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Small molecule GSK-3 inhibitors increase neurogenesis of human neural progenitor cells

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ABSTRACT

Human neural progenitor cells provide a source for cell replacement therapy to treat neurodegenerative diseases. Therefore, there is great interest in mechanisms and tools to direct the fate of multipotent progenitor cells during their differentiation to increase the yield of a desired cell type. We tested small molecule inhibitors of glycogen synthase kinase-3 (GSK-3) for their functionality and their influence on neurogenesis using the human neural progenitor cell line ReNcell VM. Here we report the enhancement of neurogenesis of human neural progenitor cells by treatment with GSK-3 inhibitors. We tested different small molecule inhibitors of GSK-3 i.e. LiCl, sodium–valproate, kenpaullone, indirubin-3-monoxime and SB-216763 for their ability to inhibit GSK-3 in human neural progenitor cells. The highest *in situ* GSK-3 inhibitory effect of the drugs was found for kenpaullone and SB-216763. Accordingly, kenpaullone and SB-216763 were the only drugs tested in this study to stimulate the Wnt/β-catenin pathway that is antagonized by GSK-3. Analysis of human neural progenitor differentiation revealed an augmentation of neurogenesis by SB-216763 and kenpaullone, without changing cell cycle exit or cell survival. Small molecule inhibitors of GSK-3 enhance neurogenesis of human neural progenitor cells and may be used to direct the differentiation of neural stem and progenitor cells in therapeutic applications.

Neural stem cells and neural progenitor cells (from hereon together referred to as neural precursor cells (NPC)) possess great promise to provide a source for transplantation to replace neurons lost from neurodegenerative diseases, stroke or spinal cord injury [21]. However, since multipotent NPC generate progeny with different cell fate, their differentiation needs to be directed to provide pure populations of the desired cell type(s) for transplantation [7].

Small molecules targeting endogenous signaling pathways of NPC can direct their differentiation [26]. For translation into clinical trials they offer several advantages over the use of secreted signaling molecules or transfection of intracellular cell fate determinants: (i) they do not require genetic manipulations, thus reducing the risk for oncogenic transformation, (ii) they are chemically synthesized *per se* without the use of animal products, (iii) they can target intracellular sub-branches of a pathway downstream of a membrane receptor and (iv) the kinetics of their application, withdrawal and concentration are under strict and continuous experimental control. These properties render small molecules an important tool for the control and direction of stem cell differentiation.

GSK-3 is a multifunctional enzyme involved in glucose metabolism, Wnt/β-catenin, Sonic Hedgehog, Notch and FGF signaling [19]. Inactivation or pharmacological inhibition of GSK-3 maintains rodent NPC at the expense of neurogenesis *in vivo* [1,18], while *in vitro*, both, increased proliferation and enhanced neurogenesis have been found upon inhibition of GSK-3 in NPC [22,25].

Abbreviations: bFGF, basic fibroblast growth factor; BSA, bovine serum albumin; DAPI, 4′,6-diamidino-2′ phenylindoldihydrochlorid; DMEM, Dulbecco's modified Eagle Medium; DMSO, dimethylsulfoxide; EGF, epidermal growth factor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GS, glycogen synthase; GSK-3, glycogen synthase kinase-3; i.e, it est; KP, kenpaullone; I3M, indirubine-3-monoxime; LRP6, low density lipoprotein receptor related protein 6; mAb, monoclonal antibody; NPC, neural precursor cell; PI, propidium iodide; SB21, SB216763; SEM, standard error of the mean; Ser, serine; TUNEL, terminal deoxynucleotidyl-transferase dUTP nick-end labeling; VPA, sodium valproate.

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Here we investigated the effects of GSK-3 inhibitors on neurogenesis in human NPC differentiation *in vitro* using the human NPC line ReNcell VM [11,15]. We treated differentiating human NPC with small molecule GSK-3 inhibitors and investigated their effect on GSK-3 enzymatic activity, GSK-3 mediated signaling and NPC differentiation. Our data suggest an enhancement of neurogenesis of human NPC by GSK-3 inhibition.

