GSK-3β inhibition/β-catenin stabilization in ventral midbrain precursors increases differentiation into dopamine neurons

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Summary

Wnts are important regulators of dopamine (DA) neuron differentiation in the developing ventral mesencephalon and could thus serve as potential tools in the treatment of Parkinson's disease. In this study, we investigate whether established intracellular Wnt signalling components could modulate the development of DA neurons. Two chemical inhibitors of glycogen synthase kinase (GSK)-3β, indirubin-3-monoxime and kenpaullone, were found to increase neuronal differentiation in ventral mesencephalon precursor cultures. In addition, the GSK-3β-specific inhibitor kenpaullone increased the size of the DA neuron population through conversion of precursors expressing the orphan nuclear receptor-related factor 1 into tyrosine hydroxylase positive neurons, thereby mimicking an effect of Wnts. We show that GSK-3β inhibitors stabilized β-catenin and that overexpression of β-catenin in ventral mesencephalic precursors resulted in increased DA differentiation. The three- to fivefold increase in DA differentiation of precursor cells by GSK-3β inhibitors suggests that such compounds could be used to improve stem/precursor cell therapy approaches in Parkinson's disease.

Key words: GSK-3β, β-catenin, Dopamine, Neuron, Wnt

Introduction

The Wnt family of secreted proteins regulates precursor proliferation, fate decisions and neuronal differentiation in the developing brain (Dorsky et al., 1998; Megason and McMahon, 2002). Deletion of the *Wnt1* gene results in loss of the midbrain-hindbrain junction and the consequent loss of dopamine (DA) neurons (McMahon and Bradley, 1990; Thomas and Capecchi, 1990; Danielian and McMahon, 1996). Another mutant mouse with a similar phenotype in the midbrain is the *low-density lipoprotein receptor related protein 6* null (Pinson et al., 2000), which lacks a receptor necessary for Wnt signalling. We have previously shown that Wnts are involved in the acquisition of a DA phenotype in the developing ventral mesencephalon (VM). Whereas Wnt1 mainly regulates the proliferation of neural precursors, Wnt5a is involved in the conversion of precursors positive for nuclear receptor-related factor 1 (Nurr1) into DA neurons (Castelo-Branco et al., 2003). These properties make Wnts attractive tools for DA cell-replacement therapy in Parkinson's disease. Wnts are palmitoylated glycoproteins (Willert et al., 2003) and as such are poorly soluble, limiting their potential use in a clinical set up. One possible way to circumvent this limitation could be the development and application of drugs that modulate Wnt signalling pathways in DA precursor/stem cell cultures.

The canonical pathway of Wnt signalling acts through stabilization of β-catenin. In the absence of Wnt, β-catenin is constantly phosphorylated by a destruction protein complex consisting of glycogen synthase kinase-3β (GSK-3β), axin, adenomatous polyposis coli and casein kinase 1, thereby targeting it for ubiquitination and degradation in the proteasome. Upon Wnt binding to Frizzled receptors, GSK-3β is inhibited, leading to stabilization of the β-catenin protein that can either enter the nucleus and initiate transcription of target genes (Huelsken and Behrens, 2002) or signal in the cytoplasm in combination with cadherins (Yu and Malenka, 2003; Bamji et al., 2003). Recently, several pharmacological GSK-3β inhibitors have been developed (Cohen and Frame, 2001; Bain et al., 2003), mimicking Wnt/β-catenin signalling in different cell systems such as adipogenesis (Bennett et al., 2002) and embryonic stem cell maintenance (Sato et al., 2004). In the present study, we examined whether pharmacological inhibition of GSK-3β in VM precursors can modulate the generation of DA neurons. We now report that GSK-3β inhibition leads to a twofold increase in neuronal differentiation and to a 2.5-fold increase in conversion of Nurr1$^+$ precursors into tyrosine hydroxylase positive (TH$^+$) DA neurons, mimicking actions of Wnts (Castelo-Branco et al., 2003). Furthermore, we show that GSK-3β inhibition in VM precursors results in stabilization of β-catenin and that direct overexpression of β-catenin in VM precursors increases the number of DA neurons. The effect of GSK-3β inhibitors on the differentiation of DA precursors suggests that these compounds could improve DA cell preparations for replacement therapy in Parkinson's disease.

