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Directed Differentiation of Neural Progenitors into Neurons Is Accompanied by Altered Expression of P2X Purinergic Receptors

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Abstract Neural differentiation has been extensively studied in vitro in a model termed neurospheres, which consists of aggregates of neural progenitor cells. Previous studies suggest that they have a great potential for the treatment of neurological disorders. One of the major challenges for scientists is to control cell fate and develop ideal culture conditions for neurosphere expansion in vitro, without altering their features. Similar to human neural progenitors, rat neurospheres cultured in the absence of epidermal and fibroblast growth factors for a short period increased the levels of β-3 tubulin and decreased the expression of glial fibrillary acidic protein and nestin, compared to neurospheres cultured in the presence of these factors. In this work, we show that rat neurospheres cultured in suspension under mitogen-free condition presented significant higher expression of P2X2 and P2X6 receptor subunits, when compared to cells cultured in the presence of growth factors, suggesting a direct relationship between P2X2/6 receptor expression and induction of neuronal differentiation in mitogen-free cultured rat neurospheres.

Keywords Purinergic receptors · Neural progenitor cells · Neurospheres · Neurogenesis · Growth factors

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Introduction

For the last 40 years, we believed that the brain was unable to regenerate after an injury. Nowadays, we know that the central nervous system (CNS) is capable to generate new neural cells from precursors present in some regions, and those cells can survive and integrate the circuitry in the adult mammalian brain (Lois and Alvarez-Buylla 1993; McKay 1997; Gage 2000; reviewed by Trujillo et al. 2009).

Neurogenesis was first evidenced in the adult brain of birds by Nottebohm and co-workers (Goldman and Nottebohm 1983; Paton and Nottebohm 1984). Some years later, Reynolds et al. (1992) demonstrated that adult mouse striatum cells differentiated in vitro in the presence of epidermal growth factor (EGF). The subgranular zone (SGZ) of the dentate gyrus and the anterior part of the subventricular zone (SVZ) along the ventricle (Ming and Song 2005) are the two major neurogenic regions of the adult mammalian brain. New neurons generated in the SVZ migrate not only to the olfactory bulb (Lois and Alvarez-Buylla 1993) but also to neocortical areas of primates (Gould et al. 1999). Many published reports showed the existence of proliferating cells in hippocampus of the intact adult CNS (Rietze et al. 2000).

Evidence of neurogenesis in specific adult brain regions brought new perspectives for cell therapy and neural regeneration (Svendsen and Smith 1999). Neural differentiation has been extensively studied in vitro. An experimental model to study neural stem cells is the heterogeneous free-floating cell aggregates, termed neurosphere (Reynolds and Weiss 1992; McKay 1997; Gage 2000). Reynolds and Weiss (1992) isolated striatum cells from adult mouse brain and induced them to proliferate as neurospheres in the presence of EGF. Each neurosphere is derived from a single stem cell that by

asymmetrical division, gives rise to another stem cell and a progenitor cell. Progenitor cell division, in turn, augments only to the progenitor population. Therefore, only a small fraction of the neurosphere corresponds to genuine stem cells (Reynolds et al. 1992). Here we use the terminology neural precursor cells (NPC) to describe both cell types within the neurosphere (Svendsen et al. 1999; Svendsen and Caldwell 2000).

In their undifferentiated stage, cells originally express nestin (stemness marker) and following attachment to culture dishes, they change their morphology and antigenic properties to those of neurons and astrocytes. The expression of neuronal marker β-3 tubulin and the glial marker glial fibrillary acidic protein (GFAP) increases during differentiation in vitro, while nestin expression is decreased (Martins et al. 2008). McLaren et al. (2001) found a similar pattern in the expression of these markers in undifferentiated and differentiated neural stem cells by flow cytometry.

