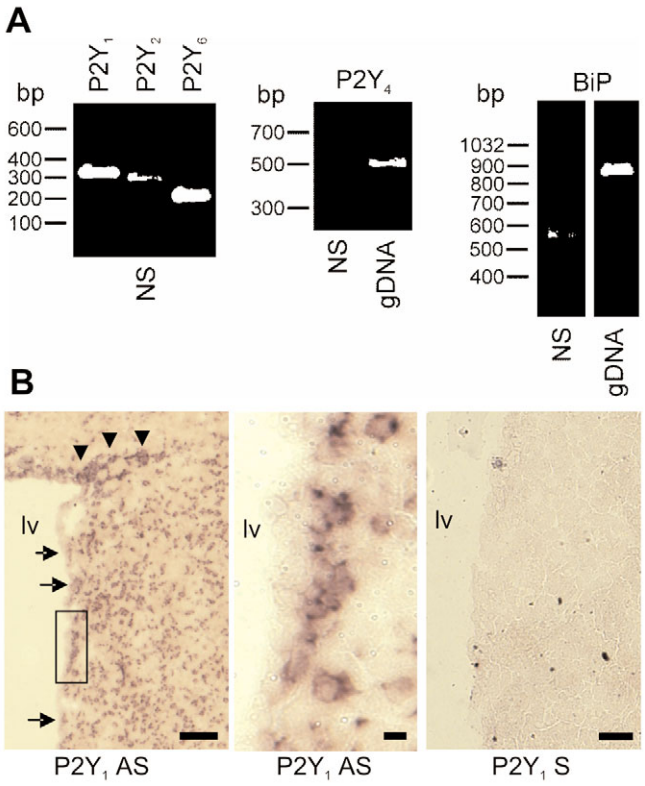

Fig. 6. P2 receptor expression. (**A**) RT-PCR. Neurospheres (NS) express mRNA for $P2Y_1$, $P2Y_2$ and $P2Y_6$ receptors, but not for the $P2Y_4$ receptor. A $P2Y_4$ PCR product could, however, be amplified from a mouse brain genomic DNA preparation (gDNA). To exclude contamination of the mRNA preparation with genomic DNA, a primer pair for the immunoglobulin heavy chain binding protein (BiP) was used. Using the mRNA preparation from the neurospheres (NS), a 560 bp sequence was amplified. With mouse brain genomic DNA (gDNA), a 876 bp PCR product was amplified that contained a 316 bp long intron sequence. (**B**) In situ hybridization. Left: Using a 319 bp fragment of the $P2Y_1$ receptor as an antisense probe, mRNA was detected in large cell bodies of the striatum and in select clusters of subependymal cells (arrows) of the lateral wall of the lateral ventricle (lv), as well as in the dorsolateral triangular area of the SVZ (arrowheads), where neuroblasts enter the RMS. A cluster of labeled subependymal cells boxed in the left image is enlarged in the middle panel. Right: Lack of staining for the corresponding sense probe. Scale bars: 50 μm in the left and right panels; 10 μm in the middle panel.

another 3 days in the presence of 5 ng/ml EGF and 2.5 ng/ml FGF2. Simultaneous addition of MRS2179 (100 μM) resulted in a reduction of cell proliferation by 35%. At present, no specific inhibitors are available for $P2Y_2$ receptors. These results suggest that constitutively released ATP may enhance EGF and FGF2-induced neurosphere cell proliferation via activation of $P2Y_1$ receptors.

To further corroborate the functional role of $P2Y_1$ receptors, and to verify the role for $P2Y_2$ receptors in neurosphere cell proliferation, we compared the effect on cell proliferation of ADPβS, UTP and their combined application (50 μM each) between adult wild-type and $P2Y_1$-receptor knockout mice (Fig. 7I). In neurospheres from C57BL/6J wild-type mice, the agonists stimulated cell proliferation as for C57BL/6N wild-type mice (comp. Fig. 7G). By contrast, no increase in cell number was obtained after application of ADPβS to neurospheres from $P2Y_1$-deficient mice. However, cell number was increased by UTP, compatible with the remaining expression of $P2Y_2$ receptors. In contrast to experiments with wild-type neurospheres, this effect was not further increased by co-application of UTP and ADPβS.

