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Impact of protein kinase CK2 inhibitors on proliferation and differentiation of neural stem cells

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Abstract

Background: Protein kinases play central roles in cell and tissue development. Protein kinase CK2, an ubiquitously expressed serine/threonine kinase has severe impacts on embryo- and spermatogenesis. Since its role in neurogenesis has so far only been investigated in very few studies, we analysed the role of CK2 in neural stem cells by using two specific inhibitors.

Methods: Neural stem cells were isolated from the subventricular zone of neonatal mice, using a neurosphere approach. Proliferation of the neurospheres, as well as their differentiation was investigated with and without inhibition of CK2. Changes in proliferation were assessed by counting the number and measuring the diameter of the neurospheres. Furthermore, the absolute cell numbers within the neurospheres were estimated. Differentiation was induced by retinoic acid in single cells after dissociation of the neurospheres. CK2 was inhibited at consecutive time points after induction of the differentiation process.

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Results: CK2 inhibition reduced the amount and size of proliferating neurospheres dose dependently. Adding the CK2 inhibitor CX–4945 at the start of differentiation we observed a dose-dependent effect of CX-4945 on cell viability and glia cell differentiation. Adding quinalizarin, a second CK2 inhibitor, at the start of differentiation led to an elevated level of apoptosis, which was accompanied by a reduced neural differentiation. Adding the CK2 inhibitors at 72 h after the start of differentiation had no effect on stem cell differentiation. Conclusion: Inhibition of CK2 influences early gliogenesis in a time point and concentration dependent manner.

General significance: The use of a CK2 inhibitor significantly affects the neural stem cell niche.

Keywords: Health sciences, Biological sciences, Biochemistry, Cell biology, Developmental biology, Neuroscience

1. Introduction

Protein kinases are key players in the regulation of cellular pathways and abnormal phosphorylation might lead to diseases. More than 500 kinases are encoded in the human genome. Due to the fact that they can easily be modulated by small molecules, the role of protein kinases as drug targets increases steadily [1, 2]. Protein kinase CK2 is involved in a broad range of signalling pathways regulating proliferation, differentiation, apoptosis or senescence [3, 4]. Although CK2 is now known for decades, its specific regulation is still largely unknown. More than 400 substrates are phosphorylated by CK2, which makes it hard to find one specific function. It has been shown that CK2 regulates, besides the above mentioned processes, also tumour development [4] and angiogenesis [5, 6]. Overexpression of CK2 seems to be correlated with oncogenesis or cardiovascular diseases, which led to numerous studies that dealt with potential inhibitors in cancer therapy (for review see: [7, 8, 9, 10]).

CK2 is much more abundant in the brain than in any other tissue [11]. According to the distribution in the body it is not surprising that there is a large number of substrates in synaptic and nuclear compartments. These CK2 substrates are somehow implicated in the development, neuritogenesis, synaptic plasticity, synaptic transmission and information storage [12, 13, 14]. On the other hand, it is known that neurotrophins regulate the activity of CK2 in the hippocampus [15, 16, 17 and papers cited within]. Huillard et al. [15] have shown that a disruption of CK2β in embryonic neural stem cells leads to a reduced differentiation of oligodendrocytes, most probably by regulating Olig-2 activity. CK2 is involved in both, Alzheimer and Parkinson's disease (PD). In Alzheimer's disease it has been shown that CK2 is associated with neurofibrillary tangles and that its activity is

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increased in the presence of the pathogenic peptide amyloid-β [16, 17]. In biopsies from PD patients CK2β subunits could be found in Lewy bodies [18].

The aim of the present study was to elucidate the role of CK2 in neurogenesis and differentiation. Neurogenesis takes place all over the life span in different neural stem cell niches in both, central and peripheral nervous system, and can be influenced by diseases, diet or inflammation. However, very little is known about the role of CK2 in stem cell differentiation. We used recently introduced small molecule CK2 inhibitors, that were applied for the treatment of cancer [19, 20, 21, 22], to study a possible influence on still ongoing neurogenesis in the central and peripheral nervous system.

Neural stem cell differentiation can be experimentally investigated by using neural stem cells, e.g. from the mouse, that were cultured as so-called neurospheres. These cells can be obtained from highly proliferative areas within the postnatal brain, for example from the subventricular zone (SVZ) or the dentate gyrus in the hippocampus. The neurospheres contain mainly neural stem cells, but also small amounts of already differentiated neurons or glial cells, and can be differentiated using e.g. retinoic acid alone or in combination with extracellular matrix proteins, such as laminin. The differentiated cells can be characterized with marker proteins that are unique for the cell types. Typical marker proteins are βIII-tubulin and PGP9.5 for neurons, glial fibrillary acidic protein (GFAP) for astrocytes and nestin for neural stem cells. Here, we investigated the role of CK2 inhibition in neurogenesis in the central nervous system concerning proliferation and differentiation of neural stem cells derived from the subventricular zone (SVZ).

2. Materials and methods

2.1. Animals

Balb/c wild-type mice of both sexes aged 2–5 days were used.

Animal preparations were conducted with the general approval for the killing of animals and the removal of organs and tissues by the LUA (Landesuntersuchungsamt) Rhineland Palatinate, according to the animal protection laws in Rhineland-Palatinate, Germany.

2.2. Isolation and cultivation of neural stem cells from the subventricular zone of newborn mice

For isolation of neural stem cells either 2 or 4 animals were used depending on the experiment. For protein extraction and proliferation experiments 4 animals were used. The brains of postnatal mice were removed after quick decapitation and stored in ice cold MEM-Hepes (Minimal essential medium, Gibco, with 1% penicillin/streptomycin). The subventricular zones were dissected from both

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hemispheres under microscopical control and transferred into 1 ml accutase solution. The digestion was performed at 37 °C for 20 min. The tissue pieces were triturated using consecutively 23 and 27 gauge needles, 3 times each. To avoid shear stress, aspiration of the tissues was performed slowly. After dissociation, the cells were centrifuged (120xg, 5 min), the supernatant removed and the pellet resuspended in 1 ml proliferation medium (DMEM/F12, 1% BSA, 2% B27 supplement without retinoic acid, 1% penicillin/streptomycin, 0.1% β-mercaptoethanol, 20 ng/ml FGF, 10 ng/ml EGF). 500000 cells from the subventricular zones (SVZ cells) were seeded per T25 culture flask and cultivated at 37 °C and 5% CO_2. The media were changed every 3 days. Neurospheres appeared within 2–4 days *in vitro*. For the individual experiments, the neurospheres had to be cultivated for 5 days to produce sufficient cell numbers for the following experiments. The neurospheres were dissociated analog to the tissue dissociation. Prior to seeding into 6- well chambers, the dissociated cells were counted and dead cells excluded by Trypan blue assay.

100000 living cells were seeded per well in 6-well plates and cultivated for up to 72 h to investigate the expansion rates. Due to the fact, that the CK2 inhibitors were dissolved in DMSO, the cells were cultivated either in the basal medium alone, or supplemented with DMSO, respectively, in ascending concentrations of CX-4945 (Selleckchem, Munich, Germany) in DMSO (0.1 μM, 1 μM, 5 μM, 10 μM, 15 μM, 20 μM, 25 μM) to analyse a dose dependent effect. Numbers and sizes of neurospheres in the individual cultures were evaluated after 24, 48 and 72 h. The diameters where quantified in three independent experiments, images were taken with an Olympus CKX microscope (Olympus, Hamburg, Germany) with a Moticam 2500 and Motic Images Plus software (Motic). To do so, 100 spheroids were measured for each individual experiment, after 24, 48 and 72 h using the freeware image J (National Institutes of Health, freeware).

2.3. Treatment of cells

For measuring the inhibition of CK2 activity, SVZ cells from 4 animals were cultivated for 5 days to produce neurospheres and a cell pool which was large enough for the following experiments. Cells were dissociated and 1 million cells per 5 ml were seeded. Cells were cultivated for two more days to generate small neurospheres prior to the addition of CX-4945 or quinalizarin (Labotest OHG, Niederschöna, Germany). As controls, cells were cultured in medium containing DMSO or in the proliferation medium alone. Cells were harvested by centrifugation at 120xg for 5 min and stored at −80 °C before they were used for protein extraction.

