Coordinate pathways for nucleotide and EGF signaling in cultured adult neural progenitor cells

Ivette Grimm1, Nanette Messem1, Matthias Stanke1, Christian Gachet2 and Herbert Zimmermann1,*

1Institute of Cell Biology and Neuroscience, Biocenter, J. W. Goethe-University, 60438 Frankfurt, Germany
2INSERM U.311, EFS-Alsace, 67065 Strasbourg, France
*Author for correspondence (e-mail: h.zimmermann@bio.uni-frankfurt.de)

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Summary
The adult subventricular zone (SVZ) contains astrocyte-like stem cells capable of generating new neurons for the olfactory bulb. Adult neurogenesis is driven by a variety of signal systems that can induce synergistic or opposing cellular responses. It is therefore important to gain insight into the underlying downstream signaling pathways. We have previously shown that the nucleotides ADPβS and UTP induce rapid Ca2+ transients in cultured SVZ-derived adult neural progenitors and augment growth-factor-mediated progenitor cell proliferation. Here, we investigated signaling pathways elicited by ADPβS, UTP and epidermal growth factor (EGF). All three agonists elicit ERK1/2 and CREB phosphorylation but the temporal characteristics differ between the nucleotides and EGF. Differentiation of the progenitors alters the receptor profile. Oligodendrocytes and young neurons, but not astrocytes, lose responsiveness to the agonists. Inhibition experiments are indicative of an ADPβS-elicited EGF receptor transactivation. Whereas UTP acts via the P2Y2 receptor, ADPβS exerts its function via the P2Y1 receptor and the P2Y13 receptor. Our data demonstrate that nucleotides and EGF induce converging, but also differential, intracellular signaling pathways and suggest that they carry the potential to act synergistically in the control of cell proliferation and cell survival in adult neurogenesis.

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Key words: ADPβS, Epidermal growth factor, Nucleotide, Neurogenesis, UTP

Introduction
New neurons continue to be formed in the adult mammalian nervous system in specific neurogenic niches, the subventricular zone (SVZ; subependymal layer SEL [Boulder Committee, 1970]) of the lateral wall of the lateral ventricles and the dentate gyrus of the hippocampus (Abrous et al., 2005). The adult SVZ contains astrocyte-like stem cells that generate neuronal precursors (neuroblasts) migrating as a network of tightly associated chains towards the olfactory bulb (OB). There, they undergo apoptosis or differentiate into interneurons (Menezes et al., 1995; Doetsch and Alvarez-Buylla, 1996; Peretto et al., 1997; Doetsch et al., 1999; Garcia et al., 2004). The molecular cues initiating the cell cycle in these multipotent stem cells, driving proliferation of intermediate precursors, directing migration of neuroblasts towards the olfactory bulb in an undifferentiated form, or finally inducing neuronal differentiation, functional integration and survival are poorly understood. Whereas the identification of transcription factors typical for the stage-specific progression has been a topic of key interest for many years (Gangemi et al., 2004), the potential multiplicity of relevant intercellular signaling pathways and the fine tuning of epigenetic gene regulation is still largely undefined (Hsieh and Gage, 2004). Neurogenesis was shown to be affected by a considerable variety of molecules, including growth factors, cell adhesion molecules, constituents of the extracellular matrix, neurotransmitter substances and other extrinsic signal substances (Riquelme et al., 2008; Zhao et al., 2008).

There is increasing evidence that nucleotides can play a role in the control of both adult and embryonic neurogenesis (Zimmermann, 2006; Neary and Zimmermann, 2009). Nucleotides such as ATP, ADP, UTP or UDP act as extracellular signal molecules via specific surface-located receptors [P2X ionotropic or P2Y metabotropic receptors (Burnstock, 2007)]. We have previously shown that neural progenitor cells cultured as neurospheres from the adult mouse SVZ express functional P2Y1 and P2Y2 receptors and that the two respective receptor agonists, ADPβS and UTP, activate rapid intracellular Ca2+ transients. In addition, these ligands augmented growth-factor-mediated (epidermal growth factor, EGF; fibroblast growth factor 2, FGF2) cell proliferation, implying overlap in the downstream mechanisms controlling the cell cycle. Studies using P2 receptor inhibitors and analysis of P2Y1 receptor knockout mice further suggested that ATP was constitutively released from neurosphere cells (Mishra et al., 2006). Recently, spontaneous ATP release from neurospheres was directly demonstrated (Lin et al., 2007).

In the developing retina, ATP was found to be essential for the coordinate proliferation and migration of neural precursors (Martins and Pearson, 2008). ATP released from the retinal pigment epithelium evokes Ca2+ transients in progenitor cells via the activation of P2Y receptors that, in turn, drive cell proliferation (Yamashita and Sugioka, 1998; Pearson et al., 2005). Propagation of ATP-induced Ca2+ waves is observed in radial glia of the proliferative murine embryonic neocortical ventricular zone. Disrupting Ca2+ waves between neuronal progenitors reduced cell proliferation in the ventricular zone during the peak of embryonic neurogenesis (Weissman et al., 2004). Furthermore, P2Y1 receptor-mediated signaling is necessary for proper migration of embryonic neural precursors from the ventricular to the subventricular zone (Liu et al., 2008). A recent study places nucleotide signaling to center...
stage of the development of an entire organ. The P2Y<sub>1</sub> receptor and the ectonucleotidase nucleoside triphosphate diphosphohydrolase 2 (NTPDase2) are crucial for eye development in *Xenopus* larvae (Massé et al., 2007).

