Nucleotides affect neurogenesis and dopaminergic differentiation of mouse fetal midbrain-derived neural precursor cells

Jasmin Delic · Herbert Zimmermann

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Abstract The fetal midbrain is a preferred source for isolating and producing dopaminergic neurons for subsequent grafting and replacement of damaged or lost dopaminergic midbrain neurons. We analysed the potential of a variety of nucleotides and of adenosine to support dopaminergic neuron formation from primary mouse fetal midbrain-derived cells, harvested at E10.5 and at E13.5 and subjected to adherent cell culture. In contrast to cells derived at E13.5, cells derived at E10.5 have the potential to produce dopaminergic neurons in culture. These neurons express tyrosine hydroxylase and the dopamine transporter. The fetal ventral midbrain contained mRNA encoding almost all P2X and P2Y receptors, all adenosine receptors as well as the ectonucleotidases nucleoside triphosphate diphosphohydrolase 2 and tissue nonspecific alkaline phosphatase. Essentially, all components of the purinergic signalling pathway were also expressed by the cultured cells. ATP, ADP β S, 2MeSATP, 2ClATP and adenosine increased neuron formation. There was, however, no preference for the formation of dopaminergic neurons—with the exception of 2ClATP that increased the relative contribution of tyrosine hydroxylase-positive neurons. In cells isolated at E13.5 UTP promoted neuron survival but ADP β S and ATP γ S essentially eliminated neurons. These data showed that the outcome of nucleotide application was different even though cells isolated at E10.5 and E13.5 expressed very similar receptor mRNA profiles. They suggest that purinergic agonists carry potential for stimulating neurogenesis and enriching the contribution of

dopaminergic neurons in vitro. Nucleotide receptor agonists may be of value for contributing to the formation and survival of dopaminergic neurons in vivo.

Keywords Adenosine · ATP · Dopaminergic neuron · Neurogenesis · Nucleotide receptor

Introduction

The discovery of adult neurogenesis has raised hopes that the insights gained into the mechanisms of stem cell activation and the integration of newly formed neurons can be applied for neurorestoration and neuroregeneration in the diseased brain [1, 2]. One of the prime targets for cell replacement therapies is Parkinson's disease that is characterised by the greatly reduced activity of dopaminergic neurons primarily of the pars compacta of the substantia nigra. These neurons project to the striatum and their loss causes alterations in the activity of the neural circuits within the basal ganglia that regulate movement [3].

To date transplanting fetal nigral grafts containing dopaminergic neurons into the brain of Parkinson's patients has met with limited success [4]. Various technical approaches have been developed to produce dopaminergic neurons for subsequent grafting. In vitro, dopaminergic neurons may be generated from embryonic stem cells or from fetal midbrain [3]. Midbrain dopaminergic neurons differ from dopaminergic neurons in other brain regions regarding their structural and chemical properties. For this reason, the fetal midbrain is the preferred source for isolating and producing dopaminergic neurons for cell replacement in Parkinson's disease. In the mouse, midbrain dopaminergic neurons develop at the ventral midbrain floor plate over a period of 3 to 4 days starting around E10.5 [5–7].

J. Delic · H. Zimmermann (✉)
Institute of Cell Biology and Neuroscience, Biocenter, J.W.
Goethe-University,
Max-von-Laue-Str. 9,
60438, Frankfurt, Germany
e-mail: h.zimmermann@bio.uni-frankfurt.de

A considerable number of factors is involved in the dopaminergic specification of fetal midbrain precursors *in vivo* and *in vitro*. These include secreted factors such as Sonic hedgehog, fibroblast growth factor 8, Wnt 1, transforming growth factors α and β , glial cell-line-derived neurotrophic factor, or brain-derived neurotrophic factor as well as a number of transcription factors and nuclear receptors [3, 8–10]. The production of midbrain-derived neurons *in vitro* proved to be difficult. Progenitors cultured as neurospheres rapidly lose the potential to create dopaminergic neurons which impairs experimental protocols for the *in vitro* expansion of progenitor cells [11, 12]. Previous studies on neural progenitors from the adult rodent brain had demonstrated the expression of a considerable variety of both ionotropic P2X and G-protein coupled P2Y nucleotide receptors. Nucleotides were found to stimulate *in vitro* progenitor cell proliferation and cell migration [13–17]. *In vitro* studies using immortalised human midbrain-derived neural progenitor cells demonstrated that nucleotides can enhance both cell proliferation and dopaminergic differentiation [18]. These cells express a variety of nucleotide receptors and respond to the application of ATP and UTP with an increase in intracellular Ca^{2+} concentrations [19]. In addition, nucleotide receptors are expressed by dopaminergic neurons of the postnatal rodent substantia nigra where they can stimulate axonal outgrowth and fibre density [20] and modulate firing activities [21]. Here we analysed the potential of nucleotides to support dopaminergic neuron formation of primary mouse fetal midbrain-derived cells subjected to adherent cell culture.