P2X purinergic receptor expression was first described by Cheung et al. (2005) during rat brain development. Functional receptors are formed by assembly of three subunits. The P2X6 subunit forms only active receptor complexes when assembled together with P2X2 or P2X4 subunits (King et al. 2000; Le et al. 1998). Except for the P2X1, all P2X subunits are highly expressed during encephalon development. P2X3 receptors were suggested to participate in axon outgrowth during early embryonic neurogenesis. P2X4, P2X5, and P2X6 subunits have their expression increased during post-natal neurogenesis (Cheung et al. 2005; da Silva et al. 2007; reviewed by Majumder et al. 2007). Our laboratory has shown before that ATP and other agonists of purinergic receptors were able to modulate proliferation and neuronal differentiation of P19 embryonic carcinoma cells in vitro (Resende et al. 2007; 2008).

The major challenge for scientists in the stem cell therapy field is to control cell fate and develop ideal culture conditions for NPC expansion in vitro, without altering their features. Previous studies suggested that NPC have a great potential for the treatment of neurological disorders. An adequate number of functional cells after transplantation is needed, to make these cells useful for cell therapy. For this purpose, in vitro manipulation before transplantation may be necessary to control the terminal phenotype of these cells.

Removal of EGF and fibroblast growth factor (FGF-2), during 10 days of neurosphere culture of three different human NPC lineages, induced differentiation into neuronal and glial cells and caused a decrease in cell proliferation together with an increase in apoptosis (Schwindt et al. 2009). Those observations originated new questions: (1) Will altering their culture conditions be critical for defining the potential for survival and integration and enhance the ability of these cells to graft and provide functional recovery? (2) Which molecular mechanisms and alterations in gene expression patterns are involved in neuronal differentiation during the starvation periods? In this work, we have analyzed patterns of purinergic P2X receptor expression, and we found a direct relationship between P2X2/6 receptor expression and increased neurogenesis in mitogen deprivation conditions.

Materials and Methods

This work was developed under the approval of the Ethics' Committee of Universidade de São Paulo. Rat NPC (rNPC) were obtained from E14 (embryonic day 14) Wistar rat embryos. The fetuses were placed in a Petri dish containing cold Dulbecco's Modified Eagle Medium (DMEM), and the dissection was made under magnifying lenses. Brains were sectioned, and obtained tissue was incubated with trypsin-EDTA solution (Invitrogen, Carlsbad, CA) for 10 min at 37°C. Trypsin was inactivated with fetal bovine serum (FBS). Following cell sedimentation, the supernatant was removed, and the cells were dissociated. The cell suspension was counted in a hemocytometer, and the cells were seeded in a 75-cm^2 flask (TPP, Trasadingen, Switzerland) at a density of 200,000 cells/mL in 20 mL. The culture media was composed of DMEM/F12 (Invitrogen) supplemented with 1% PS (penicillin streptomycin), 2% B27 (Invitrogen), 20 ng/mL EGF (Sigma-Aldrich, St. Louis, MO), 20 ng/mL FGF-2 (R&D, Minneapolis, MN), and 5 μg/mL heparin (Sigma). Cells were maintained for 10 days in an incubator at 37°C under a 5% CO_2 atmosphere. Every three days, half of the volume was replaced by fresh medium.

After neurosphere expansion (as described above), half of the population within one flask were kept in the complete media (control group—Ctr), and the other half was cultured in the absence of growth factors (EFless). For the EFless group, the spheres were transferred into conical tubes and carefully washed three times with prewarmed DMEM. If necessary, the spheres were precipitated by centrifugation at 130×*g*. The spheres were then transferred into growth factor free medium (DMEM/F12/B27) and kept for 7 days as suspension culture. Every three days, half of the volume was replaced by fresh medium.

