Prolactin Induces MAPK Signaling in Neural Progenitors without Alleviating Glucocorticoid-Induced Inhibition of *in vitro* Neurogenesis

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Key Words

Neural stem cells • Neural progenitors • Neuropeptides
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Abstract

We recently demonstrated that prolactin (PRL) prevents chronic stress-induced inhibition of adult hippocampal neurogenesis. It remained unsettled, however, whether PRL is acting directly on neural stem and progenitor cells (NPCs) or if neurogenesis is affected by an indirect mechanism, for example through the extensively described effects of PRL on the HPA axis. To address this point, we used neurosphere cultures derived from the adult rat hippocampus as an *in vitro* model for NPCs. Dexamethasone (DEX) was applied to stress the NPCs, and proliferation, survival and differentiation of cells were examined. DEX markedly inhibited proliferation of NPCs and cells entered the G₀ phase of cell cycle. Moreover, DEX reduced NPC survival and repressed astroglial differentiation, which is normally induced by serum or bone morphogenetic protein application. Even though we could demonstrate that NPCs express the PRL receptor and ERK1/2 signaling is induced by PRL, we did not observe any effect of PRL

on NPCs proliferation, differentiation or survival, neither in the presence nor during absence of DEX. In summary, our results indicate that PRL action on NPCs and neurogenesis *in vivo* occurs via an indirect mechanism.

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Introduction

Stressful life events cause the inhibition of granule cell production in the dentate gyrus in rodents and primates [1-6]. The stress-induced reduction of hippocampal neurogenesis is mediated via glucocorticoids (GCs) that are released through activation of the hypothalamo-pituitary-adrenal (HPA) axis. Hence, elevated levels of GCs reduce proliferation of hippocampal progenitor cells *in vivo* and *in vitro* [3, 4, 7-9]. In support, removal of circulating GCs by adrenalectomy leads to an increase in hippocampal neurogenesis and hippocampal plasticity [10, 11].

The effects of GCs on neuronal and glial differentiation are controversial. While GCs inhibit neuronal

differentiation of the newly generated adult hippocampal granule cells *in vivo* [12], they block astroglial differentiation in fetal mesencephalic NPCs *in vitro* [13]. In contrast, other reports did not observe any effects of GCs on the differentiation of adult or embryonic hippocampal progenitors *in vitro* [7, 14].

The neuropeptide prolactin (PRL) attenuates stress response and reduces anxiety [15, 16], most likely through a decrease in the levels of corticotropin releasing hormone (CRH), adrenocorticotrophic hormone (ACTH), corticosterone and noradrenaline [17]. PRL affects proliferation of different cell types within the central nervous system. For example, it enhances proliferation of embryonic astrocytes [18] and of oligodendrocyte precursor cells within the corpus callosum and the spinal cord [19]. Moreover, infusion of PRL increases precursor cell proliferation within the subventricular zone (SVZ) [20].

Recently, we demonstrated that PRL prevents from chronic restraint stress-induced inhibition of hippocampal neurogenesis [21]. It remains unclear, however, whether PRL acts directly on NPCs to promote neurogenesis, or if PRL influences neurogenesis via indirect mechanisms, for example through its effects on the HPA axis. To address this point, we used adult hippocampus-derived neurosphere cultures that were exposed to DEX, resembling an *in vitro* stress-neurogenesis model. We investigated the effects of DEX and of PRL on NPC proliferation, cell fate and survival. Moreover, we studied PRL-induced signal transduction in NPCs.

