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Astrocytes derived from fetal neural progenitor cells as a novel source for therapeutic adenosine delivery

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ABSTRACT

Purpose: Intracerebral delivery of anti-epileptic compounds represents a novel strategy for the treatment of refractory epilepsy. Adenosine is a possible candidate for local delivery based on its proven anti-epileptic effects. Neural stem cells constitute an ideal cell source for intracerebral transplantation and long-term drug delivery. In order to develop a cell-based system for the long-term delivery of adenosine, we isolated neural progenitor cells from adenosine kinase deficient mice ($Adk^{-/-}$) and compared their differentiation potential and adenosine release properties with corresponding wild-type cells.

Methods: Fetal neural progenitor cells were isolated from the brains of $Adk^{-/-}$ and C57BL/6 mice fetuses and expanded in vitro. Before and after neural differentiation, supernatants were collected and assayed for adenosine release using liquid chromatography–tandem mass spectrometry (LC–MS/MS).

Results: $Adk^{-/-}$ cells secreted significantly more adenosine compared to wild-type cells at any time point of differentiation. Undifferentiated $Adk^{-/-}$ cells secreted 137 ± 5 ng adenosine per 10^5 cells during 24 h in culture, compared to 11 ± 1 ng released from corresponding wild-type cells. Adenosine release was maintained after differentiation as differentiated $Adk^{-/-}$ cells continued to release significantly more adenosine per 24 h (47 ± 1 ng per 10^5 cells) compared to wild-type cells (3 ± 0.2 ng per 10^5 cells).

Conclusions: Fetal neural progenitor cells isolated from $Adk^{-/-}$ mice – but not those from C57BL/6 mice – release amounts of adenosine considered to be of therapeutic relevance.

1. Introduction

Epilepsy is a chronic neurological disorder with a prevalence between 0.5 and 1%.[1,2] Despite the availability of several anti-epileptic drugs, about 30% of patients with epilepsy still suffer from uncontrolled seizures or medication-related side effects.[3] Intracerebral delivery of anti-epileptic compounds represents a novel strategy for the treatment of refractory epilepsy.[4,5] Adenosine is a possible candidate for local delivery because of its proven anti-epileptic effects. Systemic application of adenosine is not possible because of severe side effects such as decreased heart rate, blood pressure and body temperature.[6] Adenosine is a ubiquitous neuromodulator of the brain and acts via binding to G-protein coupled adenosine receptors. The inhibitory effect of adenosine is mainly due to binding to the high-affinity A_1 receptor that is expressed in cerebral cortex, hippocampus, thalamus, cerebellum, brain stem and spinal cord.[7,8] Several in vivo studies have already proven the feasibility and anti-seizure effects of local delivery of adenosine in different animal models.[9–13] In those studies, a synthetic adenosine-releasing polymer or encapsulated adenosine-releasing cells from different sources were implanted into the lateral ventricle of kindled rats. However, the anti-seizure effect of adenosine decreased during the second week of treatment because of expiration of adenosine release from the polymer or limited long-term viability of the encapsulated cells. Further attempts to extend the adenosine release resulted in the successful development of a silk protein-based release system for adenosine. Implantation of this system in the infrahippocampal fissure of rats resulted in seizure protection and retardation of kindling acquisition.[14,15] However, these therapeutic effects lasted for a maximum of 10 days, corresponding to the duration of adenosine release from this system. Since epilepsy is a chronic disorder and lifelong treatment is required, development of an implantable adenosine source that is able to produce a sufficient and stable

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dose without the need of future replacement is necessary. A promising technique may be local delivery via implantation of stem cells.[16–18]

