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Influence of Retinoic Acid and Lithium on Proliferation and Dopaminergic Potential of Human NT2 Cells

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Our laboratory is working with the human NTera2/D1 (NT2) cell line, which has properties similar to those of progenitor cells in the central nervous system (CNS). These neural-like precursor cells can differentiate into all three major lineages, neurons, astrocytes, and oligodendrocytes. The pure neuronal population, hNT neurons, possess characteristics of dopamine (DA) cells. First, we analyzed whether the retinoic acid (RA)-treated hNT neurons and the NT2 precursor cells expressed two transcription factors required for development of the midbrain DA neurons. We report that NT2 cells endogenously expressed Engrailed-1 and Ptx3, whereas RA-treated hNT neurons did not express Engrailed-1 or Ptx3. Next we examined the influence of lithium treatment on Engrailed-1 and Ptx3 as well as another critical transcription factor, Nurr1. Previous research has shown that lithium can mimic the Wnt pathway, which is important for the induction of these transcription factors. Finally, we investigated the effect of lithium treatment on the viability and proliferation of NT2 cells, because lithium has been shown to stimulate neurogenesis in adult neural precursors. Lithium treatment increased the viability and proliferation of NT2 cells. The expression of transcription factors essential for the induction and maintenance of the DA phenotype was not increased in NT2 after lithium treatment. We conclude that the NT2 cell line is an excellent in vitro model system for studying the influence of pharmalogical agents on proliferation, differentiation, and apoptosis of a human neural progenitor cell line.

Key words: hNT; Engrailed; Nurr1; Ptx3; proliferation

The NTera2/D1 (NT2) cell line has been used as a human in vitro model system to examine neural development, because these cells are similar to neural precursor cells and can differentiate into all three neural lineages, neurons, astrocytes, and oligodendrocytes (Andrews, 1984; Andrews et al., 1984; Lee and Andrews, 1986; Pleasure et al., 1992; Langlois and Duval, 1997; Bani-Yaghoub et al., 1999; Sandhu et al., 2002). The pure neuronal population, the hNT neurons, can be generated from NT2 cells through exposure to retinoic acid (RA; 10 μM). A subpopulation of hNT neurons expresses a constellation of enzymes and receptors found in midbrain dopamine (DA) neurons, including the rate-limiting enzyme for DA synthesis tyrosine hydroxylase (TH), the essential transcription factor Nurr1, dopamine transporter (DAT), D2 receptor, and aldehyde dehydrogenase (AHD-2; Zigova et al., 1999, 2000; Misiuta et al., 2003). These neurons were shown to form functional synapses and release DA upon potassium chloride stimulation (Hartley et al., 1999; Iacovitti et al., 2001).

Recently, numerous transcription factors important for the induction and differentiation of DA neurons have been identified. Among the early transcription factors expressed during the induction of early midbrain DA neurons, also called *ventral mesencephalon (VM) precursor cells*, are two homologues, Engrailed-1 (En-1) and Engrailed-2 (En-2), which are controlled by signaling molecules in the Wnt pathway (McMahon and Bradley, 1990; Danielian and McMahon, 1996). Although TH expression is induced in Engrailed-1/-2 null mice, its expression soon diminishes, and no TH-positive neurons are found at birth (Liu and Joyner, 2001; Simon et al., 2001). During differentiation, two more transcription factors critical for DA development are expressed, Nurr1 and Ptx3. Nurr1 is an orphan steroid receptor shown to be critical for the development and differentiation of midbrain DA neurons, insofar as

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they failed to develop in Nurr1 null mice (Zetterstrom et al., 1997; Castillo et al., 1998; Saucedo-Cardenas et al., 1998; Wallen et al., 1999). Ptx3 is even more specific to midbrain neurons; Ptx3-deficient *aphakia* mice failed to develop DA neurons of the substantia nigra pars compacta (Semina et al., 1997; Smidt et al., 1997). Ptx3 was also shown to increase expression of TH by binding the promoter region of the TH gene (Smidt et al., 2000; Lebel et al., 2001). We previously showed that, prior to RA treatment, 12% of NT2 cells endogenously expressed Nurr1, whereas all 3–5-week RA-induced hNT neurons expressed Nurr1 (Zigova et al., 1999, 2000; Misiuta et al., 2003). Therefore, we first examined whether NT2 cells and RA-treated hNT neurons express the two other necessary transcription factors, En-1 and Ptx3.