Materials and Methods

Isolation, culture, and differentiation of adult hippocampal neural progenitors

Adult hippocampal progenitor cells were isolated and cultured as described before [22]. Briefly, two to four month-old female Fischer-344 rats (Charles River Deutschland GmbH, Germany) were decapitated and hippocampi were dissected. The tissue was homogenized and cells were resuspended in Neurobasal (NB) medium (Gibco BRL, Germany) containing B27 supplement (Gibco BRL, Germany), 2 mM L-glutamine (PAN, Germany), 100 U/ml penicillin/100 µg/ml streptomycin (PAN, Germany). For expansion, the NB/B27 was growth factor-supplemented with 2 mg/ml heparin (Sigma, Germany), 20 ng/ml FGF-2 (R&D Systems, Germany) and 20 ng/ml EGF (R&D Systems, Germany) (proliferation medium). The cell suspension from hippocampi of 5 rats was seeded into a T25 flask (TPP, Switzerland) in 5 ml proliferation medium. The cultures were maintained at 37°C in a humidified incubator with 5% CO₂ and half of the media was changed every 3 to 4 days.

For passaging, cells were dissociated by using accutase (PAA, Austria) and a total of 5×10^5 cells were seeded in a T75 flask (TPP, Switzerland). Neurosphere cultures from passages 3-7 were used for the experiments. The cultures consist of 11 ± 3 % nestin positive stem/progenitors and 75 ± 17 % of A2B5 positive progenitors. In some experiments, we used adult rat subventricular zone derived progenitors. These contained 32 ± 7 % Nestin expressing cells and 89 ± 13 % A2B5 positive cells.

For differentiation, 2.5×10^4 cells per well were seeded into 24 well plates (TPP, Switzerland) containing laminine (Sigma, Germany) and poly-ornithine- (250 µg/ml; Sigma, Germany) coated glass coverslips. Differentiation was induced using growth factor free NB medium containing 5% fetal calf serum (FCS) (PAN, Germany) (differentiation medium) or by using αMEM (Gibco BRL, Germany) containing 10% FCS. To induce astroglial differentiation αMEM was further supplemented with 10 ng/ml bone morphogenetic proteins (BMPs) 2 and 4 (R&D Systems). In order to rule out region- or species-specific effects, we verified some experiments also by using SVZ-derived rat NPCs and hippocampal mouse NPCs (data not shown).

Quantitative analysis of cell density and cell counts

MTS assay: cell proliferation/expansion was assessed using CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay (MTS assay) (Promega, USA). 0.5×10^4 cells per well were plated into 96 well cell culture plates (TPP, Switzerland) in a volume of 100 µl proliferation media. The cells were grown for 7 days in the presence or absence of DEX (100 nM or 1 µM), PRL (10 ng/ml, 100 ng/ml, or 1000 ng/ml) or vehicles (Veh) (control substance for DEX: 100% EtOH, for PRL: 0.01 M NaOH). Substances were added every second day. At day 7, 20 µl of the CellTiter 96® AQ_{ueous} One Solution Reagent were applied into each well. Plates were incubated at 37°C. After 1, 2, 3 and 4 hours, optic density was measured with a 96 well plate reader (Emax Precision Microplate Reader, Molecular Devices, Union City, CA, USA).

Cell counting: cells were seeded into 12 well plates (TPP, Switzerland) with a density of 5×10^4 per well in a volume of 1 ml proliferation media. Cells were grown for one week as described above, factors were added every second day but medium was not changed. At day 7 after seeding cells were dissociated using accutase (PAA, Austria), diluted with Trypan blue (Sigma, Germany) and counted using a hemacytometer (Neubauer) counting chamber.

Estimation of sphere size: Cells were grown in proliferation medium, seeded into 96 well plates and treated with DEX/PRL/vehicle for 7 days as described above. At day 7 after seeding 5 pictures per well were taken (magnification 20x) using an Olympus IX 70 microscope with a Color View documentation system (Olympus, Hamburg). Sphere number and size were analyzed using analySIS 3.2 (Soft Imaging Systems, Münster, Germany).

RNA extraction, cDNA synthesis and Real Time PCR

Cells were treated with DEX or control substance for 7 days and RNA was isolated using Qiagen RNeasy Kit following the manufacturer's protocol.