Materials and Methods

Real-time PCR and quantification of gene expression

Total RNA was isolated from embryonic day (E) 14.5 VM precursor cultures (2.5×10^6 cells in 6 cm^2 dishes) treated with the GSK-3β inhibitors for 3 days in vitro. Reverse transcription reaction, real-time RT-PCR and c-ret primer sequences were carried out/used as described (Castelo-Branco et al., 2003). Quantum RNA classical 18S internal standard was purchased from Ambion (Austin, USA) and PCR primers from DNA Technology A/S, Aarhus, Denmark. The following PCR programme was used for SYBR Green detection on the ABI PRISM 5700 Detection System (PE Applied Biosystems, Foster City, CA, USA): 94°C for 2 minutes; 35-40 cycles of 94°C for 30 seconds, 59°C for 30 seconds, 72°C for 15 seconds; and 80°C for 5 seconds. Statistical analysis of the results was performed using a two-tailed Wilcoxon signed rank test. Significance for all tests was assumed at the level of $P<0.05$ (*$P<0.05$; **$0.01<P<0.001$; ***$P<0.001$).

Precursor and MN9D cultures and treatments

E14.5 VMs obtained from time-mated Sprague-Dawley rats (ethical approval for animal experimentation was granted by Stockholm Norra Djurförsöks Etiska Nämnd) were dissected, mechanically dissociated and plated at a final density of 1×10^5 cells per cm^2 on poly-D-lysine (10 μM) coated plates (Falcon). Serum-free N2 medium was added consisting of a 1:1 mixture of F12 and MEM (Invitrogen) with 15 mM Hepes buffer, 1 mM glutamine, 5 μg/ml insulin, 100 μg/ml transferrin, 100 μM putrescine, 20 nM progesterone, 30 nM selenium, 6 mg/ml glucose and 1 mg/ml BSA (all purchased from Sigma). Cultures were incubated for 3 days in vitro, in a 37°C incubator with 5% CO_2, before fixation. MN9D and SN4741 cells were grown as described (Choi et al., 1991; Son et al., 1999). MN9D cultures were fixed after 4 days in vitro. I3M and KP (Alexis Biochemicals, Göteborg, Sweden) were diluted in DMSO and added to the cultures at the time of plating. DMSO alone did not increase the number of TH^+ neurons and did not affect morphological differentiation of MN9D (data not shown). 10 μM BrdU was added to the culture media 6 hours prior to fixation.

Constructs and transfections

Human β-catenin cDNA (a gift from Dr Steven Byers) was subcloned into the pCAIP2 vector containing the chicken β-actin promoter under the CMV enhancer. An empty pCAIP2 vector was used as a control. Primary cells were transfected in N2 medium using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations and assessed by immunocytochemistry after 2 days in vitro.

Immunocytochemistry

Cells were fixed for immunocytochemistry in ice-cold 4% paraformaldehyde for 15-20 minutes and washed in PBS as described (Castelo-Branco et al., 2003). The following primary and secondary antibodies were used: mouse α-TH (1:1000 dilution; Immunostar); rabbit α-TH (1:250; Pelfreeze); mouse α-β-III-tubulin (1:1000; Promega); rabbit α-Nurr1 (1:1000; Santa Cruz); mouse α-MAP2 (1:200; Sigma); rabbit α-GFAP (1:400; Dako); rabbit α-active-caspase III (1:100; Cell Signalling); mouse α-BrdU (1:100; Dako); rat α-BrdU (1:100; Abcam); mouse α-vimentin (1:100; Dako); mouse α-nestin (Rat401) and mouse 3CB2 (both at 1:100; Developmental studies hybridoma bank, Iowa); biotinylated α-mouse IgG and α-rabbit IgG (1:500; Vector); cyanine-2 or rhodamine-coupled horse-α-mouse IgG or goat α-rabbit IgG (1:200; Jackson Laboratories). At the end of the staining procedure, cultures were incubated with Hoechst 33258 reagent for 10 minutes. BrdU immunocytochemistry included incubation for 30 minutes with 2 N HCL. Images were acquired from stained cells in PBS at room temperature with a Zeiss Axioplan 100M microscope (LD Achrostigmat 20×, 0.3 PH1 ∞ 0-2 and LD Achroplan 40×, 0.60 Korr PH2 ∞ 0-2) and collected with a Hamamatsu camera C4742-95 (with QED imaging software).