In brain, adenosine can be metabolized and removed by three enzymes that control levels of ambient intracellular adenosine: adenosine kinase (ADK), adenosine deaminase (ADA) and S-adenosyl-homocysteine hydrolase (SAHH). Based on its low K_M for adenosine, the key enzyme for the control of adenosine levels is ADK,[19,20] which, in adult brain, is almost exclusively expressed in astrocytes.[21] Overexpression of ADK is a pathological hallmark of the epileptic brain and associated with a deficiency of the endogenous anticonvulsant adenosine and the expression of spontaneous seizure activity.[22–25] Therefore, inhibition of ADK constitutes a neurochemical rationale to increase adenosine levels and to provide seizure control. To this end adenosine-releasing embryonic stem cells have been engineered based on a bi-allelic genetic disruption of the *Adk*-gene,[26] and successfully been used to suppress kindling acquisition in rats[27] and the development of epilepsy in mice.[24] In these experiments it was necessary to differentiate the embryonic stem cells before implantation to avoid teratoma formation and genetic alterations by undifferentiated embryonic stem cells.[28,29] Several strategies are available to improve functional and safety concerns of embryonic stem cells. Differentiation of embryonic stem cells can be controlled by specific selection and culture procedures or genetic modification. Genetic aberrations must be excluded by early passage karyotyping and tumor formation can be excluded by depletion of tumorigenic cells or enrichment of non-tumorigenic cells.[30] Nevertheless it remains to be proven whether these strategies are sufficient to guarantee long-term survival and therapeutic action. Isolation of fetal or adult stem cells could avoid these ethical and practical problems coupled to embryonic stem cells and improve the safety of neural transplantation approaches. In addition, the transplantation of neural stem cells may have additional benefits since they have the potential ability to integrate in the neural network.[31] Combination with local delivery of an anti-epileptic substance like adenosine may lead to a promising and effective therapy. As a potential cell source, an adenosine kinase knockout mouse ($Adk^{-/-}$) has been developed.[32] Since $Adk^{-/-}$ mice die within 14 days due to hepatic steatosis, the isolation of adult neural $Adk^{-/-}$ stem cells is not a possibility.[32] In this paper we describe the isolation and characterization of $Adk^{-/-}$ fetal neural progenitor cells as a source for therapeutic adenosine delivery. Neural differentiation in vitro and adenosine secretion is compared with wild-type cells derived from C57BL/6 mice.

2. Materials and methods

2.1. Animals

Mice [$Adk^{+/-}$ – breeders in the C57BL-6 background – (Robert Stone Dow Neurobiology Laboratories, Legacy Research, USA) and matching wild-type C57BL/6 mice (Harlan, The Netherlands)] were housed according to the guidelines approved by the European Ethics Committee (decree 86/609/EEC). The study protocol was approved by the Animal Experimental Ethics Committee of Ghent University Hospital (ECP 07/25). The animals were kept under environmentally controlled conditions (12 h normal light/dark cycles, 21–22 °C and 50% relative humidity) with food and water ad libitum.

2.2. Isolation of neural stem cells

Fetal neural stem cells were isolated from $Adk^{-/-}$ and wild-type C57BL/6 mice fetuses at embryonic day 14 (E14). Four pregnant mice from $Adk^{+/-} \times Adk^{+/-}$ matings (all mutants were maintained in the C57BL/6 background) and two pregnant C57BL/6 mice were sacrificed by cervical dislocation; the uteri were removed and transferred to a dish with ice-cold phosphate buffered saline (PBS). The uterine horns were opened and the fetuses removed. Their brains were removed and placed in separate dishes with ice-cold PBS. The cortex was isolated via microdissection and put into culture medium (see below). The tissue was dissociated by trituration, followed by centrifugation at 80 *g* for 10 min. The pellet was resuspended in fresh medium and the cells were counted. Their viability was checked with the trypan blue exclusion method and cells were cultured in T25 flasks at a density of 10,000/cm^2.

The neural stem cell growth medium consisted of NS-A medium (StemCell Technologies SARL, Grenoble, France) with an additional 2 mM L-glutamine (Cambrex, Verviers, Belgium), 3 mM D-glucose (Sigma, Bornem, Belgium), 2% B_{27} (Invitrogen, Merelbeke, Belgium), 1% N_2 supplement (Invitrogen), 100 U/ml penicillin (Cambrex), 100 U/ml streptomycin (Cambrex), 20 ng/ml of human recombinant epidermal growth factor (EGF, Sigma) and 20 ng/ml of recombinant human basic fibroblast growth factor (bFGF, R&D, Abingdon, UK). Cells were grown at 37 °C in 5% CO_2 and 95% air with saturated humidity. They were passaged once cell clusters, with a diameter of about 100 μm, were formed, approximately 2 weeks after initial isolation. Subsequent passages were done every 4–5 days. When sufficiently large cell numbers were obtained, cells were further cultured in T75 flasks at a density of 10,000 cells/cm^2.

2.3. PCR of adenosine-releasing cells

Since the fetuses were derived from two heterozygote $Adk^{+/-} \times Adk^{+/-}$ matings, cell colonies of three different genotypes ($Adk^{+/+}$, $Adk^{+/-}$ and $Adk^{-/-}$) were isolated and genotyped by PCR. DNA from expanded cell colonies was extracted using the GenElute Mammalian Genomic DNA Purification Kit (Sigma) and subjected to PCR with allele-specific primer sets. PCR reactions were performed under standard conditions using three primers simultaneously: o107, 5′-CTC ACT TAA GCT GTA TGG AGGTGACCG-3′- (sense primer specific for wild-type Adk), o108, 5′-AGT CAC AGA TGC ATC TGC AGA GGT GAG-3′- (antisense primer specific for wild-type Adk), and o109, 5′-ACT GGG TGC TCA GGT AGT GGT TGT CG-3′- (antisense primer specific for targeting construct).

