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ORIGINAL RESEARCH

Rapid Differentiation of Human Embryonal Carcinoma Stem Cells (NT2) into Neurons for Neurite Outgrowth Analysis

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Abstract Human neurons derived from stem cells can be employed as in vitro models to predict the potential of neurochemicals affecting neurodevelopmental cellular processes including proliferation, migration, and differentiation. Here, we developed a model of differentiating human neurons from well characterized human embryonal carcinoma stem cells (NT2). NT2 cells were induced to differentiate into neuronal phenotypes after 2 weeks of treatment with retinoic acid in aggregate culture. Nestin positive progenitor cells migrate out of NT2 aggregates and differentiate into βIII-tubulin expressing neuronal cells. Culturing the NT2 cells for an additional 7–14 days resulted in increased percentage of βIII-tubulin expressing cells, elaborating a long neurite that positively stained for axonal marker (Tau) and presynaptic protein (synapsin). We then asked whether neurite outgrowth from NT2 cells is modulated by bioactive chemicals. Since the cAMP/PKA pathway has been widely investigated as a regulator of neurite outgrowth/regeneration in several experimental systems, we used chemical activators and inhibitors of cAMP/PKA pathway in the culture. The adenylyl cyclase activator, forskolin, and cell-permeable analog of cAMP, 8-Br-cAMP increased the percentage of neurite bearing cells and neurite extension. Application of the protein kinase A inhibitors, H-89 and Rp-cAMP, blocked neurite formation. Taken together, NT2 aggregates undergo migration, differentiation, and neurite elaboration and can be used as a model of differentiating human neurons to screen neurochemicals and to understand cellular mechanisms of human nerve cell development.

Keywords Neurite development · Stem cells · NTera2 · Human nerve cell · cAMP signaling

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Introduction

Neuronal growth cones, specialized fan-shaped structures at the tips of growing neurites, respond to various extracellular cues during neurite elongation and guidance (Kater and Rehder 1995). Unlike the developing nerve cells, adult CNS lacks neurite regeneration capacity after injury or neurodegeneration. Thus, there is a growing interest to develop therapeutic agents that enhance the regeneration of nerve cells. Screening for potential neurite outgrowth modulating agents usually involves the use of primary cells from rodent or the widely used rat pheochromocytoma (PC12) cell line (Radio and Mundy 2008; Meldolesi 2010). Since there are considerable differences in the response to neurochemical substances between different species, human nerve cells are especially desirable for cell-based screening assays (Paquet-Durand and Bicker 2007). Moreover, whether this difference in response to neurochemicals is due to specific differences in cellular signaling pathways is largely unknown. In this regard, human neurons obtained from stem cells are of particular interest.

The embryonal carcinoma stem cells Ntera2 (NT2), derived from a human testicular cancer, can be induced to differentiate into fully functional postmitotic neurons and other cell types of the neuronal lineages (Andrews 1984; Pleasure et al. 1992; Paquet-Durand and Bicker 2007). NT2 cells shares many similarity with human embryonic stem cells (Schwartz et al. 2005) and differentiation of NT2 cells into neurons has been suggested to resemble vertebrate neurogenesis (Przyborski et al. 2000, 2003; Houldsworth et al. 2002; Smith et al. 2010). Moreover, fully differentiated NT2 neurons have been shown to express a variety of neurotransmitters in vitro (Guillemain et al. 2000; Podrygajlo et al. 2009) and form functional synapses (Hartley et al. 1999; Podrygajlo et al. 2010). Thus, NT2 lines provide a robust and amenable culture system to study certain aspects of neurodevelopmental process that mimic vertebrate neurogenesis. In line with this, we have recently used spherical aggregates derived from NT2 cells as a model of developing human neurons and demonstrated that the gaseous messenger nitric oxide regulates neuronal progenitor cell migration (Tegenge and Bicker 2009), a finding that was confirmed in primary human fetal progenitor cells (Tegenge et al. 2010).

The originally established method of NT2 cell differentiation involves 6 weeks of retinoic acid treatment, 2 days of expansion, 7–10 days of mitotic inhibitors treatment, and several selective trypsinization steps (Andrews 1984, Pleasure et al. 1992). The entire process requires about 2 months of cell culturing, a major disadvantage of the NT2 cell line. In recent years, this lengthy differentiation method was significantly shortened by employing a free-floating aggregate culture method (Paquet-Durand et al. 2003; Podrygajlo et al. 2009, Tegenge et al. 2009).

Here, we further improve upon the differentiation of NT2 cells into neurons in aggregate culture, allowing us to use NT2 cells as a model of human neurons for neurite outgrowth analysis. Moreover, we show that neurite outgrowth from the differentiating human neuron is modulated by cAMP/Protein kinase A signal transduction.

Materials and Methods

The PKA antagonists H-89 (N-[2-((*p*-Bromocinnamyl) amino) ethyl]-5-isoquinolinesulfonamide, 2HCl) and Rp-cAMPS (Adenosine 3′,5′-cyclic monophosphorothioate, Rp-isomer, triethylammonium salt) were purchased from Calbiochem (Darmstadt, Germany). 8-Br-cAMP (8-Bromoadenine-3′,5′-cyclic monophosphate, sodium salt) was purchased from Alexis Biochemicals (Lörrach, Germany). All other materials were obtained from Sigma (Taufkirchen, Germany) unless otherwise noted.