Immunoblotting

Cell were lysed for 15 minutes in a modified RIPA buffer containing 20 mM Tris (pH 7.5), 140 mM NaCl, 10% glycerol, 1% IGEPAL, 1 mM β-glycerol-phosphate, 1 mM Na_3VO_4 and complete protease inhibitors (Roche). Plates were scraped and the cell lysate was centrifuged in a microfuge for 5 minutes. The supernatant was transferred to a clean tube for protein measurement using the BCA kit (Pierce) and stored in Laemmli buffer. Equal amounts of protein were analysed by polyacrylamide gel electrophoresis (10% polyacrylamide). The proteins were transferred onto a polyvinylidene difluoride membrane. After blocking in PBS with 0.1% Tween and 3% BSA, the membrane was incubated with the following primary antibodies overnight at 4°C: mouse α-β-catenin

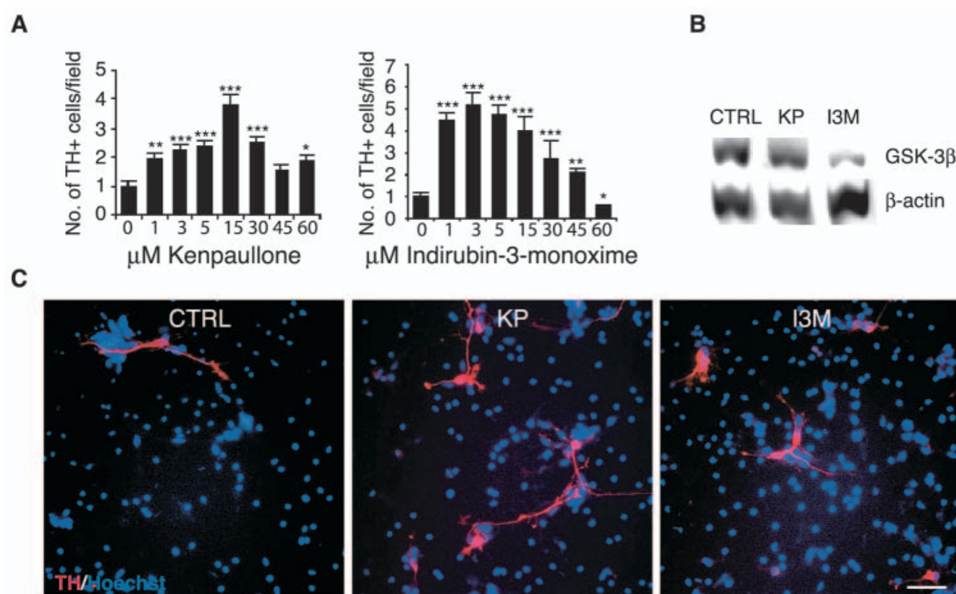

Fig. 1. Indirubin-3-monoxime (I3M) and kenpaullone (KP) increase the number of tyrosine hydroxylase positive (TH^+) neurons in ventral mesencephalon (VM) precursor cultures. (A) Dose-response experiments in E14.5 VM precursors indicate that 15 μM KP and 3-5 μM I3M are optimal doses to increase TH^+ cell numbers. Statistical analysis was performed using one-way ANOVA with Fisher's post-hoc test. *$P<0.05$; **$0.01<P<0.001$; ***$P<0.001$. (B) Treatment of E14.5 VM precursors with 15 μM KP or 5 μM I3M for 24 hours decreases GSK-3β protein levels as assessed by immunoblotting. β-actin was used as a loading control. (C) TH/Hoechst 33258 immunostaining shows an increase in the number of TH immunoreactive cells 3 days after treatment with 15 μM KP or 5 μM I3M. Bar, 50 μm.

(1:500; BD); rabbit α-GSK-3β (1:1000; Cell Signalling); rabbit α-TH (1:500; PelFreeze) and mouse α-β-actin (1:500; Abcam). After washing, the membrane was incubated with an alkaline phosphatase-conjugated secondary α-mouse antibody (1:10,000; Pharmacia Amersham) for 1 hour at room temperature and subsequently developed according to manufacturer's instructions for enhanced chemiluminescence.

Statistical analysis

Quantitative immunocytochemical data represent the means±s.e.m. of counts from ten non-overlapping fields, in three wells per condition from three to five separate experiments. For the RT-PCR experiments, five separate experiments were analysed and for the GSK-3β inhibitors dose response, two sets of experiments were analysed. Statistical analysis was performed in Prism 4 (GraphPad software, San Diego, USA) as described in the figure legends, with significance for all tests assumed at the level of $P<0.05$ (*$P<0.05$; **$0.01<P<0.001$; ***$P<0.001$). Statistical tests were chosen according to the distribution of the sample population.