The preparation of the cells for the differentiation experiments was performed in the same way as for the proliferation experiments. The isolated neural stem cells

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were cultivated for 4 to 5 days to form small neurospheres, which were dissociated and seeded in a density of 1 million cells per 5 ml in differentiation medium. After 0 h or after 72 h 10 or 20 μM CX-4945 or 40 μM quinalizarin were added. As controls DMSO (added at 0 h) or the differentiation medium were used. Five days after starting the differentiation process, cells were harvested by utilizing accutase to detach the cells from the bottom of the culture flask. After centrifugation the supernatants were discarded and the pellets stored at −80 °C.

2.4. Extraction of cellular proteins

Pellets were lysed with 40–60 μl of RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% sodium desoxycholate, 1% Triton X–100, 0.1% sodium dodecyl sulfate (SDS) supplemented with the protease inhibitor cocktail Complete^{TM} according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany)). The cell lysate was left on ice for 30 min, before being subjected to sonification (3 × 30 s) at 4 °C. Then the cell debris was removed by centrifugation at 16100xg at 4 °C for 30 min. The protein content of the supernatant was determined according to the Bradford method using the Bio-Rad protein assay reagent (Bio-Rad, Munich, Germany).

2.5. Protein kinase CK2 assay

To study the CK2 kinase activity *in vitro*, 30 μg of total protein were mixed with kinase buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT)) to a final volume of 20 μl. 30 μl of CK2 mix (25 mM Tris-HCl, pH 8.5, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 50 μM ATP, 0.19 mM (final concentration) CK2 specific substrate peptide with the sequence RRRDDDSDDD and 10 μCi/500 μl [^{32}P]γATP) were added and the reaction mix was incubated at 37 °C for 10 min. The reaction was stopped on ice and the sample pipetted onto Whatman-P81 cation-exchange paper and washed for 3 × 5 min with 85 mM phosphoric acid and 1 × 5 min with ethanol. The filter paper was dried and counted for Čerenkov radiation in a scintillation counter (Liquid Scintillation Analyser 190S AB/LA; Canberra-Packard GmbH, Dreieich, Germany).

2.6. SDS polyacrylamide gel electrophoresis and Western Blot analysis

Proteins were separated on a 7.5 or 12.5% sodium dodecylsulfate polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane (PVDF) by tank blotting using a transfer buffer containing 20 mM Tris-HCl, pH 8.3 and 150 mM glycine. The membrane was blocked with 5% dry milk in PBS containing 0.05% Tween-20 for 1 h at room temperature and then incubated with the specific

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antibody, which was diluted in PBS with 0.05% Tween-20 containing 1% dry milk powder for 1 h at room temperature or overnight at 4 °C. The membrane was washed with PBS Tween-20 containing 1% skimmed milk (2 × 10 min), before being incubated with a peroxidase-coupled secondary antibody (anti-rabbit 1:30,000 or anti-mouse 1:10,000) for 1 h at room temperature. The membrane was washed again in PBS Tween-20 (2 × 10 min). Signals were developed and visualized by the Lumilight system from Roche Diagnostic (Mannheim, Germany). The following primary antibodies were used: GFAP (1:5000, DAKO, Hamburg, Germany), βIII-tubulin (1:500, MAB1637, Millipore, Darmstadt, Germany), PGP9.5 (1:1000, DAKO, Hamburg, Germany), nestin (1:1000, MAB353, PAN, Aidenbach, Germany), CK2α (1:250, 1A5, monoclonal mouse antibody), CK2α' (1:1000, serum #30, polyclonal rabbit antibody) and CK2β (1:1000, Santa Cruz Biotechnology Inc., Heidelberg, Germany).

2.7. FACS analysis

To investigate the influence of CX-4945 on the cell cycle the progenitor cells were cultured for 5 days to generate sufficient amounts of cells. Initially, they were dissociated as described and seeded at a density of 1 million cells per 5 ml medium. Cells were treated with 10 or 20 μM CX-4945 for 24 h in proliferation medium. As controls, DMSO and the usual proliferation medium were used. After 24 h, the cells were harvested for cell cycle analysis. Cells were centrifuged at 120xg for 5 min and dissociated carefully with accutase to avoid clustering during the cell cycle analysis.

Cells were centrifuged, the supernatant was discarded and 2 ml cold 70% ethanol per 1 million cells were added with gentle vortexing. Cells were stored for 2 h at 4 °C. Subsequently, the samples were centrifuged at 300xg for 1 min to remove the ethanol. To wash the cells 1 ml PBS was added and cells centrifuged again as described before. After this washing procedure, cells were stained with FxCycle PI/RNase staining (Life technologies) solution and incubated for 15 to 30 min at room temperature.

2.8. Induced differentiation

Differentiation was induced by plating the cells on extracellular-matrix coated coverslips (ECM gel, Sigma, 1:100) and adding 20 μM retinoic acid to the culture medium. 10 or 20 μM CX-4945 or 40 μM quinalizarin were added either immediately at the start of the differentiation process or at 72 h after inducing differentiation. Since neurons and astrocytes are the major cell types in the differentiated cell cultures, differentiation success was evaluated by assessing a glial (GFAP), a neuronal (βIII-tubulin) and a neural stem cell marker (nestin). After 5 days cells were fixed and the amounts of GFAP-, βIII-tubulin- and nestin-positive cells analysed by immunofluorescence staining. To examine whether CX-4945 had an influence on the

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differentiation at later time points, 20 μM CX-4945 were added after 14 days of differentiation. The cells were incubated with CX-4945 for two more days before fixation with formaldehyde. In these experiments the differentiation medium was replaced every 3 to 4 days to assure a constant availability of nutrition and essential molecules. Subsequently, cells were fixed and stained for immunofluorescence. The differentiation experiments were performed at least three times. To analyse the influence of CX–4945 at the different stages of differentiation, the amount of GFAP-positive, βIII-tubulin-positive and nestin-positive cells were compared to the total cell number, which was assessed by the DAPI-staining. The percentages were calculated for every experiment by counting cells of 25 independent images per preparation (magnification 400 x).

2.9. Immunohistochemistry and TUNEL staining

The individual cultures were fixed with 4% formaldehyde (AppliChem, Darmstadt, Germany) for 20 min and stored in PBS (phosphate buffered saline). Prior to the immunohistochemical staining, cells were permeabilised with Triton X–100 (0.5%, 10 min, Merck, Darmstadt, Germany), blocked with 10% normal donkey serum (Millipore, Temecula, California, USA) for 30 min and stained for βIII-tubulin (1:200, MAB1637, Millipore, Darmstadt, Germany), GFAP (1:500, DAKO, Hamburg, Germany) and nestin (1:250, MAB353, PAN, Aidenbach, Germany). The incubation time of the primary antibody was 1 h at room temperature. Then, the neurospheres were incubated with Alexa-488- or Alexa-594- tagged secondary antibodies for one hour. Finally, the cultures were stained with DAPI (1:500, Sigma-Aldrich, St. Louis, Missouri, USA) for 5 min and mounted with fluorescent mounting medium (DAKO, Hamburg, Germany). Apoptotic cells were visualized with the "*In Situ* Cell Death Detection Kit, Fluorescein" from Roche Diagnostics GmbH (Mannheim, Germany) according to the manufactures' instructions. Briefly, fixated cells were permeabilized using 0.1% Triton X–100 in 0.1% sodium citrate for 2 minutes on ice and washed twice with PBS. Cells were incubated with TUNEL reaction mix for 1 h at 37 °C, washed 3 times with PBS and embedded with ProLong® Diamond Antifade Mountant with DAPI (Thermo Fisher Scientific, St. Leon-Rot, Germany). Cells were analysed with the cell observer Z1 or an Axiovert 100 microscope (Zeiss, Jena, Germany).

2.10. Statistics

The statistical analysis of the proliferation measurements was performed utilizing the Kruskal Wallis test, a non-parametric test, by applying MedCalc 12 software (MedCalc Software bvba, Ostend, Belgium). The significances were set as * p-value ≤ 0.05, ** p-value ≤ 0.01 and ***p-value ≤ 0.001.

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To assess the significant differences in the individual, we used a non-parametric Mann-Whitney test by comparing the groups with the DMSO control by applying the statistical software SigmaPlot 13.0. The significances were set: * p-value ≤ 0.05, ** p-value ≤ 0.01 and ***p-value ≤ 0.001.

