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Unlocking mechanisms in interleukin-1β-induced changes in hippocampal neurogenesis—a role for GSK-3β and TLX

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Glycogen synthase kinase-3β (GSK-3β) and the orphan nuclear receptor tailless homolog (TLX) are key regulators of hippocampal neurogenesis, which has been reported to be dysregulated in both neurodegenerative and psychiatric disorders. Inflammation is also implicated in the neuropathology of these disorders because of increased levels of the pro-inflammatory cytokine interleukin-1β (IL-1β) in the brain. At elevated levels, IL-1β signaling through the IL-1 receptor type 1 has been shown to be detrimental to hippocampal neurogenesis. TLX is required to maintain neural stem/progenitor cells (NSPCs) in an undifferentiated state and is involved in NSPC fate determination, while GSK-3β negatively regulates Wnt signaling, a vital pathway promoting neurogenesis. This study shows that GSK-3β inhibition using a small-molecule inhibitor and the mood stabilizer lithium restores the IL-1β-induced decrease in NSPC proliferation and neuronal differentiation of embryonic rat hippocampal NSPCs to control levels. The IL-1β-induced effect on NSPCs is paralleled by a decrease in TLX expression that can be prevented by GSK-3β inhibition. The present results suggest that GSK-3β ameliorates the anti-proliferative and pro-gliogenic effects of IL-1β, and that TLX is vulnerable to inflammatory insult. Strategies to reduce GSK-3β activity or to increase TLX expression may facilitate the restoration of hippocampal neurogenesis in neuroinflammatory conditions where neurogenesis is impaired.
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Introduction

Neurogenesis, the birth of new neurons from multipotent neural stem/progenitor cells (NSPCs), occurs throughout the developing brain and in distinct regions of the adult brain, including the dentate gyrus (DG) of the hippocampus. Dysregulated hippocampal neurogenesis in the adult brain has been implicated in neurodegenerative and psychiatric disorders, including major depression disorder, anxiety-related behaviors and Alzheimer's disease,[1–7] and it is well documented that antidepressant treatment increases hippocampal NSPC proliferation and neuronal differentiation.[8,9] Lithium chloride (LiCl) is currently the most widely used treatment for bipolar disorder, and may be beneficial for recurrent or treatment-resistant major depression,[10] however, its mechanism of action remains unclear. It is known that it expands the pools of both adult[11] and embryonic[12] rat NSPCs, and enhances neuronal differentiation at therapeutic concentrations.[11,12] LiCl inhibits the serine-threonine kinase, glycogen synthase kinase-3β (GSK-3β),[13–15] a negative regulator of Wnt signaling,[16] which itself is a key regulator of hippocampal neurogenesis.[17] In the absence of a Wnt ligand, GSK-3β forms part of the destruction complex resulting in the degradation of transcription factor β-catenin, thus inhibiting its translocation to the nucleus to induce downstream gene transcription.[18] β-catenin itself has been shown to be involved in maintaining NSPC proliferation in the central nervous system.[19] GSK-3β has a role in various other signaling pathways, including nuclear factor-kappa B (NF-κB) signaling, and it has been proposed as a regulator of cytokine production.[19,20] Inflammation is also associated with the neuropathology of several psychiatric and neurodegenerative disorders because of increased levels of pro-inflammatory cytokines such as interleukin-1β (IL-1β), tumor necrosis factor − α and IL-6.[21–24] Exposure of the developing fetus to inflammation *in utero*, because of maternal infection has also been shown to be associated with memory impairments[25] and increased incidences of psychiatric disorders such as schizophrenia, anxiety and depression in later life.[26–28] Studies have also demonstrated that maternal inflammation, which is known to induce IL-1β production in the fetus,[29] is detrimental to hippocampal neurogenesis.[25,30] Thus maternal infection-induced deficits in neurogenesis in the brains of offspring may have consequences in later life for neurodegenerative and psychiatric disorders. It should be noted, however, that although some studies report no effect or indeed a positive effect of IL-1β on NPSC proliferation and neuronal differentiation,[31,32] others have demonstrated it to have an anti-proliferative and detrimental effect on hippocampal neuronal differentiation and survival.[33–36] It is now established that the IL-1 type-1 receptor (IL-1R1) is expressed on NSPCs and mature neurons.[33,37,38] However, little is currently known about the role of GSK-3β in IL-1β-induced changes in hippocampal neurogenesis, or the effect of therapeutic concentrations of LiCl on NSPC proliferation and differentiation in the presence of IL-1β.

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The orphan nuclear receptor tailless homolog (TLX) has recently been identified as a key regulator of adult hippocampal neurogenesis[39,40] and also has a role in hippocampal-dependent learning and memory.[40,41] It has now been elucidated that the Wnt/β-catenin signaling pathways mediate, in part, the regulatory actions of TLX on the proliferation and self-renewal of adult NSPCs.[42] Although data is emerging on the role of TLX in neural development[43] and adult hippocampal neurogenesis,[39,40] the effect of inflammatory modulators (such as IL-1β) or the role of intracellular neurogenic regulators (such as GSK-3β) on TLX expression has not yet been explored. In order to promote the activation of endogenous NSPCs in the inflamed hippocampus, we must first understand the mechanisms by which these neurogenic modulators act.

It has been shown that NSPCs are a distinct population of cells in the embryonic DG, which share essential features with the NSPCs that reside in the DG through to adulthood.[44] Moreover, this group suggested that the molecular mechanisms regulating NSPCs cell fate specification in the DG are functional throughout all stages of development. Thus, to elucidate the mechanistic questions posed in this study, we carried out our experiments using embryonic NSPCs and so circumvented the technological limitations involved in culturing adult NSPCs.[45] The aim of this study therefore was to examine if GSK-3β inhibition using LiCl and a specific small-molecule inhibitor, could abrogate the detrimental effects of IL-1β on proliferation and differentiation of embryonic rat hippocampal NSPCs and assess the effect on TLX expression.

Materials and methods

Preparation and treatment of rat hippocampal NSPCs. Hippocampi from 18 rat embryos with an embryonic age (E) (Biological Services Unit, UCC, Cork, Ireland) were cultured for 7 *days in vitro* (DIV) as proliferating neurospheres, as previously described.[33,46] For differentiation studies, untreated neurospheres were dissociated, seeded at 5×10^4 cells/coverslip and allowed to differentiate for 7 DIV in differentiation medium (DMEM-F12; 1% antibiotic-antimycotic solution; 200 mM L-glutamine; 33 mM D-glucose; 2% B-27; 1% fetal calf serum).[33,46] Neurospheres or differentiated cells were treated with IL-1β (10 ng ml^{-1}), LiCl (2 mM), the small-molecule inhibitor SB216763 (10 μM in 0.3% ethanol), or co-treated with IL-1β and LiCl (10 ng ml; 2 mM) or IL-1β and SB216763 (10 ng ml^{-1}; 10 μM). For differentiation studies, co-treatments were carried out after a 1-h pretreatment with the respective GSK-3β inhibitor. We have previously shown that 10 ng ml^{-1} is the lowest concentration of IL-1β to affect NSPC proliferation and differentiation,[33] 2 mM LiCl is within therapeutic range[11] and 10 μM SB216763 reduces GSK-3β activity to 4%.[47] Ethanol (0.3%) had no effect on NSPC proliferation or differentiation (data not shown). For proliferation and studies, cultures were treated for 4 DIV under proliferating conditions and pulsed with 5-bromo-2′-deoxyuridine (BrdU, 10 μM) for the final 4 h of culture.[48] Neurospheres were dissociated to a single-cell suspension and the number of viable cells was calculated using the trypan blue exclusion assay. Cells were then seeded at 5×10^4 viable cells per coverslip, and were allowed to adhere to glass coverslips for 1 h before immunocytochemical analysis.