LiCl, SB-216763 (SB21) and dimethylsulfoxide (DMSO) were purchased from Sigma (Taufkirchen, Germany). Kenpaullone (KP), sodium valproate (VPA) and indirubine-3-monoxime (I3 M) were from AXXORA GmbH (Lörrach, Germany). Stock solutions of SB21, KP and I3M were dissolved in DMSO, LiCl and VPA were dissolved in PBS. ReNcell VM is a conditionally immortalized cell line derived from the ventral midbrain of a 10 weeks old human fetus [11,15] from ReNeuron Ltd. (Guildford, UK).

ReNcell VM were cultivated as monolayer under proliferative (presence of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF)) and differentiation (withdrawal of EGF and bFGF) conditions, respectively, as described [15]. With each medium change the drugs were added freshly diluted in cell culture medium. The maximum final DMSO concentration used for drug application was 0.1%.

Subcellular fractions were obtained using the QProteome Cellular Compartment Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. The identity of the fractions was evaluated using the markers GAPDH (cytoplasm), LRP6 (membrane) and Lamin A (nucleus, data not shown). The protein amount of the fractions was determined with the BCA Protein Assay (Pierce, Rockford, IL, USA) according to manufacturer's instructions.

Western Blotting was performed essentially as described [15]. 20 μg protein (β-catenin detection) or 40 μg protein (phospho-GS detection) per sample were separated with randomized gel loading to avoid error of signal intensity from gel position effects [24]. The following primary antibodies: anti-β-catenin (1:1000 monoclonal antibody (mAb); Santa Cruz, Heidelberg, Germany), anti-phospho Ser640/41 GS (1:1000, Cell Signaling Technology, Beverly, MA, USA), anti-GS (1:1000, Cell Signaling Technology), GAPDH (1:10000, Santa Cruz) and secondary antibodies: Alexa Fluor 680 goat anti-rabbit IgG, Alexa Fluor 680 goat anti-mouse IgG (both Invitrogen, 1:5000), and IRDye 800CW goat-anti-mouse and goat-anti-rabbit IgG (BIOMOL, Hamburg, Germany; 1:10,000) were used. For quantification of the glycogen synthase (GS) phosphorylation status, Ser640/641 phosphorylated GS was detected, the blot stripped and probed again against total GS and GAPDH. The signal intensity of phosphorylated GS was normalized to the signal intensity of total GS. The signal intensity of β-catenin was normalized to that of the loading control GAPDH.

Propidium iodide (PI) staining was used for detection of the cell cycle status of ReNcell VM cells. Cells were detached and fixed in ice-cold 70% ethanol for at least 1 h at −20 °C. Fixed cells were incubated with 1 mg/ml RNaseA (Sigma) followed by incubation with 50 μg/ml PI, each for 30 min at 37 °C. DNA content was measured immediately after PI staining by flow cytometry (FACSCalibur, BD Bioscience, San Jose, CA, USA) and analyzed using the Cell Quest Pro software (BD Bioscience).

Apoptotic cells were detected by terminal deoxynucleotidyl-transferase dUTP nick-end labeling (TUNEL) using the InSitu Cell Death detection Kit, Fluorescein (Roche, Penzberg, Germany) according to the manufacturer's recommendations. 10^4 cells per condition were measured by flow cytometry (FACSCalibur, BD Bioscience, San Jose, CA, USA) and analyzed using the Cell Quest Pro software (BD Bioscience).

Immunocytochemistry was performed as described [23]. β-III-tubulin antibodies (1:500, mAb, Sigma) and Alexa Fluor 488 goat anti-mouse antibodies (1:1000, Invitrogen) were used as primary and secondary antibodies, respectively. Images were obtained with Olympus BX51 microscope (Olympus Deutschland, Hamburg, Germany).

The number of DAPI positive nuclei was determined by a morphometric counting procedure based on the two-dimensional unbiased counting frame [12]. All DAPI nuclei in a 150 μm × 150 μm counting frame were counted for 64 frames in one well of a culture (4 wells per condition, 3 independent experiments). To count the number of β-III-tubulin immunoreactive neurons the dimensional unbiased counting frame was applied in combination with a projected circle of 10 μm diameter. Cells where fibers had a free ending within the circle around a DAPI positive nucleus were counted. Stereo Investigator v8.0 (Microbrightfield) was used for defining sampling areas and evaluating the cell culture frames.