DISCUSSION

We demonstrate here that neurospheres cultured from the adult SVZ in the presence of EGF and FGF2 express functional nucleotide receptors as well as enzymes for the degradation of nucleoside triphosphates to the respective nucleoside. Nucleotides evoke rapid Ca^{2+} transients and augment cell proliferation in the presence of EGF and FGF2. We identify the responsible nucleotide receptors as $P2Y_1$ and $P2Y_2$. A $P2Y_1$-receptor antagonist and lack of the receptor in $P2Y_1$ knockout mice reduce both Ca^{2+} transients and cell proliferation.

Neurospheres cultured from the adult SVZ expressed two different ecto-nucleotidases. NTPDase2, previously identified on type B-cells of the adult SVZ in situ (Braun et al., 2003), preferentially hydrolyses nucleoside triphosphates. TNAP, one of the four members of the alkaline phosphatase protein family hydrolyzes extracellular nucleoside tri-, di- and monophosphates, and thus generates extracellular nucleosides (Zimmermann, 2001). TNAP is not associated with type B cells in situ (H.Z., unpublished), but it is generally associated with cultured embryonic stem cells and widely used as a marker for undifferentiated embryonic stem cells (Annerén et al., 2004). Based on our data with neurospheres from adult neuronal stem cells, we hypothesize that purinergic signaling pathways may also be employed by embryonic stem cells.

We further demonstrate that neurospheres derived from the adult rodent SVZ express functional $P2Y_1$ and $P2Y_2$ receptors. Interestingly, the Ca^{2+} signal evoked via the $P2Y_1$ receptor was considerably stronger than that evoked via the $P2Y_2$ receptor. This may result from differences in receptor abundance or from a different coupling efficiency to intracellular signaling pathways. UDP ($P2Y_6$) did not elicit a significant Ca^{2+} signal, even though the encoding mRNA was detected. The dominating role of the $P2Y_1$ receptor in neurosphere Ca^{2+} signaling was corroborated by the inhibitory effect of the $P2Y_1$-receptor antagonist MRS2179 and the lack of the agonistic effect of ADP in $P2Y_1$-receptor knockout mice.

Neurospheres from the adult SVZ cultured in the presence of EGF and FGF2 revealed augmented cell proliferation when $P2Y_1$- or $P2Y_2$-receptor agonists were co-applied. Inversely, the $P2Y_1$-receptor antagonist MRS2179 reduced cell proliferation and cell numbers were reduced in neurospheres cultured from $P2Y_1$-knockout mice, implying that the synergistic effect of $P2Y_1$-receptor activation on growth factor-mediated cell proliferation was abrogated. $P2Y_1$ and $P2Y_2$ receptors are broadly distributed in the adult brain (Burnstock and Knight, 2004). The quality of the antibodies presently available hampers a reliable allocation of the receptors in the murine SVZ in situ and their relation to the known in situ ecto-nucleotidase distribution (Braun et al., 2003). The presence of mRNA encoding the $P2Y_1$ receptor in select clusters of SVZ cells in situ suggests, however, its involvement in adult neurogenesis.

Constitutive release of nucleotides has been described for many cellular systems and apparently represents a release mechanism additional to the stimulus-evoked and regulated release pathway (Schwiebert et al., 2003). Because even slight mechanical distortion results in the cellular release of ATP (Schwiebert et al., 2003), we have not analyzed ATP release from neurospheres. ATP may be constitutively released inside cultured neurospheres and enhance cell proliferation via $P2Y_1$ receptors in an immediate autocrine or paracrine manner. Adenosine revealed a differential effect. At low

