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Glycogen Synthase Kinase 3 Inhibition Promotes Adult Hippocampal Neurogenesis in Vitro and in Vivo

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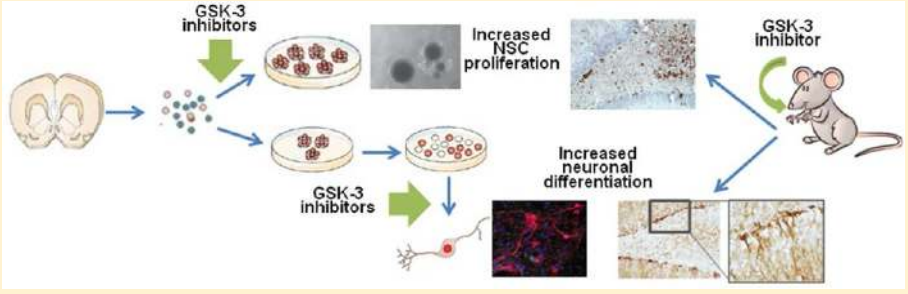
Supporting Information



ABSTRACT: Glycogen synthase kinase-3 (GSK-3) is a serine/threonine kinase originally identified as a regulator of glycogen metabolism but it also plays a pivotal role in numerous cellular functions, including differentiation, cell cycle regulation, and proliferation. The dentate gyrus of the hippocampus, together with the subventricular zone of the lateral ventricles, is one of the regions in which neurogenesis takes place in the adult brain. Here, using a chemical genetic approach that involves the use of several diverse inhibitors of GSK-3 as pharmacological tools, we show that inhibition of GSK-3 induces proliferation, migration, and differentiation of neural stem cells toward a neuronal phenotype in in vitro studies. Also, we demonstrate that inhibition of GSK-3 with the small molecule NP03112, called tideglusib, induces neurogenesis in the dentate gyrus of the hippocampus of adult rats. Taken together, our results suggest that GSK-3 should be considered as a new target molecule for modulating the production and integration of new neurons in the hippocampus as a treatment for neurodegenerative diseases or brain injury and, consequently, its inhibitors may represent new potential therapeutic drugs in neuroregenerative medicine.

KEYWORDS: *Adult brain, GSK-3, differentiation, hippocampus, neural stem cells, neurogenesis*

In the central nervous system, developing neurons are derived from quiescent multipotent or neural stem cells.[1] Throughout life, neural progenitors in the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus (DG) of the hippocampus give rise to interneurons of the olfactory bulb and neurons of the granule cell layer of the DG respectively.[2] The hippocampus is a unique structure in that it is one of the two brain regions where adult neurogenesis persists throughout adulthood. New neurons are continuously generated in the subgranular zone (SGZ) of the dentate gyrus (DG) of the hippocampus, migrate into the granule layer, and differentiate into new dentate granule cell neurons.[1,3] It has recently been demonstrated that these new generated neurons subsequently integrate into memory networks,[4] become functionally mature within 2 months,[5,6] and contribute to hippocampal-dependent learning and memory functions.[7−9]

As the maintaining and/or restoration of cognitive health and memory is the ultimate goal in the treatment of different cognitive disorders such as Alzheimer's disease (AD), an effective therapeutic strategy may be to take advantage of this regenerative ability of the brain and look for new drugs able to shift the balance from neurodegeneration to neurogenesis and neuronal plasticity. Recently, treatment with ciliary neurotrophic factor-based tetrapeptide has shown beneficial effects on adult hippocampal neurogenesis and spatial memory in mice, demonstrating the therapeutic potential of the above-mentioned hypothesis and, specifically of this peptide, for regeneration of the brain and improvement of cognition.[10,11]

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There is also evidence linking adult hippocampal neurogenesis to some psychiatric disorders including depression and schizophrenia.[12,13] Additionally, hippocampal neurogenesis has been shown to play a role after different brain injuries, such as stroke, ischemia, hypoxic injury, or excitotoxic damage[14−17] and also in some neurodegenerative diseases such Parkinson's disease.[18,19]

Glycogen synthase kinase 3 (GSK-3) is a serine/threonine kinase originally identified as a regulator of glycogen metabolism but also plays a pivotal role in numerous cellular functions including microtubule dynamics and regulation of cell proliferation, differentiation, and cell death.[20−22] There are two isoforms of this enzyme, GSK-3α and GSK-3β, and one splice variant GSK-3β2. All these forms are expressed in nervous cells in a constitutively active form. However, their activity is regulated negatively and positively by phosphorylation at serine and tyrosine residues.[20] There are some recent reports suggesting that lithium, an inhibitor of GSK-3, could regulate neurogenesis in the dentate gyrus of adult rats.[23] Moreover, it has been also shown that modulation of GSK-3β-catenin signaling pathways regulate neuronal progenitor proliferation and behavioral defects in a well established model of schizophrenia,[24] providing new insights into the mechanisms by which alterations in GSK-3-catenin signaling affect adult murine neurogenesis, a cellular process thought to play an important role in the etiology of neuropsychiatric disorders. Also conditional deletion of GSK-3 in mouse neural progenitors increases proliferation,[25] and GSK3 inhibition increases neurogenesis in the human neural progenitor cell line ReNcell VM.[26]

Considering the important well-known activity of GSK-3 in neuroprotection and its role in regulating neuronal differentiation, the main objective of the present study was to determine whether pharmacological inhibition of the GSK-3 pathway is capable of inducing neurogenesis in the adult rat brain.