Materials and methods

Preparation and culture of neural progenitors

All animal procedures were performed in accordance with the European Guidelines for the Care of Laboratory Animals. Timed-mated pregnant C57BL/6 N wild-type mice (*Mus Musculus*) were sacrificed after 10.5 or 13.5 days. Fetal brains were collected in ice cold phosphate-buffered saline (PBS) (137 mM NaCl, 3 mM KCl, 15 mM Na^+/K^+ -phosphate buffer, pH 7.4). The tissue from the ventral mesencephalon was excised and enzymatically dissociated for 10 min at 37°C with 0.5 mg/ml papain (14 U/mg) dissolved in DMEM/F12 media (Invitrogen, Karlsruhe, Germany) containing 0.1 mM ethylenediaminetetraacetic acid. The tissue was transferred into the same volume of trypsin inhibitor (0.7 mg/ml in DMEM/F12) with 1,000 U/ml of DNase I (Sigma-Aldrich, Steinheim, Germany) and mechanically dissociated by triturating with a micropipette. Cells were centrifuged at $260\times g$ for 3 min, resuspended in growth media and transferred into

glass cover slips or culture dishes coated with poly-L-ornithine and fibronectin (0.5 mg/ml and 10 $\mu\text{g}/\text{ml}$, respectively, Sigma-Aldrich).

The cells were expanded for 1 to 1.5 d in growth factor-containing media (DMEM/F12, B27 as supplement, 10-mM HEPES buffer, pH 7.2 (all Invitrogen), 100 U/ml penicillin and 10 $\mu\text{g}/\text{ml}$ streptomycin (both Sigma-Aldrich), containing 20 ng/ml of human recombinant epidermal growth factor and 20 ng/ml fibroblast growth factor 2 (both from PeproTech, London, UK)). Differentiation was initiated by a change to differentiation media (Neurobasal, 2% FCS, 5 μM forskoline, 100 pg/ml interleukin-1 β , B27, pen/strep, Glutamax). Cell cultures were incubated in reduced atmospheric oxygen (37°C, 3% O_2 , 5% CO_2 , 95% humidity). Single dose (5 μl) of nucleotides or of adenosine (all 50 μM) dissolved in sterile double-distilled water were applied to the culture medium (1 ml/well) at three different time points. Agonists were first applied 3 h after initiation of differentiation. Since a single agonist application only had no effect on cell proliferation and differentiation, additional applications followed at differentiation days 3 and 5. Application of carrier served as a control. The medium was not changed during the course of the experiment. Analysis was performed on differentiation day 7.

Immunocytochemistry

Cells were fixed for 30 min with 3% paraformaldehyde in PBS. The cells were then washed three times in PBS and permeabilized with 0.1% TritonX-100 in PBS. Sections were blocked with 5% bovine serum albumin (BSA) in PBS, containing sodium azide (45 min, RT). Immunolabeling was performed with monoclonal antibodies against nestin, the glial glutamate transporter GLAST, O4 (all Chemicon International, Hofheim, Germany), glial fibrillary acidic protein (GFAP), neuron-specific tubulin TUJ1 (both Sigma-Aldrich), calbindin, tyrosine hydroxylase (TH), dopamine active transporter (DAT) (all Santa Cruz Biotechnology, Heidelberg, Germany) and polyclonal antibodies against TH, nuclear receptor related 1 (nurr1) protein, doublecortin, G-protein regulated inward-rectifier potassium channel 2 (GIRK2), corin and LMX1A (all Santa Cruz Biotechnology). For double immunofluorescence analysis, primary antibodies from different host animals were sequentially applied for 1 h at RT. The cells were then washed 3 times in PBS and incubated with the appropriate Cy3- or Alexa Fluor488-coupled secondary antibodies (Dianova, Hamburg, Germany). Nuclei were visualised by binding of 4'-6-diamidino-2-phenylindole (DAPI; 1 $\mu\text{g}/\text{ml}$). Each incubation step was followed by washes in PBS, except for the initial incubation with BSA. The cells were finally embedded in Aqua-Poly/Mount (Polysciences,

Eppelheim, Germany) and mounted on glass slides. The labelled cells were examined using a Zeiss Axiophot microscope equipped with a Zeiss AxioVision imaging analysis system and a Zeiss AxioCam MRm CCD camera. Images were processed using Adobe Photoshop software.

For quantitative evaluation of immunolabeling, photographs were taken at $\times 20$ objective magnification across the axis of the coverslip (two coverslips per experiment). Neurons (TUJ1) and nuclei (DAPI) were counted in 20 consecutive optical fields per cover slip. TH-positive neurons were counted on the entire corresponding cover slips.

Immunoblotting and quantitative evaluation

For Western blot analysis cells were sonicated for 1 min using a Branson Ultrasonic Cleaner in the presence of lysis buffer, containing 62 mM Tris, 2% sodium dodecyl sulfate 10% glycerol (all AppliChem, Darmstadt, Germany), 0.01% bromophenol blue (Sigma) and dithiothreitol 100 mM (Serva, Heidelberg, Germany), pH 6.8. Subsequently, samples were heated at 95°C for 5 min and stored at -20°C . Cell lysates were separated on mini gels (10% acrylamide). Immunoblotting was performed using an enhanced chemiluminescence system (Amersham Biosciences, Freiburg, Germany) and

antibodies against doublecortin, TH and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; monoclonal; Chemicon, Temecula, CA). Bands were analysed on the identical blot using the KODAK 1D Image Analysis Software (Eastman KODAK). Levels of analysed proteins were standardised to levels of GAPDH.