We report that NT2 cells endogenously express En-1 and Pt3x; however, RA-induced hNT neurons do not express Ptx3 or En-1. We next examined the effect of lithium, another pharmacological agent known to influence the dopaminergic phenotype, on the expression of the three essential transcription factors as well as the viability and proliferation NT2 cells (Zigova et al., 1999). Lithium is widely used as a mood-stabilizing drug to treat bipolar (manic depressive) disorders (Goodwin and Jamison, 1990; Price and Heninger, 1994; Manji and Lenox, 2000; Manji et al., 2000a). Lithium has dramatic effects on the morphogenesis in early development in vivo as well as embryonic stem cell differentiation in vitro (Lallier, 1952; Maeda, 1970; Kao et al., 1986; Van Lookeren Campagne et al., 1988; Livingston and Wilt, 1989, 1990; Hansen et al., 1990; Stachel et al., 1993; Schmidt et al., 2001; Kim et al., 2004). Recently, some developmental abnormalities have been reported in children whose mothers were treated with lithium for bipolar disease during pregnancy, such as thyroid dysfunction, hypoglycemia, hypotonia, and Ebstein's anomaly, which is a defect in the tricuspid valve (for review see Pinelli et al., 2002).

One reason why the molecular mechanism underlying the therapeutic effects of lithium has been difficult to characterize is that the lithium affects multiple cellular targets (Manji et al., 1995). Lithium directly inhibits glycogen synthase kinase-3 (GSK3; Klein and Melton, 1996). GSK3 has numerous roles in cellular signaling from regulating neural plasticity to neuroprotection (for review see Grimes and Jope, 2001). In particular, GSK3β is inhibited by the Wnt pathway, which is important during DA neurogenesis and differentiation (Danielian and McMahon, 1996; Castelo-Branco et al., 2003). The inhibition of GSK3β allows for the accumulation of β-catenin, which then enters the nucleus and forms complexes to activate transcription of Wnt target genes. Because lithium treatment inhibits GSK3β, it might mimic the Wnt pathway and increase the expression of Nurr1 and Ptx3. Therefore, we decided to examine the effects of lithium treatment on the transcription factors essential for DA differentiation in NT2 cells.

MATERIALS AND METHODS

Cell Culture

For the first experiments we examined the effects of RA on DA transcription factors in NT2 and hNT cells. The NT2 cells were obtained from the American Type Culture Collection (Manassas, VA), and the hNT neurons were obtained from Layton Bioscience, Inc. (Sunnyvale, CA). To produce the hNT neurons, NT2 cells were exposed to 10 μM RA treatment for 3, 4, or 5 weeks (3w, 4w, 5w). After RA induction, the hNT neurons were replated with mitotic inhibitors for 10 days. All cells were cryopreserved and stored at −180°C prior to use. The cells were thawed rapidly at 37°C and transferred to a 15-cc tube containing Dulbecco's modified Eagle's medium (DMEM; Invitrogen, La Jolla, CA), 10% fetal bovine serum (FBS; Invitrogen), and Gentamicin (50 μg/ml; Sigma, St. Louis, MO). The cells were centrifuged (1000 rpm/7 min) and resuspended in 1 ml of fresh media, and the cell number and viability were assessed with the trypan blue exclusion method. The results from this study suggested that RA did not induce the dopamine phenotype. Therefore, in subsequent studies, we examined inducibility of the NT2 cells, but the hNT neurons were not studied further, because they had been exposed to RA in the differentiation process.

In the second group of experiments, we used Li as the neural differentiation agent. In these studies, NT2 cells were plated for 1 or 4 DIV. Immunocytochemical analysis for cell counts was performed at 1 DIV, and Western blot analysis was used to detect the proteins at 4 DIV, because the cell number was too great to analyze at 4 DIV. The NT2 cells were plated in triplicate in 100-mm plates at a seeding density of 50,000 cells/cm^2 and maintained at 37°C in 5% CO_2 with 95% humidity. In the experiment determining the dosage concentration of lithium chloride (Li; Sigma), the DMEM/FBS/Gentamicin medium was supplemented with 0.5 mM, 1.0 mM, 3.0 mM, 6.0 mM, or 10 mM of Li for 4 DIV, and the viability and total cell number were determined by using the Beckman Coulter Cell Counter. In the second series of experiments, we opted to use the clinically applicable dose of 1.0 mM Li not only for its therapeutic relevance but also because this dose had no detrimental influence on the viability of the cells and had a profound effect on stimulating the cells. In addition, in previous studies with hNT neurons, 0.5–2.0 mM Li increased the dopaminergic phenotype as assessed by the number of TH-positive neurons (Zigova et al., 2000). The NT2 cultures were treated with Li (1 mM), potassium chloride (KCl; 1 mM;, Sigma) and RA (10 μM; Sigma) for 1 DIV. To assess the survival and total cell number of cultured NT2 cells, we used the fluorescein diacetate (FDA)-propidium iodide (PI) staining live/dead assay on living cultures. We used KCl as the salt cation-positive control and RA because it is known to be important during the induction of neuronal differentiation.