Together, these data attribute a functional role to purinergic signaling in the control of neurogenesis. They further suggest that nucleotides together with growth factors and other signal substances can have a synergistic impact on neurogenesis. We therefore probed for the possibility that the nucleotide- and growth-factor-induced intracellular signaling pathways converge or interact in the neural progenitor cells. Our results imply that both EGF and the nucleotides ADPβS and UTP induce coordinate intracellular signaling pathways that would allow synergism in the control of adult neurogenesis.

**Results**

**Adherent progenitors are nestin-positive and multipotent**

Neural progenitors isolated from the SVZ of adult mice were grown as neurospheres in the presence of growth factors (EGF and FGF2). After 7 days, the progenitors were dissociated and cultured in the same media as adherent cells to facilitate both immunocytochemical and biochemical analysis. Undifferentiated adherent cells were analyzed after 4 days; 98.9±0.2% (n=4) of the cells were immunopositive for the intermediate filament nestin, which is expressed by undifferentiated neural stem cells (Fig. 1Aa,Ba). These adherent nestin-positive cells have the properties of multipotent precursors. Culturing of adherent cells for an additional 6 days in the absence of growth factors and in the presence of 2% FCS resulted in the formation (relative to the total of DAPI-labeled nuclei) of GFAP-positive astrocytes (51.5±3.2%), Tuj1-positive neurons (29.6±3.2%) and O4-positive oligodendrocytes (0.3±0.1%) (means ± s.d. of four to six experiments, total number of nuclei counted 36,167 for nestin, 11,345 for GFAP, 12,518 for Tuj1 and 10,282 for O4).

ADPβS, UTP and EGF induce ERK and CREB phosphorylation in undifferentiated nestin-positive precursors

ERK1/2 and CREB phosphorylation were analyzed as potential intracellular targets of nucleotides and EGF. To avoid constitutive ERK1/2 and CREB phosphorylation by growth factors contained in the medium, the cells were grown in the absence of growth factors for 24 hours prior to analysis. Cells were stimulated with the P2 receptor agonists ADPβS (50 μM), UTP (50 μM) or the growth factor EGF (20 ng/ml) for 5 minutes followed by cell fixation and immunocytochemical analysis. Carrier-treated controls revealed low phosphorylation for pERK1/2 in the cytosol of the majority of cells (Fig. 1Aa). Treatment with ADPβS, UTP and EGF considerably increased the immunostaining for pERK1/2 in nestin-positive cells (Fig. 1Ab-d). Immunostaining was most prominent in the cytosol. In contrast to the soluble pERK1/2, the filamentous nestin was unevenly distributed throughout the cytosol. Furthermore, the expression levels of nestin varied between cultured cells. Because this obscures the extent of double-labeling in the overlays (Fig. 1A), supplementary material Fig. S1 shows the individual color channels. We further noticed flattening of the cells following stimulation with EGF (Fig. 1Ad), which is indicative of cytoskeletal alterations; but this phenomenon was not further analyzed.

The quantitative evaluation of pERK1/2 immunostaining (integrated density per nestin-positive cell) revealed an increase in ERK1/2 phosphorylation relative to controls by a factor of 3.2 for ADPβS, 2.2 for UTP and 5.7 for EGF (Fig. 1C). Similarly, all three agonists stimulated the phosphorylation of nuclear CREB

![Fig. 1. ADPβS, UTP and EGF induce ERK1/2 and CREB phosphorylation in undifferentiated cultures. Adherent undifferentiated cells cultured for 4 days after dissociation of primary neurospheres were immunostained for nestin and ERK1/2 (Aa-d) or CREB (Ba-d) phosphorylation following stimulation for 5 minutes with either ADPβS (50 μM) (Ab,Bb), UTP (50 μM) (Ac,Bc) or EGF (20 ng/ml) (Ad,Bd). Carrier was added to controls (Aa,Ba). The images represent mergers of immunolabeling for nestin (green), pERK1/2 (red) and nuclear DAPI staining (blue). The bar in (Bd) applies to all images. (C,D) Quantification of the mean integrated density of pERK1/2 phosphorylation in undifferentiated (C) or pCREB-positive cells (D) in nestin-positive undifferentiated cells. In the controls, the contribution of nestin-positive cells with a pCREB-positive nucleus corresponded to 1.0±0.3% of the total nestin-positive cells. Values are means ± s.d. for ERK-phosphorylation or CREB phosphorylation (n=3). Note that for pCREB analysis differences in the staining intensity between individual nuclei are not taken into account. *P<0.05, **P<0.01; ***P<0.001 relative to control; unpaired t-test.](image-url)
quantitative assessment of CREB phosphorylation, the relative number of all nestin-positive cells with pCREB-positive nuclei was determined. The contribution of cells with pCREB-positive nuclei was increased by a factor of 42 for ADPβS, 29 for UTP and 49 for EGF (Fig. 1D). These results show that, in addition to EGF receptors, the undifferentiated nestin-positive precursors express functional P2 receptors whose activation by ADPβS or UTP initiates intracellular signaling cascades involving the phosphorylation of ERK1/2 and CREB.