RT-PCR analysis

Reverse transcription polymerase chain reaction (RT-PCR) analysis was accomplished on freshly excised tissue, on undifferentiated and on differentiated cells. Undifferentiated cells were collected after 4 DIV in growth factor-containing expansion media. Differentiated cells were plated into expansion media for 1 day and collected after 7 DIV in differentiation media. mRNA was isolated using the RNeasy Mini Kit followed by an on-column digestion of genomic DNA with RNase-free DNase I (Qiagen, Hilden, Germany). cDNA was synthesised with the Omniscript RT Kit (Qiagen) using a combination of oligo-dT primers and random hexamer oligonucleotides. A PCR with 35 cycles was performed using GoTaq Hot Start Polymerase (Promega, Madison, WI) and a $5\times$ concentrated enhancer solution (2.7 M betaine, 6.7 mM dithiothreitol, 6.7% dimethylsulfoxide and 55 $\mu\text{g/ml}$ BSA [22]). A primer pair for the house keeping

Table 1 Primer sequences and lengths of the expected amplification product used for detection of P2 receptors, TNAP and NTPDase2

Gene name	Forward primer sequence	Reverse primer sequence	Amplicon (bp)
P2RX1	GAGAGTCGGCCAGGACTTC	GCGAATCCCAAACACCTTGA	233
P2RX2	CCCCAAATTTACCATCC	GCACTCTGATTACAGACAAGTCC	263
P2RX3	GAAACCTCCTTCCTAACCC	ATACCAGCACATCAAAGC	363
P2RX4	AATATCCTCCCCAACATTACC	CTGCCATCTCCTGAAAGC	133
P2RX5	TGGACAACAAACACACACAATCC	TCACAGAAGAAAGCCCCAGC	223
P2RX6	TCTGGATTACAAGACGGAGAA	AGTTGGTTACCAGGAAGAACA	281
P2RX7	TCCATATGACAAGACAAGG	TTGTGAAAGGTACAAGAGC	201
P2RY1	TGCTATTGAGTGGTATGGAACAG	ACACGAGCAAAGTCACAGAAA	202
P2RY2	TCTTACCCTCTTGCCCTCCATC	CCCATTCTGCCTAACTCC	205
P2RY4	GGCATTGTGACACACCTTGTA	AAGGCACAAAGCAGACAGCAA	213
P2RY6	ATCAGCTTCTGCCTTTCC	CTGTGAGCCTCTGTAAGAGATCG	214
P2RY12	TAACCATTGACCGCTACC	TTCGTGCCAACTAGACC	212
P2RY13	CTGCCCTTTCAAATCCTTTCC	TGCTGTCTTACTCCTAACTTCC	454
P2RY14	ACCACAGACCTCCAAACC	TGCACACAAACACATTACCC	268
Adora1	GTCTGTCTCTCATCTCACC	GGCTATCCAGGCTTGTTCC	221
Adora2	CACCATCAGCACTGGCTTC	TGACAGCACCCAGCAAATC	205
Adora2b	CTTCTGCACGGACTTCCAC	CAAAGGCAAGGACCCAGAG	194
Adora3	GCCTGCCTTTTCATGTCC	CCACCAGAAAGGAACTAGCC	181
Aplp	TGACCTTCTCTCCTCCATCC	GTGTGTGTGTGTCTCTGTCC	205
Entpd2	CTGTCTGTGACCTCGTCTCTG	GTCTGGTTGCAGGTGGTCTC	213
GAPDH	AAGGGCATCTGGGCTACACT	TCCAGGGTTTCTTACTCCTTG	209
MBIP	ACACTTGGTATTGAACTG	GTGGCTTTCCAGCCATTC	560

gene 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 [13]. In the BiP gene, binding sites of the primers are

Fig. 1 Immunocytochemical characterization of E10.5-derived cells. Images are derived from stage day in vitro 1 (*DIV 1*) (a, c, e, g) and stage day in vitro 7 (*DIV 7*) (b, d, f, h–j). The images represent overlays of double staining for nestin (green) and DAPI (blue) (a, b), GLAST (red) and DAPI (blue) (c, d), TUJ1 (green) and DAPI (blue) (e, f), tyrosine hydroxylase (red, TH) and DAPI (blue) (g, h) and GFAP (red) and DAPI (blue) (j). i Triple labelling for TH (red), DAPI (blue) and TuJ1 (green)). Scale bars=30 μm

Fig. 2 Immunocytochemical analysis of proteins expressed in midbrain dopaminergic neurons in E10.5-derived cells. All images are derived from stage day in vitro 7 (*DIV 7*). They represent overlays of triple labelling for calbindin (green), doublecortin (DCX) (red) and DAPI (blue) (a), calbindin (green), TH (red) and DAPI (blue) (b), TH (green) GIRK2 (red) and DAPI (blue) (c), TH (green), nurr1 (red) and DAPI (blue) (d), and DAT (green), TH (red) and DAPI (blue) (e, f). Scale bars=30 μm

separated by an intron. Only a 560 bp sequence (that excludes the 316 bp long intron sequence) was amplified. Primer sequences and length of the expected amplification product are presented in Table 1.