Statistical analyses were performed using SPSS 15.0 for Windows. For normal distribution (Shapiro–Wilk) one-factor stages as well as homoscedasticity (Levene) and balanced samples, the one- or two-way (treatment and time), univariate analysis of variance with repeated measurements was performed, assuming the general linear model (fixed effects). A pair-wise *t*-test for dependent samples with a Bonferroni-adjusted *p*-value was added to detect differences between treatment- and time points, respectively.

Otherwise, the respective nonparametric procedures (Friedman-test followed by Wilcoxon pair-wise test) were used. $P < 0.05$ (0.01) was considered to indicate a statistically (highly **) significant (*) difference. Data are presented as mean ± standard error of the mean.

The activity of the serine/threonine kinases GSK-3α and GSK-3β (here together referred to as GSK-3) has been implicated in a wide array of biological processes, including apoptosis [8,10]. To investigate whether small molecule inhibitors of GSK-3 affect the viability of proliferating human NPCs, we measured the metabolic activity of proliferating ReNcell VM human NPC in the presence of different concentrations of SB216763 (SB21), LiCl [6], sodium valproate (VPA) [4], indirubine-3-monoxime (I3M) [20] and kenpaullone (KP) [27] (see Supplementary Data).

On the basis of viability testing the following concentrations of GSK-3 inhibitors were chosen for investigating physiological effects of the drugs on human NPC: 3 μM SB21, 3 mM LiCl, 1 mM VPA, 3 μM I3 M and 1 μM KP.

To determine the ability of GSK-3 inhibitors to reduce GSK-3 activity in human NPCs, we measured their influence on the phosphorylation status of glycogen synthase (GS) Ser640/41 a specific substrate of GSK-3 [19]. Confluent ReNcell VM cells were exposed for 3 h to cell culture medium lacking EGF and bFGF (differentiation conditions) in the presence or absence of 3 μM SB21, 3 mM LiCl, 1 mM VPA, 3 μM I3 M, and 1 μM KP, respectively, and lysed for preparation of subcellular extracts. Cytoplasmic extracts were probed for total GS and phospho-Ser 640/41 GS by Western blotting (Fig. 1a).

Treatment with SB21 and KP reduced GS phosphorylation normalized to total GS by 76% and 70%, respectively, whereas I3M and VPA reduced the phosphorylation by 37% and 32%, respectively, while for LiCl no significant effect was detected (Fig. 1b). All drug treatments had only minor effects on the expression of GS (Fig. 1a). Similar results were obtained using whole cell lysates (data not shown).

These results demonstrate a differential ability of the investigated GSK-3 inhibitors to interfere with the enzymatic activity of GSK-3 in intact human NPCs at the respective concentrations.

To investigate whether GSK-3 inhibition was sufficient to affect GSK-3 mediated signaling we next determined whether GSK-3 inhibitors interfered with the GSK-3 mediated degradation of β-catenin to stabilize β-catenin in human NPC. Therefore, we examined the free cytosolic pool of β-catenin by Western blotting. Again, confluent ReNcell VM cells were cultured for 3 h in