Immunocytochemistry. Cells were incubated in antibodies that target nestin (1:200; goat polyclonal, NSPCs), BrdU (1:100; mouse monoclonal, proliferating cells), βIII-tubulin (1:300; mouse monoclonal, young neurons[11,12]), doublecortin (DCX) (1:200; goat polyclonal, newly born neurons[49]), glial fibrillary acidic protein (GFAP) (1:300; rabbit polyclonal, astrocytes[11,12]), GSK-3β (1:200; rabbit monoclonal), IL-1R1 (1:200; rabbit polyclonal) or TLX (1:150; goat polyclonal) overnight at 4 °C and subsequently incubated in the appropriate secondary antibody, as previously described.[20,33] Cells were counterstained with 4′6-diamidino-2-phenylindole (DAPI) (1:2500) to identify the nuclei. For each antibody, the cells from one well were incubated in blocking solution and secondary antibody, without primary antibody (to account for nonspecific binding of the secondary antibody), and showed a complete absence of immunoflouresencent staining (data not shown).

Cell counts and densitometry. Immuno-positive cells were viewed with an upright microscope (AX70, Olympus, Hamburg, Germany). Immuno-positive cells were counted in five randomly chosen fields of view from each of the four coverslips, and divided by the total number of cells per five fields of view to give an average percentage for each coverslip. Only βIII-tubulin or GFAP-positive cells with a differentiated phenotype were counted. Each experiment was independently repeated two or three times. Each experiment consisted of the hippocampi of at least five rat E18 embryos, pooled and prepared as already described. The densitometry of eight TLX-positive cells (selected as a systematic random sample) per image was carried out using Image J software (Version 1.38X, NIH, Bethesda, MD, USA). Twenty micrographs per treatment were analyzed from either two or three independent experiments. For each photomicrograph, background measurements were subtracted from each TLX-positive cell value to obtain a corrected fluorescence measurement.

PCR. Total cellular RNA was extracted from neurospheres and differentiated NSPCs using an RNeasy kit (Roche, Hertfordshire, UK), according to the manufacturer's instructions. Complimentary DNA synthesis was performed on RNA using oligo (dT)s, random primers and reverse transcriptase at 37 °C for 1 h. RNA was incubated with DNAse for 30 min to exclude genomic DNA contamination. PCR was carried out on a light cycler 480 (Roche) using the following primers; TLX F: GCTTTCTTCACAGCGGTCAC, R: GCAGACACAGCGGTCAACT and involved the following steps: 90 °C for 10 min, 45 cycles of 90 °C for 10 sec, 60 °C for 30 sec and 72 °C for 1 sec. Each reaction contained 2 μl of complimentary DNA (0.1 volume), primers (0.5 μm), light cycler master mix (0.2 volume; Roche), and made up to 20 μl with molecular grade H_20. Samples lacking the reverse transcriptase (RT) enzyme were run to ensure the samples were free of genomic DNA contamination. PCR products were electrophoresed on a 1% agarose gel and visualized on a UV transilluminator.

Immunoblotting. Cells were lysed, separated by SDS-polyacrylamide gel electrophoresis and electrophoretically

transferred to nitrocellulose membrane, as previously described.[20,50] The membrane was incubated in primary antibodies that target GSK-3β (rabbit monoclonal, 1:1000), p-GSK-3β (serine-9; rabbit monoclonal 1:500), β-catenin (rabbit monoclonal, 1:500) and β-actin (mouse polyclonal, 1:500) and subsequently with the appropriate secondary antibodies; donkey IRdye680 anti-rabbit and donkey-IRdye800 anti-mouse. Proteins were visualized using the Odyssey infrared imaging system (Li-Cor Biosciences Ltd, Lincoln, NE, USA).

Statistical analyses. An unpaired Student's *t*-test or a two-way analysis of variance (ANOVA), followed by the Bonferroni *post hoc* analysis, were performed as appropriate. Results were expressed as means with s.e.m and deemed significant when $P<0.05$.

Results

GSK-3β inhibition ameliorates the effects of IL-1β on proliferating cells. Almost all cells expressed GSK-3β under proliferation conditions (Figure 1a), as demonstrated by immunocytochemistry (Figure 1b). IL-1R1 was also expressed in almost all NSPCs (Figure 1c). When neurospheres were exposed to IL-1β for 7 DIV under proliferation conditions, there was no effect of IL-1β on the percentage composition of nestin-positive cells (Figure 1d), but it significantly increased the percentage of NSPCs expressing GSK-3β ($P<0.001$) (Figure 1e). IL-1β treatment also increased GSK-3β activity in NSPCs under proliferation conditions; IL-1β significantly decreased β-catenin in total cell lysate ($P<0.001$; Figures 1f and g) and significantly decreased the p-GSK-3β (serine 9) to GSK-3β ratio in total cell lysate ($P<0.05$; Figures 1h and i). GSK-3β expression was confirmed on BrdU-positive cells in the presence and absence of IL-1β (Figure 1j). LiCl partially attenuated the IL-1β-induced decrease in BrdU-positive cells, whereas SB216763 completely attenuated the IL-1β-induced decrease (Figure 1k). Two-way ANOVA revealed a significant main effect of IL-1β ($F_{1,\ 28}=143.83$; $P<0.001$), LiCl ($F_{1,\ 28}=117.13$; $P<0.001$) and SB216763 ($F_{1,428}=85.55$; $P<0.001$) and a significant interaction effect of IL-1β × LiCl ($F_{1,28}=72.98$; $P<0.001$) and IL-1β × SB216763 ($F_{1,28}=74.47$; $P<0.01$). *Post hoc* analysis showed that IL-1β significantly decreased the percentage of BrdU-positive cells compared with untreated cultures ($P<0.001$); IL-1β in the presence of LiCl significantly decreased the percentage of BrdU-positive cells compared with cultures treated with LiCL alone ($P<0.001$) and that both LiCl and SB216763 treatment in the presence of IL-1β increased the percentage of BrdU-positive cells compared with cultures treated with IL-1β alone ($P<0.001$) (Figure 1k).

IL-1β increases GSK-3β activity in differentiated cells. Immunocytochemistry revealed that GSK-3β was ubiquitously expressed on DCX-positive cells, βIII-tubulin-positive cells (neuronal lineage restricted cells; newly born neuronal progenitor cells and young neurons) and GFAP-positive cells (astrocytic lineage restricted cells) cultured for 7 DIV in the presence or absence of IL-1β (Figure 2a). IL-1β significantly decreased the level of β-catenin ($P<0.001$; Figures 2b and c) and the p-GSK-3β (serine 9) to total GSK-3β ratio ($P<0.05$; Figures 2d and e), indicating an increased GSK-3β activity after IL-1β treatment.