The response pattern of adherent cells is altered following induced differentiation

We further analyzed the responsiveness of adherent cells to the P2 receptor agonists and to EGF following differentiation induced by growth factor withdrawal and addition of fetal calf serum (FCS). Astrocytes, oligodendrocytes and neurons were identified by immunostaining for GFAP, O4 and Tuj1, respectively (Fig. 2A-F). The vast majority of GFAP-positive astrocytes coexpressed nestin (supplementary material Fig. S3). Because astrocytes from murine brain in primary culture or in cultured hippocampal slices equally coexpress nestin and GFAP (Pekny et al., 1998; Schmidt-Kastner and Humpel, 2002), similarly to reactive astrocytes in situ (Clarke et al., 1994; Frisén et al., 1995), nestin expression in the progenitor cell-derived astrocytes presumably reflects a reactive rather than an undifferentiated phenotype. As observed in cultures of primary astrocytes (Pekny et al., 1998), there was considerable heterogeneity regarding GFAP and nestin immunoreactivity and the relative intensity of the coexpressed proteins.

Cultured cells were stimulated with ADPβS, UTP or EGF (Fig. 2). GFAP-positive astrocytes revealed large round to oval nuclei and responded to all three agonists. The immunoreactivity for pERK1/2 was mainly located in the cytosol and was increased after addition of agonists (Fig. 2Aa-d; individual color channels in supplementary material Fig. S4). Oligodendrocytes (Fig. 2Ba-d) revealed a light constitutive immunostaining for pERK1/2 that was not enhanced by any of the agonists. Tuj1-positive neurons (Fig. 2Ca-d) revealed smaller nuclei with intense DAPI staining. There was only faint pERK1/2 immunoreactivity and the cells were non-reactive to the nucleotides and EGF. The quantitative evaluation of pERK1/2 immunostaining in astrocytes revealed an increase in

Fig. 2. Differential effect of ADPβS, UTP and EGF on ERK and CREB phosphorylation in differentiated cells. Cells initially grown as neurospheres for 7 days were dissociated and transferred into adherent culture for 4 days, followed by growth factor withdrawal-induced differentiation for 6 days. Cells were stimulated for 5 minutes with either ADPβS (50 μM), UTP (50 μM) or EGF (20 ng/ml). Carrier was added to controls. The images represent mergers of immunolabeling for GFAP, O4 or Tuj1 (green), pERK1/2 or pCREB (red) and nuclear DAPI staining (blue). (A-C) Immunocytochemical analysis of ERK1/2 phosphorylation in GFAP-positive (Aa-d), O4-positive (Ba-d) and Tuj1-positive (Ca-d) cells. (D-F) Immunocytochemical analysis of CREB phosphorylation in GFAP-positive (Da-d), O4-positive (Ea-d) and Tuj1-positive (Fa-d) cells. Nuclei double-labeled for pCREB and DAPI appear in purple. The bar in Fd applies to all images. (G) Quantification of pERK1/2 labeling. The mean integrated density of pERK1/2 labeling in GFAP-positive cells treated with carrier (control) is set to 100%. (H) Quantification of pCREB labeling. The contribution of GFAP-, O4- or Tuj1-positive cells with a pCREB-positive nucleus in carrier-treated controls is set to 100% (corresponding to 8.7±1.5%, 25.1±11.0% and 6.4±1.8% of the total GFAP-, O4- or Tuj1-positive cells, respectively). Values are means ± s.d. (n=4–6). *P<0.05, **P<0.01; ***P<0.001 relative to control; unpaired t-test.
ERK1/2 phosphorylation (integrated density per GFAP-positive cell) relative to carrier-treated controls by a factor of 3.0 for ADPβS, 1.8 for UTP and 2.8 for EGF (Fig. 2G). No quantitative evaluation for pERK immunolabeling was performed for oligodendrocytes and neurons.

The general response pattern of differentiated cells was similar for CREB phosphorylation. Occasional CREB-positive nuclei were identified in astrocytes of unstimulated controls (Fig. 2Da). UTP, EGF and, in particular, ADPβS increased pCREB labeling in astrocytes (Fig. 2Db-d; individual color channels in supplementary material Fig. S5). Under control conditions, O4-positive oligodendrocytes revealed little staining for pCREB and this was not altered by addition of agonists (Fig. 2Fa-d). Similarly, Tuj1-positive neurons revealed very little pCREB immunostaining and were not affected by agonist application (Fig. 2Fa-d). The quantitative assessment of the relative number of GFAP-positive cells with pCREB-positive nuclei revealed an increase by a factor of 9.2 for ADPβS, 2.7 for UTP and 3.0 for EGF (Fig. 2H). These results suggest that the astrocytes formed following differentiation of nestin-positive precursors retain the reactivity for ADPβS, UTP and EGF, whereas the newly formed oligodendrocytes and neurons lack functional receptors for these agonists.

The ERK1/2 and CREB phosphorylation induced by P2 receptor agonists differs in onset and duration from that induced by EGF.