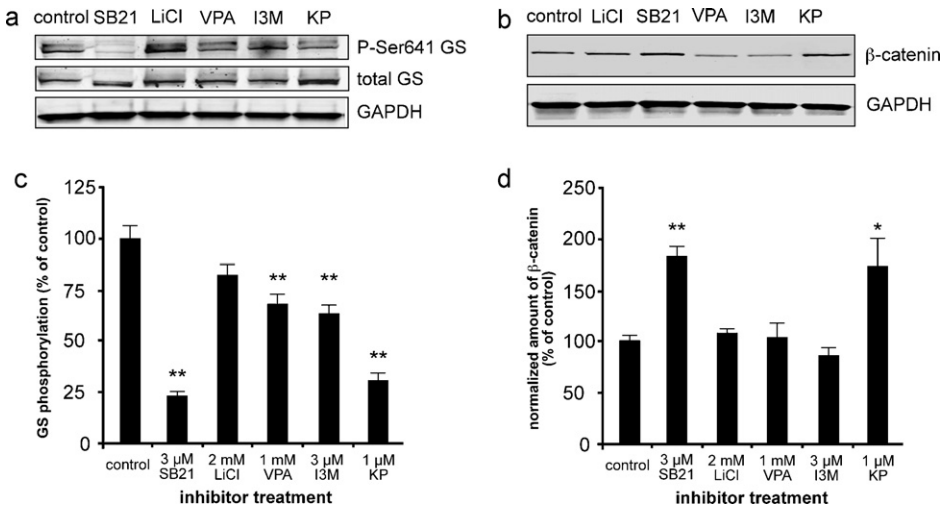

Fig. 1. Inhibition of GSK-3 enzymatic activity and GSK-mediated signaling by GSK-3 inhibitors in human NPC. Human NPC line ReNcell VM was cultured under differentiation conditions in the absence and presence of GSK-3 inhibitors at the indicated concentrations for 3 h. (a) Cytoplasmic lysates were immunoblotted with antibodies against phospho Ser-641 GS (top), total GS (middle) and for control of loading with antibodies against GAPDH (bottom). Note the appearance of a second smaller GS-band in the total GS blot representing unphosphorylated GS. (b) Quantification of immunoblot results of three independent experiments as illustrated in (a), each done in triplicate. The amount of phosphorylated GS was normalized to the amount of total GS for each GSK-3 inhibitor tested. $n = 9$. (c) Cytoplasmic lysates were immunoblotted with antibodies against β-catenin (top) and for control of loading with antibodies against GAPDH (bottom). (d) Quantification of immunoblot results of three independent experiments as illustrated in (c), each done at least in triplicate. $n = 10$–11. (b and d) Data are expressed relative to the control levels of untreated cells, which were defined as 100% (means ± SEM). Values were significantly different from controls at $^{*}p < 0.05$ and $^{**}p < 0.01$, respectively.