Cell Culture

NT2/D1 precursor cells were purchased from American Type Culture Collection (ATTC, Manassas, VA20108 USA) and treated as indicated in the ATTC instruction manual. NT2 cell aggregates were cultured as free floating spherical aggregate as by modifying previously described method (Paquet-Durand et al. 2003; Podrygajlo et al. 2009). The NT2 precursor cells (passages 24–32) were seeded in 95 mm, bacteriological grade Petri dishes (Greiner, Hamburg, FRG) at a density of 5×10^6 cells/dish in 10 ml of Dulbecco's modified eagle medium (DMEM/F-12, Gibco-Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal bovine serum (Invitrogen) for 24 h. A differentiation medium, DMEM containing retinoic acid at a final concentration of 10 μM was used with medium change every 2–3 days for 14 days. The aggregates were further cultured for 2–14 days on poly-D-lysine/Matrigel coated 12-mm cover glass in 24-wells plate in the presence of DMEM medium with mitotic inhibitors (1 μM 1-6-D-arabinofuranosylcytosine, 10 μM 2′-deoxy-5-fluorouridine, and 10 μM 1-β-D-ribofuranosyluracil).

Imunocytochemistry

Immunocytochemical stainings were performed on NT2 spherical aggregate or after mechanical dispersion into single cells as previously described (Tegenge and Bicker 2009). In brief cultures were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Monoclonal primary antibodies against neuronal progenitor marker nestin (1:400, Calbiochem), neuronal marker βIII-tubulin (1:10000, Sigma), axonal marker Tau (1:500, Millipore International), and pre-synaptic protein synapsin (1:500, Synaptic Systems, Göttingen, Germany) were applied overnight at 4°C. Secondary biotinylated antibodies (Vector, Burlingame, Mass., USA) were applied for 1 h at room temperature. Immunofluorescence was detected by applying streptavidin-CY3 (Sigma) or streptavidin-Alexa Fluor 488 (Mobitec, Göttingen, Germany) for 1 h at room temperature. DAPI (4′,6-diamidino-2′-phenylindoldihydrochloride) at 1 μg/ml was used as a nuclear counterstain.

Drug Application

All chemicals were diluted in the medium with mitotic inhibitors at the following final concentrations: forskolin (10 and 50 μM), 8-Br-cAMP (100–1000 μM), Rp-cAMP (10 μM), and H-89 (5 μM). Forskolin and H-89 were prepared from stocks in dimethyl sulfoxide (DMSO),

which were further diluted in the medium to result in a maximum concentration of 0.5% DMSO. Solution controls that contain 0–0.5% DMSO were used as a control.

Analysis of Neuronal Differentiation and Neurite Outgrowth

NT2 aggregates were mechanically dispersed into single cells. About 5,000 cells were allowed to attach for 1 h on poly-D-lysine/Matrigel coated 12-mm cover glass in 24-wells plate. Cultures were then treated with chemicals diluted in the medium containing mitotic inhibitors for 48 h. Cultures were fixed with 4% PFA and stained for β-III-tubulin. For evaluation of neuronal differentiation, the percentage of neuronal cells was determined by dividing β-III-tubulin positive cells by DAPI stained cells. For analysis of neurite outgrowth, β-III-tubulin stained cells that bear neurite greater than one times soma diameter were counted from at least five randomly taken images per wells. The percent of cells with neurite was calculated by dividing neurite bearing cells by β-III-tubulin positive cells. Neurite length was determined by measuring the longest outgrowing neurite from the cell bodies of the evaluated neurons. Each experiment was performed at least in duplicate wells and repeated three times.

Cell Viability Assay

The Alamar Blue viability assay (Trek Diagnostic Systems, East Grinstead, UK) were used to monitor the viability of the cells. About 5,000 cell/well were seeded into poly-D-lysine/Matrigel coated 24-well plate in the presence of chemical compounds and incubated at 37°C/5% CO_2. After 48 h, the medium was completely changed into a cell culture medium containing 3% Alamar Blue. After 3 h of incubation, 150 µl of aliquots from each wells were transferred into duplicate wells of 96-well plates and fluorescence intensity of the Alamar Blue was detected at excitation/emission wavelength of 530/590 nm using a microplate reader (Infinite M200, Tecan).

Microscopy and Statistics

Preparations were viewed with a Zeiss Axiovert 200 (Göttingen, Germany), equipped with a CoolSnap camera (Photometrics, Tucson, AZ, USA) and MetaMorph software (Molecular Devices, Sunnyvale, CA, USA). Data were presented as mean ± SEM. Statistical analysis was performed using unpaired Student's t test. Levels of significance were: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Results

Rapid Differentiation of NT2 Cells into Neurons

NT2 precursor cells were initially maintained as adherent culture and transferred into Petri dishes upon 90% confluency. NT2 cells were treated with retinoic acid for 2 weeks in aggregate culture and directly plated on poly-D-lysine/Matrigel-coated cover glasses placed in 24-well plates. After 2 days of culturing, cells inside the sphere and migrating cells positively stained for both progenitor (nestin) and neuronal markers (β-III-tubulin) (Fig. 1a, b). More cells migrated out of the aggregates and differentiated into neuronal phenotypes upon further culturing for 7 days (Fig. 1c, d). After 14 days in culture, cells almost completely moved out of the NT2 aggregates and formed neuronal networks labeled by β-III-tubulin (Fig. 1e, f) and elaborated long neurites that positively stained for the axonal marker, Tau (Fig. 2a). Moreover, at 14-day in vitro culture, the differentiated cells express the pre-synaptic marker, synapsin (Fig. 2b).