Using quantitative western blotting, we performed a detailed analysis of the cellular response to ADPβS, UTP, its hydrolysis product UDP, and EGF. This approach would not differentiate between individual cell types in differentiated cultures, therefore the investigation was restricted to the nestin-positive undifferentiated precursors. ADPβS, UTP and EGF activate ERK1/2 phosphorylation in a concentration-dependent manner (Fig. 3). Maximal responses for ADPβS and UTP were elicited at a concentration of 50 μM with an approximately eightfold and fivefold increase, respectively (Fig. 3A,B). At the concentrations applied, EGF induced a considerably stronger ERK1/2 phosphorylation (approximately 20-fold increase) (Fig. 3C). Similar results were obtained for CREB phosphorylation with an approximately fivefold and sixfold increase for ADPβS and UTP, respectively (Fig. 4A,B). The relative increase in CREB phosphorylation with EGF (approximately 11-fold) (Fig. 4C) was considerably lower than that for ERK1/2 phosphorylation. Notably, the onset of ERK1/2 and CREB phosphorylation was faster for P2 receptor stimulation than for activation of EGF receptors (Figs 3 and 4). A significant activation of ERK1/2 and CREB by ADPβS and UTP was observed after 1 minute and after reaching a maximum (2.5-5 minutes) phosphorylation levels started to decline (Fig. 3D,E; Fig. 4D,E). Significant ERK1/2 and CREB activation by EGF was obtained only after 2.5 minutes, and phosphorylation levels were still increasing at 10-20 minutes (Fig. 3F; Fig. 4F).

Because neural progenitors express ectonucleotidases (Mishra et al., 2006) that can hydrolyze UTP to the P2Y6 receptor agonist UDP, we analyzed the potential of UDP to induce ERK1/2 and CREB phosphorylation. No significant increase in phosphorylation levels was obtained (supplementary material Fig. S6).

Neural progenitors express multiple P2Y receptors

We had previously identified receptors for ADPβS and UTP in adult SVZ-derived neurospheres (Mishra et al., 2006). In order to define the P2 receptor repertoire of the adherent neural progenitors employed in the present experiments, we performed RT-PCR using mRNA extracted from neurosphere-derived 4-day-old adherent progenitors (Fig. 5). We obtained strong bands for P2Y1 (ADP, ATP), P2Y2 (UTP, ATP) and P2Y6 (UDP) receptor mRNA; weaker bands for the P2Y12 (ADP), P2Y13 (ADP) and P2Y14 (UDP glucose) receptor; and no signal for the P2Y4 (UTP) receptor. Strong PCR signals were also observed for P2X1 to P2X6 receptors, but only a...
faint signal was obtained for the P2X7 receptor (all ATP). In addition, we identified the mRNA encoding the ectonucleotidases nucleoside triphosphate diphosphohydrolase (NTPDase2) and the tissue-nonspecific form of alkaline phosphatase (TNAP) (Zimmermann, 2000) that we had previously identified in both neurospheres and progenitors of the SVZ in situ (Mishra et al., 2006; Langer et al., 2007).

These results suggest that the neural precursors contain a large repertoire of mRNAs for P2Y and P2X nucleotide receptor subtypes. The absence of the P2Y4 receptor implies that the UTP-mediated response was solely due to P2Y2 receptors. Because UDP did not evoke significant ERK1/2 or CREB phosphorylation (supplementary material Fig. S6), functional protein levels of the (phospholipase-C-coupled) P2Y6 receptor are apparently negligible. ADP is the cognate agonist of P2Y1, and the closely related P2Y12 and P2Y13 receptors. In the mouse, all three P2Y receptors are activated by ADPβS (von Kügelgen, 2006). The P2Y1 receptor is coupled to Gq and activates phospholipase C. The P2Y12 and P2Y13 receptors are coupled to Gi. However, activation of the P2Y13 receptor can induce an increase in intracellular Ca²⁺, activation of phospholipase C, and ERK1/2 phosphorylation (Communi et al., 2001). Also, P2Y12 receptors have been suggested to induce Ca²⁺ elevation and phospholipase C activation (Irino et al., 2008; van der Meijden et al., 2008).

P2Y1 and P2Y13 contribute to the ADPβS-mediated cellular response

In order to further identify the receptor subtype(s) responding to ADPβS, we first applied the selective P2Y1-receptor-specific agonist MRS 2365 (Bourdon et al., 2006) (Fig. 6A,B). MRS2365 (50 μM) induced an increase in both ERK1/2 and CREB phosphorylation corresponding to 32% and 73% of the ADPβS-induced (50 μM) phosphorylation. Accordingly, the competitive P2Y1 receptor antagonist MRS2179 reduced ADPβS-mediated ERK1/2 and CREB phosphorylation to 66% and 52%, respectively (Fig. 6C,D), demonstrating a significant contribution of the P2Y1 receptor but implicating the presence of additional P2Y receptors. Application of the P2Y12-specific antagonist MRS2395 (100 μM) had no effect on ADPβS-induced (50 μM) ERK1/2 and CREB phosphorylation (n=7). However, cangrelor (AR-C69931MX; 100 μM), a selective competitive antagonist of both P2Y12 and P2Y13 receptors (Fumagalli et al., 2004) induced a reduction of about 30% of the ADPβS-induced (50 μM) ERK and CREB phosphorylation (Fig. 6C,D). These data suggest that the P2Y1 and the P2Y13 receptor are the major mediators of the ADPβS-induced ERK1/2 and CREB responses.