For immunocytochemistry, cells fixed on coverslips were treated with blocking solution and permeabilized for 30 min in 5% normal goat serum and 0.1% Triton X-100 in phosphate buffered saline (PBS). Primary antibodies for β-3 tubulin (Sigma, mouse IgG, 1:500), GFAP (Dako, Carpinteria, CA, rabbit IgG, 1:500), and nestin (Millipore, Billerica, MA, mouse IgG3, 1:500) were added, and cells were incubated overnight at 4°C. The coverslips were washed in PBS and incubated with secondary antibodies (Alexa 546 anti-mouse

IgG or Alexa 488 anti-rabbit IgG, Invitrogen, 1:500). Following another washing step with PBS, 4'-6-diamidino-2-phenylindole solution (Sigma, 0.3 μg/mL) was used as a nuclear stain, and cells were analyzed under a fluorescence microscope. β-3 tubulin was considered a reliable marker for neuronal differentiation because it was shown previously that besides being expressed by both mature neurons and neural progenitors, β-3 tubulin$^+$ migrating cells from neurospheres adopt a neuronal phenotype and express neurochemical markers (GABA, glutamate) and mature neuronal markers (Neu-N, MAP-2ab). GFAP expression was detected as a marker for astrocytic cells.

Cell extracts obtained in RIPA buffer containing protease inhibitors (sodium pyrophosphate, sodium fluoride, phenylmethanesulfonyl fluoride, pepstatin A, aprotinin, leupeptin, antipain, benzamidine—all from Sigma) were used for Western blot experiments. Fifty micrograms of protein extract were electrophoretically separated on a 10% polyacrylamide gel containing SDS and transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA). The blot was probed for β-3 tubulin (Sigma, 1:1,000), GFAP (Dako, 1:1,000), nestin (Millipore, 1:1,000) and β-actin expression (Sigma, 1:2,000). Immunostaining by secondary antibodies conjugated to horseradish peroxidase (1:2,000 HRP, Jackson Laboratories, West-Grove, PA) was detected with Western blot luminol reagents (Santa Cruz Biotechnology, Santa Cruz, CA). HRP-antibodies were diluted 1,000-fold for nestin staining.

Total RNA was isolated by using the TRizol reagent (Invitrogen) according to the manufacturer's protocol. Total RNA concentration and integrity were determined by spectrophotometer readings at absorbance of 260 and 280 nm and by observation of ribosomal RNA bands in a 1% agarose gel, respectively. RNAs were previously treated for 30 min at 37°C with 1 U RQ1 RNase-Free DNase (Promega, Madison, WI) in presence of 20 U RNAseOUT (Invitrogen) RNAse inhibitor for avoiding DNA contamination. First strand cDNA synthesis from 5 μg of total RNA was performed using SuperScript II Reverse Transcriptase (Invitrogen). Resultant cDNA was then used for real-time PCR amplification as described below.

Expression analysis of mRNA was performed in the ABI Step One Plus Instrument (Applied Biosystems, Foster City, CA) using the SYBR-Green amplification detection system. Each reaction was performed in a final volume of 25 μL using cDNA reversed transcribed from 25 ng of the RNA, 10 μL of the SYBR-Green Universal PCR Master Mix (Applied Biosystems, Foster City, CA), and 1 μL of each forward and reverse primers (10 μM each) shown below. The real-time PCR reactions were performed using the temperature protocol 50°C–2 min, 95°C–10 min, and 50 cycles of 95°C–15 s and 60°C–1 min, followed by a dissociation curve protocol for evaluation of the specificity of the amplicon produced in each reaction. Standard curves were measured for each primer set and cDNA sample to verify reaction efficiency. As the efficiency of all reactions was >95%, we were able to use the $2^{-\Delta\Delta Ct}$ parameter to express relative expression data, taking glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene expression as endogenous control. Comparisons of real-time PCR data were made by two-way analysis of variance with Bonferroni's post-test using GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA). The criteria for statistical significance were set at $P<0.05$. Forward and reverse primers, respectively, are represented in the 5'→3'direction:

β-3 tubulin: AGACCTACTGCATCGACAATGAAG and GCTCATGGTAGCAGACACAAGG;

GFAP: AAGAGTGGTATCGGTCCAAGTTTG and CAGTTGGCGGCGATAGTCAT;