During the last years, much effort has been focused on discovering new and potent inhibitors of GSK-3. Our group has great experience in design and development of chemically diverse GSK-3 inhibitors[27,28] and with many different binding modes to this important kinase.[29−31] We have here used a chemical genetic approach to show that GSK-3 inhibition is involved in neurogenesis and that its inhibitors should be a therapeutic effective way to recover the damaged brain in some important pathologies such as Alzheimer's disease where the hippocampal neurogenesis is affected.

Usually, the study of the role of a specific protein in molecular physiology and/or pathology is done following a classical genetic approach trough mutation of the corresponding gene. Complementarily, the chemical genetic approach exploits the use of small molecules as pharmacological tools to not only show the biological role of a specific protein, but also allow defining some new drug leads for further pharmaceutical development.[32] In this study, we have selected various structurally different GSK-3 inhibitors. Among them are thiadiazolidinediones (TDZDs), compounds first described as non-ATP competitive GSK-3β inhibitors[33] and later as irreversible inhibitors[29,34] and different heterocyclic compounds with thiazole or maleimide scaffolds, recently reported as reversible and irreversible inhibitors of GSK-3β.[35] In all the cases, the molecular weights of the different small probes are below 500 Da and the IC_{50} value regarding GSK-3 is between 0.05 and 10 μM (Figure 1). In the in vitro studies, the concentration used for all GSK3 inhibitors was 10 μM. Here we demonstrate that different chemically and biologically diverse GSK-3 inhibitors increased the number, migration, and differentiation capacity of neural stem cells forming neurospheres. Moreover, NP03112, an inhibitor of GSK-3 belonging to the TDZDs chemical family and now called tideglusib, is an effective inducer of proliferation and differentiation in the SGZ of adult rats. Altogether, these findings suggest that inhibition of GSK-3 may regulate neural progenitor cell proliferation, migration, and differentiation in the adult hippocampus. Thus, our work provides empirical support for the potential relevance of GSK-3 inhibitors in the development of therapeutic approaches to treat different brain disorders and may offer great potential for the recovery of damaged brain in different cognitive disorders, such as Alzheimer's disease.

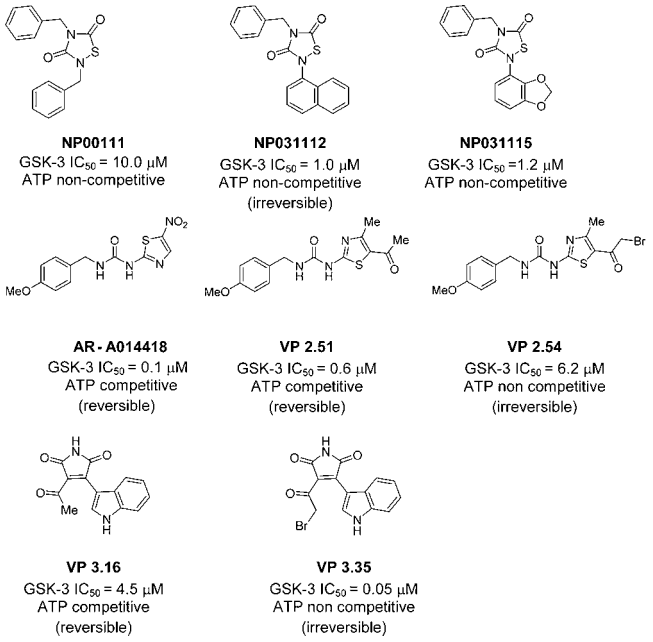

Figure 1. Chemical structures, IC_{50} values regarding GSK-3β inhibition, and binding mode of the small heterocycles GSK-3 inhibitors used as molecular probes.

RESULTS

GSK-3 Inhibition Regulates Proliferation and Growth of Neurospheres in Vitro. One of the characteristics of neural stem cells is their ability of growing in vitro as spheres.[36] Therefore, we first performed in vitro studies using primary and secondary neurosphere (NS) cultures. Primary NS were obtained from adult rats and maintained and expanded as commented in the Methods. First, we determined whether neural stem cells expressed GSK-3 when isolated and cultured in vitro (Figure 2A). Western blot analysis of NS established from hippocampus of adult rats confirmed that these cells indeed express GSK-3, being then a druggable for target in neurogenesis signaling. Next, we investigated whether addition of different small molecule GSK-3 inhibitors would increase their formation and/or their size. As shown in Figure 2B/C, the number and size of primary NS were significantly higher in those cultures treated with the different GSK-3 inhibitors, compared to controls. Also, cultures grown in the presence of the inhibitors exhibited an enhanced self-renewal capacity, as suggested by the increased number in the secondary neurospheres formed in these cultures. These results demonstrate