2.4. Western Blot

Western Blot analysis for ADK expression was performed on cell lysates of undifferentiated and differentiated $Adk^{-/-}$ cells. Corresponding wild-type cells served as control. GAPDH expression was used to control for protein concentration.

Following harvesting and washing the cells with PBS, total cell lysates were made by adding Laemmli buffer followed by sonication. Protein concentration was measured and protein extracts were applied to a 4–12% Bis–Tris gel (Invitrogen) for electrophoresis by using MES/SDS running buffer (Invitrogen). Then proteins were transferred to Protran Nitrocellulose Hybridization Transfer membrane (0.2 μm pore size; PerkinElmer, Belgium) with transfer buffer (Invitrogen). After the blotting process, the membranes were blocked for 1 h in Tris Buffered Saline (TBS) containing 0.075% Tween 20 (Invitrogen) and 5% nonfat milk, followed by addition of the primary antibodies: rb anti-ADK 1:6000 (custom made at RS Dow Neurobiology Laboratories, Legacy Research, Portland, USA) or ms anti-GAPDH 1:1000 (Santa Cruz Biotechnology, sc-47724). Primary antibodies were incubated for 1 h. After rinsing, secondary antibodies anti-rb/ms IgG Horseradish Peroxidase 1:3000 were added during 90 min at room temperature (GE Healthcare, Buckinghamshire, UK, NA934/NA931). After thorough rinsing, signals were visualized using a commercial enhanced bioluminescence detection method (ECL) kit (Invitrogen).

2.5. Immunocytochemistry

To evaluate the expression of neural stem cell markers (nestin, GFAP and Sox-2), *Adk*$^{-/-}$ cells and corresponding wild-type cells were plated at a density of 20,000 cells/cm^2 on laminine coated (3 μg/cm^2, Roche Diagnostics, Vilvoorde, Belgium) chamber slides (VWR International, Leuven, Belgium). The cells were plated in neural stem cell growth medium without addition of the growth factor EGF for 24 h. Then they were fixed with 4% paraformaldehyde (PFA) for 15 min and subjected to immunocytochemistry (see below).

For neural differentiation, *Adk*$^{-/-}$ cells and corresponding wild-type cells were plated for 24 h in neural stem cell growth medium without the addition of EGF at a density of 50,000 cells/cm^2 on laminine coated (3 μg/cm^2) chamber slides. Then the medium was changed to neural stem cell growth medium without added growth factors but with the addition of 3% Fetal Calf Serum (FCS, Serum Supreme, Cambrex). On day 7 cells were fixed with 4% PFA and subjected to immunocytochemistry for the three different neural phenotypes: neurons (tau), glial cells (GFAP) and oligodendrocytes (RIP). The fixed cells were rinsed with 50 mM NH_4Cl for 10 min (quenching), permeabilized and blocked in PBS containing 0.4% fish skin gelatine (FSG) and 0.3% Triton X-100 (PBS/FSG/TX100). This was followed by incubation overnight at 4 °C with primary antibodies (ms, mouse monoclonal; rb, rabbit polyclonal): rb anti-Sox2 1:1000 (Chemicon, Brussels, Belgium, AB5603), rb anti-nestin 1:5000 (Eurogentec, Seraing, Belgium, PRB-315C-0100), rb anti-tau 1:2000 (Dako, Heverlee, Belgium; A0024), rb anti-GFAP 1:400 (Dako, Z0334), ms anti-RIP 1:5000 (Chemicon, MAB1580). After rinsing, the cells were incubated with the secondary antibodies Alexa Fluor 594 goat anti-rabbit IgG 1:1000 (Invitrogen, A11072) or Alexa Fluor 594 goat anti-mouse IgG 1:1000 (Invitrogen, A11020) diluted in PBS/FSG/TX100 for 2 h each. Chamber slides were then rinsed and nuclei were stained with 0.3 μM 4′-6-diaminido-2-phenylindole (DAPI, Invitrogen) for 1 min. After additional rinsing, slides were mounted with Vectashield mounting medium (Labconsult, Brussels, Belgium). To assess the fraction of cells expressing a specific marker, immunopositive cells were counted in nine randomly selected high power fields. The relative amount of cells positive for a specific marker was obtained by dividing the total number of immunopositive cells by the total number of nuclei counted. The cells were evaluated under a fluorescence microscope. In control slides the primary antibody was omitted and no immunostaining was detected in these controls.