P2X1: GAGAGTCGGGCCAGGACTTC and GCGAATCCCAAACACCTTGA

P2X2: TCCCTCCCCCACCTAGTCAC and CACCAC CTGCTCAGTCAGAGC

P2X3: CTGCCTAACCTCACCGACAAG and AATAC CCAGAACGCCACCC

P2X4: CCCTTTGCCTGCCCAGATAT and CCGT ACGCCTTGGTGAGTGT

P2X5: GGATGCCAATGTTGAGGTTGA and TCCTGACGAACCCTCTCCAGT

P2X6: CCCAGAGCATCCTTCTGTTCC and GGCAC CAGCTCCAGATCTCA

Nestin: TGGAGCGGGAGTTAGAGGCT and ACC TCTAAGCGACACTCCCGA

GAPDH: TGGCCTCCAAGGAGTAAGAAAC and GGCCTCTCTCTTCCTCTCAGTATC

For flow cytometry experiments, undifferentiated and differentiated neurospheres were collected by centrifugation for 5 min at 200×*g* and dissociated to a single-cell suspension using trypsin. Then the cells were again precipitated by centrifugation for 5 min at 200×*g* and fixed in PBS–1% formaldehyde for 20 min on ice, and washed with PBS–2% FBS. Cells were incubated with 1:500 dilutions of primary antibodies specific for neural markers (GFAP, Dako; β-3 tubulin, Sigma; nestin, Millipore). Cells were washed with PBS, incubated with a 1:500 dilution of the secondary antibodies (Alexa 488, Invitrogen), and analyzed by using a flow cytometer (Beckman Coulter, Fc500, Fullerton, CA). An argon laser line (550–600 nm) was used for fluorescence excitation of Alexa 488. Data were analyzed using the Cyflogic software available at http://www.cyflogic.com. Fifty-thousand events were acquired per sample with fluorescence measured in logarithmic scales. Forward and side light-scatter gates were used to exclude dead cells and debris. Data were gated using side scatter (linear scale) and fluorescence signals (logarithmic scale). Negative controls were carried out in the absence of primary antibodies.