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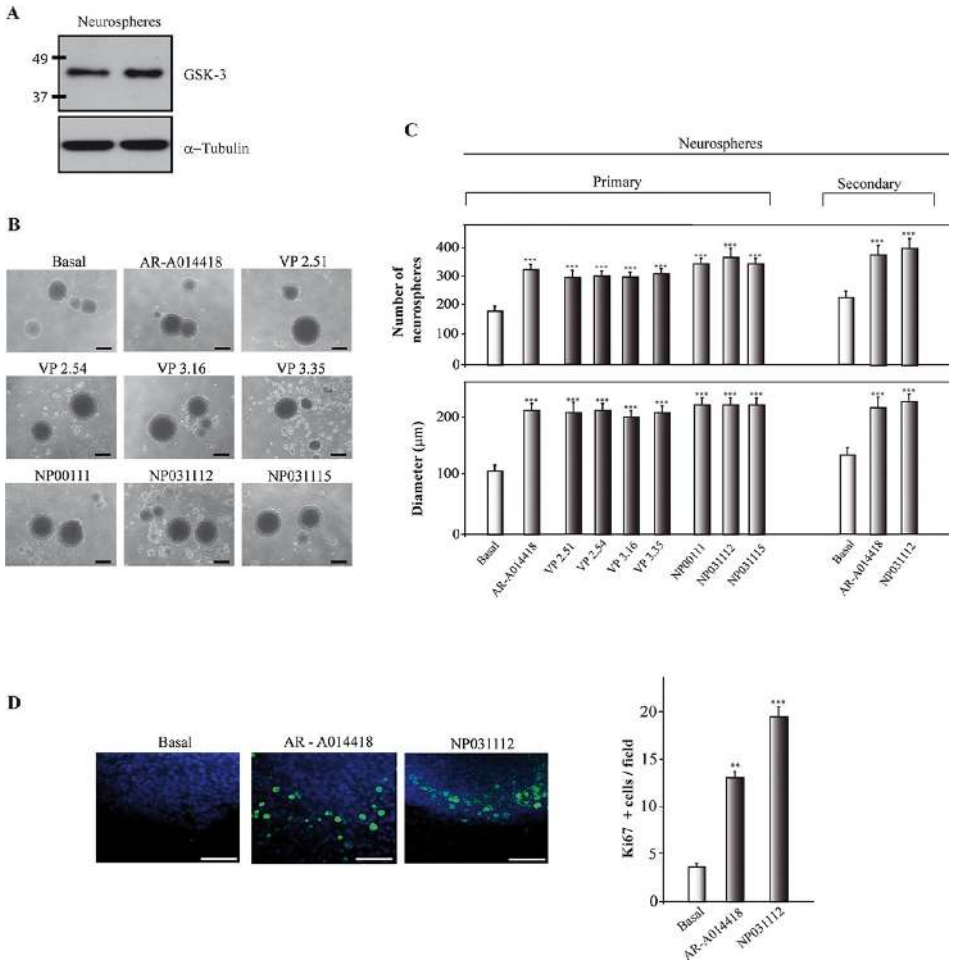

Figure 2. Effects of GSK-3 inhibitors on neurosphere formation. (A) Representative Western blot showing expression of GSK-3 in neurospheres derived from hippocampus. (**B**) Representative phase-contrast micrographs showing the size of primary neurospheres after 7 days in culture in the presence or absence of the different inhibitors. Scale bar = 50 μm. (C) Quantification analysis of the primary and secondary neurosphere number. The number of neurospheres was counted and their diameter measured. The diameter of at least 50 neurospheres and the total number of neurospheres was determined in control and GSK-3 inhibitor-treated cultures. Results are mean values ± SD from three independent experiments. $^{**}P \leq 0.01$; $^{***}p \leq 0.001$. (D) Representative confocal images of Ki67 immunoreactivity (green) in primary neurospheres. DAPI staining (blue) was used as a nuclear marker. Quantification of Ki67-positive cells is shown. Results are mean values ± SD from three independent experiments performed in triplicate. $^{**}P \leq 0.01$; $^{***}P \leq 0.001$. Scale bar = 50 μm.

that reversible and irreversible inactivation of GSK-3 enhances the number as well as the size of neurospheres generated from hippocampus, suggesting that these inhibitors regulate the proliferation of the neurosphere forming progenitors and confirming their therapeutic potential. Hence, proliferation was assessed by Ki67 (a marker of dividing cells) staining, 7 days after treatment with the different compounds. As expected, treatment of the cultures with GSK-3β inhibitors resulted in an increase in the number of Ki67-positive cells, indicating a direct effect of these compounds on proliferation (Figure 2D).

GSK-3 Inhibition Enhances Migration out of the Neurospheres. In order to analyze whether GSK-3 inhibitors altered the cell migration pattern from neurospheres, selected diverse GSK-3 inhibitors were added to the culture medium during 24 h and the new cell migration was monitored by live-scanning microscopy. The results shown in Figure 3 (and in Supporting Information, videos 1−4) show that incubation of the NS cultures with these compounds resulted in a significant increase in migration. The neural stem cells moved long distances out of the neurosphere body to create overlapping zones of migration between adjacent NS. On the contrary, cells in control cultures remained close to the neurosphere body.

GSK-3 Inhibition Induces Differentiation of Neural Stem Cells. Next, we analyzed whether GSK-3 inhibition could regulate cell differentiation after adhesion of neurospheres. To this end, we performed immunocytochemistry analysis using specific antibodies to identify the different nervous system cell types. Neurospheres were allowed to adhere to the substrate and then incubated for 24 h in the absence of EGF and FGF and in the presence or absence of the different GSK-3 inhibitors. As shown in Figure 4, in control cultures, only scattered cells stained with GFAP (to identify astrocytes) or MAP-2 (to identify neurons) were observed. However, the number of MAP-2-positive cells was significantly increased in those cultures treated with the GSK-3 inhibitors. Almost no differentiation toward a glial phenotype was detected. These results suggest that GSK-3 inhibition results in an induction of neuronal differentiation of neural stem cells toward mature neurons.