Results

Analysis of neuronal and dopaminergic differentiation at E10.5

Acutely isolated cells derived from the ventral mesencephalon at E10.5 were subjected to adherent culture. In a first series of experiments we performed an analysis of the spontaneous differentiation and survival of the cultured cells subjected to differentiation media for up to 7 days. The immunocytochemical analysis of the cultured cells revealed that at 1 DIV the culture contained largely nestin-positive cells whose contribution increased with increasing culture time (Fig. 1a, b). Similarly, cells positive for the radial glia marker GLAST [23] were identified at 1DIV. GLAST expression was strongly enhanced after 7DIV (Fig. 1c, d). At 1 DIV very few TUJ1-positive neurons

(often occurring in clusters) were identified (Fig. 1e) some of which were also TH positive (not shown). The contribution of either cell type considerably increased with culture time (Fig. 1f–h). Many of the new neurons formed were TH positive (Fig. 1i). The TH-positive cells extended multiple and long neuritic processes. No cells were immunopositive for GFAP at the beginning of the culture period and only few GFAP-positive cells were detected after the end of the culture period (Fig. 1j). These data suggested that cells isolated from the E10.5 embryonic mouse midbrain largely contained undifferentiated neural progenitors that can produce progeny in culture. With increasing culture time these cells mainly generated neurons, part of which were TH positive.

It has previously been recognised that the ventral mesencephalon contains several types of dopaminergic neurons. The calcium-binding protein calbindin is expressed in the vast majority of TH-positive A10 neurons which innervate the tegmental area whereas the G protein-gated inwardly rectifying K^+ channel GIRK2 is expressed almost exclusively by TH-positive A9 neurons which innervate the striatum [24, 25]. In order to identify the type of TH-positive neurons generated in our culture we performed double labelling for TH and calbindin or GIRK2 of neurons at 7 DIV. In addition, we probed for the presence of *nurr1*, a transcription factor belonging to the nuclear receptor family that is essential for the generation of midbrain dopaminergic neurons during embryonic development [26] as well as for DAT, the transporter responsible for the cellular reuptake of dopamine [27]. The majority of doublecortin-positive young neurons were calbindin positive (Fig. 2a), but the majority of TH-positive cells lacked expression of calbindin (Fig. 2b). Similarly, GIRK2 was essentially absent from TH-positive cells (Fig. 2c). The transcription factor *nurr1* was expressed by almost all TH-positive cells (Fig. 2d). Similarly DAT was expressed by TH-positive neurons, but DAT immunoreactivity was also identified in TH-negative neurons (Fig. 2e, f). These data suggested that the young TH-positive neurons formed in culture only occasionally expressed calbindin and GIRK2. But they revealed expression of the dopaminergic neuron markers *nurr1* and DAT. *Corin* a transmembrane protein expressed in the floor plate [28] and the homeodomain protein *LMX1a*, a determinant of midbrain dopamine neurons [29], could no longer be identified in the cultures.

The qualitative assessment of TUJ1- and TH-positive neurons was substantiated by the quantitative analysis shown in Fig. 3. The formation of TUJ1-positive neurons revealed a very slow onset and steeply rose after 5 DIV (Fig. 3a). This also applied to the TH-positive neurons (Fig. 3b). The contribution of the total cell population (as revealed by DAPI-staining) already began to increase at DIV 4 (Fig. 3c). Due to the high density, the total number

Fig. 3 Developmental expression of TUJ1 and TH in E10.5 ventral mesencephalon-derived cells. Dissociated cells were plated onto coverslips and first expanded for 1.5 days in growth factor-containing media before initiation of differentiation by a change to differentiation media. Immunocytochemical analysis was performed on day 7. Neurons (*TUJ1*) (a) and nuclei (*DAPI*) (c) were counted in 20 consecutive optical fields per cover slip. Tyrosine hydroxylase (*TH*)-positive neurons (b) were counted from the entire corresponding cover slips. Values are means ± SEM, three independent experiments

of cultured cells could no longer be determined after DIV5. These data clearly revealed that there is ongoing neurogenesis in cell cultures derived from the E10.5 midbrain and that a subpopulation of these newly formed cells acquires a TH-positive phenotype.