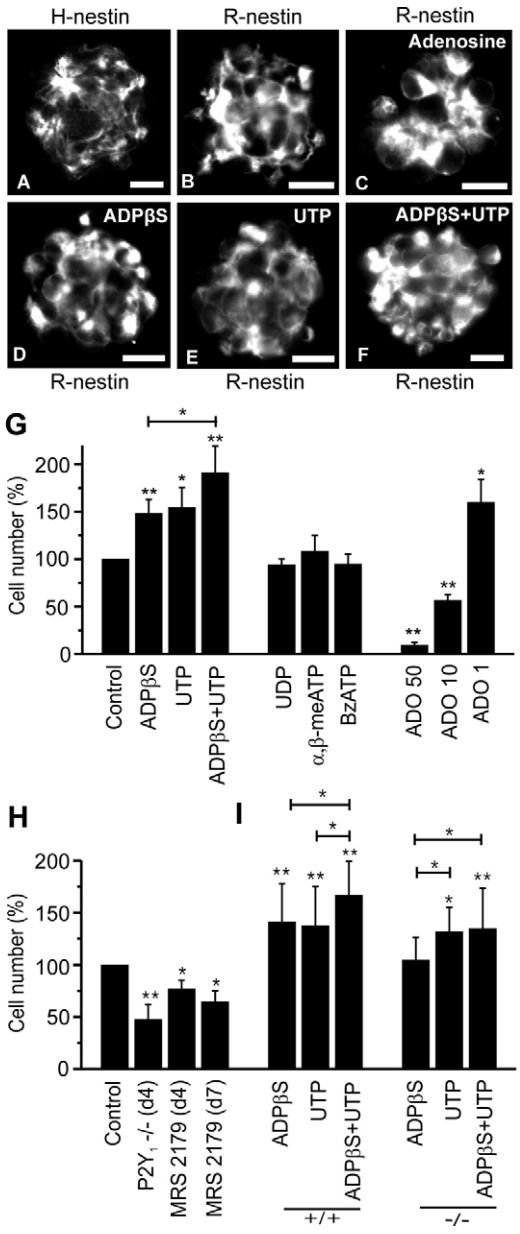

Fig. 7. Modulation of cell proliferation by agonists of P2Y and P1 receptors. (**A**,**B**) Immunocytochemistry (representative images). Neurospheres from C57BL/6N mice were dissociated and expanded in the presence of either 20 ng/ml EGF and 10 ng/ml FGF2 (A) or 5 ng/ml EGF and 2.5 ng/ml FGF2 (B). Equal expression of nestin was observed in 4-day-old neurospheres whether cultured at high (A, H-nestin) or reduced (B, R-nestin) growth factor concentrations. (**C-F**) The daily addition to reseeded cells of adenosine (C), the $P2Y_1$-receptor agonist ADPβS (D), the $P2Y_2$ receptor agonist UTP (E) or of ADPβS and UTP (F) (50 μM each) had no effect on nestin expression. Scale bars: 10 μm. (**G**) Agonist-dependent effect on cell proliferation after daily application of ADPβS+UTP, ADPβS, UTP, UDP, α,βmeATP, BzATP (50 μM each) or adenosine (ADO) (1 μM to 50 μM) to cells dissociated from neurospheres and expanded in the presence of reduced growth factor concentrations. The cell number was determined after 4 days. (**H**) Cell proliferation in neurospheres derived from wild-type (+/+) and $P2Y_1$-receptor knockout (–/–) mice. First, the cell number obtained after 4 days was compared between wild-type (control, 100%) and $P2Y_1$-knockout mice (–/–) (both C57BL/6J). In addition, the effect of the $P2Y_1$-receptor antagonist MRS2179 (100 μM) on neurospheres derived from wild-type mice was analyzed under two different experimental conditions. In one case [MRS2179(d4)], the antagonist was added immediately after reseeding and then daily for 4 days. In the other case [MRS2179(d7)], neurospheres were first cultured for 4 days in the presence of high growth factor concentrations, washed and reseeded at reduced growth factor concentrations. A single dose of MRS2179 (100 μM) was then applied and the cell number was determined at 7 days. (**I**) Comparison of the proliferative effect of P2-receptor agonists on neurosphere cells from wild-type (+/+) and $P2Y_1$-receptor knockout (–/–) mice. Processing of cells was as for G. Values in G-I represent mean±s.d., n=3-7, $*P<0.05$, $**P<0.01$. The average cell number derived from a single well in the control experiments (100%) amounted to $4.0\pm1.2\times10^6$ (s.d.) for G, to $2.8\pm1.3\times10^6$ (s.d.) for H, and to $3.3\pm1.3\times10^6$ (s.d.) for I.

concentrations (1 μM), adenosine increased cell number to a similar extent as P2Y-receptor agonists. A receptor-mediated proliferative effect of adenosine receptor agonists on cultured astrocytes has previously been described (Ciccarelli et al., 1994). At high concentrations, adenosine dramatically reduced cell number, compatible with the previously observed apoptosis-inducing effect of the nucleoside in astrocytes at concentrations of 10 μM and higher (Di Iorio et al., 2002). We have not identified the mechanism by which adenosine stimulates cell proliferation. The presence of the ecto-nucleotidase pathway leading from ATP to adenosine could invoke parallel and synergistic ATP- and adenosine-mediated effects on neurosphere cell proliferation.