Downstream signaling to ERK1/2 differs between ADPβS and UTP

Our results suggested that the two nucleotides ADPβS and UTP, as well as EGF, induce a partially overlapping receptor-mediated phosphorylation of intracellular targets. This could imply either parallel activation of converging pathways or transactivation, whereby the primary nucleotide agonist, via binding to its receptor, would activate the EGF receptor. We therefore applied a series of inhibitors to further characterize the signaling pathways. In order to investigate the possibility that a transactivation of the EGF receptor is involved in ADPβS-induced ERK1/2 phosphorylation, we inhibited the phosphorylation of the EGF receptor with AG1478 (Fig. 7A). This resulted in a reduction in the ADPβS-induced ERK1/2 phosphorylation by 44%. GF109203X, which non-selectively inhibits all forms of serine/threonine protein kinases with similarity to staurosporine and the isoforms of protein kinase C (total PKCs) (Mackay and Twelves, 2007), almost completely abolished ADPβS-induced ERK1/2 phosphorylation. By contrast, Gö6976, which selectively inhibits the α- and β-isoforms of conventional

**Fig. 4.** ADPβS, UTP and EGF induce time- and concentration-dependent phosphorylation of CREB. Experiments were performed with adherent undifferentiated cells 4 days after dissociation of primary neurospheres. (A–C) For analysis of concentration dependence the agonist concentrations indicated (ADPβS, UTP or EGF) were applied for 5 minutes. (D–F) For analysis of time dependence cells were stimulated for the indicated times with either ADPβS (50 μM), UTP (50 μM), or EGF (10 ng/ml). Carrier was added to controls. GAPDH served as a loading control. Values are means ± s.e.m. (n=3–5). *P<0.05, **P<0.01; ***P<0.001 relative to control; ANOVA, Tukey-Kramer multiple comparisons test (unpaired). Representative immunoblots probed for pCREB and GAPDH as a loading control are shown at the bottom of each graph.
PKCs (cPKCs; often referred to as Ca²⁺-dependent isoforms of PKC), reduced ERK1/2 phosphorylation to only about half. Inhibition of the upstream located kinase MAP/ERK kinase (MEK) by U0126 abrogated ERK1/2 phosphorylation. Because it has previously been suggested that P2Y receptor activation can induce growth-factor-receptor transactivation via activation of metalloproteinases (Camden et al., 2005; Yin et al., 2007), we applied the metalloproteinase inhibitor GM6001 together with ADPβS. No significant reduction in ERK1/2 phosphorylation was obtained. As compared to application of Gö6976 alone, coapplication of AG1478 with Gö6976 had no additional impact on ERK1/2 phosphorylation, suggesting that the cPKC-dependent component of ADPβS-induced ERK1/2 activation is downstream of EGF receptor activation.

Interestingly, the intracellular signaling pathways initiated by ADPβS and UTP differ. Inhibition of the EGF receptor (AG1478) had no significant effect on ERK1/2 activation by UTP (Fig. 7B). Whereas Gfi109203X reduced ERK1/2 phosphorylation by 68%, the cPKC inhibitor Gö6976 had no effect on UTP-induced ERK1/2 phosphorylation. As for the stimulation with ADPβS, UTP-induced ERK1/2 phosphorylation was abrogated by MEK inhibition (using U0126). These data imply that the ADPβS and UTP pathways for ERK1/2 phosphorylation converge on MEK but differ in the upstream signaling pathways. In particular, ERK1/2 phosphorylation induced by UTP-mediated activation of the P2Y₁ receptor could not be inhibited by AG1478, an inhibitor of the EGF receptor. Furthermore, cPKCs were not involved in UTP-induced intracellular signal propagation.

Downstream signaling to CREB differs from that to ERK1/2. As for ERK1/2 activation, ADPβS-induced CREB phosphorylation was significantly reduced by application of the EGF receptor inhibitor AG1478 (Fig. 7C). Similarly, the total PKC inhibitor GF109203X nearly abolished ADPβS-induced CREB phosphorylation. In contrast to ERK1/2 phosphorylation, inhibition of cPKCs (using Gö6976) completely inhibited CREB phosphorylation. Inhibition with U0126 showed that MEK activation was only partially (60%) involved in CREB phosphorylation. No effect on ADPβS-induced CREB phosphorylation was observed on inhibition of metalloproteinases (using GM6001).

UTP-induced CREB phosphorylation was not affected by inhibiting the EGF receptor (Fig. 7D). As for application of ADPβS, UTP-induced CREB phosphorylation was largely inhibited by the PKC inhibitors GF109203X and Gö6976, whereas MEK activation was only partially involved in CREB phosphorylation (shown using U0126). These data suggest that (except for a potential contribution of EGF-receptor transactivation by ADPβS) the signaling pathways leading to CREB phosphorylation are very similar for ADPβS- and UTP-induced P2 receptor activation. There are, however, significant differences to the signaling pathways leading to ERK1/2 phosphorylation. Notably, CREB phosphorylation appears to be activated by parallel downstream pathways that only partially involve MEK activation.

The reduction in ADPβS-induced ERK and CREB phosphorylation by AG1478 provides indirect evidence for EGF-receptor transactivation. We further explored the possibility of ADPβS-induced EGF-receptor transactivation by western-blot analysis using a variety of antibodies against phosphorylation sites of the EGF receptor (phosphorylation sites Y845, Y1068 and Y1173). No increase in EGF-receptor phosphorylation could be obtained following application of ADPβS (not shown).

**Discussion**

Adult neurogenesis is driven by a variety of signal systems that potentially induce converging, synergistic or opposing cellular responses (Abrous et al., 2005). It is therefore important to gain insight into the underlying signaling pathways. Here, we show how nucleotides and EGF induce converging but differential intracellular signals in cultured multipotent adult neural progenitor cells.
Furthermore, differentiation of the progenitors alters the receptor profile, and oligodendrocytes and neurons lose responsiveness to the agonists. Our data imply that, via converging intracellular signaling pathways, nucleotides and EGF carry the potential for synergism in controlling adult neurogenesis.