To accurately quantify neuronal differentiation, NT2 cell aggregates were mechanically dispersed into single cells and stained for nestin and β-III-tubulin at different time points. Two days after plating, about 55% of cells were nestin positive while 30% were β-III-tubulin positive (Fig. 1g). The level of nestin-labeled cells decreased to about 34% while the β-III-tubulin positive cells were increased to about 63% at 7 days in vitro culture (Fig. 1g, h). Upon further culturing for 14 days, the level of β-III-tubulin positive cells reached almost 80% (Fig. 1h). Thus, the present differentiation method considerably reduced the time required to generate postmitotic neurons from NT2 cells. This was achieved by prolonging the aggregate culture length to 2 weeks and avoiding intermediate re-plating steps and selective trypsinization method (Pleasure et al. 1992; Paquet-Durand et al. 2003; Podrygajlo et al. 2009).

Elevation of cAMP Level Promotes Neurite Outgrowth

For the analysis of neurite outgrowth, we mechanically dispersed NT2 aggregates into single cells and cultured for 48 h on poly-D-lysine/Matrigel-coated substrates in the presence of chemicals that activate or inhibit cAMP pathways.

Initially we used the adenylyl cyclase activator, forskolin, which elevates the endogenous level of cAMP. Application of 10–50 µM forskolin for 48 h in culture significantly enhanced the percentage of neurite bearing cells (Figs. 3a, b; 4a). Moreover, 50 µM of forskolin significantly increased neurite extension (Fig. 4b). Next we used a cell-permeable exogenous cAMP analog,