Identification of mRNA for proteins of the purinergic signalling pathway (E10.5)

In order to probe for a potential susceptibility of the cultured cells to nucleotides and also to adenosine we performed RT-PCR. The mRNA was extracted from the freshly dissected parent ventral mesencephalon or from cells cultured for 4 days under expansion conditions or for 7 days under differentiation conditions. mRNA was probed for all P2X (P2X1 to P2X7) and P2Y (P2Y1, P2Y2, P2Y4,

P2Y6, P2Y12, P2Y13, and P2Y14) nucleotide receptors, the adenosine (P1) receptors (A_1 , A_{2A} , A_{2B} , and A_3) and for the ectonucleotidases nucleoside triphosphate diphosphohydrolase 2 (NTPDase2) and tissue nonspecific alkaline phosphatase (TNAP). To exclude contamination with genomic DNA, a primer pair for the immunoglobulin heavy chain BiP was used.

The PCR data (Fig. 4) revealed that the E10.5 mouse midbrain in situ contained mRNA encoding a multitude of purinergic receptors. These included all P2X receptor subunits, all P2Y receptors except for P2Y4 (P2Y11 is absent from rodent tissue), all adenosine receptors as well as the ectonucleotidases NTPDase2 and TNAP. The P2X2 receptor revealed PCR products of varying length, indicating the presence of splice variants, in accordance with earlier studies that demonstrated abundant expression of P2X2 variants in the rat and mouse brain [30, 31]. This wide mRNA expression was largely maintained when cells were transferred into culture. When cultured under expansion conditions the mRNA of the P2X4 and P2X7 receptors were lost. In differentiated cells P2X4 revealed a strong signal and there was a very faint band for P2X7. Expression of all P2Y receptors was maintained when cells were subjected to culture. Similarly, all adenosine receptors and NTPDase2 and TNAP mRNA could be identified under all experimental conditions. The presence of only a 560-bp sequence (and the absence of a potential 316-bp-long intron sequence) for BiP excluded contamination with genomic

DNA. These data corroborate the impact of purinergic signalling in the developing mammalian brain [32].

Analysis of the effect of nucleotides on neuronal and dopaminergic differentiation (E10.5)

The data shown in Fig. 4 suggest that the isolated cells maintain the capacity to respond to a broad range of nucleotides and to adenosine. For this reason we applied quantitative immunoblotting to analyze the effect of the P2 receptor agonists ADP β S, 2MeSATP, ATP γ S, 2CIATP, UTP, ATP, and of adenosine on neuronal and dopaminergic phenotype formation. P2 receptors reveal a certain degree of promiscuity regarding the activation by endogenous ligands and nucleotide analogues [33–35]. ATP activates all homomeric and heteromeric P2X receptors and in addition P2Y1 and P2Y2 receptors and it is a partial agonist at P2Y4 and P2Y12 receptors. UTP is an agonist of P2Y2 and P2Y4 receptors. ADP β S activates P2Y1, P2Y12 and P2Y13 receptors whereas 2MeSATP activates these receptors and in addition essentially all P2X receptors. ATP γ S is an agonist of P2Y1 and P2Y12 receptors as well as of several P2X receptors and 2CIATP activates P2Y1, P2Y2 and P2Y12 receptors. Single doses of agonists were applied at differentiation days 1, 3 and 5 (all 50 μ M) and analysis was performed at differentiation day 7.

Nucleotides significantly affected the contribution of TH-positive cells (Fig. 5a). There was a small but significant

Fig. 4 RT-PCR of ventral mesencephalon-excised tissue at E10.5 and of mesencephalon-derived cells. Cells were kept in expansion medium for 4 days or in differentiation medium for 7 days. Primers were employed to target all P2X and P2Y nucleotide receptors and P1 adenosine receptors (A_1 to A_3) as well as the ectonucleotidases NTPDase2 (*N*) and tissue nonspecific form of alkaline phosphatase (*T*). GAPDH (*G*) was analysed as a control. For the

immunoglobulin heavy chain binding protein (*BiP*) only a single band at 560 bp could be detected, excluding contamination with genomic DNA. The shadow in the lane for P2Y4 does not represent a PCR product (expected to be 213 bp, Table 1) but a contaminant of the PCR reaction. The results are representative of three independent experiments

Fig. 5 Nucleotide-induced neuronal and dopaminergic differentiation of E10.5-derived cells. Cells derived from the ventral mesencephalon were plated on 24-well plates (30,000 cells/well) and expanded for 1.5 days in growth factor-containing media. Differentiation was initiated by a change to differentiation media. Nucleotides and adenosine (50 μ M) were added on differentiation days 1, 3 and 5. Vehicle was added to controls. Cells were harvested for quantitative immunoblotting on day 7 and analysed for TH (**a**) or doublecortin (DCX) (**b**). **c** Ratio of TH- and DCX-positive neurons. Values are means $\pm$ SEM ($n=11-13$). Significantly different from control * $p<0.05$; ** $p<0.01$ (ANOVA one way t test). *Bottom of the graph:* representative immunoblots probed for TH and DCX and for GAPDH as a loading control. Bands correspond to 60 kDa for TH, 40 and 43 kDa for DCX and 35 kDa for GAPDH

increase (up to 29%) in the contribution of TH-expressing cells after addition of ATP, ADP β S, 2MeSATP, and of adenosine. The strongest increase (90%) was obtained with 2CIATP. ATP γ S induced a minor decrease in TH immunoreactivity. Similar results were obtained for doublecortin-positive neurons (Fig. 5b). In this case, however ADP β S, 2MeSATP, ATP γ S and UTP induced a significant increase whereas 2CIATP induced a loss of 58%. When the contribution of TH-positive neurons to the total of doublecortin-expressing neurons was analysed (Fig. 5c), the effects were largely cancelled out—with the exception of 2CIATP which increased the ratio by a factor of 4.5. This suggests that in general the contribution of TH-positive neurons was increased in parallel to the total of young neurons but that TH-expressing neurons were resistant to the deteriorating effects of 2CIATP on neuron formation. No apparent effect was observed of nucleotides and adenosine on either process length or ramification.