The GSK-3 Inhibitor NP031112 (Tideglusib) Regulates Adult Neurogenesis in Vivo. We next investigated whether

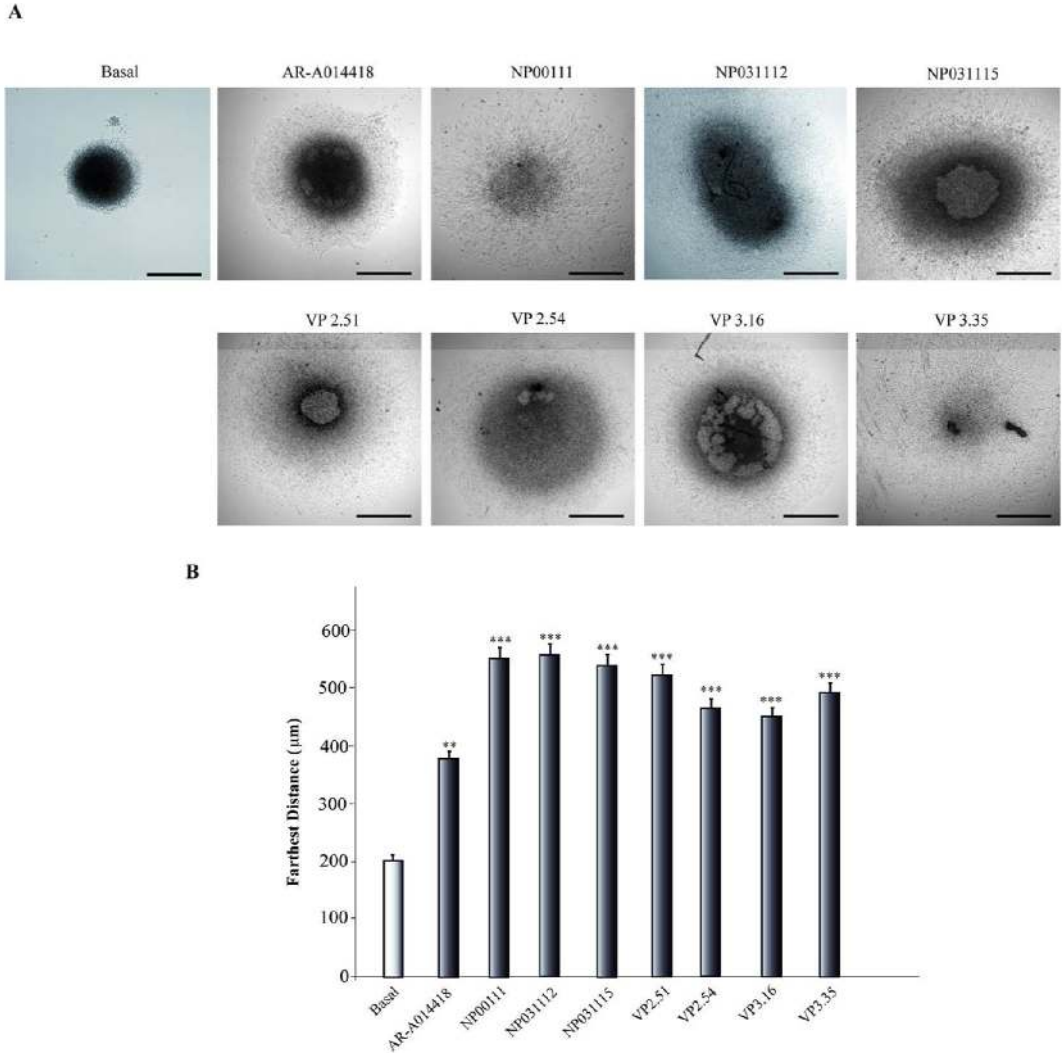

Figure 3. Effects of GSK-3β inhibitors on cell migration out of the neurospheres. (A) Single neurospheres were plated on polylysine-coated coated culture dishes in the presence or absence of the inhibitors and the cell migration out of the sphere was monitored 24 h later. Representative photomicrographs are shown. Scale bars = 50 μm. (B) Quantitative data of the furthest distance of cell migration. $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

NP031112, called tideglusib, affected cell proliferation in the DG of the hippocampus. Adult rats were orally treated with this compound for 7 or 14 days. To label proliferating cells, animals were injected with BrdU 24 h before being sacrificed (Figure 5). We observed a significant increase in the number of BrdU-positive cells in the DG of NP031112-treated animals. Interestingly, this increase was present not only in the SGZ of the DG but also in the hilus. Quantification of the results indicated that NP031112 treatment increased BrdU-labeled cell number above control values, 7 and 14 days after the last injection. BrdU-labeled cells in the hilus of the hippocampus have also been found by other authors and in different paradigms[37−39] playing a critical role in network excitability.[40]

Doublecortin (DCX) is a microtubule-associated protein expressed by neuroblasts and is considered to be a reliable marker of neurogenesis.[41,42] This protein is highly expressed in both cell body and dendrites of the newly generated neuroblasts. To test the hypothesis that inhibition of GSK-3 by NP031112 may play a role in adult neurogenesis, adult rats received daily intragastrical administration of this compound, sacrificed at the indicated times, and brain sections were stained for DCX. As shown in Figure 6A, there is a considerable increase in DCX staining in those animals treated with NP031112. Besides, these cells exhibited extensive dendritic arborizations.

To further substantiate these findings, we also studied the effect of NP031112 on the expression of polysialylated neural cell adhesion molecule (PSA-NCAM), a molecule that is involved in migration and process elongation of developing neurons. The presence of PSA-NCAM in adult brain is highly correlated with neuronal precursor migration and differentiation.[43,44] Almost no PSA-NCAM-positive cells were observed in control animals (Figure 6B). However, administration of NP031112 increased the number of the migrating neuroblast subpopulation stained with this marker.