Results

GSK-3β inhibition increases the number of DA neurons in VM precursor cultures

Precursor cells in the rat VM acquire a DA phenotype between embryonic day 11 and 16 (E11-E16) (Perrone-Capano et al., 2000). As proliferating precursors in the neuroepithelium begin migrating ventrally, they start expressing Nurr1 (Wallén et al., 1999), thereby withdrawing from the cell cycle and acquiring a DA phenotype by a mechanism involving the cyclin dependent kinase (CDK) inhibitor p57 (Joseph et al., 2003). We have previously shown that Wnts promote DA differentiation in VM precursors (Castelo-Branco et al., 2003). In order to investigate whether established intracellular Wnt signalling components can modulate DA neuron development, we treated rat VM E14.5 precursor cultures with increasing doses of two ATP-competitive inhibitors of GSK-3β, a critical enzyme for β-catenin degradation. Addition of indirubin-3-monoxime (I3M) (Hoessel et al., 1999; Leclerc et al., 2001) or kenpaullone (KP) (Bain et al., 2003) to precursor cultures increased the number of TH^+ cells in a dose-dependent manner, with maximal effects at 3-5 μM for I3M (fivefold increase) and 15 μM for KP (threefold increase) (Fig. 1A,C). At these concentrations, KP and I3M differ in that KP is a GSK-3β selective inhibitor whereas I3M inhibits both GSK-3β and CDKs (Leclerc et al., 2001; Bain et al., 2003). KP at 15 μM increased the percentage of DA neurons (TH^+) out of the total number of cells (Hoechst 33258^+) present in the culture after 3 days, from 0.3±0.06 to 1.1±0.01. Interestingly, we found that treatment of VM precursors with KP or I3M led to a downregulation of GSK-3β protein levels (Fig. 1B). Similar results (data not shown) were observed in the DA precursor cell line SN4741, derived from the developing substantia nigra (Son et al., 1999).

GSK-3β inhibition increases the differentiation of VM precursors

VM precursor cultures are heterogenous preparations that mainly consist of neuronal ($nestin^+$) and glial ($vimentin^+$) precursors. Upon culture in serum-free medium for 3 days, these precursors can remain in a proliferative precursor state, undergo differentiation into neurons or astrocytes, or initiate programmed cell death. We thus asked how inhibition of GSK-3β affected differentiation of VM precursors. We found, by immunocytochemistry, that I3M reduced the amount of $nestin^+$ precursors (Fig. 2A), consistent with previous reports showing that I3M inhibits CDKs (Leclerc et al., 2001; Bain et al., 2003), thereby inducing cell cycle exit. However, the selective GSK-3β inhibitor KP did not alter this population (Fig. 2A) or the radial glial population, identified by vimentin (Shults et al., 1990) (Fig. 2B) and 3CB2 (data not shown). Very low numbers of astrocytes were detected in our serum-free cultures (0.06±0.02%; $GFAP^+$/Hoechst 33258^+) and only KP was found