Analysis of neuronal and dopaminergic differentiation at E13.5

For comparison we isolated and cultured cells from the embryonic midbrain at stage E13.5, when dopaminergic neurons in this brain region are largely postmitotic [5, 6]. As shown in Fig. 6a, b, nestin-positive precursors were abundant in the culture at 1 DIV. After 7 DIV these cells had proliferated to cover the entire coverslip. Already from the beginning the culture contained a considerable contribution of differentiated neurons (as revealed by immunostaining for β III tubulin, TUJ1) (Fig. 6c). Their contribution did not further increase during subsequent days of culturing (Fig. 6d). Similarly, TH-positive neurons were already present in the acutely isolated cell population (Fig. 6e). The contribution of TH-positive neurons remained unaltered on continued cell culture (Fig. 6f). A small contribution of TUJ1-positive cells also expressed TH (Fig. 6h). No GFAP-positive astrocytes could be detected at DIV 1 (not shown) but few clusters of GFAP-positive cells were observed at 7 DIV (Fig. 6g). We further identified coexpression of calbindin and GIRK2 in subpopulations of TH-positive neurons (Fig. 6i, j). These data suggested that at 13.5 DIV mainly mature neurons are isolated from the ventral midbrain. Nestin-positive cells are isolated at E13.5 but they do not carry significant potential for the formation of neurons and of astrocytes or oligodendrocytes (not shown).

The lack of new neuron formation is also revealed by the quantitative analysis (Fig. 7). The contribution of TUJ1-positive neurons (Fig. 7a) rather decreases and the number of TH-positive cells remains constant (Fig. 7b). As revealed by the analysis of DAPI-stained nuclei, the contribution of total cells remained constant during the initial culture

Fig. 6 Immunocytochemical characterization of E13.5-derived cells. Images are derived from stage day in vitro 1 (*DIV 1*) (**a**, **c**, **e**) or stage day in vitro 7 (*DIV 7*) (**b**, **d**, **f**, **g–j**). They represent overlays of double staining for nestin (red) and DAPI (blue) (**a**, **b**), TUJ1 (green) and DAPI (blue), (**c**, **d**), tyrosine hydroxylase (TH) (red) and DAPI (blue) (**e**, **f**) and GFAP (red) and DAPI (blue) (**g**). **h** Triple labelling for TH (red), TUJ1 (green) and DAPI (blue), **i**, **j** Triple labelling for calbindin (green), TH (red) and DAPI (blue) and GIRK2 (red), TH (green) and DAPI (blue), respectively. Scale bars=30 μ m

Fig. 7 Developmental expression of TUJ1 and TH in E13.5 ventral mesencephalon-derived cells. Dissociated cells were plated onto coverslips and first expanded for 1.5 days in growth factor-containing media before initiation of differentiation by a change to differentiation media. Immunocytochemical analysis was performed on day 7. Neurons (*TUJ1*) (**a**) and nuclei (*DAPI*) (**c**) were counted in 20 consecutive optical fields per cover slip. Tyrosine hydroxylase (*TH*)-positive neurons (**b**) were counted from the entire corresponding cover slips. $n=3$

period but was considerably increased after 6 days (Fig. 7c). Together with the immunocytochemical analysis (Fig. 6b) this suggests that the proliferating cells represent mainly nestin-positive undifferentiated precursors.

Identification of mRNA encoding proteins of the purinergic signalling pathway (E13.5)

As for stage E10.5 the ventral midbrain contained the mRNA for a multitude of purine receptors (Fig. 8). These included the P2X receptor subunits, the P2Y receptors except for P2Y4, all adenosine receptors and the ectonucleotidases NTPDase2 and TNAP. In cultured cells this pattern was maintained. The specificity of the PCR was again confirmed using primers for BiP. The presence of

Fig. 8 RT-PCR of ventral mesencephalon at E13.5 and of mesencephalon-derived cells. Cells were kept in expansion medium for 4 days or in differentiation medium for 7 days. Primers were employed to target all P2X and P2Y nucleotide receptors and P1 adenosine receptors (A1 to A3) as well as the ectonucleotidases NTPDase2 (*N*) and tissue nonspecific form of alkaline phosphatase (*T*). GAPDH (*G*) was

analysed as a control. For the immunoglobulin heavy chain binding protein (*BiP*) only a single band at 560 bp could be detected, excluding contamination with genomic DNA. The shadow in the lane for P2Y4 does not represent a PCR product (expected to be 213 bp, Table 1) but a contaminant of the PCR reaction. The results are representative of three independent experiments

only a 560 bp sequence for BiP excluded contamination with genomic DNA. When genomic DNA isolated from the cultured cells was analysed a strong 876 bp PCR product was amplified (that contained the 316 bp long intron sequence) (not shown).