For cultures that were maintained for 4 DIV, the NT2 were prepared by first removing the media and adding 0.25% trypsin (Sigma) with EDTA (Mediatech, Inc., Herndon, VA) for 2 min. About 25 ml of DMEM/FBS with 0.05% DNase (Sigma) was then added to the cultures, and the cells were then aspirated from the culture wells and centrifuged for

5 min at 1,000 rpm. The cells were washed and centrifuged three times in the DMEM/FBS/0.05% DNase, and the pellet was resuspended in 1 ml of media. The cells were counted in the Beckman Coulter Cell Coutner.

Immunocytochemistry

The cultures were first washed in 0.1 M phosphate-buffered saline (PBS; pH 7.4), then fixed with cold 4% paraformaldehyde for 15 min and washed three times with cold PBS. The slides were treated for 1 hr at room temperature with 10% normal goat serum, 0.03% Triton X-100 in PBS. Cultures were then incubated overnight at 4°C with the following primary antibodies, Nurr1 (1:100, mouse; BD Biosciences, San Jose, CA), Ptx3 (1:250, rabbit; Abcam), and Ki67 (1:100, mouse; Abcam). The primary antibody was omitted from the control slides.

After several PBS washes, the slides were incubated in the appropriate secondary antibodies (Molecular Probes, Eugene, OR; Alexa Fluor 594 goat anti-rabbit, 1:2,000; Alexa Fluor 546 goat anti-mouse, 1:1,000; Alexa Fluor 488 goat anti-mouse, 1:800). The slides were washed with cold PBS and coverslipped with 95% glycerol. To visualize cell nuclei, some cultures were coverslipped by using Vectashield with DAPI counterstain (Vector Laboratories, Burlingame, CA). The sections were observed under epifluorescence and photographed on the Olympus BX60 microscope.

Western Blot

Western blots were used to determine the presence of transcription factors in NT2 and hNT neurons. Protein samples were prepared immediately after the cells were thawed. The cells were washed and centrifuged three times in the DMEM/FBS/0.05% DNase, and the pellet was resuspended in cold PBS. Protein samples were suspended in lysis buffer and 1 μM dithiothreitol and sonicated. Protein samples (20 μg) and full-range molecular weight markers (Amersham Bioscience, Arlington Heights, IL) were resolved on 10% SDS-PAGE gel, and transferred to Invitrolon PVDF membranes (Invitrogen). The membranes were incubated in Tris-buffered saline (TBS) containing 5% nonfat milk and 0.1% Tween-20 for 1 hr at room temperature to block nonspecific binding, then incubated overnight in appropriate antibody at 4°C. The membranes were washed three times, for 10 min each, in TBS with 0.1% Tween-20 and incubated in peroxidase-conjugated anti-mouse IgG (1:30,000; Jackson Immunoresearch, West Grove, PA) or peroxidase-conjugated anti-rabbit (1:20,000) for 1 hr at room temperature. Primary and secondary antibodies were diluted in TBS, 5% nonfat milk, and 0.1% Tween-20. Immunoreactivity was visualized by using a West Pico Chemiluminescent Kit (Pierce Biotechnology, Rockford, IL). Digitized images of the films were analyzed in Image Pro-Plus (Media Cybernetics, Silver Springs, MD). The primary antibodies and their appropriate concentrations were Nurr1 (1:1,000, mouse; BD Biosciences), Ptx3 (1:500, rabbit; Abcam), En-1 (1:500, rabbit; Chemicon, Temecula, CA), β-catenin (1:5,000, rabbit; BD Biosciences), glycogen synthase kinase-3β (1:1,000, rabbit; Cell Signaling Technology, Beverly, MA), and Bcl-2 (1:500, mouse; Calbiochem, La Jolla, CA). In addition, we used b-actin (1:2,500, rabbit; Abcam) as a loading control to ensure the same amount of protein in each lane of the gel (data not shown).