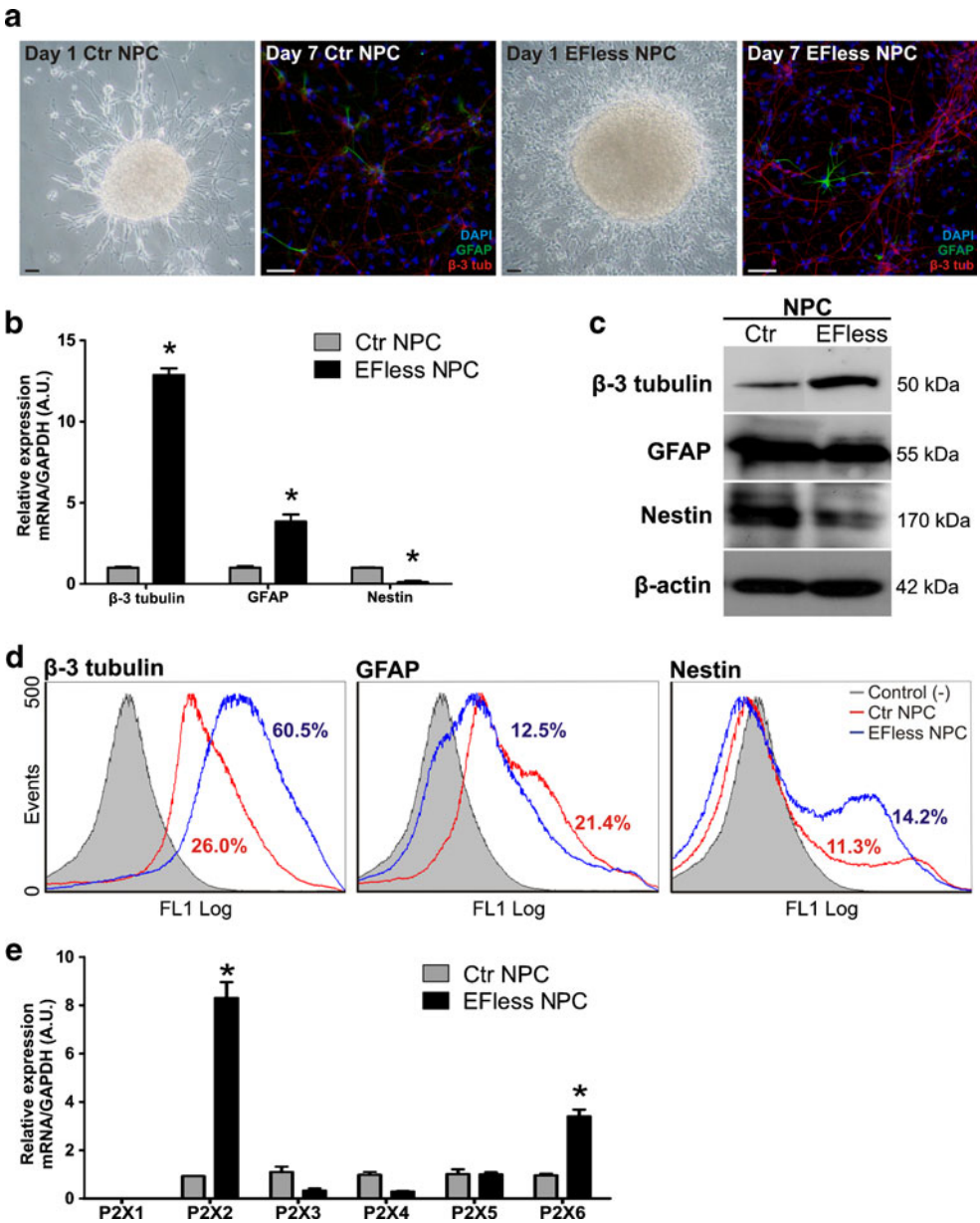

Figure 1 Changes in gene expression patterns of P2X receptors following neurogenesis induction of NPC by growth factors deprivation. Following neurosphere expansion, half of the obtained NPC population was maintained in culture medium containing EGF and FGF-2 (control group—Ctr), while the other half of the population was cultured in the absence of growth factors (EFless). **a** Cell migration and differentiation. Following one day of plating and induction of differentiation, cells migrated out from neurospheres. Migration was enhanced in the EFless condition, when compared to the Ctr group. Immunofluorescence staining after 7 days differentiation revealed an increase in neuronal differentiation in the EFless group. **b** Real-time PCR analysis of neural genes. Short-term growth factor removal (EFless group) significantly increased β-3 tubulin and GFAP gene expression and reduced nestin expression. **c** Western blot analysis of neural proteins. β-3 tubulin protein is increased in neurospheres from the EFless group, while GFAP and nestin levels in the same group decrease. **d** Flow cytometry analysis of stemness and neural marker expression in Ctr and EFless neurospheres. Neurospheres from Ctr and EFless groups were stained for the neural markers β-3 tubulin and GFAP and for the stemness marker nestin, revealing augmented neurogenesis in EFless neurospheres. Control (-): Filled areas indicate fluorescence obtained in the absence of primary antibodies. **e** Real-time PCR analysis of P2X1–P2X6 purinergic receptor subunit gene expression in Ctr and EFless neurospheres. An increase in neurogenesis was accompanied by augmented expression of P2X2 and P2X6 receptor subunits and decreased expression of P2X3, P2X4, and P2X5 receptor subunits. **b**, **c, e** Glyceraldehyde-3-phosphate (GAPDH) and β-actin expression, which are not supposed to vary under the experimental conditions, were determined in real-time PCR and Western blot assays, respectively, for normalization and relative quantification of expression