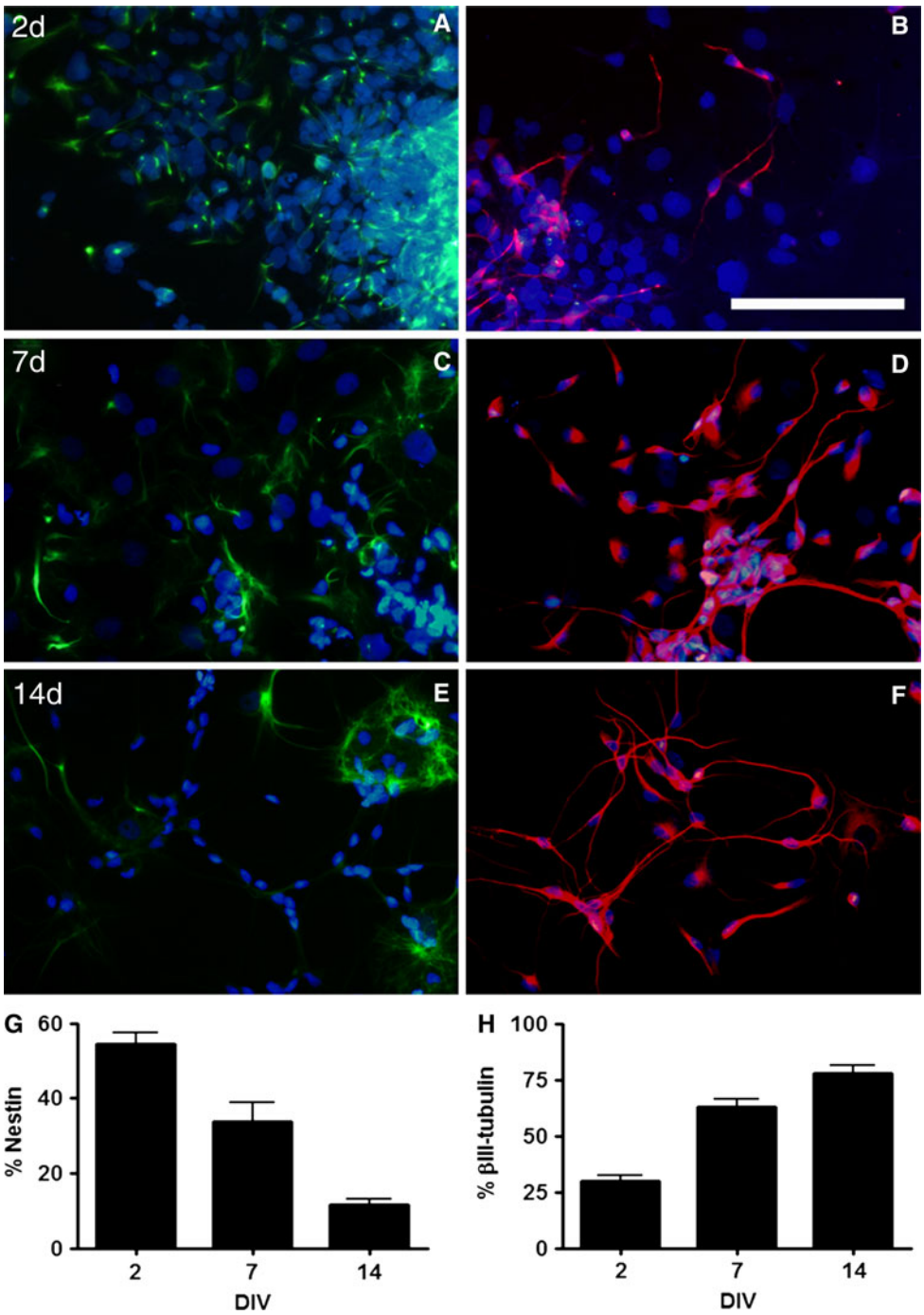

Fig. 1 Rapid differentiation of human embryonal carcinoma stem cells into neurons. NT2 cells were treated in 10 μM of retinoic acid for 14 days and plated into poly-D-lysine/Matrigel coated cover glasses. Two days after plating migrated cells and cells inside sphere positively stained for **a** a marker of neuronal progenitor, nestin (*green* immunofluorescence) and **b** a marker of early neuronal differentiation, *β*-III-tubulin (*red*). **c**, **d** After 7 days in vitro culture the level of nestin positive cells decreased while *β*-III-tubulin expressing cells increased. **e**, **f** Upon 14 days in vitro culture less cells were stained for nestin while majority of cells expressed *β*-III-tubulin. **g**, **h** The percentage of nestin and *β*-III-tubulin positive cells was displayed at different time points. *Blue* (DAPI) indicates nuclear counterstaining. *Scale bar* 100 μm (**a**–**g**). Data represent mean ± SEM of three independent experiments (**g**, **h**) (Color figure online)

8-Br-cAMP. Application of 8-Br-cAMP significantly increased the percentage of cells bearing neurites in a dose-dependent manner (Figs. 3c, d; 4c). One millimolar 8-Br-cAMP significantly promoted neurite extension (Fig. 4d). To determine whether cell survival was affected under these conditions, we conducted Alamar blue cell viability assay after 48 h of forskolin and 8-Br-cAMP application. Both forskolin and 8-Br-cAMP did not significantly alter the viability of cells at the highest concentration that facilitated neurite outgrowth (Fig. 4e). Thus, the observed increase in neurite outgrowth was not as a result of effects on cell viability.

We tested whether the effect on neurite outgrowth was as a result of change in the percentage of total neuronal cells. For this purpose, we have quantified the percentage of *β*-III-tubulin cells after 48 h of forskolin and 8-Br-cAMP application. As shown in Fig. 4f, neither chemical altered the overall percentage of *β*-III-tubulin

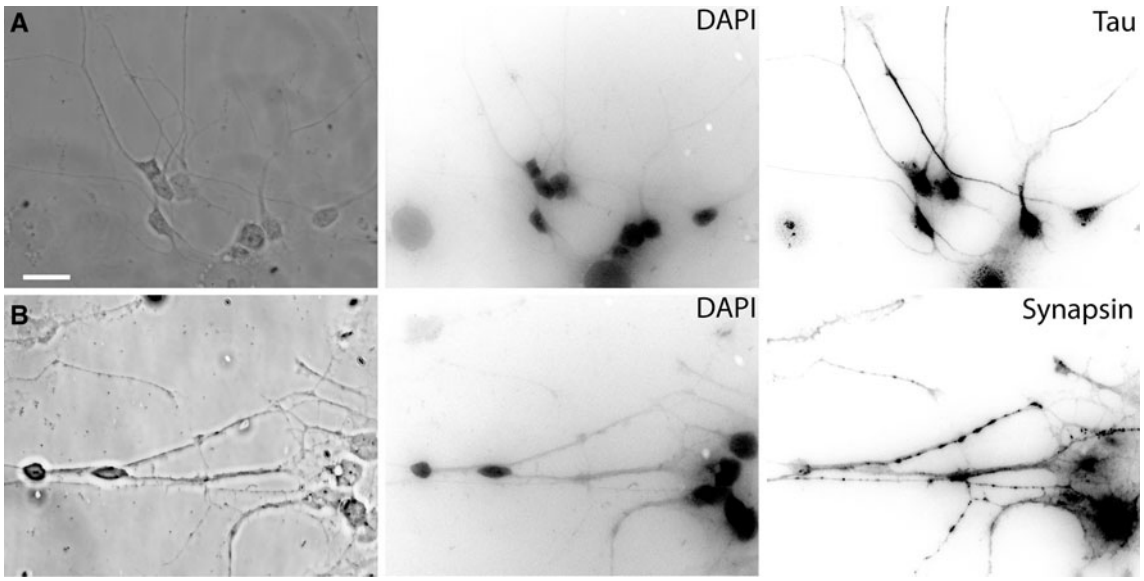

Fig. 2 Rapidly differentiated NT2 cells elaborate long axons and express pre-synaptic protein. NT2 cells were treated in 10 μM of retinoic acid for 14 days and plated into poly-D-lysine/Matrigel-coated cover glasses. Cultures were maintained for additional 14 days in the presence of mitotic inhibitors. **a**, **b** Representative phase contrast pictures of NT2 neurons, nuclear counter staining (DAPI) and long neurites positively stained for the axonal marker, Tau and a pre-synaptic protein, synapsin. *Scale bar* 25 μm (**a**, **b**)