■ DISCUSSION

A therapeutic strategy for neurodegenerative disorders such as Alzheimer's disease is to consider the regenerative ability of the adult brain to shift the balance from neurodegeneration to neurogenesis and neuronal plasticity. GSK-3 has emerged as an important therapeutic target in this area.[45] However, there is almost no information regarding the effect of GSK-3 inhibition on neurogenesis in the adult brain. In the present study, we

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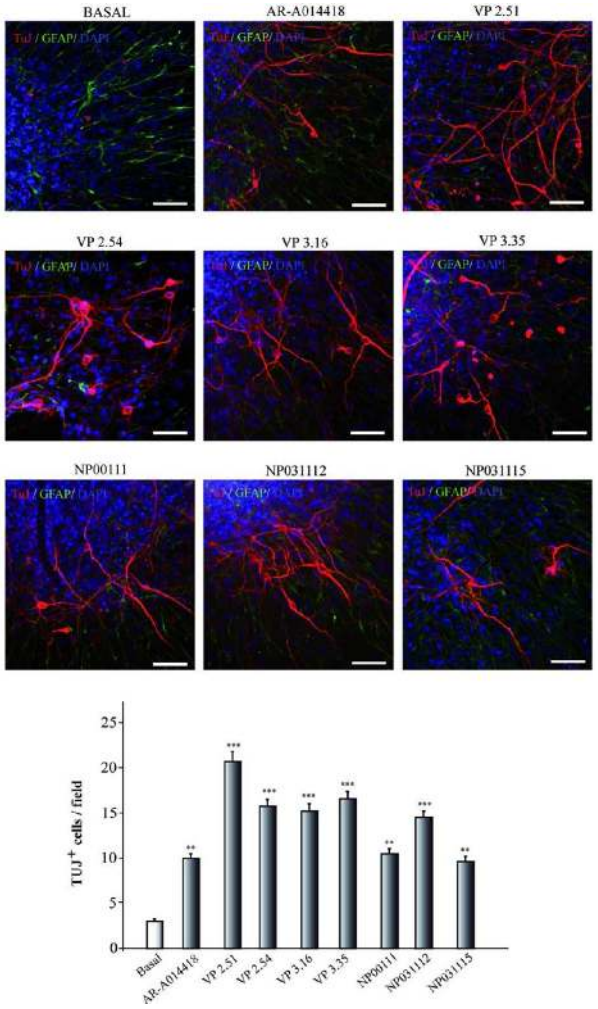

Figure 4. Effects of GSK-3 inhibitors on neural stem cell differentiation. The neurospheres were grown for 7 days in the presence or absence of GSK-3 inhibitors and then adhered for 2 days to allow differentiation. Neuronal cells were detected using an anti-β-tubulin antibody (TuJ clone, red) and astrocytes using an anti-GFAP (green) antibody. DAPI marker (blue) was used for nuclear staining. Scale bar = 30 μm.

have used chemically and biologically diverse, previously known and novel, GSK-3 inhibitors to demonstrate that inactivation of this enzyme leads to an enhancement of proliferation, migration, and differentiation of neural stem cells in primary neurosphere cultures in vitro. We also show that one of these compounds, the TDZD named NP03112 (tideglusib), induces cell proliferation and differentiation of neural stem cells in the adult dentate gyrus of the hippocampus in vivo. These studies reveal an important role for GSK-3 in neuroreparation and may represent a potential therapeutic strategy for stem cell activation through the use of small molecules that inhibit this enzyme. As tideglusib is currently in clinical trials for AD, these results may shed some light and new ideas in the future patient results analysis.

During development, the Wnt signaling pathway influences neural stem cell proliferation and differentiation. More recently, diverse studies have also identified the Wnt signaling pathway as a key regulator of adult hippocampal neurogenesis.[46,47] The autocrine signaling of Wnt supports the proliferation and multipotency of neural stem cells through the canonical pathway involving GSK3/β-catenin.[47] Here we show that specifically targeting GSK-3 by using different inhibitors results in an enhancement of proliferation and differentiation toward a neuronal phenotype, both in vitro, in neural stem cell cultures, and in vivo, in the adult hippocampal DG.

Our results show that neural progenitor cell cultures treated with GSK-3 inhibitors presented an increase in the number of primary and secondary neurospheres formed. The higher number of secondary neurospheres formed in GSK-3-treated cultures indicates that the neural stem cells generated in the presence of these compounds underwent a higher proportion of self-renewing, leading to the expansion of the stem cell pool. This lends support to the view that GSK-3 inactivation might stimulate the capability to maintain stemness. These results are in agreement with those of Nedachi et al. showing that progranulin, an estrogen-inducible growth factor, enhances neural progenitor cell proliferation, apparently by a mechanism involving indirect inactivation of GSK-3.[48] Here, we also show that GSK-3 is involved in the control of neural stem cell differentiation in vitro. Our results indicate that inhibition of GSK-3 causes neural stem cells to differentiate mainly into neurons. Finally, our results clearly demonstrate that the different inhibitors of GSK-3 used induces migration of cells out of the neurospheres. These results are especially relevant in a clinical setting, since the identification of factors that not only promote neural stem cell proliferation and differentiation but also have a significant effect on their migration capacity might have an important regulatory role in hippocampal migratory events in a brain injury context.