GSK-3β inhibition ameliorates the effects of IL-1β on NSPC differentiation. The effect of IL-1β and GSK-3β inhibition on the cell fate specification of NSPCs under differentiation conditions was subsequently investigated. DCX and βIII-tubulin were used as neuronal progenitor cell markers, with DCX and βIII-tubulin-positive cells in our culture system exhibiting a neuronal phenotype with neuronal processes (Figure 3a). Although they are not mature neurons, their neuronal morphology in addition to their expression of neuronal specific microtubules indicate that they have become lineage restricted to a neuronal fate. GFAP-positive cells within our culture system exhibited a characteristic astrocytic phenotype and show signs of differentiation when compared with undifferentiated nestin-positive cells (arrow heads) (Figure 3a). Previous studies from our group and others have shown that IL-1β induces undifferentiated embryonic hippocampal NSPCs to pursue an astroglial rather than a neuronal fate under differentiation conditions,[33,35,51] thus we examined if the increased GSK-3β activity was involved in this process and if GSK-3β inhibition could ameliorate the effect. Viable cells were plated at equal densities to eliminate the possibility of unequal cell numbers skewing the percentages. There was no significant difference in the total number of cells after 7 DIV under differentiation conditions (as determined by DAPI staining), between the IL-1β-treated, LiCl-treated, SB216763-treated or co-treatment groups and untreated cultures (data not shown). IL-1β significantly decreased the percentage of DCX-positive cells ($P<0.05$; mean difference 12.54%; Figures 3b and c). Two-way ANOVA revealed a significant main effect of IL-1β ($F_{1,\ 44}=9.06$; $P<0.01$), LiCl ($F_{1,44}=9.10$; $P<0.01$) and SB216763 ($F_{1,\ 44}=25.74$; $P<0.001$), but not of IL-1β × LiCl or IL-1β × SB216763. *Post hoc* analysis showed that IL-1β significantly decreased the percentage of DCX-positive cells compared with untreated cultures ($P<0.05$) (Figure 3b). Both LiCl and SB216763 treatment prevented the IL-1β-induced decrease in the percentage of βIII-tubulin-positive cells (Figures 3d and f). Two-way ANOVA revealed a significant main effect of IL-1β ($F_{1,\ 44}=38.17$; $P<0.001$), LiCl ($F_{1,\ 44}=11.17$; $P<0.001$) and SB216763 ($F_{1,\ 44}=97.18$; $P<0.001$), and an interaction effect of IL-1β × LiCl ($F_{1,44}=5.42$; $P<0.05$) and IL-1β × SB216763 ($F_{1,44}=5.05$; $P<0.05$). *Post hoc* analysis showed that IL-1β significantly decreased the percentage of βIII-tubulin-positive cells compared with untreated cultures ($P<0.001$), that SB216763 increased the percentage of βIII-tubulin-positive cells compared with untreated cultures ($P<0.001$), and that both SB216763 and LiCl treatment in the presence of IL-1β increased the percentage of βIII-tubulin-positive cells compared with cultures treated with IL-1β alone ($P<0.01$) (Figure 3d). In contrast to this, an IL-1β-induced increase in the percentage of GFAP-positive cells was blocked by both LiCl and SB216763 (Figures 3e and f). Two-way ANOVA revealed a significant main effect of IL-1β ($F_{1,\ 44}=13.03$; $P<0.001$), LiCl ($F_{1,44}=11.01$; $P<0.01$) and SB216763