Analysis of the effect of nucleotides on neuronal and dopaminergic differentiation (E13.5)

Cells isolated at E13.5 maintained the capacity to respond to a broad range of nucleotides (Fig. 9). Single doses of agonists were again applied at differentiation days 1, 3 and 5 (all 50 μ M) and analysis was performed at differentiation day 7. The outcome of nucleotide application differed considerably from that of cells isolated at stage E10.5. ADP β S and ATP γ S had a deleterious effect on TH-positive neurons but 2MeSATP (factor of 2.3) and adenosine (factor of 1.5) enhanced the contribution of TH-positive neurons (Fig. 9a). 2MeSATP and UTP increased the contribution of (TUJ1-positive neurons) whereas adenosine was without effect. 2CIATP diminished the contribution of neurons and ADP β S and ATP γ S nearly eliminated the neurons (Fig. 9b). This is reflected by the ratio of TH-expressing cells to the total of TUJ1-expressing neurons (Fig. 9c). Whereas ADP β S and ATP γ S exerted an overall deleterious effect, 2CIATP induced a relative enrichment of dopaminergic neurons. In order to analyze the overall effect of the agonists on total cell number, we analysed in addition the total number of DAPI-stained nuclei (Fig. 9d). The data

reveal that ADP β S, ATP γ S and 2CIATP diminished the number of cultured cells, but the survival of neurons was preferentially deteriorated.

Taken together, nucleotides have little effect on promoting neurogenesis and dopaminergic differentiation when cells are derived from E13.5 midbrains and ADP β S and ATP γ S strongly decrease the survival and of cultured neurons.

Discussion

Our study reveals that neural progenitors derived from the E10.5 mouse midbrain proliferate and have the potential to produce dopaminergic neurons when subjected to adherent cell culture. These neurons not only express TH, a hallmark of catecholaminergic neurons but also the dopamine transporter suggesting that they represent functional dopaminergic neurons [27]. Most of the TH-positive neurons did not express calbindin or GIRK2, implicating that they have not yet acquired all characteristics typically displayed by dopaminergic neurons in the subfields A9 and A10 of the adult murine brain [8, 24, 25]. In accordance with previous investigations [7], our data show that at E13.5 the ventral mouse midbrain contains mainly postmitotic dopaminergic neurons. Most of these TH-positive neurons also express calbindin or GIRK2. These cells survived in culture for several days but no significant neurogenesis could be observed.

One of the significant findings of our study concerns the presence in fetal mouse ventral midbrain of mRNA encoding essentially all P2X and P2Y receptors (except for P2Y4) as well as all adenosine receptors. This is in accord with previous reports that nucleotide and nucleoside receptors are abundantly expressed in the developing mammalian brain (for review see [32]). This broad

Fig. 9 Nucleotide-induced neuronal and dopaminergic differentiation of E13.5-derived cells. Cells derived from the ventral mesencephalon were plated on coverslips (50,000 cells/well) and expanded for 1.5 days in growth factor-containing media. Differentiation was initiated by a change to differentiation media. Nucleotides and adenosine (50 μ M) were added on differentiation days 1, 3 and 5. Vehicle was added to controls. Immunocytochemical analysis for tyrosine hydroxylase (TH) (a) or TUJ1 (b) was performed on day 7. c Ratio of TH- and TUJ1-positive neurons. d Total number of DAPI-stained nuclei relative to control. Neurons (TUJ1) and nuclei (DAPI) were counted in 20 consecutive optical fields per cover slip. TH-positive neurons of the entire corresponding cover slips were counted. Values are means $\pm$ SEM ($n=3-11$). Significantly different from control * $p<0.05$; ** $p<0.01$ (ANOVA one way t test)

expression profile is maintained in the cultured fetal progenitor cell population. In accordance with our results, RT-PCR identified all P2Y receptors except for P2Y4 as well as all P2X receptors in cultured and adherent adult neural progenitor cells [16]. Adult mouse subventricular zone-derived progenitors cultured as neurospheres were also found to express all P2Y receptors with the exception of P2Y4 [36]. In addition several studies report the presence of mRNA encoding P2 receptors in astrocytes cultured from rodent brain. While the extent of analysis varies between studies, mRNAs for all subtypes of P2Y and P2X receptors have been reported [37, 38]. The presence of mRNA does not prove the presence of functional protein. But functional P2Y1, P2Y13 and P2Y2 receptors activated by ADPβS or UTP, respectively, and A1 adenosine receptors were previously identified in cultured adult progenitors derived from the rodent subventricular zone [13, 14, 16, 36, 39]. Similarly, mouse embryonic stem cell-derived neuronal cultures contained mRNA for P2X1-7 receptors as well as P2Y1,2 and P2Y6 receptors, whereby P2X2, P2X4 and P2Y1 receptors elevated intraneuronal Ca^{2+} [40].