For cDNA synthesis we used Promega reverse transcription PCR Kit. Master mix-contained 1 µg RNA, 4 µl MgCl₂ (25 mM; final 4 mM), 2 µl dNTP (10 mM; final 800 µM), 2 µl Reverse Transcription 10x buffer (100 mM Tris-HCl, (pH 8.8); 500mM KCl, 1% Triton X-100), 1 µl Random Hex Primer (500 ng/µl; final 20 ng/µl), 0.7 µl AMV Reverse Transcriptase (5U/ml), 0.5 µl RNase Inhibitor (40U/ml), 0.8 µl H₂O. Quantitative Real time PCR was performed using Stratagene, Mx3000P qPCR System. Samples were analyzed in reaction volume of 20 µl, which contained 10 µl SYBR-Green jump start taq master mix (Sigma), 5 µl H₂O, 0.5 µl of each primer (2.5 µM), and 4 µl of a 1:4 dilution (50 ng) of the respective cDNA. Data was analyzed using the Mx3000P analysis software. Primer sequences: p21 fwd: TGT TCC ACA CAG GAG CAA AG, p21 rev: AAC ACG CTC CCA GAC GTA GT; p27 fwd: GCG TTT CGC TTT TGT TTG GT, p27 rev: ACG TTT GAC ATC TTC CTC CCC.

Cell cycle analysis

The cell cycle was analyzed by fluorescence activated cell sorting (FACS) using a modified Ki67/PI staining protocol from Endl and colleagues [23]. Cells were seeded into T25 flasks (TPP, Switzerland) and stimulated with 1 µM DEX or EtOH for 6 days. DEX was added every second day. On day 7, cells were spun down, medium was removed and cells were fixed with 1 ml ice cold 70% EtOH. After fixation cells were washed with ice cold PBS and then resuspended in 500 µl ice cold PBS containing 0.1% TritonX-100 and incubated for 5 min on ice. After two subsequent washing steps, cells were resuspended in 100 µl of a 1:6 dilution of the antibodies in PBS (100 µl PBS + 20 µl antibody IgG or Ki67 BD Biosciences Pharmingen, FITC conjugated antibodies). After 30-40 min incubation at 4°C, cells were washed with 1 ml PBS and then resuspended in PBS for staining. In case of PI staining, cells were resuspended in 470 µl PBS. Cells without PI staining were resuspended in 495 µl. All samples were treated with 5 µl of RNase for 1 hour to eliminate the RNA, which would interfere with the PI staining. After this 1h incubation at 37°C, 25 µl of PI were added to the respective samples. The stained cells were analyzed with the Flow Cytometer (FACS Calibur, Becton Dickinson, Heidelberg). Data was analyzed using Win MDI 2.8 software.

Differentiation and immunostainings

Immunocytochemistry was performed as described before [24]. Before starting the treatments, cells were seeded and allowed to attach for one day, and then DEX (1 µM) and/or PLR (1 µg/mL) and/or BMP-2 and BMP-4 (10 ng/ml each) were added to the culture medium. Medium was changed every second day and after 7 days cells were fixed with 4% paraformaldehyde (PFA) for 30 minutes.

After fixation, PFA was removed and cells were washed 3 times with PBS. For blocking, cells were incubated in fish skin gelatin buffer (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 1% BSA, 0.2% fish skin gelatin (Sigma, Germany)) was applied to the cells for two hours. For staining of intracellular markers, 0.1% Triton X-100 was added to the solution. This buffer was also used for all further washing steps, as well as for dilution of

antibodies. Following blocking, cells were incubated with the primary antibody at 4°C overnight. The following primary antibodies were used: mouse anti-microtubule associated protein 2ab (Map2ab) (dilution 1:250; Sigma, Germany), rabbit anti-glial fibrillary acidic protein (GFAP) (dilution 1:1000; DAKO, Denmark) and, myelin basic protein (MBP) (dilution 1:500, Covance, CA, USA).