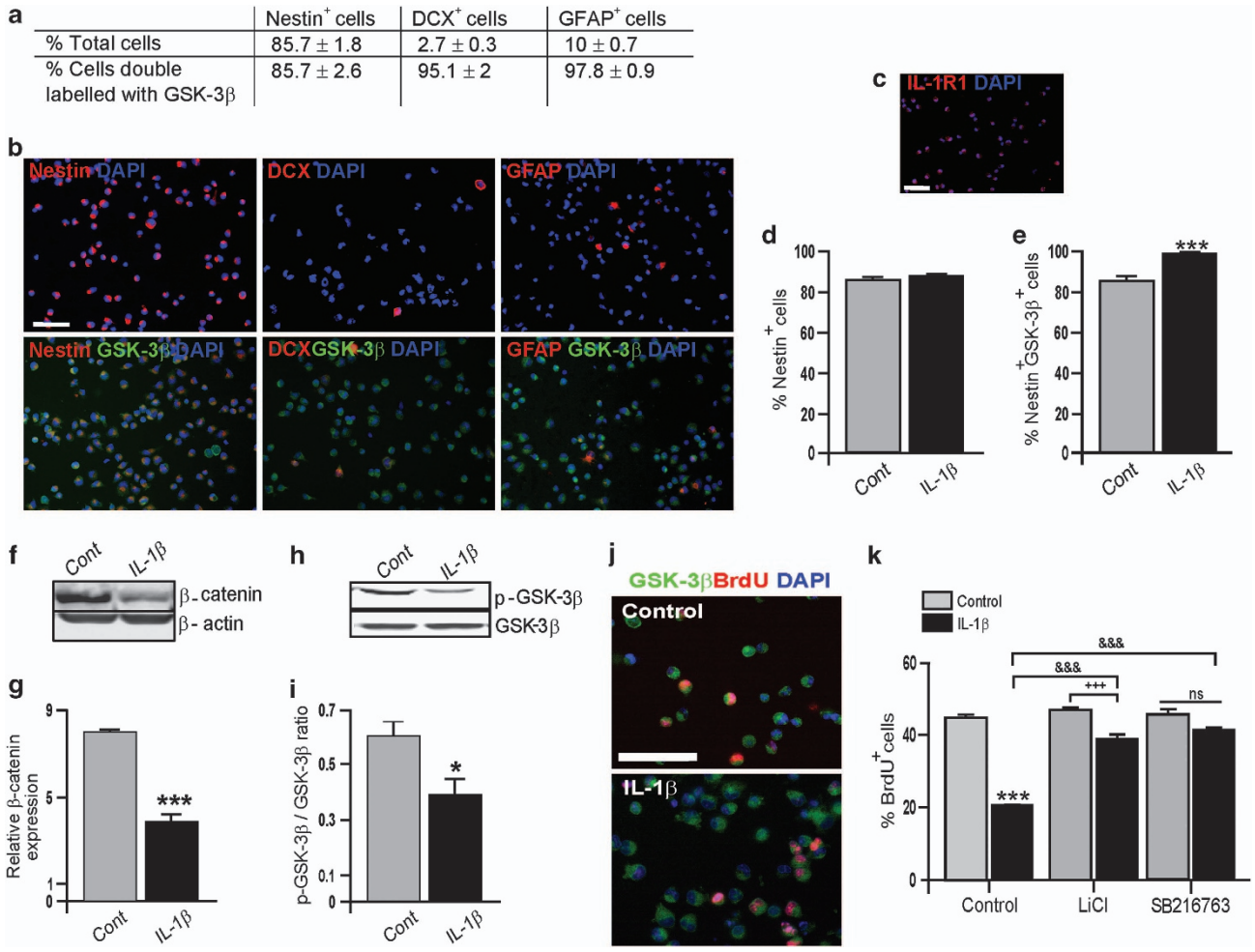


Figure 1 IL-1β-induced changes in neural stem/progenitor cell (NSPC) proliferation are prevented by glycogen synthase kinase-3β (GSK-3β) inhibition. (**a**) Characterization of neurosphere cell phenotype composition. (**b**, **c** and **j**) Representative photomicrographs of cells immunocytochemically stained for nestin (red), doublecortin (DCX) (red), glial fibrillary acidic protein (GFAP) (red), 5-bromo-2′-deoxyuridine (BrdU) (red) with GSK-3β (green) or interleukin-1 type-1 receptor (IL-1R1) (red) in untreated cultures after 7 DIV under proliferation conditions. Cells were counterstained with DAPI (blue) to visualize the nuclei. Scale bar = 50 μm. Percentage composition of nestin$^+$ (**d**) and nestin$^+$GSK-3β$^+$ (**e**) cells in untreated control and IL-1β-treated cultures, ***$P<0.001$ vs untreated control (Student's *t*-test; $n=3$). (**f** and **h**) Immunoblot analysis of β-catenin, *P*-GSK-3β (serine 9), total GSK-3β and β-actin protein levels in total cell lysates from cells cultured in the presence or absence of IL-1β. Protein bands of 92, 46, 46 and 42 kDa indicate β-catenin, p-GSK-3β (serine 9), total GSK-3β and β − actin protein expression, respectively. Mean densitometry analysis of β-catenin (**g**) and p-GSK-3β (**i**) equalized to β-actin or total GSK-3β, respectively, from three independent experiments, *$P<0.05$, ***$P<0.001$ vs untreated control (Student's *t*-test). (**k**) Percentage composition of BrdU$^+$ cells in untreated, IL-1β-treated, LiCl-treated, SB216763-treated, IL-1β + LiCl-treated and IL-1β$^+$ SB216763-treated cultures ***$P<0.001$ vs untreated control; $^{\&\&\&}P<0.001$ vs IL-1β treatment alone; $^{+++}P<0.001$ vs LiCl treatment alone, ns = not significant (two-way analysis of variance (ANOVA) with Bonferroni *post hoc* test; $n=2$). (**a**, **d**, **e**, **g**, **i** and **k**) Data are expressed as mean ± s.e.m.

($F_{1,\ 44}=38.56$; $P<0.0001$), and an interaction effect of IL-1β × LiCl ($F_{1,44}=6.94$; $P<0.01$) and IL-1β × SB216763 ($F_{1,\ 44}=7.36$; $P<0.01$). *Post hoc* analysis showed that IL-1β significantly increased the percentage of GFAP-positive cells compared with untreated cultures ($P<0.01$), and that both SB216763 and LiCl treatment in the presence of IL-1β decreased the percentage of GFAP-positive cells compared with cultures treated with IL-1β alone ($P<0.01$, $P<0.001$, respectively) (Figure 3e).

GSK-3β inhibition ameliorates the effect of IL-1β on TLX expression in proliferating NSPCs. We identified that TLX is expressed on embryonic rat NSPCs at the transcriptional and translational level (Figures 4a and b), and further show that TLX is expressed on nestin-positive NSPCs, BrdU-positive and IL-1R1-positive cells (Figure 4c), indicating that TLX may be susceptible to an IL-1β-induced inflammatory insult. The percentage of cells expressing TLX was not altered after IL-1β treatment (data not shown), however, densitometric analysis of TLX from the total population of cells in neurosphere cultures revealed that IL-1β significantly reduced TLX expression in NSPCs after 7 DIV ($P<0.001$; Figures 4b and d). This decrease was also evident in nestin-positive cells ($P<0.001$; Figure 4e) and in BrdU-positive cells ($P<0.01$; Figure 4f). Because the anti-proliferative effect of IL-1β on NSPCs can be blocked by specific GSK-3β inhibition, we examined if specific GSK-3β inhibition could affect the IL-1β-induced decrease in TLX expression in

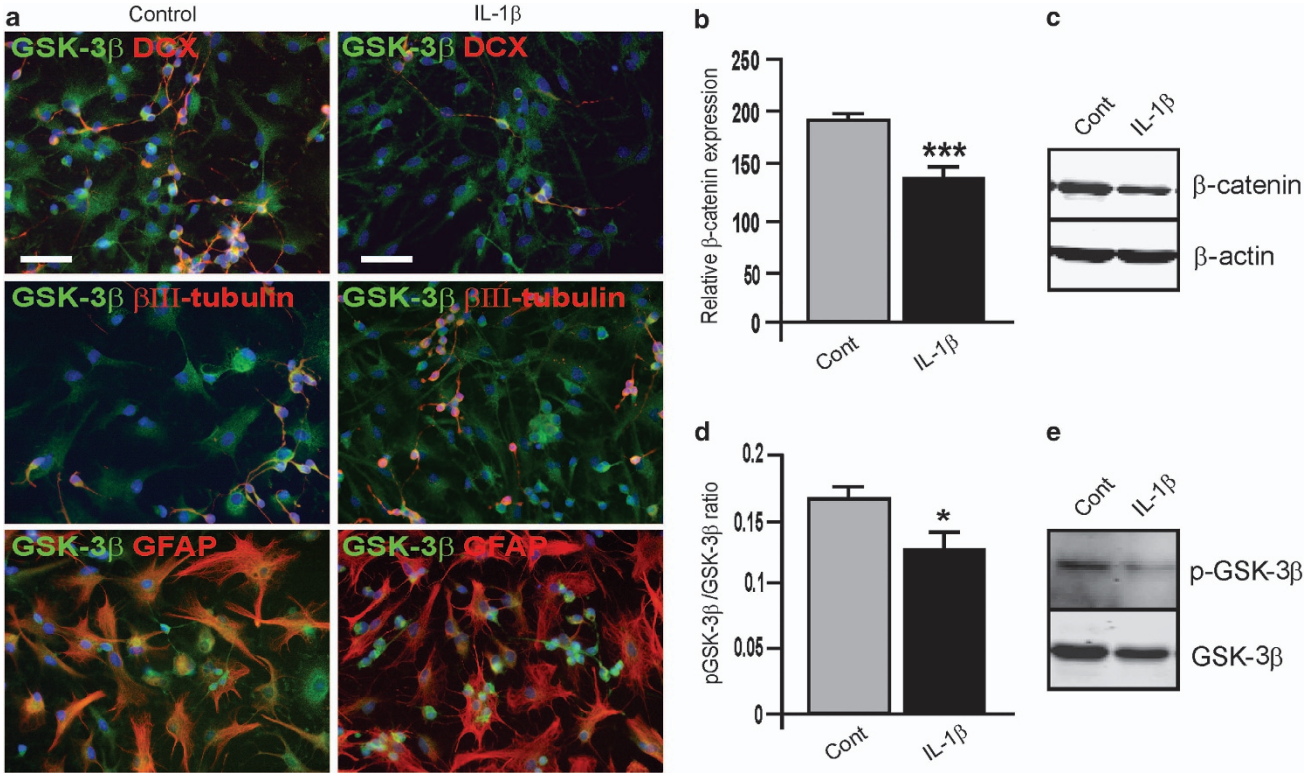
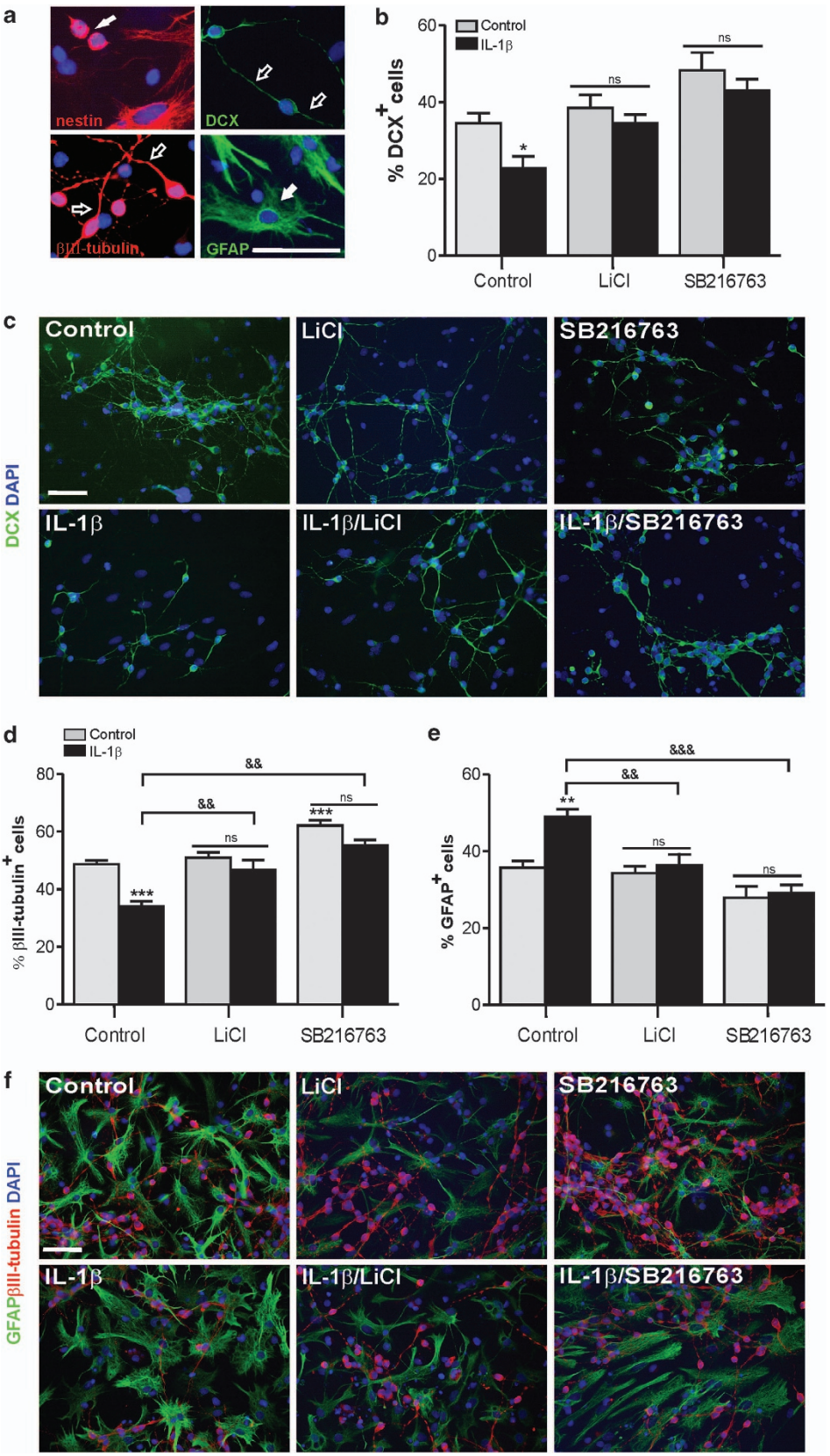