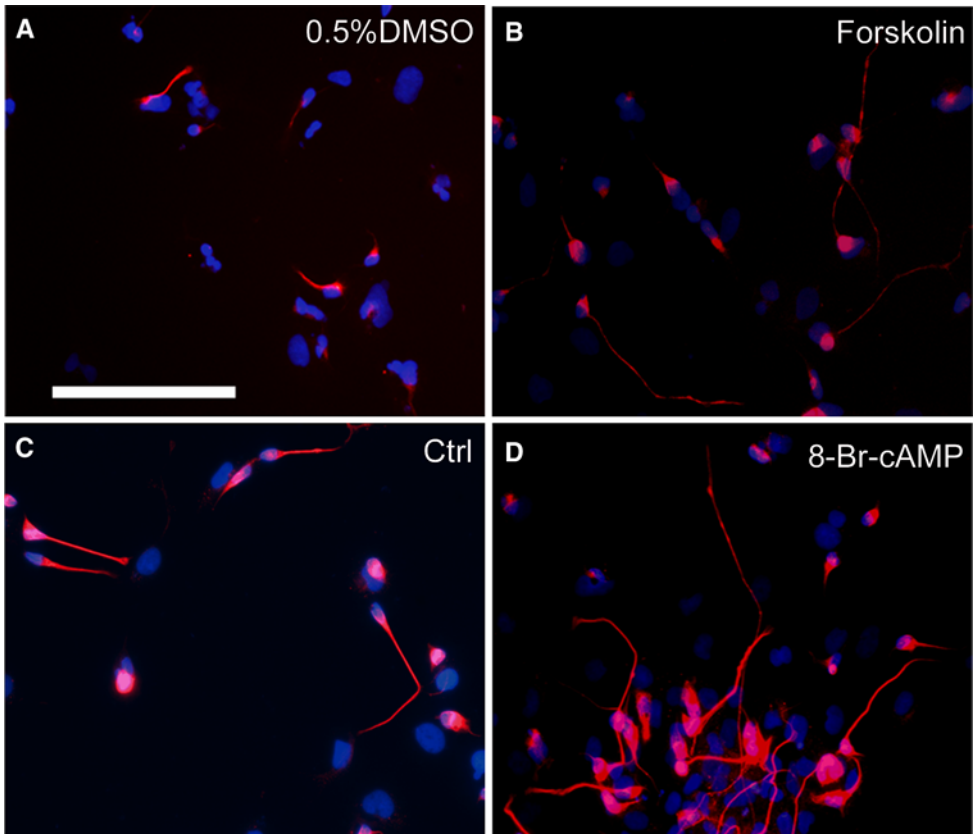

Fig. 3 Representative images of differentiating human NT2 cells in the presence of adenylyl cyclase activator (forskolin) and a cell-permeable analog of cAMP (8-Br-cAMP). After 14 days of retinoic acid treatment in Petri dishes, NT2 aggregates were mechanically dispersed into single cells. After 48 h of incubation with chemicals, cultures were fixed and stained for *β*-III-tubulin. **a**, **c** Under control condition *β*-III-tubulin positive cells elaborate neurites. In the presence of **b** forskolin and **d** 8-Br-cAMP, more cells bears neurites and elaborate long neurites. *Blue* (DAPI) indicates nuclear counterstaining. *Scale bar* 50 μm (**a**–**d**) (Color figure online)

positive cells. Taken together, the results indicate that exogenous elevation of cAMP via stimulating adenylyl cyclase or direct application of cAMP promote neurite outgrowth in a model of differentiating human neurons.

PKA Inhibitors Blocks the Formation of Neurite

Since cAMP exerts most of its cellular action via activating protein kinase A (PKA), we used two inhibitor of PKA.