3. Results

Protein kinase CK2 plays a central role for the regulation of eukaryotic cell proliferation. Inhibition of the kinase activity of CK2 leads to severe disturbances of cell proliferation and the induction of apoptosis in nearly all tumour cells. So far only little is known about a role of CK2 in stem cell proliferation and differentiation. Here, we investigated the impact of CK2 inhibition on neural stem cells, derived from the early postnatal mouse brain. Over the last 10 years a great number of different inhibitors of the kinase activity of CK2 have been described [7, 23]. Among these inhibitors, CX-4945 is the most interesting one being already tested on 235 kinases and found to be specific for CK2 [24]. Due to its specificity and bioavailability CX-4945 is now used in clinical trials [20]. A pilot experiment was used to evaluate whether CX-4945 had a significant effect on neural stem cells. To evaluate the critical amount of inhibition of CK2 on the proliferation of SVZ cells in neurospheres, increasing concentrations of CX-4945 were added, starting with a relative low dosage of 0.1 μM (Fig. 1). The size and amount of neurospheres (Fig. 1A–E), as well as the number of cells within the neurospheres (Fig. 1F) decreased when the cells were cultured with increasing concentrations of the inhibitor. Already after 24 h, there were significant differences in neurosphere sizes. At each individual time point the average size of the control (DMSO) neurospheres was not different from those cultivated with 0.1 to 5 μM CX-4945, while all higher concentrations showed a reduced sphere size of up to only 45% of the control diameters (5 μM: 95%, 10 μM: 85%, 15 μM: 75%, 20 μM: 64%, 25 μM 45%). The reductions in the size of the neurospheres were very similar for 48 h and 72 h. Although, these reductions in sphere sizes are impressive, they are even more impressive in relation to real cell numbers. The neurosphere diameter is only a 2-dimensional representation of the proliferative effect. Based on average cell diameters measured at day one (12.8 μm +/− 2.2 μm) the volumes of the neurospheres were calculated and divided by the average cell volume. While the average neurosphere diameters dropped to 45% of the control value, the cell number in the spheres dropped by a factor of 20, from an average of 226 cells per sphere, to only 11, after treatment of the cells with 25 μM CX-4945 for 72 h (Fig. 1F and G). Fig. 1A–D impressively demonstrate the reduction of sphere size from control (Fig. 1A) to 25 μM (Fig. 1D) in a dose-dependent manner. We show representative values of one single experiment in Fig. 1E and F; the bar graph in Fig. 1G shows the median relative spheroid diameters of all results after normalization to control spheroids.

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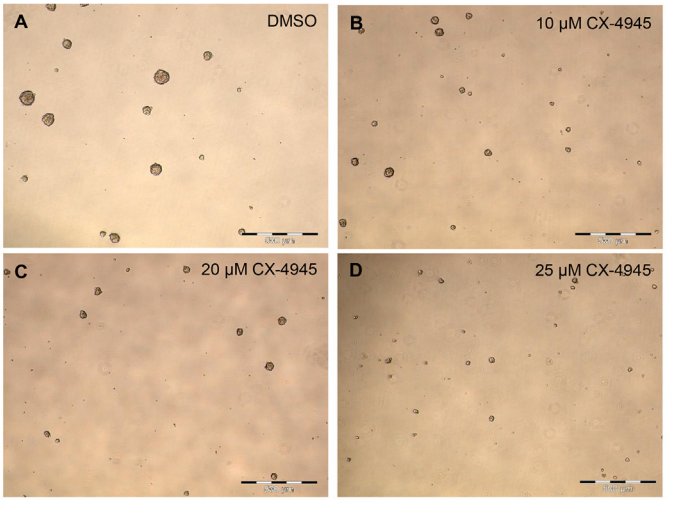

E	un-treated	DMSO	0.1 µM CX-4945	1 µM CX-4945	5 µM CX-4945	10 µM CX-4945	15 µM CX-4945	20 µM CX-4945	25 µM CX-4945
24 h	40.73	43.47	41.36	43.02	45.91	32.40	25.56	17.80	12.80
48 h	54.81	59.12	63.24	59.33	54.66	43.09	36.38	31.75	26.45
72 h	72.99	77.93	76.94	81.51	72.30	60.37	46.10	37.88	28.10

F	un-treated	DMSO	0.1 µM CX-4945	1 µM CX-4945	5 µM CX-4945	10 µM CX-4945	15 µM CX-4945	20 µM CX-4945	25 µM CX-4945
24 h	32	39	34	38	46	16	8	3	1
48 h	79	99	121	100	78	38	23	15	9
72 h	185	226	217	258	180	105	47	26	11

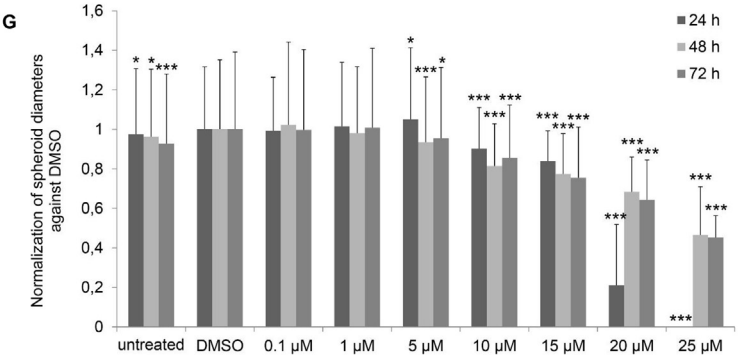

Fig. 1. Neurosphere growth in dependence of CK2-inhibition. Size of SVZ neurospheres after 72 h treatment with DMSO (dimethylsulfoxide) as a control (A) and increasing concentrations of the CK2-inhibitor CX-4945: 10 µM (B), 20 µM (C) and 25 µM (D). Tables E and F display two-dimensional diameter of the individual spheres of one representative experiment in µm (E) and the calculated number of cells within a sphere (F), thus underlining the large differences between the untreated, DMSO treated and CK2-inhibited neurospheres. The graph in G shows normalised neurosphere diameters after 24, 48 and 72 h averaged over all experiments. The diameters were normalised against DMSO (control). For the analysis of a statistical significance a non-parametric Kruskal-Wallis test was applied. Lower limit of measured neurosphere sizes was ≤20 µm. Scale bar 500 µm.

To assure that the observed effects are in fact due to a reduction in the CK2 kinase activity we used a second well-established CK2 inhibitor, namely quinalizarin [25]. Quinalizarin was tested on 72 kinases and found to be specific for CK2 as well [25, 26]. Different concentrations were tested (data not shown) and the most suitable one, which was a compromise between apoptosis induction and CK2 inhibition, namely

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40 μM, was used for the following experiments. To prove that these concentrations are appropriate to inhibit CK2 activity, proliferating SVZ were left untreated or treated with the given concentrations of the inhibitors for 24 h. Cells were harvested and either subjected to an *in vitro* phosphorylation assay with the CK2 specific substrate peptide RRRDDDSDDD [27] or a Western blot analysis. Fig. 2A demonstrates that both CK2 inhibitors reduced the CK2 activity, albeit to a different degree. At lower concentrations CX-4945 was more potent than quinalizarin. CX-4945 reduced the CK2 activity to 31% residual activity at 10 μM and to 18% at 20 μM, whereas 40 μM quinalizarin reduced the activity only to 61%. The Western blot analysis with antibodies recognizing the different subunits of CK2 (Fig. 2B) shows that the expression of these proteins is not influenced by the inhibition.