2.6. In vitro adenosine release

The amount of adenosine released from the cells was assessed from plated cells. Adenosine release from *Adk*$^{-/-}$ cells was compared with adenosine release from corresponding wild-type cells and this under different experimental conditions. First, samples were collected from supernatants of non-differentiated cells. Therefore cells were plated at densities of 10,000 cells/cm^2 and 100,000 cells/cm^2 in a laminine coated (3 μg/cm^2) 96-well plate (200 μl medium/well) in neural stem cell growth medium without EGF. After 24 h, medium was replaced with fresh medium without growth factors but with addition of the adenosine deaminase inhibitor erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride (EHNA, 50 μM, Sigma). The addition of EHNA was necessary to prevent adenosine breakdown in the culture medium. It has been shown that addition of EHNA to cultured neurons or astrocytes is not toxic for the cells.[33] At different time points after medium replacement (1 h, 2 h, 4 h, 8 h, 12 h and 24 h) samples of 200 μl supernatants were taken, each time from a different well. Samples were subsequently frozen at −20 °C until later analysis. At each time point six samples were taken.

Second, a similar protocol was performed to collect samples from differentiated cells. For this condition, cells were plated at a density of 50,000 cells/cm^2 in a laminine coated (3 μg/cm^2) 96-well plate (200 μl medium/well) in neural stem cell growth medium without EGF. After 24 h the medium was changed to neural stem cell growth medium without growth factors but with addition of 3% FCS. Seven days later medium was replaced with fresh medium without growth factors or FCS but with the addition of EHNA (50 μM) and samples of supernatants were collected (1 h, 2 h, 4 h, 8 h, 12 h and 24 h after medium replacement; six samples per time point) and frozen for later analysis.

At each sample collection, cells were counted with the trypan blue exclusion method and averaged. These data were used for normalization and quantification of adenosine release per cell number.

2.7. Sample analysis

Samples were analyzed using liquid chromatography–tandem mass spectrometry (LC–MS/MS) according to an earlier described protocol.[34] Liquid chromatographic separation was performed using an Agilent LC 1100 HPLC system (Agilent Technologies, Santa Clara, US) equipped with a quaternary pump, a column oven and a 100 well-plate autosampler. The LC was coupled to an API 2000 Triple Quadrupole system (Applied Biosystems/MDS Sciex, Foster City, US) equipped with an APCI interface. Chromatographic separation was done using a reversed phase column, the XBridge C_8 column 4.6 mm × 75 mm, 3.5 μm (Waters, Zellik, Belgium) and the temperature of the column oven was set at 40 °C. The solvent system consisted of 2 mM ammonium acetate in water (A) and 2 mM ammonium acetate in methanol (B). An isocratic elution with 65% A and 35% B (v/v) was used with a flow rate was of 500 μl/min and an injection volume of 5 μl. The total run time was 4.5 min per analytical run. Mass spectrometer analysis was set up in selected reaction monitoring (SRM) in positive polarity. Based on the component dependent parameters the SRM transitions of m/z 268.2/136.1 and 302.2/170.0 were selected, respectively for adenosine and IS.

Before analysis of the samples, 2-chloroadenosine (1000 ng/ml, 20 μl) was added as internal standard to each sample. At each analytical run a new calibration curve was made consisting of a dilution series of 10 concentrations of adenosine in medium with addition of internal standard.

2.8. Data analysis and statistics

At each sample collection, six different replicates were taken and the mean was calculated. Adenosine secretion from differentiated and non-differentiated cells was normalized to secretion per 10^5 cells. Statistical evaluation (SPSS 15.0) was performed using parametric tests. All data were expressed as means and standard errors of the mean. $p < 0.05$ indicates a significant difference.

3. Results

Neurosphere-forming cells were isolated as described above from the cortex of E14 fetal mice derived from *Adk*$^{+/-}$ × *Adk*$^{+/-}$ matings and from respective wild-type dams. Approximately 2 weeks after initial isolation – when their diameter ranged from 100 to 150 μm – neurospheres could be passaged mechanically and further passages were performed every 4–5 days. At passage 7, PCR analysis with allele-specific primer sets (o107, o108 and o109) was performed (Fig. 1A). The wild-type specific primer set (o107/o108) gave rise to a 640 bp band, whereas the knockout specific primer set (o107/o109) showed a 840 bp band. Based on PCR results one cell clone homozygous for *Adk*$^{-/-}$ and one from the C57BL/6