The nucleotides ADPβS and UTP and EGF elicit converging but differential signaling pathways

Growth factors such as EGF and FGF2 that act via receptor tyrosine kinases expand neural progenitors both in vivo and in vitro, either as neurospheres or as adherent cultures (Temple, 2001). Because we have previously shown that the nucleotide agonists ADPβS and UTP induce Ca²⁺ transients and augment growth-factor-stimulated proliferation in cultured adult neural stem cells (Mishra et al., 2006), we analyzed intracellular signaling pathways induced by these nucleotides and compared them to EGF-receptor activation. Depending on the subtype of P2Y receptor, nucleotides such as ATP, ADP, UTP and UDP activate a variety of intracellular signaling cascades, most notably phospholipase C and an increase in [Ca²⁺], activation or inhibition of adenyl cyclase, coupling to ion channels, activation of the mitogen-activated (MAP) kinase pathways, and induction of immediate early genes (Erb et al., 2006; Majumder et al., 2007). Importantly, the signaling pathways elicited and the functional outcome vary not only between P2Y receptors but also with the cell context (Fam et al., 2003; Fam et al., 2005; Baker et al., 2006; Kudirka et al., 2007).

Our data demonstrate that activation of P2Y₁ and P2Y₁₃ receptors via ADPβS, as well as of the P2Y₂ receptor via UTP, induces a rapid, parallel and transient activation of both ERK1/2 and CREB. Although EGF activates identical targets, the temporal pattern differs. A delayed onset is followed by prolonged activation. This suggests that co-activation of the two signaling pathways could lead to intensified but differential physiological effects in situ, with the nucleotides as fast but transient effectors, and EGF as a delayed but more persistent effector. We have not investigated the mechanism underlying the decay of the response to nucleotides, but agonist-induced desensitization accompanied by receptor internalization has been demonstrated (Hoffmann et al., 2008; Sanabria et al., 2008).

Differences between P2 receptor agonists and targets

Our data reveal that activation of the intracellular signaling pathways differs regarding the activation of ERK1/2 and CREB and also regarding the agonists ADPβS and UTP. ADPβS- and UTP-induced ERK1/2 activation proceeds exclusively via MEK activation. CREB can also be activated by a pathway bypassing MEK and it fully relies on cPKCs. PKCs play a major role also in ERK1/2 activation, whereby cPKCs are involved only following stimulation with ADPβS.

The reduction in ADPβS-induced ERK1/2 and CREB phosphorylation observed on inhibition of EGF-receptor phosphorylation is suggestive of EGF-receptor transactivation. However, following application of ADPβS, no increase in EGF-receptor phosphorylation could be obtained by western blotting. As revealed in Fig. 3, ERK phosphorylation induced by ADPβS amounted to only about a third of that induced by EGF. It therefore appears possible that the small contribution of ADPβS to EGF-receptor transactivation escaped detection by quantitative western blotting. Although a non-specific effect of AG1478 on downstream signaling pathways cannot be excluded, it is worth noting that application of this inhibitor had no effect on UTP-induced ERK1/2 or CREB phosphorylation.

The extent of nucleotide-receptor-mediated transactivation of growth factor receptor might vary between cell types and between species investigated. The UTP-activated recombinant human P2Y₂ receptor was previously found to induce a transactivation of several growth factor receptors, including the EGF receptor. This involved Src activation via proline-rich SH3-domain-binding motifs at the C-terminus of the P2Y₂ receptors (Liu et al., 2004). Src family kinases have also been implicated in the P2Y₂-receptor-mediated activation of TrkA receptor tyrosine kinase (Arthur et al., 2006a). It remains questionable whether the consensus motif-like C-terminal domain KPPTEPTSP (aa 321-330) of the mouse P2Y₂ receptor is functional. P2Y₁-induced transactivation of the EGF receptor was recently reported for tumoral HeLa cells and FRT epithelial cells (Buvinic et al., 2007). At present, the mechanism leading to the potential transactivation of the EGF receptor by ADPβS has not been defined. Inhibition of metalloproteinases neither affected ERK1/2 nor CREB phosphorylation, suggesting that shedding of growth factor precursors would not be involved (Wong and Guillaud, 2004; Ohtsu et al., 2006).

Following differentiation, responsiveness of astrocytes is maintained but not that of neurons and oligodendrocytes

Young neurons and oligodendrocytes derived from cultured progenitors no longer responded to ADPβS, UTP or EGF, suggesting that the relevant receptors become downregulated on differentiation.
Similarly, neuronal maturation was found to reduce the response to purinergic stimulation in both the neural retina (Martins and Pearson, 2008) and in cultured SVZ-derived cells (Lin et al., 2007). By contrast, P2Y2 receptors are expressed in the immortalized PC12 cells or in primary cultures of dorsal root ganglion neurons (Arthur et al., 2006b). Because both activated ERK1/2 and CREB convey survival signals and neuroprotective effects (Bonni et al., 1999; Lonze and Ginty, 2002), the loss of responsiveness to EGF and the nucleotide agonists might render the differentiated neurons and oligodendrocytes susceptible to apoptosis. However, the differentiated cells survived well in the growth-factor-depleted medium. We did not investigate the potential of other agonists to induce survival pathways in these cells. The maintenance of the response to nucleotides and growth factor in cultured progenitor-derived astrocytes concurs with the principal properties previously observed for cultured primary astrocytes derived from postnatal brain (Neary et al., 2001).