A reduction in the size of the neurospheres as well as the reduction in the number of cells in neurospheres might be induced by cell cycle arrest or the induction of

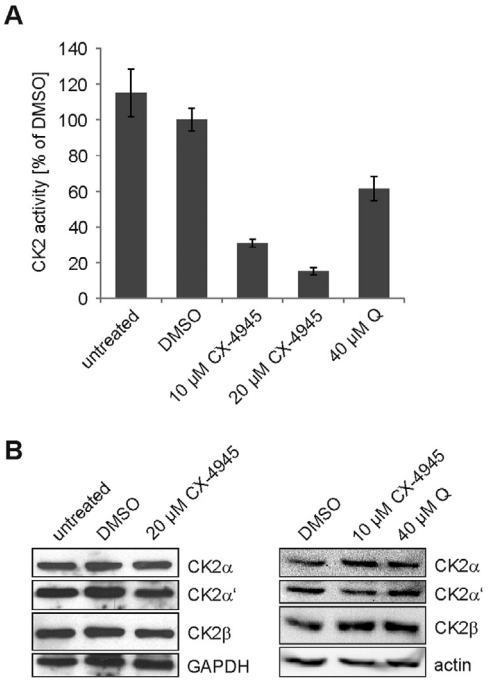

Fig. 2. Assessment of CK2 kinase activity. CK2 inhibitors efficiently inhibit CK2 kinase activity in SVZ cells without altering expression of CK2 subunits. (A) Endogenous CK2 kinase activity in undissociated SVZ cells was determined 24 h after treatment with DMSO, 10 μM or 20 μM CX-4945 or 40 μM quinalizarin. Untreated cells were used as a control. Relative CK2 kinase activities measured in DMSO-treated cells were set to 100%. The mean of at least two independent experiments is shown. (B) SVZ cells were kept in normal medium (untreated) or treated with DMSO (solvent control), 10 μM or 20 μM CX-4945 or 40 μM quinalizarin for 24 h. Cell lysates were prepared and analysed on a 12.5% SDS-polyacrylamide gel followed by Western Blotting using anti-CK2α, anti-CK2α' and anti-CK2β specific antibodies. GAPDH or actin was used as a loading control. One representative of at least 2 Western Blots is shown here. Full, uncropped versions of the Western Blots are available as supplementary material.

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apoptosis just to mention but a few. To address this question we performed a cytofluorimetric analysis of cells treated with a CK2 inhibitor or treated with the solvent control DMSO. Fig. 3 demonstrates that CX-4945 treatment of SVZ cells led to a considerable number of cells in the subG1 phase, when a concentration of 20 μM was used. At the 10 μM concentration there were hardly any cells in subG1 phase, similar to the DMSO control, indicating that there is almost no apoptosis.

To investigate the role of CK2 inhibition on neuronal and glial differentiation, the neural stem cells were selectively differentiated for five days by using retinoic acid and extracellular matrix coatings. Neurons were detected by staining the cells with an antibody against βIII-tubulin and astrocytes were identified by staining with an antibody against glial fibrillary acidic protein (GFAP). Neural stem cells were stained with an antibody against the **n**euro**e**pithelial **st**em cell prote**in** (Nestin). Nestin is a general marker for neural stem cells and gives a first impression of neural stem cell concentrations. It is phosphorylated at Thr316 by cdc2 during mitosis to enable remodelling of the cytoskeleton [28]. The use of antibodies for both phosphorylated and non-phosphorylated nestin allows to identify nestin-positive cells that are in the mitotic circle, and thus most probably expressing a neuronal stem cell character. Due to the fact, that nestin can also be expressed in regenerating neurons and glial cells, the unphosphorylated nestin gives rather an impression of ongoing regeneration seen in tissue culture.

First, we checked undifferentiated stem cells for the expression of the marker proteins. Fig. 4A–D compares the characteristics of undifferentiated SVZ neurospheres and neurospheres that were differentiated for 5 days. The immunofluorescence analysis demonstrates that undifferentiated neurospheres already contained some GFAP-positive glia cells (red), but only a few neurons (green, Fig. 4A), whereas in the differentiated cultures the number of neurons was significantly elevated and nearly all non-neural cells were GFAP-positive glial

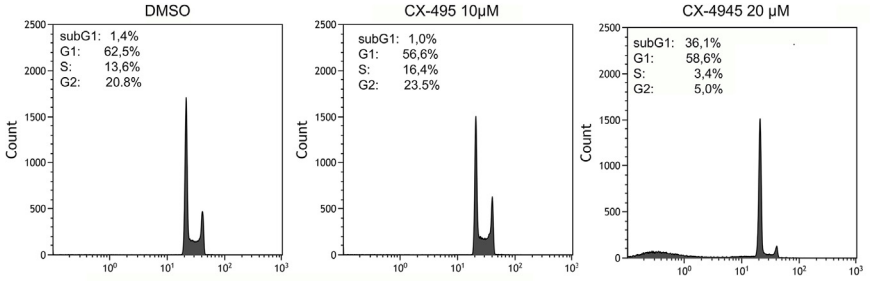

Fig. 3. FACS analysis of DMSO-control and inhibitor treated cells. Proliferating SVZ cells were seeded at an equal density and treated with DMSO (control), 10 μM or 20 μM CX-4945 in proliferation medium for 24 h. Then cells were harvested, carefully dissociated, fixed with ethanol and stained for 15–30 min using the FxCycle PI/RNase staining kit (Life Technologies). 10.000 cells per treatment were counted in a flow cytometer. Percentages of individual cell cycle phases were calculated according to the total of all phases.

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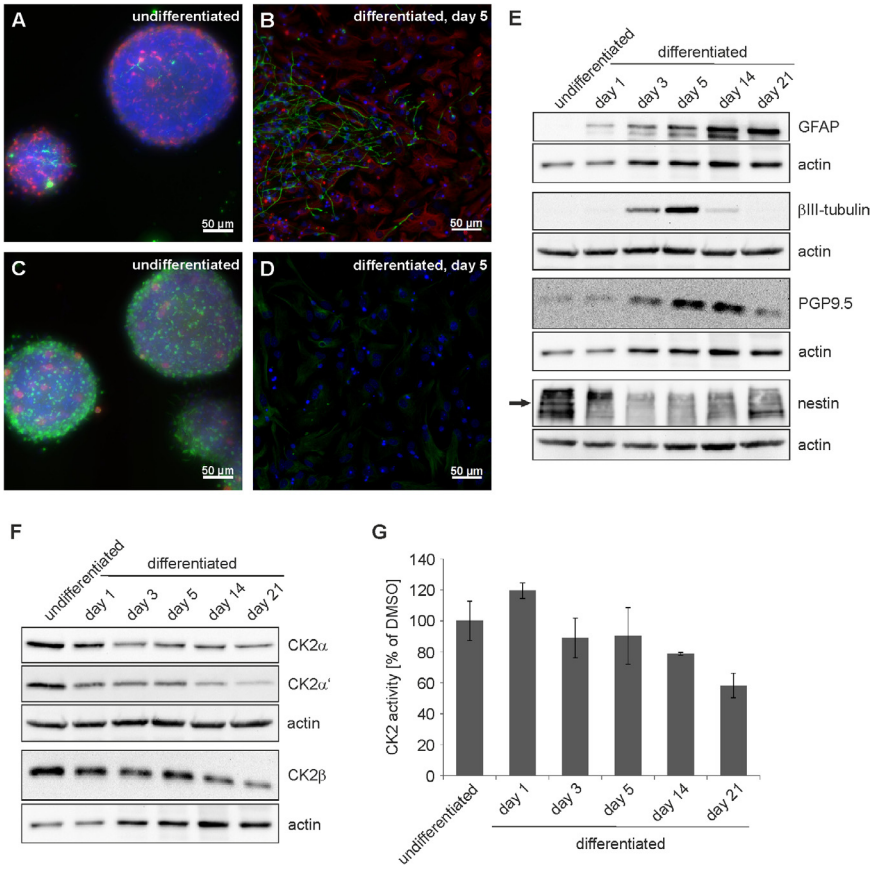

Fig. 4. Comparison of stem cell and differentiation markers, as well as expression and activity of CK2 in undifferentiated and differentiated neurospheres. (A-D) Analysis of GFAP-, βIII-tubulin- and nestin expression in undifferentiated and 5 days differentiated neurospheres. Neurospheres were seeded on matrix-coated coverslips and either fixated directly after sedimentation (undifferentiated) or differentiated by adding retinoic acid to the medium (differentiated, day 5). Neurospheres were stained for GFAP (red) and βIII-tubulin (green) (A, B) or for total nestin (green) and phosphoThr316-nestin (red) (C, D). Exposure time was adjusted to the strongest signal and was identical for undifferentiated and differentiated samples. Scale bar 50 µm. (E) Neurospheres were harvested during proliferation (undifferentiated) or after 1, 3, 5, 14 or 21 days of differentiation with retinoic acid. Cell lysates were analysed for GFAP, βIII-tubulin, PGP9.5 and nestin expression (E) and for the expession of the CK2 subunits (F) by Western Blot. Actin was used as loading control. Cell lysates were also analysed for CK2 activity (G).Full, uncropped versions of the Western Blots are available as supplementary material.