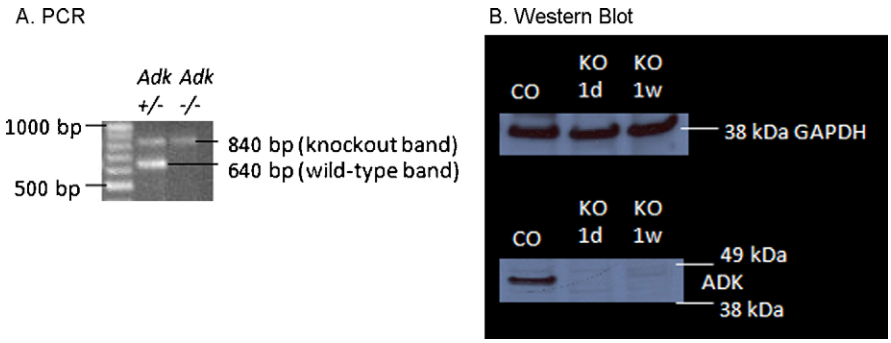

Fig. 1. PCR and Western Blot. (A) PCR: results of isolated cells from $Adk^{+/-} \times Adk^{+/-}$ matings. Based on these results, an $Adk^{-/-}$ clone needed for the experiment could be detected. The genomic DNA was amplified using the three allele-specific primers o107, o108 and o109. DNA from $Adk^{+/-}$ cells gives a combination of the wild-type specific 640 bp band (primers o107/o108) and the knockout-specific 840 bp band (primers o107/o109), while DNA from $Adk^{-/-}$ cells gives rise to only a 840 bp band (primers o107/o109), demonstrating the bi-allelic genetic disruption of the *Adk* locus. (B) Western Blot: above the GAPDH immune reactivity for protein control is shown (38 kDa). Beneath ADK immune reactivity is shown (44–46 kDa). A clear reactivity is found in wild-type cells, whereas no reactivity is found in non-differentiated and differentiated $Adk^{-/-}$ cells (CO = control wild-type; KO 1d = $Adk^{-/-}$ after 24 h; KO 1w = $Adk^{-/-}$ after 1 week).

control were selected for all subsequent experiments. Additionally protein extracts from both cell clones were investigated with Western Blot analysis for ADK expression (Fig. 1B). The control cells showed expression for ADK whereas no expression was found in the $Adk^{-/-}$ cell clones, confirming the PCR results.

To analyze stem cell properties for both cell clones, we performed immunohistochemical staining of the undifferentiated isolated cells. Three neural stem cell markers (nestin, GFAP and Sox-2) were evaluated and expression of all markers was found in both $Adk^{-/-}$ and wild-type cells (Fig. 2A–D). For the nuclear marker Sox-2, we performed a quantitative analysis by randomly selecting nine high power fields (200×) under a fluorescence microscope. The amount of immunopositive cells was counted together with the total number of nuclei. The results revealed Sox-2 expression in 361/570 (63%) of the $Adk^{-/-}$ cells and in 324/528 (61%) of the corresponding wild-type cells.

Since $Adk^{-/-}$ cells were obtained by genetically manipulation, their differentiation potential was evaluated and compared with wild-type cells. Therefore cells were subjected to growth in differentiation medium for 7 days followed by immunohistochemical staining for the three different neural phenotypes: neurons (tau), glial cells (GFAP) and oligodendrocytes (RIP). Both $Adk^{-/-}$ cells and wild-type cells expressed tau and GFAP (Fig. 3A–D). Quantitative analysis – as described above for the Sox-2 marker – demonstrated expression of tau in 6% (42/694) and GFAP in 28% (178/640) of the $Adk^{-/-}$ cells. Similar ratios were obtained in wild-