Quantitative Analysis

For immunocytochemical analysis, the cells were coded and screened at random with a ×20 objective and a photographic frame (field = 0.3 mm^2). Counts were performed from 30 fields per condition (five pictures per well times six wells per condition) from three independent platings. To quantify the viability and total cell number for NT2 cells at 4 DIV, the Beckman Coulter Cell Counter was used with three samples from three independent platings. For Western blot analysis, three different protein samples were used, and Image Pro-Plus was used to determine the optical densities of the different samples. The values for each experiment were reported as mean ± SEM. Data were analyzed using analysis of variance (ANOVA), and the Newman-Keuls test was employed for the post hoc comparisons.

RESULTS

Expression of Dopaminergic Transcription Factors Engrailed-1 and Ptx3 in NT2- and RA-Treated hNT Cells

We used Western blot analysis to examine whether NT2 precursor cells and the pure neuronal population, hNT cells, express two essential midbrain DA transcription factors, En-1 and Ptx3. The correct band sizes of both En-1 and Ptx3 were detected in the NT2 precursors, however, the 3-, 4-, or 5-week RA-treated hNT cells did not express either of the two proteins (Fig. 1).

We next exposed NT2 cells to RA, Li, or KCl (a positive control) for 1 day and quantified the expression of Nurr1 and Ptx3 (Fig. 2). After 1 DIV, approximately 15.9% ± 0.098% and 3.1% ± 0.098% of untreated NT2 expressed Nurr1 and Ptx3, respectively. ANOVA revealed significant differences in the Nurr1 expression between the untreated and the treated groups (Fig. 2A; $F_{3,83} = 4.6$, $p < 0.0054$). After 24 hr of treatment, only the RA-treated group had significantly more Nurr1 expression ($w = 6.7$, $p < 0.05$), whereas no difference in Nurr1 expression was observed between the control Li ($w = 1.3$, $p > 0.05$)- and the KCl ($w = 0.8$, $p > 0.05$)-treated NT2 cells. None of the treatments influenced Ptx3 expression in NT2 cells (Fig. 2B; $F_{3,39} = 1.0$, $p = 0.40$).

These results show that the standard induction protocol for NT2 cells using RA increased only one of the essential transcription factors for DA (Nurr1) and either had no effect or decreased the other examined transcription factors. Therefore, in all subsequent studies in which we were trying to determine whether Li could induce DA transcription factors, we did not use the RA-treated hNT cells in which the transcription factors of interest had already been inhibited, but used the NT2 cells only.