After washing, cells were incubated with the secondary antibody for 2 hours at room temperature. Secondary antibodies used were donkey anti-rabbit Alexa Fluor 488 conjugated antibody and donkey anti-mouse Alexa Fluor 488 conjugated antibody (dilutions 1:1000, respectively, Invitrogen Molecular Probes) and donkey anti-mouse antibody Rhodamine Red-X conjugated (dilution 1:500, Jackson ImmunoResearch, Newmarket, UK). Nuclei were stained with 0.25 µg/ml DAPI diluted in PBS. After 3 additional washing steps cells were mounted on slides using Prolong Gold Antifade reagent (Invitrogen Molecular Probes, Oregon, USA).

Survival Assay

Cells were seeded on laminin/poly-ornithine coated coverslips in 24 well plates with a density of 2.5×10^4 cells per well in a volume of 500 µl differentiation media. Cells were treated with 1 µM DEX and/or 1 µg/mL PRL or vehicle and grown for 3, 5, or 7 days. For cell death analysis, cells were incubated for 8 minutes with 50 µg/ml propidiumiodide (PI). Then, cells were fixed with 4% PFA, washed and stained with DAPI as described above. For each well, five randomly selected visual fields were photomicrographed using an inverse Fluorescence microscope (Olympus, Hamburg) using analySIS 3.2 software (Soft Imaging Systems, Münster, Germany) and the percentage of PI-positive cells was determined.

Western Blotting

Cells were lysed in protein isolation buffer as described before [25]. The suspension was then passed several times through a 20-gauge needle fitted to a 10 ml syringe to lyse the cells. The homogenate was shaken 30-60 min at 4°C. Afterwards, it was centrifuged at 17900xg for 20 min. The supernatant was collected into a new tube. Protein concentration was determined using BCA test (Sigma, Germany). Proteins were separated on a 12% polyacrylamide gel (20 µg per lane) and blotted onto a nitrocellulose membrane for Western Blotting. Membrane washes were performed with TBS containing 0.1% Tween-20 (Sigma, Germany) (TBST). For blocking and antibody dilution TBST containing 5% bovine serum albumin (BSA) (Biomol, Germany) was used. The primary antibody (mouse anti-prolactin receptor, Affinity Bioreagents, Golden, Co, USA) was diluted 1:1000 and applied overnight at 4°C. Secondary antibody (horseradish peroxidase-conjugated goat anti-mouse antibody, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was diluted 1:5000 and applied to the membrane for one hour at room temperature. Immuno-complexes were detected using ECL-Plus Western Blotting Detection System (Amersham-Pharmacia, Freiburg) and Amersham HyperfilmTM ECL (GE Healthcare Amersham, UK).

Fig. 1. DEX inhibits NPC proliferation. NPCs were treated for one week with DEX. A) Determination of cell number and B) MTS assay demonstrates that 1 μ M and 10 μ M DEX limit the expansion of the NPC culture. One way ANOVA, post hoc test: Tukey, * p <0.05, ** p <0.1, *** p <0.001, n =3. C) Phase contrast image of control and DEX-treated neurospheres showing reduced sphere size in DEX-treated cultures; scale bars: 100 μ m. D) Treatment of NPCs with 1 μ M DEX significantly reduced the number of spheres larger than 60 μ m in diameter size, but did not change overall sphere number (E). One way ANOVA, post hoc test: Tukey, *** p <0.001, n =3. F) RT-PCR results on DEX induced elevation of p21 mRNA expression; n =3; control: 100%. G) Plotting of Ki67 signal against PI signal area. Cells in the lower left quadrant are Ki67 negative and reflect cells in G_0 arrest. Cells in the upper left quadrant are G_1 phase cells; cells in the upper right quadrant are cells at S and G_2/M phase. H) FACS analysis of Ki67/PI stained cells revealed a strong shift of DEX-treated cells into G_0 phase of the cell cycle. Two way ANOVA, Bonferroni post hoc test, * p <0.05, ** p <0.01. n =3. Significant differences in respect to control are marked with asterisks.