Figure 2 The effect of IL-1β on glycogen synthase kinase-3β (GSK-3β) activity in differentiated hippocampal neural stem/progenitor cells (NSPCs). (**a**) Representative photomicrographs of cells immunocytochemically stained for doublecortin (DCX) (red), βIII-tubulin (red) or glial fibrillary acidic protein (GFAP) (red) with GSK-3β (green) in untreated control and interleukin-1β (IL-1β)-treated cultures after 7 DIV under differentiation conditions. Cells were counterstained with DAPI (blue) to visualize the nuclei. Scale bar = 50 μm. (**c** and **e**) Immunoblot analysis of β-catenin, p-GSK-3β (serine 9), total GSK-3β and β-actin protein levels in total cell lysates from cells cultured in the presence or absence of IL-1β. Protein bands of 92, 42, 46 and 46 kDa indicate β-catenin, β − actin (**c**) p-GSK-3β (serine 9) and total GSK-3β (**e**) expression, respectively. (**b** and **d**) Mean densitometry analysis of β-catenin equalized to β-actin (**b**), and p-GSK-3β equalized to total GSK-3β (**d**) from three independent experiments. Data are expressed as mean ± s.e.m. *$P<0.05$, ***$P<0.001$ vs untreated control (Students *t*-test).

BrdU-positive cells. SB216763 treatment prevented the IL-1β-induced decrease in TLX expression in BrdU-positive cells (Figure 4f). This finding was revealed by a main effect of IL-1β ($F_{1,\ 152}=4.34$; $P<0.05$) and SB216763 ($F_{1,\ 152}=31.64$; $P<0.001$), and an interaction effect of IL-1β × SB216763 ($F_{1,\ 152}=6.73$; $P<0.05$). *Post hoc* analysis showed that IL-1β significantly reduced TLX expression in BrdU-positive cells compared with untreated cultures ($P<0.01$), and that SB216763 treatment in the presence of IL-1β increased TLX expression in BrdU-positive cells compared with cultures treated with IL-1β alone ($P<0.001$) (Figure 4f).

GSK-3β inhibition ameliorates the effect of IL-1β on TLX expression in neurons. We established that TLX expression was retained on differentiated cells cultured in the presence and absence of IL-1β for 7 DIV; TLX is expressed in DCX-positive and βIII-tubulin-positive neurons, and in GFAP-positive astrocytes (Figure 5a). Cells undergoing mitosis under differentiation conditions, apparent from DAPI staining, appear to strongly express TLX within the cytoplasm (Figure 5b). We also show the co-localization of GSK-3β and TLX on differentiated NSPCs in the presence and absence of IL-1β (Figure 5c). SB216763 prevented the IL-1β-induced decrease in TLX expression in DCX-positive cells (Figure 5d). Two-way ANOVA revealed a main effect of SB216763 ($F_{1,\ 152}=29.73$; $P<0.001$) but not of IL-1β, and an interaction effect of IL-1β × SB216763 ($F_{1,\ 152}=18.98$; $P<0.001$). *Post hoc* analysis showed that IL-1β significantly reduced TLX expression in DCX-positive neurons compared with untreated cultures ($P<0.05$), and that SB216763 treatment in the presence of IL-1β increased TLX expression in DCX-positive neurons compared with cultures treated with IL-1β alone ($P<0.001$) (Figure 5d). SB216763 also prevented the IL-1β-induced decrease in TLX expression in βIII-tubulin-positive neurons (Figure 5e). Two-way ANOVA revealed a main effect of SB216763 ($F_{1,\ 152}=72.28$; $P<0.001$), but not of IL-1β, and an interaction effect of IL-1β × SB216763 ($F_{1,\ 152}=35.31$; $P<0.001$). *Post hoc* analysis showed that IL-1β reduced TLX expression in βIII-tubulin-positive neurons compared with untreated cultures ($P<0.05$), and that co-treatment of cultures with SB216763 and IL-1β increased TLX expression in βIII-tubulin-positive neurons compared with cultures treated with IL-1β alone ($P<0.001$) (Figure 5e). When analysis of TLX expression in GFAP-positive cells was carried out, the IL-1β-induced decrease in TLX expression was not blocked by SB216763 treatment (Figure 5f). This finding was revealed by a main effect of IL-1β ($F_{1,\ 152}=10.04$; $P<0.05$) and SB216763 ($F_{1,\ 152}=4.30$; $P<0.05$), but no interaction effect of IL-1β × SB216763. *Post hoc* analysis showed that IL-1β reduced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TLX expression in GFAP-positive cells compared with untreated cultures ($P<0.05$) (Figure 5f).