Our study clearly demonstrates the presence of functional nucleotide and adenosine receptors in progenitors cultured from mouse fetal midbrain. However, the outcome on neuronal and dopaminergic differentiation and cell survival differed considerably between cells isolated at E10.5 and E13.5. At E10.5 all agonists tested except for 2ClATP increased neuron formation but did not specifically enrich TH-expressing cells. 2ClATP in turn considerably reduced the contribution of neurons but induced a relative increase in the contribution of TH-positive neurons. At E13.5 2ClATP and also ADPβS and ATPγS reduced total cell number and the contribution of neurons whereas 2MeSATP increased the contribution of TUJ1- and TH-positive neurons.

In principle, the outcome of agonist application could be governed by the presence or absence of individual functional P2 receptor subtypes, the varying cell-specific expression of intracellular signalling cues or also by the conditions of culture and agonist application. For example, at a concentration of 50 μ M, the P2Y1 receptor can be

activated by ADP β S, ATP γ S, 2MeSATP and 2ClATP. But each of these receptors can activate at these concentrations additional P2Y receptors and in the case of ATP γ S and 2MeSATP also P2X receptors [33–35]. Furthermore, it is possible that indirect effects are involved. In several neuronal and glial cell lines 2ClATP was found to induce either cell cycle arrest or cell death, acting neither on P2 nor on P1 receptors, but being extracellularly metabolised into 2-Cl-adenosine, intracellularly transported and re-phosphorylated [41]. But the outcome differed in other cellular systems. 2ClATP enhanced neurite outgrowth in PC12 cells and sustained cell survival after serum deprivation [42]. In mouse olfactory epithelium, ATP γ S induced proliferation and neuronal differentiation [43] whereas in our study it abrogated E13.5-derived progenitor cells, in contrast to 2MeSATP. 2MeSATP was found to stimulate catecholamine release from chromaffin cells [44] or to promote neurite outgrowth in entorhino-hippocampal slice cocultures [45]. Thus, even though ADP β S, ATP γ S and 2ClATP share their deleterious effect on progenitors they may exert their function via different mechanisms.

The P2X7 receptor is another candidate for induction of cell death. ATP has been reported to induce P2X7 receptor-mediated necrotic swelling in a dopaminergic cell line (SN4741 [46]) and to induce necrosis of cultured neural precursor cells [47]. Whereas mRNA for P2X7 receptors was expressed in the intact midbrain tissue, P2X7 mRNA was almost absent from E10.5-derived cells but it was present in the more labile E13.5-derived cells.

The outcome of nucleotide stimulation on in vitro neurogenesis and dopaminergic differentiation observed in this study differed from that of immortalised human midbrain-derived neural progenitors [18]. In these cells, UTP augmented cell proliferation and both UTP and UDP increased the pool of dopaminergic cells. In contrast, ATP and ADP exhibited antiproliferative effects and ADP reduced the contribution of dopaminergic cells. Microarray analyses revealed the presence of P2Y1 (ADP, ATP), P2Y4 (UTP) and P2X4 (ATP) receptors. RT-PCR confirmed the mRNA for these three subtypes of P2 receptors and further revealed the presence of P2Y6 (UDP) and the absence of P2Y2 (UTP, ATP) mRNA. While in this study the effects of UTP apparently were solely mediated by P2Y4 receptors in our study only P2Y2 receptors could have contributed to the UTP-mediated effects. mRNA for the UDP-activated P2Y6 receptor was present in our cellular systems but UDP had no effect on the cultures (not shown). Typically P2Y1, P2Y2, P2Y4 and P2Y6 receptors couple to Gq leading to activation of phospholipase C β and the MAP kinase pathway. But additional protein interactions as well as $\beta\gamma$ subunit-mediated activation pathways have been reported and may lead to cell-specific differences in experimental outcome [34]. In contrast, P2Y12 (ADP), P2Y13 (ADP)

and P2Y14 (nucleotide sugars) receptors inhibit adenylate cyclase and reduce intracellular levels of cAMP. Finally, all activated P2X receptors increase the permeability for Na⁺, K⁺ and Ca²⁺. Accordingly, any effects of the endogenous ligands ATP, ADP (as a hydrolysis product of ATP), UTP or UDP (as a hydrolysis product of UDP) will depend on the relative potency and cell-specific signal characteristics of the functionally expressed receptors. These data clearly show that care has to be taken when comparing agonist-dependent effects of cells from different donor species and cultured under differing conditions.

Taken together our results reveal that the fetal dopaminergic ventral midbrain expresses essentially all receptor systems as well as ectonucleotidases required for extracellular nucleotide/nucleoside signalling. This applies also to the progenitors subjected to adherent cell culture. Purinergic agonists carry potential for stimulating neurogenesis whereby 2ClATP causes a relative enrichment of dopaminergic neurons. Nucleotide receptor agonists may be of value to contribute to the formation and survival of dopaminergic neurons in vivo.

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