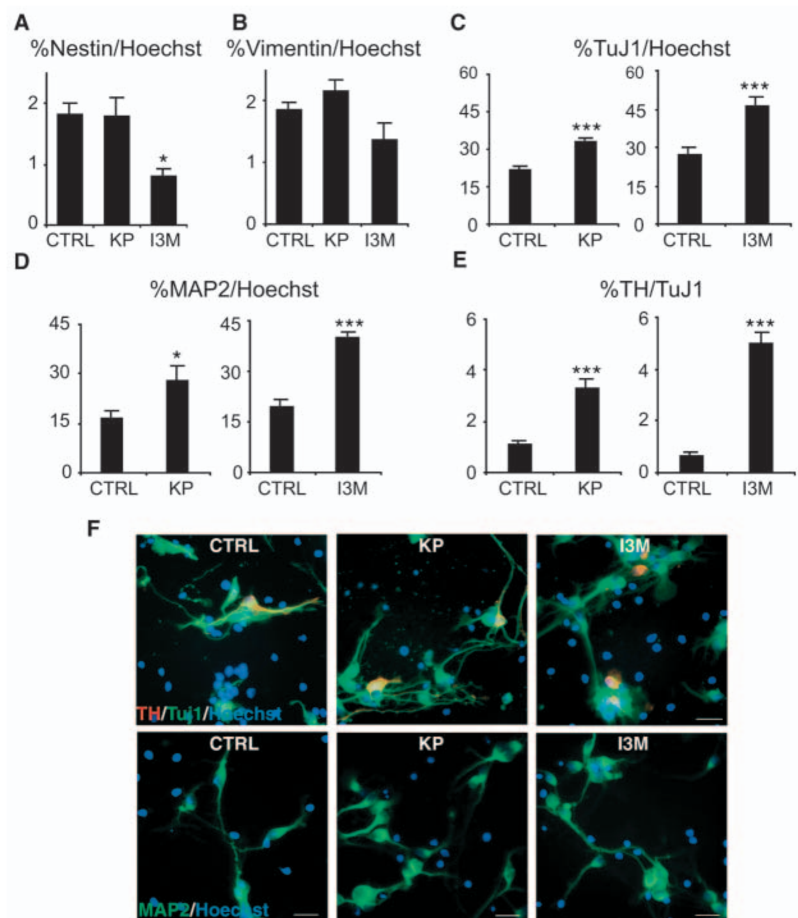

Fig. 2. Treatment with KP or I3M increases neuronal differentiation of VM precursors, particularly into DA neurons. (A,B) Immunostaining with antibodies against nestin/Hoechst 33258 (A) and vimentin/Hoechst 33258 (B), showed a reduction in the number of neuronal precursors ($nestin^+$) after treatment with I3M, with no effect on glial precursors ($vimentin^+$). (C-F) 15 μM KP or 5 μM I3M increased the number of immature $Tuj1^+$ (C,F) and mature $MAP2^+$ (D,F) neurons and the proportion of TH^+ DA neurons (E,F) after 3 days in vitro. Statistical analysis was performed using one-way ANOVA with Boneferroni's multiple comparison test (A,B) and two-tailed unpaired *t*-tests (C-E). Significance levels as for Fig. 1. Bar, 25 μm.

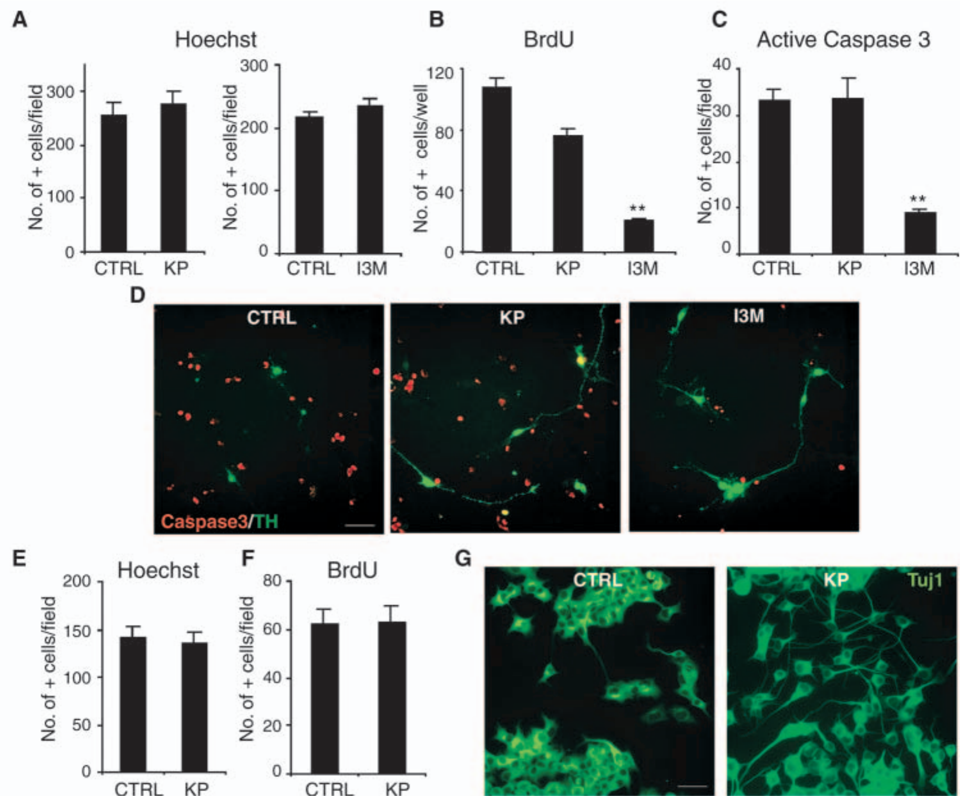

Fig. 3. GSK-3β inhibition does not regulate cell survival or proliferation of VM precursors or MN9D cells but induces their morphological differentiation. (A) Treatment with 15 μM KP or 5 μM I3M did not change the total number of VM cells after 3 days in culture, as assessed by Hoechst 33258 staining. (B) Proliferation, as assessed by BrdU incorporation, was not affected by 15 μM KP, but was significantly decreased with 5 μM I3M. (C,D) Immunostaining with α-Active Caspase 3 showed a decreased number of positive cells 3 days after treatment with 5 μM I3M, but not with KP. (E,F) Treatment of MN9D cells with 15 μM KP for 4 days did not alter the total number of cells (Hoechst 33258) or their proliferation (BrdU incorporation). (G) Treatment with 15 μM KP for 4 days induced morphological differentiation of MN9D cells. Statistical analysis was performed using two-tailed unpaired *t*-test (A, E and F), Kruskal-Wallis test (B) and one-way ANOVA with Boneferroni's multiple comparison test (C). Significance levels as for Fig. 1. Bar, 50 μm.