Discussion

The present study demonstrates that GSK-3β has an important role in the IL-1β-induced anti-proliferative and pro-gliogenic effects on hippocampal NSPCs. Specifically, we show that GSK-3β inhibition increases the number of young neurons, and that it abolishes the negative effect of IL-1β on NSPC proliferation and neuronal differentiation *in vitro*. We show that IL-1β decreases TLX expression in both proliferating and differentiated cells, and that GSK-3β inhibition has a differential effect on TLX expression depending on cell type.

To examine the role of GSK-3β in neural cells exposed to IL-1β, we first confirmed that it was expressed in cells under proliferation conditions. Previous studies have demonstrated that the IL-1β cell surface receptor IL-1R1 is evident on neural progenitors in the DG of adult rats,[35] E18 rat hippocampal neurons,[52] NSPCs from the E16 rat forebrain[53] and E18 hippocampal NSPCs,[33] facilitating IL-1β intracellular signaling, and here we show that NSPCs express both IL-1R1 and GSK-3β. Thus, GSK-3β may be susceptible to or indeed involved in the effects of IL-1β treatment. We observed an increase in the percentage of nestin-positive cells expressing GSK-3β and moreover an increase in GSK-3β activity in response to IL-1β. GSK-3β, when part of the destruction complex, causes the phosphorylation and subsequent degradation of β-catenin; conversely, when GSK-3β is phosphorylated on serine 9, the active site is blocked, inhibiting enzyme activity. Hence, decreased levels of β-catenin and/or p-GSK-3β indicate enhanced GSK-3β activity and conversely,

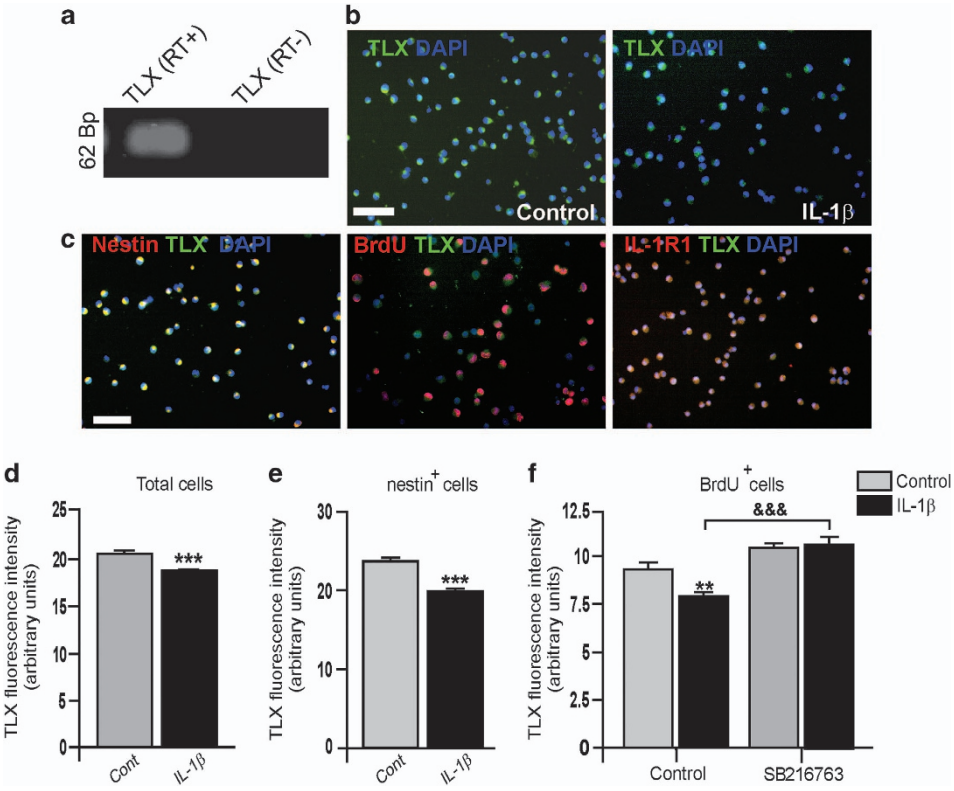

Figure 4 Interleukin-1β (IL-1β)-induced changes in TLX expression in hippocampal neural stem/progenitor cells (NSPCs) are prevented by glycogen synthase kinase-3beta (GSK-3β) inhibition under proliferation conditions. (**a**) Reverse transcriptase PCR (RT-PCR) analysis of TLX expression in NSPCs in untreated cultures after 7 DIV under proliferation conditions. PCR product of 62 bp indicates TLX mRNA expression. (**b** and **c**) Representative photomicrographs of cells immunocytochemically stained for TLX (green) in untreated and IL-1β-treated cultures (**b**), or nestin (red), 5-bromo-2′-deoxyuridine (BrdU) (red) and interleukin-1 type-1 receptor (IL-1R1) (red) with TLX (green) (**c**) in untreated cultures. Cells were counterstained with DAPI (blue) to visualize the nuclei. Scale bar = 50 μm. (**d**, **e** and **f**) Mean densitometry analysis of TLX protein in total NSPCs (**d**), nestin$^+$ cells (**e**) and BrdU$^+$ cells (**f**). (**d** and **e**) NSPCs cultured in the presence or absence of IL-1β, $^{***}P<0.001$ vs untreated control (Student's *t*-test; $n=3$). (**f**) BrdU$^+$ cells in untreated, IL-1β-treated, SB216763-treated and IL-1β + SB216763-treated cultures. $^{**}P<0.01$ vs untreated control; $^{\&\&\&}P<0.001$ vs IL-1β treatment alone (two-way analysis of variance (ANOVA) with Bonferroni *post hoc* test; $n=3$). (**d**-**f**) Data are expressed as means ± s.e.m.

Figure 3 Interleukin-1β (IL-1β)-induced changes in the differentiation of hippocampal neural stem/progenitor cells (NSPCs) are prevented by glycogen synthase kinase-3beta (GSK-3β) inhibition. (**a**, **c** and **f**) Representative photomicrographs of cells immunocytochemically stained for nestin (red, **a**) doublecortin (DCX) (green, **a** and **c**), βIII-tubulin (red, **a** and **f**) and glial fibrillary acidic protein (GFAP) (green, **a** and **f**). Cells were counterstained with DAPI (blue) to visualize the nuclei. Scale bar = 50 μm. Percentage composition of DCX$^+$ neurons (**b**), βIII-tubulin$^+$ neurons (**d**) and GFAP$^+$ astrocytes (**e**) in untreated, IL-1β-treated, LiCl-treated, SB216763-treated, IL-1β$^+$ LiCl-treated and IL-1β$^+$ SB216763-treated cultures after 7 DIV under differentiation conditions. Data are expressed as means ± s.e.m. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs untreated control; $^{\&\&}P<0.01$, $^{\&\&\&}P<0.001$ vs IL-1β treatment alone, ns = not significant, (two-way analysis of variance (ANOVA) with Bonferroni *post hoc* test; $n=3$).