The molecular features of neural progenitors only partially overlap with those of astrocytes

Our data also shed light on functional differences between neural progenitors and differentiated astrocytes. Adult mammalian neural stem cells of the SVZ carry features of astrocytes such as the expression of glial fibrillary acidic protein (GFAP) or the astrocyte-specific glutamate transporter (GLAST) (Ninkovic and Götz, 2007; Wang and Bordey, 2008), and are sometimes also referred to as the astrocytes of the SVZ. We therefore compared receptor profiles and induced signaling pathways between the cultured neural progenitors and cultured astrocytes. The GFAP-positive progenitor cells investigated in this study were multipotent and expressed the stem-cell marker nestin as well as the type-B cell-associated ectonucleotidases NTPDase2 and TnAP (Mishra et al., 2006; Langer et al., 2007), suggesting that they represent type-B cell-like neural precursors. Both RT-PCR and pharmacological experiments suggested that cultured astrocytes express a variety of P2Y and P2X receptors (Jacques-Silva et al., 2004; Washburn and Neary, 2006) whose functional impact is only partially understood. We have identified several P2 receptors (P2X and P2Y) in cultured adult neural progenitor cells but the RT-PCR data alone do not allow conclusions regarding the extent of translation and amount of functional protein. For example, we identified the mRNA for the P2Y6 receptor but [as previously shown for cultured neurosphere cells (Mishra et al., 2006)] the corresponding ligand UDP was ineffective. In murine astrocytes, P2Y6 mRNA was found to be absent (Lenz et al., 2000) whereas P2Y4 receptor mRNA could be identified, in contrast to neural progenitors. Cultured astrocytes express the P2X2 receptor and activation of this receptor stimulates Akt phosphorylation (Jacques-Silva et al., 2004), but P2X2 receptor mRNA is essentially absent from cultured neural progenitors. The notion that the molecular features of neural progenitors only partially overlap with those of astrocytes is also supported by our observation that the fingerprint of ERK1/2 and CREB phosphorylation induced by ADPβS, UTP and EGF differs between neural progenitors and progenitor-derived astrocytes. These data underline the notion that the adult astrocyte-like neural progenitors differ in their molecular profile from bona fide astrocytes.

Functional implications

In cultured astrocytes, extracellular ATP and fibroblast growth factor synergistically activate cell proliferation (Neary et al., 1994) and, as found in neural progenitors, ATP induces phosphorylation of ERK1/2 (Neary et al., 1999). Activation of P2Y2 receptors in astrocytoma cells activates CREB, leading to an upregulation of genes encoding anti-apoptotic and neurotrophic proteins (Chorna et al., 2004). Here, we show that activation of either P2Y2 or P2Y1 and P2Y13 receptors induces ERK1/2 and CREB phosphorylation in cultured neural progenitors. ERK1/2 phosphorylation plays a central role in control of the cell cycle (Melone and Pouyssegur, 2007), including that in adult murine neural stem cells (Kalluri et al., 2007). CREB phosphorylation can lead to the induction of numerous target proteins (Impey et al., 2004). Interestingly, CREB phosphorylation was shown to support differentiation and survival of newly formed neurons in the olfactory bulb (Giachino et al., 2005). Assuming receptor coexpression in situ, the physiological outcome of the parallel activation of both the ERK1/2 and CREB signaling pathways is difficult to predict. Within the SVZ in situ, EGF receptors have mainly been attributed to the transit-amplifying type C cells (Doetsch et al., 2002), whereas the location of the P2Y receptors has to await the availability of reliable antibodies. Furthermore, the nucleotide-induced increase in intracellular Ca2+ can elicit additional cell responses such as exocytosis, including the exocytosis of more ATP (Pryazhnikov and Khirogu, 2008). It should be noted that ATP, via P2X receptors, might induce additional responses in the progenitor cells, but this has not been investigated in this study.

These data suggest that parallel activation of neural precursors via EGF receptors and P2Y1, P2Y2 and P2Y13 receptors has the potential for synergism in progenitor proliferation, growth and survival. The data highlight the ability of nucleotides to interact with other signal molecules via downstream signaling pathways, including those elicited by growth factors.