cells (red) (Fig. 4B). The nestin staining in Fig. 4C and D shows that the total nestin (green) signal was stronger in the undifferentiated neurospheres compared to the differentiated ones, indicating down-regulation during differentiation. In the differentiated cultures also no phosho-nestin (red, indicating mitotic cells) could be detected. Next, we analysed the expression of glial, neuronal and stem cell markers between day 0 (undifferentiated) and day 21 of differentiation (Fig. 4E). We could clearly see that neuronal and glial markers increased with differentiation, whereas nestin expression was strongest in the undifferentiated stage. Interestingly,

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βIII-tubulin expression was transient with a maximum at day 5 of differentiation. Due to this transient expression we used the pan-neuronal protein Ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCH-L1 or PGP9.5) as a second neuronal marker. The expression of PGP9.5 increased steadily until day 14 and was slightly reduced at day 21, probably due to beginning neuronal die-off often observed in long time cultures. To assay a possible role for CK2 we then analysed the expression of the individual subunits during the differentiation process. As shown in Fig. 4F there was a decrease in the amount of CK2α and CK2α' with the progress of differentiation. The decrease of CKβ started at later time points of differentiation. The analysis of the CK2 kinase activity is shown in Fig. 4G. CK2 kinase activity remained nearly the same until day 5 and decreased thereafter.

Thus, we have shown that in the presence of retinoic acid, SVZ cells differentiate from neural stem cells to glial and neuronal cells and that this differentiation goes along with a decrease in the level of the CK2 subunits and CK2 kinase activity.

In a next step we analysed the differentiation of the SVZ in the presence or absence of a CK2 inhibitor. We treated the cells either with the solvent DMSO as a control or with 10 or 20 μM CX-4945 or with 40 μM quinalizarin. The CK2 inhibitor was added either immediately at the beginning of the differentiation process (0 h) or at 72 h after induction of differentiation. In pilot experiments (data not shown) we saw, that the effects of the inhibitors decreased, the later they were added to the differentiating cultures. At 72 h this effect was, if ever, very small. We therefore used 72 h as the second time point. Independently of the time point when the inhibitor was added, immunofluorescence was always performed five days after start of differentiation (Fig. 5B–I) and βIII-tubulin positive neurons (green) or GFAP positive astrocytes (red) were counted and presented in bar graphs (Fig. 5A). In the control cultures (DMSO or normal medium) we found 40% βIII-tubulin-positive neurons (green) and 50% GFAP-positive astrocytes (red) after 5 days under differentiation conditions (Fig. 5A–C). Both neurons and glial cells showed a normal morphology with extended neurites in neurons (green) and star-like processes in astrocytes (red). Inhibiting the CK2 activity with CX-4945 or quinalizarin resulted at least in part in a failure of differentiation.

Inhibition of CK2 kinase activity with 10 μM CX-4945 from the beginning of differentiation reduced the signal of GFAP staining (Fig. 5D) indicating reduced glial differentiation, whereas neuronal differentiation was not affected. When CK2 was inhibited with the same concentration 72 h after start of differentiation (Fig. 5E), the numbers for both cell types were comparable to those in control cultures. The inhibition of CK2 with 20 μM CX-4945 from the start of differentiation resulted in no detectable differentiation of neurons (lack of green βIII-tubulin staining (Fig. 5F). The overall cell number was reduced, probably due to apoptosis, but the majority of the remaining cells showed a GFAP-staining,

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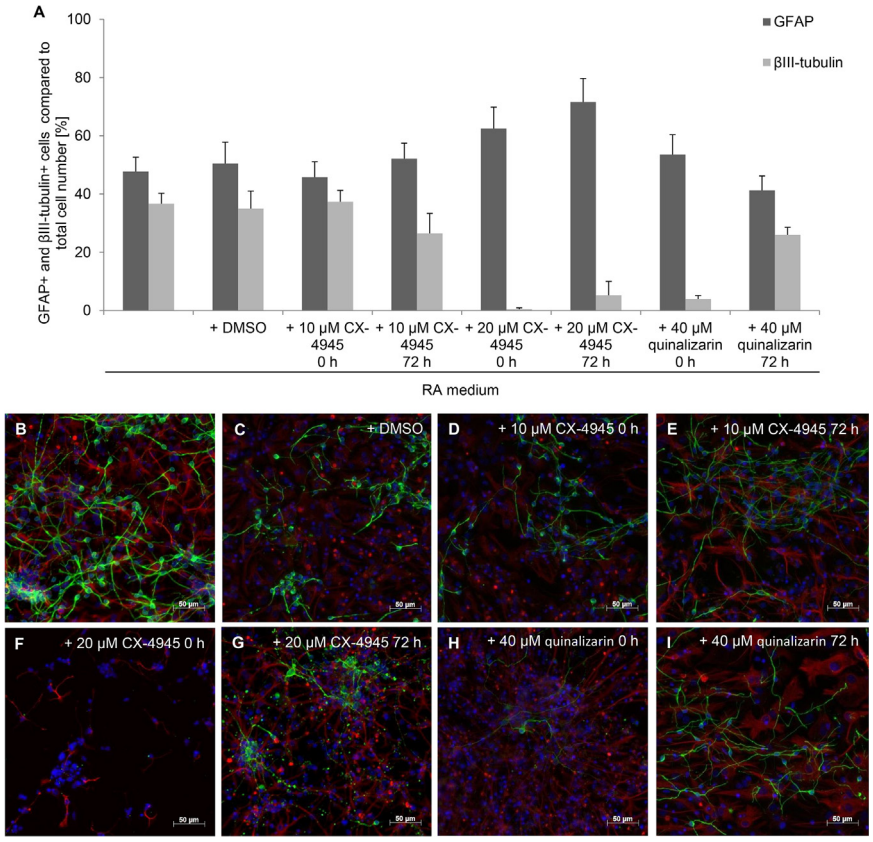

Fig. 5. Influence of CK2 inhibition on neural and glial differentiation. GFAP- and βIII-tubulin staining of differentiated neurospheres at day 5 *in vitro*. SVZ cells were seeded on matrix-coated coverslips and differentiation was induced by adding retinoic acid (RA) to the medium. 10 µM or 20 µM CX-4945 or 40 µM quinalizarin was added to the RA-containing medium at the indicated time points after start of differentiation. DMSO served as solvent control. (A) GFAP+ and βIII-tubulin+ cells compared to the total cell number (DAPI). (B) to (I) demonstrate the impact of CK2 inhibition upon glial (red GFAP staining) and neural (green βIII-tubulin staining) differentiation: (B) RA alone, (C) RA plus DMSO, (D) RA plus 10 µM CX-4945: 0 h, (E) RA plus 10 µM CX-4945: 72 h, (F) RA plus 20 µM CX-4945: 0 h, (G) RA plus 20 µM CX-4945: 72 h. (H) RA plus 40 µM quinalizarin: 0 h, (I) RA plus 40 µM quinalizarin: 72 h. Scale bar 50 µm.

although the intensity of the expression was very low, and the cells were very small with short processes (Fig. 5G, red staining). When CK2 was inhibited from 72 h after start of differentiation, the glial population did not change much in relative numbers of glial cells to neurons. The amount (Fig. 5A) and quality (Fig. 5G) of neurons increased, but it did not reach the control samples. Although there was a beginning of neurite outgrowth, the neurites exhibited pathological swellings (Fig. 5G). Cultures that where inhibited with 40 µM quinalizarin from the beginning of differentiation showed a reduced outgrowth of the neurospheres and slight apoptosis, but not as strong as seen with 20 µM CX-4945. The number of neurons was reduced significantly, but the neurons had a normal morphology (Fig. 5H). Glial cell differentiation was not affected. When the CK2 kinase activity

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was inhibited with quinalizarin 72 h after start of differentiation cell numbers and morphologies of both cell types were comparable to control cultures (Fig. 5I).