The SGZ of the adult DG represents one of the two major neurogenic niches in the adult brain and contains a population of radial glia-like cells which express nestin and GFAP, exhibit proliferative activity, and are capable of self-renewal.[49] These cells have been demonstrated to act as primary precursors of the granule cell lineage.[49] They give rise to more rapidly dividing progenitors, which express markers of immature neurons (PSA-NCAM and doublecortin) and then exit the cell cycle to differentiate into granule cells. The data presented here show that, in adult animals, the GSK-3 inhibitor NP031112 increases proliferation, since BrdU incorporation was significantly enhanced. We also present evidence that GSK-3 inactivation increases the number of newly generated neurons in the DG of the hippocampus, as suggested by the enhancement in the number of DCX- and PSA-NCAM-positive cells. Thus, our results suggest that inhibition of GSK-3 can be an important regulator for the neural stem cells to become neurons in the hippocampus of the rodent adult brain, thereby identifying a new function for this enzyme in this area of the brain. Moreover, the therapeutic potential of GSK-3 inhibitors, specifically NP031112 (tideglusib), to increase endogenous hippocampal neurogenesis shown here may represent a new promising line of treatment of brain disorders which involve neurogenesis deficits.

The role of neurogenesis in the hippocampus is not completely clear, but it has been suggested to be involved in memory formation.[7,9] Poor performance in hippocampal-dependent memory tasks has been found in mice where adult neurogenesis had been depleted.[50] It has been also shown that enhanced adult hippocampal neurogenesis is required for antidepressant drug efficacy.[51,52] Finally, an age-related decrease in neurogenesis has been postulated to be linked to age-related memory deficits, including neurodegenerative diseases.[53] Interestingly, recent evidence suggests that, in

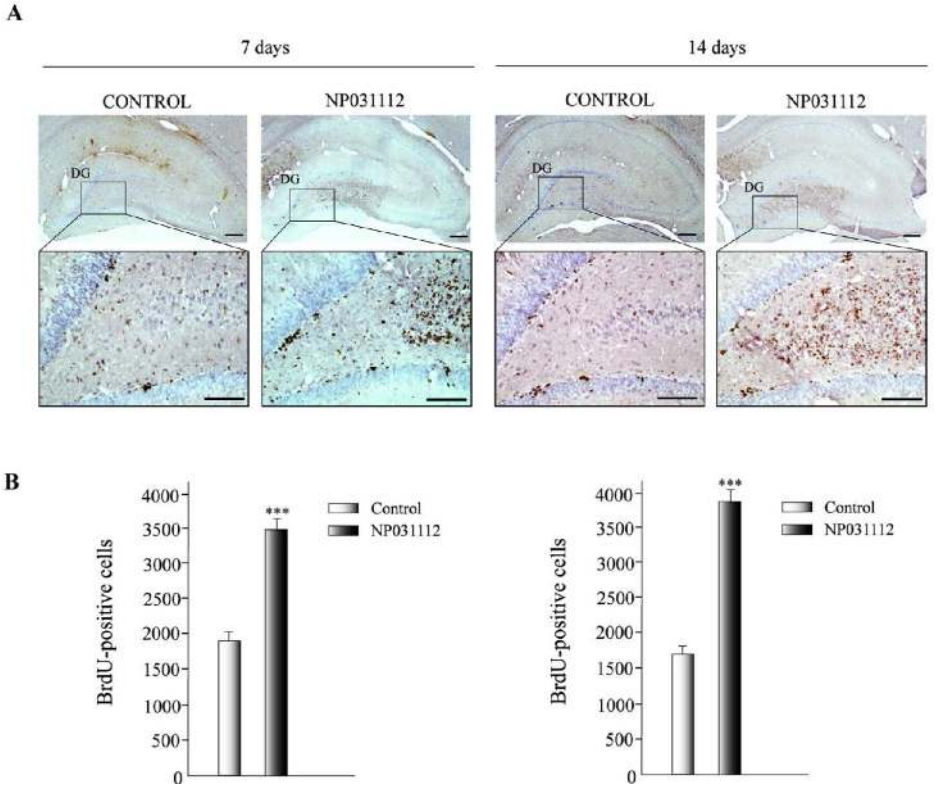

Figure 5. Effects of GSK-3 inhibitors on proliferating cells in the hippocampus. (A) Representative coronal sections showing BrdU-labeled cells in the hippocampus. Scale bar = 100 μm. Insets show higher magnifications of representative areas in the dentate gyrus. Scale bar = 50 um. (B) Quantification of BrdU positive cells in the hippocampus. Values are the mean ± SD from five different animals. ***$P \leq 0.05$.

patients and animal models of Alzheimer's disease, there might be alterations in hippocampal neurogenesis that contribute to cognitive impairment (review in ref 54). Therefore, the targeting of endogenous hippocampal NSCs by GSK-3 inhibitors might be a promising line of treatment for different brain disorders in the future.

Thus, our data provide evidence for a general role of GSK-3 on hippocampal neurogenesis and indicate that GSK-3β inhibitors might be particularly well suited for potential novel therapies in the treatment of neuropsychiatric disorders and neurodegenerative diseases, particularly Alzheimer's disease, where neurogenesis is compromised.

METHODS

Animals. Adult (8−12 weeks old) male Wistar rats (n = 6 per group), housed in a 12 h light−dark cycle animal facility, were used in this study. All procedures with animals were specifically approved by the "Ethics Committee for Animal Experimentation" of the Consejo Superior de Investigaciones Científicas and carried out in accordance with the protocols issued which followed National (normative 1201/2005) and International recommendations (Directive 2010/63 from the European Communities Council). Special care was taken to minimize animal suffering.