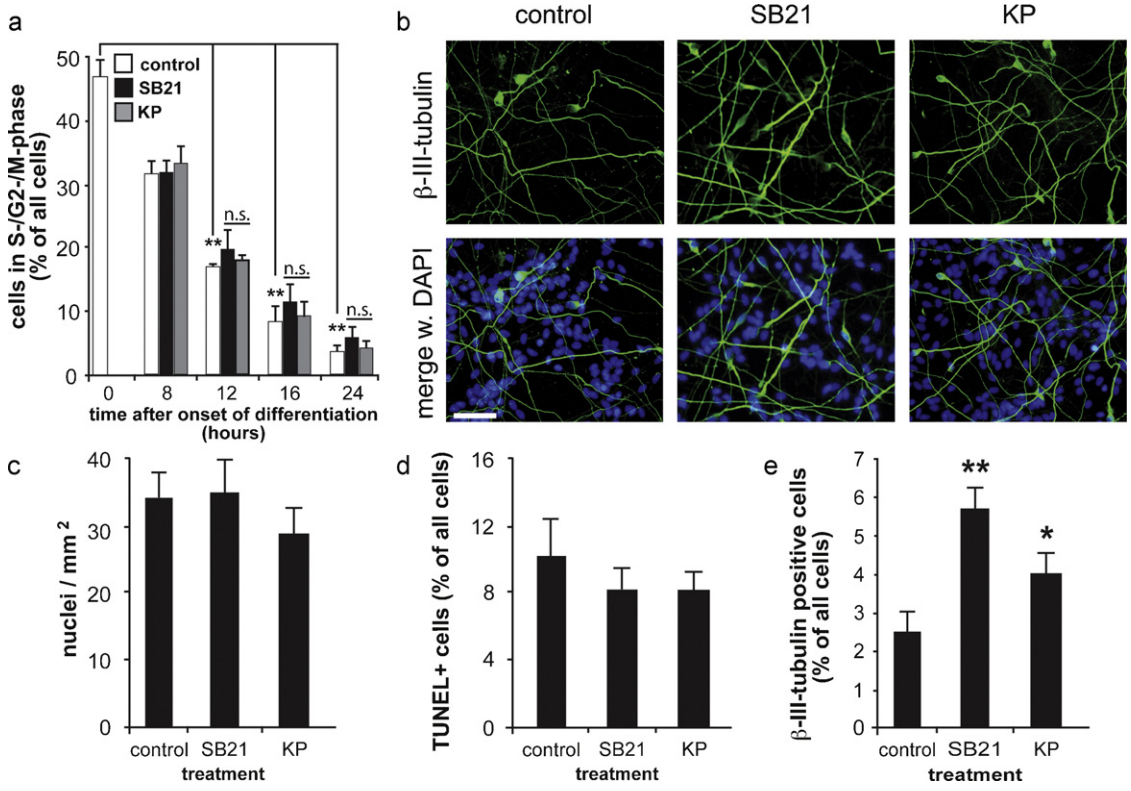

Fig. 2. Influence of GSK-3 inhibitors on the differentiation of human NPC. The human NPC line ReNcell VM was cultured under differentiation condition for the indicated time (a) or for 4 days (b–d). (a) Combined percentage of cells in the S-, G2- and M-phase for cells without inhibitor treatment (control, white bars), treated with 3 μM SB21 (black bars) or 1 μM KP (grey bars), respectively for the indicated time periods after onset of differentiation. $n = 4$. n.s. = not significantly different from control. (b) Representative photomicrographs of human NPC differentiated without inhibitor (control, left), 3 μM SB21 (middle) and 1 μM KP (right) and stained with β-III-tubulin (top) and counterstained with DAPI (bottom). Bar = 40 μm. (c) Quantification of DAPI stained cell nuclei per mm^2 in cultures of human NPC differentiated without inhibitor (control, left), 3 μM SB21 (middle) or 1 μM KP (right). $n = 12$. (d) Quantification of TUNEL-positive human NPC differentiated without inhibitor (control, left), 3 μM SB21 (middle) or 1 μM KP (right). $n = 3$. (e) Quantification of β-III-tubulin positive cells after differentiation of human NPC without inhibitor (control, left), 3 μM SB21 (middle) or 1 μM KP (right). $n = 12$. Values were significantly different from controls at $^{*}p < 0.05$ and $^{**}p < 0.01$, respectively.

medium lacking EGF and bFGF (differentiation conditions) but containing 3 μM SB21, 3 mM LiCl, 1 mM VPA, 3 μM I3 M, 1 μM KP or no inhibitor (control), respectively, until lysis. We prepared cytoplasmic, membrane, nuclear and cytoskeletal lysates to separate the pool of free β-catenin in the cytoplasm from the much more abundant membrane-bound β-catenin involved in cell adhesion.

Compared with the control, the β-catenin concentration in the cytosolic fraction was significantly increased in cell cultures treated with SB21 or KP by 82% and 74% respectively, whereas the other inhibitors had no significant effect (Fig. 1c and d).

These results are in agreement with the higher inhibitory effect of SB21 and KP and the lower or no effect of VPA, I3 M or LiCl on GS phosphorylation in the same cells (see Fig. 1a and b) and provide evidence that the strong inhibition by SB21 and KP is sufficient to interfere with GSK-3 mediated signaling in human NPC.

Finally, we analyzed whether potent GSK-3 inhibition by SB21 and KP influenced the cell cycle exit and cell fate acquisition during differentiation of human NPCs. For this purpose, confluent ReNcell VM cells were differentiated by withdrawal of EGF and bFGF in the presence of 3 μM SB21, 1 μM KP or solvent control respectively. The cell cycle status of differentiating cells was analyzed at 0, 8, 12, 16, and 24 h after onset of differentiation. In the absence of GSK-3 inhibitors the proportion of cycling cells in S- and G2-/M-phase was reduced from averaged 47.1% under proliferation conditions to 31.6%, 17.1%, 8.5%, and 3.8% after 8, 12, 16 and 24 h differentiation, respectively (Fig. 2a). Similar results were found after treatment with SB21 or KP during the first 24 h of differentiation, whereas no differences between treatments (control, SB21 and KP) were detectable (Fig. 2a). These data demonstrate that growth factor withdrawal in ReNcell VM induces the vast majority of cells to exit the cell cycle during the first 24 h of differentiation, irrespective of GSK-3 inhibitor treatment.

Finally, we investigated the effect of GSK-3 inhibition on neurogenesis during the differentiation of human NPCs. Confluent ReNcell VM cells were differentiated by withdrawal of EGF and bFGF for 4 days in the presence of 3 μM SB21, 1 μM KP and solvent control, respectively. Cells were stained with DAPI, TUNEL and the neuronal marker β-III-tubulin (Fig. 2b) and the proportions of TUNEL+ apoptotic cells and β-III-tubulin immunoreactive neurons were quantified.. No significant changes were detected neither for cell density (Fig. 2c) nor for apoptosis (Fig. 2d) after 4 days differentiation in the presence of GSK-3 inhibitors compared to control differentiation. In contrast, treatment of differentiating cells with SB21 and KP significantly increased the average proportion of neurons from 2.6% under control conditions by more than twofold to 5.7% for SB21 or by more than 1.5-fold to 4.1% by KP, respectively (Fig. 2d). The vast majority of of β-III-tubulin negative cells were positive for the astrocytic markers GFAP and S100-β (data not shown).