Results

Dexamethasone induces growth arrest in adult neural progenitor cells

As previously shown hippocampal neural progenitors express glucocorticoid and mineralocorticoid receptors [26]. Apparently, glucocorticoid receptors are activated by high levels of glucocorticoids that typically occur during stress response (for review see [27]). With the aim to mimic the impact of stress on neurogenesis, we treated neurosphere cultures [22] with the glucocorticoid receptor agonist DEX and analyzed cell proliferation, differentiation and survival. NPCs were seeded and grown in the presence of 0 μ M (vehicle only), 1 μ M or 10 μ M DEX for 7 days. DEX or vehicle was added to the culture every second day. Both, the 7-day incubation of NPCs with 1 μ M or with 10 μ M DEX significantly reduced the total number of cells, compared to vehicle-treated cultures, as determined by direct cell counting and by

MTS assay (Fig. 1A, B).

The total number of neurospheres, which is a rough estimation of the amount of sphere forming units or of proliferation-competent cells, was not affected by DEX treatment, even though neurospheres appeared to be smaller than those seen in vehicle-treated cultures (Fig. 1C-E). The quantitative analysis revealed that DEX significantly reduced sphere size (ctrl 32.02% > 60 μ m; DEX 1 μ M: 11.39% > 60 μ m, p <0.05; DEX 10 μ M: 4.845% > 60 μ m, p <0.001), whereas the total number of spheres within the cultures was not affected (Fig. 1D, E). In all experiments described in this report, 1 μ M DEX was as effective as 10 μ M DEX. Thus, for all the following experiments, a dose of 1 μ M DEX was used.

Next, we hypothesized that the DEX induced reduction in NPC numbers might be caused by an inhibition of the cell cycle. Therefore, we analyzed the expression of p27kip2 and of p21, two major regulators of the cell cycle, 7 days after DEX or vehicle treatment. While the

Fig. 2. PRL signals in NPCs but does not promote NPC proliferation. A) Western Blot of 20 μ g NPC protein samples per lane showing that NPCs express the short form of PRLR (~ 40 kDa). B) Western Blot of NPCs for pERK1/2 (upper blot) and ERK1/2 (lower blot) demonstrating that PRL activates MAP-Kinase signaling in NPCs. Hypothalamus (Ht) as control. C) PRL treatment does not affect NPC proliferation: cells were treated with 10 ng/ml, 100 ng/ml and 1 μ g/ml of PRL for 7 days. Analysis of cell number using the MTS assay showed no effect of PRL on proliferation. n=3. D) PRL treatment does not block the anti-proliferative effect of DEX on NPCs: cells were treated with DEX and additionally with PRL for 7 days. Analysis of cell number by MTS assay showed that PRL did not prevent from DEX induced inhibition of NPC proliferation. One Way ANOVA; post hoc test: Tukey **p<0.05, ***p<0.01. n=3. E) NPCs were pretreated with 1 μ g/ml PRL for 6 days and then grown in the presence or absence of PRL and DEX. The pretreatment with PRL did not prevent DEX-induced inhibition of NPC proliferation. One way ANOVA; post hoc test: Tukey, ***p<0.001. n=3. Significant differences in respect to control are marked with asterisks.

expression of p27kip2 was not significantly changed after DEX stimulation (data not shown), DEX induced a significant up-regulation of p21 mRNA (Fig. 1F).

The reduction in the total number of cells and inhibition of cell cycle observed after DEX treatment might either result from induced cell quiescence or a general lengthening of the cell cycle. Thus, we analyzed the cell cycle by propidium iodide (PI)/Ki67 FACS analysis. This method allows the dissection of G_0 and G_1 , since Ki67 is expressed throughout the cell cycle except in G_0 [23]. Figure 1G shows a representative plot of the FACS analysis of DEX-treated cells. The quantitative analysis revealed that DEX treatment induced a strong shift from the S and G_2 /M towards the G_0 phase of the cell cycle (G_0 : ctrl, 24.7%; DEX, 43%, p<0.01) (Fig. 1H).