Results and Discussion

In this manuscript, we demonstrate significant differences in cell migration after plating and in the expression of neural proteins in rNPC subjected to deprivation of EGF and FGF-2, when compared with those cultured in standard conditions. Cell migration after plating is clearly favored in the EFless group, as seen in Fig. 1a. Moreover, immunofluorescence studies performed following seven days of plating and differentiation revealed increased NPC differentiation into neurons, when neurospheres had been cultured in the absence of growth factors (EFless group), compared to neurospheres from the Ctr group (Fig. 1a). Flow cytometry analysis did not reveal any reduction in the percentage of nestin-expressing cells from the EFless group. However, overall nestin expression levels were reduced as shown by real-time PCR and Western blot analysis, indicating that cells expressed less nestin. Real-time PCR analysis revealed that β-3 tubulin gene expression was seven times higher in its expression when compared to the control group (Fig. 1b). Different from protein expression levels, mRNA transcription of the GFAP gene was four times enhanced in the EFless group when compared to the Ctr group (Fig. 1b). This discrepancy is explained by previous observations that mRNA and protein levels are not necessarily correlated. For instance, Magnaghi and co-workers reported a lack in the correlation between GFAP-mRNA and protein levels in spinal cord of cobalamin-deficient rats (Magnaghi et al 2002). In our work, we found distinct mRNA and protein levels for GFAP in the EFless group, with an increase in mRNA and a decrease in protein expression, as detected by real-time PCR, Western blot, and flow cytometry, respectively. This can be due to a difference in the post-transcriptional control of GFAP and/or to an alteration in mRNA stability between Ctr and EFless groups.

Western blot experiments revealed increased β-3 tubulin and reduced GFAP and Nestin protein expression in the EFless group (Fig. 1c). Flow cytometry analysis showed that when rNPC are cultured in the absence of FGF-2 and EGF for a short period, they express significant higher levels of the neuronal marker β-3 tubulin (Fig. 1d). Cultures contained 60.5% of β-3 tubulin positive cells following short-term mitogen deprivation of rNPC cultures, while cells cultured in standard condition displayed 26.0% of neurons. The percentage of GFAP-positive cells decreased from 21.4% in the Ctr group to 12.5% in the EFless group, and the percentage of nestin positive cells did not significantly change between Ctr and EFless groups, from 11.3% to 14.2% (Fig. 1d).

We have shown in a previous work that purinergic receptor expression and activity is regulated during neuronal differentiation using P19 embryonic carcinoma cells as an in vitro model (Ulrich and Majumder 2006; Resende et al. 2007, 2008). Purinergic receptor activity participated in induction of proliferation in embryonic and NPC-stage P19 and also promoted differentiation into neurons (Resende et al. 2008). Here, we have used NPC from primary embryonic telencephalon, whose patterns of migration and differentiation closely resemble events occurring during cortex development. In conditions of increased neurogenesis, P2X2 and P2X6 receptor subunit expression was significantly increased (5-fold and 2.5-fold, respectively) being in agreement with our previous studies in P19 cells (Resende et al. 2008). P2X3 and P2X4 receptor subunits had their expression reduced in the EFless group, and P2X1 receptor expression was below detection limit of the real-time PCR assay (Fig. 1e). These results suggest that homomeric P2X2 and heteromeric P2X2/6 receptors are active in NPC differentiated into neurons, whereas P2X3, P2X4, and P2X5 receptors did not have major participation in this process, being in agreement with our previous work (Resende et al. 2007; 2008). Further studies may now concentrate on the participation of P2X2 and P2X2/6 receptors in the progress of neurogenesis using pharmacological approaches and/or specific suppression of purinergic receptor subunit expression. The paper shows for the first time an intimate correlation between regulation of P2X receptor expression and induction of neurogenesis. P2X2 and P2X6 receptor subunits have their expression increased when neurogenesis is induced by the removal of EGF and FGF-2. Our results suggest that P2X2 and P2X6 receptor subunits are marker proteins for neurogenesis and also participate in neuronal differentiation of fetal brain rNPC. Developmental expression of P2X2 and P2X6 receptors and their functions during in vivo neurogenesis should be further investigated.

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