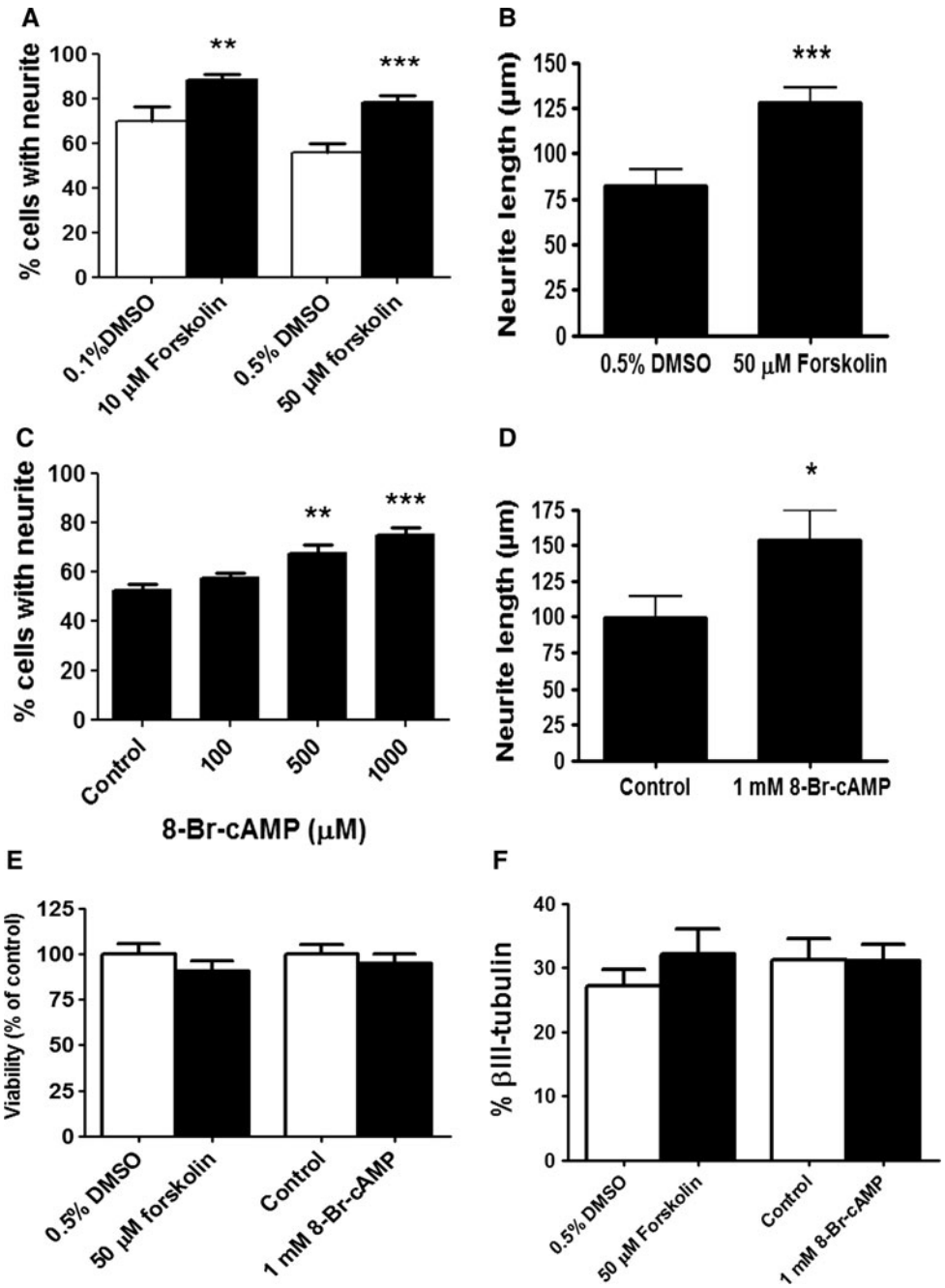

Fig. 4 Elevation of cAMP level facilitates neurite outgrowth. **a** The percentage of neurite bearing cells was significantly increased in the presence of forskolin. **b** 50 μM of forskolin significantly increased neurite extension. **c** In the presence of 8-Br-cAMP the percentage of neurite bearing cells was increased in a dose-dependent manner. **d** 1 mM of 8-Br-cAMP facilitates neurite extension. **e** The viability of cells was not significantly altered in the presence of forskolin and 8-Br-cAMP. **f** The overall percentage of neuronal cells as determined from β-III-tubulin positive cells was not significantly changed in presence of forskolin and 8-Br-cAMP. Data represent mean ± SEM from at least three independent experiments

H-89 is a specific and potent inhibitor of PKA that blocks cAMP-dependent phosphorylation of PKA substrates while Rp-cAMP is a competitive inhibitor of PKA (Murray 2008). Application of Rp-cAMP and H-89 for 48 h significantly reduced the percentage of neurite bearing cells (Figs. 5a–d, 6a). Moreover, both Rp-cAMP and H-89 inhibited neurite extension (Fig. 6b) suggesting that activation of PKA is required for the formation of neurite in differentiating human neurons. When used at concentrations that modulated neurite outgrowth, neither Rp-cAMP nor H-89 altered cell viability (Fig. 6c). Thus, the observed decrease in neurite outgrowth upon application of the two PKA inhibitors was not as a result of change in cell survival. In addition, the percentage of β-III-tubulin positive cells were not significantly altered (Fig. 6d).

Discussion

The human embryonal carcinoma stem cell line Ntera-2/cl.D1 (NT2), is extensively studied as a model of human neuronal differentiation in vitro. The NT2 cells have been