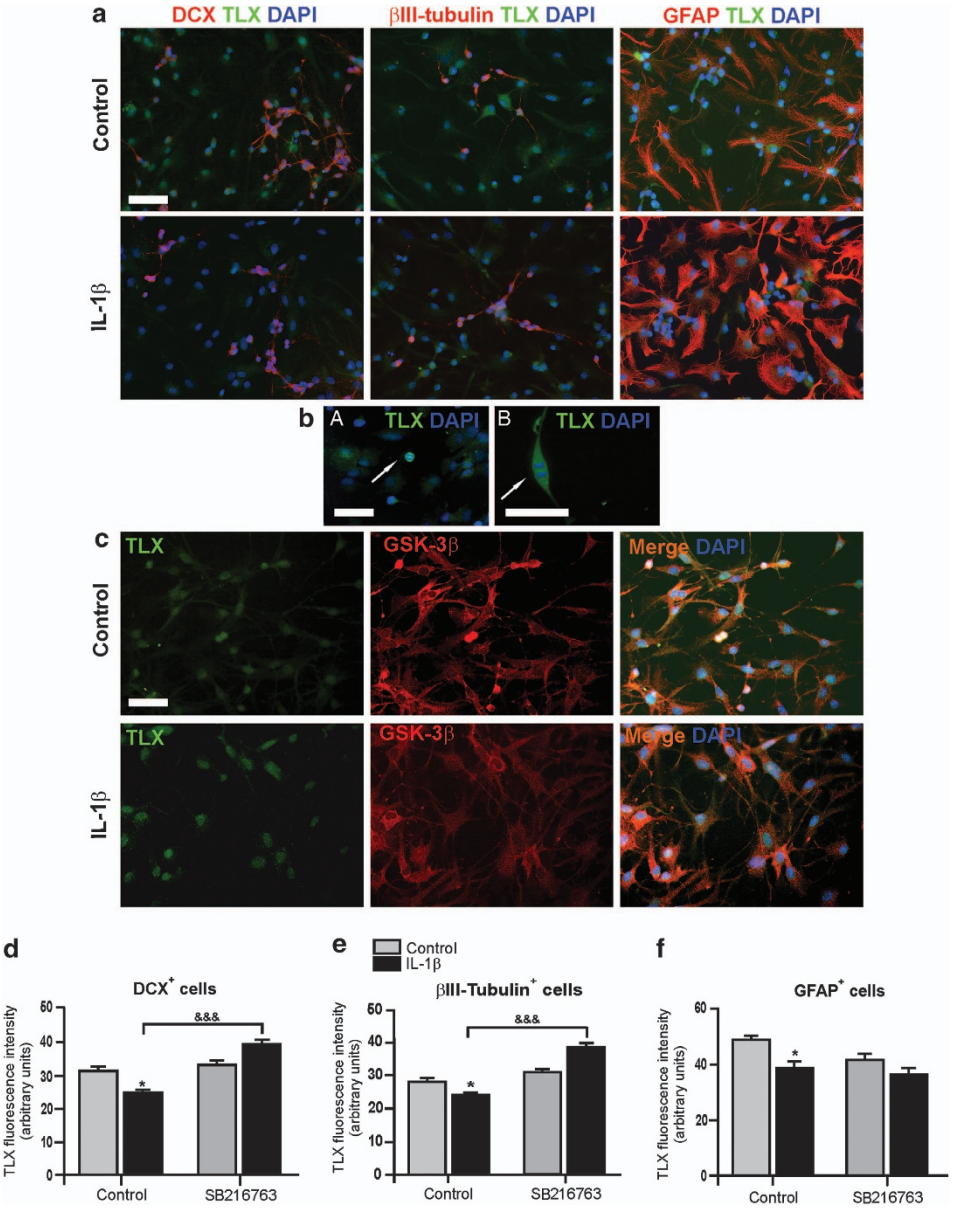

Figure 5 GSK-3β inhibition blocks the effect of IL-1β on TLX expression in differentiated hippocampal neurons. Representative photomicrographs of cells immunocytochemically stained for doublecortin (DCX) (red), βIII-tubulin (red), and glial fibrillary acidic protein (GFAP) (red) with TLX (green) (**a** and **b**) or glycogen synthase kinase-3beta (GSK-3β) (red) with TLX (green) (**c**) in untreated and interleukin-1β (IL-1β)-treated cultures under differentiation conditions for 7 DIV. (**b** A and B) Arrow indicates cell undergoing mitosis. Cells were counterstained with DAPI (blue) to visualize the nuclei. Scale bar = 50 μm. Mean densitometry analysis of TLX protein in DCX$^+$ neurons (**d**), βIII-tubulin$^+$ neurons (**e**) and GFAP$^+$ astrocytes (**f**) in untreated, IL-1β-treated, SB216763-treated and IL-1β + SB216763-treated cultures. Data are expressed as means ± s.e.m. $^*P<0.05$, vs. untreated control; $^{\&\&\&}P<0.001$ vs IL-1β treatment alone (two-way analysis of variance (ANOVA) with Bonferroni *post hoc* test; $n=3$).

inhibition of GSK-3β restores β-catenin levels. In support of a role for β-catenin in neurogenesis, Zechner *et al.*[54] have shown that β-catenin is involved in the maintenance of NSPC proliferation: mice with β-catenin-loss-of-function mutations have smaller nervous systems due to reduced cell proliferation. Thus in our culture system, decreased levels of β-catenin owing to elevated GSK-3β activity may have a role in the observed negative effects of IL-1β on hippocampal neurogenesis. Similarly, a recent study by Garza *et al.*[55] demonstrated that the ability of leptin to ameliorate the negative effects of chronic stress on hippocampal neurogenesis may be mediated via the GSK-3β/β-catenin signaling pathway. In agreement with the results presented here, Hu [59] found that β-amyloid, which promotes the activation of microglia and increases pro-inflammatory cytokine release,[56–58] caused an increase in GSK-3β activity in the rat hippocampus *in vivo*. In contrast, Martin *et al.*[19], showed that lipopolysaccharide, which induces pro-inflammatory cytokine production, increased p-GSK-3β and thus decreased GSK-3β activity in human monocytes, while we have recently reported that

lipopolysaccharide induced an increase in GSK-3β expression and activity in rodent cortical glia.[20] It is thus possible that the cell lineage, location, species or the environment in which the cells reside may account for the differences observed. As overexpression of GSK-3β has been shown to induce apoptosis[60] and inhibit neuronal survival,[61] the increase in GSK-3β activity we observed in NSPCs may affect their proliferation capabilities and/or cell fate specification. We therefore assessed and confirmed that GSK-3β is expressed on proliferating cells under basal conditions and when stimulated with IL-1β. Previous work has shown that IL-1β decreases NSPC proliferation in the DG *in vitro* and *in vivo*.[33,35,62] Furthermore, we have shown that after 4 DIV, IL-1β decreases both the number of cells present in neurosphere cultures and the size of spheres but does not induce apoptosis until 7 DIV.[33] GSK-3β was inhibited using the small-molecule inhibitor SB216763, which abolishes almost all (96%) GSK-3β activity at 10 μM (48), and to a lesser extent and nonspecifically using the mood stabilizer LiCl.[13,14,63] LiCl partially abolished the negative effect of IL-1β on proliferating cells, whereas small molecule inhibition of GSK-3β reversed the IL-1β-induced decrease in the percentage of BrdU-positive cells at 4 DIV, indicating that GSK-3β may have a role in the anti-proliferative effect of IL-1β. LiCl is a less potent inhibitor of GSK-3β and may require a longer treatment time to abolish the anti-proliferative effect of IL-1β. Thus, GSK-3β activity may be a promising target for further *in vivo* experiments exploring the restoration of reduced NSPC proliferation associated with neuroinflammation.