to significantly increase that proportion to 0.23±0.02% (P<0.0001 unpaired two-tailed student's t-test). Interestingly, a marked effect on the number of neurons was detected and both inhibitors were found to increase the proportion of immature neurons [beta-tubulin type III $(TuJ1)^+$/Hoechst 33258^+] (Fig. 2C,F) and of mature neurons [microtubule-associated protein 2 $(MAP2)^+$/Hoechst 33258^+] (Fig. 2D,F). Moreover, both inhibitors gave rise to a pronounced increase in DA neuron differentiation, as shown by a three- to fivefold increase in the proportion of $TH^+/TuJ1^+$ cells in the VM (Fig. 2E and images in Fig. 2F). These results suggested that selective inhibition of GSK-3β does not alter the proportion of precursors but rather modulates their differentiation, resulting in increased numbers of DA neurons.

GSK-3β does not regulate cell survival or proliferation in VM precursor cultures

In order to determine whether GSK-3β inhibition was promoting proliferation, we examined the total number of cells in the culture and the number of cells that incorporate BrdU, a marker of proliferation. Treatment with the GSK-3β inhibitors did not change the total number of cells present after three days in vitro, as assessed by Hoechst 33258 staining (Fig. 3A). Moreover, when VM precursors were exposed to a 6-hour BrdU pulse before fixing, KP did not significantly affect BrdU incorporation (Fig. 3B). However, consistent with previous reports showing that I3M inhibits CDKs (Leclerc et al., 2001; Bain et al., 2003), we found that I3M treatment dramatically reduced the number of $BrdU^+$ cells (Fig. 3B). Thus, our results suggest that the increase in TH^+ neurons by GSK-3β inhibition was not achieved through a general proliferation mechanism. We next examined whether GSK-3β inhibitors could increase the number of DA cells through survival. Whereas the GSK-3β/CDK inhibitor, I3M, reduced the number of active Caspase-3 immunoreactive cells, KP, the selective GSK-3β inhibitor, had no effect (Fig. 3C,D). More importantly, we found that about 5% of the TH^+ cells stained positive for active Caspase 3 but neither of the two GSK-3β inhibitors decreased the

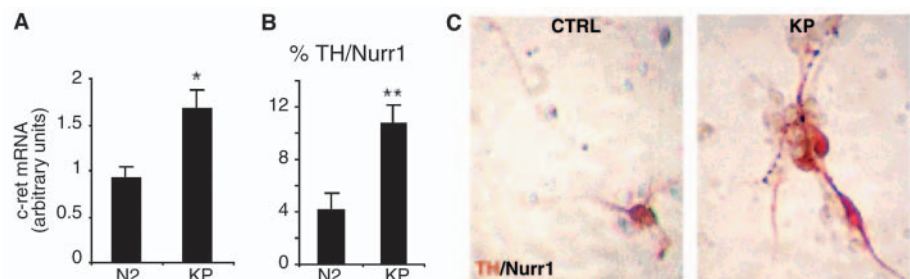

Fig. 4. KP upregulates c-ret mRNA and increases conversion of $Nurr1^+$ precursors into TH^+ neurons. (A) Real time RT-PCR analysis revealed that c-ret mRNA levels were upregulated upon treatment with 15 μM KP. (B) 15 μM KP increased the conversion of VM $Nurr1^+$ precursors into TH^+ neurons. (C) TH/Nurr1 double immunostaining showed an increase in the number of immunoreactive cells 3 days after treatment with 15 μM KP. Statistical analysis was performed using a two-tailed Wilcoxon's signed rank test (A) and a two-tailed unpaired t-test (B). Significance levels as for Fig. 1.