In order to confirm the experiments obtained by immunofluorescence we repeated the experiments but in this case, proteins were extracted from differentiated cells and analysed by Western blotting. Cells were treated with CX-4945 (10 or 20 μM) either at the start of the differentiation or 72 h after the start of the differentiation. In order to demonstrate CK2 inhibition during differentiation, we first measured CK2 kinase activity in cell extracts. As shown in Fig. 6A, in differentiated cultures 10 μM CX-4945 reduced the CK2 activity to 61% (0 h) and 49% (72 h) residual activity and 20 μM reduced it to 19% (0 h) and 25% (72 h). In a next step, we subjected the extracts to SDS-polyacrylamide gel electrophoresis followed by Western Blot analysis where we compared GFAP and βIII-tubulin marker protein expression in the absence (DMSO) or presence of the inhibitor. As shown in Fig. 6B in the presence of retinoic acid alone or retinoic acid and the solvent control, GFAP was strongly expressed which correlated with ongoing glial differentiation. When 10 or 20 μM CX-4945 were added together with retinoic acid there was a reduced expression of GFAP, indicating a block in differentiation. When 10 or 20 μM CX-4945 were added to the cells 72 h after the start of the differentiation we detected a protein band for GFAP indicating that at this time point CX-4945 was unable to inhibit differentiation into glial cells completely.

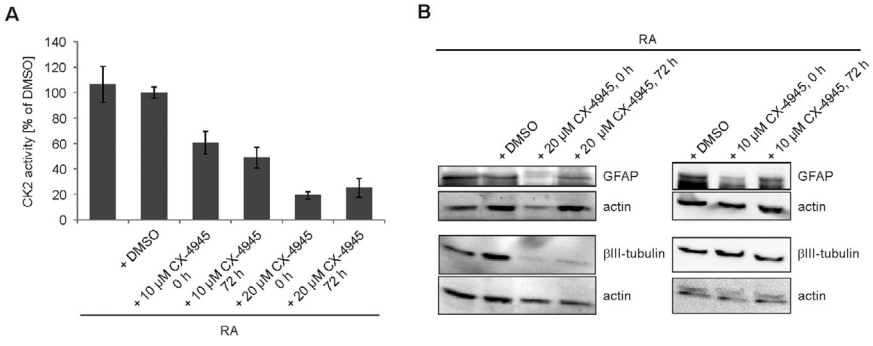

Fig. 6. Dose and time dependent inhibition of CK2 and its influence on glial and neurogenic differentiation. (A) Endogenous CK2 kinase activity in SVZ cells was determined at day 5 of differentiation after treatment with retinoic acid (RA control), RA plus DMSO or RA plus 10 μM or 20 μM of CK2 inhibitor CX-4945. CX-4945 was added either directly with the differentiation mix (0 h) or 72 h after start of differentiation (72 h). Relative CK2 kinase activities measured in DMSO-treated cells were set to 100%. The mean of at least two independent experiments is shown. (B) SVZ cells were differentiated for 5 days with retinoic acid (RA) alone or with RA plus DMSO or 10 μM or 20 μM CX-4945 as indicated. Cell lysates were prepared and analysed on a 12.5% SDS-polyacrylamide gel followed by Western Blotting using anti-GFAP and anti-βIII-tubulin specific antibodies. GAPDH or actin was used as a loading control. One representative of at least 2 Western Blots is shown. Full, uncropped versions of the Western Blots are available as supplementary material.

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A very similar observation was made for the βIII-tubulin expression after treatment with 20 μM CX-4945 indicating that CK2 inhibition at the beginning of differentiation blocked neural differentiation completely, while CK2 inhibition after 72 h of differentiation resulted in a reduced but detectable neuronal differentiation compared to the control. In contrast, the inhibition of CK2 activity using 10 μM CX-4945 had no effect at all on neuronal differentiation i.e. protein bands for βIII-tubulin showed the same intensity as the DMSO control, whether the inhibitor was added at the beginning or after 72 h of differentiation. These results confirm the observation of the immunofluorescence studies (Fig. 5). As a general conclusion of these data, we can say that CK2 inhibitors have a very strong toxic effect on neural stem cells, and can inhibit, even at non-toxic concentrations, the glial differentiation.

As the induction of apoptosis might also play a role during differentiation we performed an *in situ* TUNEL assay. The TUNEL reaction preferentially labels DNA strand breaks generated during apoptosis. SVZ cells were induced to differentiate and either treated with 10 μM CX-4945, 20 μM CX-4945 or with 40 μM quinalizarin either immediately (0 h) or 72 h after the start of differentiation. Five days after the start of differentiation cells were subjected to a TUNEL assay (Fig. 7A–H). TUNEL positive cells (green) were counted, the counts were normalised to the DMSO control and presented as bar graphs. As demonstrated in Fig. 7 treatment of SVZ with 10 μM CX-4945 either at 0 h or at 72 h showed no indication for apoptosis (Fig. 7A–D), while a concentration of 20 μM CX-4945 led to an elevated level of apoptosis (E and F). Treatment of SVZ with quinalizarin however resulted at least in part in the induction of apoptosis when applied at 0 h, but it had no effect when added at 72 h after start of differentiation.

We also investigated the immediate impact of CK2 inhibition on neural stem cell numbers and properties, based on nestin staining. DMSO alone did not alter the number of nestin-positive cells compared to the basic culture medium. Fig. 8 shows the nestin expression in control cells (8B with retinoic acid, 8C with both retinoic acid and DMSO), in cells treated immediately (0 h) or 72 h after the start of the differentiation with 10 μM CX-4945 (8 A, D-E), 20 μM CX-4945 (8 A, F-G) or 40 μM quinalizarin (8 A, H-I) either as bar graph showing the median percentage of nestin positive cells over all experiments or as representative immunofluorescence images. Counting numbers of nestin-positive cells in the cultures showed a reduction of nestin-positive cells when either CX4945 or quinalizarin was used in toxic concentrations (Fig. 8A). The number of nestin-positive cells dropped significantly when 20 μM CX-4945 was added at the start of the differentiation process. Similar to what has been seen with the GFAP-positive astrocytes, the nestin-positive cells were very small and hard to discriminate (Fig. 8F). Even when the compound was added at 72 h, the morphology of the nestin positive cells was different compared to the control (Fig. 8G). Quinalizarin led to a similar result when added at 0 h, while a late addition of the inhibitor had

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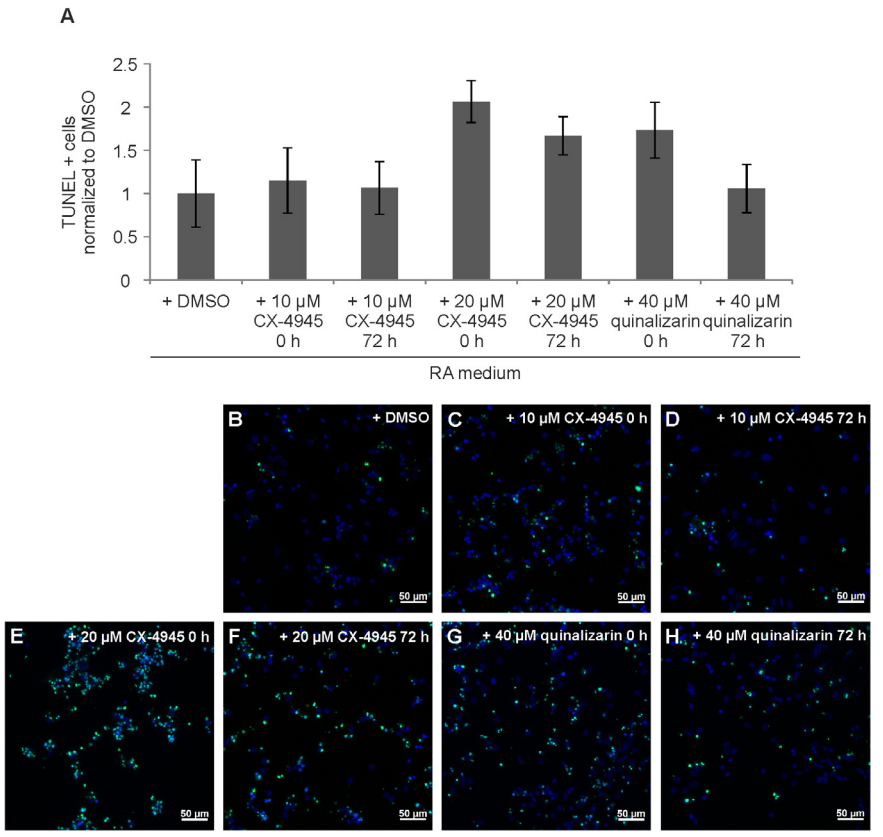

Fig. 7. Influence of CK2 inhibition on apoptosis induction. TUNEL-staining of differentiated neurospheres at day 5 *in vitro*. SVZ cells were seeded on matrix-coated coverslips and differentiation was induced by adding retinoic acid (RA) to the medium. 10 or 20 µM CX-4945 or 40 µM quinalizarin was added to the RA-containing medium at the indicated time points after start of differentiation. DMSO served as solvent control. (A) TUNEL positive cells compared to the total cell number (DAPI), normalized to DMSO. (B) to (H) demonstrate the impact of CK2 inhibition upon apoptosis induction: (B) RA plus DMSO, (C) RA plus 10 µM CX-4945: 0 h, (D) RA plus 10 µM CX-4945: 72 h, (E) RA plus 20 µM CX-4945: 0 h, (F) RA plus 20 µM CX-4945: 72 h, (G) RA plus 40 µM quinalizarin: 0 h, (H) RA plus 40 µM quinalizarin: 72 h. Scale bar 50 µm.

no effect at all (Fig. 8H–I). The non-toxic concentration of CX-4945 (10 µM) did not influence nestin positive cell numbers significantly (Fig. 8D and E).