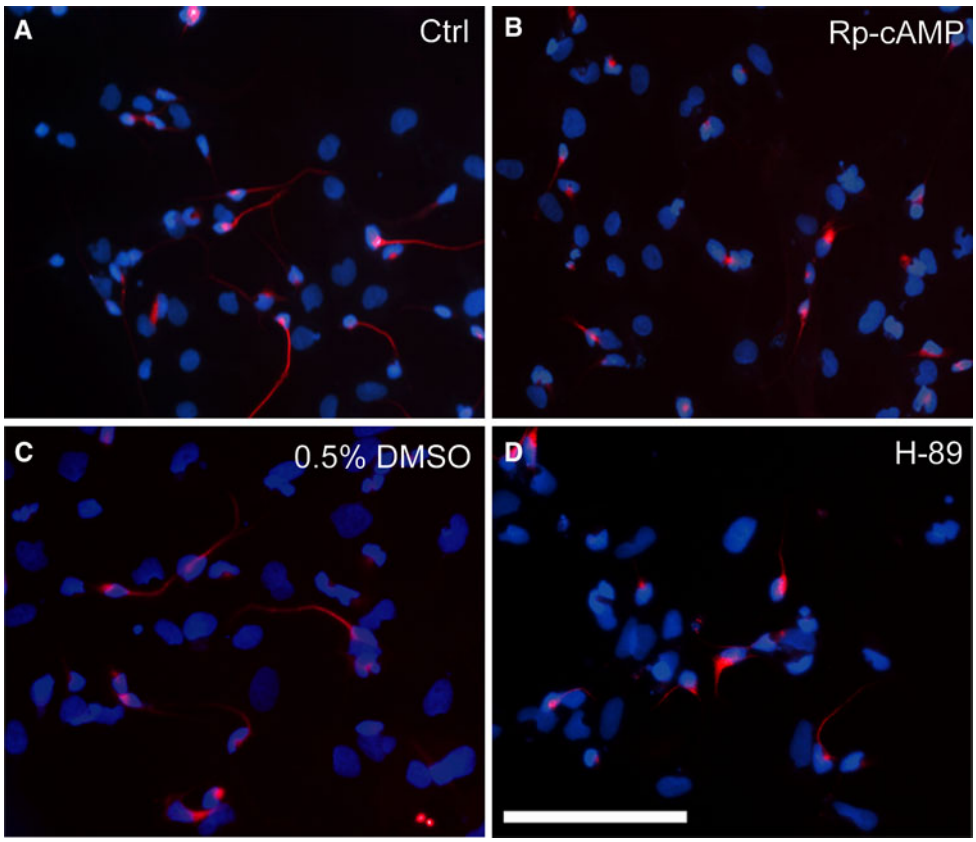

Fig. 5 Representative images of differentiating human NT2 cells in the presence of PKA inhibitors stained for β-III-tubulin (*red*). **a** Neurite bearing NT2 cells under control condition and **b** in the presence of 10 μM Rp-cAMP. **c** A solution control (0.5% of DMSO) and **d** 5 μM of H-89. *Blue* (DAPI) indicates nuclear counterstaining. *Scale bar* 50 μm (**a**–**d**) (Color figure online)

demonstrated to terminally differentiate into functional postmitotic neurons in vitro (Andrews 1984; Pleasure et al. 1992; Paquet-Durand and Bicker 2007). The major drawback was the rather lengthy differentiation protocol, which has been shortened significantly in recent years by employing the aggregate culture method (Paquet-Durand et al. 2003; Podrygajlo et al. 2009). In this study, we presented a rapid differentiation of the human NT2 cells into neurons by modifying the previous aggregate culture method (Podrygajlo et al. 2009). We obtained postmitotic neurons by prolonging the aggregate culture length to 2 weeks and avoiding intermediate re-plating steps (Paquet-Durand et al. 2003; Podrygajlo et al. 2009). Following this improved differentiation method, we obtained about 80% pure β-III-tubulin positive cells within 4 weeks (i.e., 2 weeks in aggregate culture and additional 2 weeks on Matrigel-coated substrates). Previous methods yielded up to 90% pure neurons after 6 weeks, at which point intense expression of Tau and synapsin can be observed (Podrygajlo et al. 2009; Tegenge et al. 2009). Thus, the present method allows for relatively rapid differentiation into neurons without significantly compromising the efficacy of neuronal differentiation. In addition, we found that if a relatively pure neuronal culture is not needed, neurons can be studied even earlier. After just 2 weeks of retinoic acid treatment, more than 30% of the cells were β-III-tubulin positive (Fig. 1h) and elaborate neuronal processes within a few hours of plating (data not shown).

After establishing a rapid human neuronal culture system that encompasses cell proliferation, neuronal migration, and neurite outgrowth, we next sought to determine whether it may be employed as a model to screen compounds that influence neurite outgrowth/regeneration. As a proof of principle, we have used small bioactive chemicals that modulate the cAMP/PKA signaling pathway.

In a developing neuron, the second messenger cAMP has been implicated to mediate the attractive signal of netrin-1 during axon guidance (Song et al. 1997; Ming et al. 1997). During the course of development, levels of cAMP decline; this decline has been implicated in the lack of axon regeneration in the adult CNS (Cai et al. 2001). In line with this, elevation of cAMP level has been shown to promote neurite outgrowth in models of adult CNS injury (Qiu et al. 2002a, b; Neumann et al. 2002; Hannila and Filbin 2008; Cai et al. 2001). This neurite outgrowth facilitatory action of cAMP has been attributed, in part, to counteracting the action of the growth inhibitory protein myelin. For example, high levels of cAMP permit neurite extension of neonatal dorsal root ganglion neuron (DRG) on CNS myelin suggesting a role of cAMP in neurite outgrowth on non-permissive substrates (Cai et al. 1999).