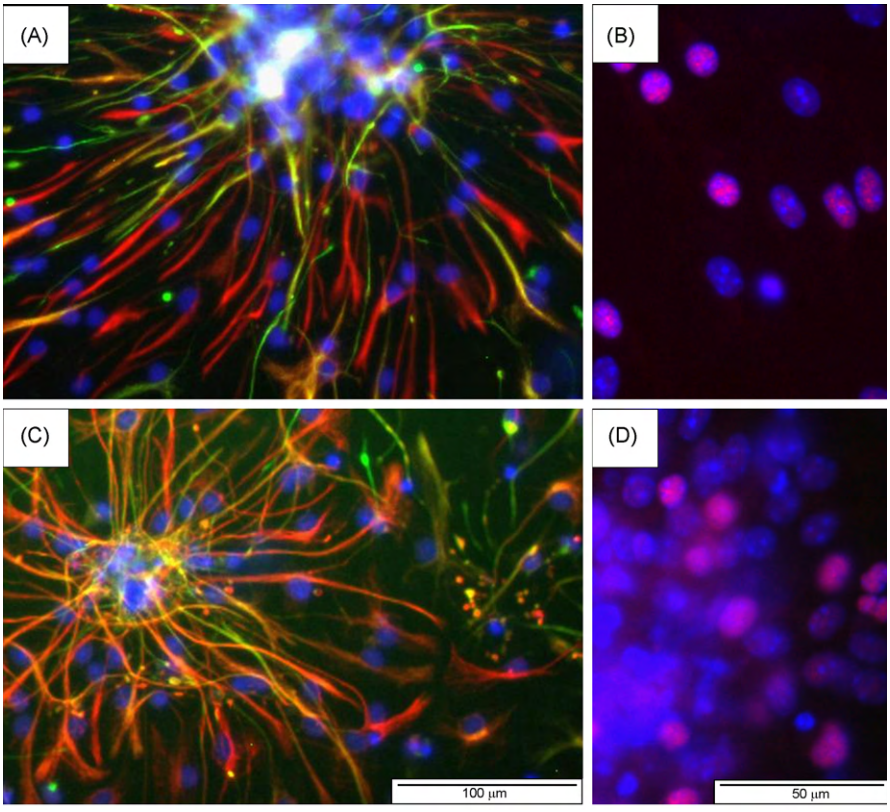

Fig. 2. Expression of neural stem cell markers. *Left*: Immunostaining with nestin (red) and GFAP (green) in $Adk^{-/-}$ cells (A) and wild-type cells (C). Scale bar = 100 µm. *Right*: Immunostaining with Sox2 (red) in $Adk^{-/-}$ cells (B) and wild-type cells (D). Scale bar = 50 µm. Nuclei are stained with DAPI (blue).

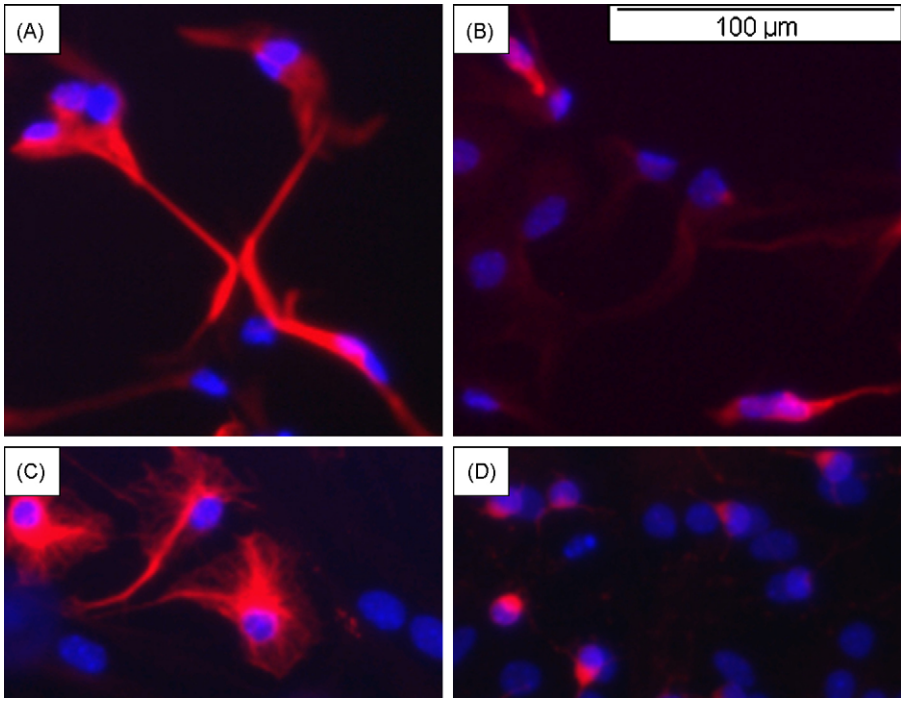

Fig. 3. Glial and neural differentiation of neural progenitor cells in vitro. *Left*: Immunostaining with GFAP (red) in *Adk*$^{-/-}$ cells (A) and wild-type cells (C). *Right*: Immunostaining with tau (red) in *Adk*$^{-/-}$ cells (B) and wild-type cells (D). Nuclei are stained with DAPI (blue). Scale bar = 100 µm.

type cells: 7% (29/441) tau^{+} and 29% (200/679) GFAP^{+}. The remaining cells did not express differentiation markers and none of the cells expressed the oligodendrocyte marker RIP. The isolated fetal cells therefore have a bipotential differentiation capacity consistent for neural progenitor cells.