Prolactin activates MAPK signaling in NPCs

One goal of the present study was to analyze direct effects of PRL on NPCs under normal and stressed conditions. In a first set of experiments, we analyzed PRL signaling in NPCs. PRL receptors (PRLR) exist in three different isoforms, which differ in length and composition of their intracellular domain and are referred to as short, intermediate, and long isoform respectively [28], Western Blot analysis demonstrates that NPCs express the short PRLR (Fig. 2A). Since the short PRLR is known to signal via MAP-Kinase pathway [28], and PRL has recently

been shown to activate the ERK1/2 MAP kinase cascade in neurons *in vitro* and *in vivo* [29] we analyzed ERK1/2 phosphorylation at different time points after PRL stimulation. The level of pERK1/2 was significantly elevated as early as five minutes after PRL stimulation and peaked at 30 minutes (Fig. 2B). Thus, NPCs possess a functionally active isoform of the PRLR, which activates the MAPK cascade following PRL stimulation.

Prolactin does not prevent DEX-induced effects on NPCs

NPCs were treated with PRL (10 ng/ml, 100 ng/ml, or 1000 ng/ml) alone or in combination with 1 μ M DEX. Substances were re-added every second day and one week after seeding proliferation of cells was analyzed using the MTS assay. PRL did not have any detectable effects on NPC proliferation (Fig. 2C). Moreover, in cases of PRL treatment in combination with DEX, PRL did not prevent from the DEX-mediated reduction in cell number (Fig. 2D). Next, we asked whether pre-stimulation with PRL might prevent from DEX-induced inhibition of cell proliferation. As shown in Fig. 2E, a 6-day PRL pretreatment did neither increase proliferation on its own nor attenuate the anti-proliferative effect of DEX, even when DEX was used in a lower concentration (100 nM DEX) (Fig. 2E).

Fig. 3. PRL does not prevent DEX-induced NPC cell death. NPCs were treated with DEX and/or PRL for 1 (A), 3 (B), 5 (C), and 7 (D) days and the percentage of PI-positive cells was determined. DEX significantly induced cell death beginning at day 5 (d5) after starting the treatment. PRL did not prevent from DEX-induced cell death nor did it increase survival if applied alone. One way ANOVA; post hoc test: Tukey, * $p < 0.05$, ** $p < 0.01$. $n = 3$. Significant differences in respect to control are marked with asterisks.

Effects of PRL and DEX on NPC survival and differentiation

To examine whether PRL affects survival of NPCs during differentiation, cells were seeded and grown for 1, 3, 5, or 7 days in the presence or absence of DEX and/or PRL. At the end, cells were stained with PI and DAPI to determine the percentage of dead cells. DEX significantly induced cell death as demonstrated by an increase in the percentage of apoptotic cells after 5 and 7 days of treatment (Fig. 3C, D). PRL could neither prevent spontaneous nor DEX-induced cell death according to the number of PI-positive cell profiles detected in vehicle and PRL treated cultures. Thus, our results indicate that PRL has no anti-apoptotic or survival promoting effect during differentiation of NPCs *in vitro*.

Next, we investigated the PRL effects on NPC differentiation. NPCs were seeded in differentiation medium and incubated overnight to allow the cells to attach, before DEX and/or PRL was added. Cells were grown for 7 days and media was changed every second day. Then cells were fixed and stained for the astroglial marker glial fibrillary acidic protein (GFAP), the oligodendroglial marker myelin basic protein (MBP) (mature oligodendrocytes), and the neuronal marker microtubule-associated protein 2 isoforms a and b (Map2ab). We did not observe any effect of PRL on the differentiation of NPCs. Consistent with previous reports however, 1 μ M DEX significantly decreased the frequency

of astrocytic differentiation in culture as reflected by fewer GFAP-positive cells (Fig. 4A, B) (ctrl: 29.56%; 1 μ M DEX: 10.35%; PRL+DEX: 4.76%; $p < 0.001$). Co-treatment with PRL did not prevent this DEX-induced loss of GFAP positive cells. The amount of oligodendrocytes generated did not differ after any of the treatments (Fig. 4A, C). The percentage of Map2ab positive cells was very low ($< 1\%$) and was also not changed in any of the conditions (data not shown).