GSK-3 Inhibitors. The commercial ATP competitive and reversible GSK-3β inhibitor, named AR-A01441, was purchased from Calbiochem. Thiadiazolidindiones (TDZDs) NP00111,[55] NP031112,[34] and NP031115[56] were kindly supplied by Noscira S.A. Tres Cantos, Spain (Martinez et al., WO2006/084934). The TDZDs were first described as non-ATP competitive GSK-3 inhibitors [33] and later as irreversible inhibitors.[29,34] Thiazoles, VP 2.51 and VP 2.54,[57] and maleimides, VP 3.16 and VP 3.35,[31] were synthesized in the Instituto de Quimica Medica-CSIC following described procedures (Martinez et al., PCTES2012070119). They are reversible and irreversible inhibitors of GSK-3.[35] All these GSK-3 inhibitors were used at a concentration of 10 μM, since this concentration has been shown to be effective in a dose−response curve (Supporting Information Figure 1).

Neurosphere Cultures. NS cultures were derived from the hippocampus of adult rats and induced to proliferate using established passaging methods to achieve optimal cellular expansion according to published protocols.[58,59] Rats were decapitated, brains were removed, and the hippocampus was dissected as described.[57] Briefly, cells were seeded into 12-well dishes and cultured in Dubecco's modified Eagle's medium (DMEM)/F12 (1:1, Invitrogen) containing 10 ng/mL epidermal growth factor (EGF, Peprotech, London, U.K.), 10 ng/mL fibroblast growth factor (FGF, Peprotech), and B27 medium (Gibco). After 3 days in culture, some primary NS cultures were treated with different GSK-3 inhibitors. Four days later, primary neurospheres were dissociated and replated in normal proliferative conditions for another 7−9 days to score the number of secondary neurospheres generated. Six to eight wells per condition tested were counted.

In order to determine the ability of GSK-3 to induce differentiation, NS from 10 day old cultures were plated for 72 h onto 100 μg/mL poly-L-lysine-coated coverslips in the absence of exogenous growth factors. Then, cells were processed for immunocytochemistry for glial fibrillary acidic protein (GFAP), to identify astrocytes and β-tubulin to identify neurons.

For measurements of growth and proliferation, primary and secondary NS were counted and their size was analyzed using the Nikon Digital Sight, SD-L1 software (Nikon, Japan). Proliferation assays were carried out by culturing whole primary NS, in the presence or absence of the indicated compounds, during 10 days. Afterward, they were plated onto poly L-lysine coated coverslips for 24 h. Cells were then fixed in cold methanol, stained with anti-Ki67 antibody (Novo Castra), and processed for immunocytochemistry.

Assay of Cell Migration. Single spheres were plated onto poly-L-lysine μ-Slide 8-well plates (Ibidi, Martinsried, Germany), and

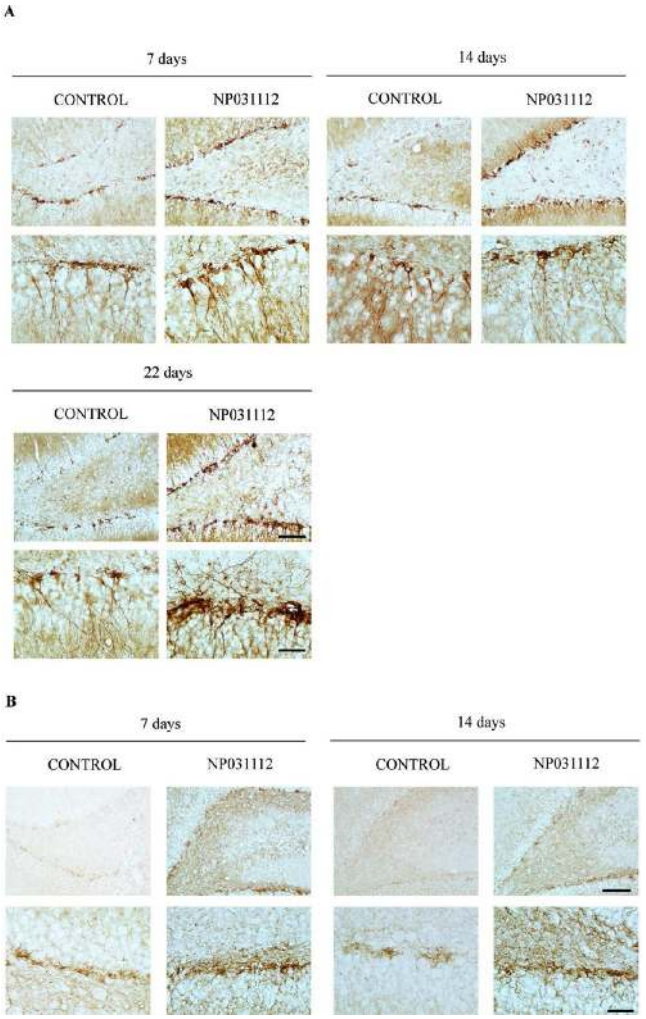

Figure 6. Effects of GSK-3 inhibitors on neuronal differentiation of hippocampal neural stem cells. Representative coronal sections of the hippocampus showing the expression of DCX (A) and PSA-NCAM (B) in the subgranular zone of the dentate gyrus (DG). Scale bar = 100 μm. Insets show higher magnification of the DG. Scale bar = 50 μm.

migration was monitored for 24 h. Images were acquired with a Cell Observer system from Zeiss (Jena, Germany) using a Zeiss Observer Z1 microscope, equipped with a Cascade 1K camera, a monitorized X/Y stage, and a Module S incubator with equipment for temperature and CO_2 control. During all experiment,s cells were kept in a humidified atmosphere of 5% CO_2 in air at 37 °C. Axio Vision Rel. 4.8 software (Zeiss) for time-lapse imaging and cell tracking was used. Phase contrast images of cells were taken every 60 min using a ×4 objective (Achromatic, Zeiss). Cell migration was scored in at least 10 independent experiments per condition. The farthest distance of cell migration was calculated from the edge of the sphere. Resulting movies were collected and exported as Quicktime and are shown at 5 frames per second (Supporting Information videos 1−4).