To analyze the amount of adenosine release, cells were plated at different densities and cultured in undifferentiated (10,000 and 100,000 cells/cm^2) and differentiated (50,000 cells/cm^2) conditions. After sample collection, cells from different wells per condition were counted and averaged. These data were used for normalization of adenosine release per cell number. Already 1 h after medium replacement adenosine was measured in samples from undifferentiated (3.40 ± 0.3 ng per 10^5 cells) and differentiated (3 ± 0.2 ng per 10^5 cells) *Adk*$^{-/-}$ cells, whereas no adenosine was found in samples from wild-type cells. Comparison of adenosine release at the different time points after medium replacement showed an accumulation of adenosine over time in (un)differentiated *Adk*$^{-/-}$ cells, whereas corresponding wild-type cells did not release significant amounts of adenosine (Table 1). Fig. 4 shows the adenosine release in samples taken 24 h after medium replacement. A significant difference is seen between adenosine release from *Adk*$^{-/-}$ cells compared to wild-type, both in undifferentiated (Fig. 4A) and differentiated (Fig. 4B) conditions: 137 ± 5 ng compared to 11 ± 1 ng adenosine per 10^5 cells (non-differentiated cells, $p < 0.05$) and 47 ± 1 ng compared to 3 ± 0.2 ng adenosine per 10^5 cells (differentiated cells, $p < 0.05$). In undifferentiated conditions, results of *Adk*$^{-/-}$ cells plated at 10,000/cm^2 showed a release of 152 ± 4 ng per 10^5 cells after 24 h, indicating that cell density has no effect on the amount of adenosine secretion. These results indicate that *Adk*$^{-/-}$ cells release significantly more adenosine compared to wild-type cells. Furthermore the higher amount of adenosine release is sustained in differentiated cells, meaning that they can be used as a therapeutic source for adenosine release.

4. Discussion

In this experiment we successfully isolated a neural novel line of *Adk*$^{-/-}$ neural progenitor cells. We demonstrated their ability to secrete amounts of adenosine – in contrast to matching wild-type cells – which are considered to be of therapeutic relevance. Sample analysis revealed adenosine release from 47 ± 1 ng (differentiated *Adk*$^{-/-}$ cells) to 137 ± 5 ng (undifferentiated *Adk*$^{-/-}$ cells) per 10^5 cells per 24 h. These results are comparable with adenosine secretion from *Adk*$^{-/-}$ embryonic stem cell derived glial cells.[26] In those former

Table 1
Adenosine release in non-differentiated and differentiated conditions from Adk$^{-/-}$ cells and corresponding wild-type cells. After medium replacement six samples per time point per condition were collected and the mean was calculated. An accumulation of adenosine release is seen over time in Adk$^{-/-}$ cells in both conditions, whereas wild-type cells do not release significant amount of adenosine. Adenosine release is expressed in ng per 10^5 cells.

Time after medium replacement	Non-differentiated cells		Differentiated cells	
	Adk$^{-/-}$ cells (ng adenosine/10^5 cells)	Wild-type cells (ng adenosine/10^5 cells)	*Adk*$^{-/-}$ cells (ng adenosine/10^5 cells)	Wild-type cells (ng adenosine/10^5 cells)
1 h	3.4 ± 0.3	0	3 ± 0.2	0
2 h	9 ± 1	0.4 ± 0.2	5 ± 0.3	0.4 ± 0.3
4 h	19 ± 1	2 ± 0.1	11 ± 1	1 ± 0.4
8 h	42 ± 2	4 ± 0.4	19 ± 1	3 ± 0.5
12 h	63 ± 3	6 ± 0.3	25 ± 1	3 ± 0.7
24 h	137 ± 5	11 ± 1	47 ± 1	3 ± 0.2