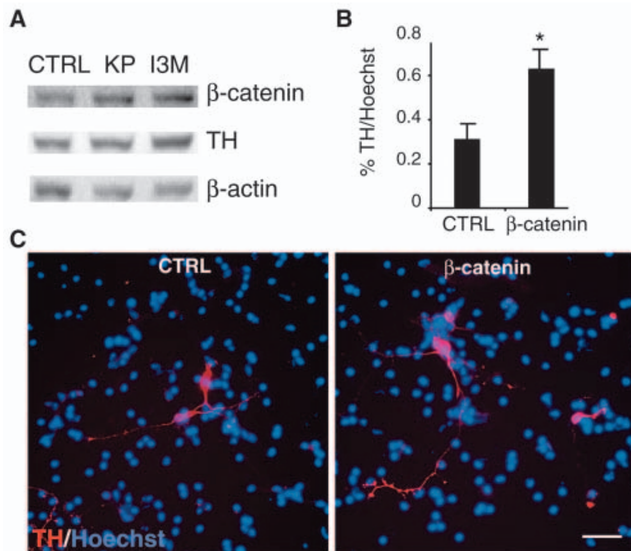

Fig. 5. GSK-3β inhibitors stabilize β-catenin and overexpression of β-catenin increases the number of TH^+ neurons. (A) Treatment of VM precursors with 15 μM KP or 5 μM I3M for 24 hours increased both β-catenin and TH protein levels, as assessed by immunoblotting. β-actin was used as a loading control. (B,C) TH immunostaining shows a twofold increase in the number of positive cells out of the total number of cells (Hoechst 33258) two days after transfection with β-catenin, compared to transfection with an empty control vector. Statistical analysis was performed using a two-tailed unpaired *t*-test. Significance levels as for Fig. 1. Bar, 50 μm.

number of $Caspase^+/TH^+$ cells (data not shown), indicating that GSK-3β inhibition does not lead to increased numbers of TH^+ neurons through decreased apoptosis. In order to confirm the effects of GSK-3β inhibition on DA differentiation, we investigated the effects of the selective GSK-3β inhibitor KP on MN9D cells. This cell line, derived from mouse embryonic VM, expresses TH, synthesizes and stores dopamine and can mature and extend neurites under specific differentiation conditions (Choi et al., 1991; Castro et al., 2001). As in the VM precursor cells, treatment with KP did not change the total number of cells (assessed by Hoechst 33258) or the level of BrdU incorporation (Fig. 3E,F). KP treatment did however induce morphological differentiation and neurite extension of MN9D cells (Fig. 3G), which was similar to that found in the VM precursor cultures (Fig. 2C). Comparable effects on morphological differentiation of MN9D cells have been reported after Nurr1 overexpression (Castro et al., 2001), suggesting that these cells and VM precursors share the capacity to differentiate in response to Nurr1 and GSK-3β inhibitors. Combined, our results indicated that the increase in the number of TH^+ neurons was not achieved through proliferation or survival and prompted us to examine whether GSK-3β inhibition promotes DA differentiation in VM precursors.

Inhibition of GSK-3β increases DA cell numbers through conversion of $Nurr1^+$ precursors into DA neurons

We first asked whether GSK-3β inhibition promoted the expression of genes characteristic of differentiated VM DA neurons. Real-time RT-PCR analysis showed that KP treatment resulted in increased mRNA levels of the protooncogene *c-ret* (Fig. 4A). *C-ret* has also been reported to be upregulated upon treatment of VM precursor cultures with Wnt5a (Castelo-Branco et al., 2003) or in catecholaminergic PC12 cells treated with Wnt1 (Zheng et al., 1996). We next examined whether the number of $Nurr1^+$ cells changed upon treatment with KP but found no significant increase (control: 149.7±22.85, KP: 188±26.95; unpaired two-tailed student's *t*-test). These results suggested that GSK-3β inhibition might cause an increase in the differentiation of DA precursors rather than the proliferation and/or survival of Nurr1-expressing DA precursors. We therefore examined the differentiation of $Nurr1^+/TH^-$ precursors into $Nurr1^+/TH^+$ DA neurons and found that the specific GSK-3β inhibitor KP increased the number of $TH^+/Nurr1^+$ cells 2.5-fold (Fig. 4B,C). Thus, our results indicate that the mechanism by which GSK-3β inhibition leads to increased numbers of TH^+ neurons is not by promoting proliferation or survival of $Nurr1^+$ VM precursors but by promoting their differentiation.