Immunocytochemistry. Cells were processed for immunocytochemistry as previously described.[60] Briefly, at the end of the treatment period, NS cultures were grown on glass coverslips in 24-well cell culture plates. Cultures were then washed with phosphate-buffered saline (PBS), fixed for 30 min with 4% paraformaldehyde at 25 °C, and then permeabilized with 0.1% Triton X-100 for 30 min at 37 °C. After 1 h incubation with the corresponding primary antibody, cells were washed with PBS and incubated with an Alexa-labeled secondary antibody (Molecular Probes; Leiden, The Netherlands) for 45 min at 37 °C. Later on, images were obtained using a TCS SP5 laser scanning spectral confocal microscope (Leica Microsystems). Confocal microscope settings were adjusted to produce the optimum signal-to-noise ratio. Quantification was undertaken using the image analySIS software (Soft Imaging System Corp., Münster, Germany) and normalized to total nuclei. Primary antibodies were directed against the following: β-tubulin (rabbit; Abcam), GFAP (mouse; Sigma), and Ki67 (mouse; Novocastra). 4′,6-Diamidino-2-phenylindole (DAPI) staining was used as a nuclear marker. For quantification of the number of $Ki67^+$ cells, at least eight neurospheres per condition over three independent experiments were used.

Protein Extraction and Western Blot Analysis. Proteins were isolated from cultured NS derived from adult rat hippocampus, and a total amount of 30 μg of protein was loaded on a 10% SDS-PAGE gel. After electrophoresis, proteins were transferred to nitrocellulose membranes (Protran, Whatman, Dassel, Germany) and blots were probed with a primary specific antibody against GSK-3 (mouse; Cell Signaling) followed by a secondary peroxidase-conjugated rabbit anti-mouse antibody (Jackson Immunoresearch). For each sample, the α-tubulin level expression (Sigma) was determined as a loading control.

NP031112 and Bromodeoxyuridine Administration. At least six animals per group received daily intragastrical administration of NP031112 (50 mg/kg body weight; Noscira) or vehicle for 7 consecutive days. The dose used was chosen according to previous in vivo works.[61,62] Twenty-four hours before sacrifice, treated and nontreated rats received an intraperitoneal injection of 5-bromo-2-deoxyuridine (BrdU, 50 mg/kg) to label the population of proliferating cells in the hippocampus.

Tissue Preparation and Immunohistochemistry. After treatment, animals were perfused transcardially, as previously described.[57] Briefly, after perfusion with 4% paraformaldehyde, brains were removed, postfixed in the same fixative, cryoprotected, frozen, and finally sectioned in the coronal plane at 30 μm with a cryostat. Free floating sections were processed for immunohistochemistry as previously described.[57] For BrdU-labeling, sections were first incubated at 37 °C with 2 M HCl during 30 min before blocking for 1 h in PBS containing 5% normal serum, 0.1 M lysine, and 0.1% Triton X-100. The following primary antibodies were used: mouse monoclonal anti-BrdU (Dako), goat polyclonal anti-DCX (Sta. Cruz Biotech), and mouse monoclonal anti-PSA-NCAM (Chemicon). After several rinses in PBS, samples were incubated with the corresponding biotinylated secondary antibodies for 1 h at room temperature. Samples were washed three times and mounted with DePeX (Serva, Heidelberg, Germany). Images were obtained using a Nikon Eclipse 80i microscope equipped with a Nikon DS-Fi1 digital camera (Melville, NY). The total number of BrdU-positive cells was determined in 30 μm coronal sections. Only one in every sixth section throughout the hippocampus was used in order to avoid oversampling errors. Using this spacing ensures that the same cell will not be counted in two sections. All BrdU-labeled cells in the dentate gyrus and hilus were counted at high magnification (×40) under a light microscope (Nikon Eclipse 80i), omitting cells in the outermost focal plane. The resulting number was multiplied by 6 to have an approximate total number of BrdU-positive cells. Six animals/group were analyzed.

Statistical Determinations. The data shown are the mean mean ± SD of at least three independent experiments. Statistical comparisons for significance between cells with different treatments were performed using the Student's t test with $p \leq 0.05$ being considered significant. Analysis of variance (ANOVA) was used to analyze the data of Figure 4.

■ ASSOCIATED CONTENT

Supporting Information

Video recording of the cell migration pattern from basal and GSK-3-treated neurospheres. Dose−response of GSK-3 inhibitors on neurosphere formation. This material is available free of charge via the Internet at http://pubs.acs.org/

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Author Contributions

A.P.C. and A.M. conceived and designed the experiments, analyzed data, and wrote the manuscript. A.S. designed experiments, analyzed data, and wrote the manuscript. J.A.M.-G. helped design the experiments, analyzed data, and performed the experiments. R.L.-M., S.A.-G. and M.S.-S. performed the experiments. V.P. synthesized and optimized the compounds. C.G. synthesized and optimized the compounds and analyzed data.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

AD, Alzheimer's disease; DG, dentate gyrus; GSK-3β, glycogen synthase kinase-3β; NSC, neural stem cell; NS, neurosphere; SGZ, subgranular zone; SVZ, subventricular zone; TDZDs, thiadiazolidinediones

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