In the following experiments, we tested whether the DEX-associated reduction of the GFAP-expressing population in NPC cultures was due to inhibition of astroglial differentiation or due to direct effects on pre-existing astrocytes. Thus, prior to stimulating cultures with DEX for 7 days as described above, NPCs were pretreated with serum and/or BMP2 and BMP4 for 7 days, which induced a massive astroglial differentiation within this pre-treatment period [30]. Three different treatments were chosen: the first group received no BMP, the second group received BMP only during the first 7 days, and the third group received BMP during the entire 14 days. The results show that DEX did not influence the percentage of astrocytes in the cultures, independently of the stimulation paradigm (Fig. 5 A-C).

Thus, these results indicate that DEX affects predominantly the astroglial differentiation process, but does not impair survival of pre-differentiated astrocytes. However, differences in cellular morphology such as

Fig. 4. DEX and PRL effects on NPC differentiation. NPCs were seeded in differentiation medium and grown in the presence or absence of PRL and DEX for 7 days. A) Immunofluorescence staining of Veh, DEX-, PRL-, and DEX- plus PRL-treated cultures stained for MBP (oligodendrocytes) and GFAP (astrocytes), demonstrating that DEX-treated cultures have a reduced number of GFAP positive cells, while PRL did not influence the overall MBP or GFAP expression. Scale bar: 100 μ m. Quantitative analysis of B) % of GFAP and C) % of MBP expressing cells. One way ANOVA; post hoc test: Tukey, $**p < 0.01$. $n = 3$. Significant differences in respect to control are marked with asterisks.

Fig. 5. DEX and PRL effects on differentiated astroglia. NPCs were pretreated for 7 days with or without serum and/or BMP2 and BMP4, before stimulation with DEX for another 7 days, and the percentage of GFAP expressing cells was analyzed. A) No BMP treatment, B) BMP treatment from day 1 to day 7, C) BMP treatment from day 1 to day 14. Note that DEX did not influence the percentage of GFAP expressing cells in the cultures, independently of the stimulation paradigm. D) Immunofluorescence staining of vehicle and DEX treated cultures demonstrating DEX-induced reduction in soma size and cellular processes. Scale bar represents 100 μ m. Significant differences in respect to control are marked with asterisks.

reduction in soma size and cellular processes in DEX-treated cultures were observed (Fig. 5 D). In summary, application of DEX during the differentiation period

reduced the percentage of GFAP-positive cells, while DEX did not affect the number of already differentiated astrocytes, although it did affect astroglial morphology.

Discussion

Multiple effects of DEX on NPCs

The impact of GCs on neural cells is quite diverse and depends on the individual cell type and its developmental stage. For example, elevated levels of GCs reduce proliferation of hippocampal progenitor cells *in vitro* and *in vivo* [7], and in support, removal of circulating GCs by adrenalectomy leads to an increase in hippocampal cell proliferation [10]. The effects of GCs on progenitor fate and neuronal/glial differentiation are more controversial. For instance, elevated levels of corticosterone inhibit neuronal differentiation of newly generated granule cells in the adult rat hippocampus [12] and block astroglial differentiation in fetal mesencephalic progenitors [13]. Other reports, however, did not observe any effects of GCs on the differentiation of adult or embryonic hippocampal progenitors [7, 14].

The impacts of GCs on neuronal survival also appear to be rather diverse. Acute GC exposure promotes neuronal survival and neuroprotection through activation of neurotrophin receptors [31]. Moreover, it attenuates neuronal loss after cerebral ischemia [32]. Conversely, chronic treatment with GCs potentiates neuronal damage and loss after ischemic injury [33]. It is hypothesized that the differential effects of GCs on neuronal survival are partly explained by a dose-dependency. Physiological levels of GC concentrations provide a balanced milieu for neuronal integrity, while moderately increased levels of GCs promote neuroprotection. In contrast, lack or high concentrations of GCs cause and promote neuronal damage [34].