In order to investigate whether the influence of CK2 inhibition by CX-4945 decreases or even disappears with time of differentiation, we expanded the differentiation period up to three weeks and investigated whether there were differences between inhibitor treated and control cultures. We decided to treat the SVZ with the highest concentration of CX-4945 because it exerted the strongest effects on differentiation under these conditions. Here, the cultures were differentiated for 14 and 21 days (data only shown for 14 days), and then 20 µM CX-4945 was added to the differentiation medium for another three days. For both time points, the addition of the inhibitor did not lead to any significant changes (Fig. 9). The cultures showed similar properties

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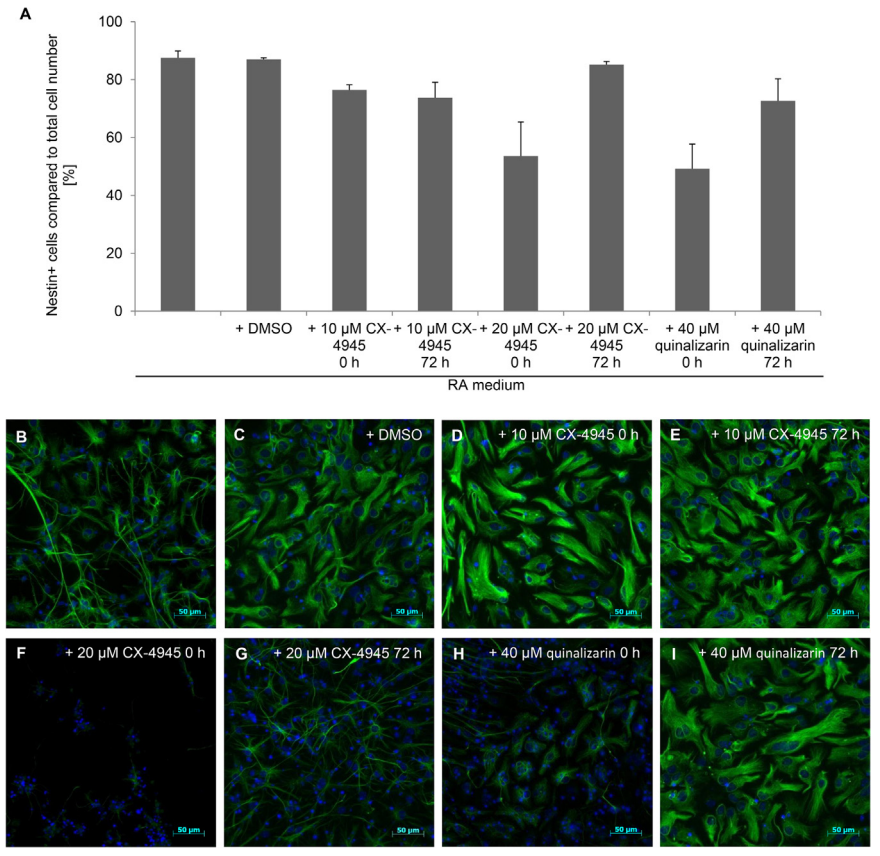

Fig. 8. Influence of CK2 inhibition on neural stem cells. Nestin staining of differentiated neurospheres after 5 days *in vitro*. SVZ cells were plated on matrix-coated coverslips and differentiation was induced by adding retinoic acid (RA) to the medium. 10 µM or 20 µM CX-4945 or 40 µM quinalizarin was added to the RA-containing medium at the indicated time points after start of differentiation. DMSO served as solvent control. (A) Nestin-positive cells compared to the total cell number (DAPI). (B) retinoic acid (RA), (C) RA plus DMSO, (D) RA plus 10 µM CX-4945: 0 h, (E) RA plus 10 µM CX-4945: 72 h, (F) RA plus 20 µM CX-4945: 0 h, (G) RA plus 20 µM CX-4945: 72 h, (H) RA plus 40 µM quinalizarin: 0 h, (I) RA plus 40 µM quinalizarin: 72 h. Scale bar 50 µm.

and glia to neuron-ratios (Fig. 9A). The expression patterns of astrocytic (GFAP, red) and neuronal (βIII-tubulin, green) cells is shown for the differentiation medium alone (Fig. 9B), for DMSO supplemented differentiation medium (Fig. 9C), or differentiation medium with CX-4945 (Fig. 9D). Similar findings were observed for the expression of nestin and the number of nestin-positive cells (Fig. 9E–H).

The experiment demonstrates that CK2 does neither have an impact on neural and glial differentiation nor on the remaining stem cell population at later time points during differentiation. Overall, we conclude that CK2 inhibitors have a crucial influence upon neural stem cell survival, when used in concentrations from 20 µM (CX-4945) or 40 µM (quinalizarin) onwards. This effect leads to significantly reduced neuronal and glial cell numbers. Moreover, the CK2 inhibition of CX-4945 in a non-toxic (10 µM) concentration leads to a decrease in glial differentiation.

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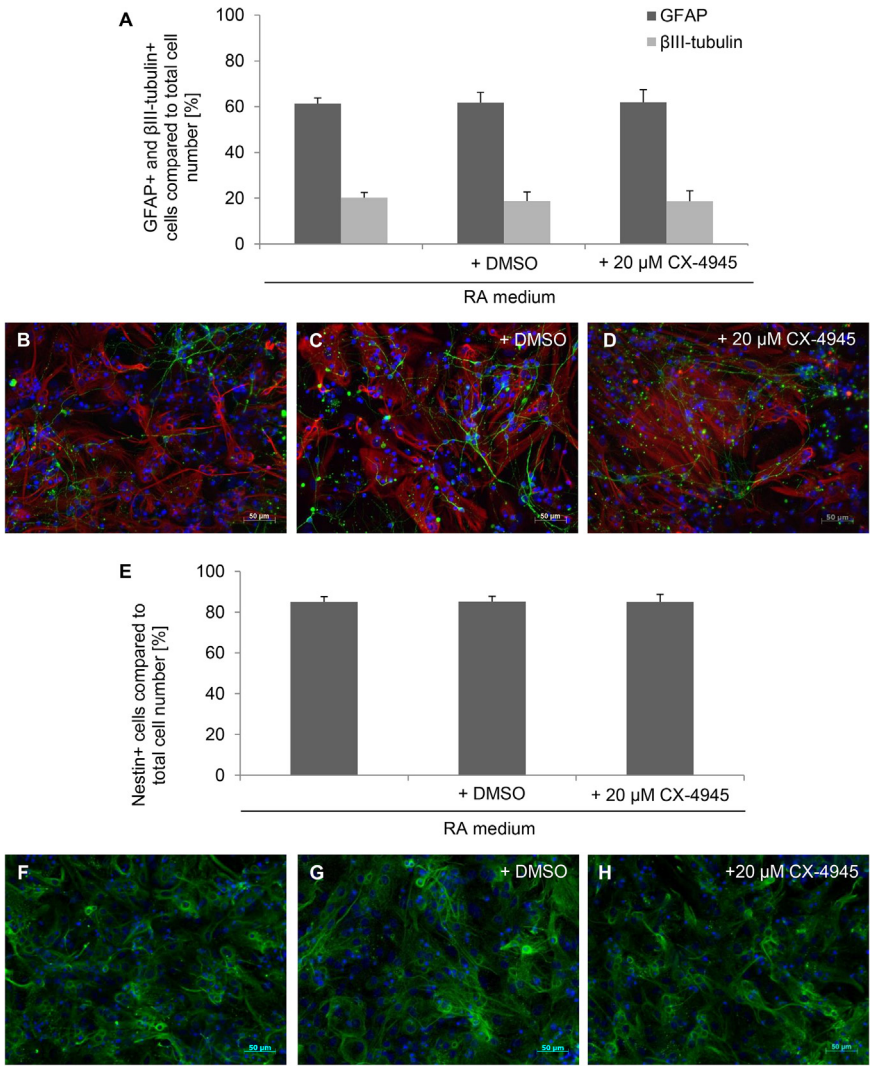