In situ, nucleotides could be released from any cell type associated with the neurogenic pathway and co-activate EGF-expressing progenitors, which have been identified on type C and to a small extent on type B cells (Doetsch et al., 2002). The tight association of type C cells, the ensheathing type B cells and the migrating type A cells would allow for intense paracrine interaction. But nucleotide release may also be triggered from nerve terminals entering the SVZ (Höglinger et al., 2004). Furthermore, nucleotides may play a significant role in the activation of neural progenitors following central lesions, including stroke, that result in the release of cellular nucleotides (Zhang et al., 2004). Ecto-nucleotidases can selectively modulate the effective agonist concentration at P2Y receptors on identical or also on neighboring cells, either by degrading ATP/UTP or by generating ADP/UDP (Alvarado-Castillo et al., 2005; Jhandier et al., 2005). The strong expression of NTPDase2 in type B cells in situ suggests that the effective concentration of ATP/UTP is reduced, whereas that of ADP/UDP is increased in the immediate environment of these progenitors.

Purinergic signaling pathways presumably are also involved in embryonic neurogenesis. $P2Y_1$-receptor-mediated Ca^{2+} transients could be evoked in neurospheres from the embryonic mouse striatum (E14) (Scemes et al., 2003). ATP induced an elevation of cytosolic Ca^{2+} levels and proliferation of precursor cells cultured from immortalized human stem cells derived from the embryonic

telencephalon as well as from mouse embryonic neurospheres (Ryu et al., 2003; Tran et al., 2004). A functional role of nucleotides in embryonic neurogenesis is further corroborated by the observation that Ca^{2+} waves through radial glial cells in slices of the embryonic rat ventricular zone are mediated by $P2Y_1$ receptors. Disrupting Ca^{2+} waves between these embryonic neuronal progenitors reduced ventricular zone cell proliferation during the peak of embryonic neurogenesis (Weissman et al., 2004). In the embryonic chicken retina, ATP spontaneously released via gap junction hemichannels from the pigment epithelium, speeds division of neural retinal progenitor cells (Pearson et al., 2005).

Previous studies have unraveled intracellular pathways that would allow for an interaction of P2Y receptors and growth factor-receptor-mediated signaling cascades. In a variety of cell types, nucleotides exert a synergistic effect on cell proliferation together with growth factors, chemokines or cytokines (Huang et al., 1989; Neary et al., 1994; Lemoli et al., 2004), by parallel activation of the MAP kinase pathway and/or by transactivation of growth factor receptors (Neary and Zhu, 1994; Lenz et al., 2000). In astrocytes, extracellular signal-regulated protein kinases (ERK) can be activated via $P2Y_1$, $P2Y_2$ or $P2Y_4$ receptors (King et al., 1996; Neary et al., 1999; Lenz et al., 2000; Neary et al., 2003), as well as by P1 purinoceptor agonists (Neary et al., 1998). Interestingly, the $P2Y_2$ receptor contains two SH3-binding domains that activate Src and thus transactivate receptor tyrosine kinases, including the EGF receptor (Liu et al., 2004).

Nucleotides and nucleosides may exert more complex effects than stimulation of cell proliferation. Both nucleotides and nucleosides were found to induce differentiation in cultured neuronal cells (D'Ambrosi et al., 2001; Canals et al., 2005). The P1 purinoceptor agonist 2-chloroadenosine enhanced neurite outgrowth of cultured myenteric neurons synergistically with FGF2 (Schafer et al., 1995). By contrast, ATP, via P2X receptors, induced a reduction in neurite outgrowth from rat neural tube explant cultures (Cheung et al., 2005). No neuronal differentiation was observed by application of P2 or P1 receptor agonists under the present experimental conditions.

Taken together, our evidence supports the notion that nucleotides and nucleosides contribute to the neurogenesis in the adult SVZ. Endogenously released ATP and UTP may augment the effect of growth factors on cell proliferation via $P2Y_1$- and $P2Y_2$-receptor activation.

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