Furthermore, differential and opposite actions of GCs might be mediated via the activation of different glucocorticoid receptors. Whereas activation of mineralocorticoid receptors is sufficient to reduce apoptosis, glucocorticoid receptor activation results in an increase of apoptosis. Hence DEX, which is a glucocorticoid receptor agonist, was previously shown to increase apoptosis in the hippocampus, whereas application of corticosterone at doses that only activate the mineralocorticoid receptors decreased apoptosis [35, 36].

We revisited the question of glucocorticoid activities on neural cells by focusing on neural progenitor cells derived from the adult rat hippocampus. Our results confirm the anti-proliferative effects of DEX, which involve a cell cycle shift from S and G₂/M to G₀. This notion is supported by the fact that DEX up-regulates the cell cycle inhibitors p21 and p27 in a variety of different cell types [7, 37, 38], which we could partly confirm.

Cell cycle exit of NPCs might be understood as stem cell quiescence and consequently as protection of the stem cell niche. Such an explanation was recently proposed for the hematopoietic stem cell niche, where DEX provides bone marrow- and hemato-protection via fostering quiescence of the hematopoietic stem cell pool [39]. In the present work, although DEX inhibited the proliferation rate, the number of neurosphere forming units and thus, also the pool of active progenitors, was maintained. Paradoxically, we also demonstrated that DEX induced cell death in NPCs, thus being potentially detrimental for the stem cell niche. Similarly, DEX has been previously shown to increase apoptosis in fetal and adult hippocampal progenitor cell culture [7, 14].

Apart from the anti-mitotic effect of DEX on NPCs, we observed a strong inhibition of astroglial differentiation, which might be of therapeutic relevance in the context of axonal regeneration. Indeed, chronic psychosocial stress reduces the number of astroglia in the adult tree shrew hippocampus [40], and DEX diminishes reactive astrogliosis in the neocortex [41]. Moreover, suppression of astroglial scar formation enhances axonal regeneration and functional recovery after spinal cord injury [42]. An anti-astroglial effect was also demonstrated for methylprednisolone, a synthetic glucocorticoid, inhibiting the expression of GFAP in reactivated astrocytes [43].

The effects of PRL on neurogenesis and NPCs

We demonstrate that PRL neither affected cell proliferation nor differentiation or survival of NPCs *in vitro*. Furthermore, PRL did not protect NPCs from the antiproliferative, apoptotic and anti-differentiation effects of DEX. This lack of PRL-associated protection, however, was not due to a lack of functional PRL receptor expression, as we could demonstrate by PRL receptor protein expression and ERK1/2 signaling. Thus, it appears that the profound impact of PRL on *in vivo* neurogenesis [20, 21] is probably not mediated via direct activities of PRL on neural progenitors, but rather via indirect mechanisms such as PRL-induced attenuation of HPA axis responses [15, 16]. Our results substantiate previous work by Shingo et al., showing that *ex vivo* growth of NPCs was stimulated only after chronic PRL infusion in the animal [20].

The effects of PRL on neurogenesis are region-specific. While Shingo et al. reported a proliferative effect specifically in the SVZ, but not in the hippocampus, PRL protected hippocampal neurogenesis from chronic stress-induced impairment of

survival and neuronal differentiation of newly generated cells [21]. Thus, the two neurogenic regions appear to be differentially regulated [44, 45]. Such differences might reflect the cellular composition of the individual stem cell niche. Indeed, while the ventricular wall was shown to contain neural stem cells, the dentate gyrus might be enriched with fate-restricted progenitors [46]. In order to rule out region-specific or also species-dependent effects, we performed some of the experiments, i.e. the effects of DEX and PRL on cell proliferation, using SVZ-derived rat NPCs and hippocampal mouse NPCs. The acquired results demonstrated that DEX inhibited and PRL did not promote NPC proliferation regardless of the culture origin (data not shown).

In summary, based on the present results, modulation of hippocampal neurogenesis *in vivo* is unlikely to be

due to direct effects of PRL on neural progenitor cells. PRL might act indirectly on the neural circuitries for instance via modulation of HPA axis.

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