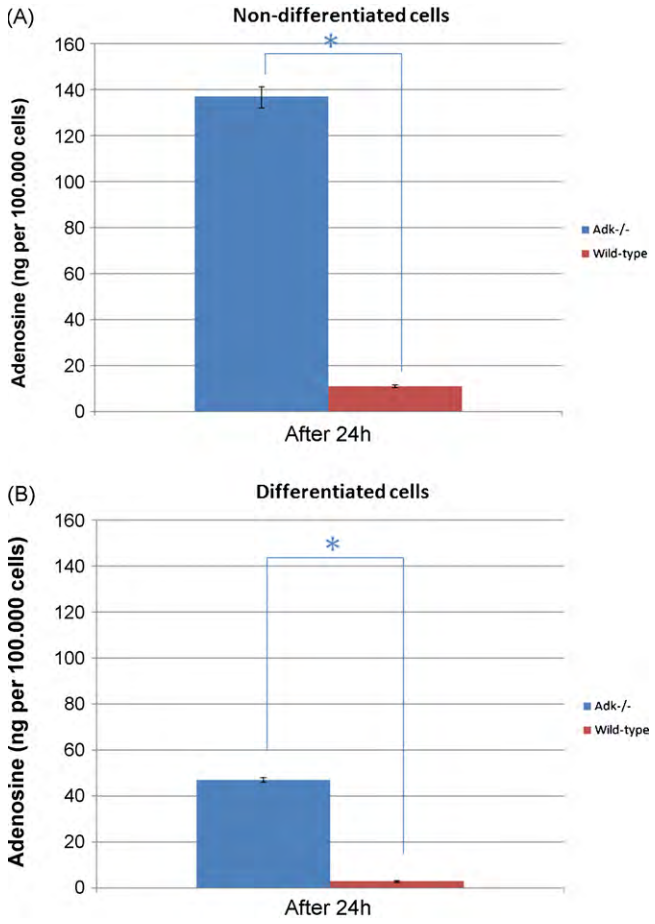

Fig. 4. Adenosine releases from $Adk^{-/-}$ cells (blue) and corresponding wild-type cells (red) in 24 h. (A) Adenosine released from 100,000 non-differentiated cells: adenosine release from $Adk^{-/-}$ cells is significantly higher compared to wild-type cells. (B) Adenosine released from 100,000 differentiated cells: adenosine release from $Adk^{-/-}$ cells is significantly higher compared to wild-type cells. Standard errors are marked with black bars. Significance is marked with an asterisk (*): $p < 0.05$.

studies non-differentiated embryonic stem cells secreted 2.6 ± 0.4 ng adenosine per 10^5 cells per hour (i.e. around 62 ng adenosine per 10^5 cells per 24 h), whereas non-differentiated glial precursor cells secreted 2.7 ± 0.8 ng to 11.7 ± 1.7 ng per 10^5 cells per hour (i.e. around 65–280 ng adenosine per 10^5 cells per 24 h) depending on the growth conditions.

The lower adenosine secretion of differentiated $Adk^{-/-}$ cells compared to undifferentiated $Adk^{-/-}$ cells during the first 24 h of sample collection was a remarkable finding. Since stem cells have a higher metabolic rate compared to differentiated cells, differences in adenosine release during the first 24 h might be due to differences in their overall metabolic rate. However, adenosine release from our differentiated cells is considered to be of therapeutic relevance since previous studies with local adenosine delivery in the lateral brain ventricle of rats revealed that a continuous release of relatively low (20–50 ng per day in vitro) adenosine concentrations lead to seizure suppression in the rat kindling model.[9,11–13] Subsequent studies with release of 1000 ng adenosine per day using a silk-based delivery system implanted into the infrahippocampal cleft showed sustained suppression of evoked seizures in the kindling model.[14,15] These studies demonstrate the therapeutic potential of focal adenosine augmentation therapies in refractory epilepsy.

Genetically engineered neural stem cells have been investigated as a tool for different neurological diseases, including refractory epilepsy.[31,35] Compared to other cell sources, such as fetal brain tissue, survival capacities of neural stem or progenitor cells are higher and more stable.[18] Therefore transplantation of neural stem/progenitor cells combined with local delivery of an anti-epileptic substance may be a successful alternative treatment option for refractory epilepsy.

Since $Adk^{-/-}$ cells are genetically manipulated, it was necessary to investigate their ability to differentiate. We subjected the $Adk^{-/-}$ and wild-type neural progenitor cells to in vitro differentiation, and compared their differentiation potential towards neurons and astrocytes. We previously demonstrated the ability of neural progenitor cells to survive in a sclerotic hippocampus in the rat kainic acid induced status epilepticus model.[36] We found that the majority of surviving cells differentiated towards astrocytes in vivo. Likewise, in our current experiment we found that neural progenitor cells differentiated towards astrocytes in vitro. This differentiation towards astrocytes may be a major advantage in transplantation strategies for epilepsy since astrocytes are key regulators of adenosine.[22] The ultimate rationale for using this cell source is transplantation of astrocytes derived from the $Adk^{-/-}$ progenitor cells after predifferentiation in vitro. These cells might be superior to integrate into the astrogliotic environment of an epileptic hippocampus and interact with the epileptogenic process by releasing adenosine. We recently demonstrated that local delivery of adenosine directly into the epileptic hippocampus has an antiseizure effect in rats with spontaneous seizures.[37] Together with the results of our former transplantation experiment[36] the new cells described here indicate that $Adk^{-/-}$ cells offer the possibility to stably integrate after transplantation into the brain, and to become a long-term source for continuous local adenosine delivery.

5. Conclusion

Compared with data from previous studies,[9,11–15] we conclude that the amount of secreted adenosine – both from non-differentiated and differentiated $Adk^{-/-}$ cells – is sufficient to obtain a therapeutic effect in the treatment of refractory epilepsy. Since astrocytes play a major role in the regulation of adenosine in the brain, astrocytes derived from $Adk^{-/-}$ neural progenitor cells seem a promising source for local delivery of adenosine in an epileptic brain. Further in vivo studies in relevant animal models with spontaneous seizures should focus on effect on seizure frequency.

Conflicts of interest

None of the authors has any conflict of interest to disclose.

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'We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines'.

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