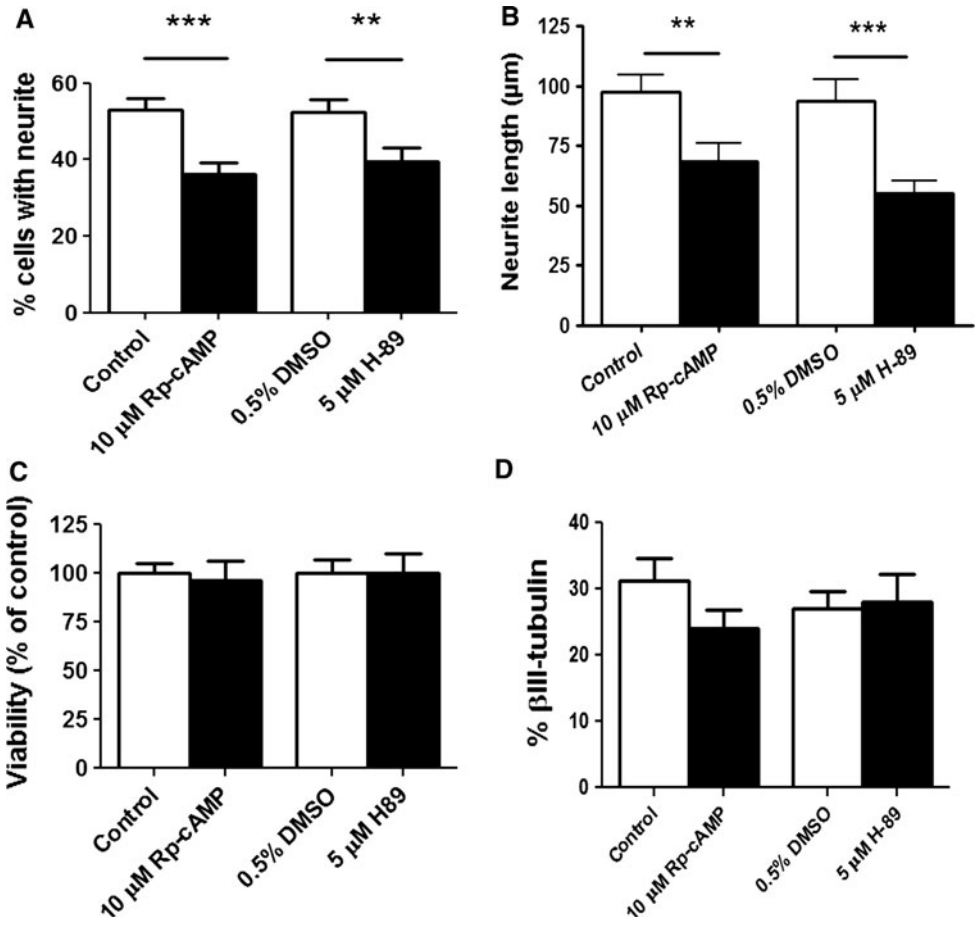

Fig. 6 PKA inhibitors block the formation of neurite in differentiating human NT2 cells. **a** The percentage of neurite bearing cells was significantly reduced in the presence of Rp-cAMP and H-89. **b** Both Rp-cAMP and H-89 block neurite extension. **c** The viability of cells was not significantly altered in the presence of Rp-cAMP and H-89. **d** The percentage of neuronal cells as determined from β-III-tubulin positive cells were not significantly altered in presence of Rp-cAMP and H-89. Data represent mean ± SEM from at least three independent experiments

However, the role of cAMP signal in promoting neurite outgrowth on permissive substrates is not fully understood, and results have been conflicting. Pharmacological manipulation of cAMP signaling has been shown to promote the rate of neurite outgrowth from DRG (Neumann et al. 2002) and embryonic motor neurons (Aglah et al. 2008) on permissive substrates. On the other hand, lack of neurite outgrowth in the presence of high levels of cAMP on permissive substrates in various model systems have been reported (Cai et al. 2001; Qiu et al. 2002a, b).

The data from the differentiating human neuron indicate that application of forskolin, which increases the level of cAMP via activating adenylyl cyclase, facilitates neurite outgrowth on permissive substrate. Direct application of a cAMP analog into the differentiating human neuronal cells also promoted neurite outgrowth. This effect of cAMP was not as a result of change in the rate of neuronal differentiation since the percentage of β-III-tubulin positive cells was not significantly altered upon both activator and inhibitor treatment for 48 h (Figs. 4f, 6d). This is in contrast to several studies conducted on PC12 cells where it has been difficult to uncouple neurite outgrowth from the actual differentiation into neurons. In PC12 cells, neurite outgrowth is a component of differentiation into neuron-like cells (Meldolesi 2010). In the NT2 cells, initial neuronal differentiation can be induced terminally by retinoic acid treatment. Application of cAMP at a later stage (i.e., after 2 weeks of retinoic acid treatment) appears to specifically stimulate neurite outgrowth without altering the fate of cells.

Most of the cellular effects of cAMP are mediated by activation of PKA. For example, chemoattractive turning of embryonic growth cones toward netrin-1 can be converted into repulsion by blocking PKA (Song et al. 1997; Ming et al. 1997) suggesting that activation of PKA is required to facilitate elongation of developing axon. The regenerative capacity of embryonic neurons has been shown to decline by blocking PKA (Cai et al. 2001). In the differentiating human neurons, the use of two PKA inhibitors resulted in significant reduction of neurite bearing cells and neurite extension suggesting that PKA activity is required for neurite formation. This result is in agreement with previous reports on NT2 cells that showed increased PKA and PKC activity following retinoic acid induced differentiation (Abraham et al. 1991). Taken together, the data implicate that the cAMP/PKA signaling pathway positively regulates neurite formation in differentiating human neurons.

Overall, the data demonstrate that rapid differentiation of NT2 cells into process-bearing neurons represents a useful in vitro model to understand cellular and molecular pathways affecting human nerve cell development and regeneration. In addition, this model may serve as a platform to screen for novel neurotherapeutics that target cellular migration, differentiation, and neurite elaboration.

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