β-catenin stabilization increases the differentiation of VM precursors into DA neurons

We have previously shown that Wnts promote DA differentiation in VM cultures and that there is Nurr1/β-catenin colocalization and TCF/LEF transcriptional activity in E10.5 VM precursors in vivo (Castelo-Branco et al., 2003). However, GSK-3β was recently described to mediate axonal remodeling through microtubule stabilization in a β-catenin-independent pathway (Ciani et al., 2004). Thus, in order to investigate the mechanism of DA differentiation further, we analysed the level of β-catenin protein in VM precursor cultures treated with KP or I3M. Immunoblotting showed increased levels of the β-catenin protein, indicating increased stabilization of β-catenin upon treatment with the inhibitors (Fig. 5A). Furthermore, both inhibitors increased the levels of TH protein (Fig. 5A). These results suggested that β-catenin could mediate some of the effects of the GSK-3β inhibitors and Wnts on DA precursors. In order to assess the role of β-catenin stabilization in early DA differentiation, we transfected VM precursors with a vector expressing β-catenin. Overexpression of β-catenin in VM precursor cells did not alter the total number of cells, as assessed by Hoechst 33258 staining (control: 470.1±12.57, β-catenin: 471.8±12.94; unpaired two-tailed student's *t*-test), but led to a twofold increase in the number of TH^+ cells, compared to an empty control vector (Fig. 5B,C). These results suggest that β-catenin might mediate the observed effects of GSK-3β inhibitors in VM precursors.

Discussion

The indirubin family of GSK-3β inhibitors was initially isolated from traditional Chinese medicines used against various chronic diseases including chronic myelocytic leukaemia (Hoessel et al., 1999). More recently, the potential application of a GSK-3β inhibitor of the indirubin family in stem cell therapy was reported (Sato et al., 2004). Our results, describing new properties of chemical GSK-3β inhibitors on the differentiation of DA precursors, may also have important

implications for the development of cell replacement therapies for Parkinson's disease. Current approaches focus on grafting human foetal midbrain DA precursors into the neostriatum of patients with Parkinson's disease (Dunnett et al., 2001). The success of these approaches critically depends on the number of grafted DA neurons and more than five fetuses per patient are required (Dunnett et al., 2001; Arenas, 2002). As DA neurons are present in very low numbers in embryonic tissue, the three- to fivefold increase in TH^+ neurons observed upon treatment with GSK-3β inhibitors could reduce the need of fetal tissue for transplantation. Similarly, GSK3-β inhibitors could also be applied to enhance the efficiency of DA differentiation in alternative cell sources for cell replacement, including stem cells. Indeed, the differentiation of human stem cells into DA neurons (Storch et al., 2001; Hornstein and Benvenisty, 2004) has proven to be more difficult than that reported for mouse stem cells (Wagner et al., 1999; Lee et al., 2000; Kim et al., 2002; Barberi et al., 2003), highlighting the need for novel differentiation signals to be implemented in stem cell preparations. Thus, our results showing that GSK-3β inhibitors efficiently promote DA differentiation of precursor cells suggest that these compounds could also be applied to improve DA cell preparations prior to transplantation in patients with Parkinson's disease.

β-catenin signalling has generally been associated with proliferation of neural progenitors in the developing central nervous system (Megason and McMahon, 2002; Chenn and Walsh, 2002; Zechner et al., 2003). Indeed, constitutive expression of β-catenin under the control of promoters active in neural stem/progenitor cells results in an expansion of the entire neural tube (Chenn and Walsh, 2002; Zechner et al., 2003), supporting a role of β-catenin in progenitor proliferation. However, these experiments do not directly address a possible role of β-catenin in later developmental stages, during neuronal differentiation. We have previously shown that Wnt-1 and Wnt-5a promote VM precursor proliferation and DA differentiation in VM precursor cultures, respectively. Furthermore, we found Nurr1/β-catenin colocalization and TCF/LEF transcriptional activity at E10.5 in the VM domain where DA precursors differentiate in vivo (Castelo-Branco et al., 2003). These results suggested that Wnt/β-catenin signaling regulates both proliferation and differentiation in DA precursors. We now report that GSK-3β inhibition and β-catenin stabilization acts on differentiation rather than proliferation in E14.5 rat VM precursors, suggesting that the proliferative effects of Wnts cannot be ascribed to β-catenin signalling alone but might involve other pathways or a cross-talk with additional signalling pathways. Importantly, we also observe a greater increase in neuronal differentiation and in the proportion of DA neurons out of the total neuronal population. Our results are in agreement with recent publications supporting a role for Wnt/β-catenin signalling in sensory neuron differentiation (Lee et al., 2004) and dendritogenesis (Yu and Malenka, 2003), as well as in the differentiation of other organs such as the skin (Huelsken et al., 2001; Merrill et al., 2001; Tumbar et al., 2004). Thus, our data provide evidence for a general role of β-catenin in cell differentiation and indicate that GSK-3β inhibitors might be particularly well suited for cell replacement therapies by promoting DA differentiation in cell preparations containing neural precursors.

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