Fig. 9. CK2 inhibition at late time points (14 days) of differentiation. SVZ cells were differentiated with retinoic acid (RA) for 14 days. Then DMSO or 20 μM CX-4945 were added to the RA-containing medium for 3 additional days. Neither GFAP- nor βIII-tubulin or nestin-expression were altered when CK2 was inhibited at late time points of differentiation (14 days) (A, E). GFAP- and βIII-tubulin staining of neurospheres after 14 days of undisturbed differentiation and three more days of either RA alone (B), RA plus DMSO (C) or RA plus 20 μM CX-4945 (D) treatment. Nestin-staining of parallel experiments did also not show any difference between RA (F), RA plus DMSO (G) or RA plus 20 μM CX- 4945 (H) treatment. Scale bar 50 μm.

4. Discussion

In the present study, we have shown for the first time that CK2 has a strong impact on the proliferation and differentiation of neural stem cells from the subventricular zone. Inhibition of the enzyme leads to a significant reduction of the proliferative capacity. During differentiation, the inhibition of CK2 interrupts the neural and glial lineages within a time window of around three days. At later stages of

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differentiation CK2 kinase activity seems to be dispensable. Although it was demonstrated that CK2 is involved in the development of the nervous system [15], information concerning the involvement of kinase-dependent pathways in neurogenesis is rare. This might mainly be due to the severe and lethal neurodevelopmental phenotypes of CK2α or CK2β knockout mice [15, 29, 30]. The study by Huillard demonstrated that the interruption of the CK2β subunit expression caused a negative regulation of oligodendrogenesis. CK2β obviously interacts with the bHLH transcription factor Olig2, which is one of the key regulators for oligodendrocyte development [15].

Ziercher et al. have demonstrated that embryonic stem cells without CK2β exhibited a defect in viability. They have further shown that CK2β is necessary for oligodendroglia differentiation of neurospheres [31]. Obviously, the influence of CK2β is due to a defect in the CK2 kinase activity of the holoenzyme in the absence of CK2β. Without CK2β, those substrates that are phosphorylated only by the holoenzyme will not be phosphorylated. Jauch et al. have shown a role of Drosophila CK2β for cell proliferation or cell survival during brain development [32]. One of the possible substrates is the nucleolar mushroom body miniature (mbm) protein. This protein is implicated in nutrient dependent signalling pathways controlling ribosomal expression [33]. The idea that the holoenzyme is the functional form of CK2 implicated in the proliferation and differentiation of neurosphres is strongly supported by our present results with inhibitors of the kinase activity of CK2.

Moreover, the genetic interruption of embryonic neurogenesis leads to a reduced proliferation and self-renewal of the neural stem cells. These findings are quite consistent with our data from inhibited postnatal neural stem cells derived from the subventricular zone. Using knock-down approaches or CK2 inhibitors can overcome the lethality problem and does allow to investigate the postnatal role of CK2 in the nervous system. Using these approaches the role of CK2 in the nervous system could be evaluated further. Thus, it turned out that CK2 is obviously involved in ion channel organization [34, 35] and synaptic transmission [36, 37].

CX-4945 is a potent inhibitor of the kinase activity of CK2 [38], which was introduced as an anti-cancer drug [20]. So far it could be demonstrated that CX-4945 provides cytotoxic activities in acute lymphoblastic leukemia cells [21], attenuates prosurvival signalling in human breast cancer cells [19] or might be used for the treatment of cancer stem cells in glioblastomas [22]. Moreover, CX-4945 seems to regulate osteoblast differentiation *in vitro* [39] and might serve as a splicing regulator [40]. In a recent study it was shown that CX-4945 treated human mesenchymal stem cells differentiate into adipocytes similar to untreated cells [41]. It turned out that the failure in inhibiting differentiation was accompanied by

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a loss of CK2 kinase inhibitory function. Here, we used CX-4945 in different concentrations to investigate the role of CK2 in neurogenesis. In our study, we have demonstrated, that the proliferation of the neurospheres is inhibited in a dose dependent manner. Two concentrations, 10 and 20 μM, were selected for differentiation experiments as CK2 can be inhibited very effectively. The higher concentration of CX-4945 obviously leads to apoptosis, which reduces the cell number significantly and targets especially the stem cells and neuronal precursor cells. So the impact on neurogenesis at this concentration is rather based on apoptosis, than on a specific inhibition. Nevertheless, the surviving cells show an unaltered cell cycle distribution. Focusing on the surviving cells during differentiation, we could show that CK2 inhibition leads to a severe suppression of neurogenesis and to a reduced gliogenesis as well. We have further demonstrated here, that the impact of CK2 lasts for several days during differentiation, and that this impact degrades slowly and continuously. Using a concentration of 10 μM CX-4945 resulted in complete absence of apoptosis showing that this concentration is non-toxic. In this case we detected an inhibition of gliogenesis but not of neurogenesis supporting the observation that neuronal precursors are more sensitive to apoptosis at higher inhibitor concentrations. Using quinalizarin in different concentrations we obtained either strong apoptosis or no inhibition of the CK2 kinase activity. 40 μM quinalizarin resulted in an inhibition of the CK2 kinase activity by around 40%. In this case we observed an inhibition of neurogenesis but not gliogenesis. From the results obtained with the two inhibitors one might speculate that the inhibition of neurogenesis is somehow connected with apoptosis induction and the inhibition of gliogenesis is directly connected to CK2 inhibition. This speculation is supported by the observation by Huillard and Ziercher who had demonstrated that the interruption of the CK2β subunit expression causes a negative regulation of oligodendrogenesis [15, 31].

The time point when CK2 kinase activity is required for differentiation seems to be crucial. While knock-out of CK2β in embryonic stem cells leads to an overall developmental breakdown [29], a timely activation or inhibition of the enzyme within a specific organ system might be important for the lineage finding, especially when the enzyme is partially up- or down-regulated. Our data have shown that, during a period of 1–3 days, the inhibition of CK2 abrogates differentiation of stem cells.

A very important aspect in the role of CK2 in neurogenesis is also the interdependencies of the individual partners, such as neurons and glial cells. It is well known that glial cells provide a broad range of trophic factors and cytokines that can positively influence the survival and differentiation of neurons [42].

The present findings in the nervous system are in line with results obtained with CX-4945 in osteoclast differentiation. Moreover, it was shown that CX-4945

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inhibited the RANKL induced osteoblast differentiation. In contrast to this finding CX-4945 enhanced the BMP2 induced osteoblast differentiation [39]. In another study, which analysed the differentiation of preadipocytes into adipocyctes it was shown that the CK2 kinase activity increased at the beginning of the differentiation process and decreased with ongoing differentiation. Moreover, inhibition of the CK2 kinase activity at the beginning of the differentiation for up to 6 days after start of differentiation prevents differentiation whereas after day 6 there was no inhibition of differentiation anymore [43]. Thus, these results are in a good agreement with the data presented here for neural differentiation.

Future studies with graded up- and down-regulations of CK2 will show whether CK2 will also be involved in fate decision of neurons and glial cells during differentiation.

Declarations

Author contribution statement

Melanie Bender, Lisa Schwind: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

David Grundmann: Performed the experiments; Analyzed and interpreted the data.

Monika Martin, Markus Klotz: Performed the experiments.

Claudia Götz: Conceived and designed the experiments; Wrote the paper.

Mathias Montenarh, Karl-Herbert Schäfer: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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