GSK-3β has been shown to be ubiquitously expressed in the adult mouse hippocampus,[64] and the results generated here demonstrate that young neuronal and astroglial cells from embryonic hippocampal neurosphere cultures retain their GSK-3β expression under differentiation conditions. Furthermore, GSK-3β activity in NSPCs under differentiating conditions is upregulated after IL-1β treatment. As previous studies have shown that IL-1β exerts an anti-neurogenic effect on the differentiation of embryonic hippocampal NSPCs,[33,35,51] we examined if GSK-3β activity was involved in this process. We firstly assessed cell viability in our culture system, and while previous studies have demonstrated apoptosis in astrocytes and neurons as a result of GSK-3β overexpression,[60,61] and that ablation of the *GSK-3β* gene is embryonic lethal in rats,[65] we did not observe a change in the total number of cells after 7 DIV under differentiation conditions. This suggests that elevated levels of GSK-3β did not affect cell viability in our culture system. It is also possible, however, that cells undergoing mitosis could be masking cell death. Our data demonstrating that SB216763 treatment increased the percentage of βIII-tubulin-positive neuronal cells is in agreement with the previous findings from rat subventricular neurospheres.[66] This effect is not apparent in DCX-positive cells, however, suggesting that the differentiation stage of the cells may influence their susceptibility to GSK-3β inhibitors. It has been shown that LiCl, at a similar dose used in the present series of experiments increased the number of neurons in cultures of adult hippocampal NSPCs,[11] suggesting a difference in behavior of adult and embryonic NSPCs. Thus, there are differing therapeutic implications of this result for embryonic/maternal inflammation and adult neuroinflammation. LiCl is a less potent inhibitor of GSK-3β than SB216763, which may also account for the absence of an effect of LiCl on the percentage of βIII-tubulin-positive cells, or indeed a longer treatment schedule may be necessary to observe a LiCl-induced change. Both GSK-3β inhibitors prevented the IL-1β-induced decrease in neuronal cell fate specification, however, advocating the notion that targeting GSK-3β activity may be beneficial for promoting neuronal differentiation in the presence of inflammatory mediators such as IL-1β. Given that SB216763 treatment alone increased the percentage of βIII-tubulin-positive neuronal cells, it is possible that the observed change in the percentage of βIII-tubulin-positive cells as a result of co-treatment with IL-1β and SB216763 may be an additive effect of the treatments. Notwithstanding, inhibiting GSK-3β in the presence of IL-1β can restore the IL-1β-induced decrease in the percentage of βIII-tubulin-positive cells to that of control levels. These results also suggest that LiCl, even at concentrations too low to exert an effect on NSPC cell fate specification, exerts a neuroprotective effect in the presence IL-1β *in vitro*. We further explored if GSK-3β is involved in the pro-gliogenic effect of IL-1β that we and others have previously demonstrated in NSPCs.[33,67,68] Unlike a previous report showing a decrease in the percentage of GFAP-positive cells differentiated from embryonic rat hippocampus after LiCl treatment,[12] we observed that GSK-3β inhibition had no effect on astrocyte cell fate specification. However, both inhibitors reversed the IL-1β-induced increase in the percentage of GFAP-positive cells under differentiation conditions. We have previously suggested that IL-1β alters the cell fate specification of the NSPCs in our culture system from a neuronal to a glial fate rather than increasing astrocytic mitosis,[33] and here we suggest that GSK-3β may be involved in mediating this effect. It is well known that IL-1β signaling results in NFκB-mediated gene transcription, and it is now also known that within the nucleus, GSK-3β activity is necessary for NF-κB target gene transcription, as it is involved in NF-κB (p65)–DNA binding.[69] Thus it may be here that the cross talk between GSK-3β and IL-1β signaling occurs. However, the spatial and temporal aspect of this interaction requires further research.

As Wnt signaling has been identified as a mediator of TLX-regulation of embryonic and adult neurogenesis,[42] we hypothesized that TLX may have a role to play in the GSK-3β-mediated changes in hippocampal neurogenesis upon exposure to IL-1β. We show that GSK-3β inhibition blocked an IL-1β-induced decrease in TLX expression thus these results allude to the possibility that the anti-proliferative effect of IL-1β may be mediated in part, by a decrease in TLX expression. As GSK-3β inhibition blocked the anti-proliferative effect of IL-1β and this was accompanied by restored TLX expression levels, our results further suggest that the ability of GSK-3β inhibition to abolish the negative impact of IL-1β on embryonic cell proliferation may be because of the restored TLX expression levels. In support of this theory, reduced TLX expression via siRNA-mediated knockdown in adult hippocampal NSPCs,[42] or via microRNA let-7b alterations in the adult forebrain NSPCs,[70] has been shown to reduce cell proliferation. Constitutive β-catenin production has also been shown to rescue a *Tlx* siRNA-induced reduction in cell proliferation of adult NSPCs *in vitro*, and active β-catenin was shown to

increase the number of proliferating cells in the subventricular zone of *Tlx*$^{-/-}$ mutant mice.[42] Thus, it is possible that β-catenin may have a role in the IL-1β-induced decrease in TLX expression in NSPCs, as we have shown that IL-1β reduces both β-catenin and TLX protein levels in cultures under proliferation and differentiation conditions, but this hypothesis requires further investigation. What is also interesting from a behavioral perspective is that the deficits in activity, learning and NSPC proliferation evident in TLX mutant mice have recently been shown to be resistant to chronic lithium treatment.[71] In our study, we demonstrated that TLX also co-localizes with GSK-3β on hippocampal NSPCs under differentiation conditions, and that GSK-3β inhibition increased TLX expression in young neurons. Indeed cells undergoing mitosis appear to have higher levels of TLX than their nondividing counterparts, suggesting that TLX may be upregulated during division of cells even under differentiation conditions. GSK-3β inhibition also ameliorated the effect of IL-1β on TLX expression in both newly-born and young neurons, indicating that the IL-1β-induced decline in differentiation toward a neuronal fate, may involve decreased TLX expression. In line with a proposed role of TLX in neurogenesis, it has recently been reported that silencing of TLX in adult rat hippocampal NSPCs reduces the percentages of neurons present, whereas TLX overexpression resulted in increased numbers of neurons.[39] Our cell fate specification experiments also show that GSK-3β inhibition blocked the pro-gliogenic effect of IL-1β. Zhao *et al.*,[70] have recently shown that a reduction in TLX expression induced an increase in glial differentiation in adult mice NSPCs. However, when we examined TLX expression in embryonic rat NSPCs, GSK-3β inhibition did not restore TLX expression levels in astrocytes, suggesting that the pro-gliogenic effect of IL-1β is independent of TLX.

Our findings suggest that GSK-3β has a role in IL-1β-induced impairment of hippocampal neurogenesis *in vitro* and thus is an important target for further investigation as a potential therapeutic target for restoration of neurogenesis in hippocampal-based disorders involving neuroinflammation. Specifically, we propose that our studies on embryonic NSPCs may have implications in elucidating the molecular mechanisms involved in both maternal infection-induced and adult neuroinflammation-induced deficits in hippocampal neurogenesis. While the limitations of experimentation using cultures of embryonic rodent cells must be taken into account, it is reasonable to suggest that the neuroprotective effect of LiCl in the presence of IL-1β may contribute to its clinical efficacy and hence warrants further research using adult rodent cells, animal models or human embryonic cells. Owing to the pleiotropic nature of GSK-3β, however, therapies that target GSK-3β will have to take into account their potential effects on interactions with other signaling pathways. For example, our data show that the orphan nuclear receptor TLX is susceptible to GSK-3β inhibition, as well as to IL-1β exposure *in vitro* and thus point to a potential role for TLX in hippocampal-based disorders involving neuroinflammation and Wnt signaling.

Conflict of interest

The authors declare no conflict of interest.

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