Taken together, these data suggest a selective induction of neurogenesis of multipotent human NPCs due to inhibition of GSK-3 by a mechanism that does not rely on prolonged proliferation of neuronal progenitors after onset of differentiation or differential cell survival.

This work reports biological effects of GSK-3 inhibition in human NPC. We found that the tested inhibitors did affect viability of proliferating ReNcell VM cells at concentrations commonly used in other cell types [4,6,20,27]. However, at least the reduction of cell viability by LiCl, VPA and I3M cannot be attributed to an adverse effect of excessive GSK-3 inhibition itself, because SB21 and KP reduced GSK-3 activity more than double as effective as LiCl, VPA and I3M without influencing cell viability. Rather, pleiotropic effects of the inhibitors might underlie the reduction of cell viability, also supported by the fact that SB21 and KP are the most selective of the tested inhibitors [2,3].

Despite the low concentrations of GSK-3 inhibitors in our study, these inhibitors reduced the enzymatic activity of GSK-3 in human NPC though with differential efficacy. The most effective inhibitors SB21 and KP reduced GSK-3 substrate phosphorylation in intact cells by circa 70%, and were the only inhibitors sufficient to induce β-catenin accumulation. These results are consistent and might be explained with the finding that deletion of at least 3 of the 4 GSK-3α/β alleles, i.e. reduction by 75%, is required for activation of β-catenin signaling in mouse ES cells [9]. Thus, we independently reproduce here in human NPC the existence of a threshold GSK-3 inhibition for activation of β-catenin signaling. Further, our data may suggest that inhibition of GSK-3 by SB21 and KP is sufficient to interfere with the role of GSK-3 in cell signaling, presumably also in pathways other than Wnt.

The most striking observation in this study is the increase of neurogenesis of multipotent human NPC by treatment with SB21 and KP. The fact that the two structurally unrelated compounds SB21 and KP, whose main though not exclusive target is GSK-3, cause the same phenotype after differentiation, strongly suggest GSK-3 inhibition, rather than off-target effects, as the cause for the increase in neurogenesis. Consistently, SB21 and KP do very limitedly overlap in the inhibition of kinases other that GSK-3 *in vitro* [3]. Clearly, we do not exclude the possibility that LiCl, I3M or VPA may also enhance neurogenesis in human NPC, however, by influencing other enzymes than GSK-3 as it has been shown for VPA and rodent NPC [16].

One hallmark of potent GSK-3 inhibition that is reproduced in this study in human NPC is the activation of β-catenin signaling and gain-of-function of β-catenin phenocopies some effects of GSK-3 inactivation in rodent NPC *in vivo* [1,5,18] and *in vitro* [13,14,22,25]. However, in contrast to treatment with SB21 and KP, overexpression of stabilized β-catenin alone in ReNcell VM cells is not sufficient to increase neurogenesis under differentiation conditions, despite its ability to activate β-catenin signaling in these cells [17]. Thus, these data suggest that in opposition to studies in rodent NPC [13], the GSK-3 inhibition induced enhancement of neurogenesis in our cellular model might not be dependent on β-catenin stabilization and points to a primary role for β-catenin-independent mechanisms downstream of GSK-3 [18] for this effect. Further studies are needed to dissect the molecular mechanisms of GSK-3 inhibition on neurogenesis of human NPC.

Our data demonstrates a role for GSK-3 in the regulation of cell fate during differentiation of human NPC. We find that two different small molecule inhibitors of GSK-3, SB21 and KP, enhance neurogenesis during the differentiation of ReNcell VM, a human NPC line. These results provide insight into the molecular mechanisms of neurogenesis of human NPC and may contribute to direct the differentiation of human NPC for therapeutic applications.

Author's contribution

AR conceived the study. CL, EM and AR planned experiments. CL, JF, JM OS KK and SO performed experiments. CL, EM, ÄG, ACS and MJF analyzed data. EM, ÄG, ACS, RH, SO, AW and AR contributed to drafting the manuscript. CL wrote the paper.

Competing interests

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neulet.2010.10.076.

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