Materials and Methods

Preparation and culture of neural stem cells

Neural stem cells were prepared from 8-12 week old C57BL/6N wild-type mice (Mus musculus). Neural stem cells were first cultured as neurospheres as previously described (Mishra et al., 2006) and subsequently replated as adherent cultures. In brief, the SVZ was excised from tissue surrounding the lateral wall of the lateral ventricles, using 400 μm thick coronal sections kept in ice cold PBS buffer (137 mM NaCl, 5 mM KCl, 15 mM NaH2PO4, pH 7.4). The tissue was enzymatically dissociated for 20-30 minutes at 37°C with 0.5 mg/ml papain (14 U/mg dissolved in DMEM/F12 media (Invitrogen) containing 0.1 mM EDTA. The tissue was transferred into the same volume of trypsin inhibitor (0.7 mg/ml in DMEM/F12) with 1000 U/ml ofDNase I (Sigma-Aldrich, Steinheim, Germany) and mechanically dissociated by triturating with a micropipette. Cells were centrifuged at 260 × g for 5 minutes, resuspended in growth media, transferred into uncoated culture dishes and cultured with growth media: DMEM/F12, B27 as supplement, 10 mM HEPES buffer, pH 7.2 (all Invitrogen), 100 U/ml penicillin and 10 μg/ml streptomycin (both Sigma-Aldrich), containing 20 ng/ml of human recombinant EGF and 10 ng/ml FGF-2 (both from PeproTech, London, UK) (5% CO2). After 7 days in vitro, the neurospheres formed were dissociated with accutase II (Alfazyme, PAA Laboratories, Köln, Germany) at 37°C for 30 minutes, and cells were seeded into 24-well plates coated with poly-L-ornithine and fibronectin (0.5 μg/ml and 10 μg/ml, respectively, Sigma-Aldrich) in media as described above. For immunocytochemical analysis, cells were plated onto cover slips coated with poly-L-ornithine and fibronectin.

Adherent cells were grown in the presence of growth factors for three days followed by 24 hours in medium without growth factors. After that time, they were either analyzed or cultured for another 6 days in the absence of growth factors and in the presence of 2% fetal calf serum (FCS, Invitrogen) (10 days). Cells cultured for 4 or 10 days were either directly subjected to biochemical analysis or stimulated with ADPβS (50 μM), UTP (50 μM), EGF (10-20 ng/ml) or MRS2365 (P2Y2 receptor agonist; Torcix Bioscience, Bristol, UK), dissolved in conditioned medium for 5 minutes for biochemical analysis or processed for immunocytochemical analysis. Carrier was used as a control.

Application of inhibitors

Inhibitors were pre-applied to cultures in growth media for 30 minutes (unless indicated otherwise) and then together with agonists or carrier for another 5 minutes. The following inhibitors were applied (concentration, target): AG1478 (10 μM, EGF receptor tyrosine kinases), GF109203X (10 μM, total PKC), Gö6976 (10 μM,
conventional PCK), U0126 (10 μM, MEK1/2), GM6001 (2.5 μM, matrix metalloproteinase inhibitor), MRS2179 (100 μM, P2Y receptor antagonist; Torcix Bioscience), MRS2365 (100 μM, P2Y receptor antagonist; Sigma-Aldrich), and cangrelor (AR-C69391MX, a P2Y1 and P2Y13 receptor antagonist; gift of AstraZeneca, Loughborough, UK). Inhibitors were added also to the carrier controls.

Immunoblotting and quantitative evaluation

For western-blot analysis, cells were sonicated (1 minute) in the presence of lysis and sample buffer containing Tris (87.5 mM; AppliChem, Darmstadt, Germany), β-mercaptoethanol (10 mM; Sigma-Aldrich), p-nitrophenyl phosphate (25 mM; Sigma-Aldrich), ethylene glycol tetraacetic acid (EGTA, 0.5 mM; Sigma-Aldrich), Triton X-100 (0.5% v/v; Sigma), phenylmethylsulphonyl fluoride (PMSF, 1 mM; Fluka; Nucléo, Ulm, Germany), leupeptin (1 μg/ml; Calbiochem), antipain (1 μg/ml; Calbiochem), Na3VO4 (1 mM; Sigma-Aldrich), pH 7.2. Subsequently samples were heated at 95°C for 5 minutes and stored at -20°C. Cell lysates were separated on minigels (10% acrylamide). Immunoblotting was performed using an enhanced chemiluminescence system (Amersham Biosciences, Freiburg, Germany) and antibodies against pCREB, pERK1/2 (polyclonal; Cell Signaling), GFAP (monoclonal), neuron-specific tubulin (Tuj1, monoclonal; both Sigma-Aldrich). For double immunofluorescence labeling, the cells were washed several times in inhibitor-containing PBS (30 minutes, RT). Immunolabeling was performed with antibodies against nestin (monoclonal), O4 (monoclonal) (both Chemicon International, Hofheim, Germany), and antibodies against pCREB, pERK1/2 (polyclonal; Cell Signaling), GAP43 (monoclonal), neuron-specific tubulin (Tuj1, monoclonal; both Sigma-Aldrich). For double immunofluorescence analysis, primary antibodies from different host animals were sequentially applied overnight at 4°C, followed by incubation with the appropriate Cy3 or Alexa Fluor 488 secondary antibody (Dianova, Hamburg, Germany). Images were processed using Adobe Photoshop and Biplane Imaris 4.1.

For quantitative evaluation of immunolabeling, photographs were taken at 20×-40× objective magnification. MRS2179 (100 μM, P2Y1 receptor antagonist) and MRS2365 (100 μM, P2Y2 receptor antagonist; Sigma-Aldrich) were used. For each of about 20 control images of an identical experiment, a threshold value was defined and the mean of the threshold values was applied for calculating the increase in the stimulated samples. The integrated density reflecting the sum of the grey values of the pixels of the entire image was calculated. Counting the number of nuclei (DAPI) or of cells labeled with cell-type-specific markers permitted the determination of the integrated density per cell or cell type.

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References


**Table S1.** Primer sequences and length of the expected amplification product used for detection of P2 receptors, TNAP and NTPDase2 in cultured neural progenitor cells.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Forward primer sequence</th>
<th>Reverse primer sequence</th>
<th>Amplicon (bp)</th>
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