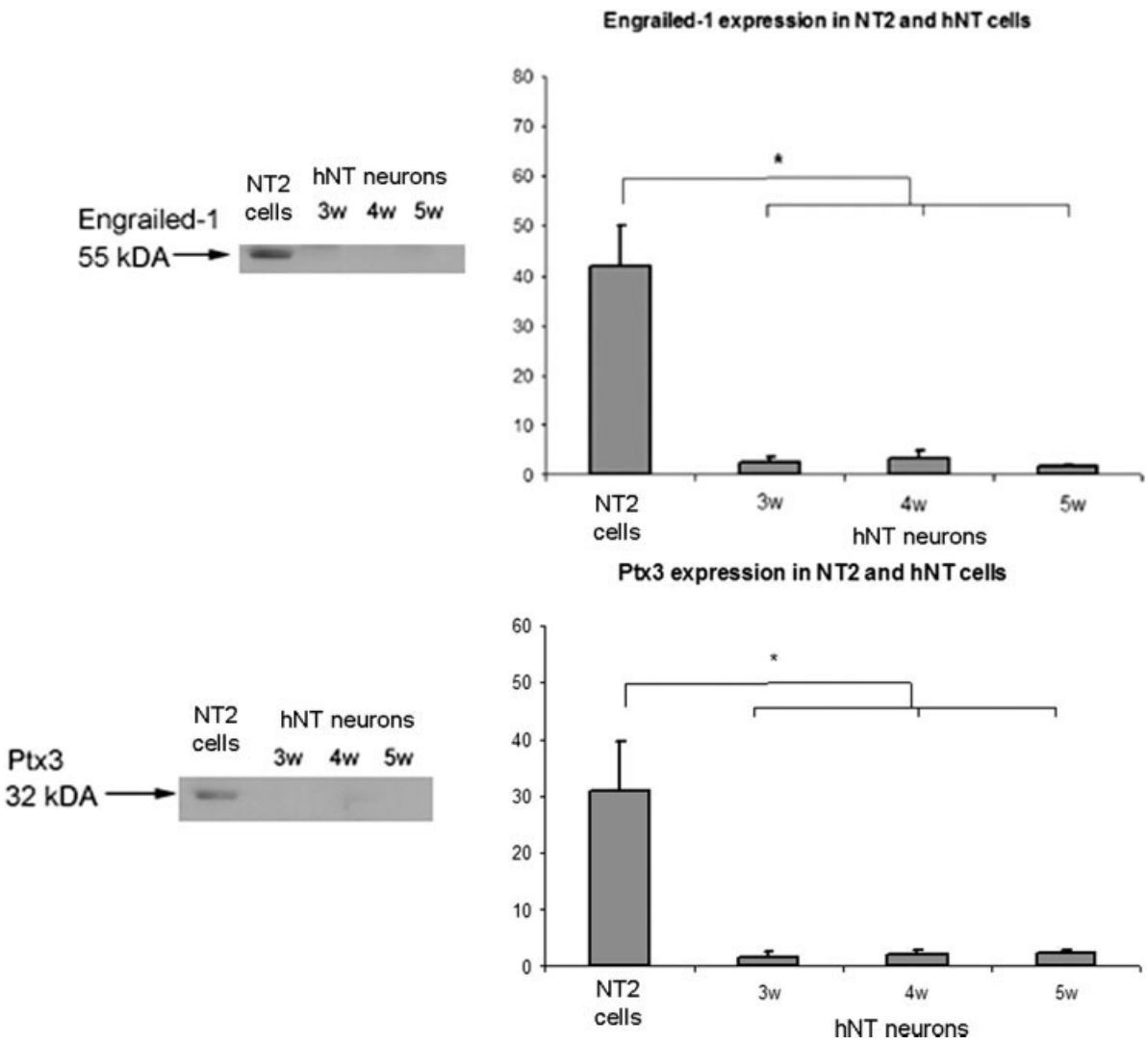

Fig. 1. Expression of En-1 and Ptx3 in NT2 and hNT cells. The correct band size of 55 kDA for Engrailed and 32 kDA for Ptx3 was observed in NT2 cells. Once hNT neurons were exposed to 3–5 weeks of RA treatment, they no longer expressed En-1 or Ptx3 protein. *$p < 0.01$.

Dose Effect of Lithium Treatment on the Viability and Total Number of NT2 Cells up to 4 DIV

In this study, we investigated whether lithium treatment could influence the expression of Ptx3 or En1 transcription factors in NT2 cells. We first determined the dose response of lithium on NT2 cells up to 4 DIV. We exposed NT2 cells to a dose range of 0.5–10.0 mM concentrations. When we performed the ANOVA, there was no significant difference in the viability between the Li-treated and the untreated NT2 cells at these concentrations (Fig. 3A). There was a significant difference in the total cell number between the untreated and treated NT2 cells (Fig. 3B; $F_{5,17} = 14.3$, $p < 0.01$).

The 1.0 mM ($w = 17.2$, $p < 0.01$), 3.0 mM ($w = 41.0$, $p < 0.01$), 6.0 mM ($w = 17.0$, $p < 0.01$), and 10 mM ($w = 11.6$, $p < 0.01$) Li-treated cultures had more cells compared with the untreated NT2 cells. There was no difference in the total cell number between the untreated and the 0.5 mM Li-treated NT2 cells ($w = 0.27$, $p > 0.05$). The number of cells peaked at 3.0 mM Li. There were significantly more NT2 cells in the 3.0 mM Li than in the untreated ($w = 41.0$, $p < 0.01$), 0.5 mM ($w = 48.0$, $p < 0.01$), and 1.0 mM Li ($w = 5.1$, $p < 0.05$) groups. Although the cell number had a tendency to decrease at 6 mM Li ($w = 5.3$, $p > 0.05$) and 10 mM Li ($w = 9.02$, $p > 0.05$), these values were not significantly different from values for the 3.0 mM Li-treated group.

Lithium's Influence on the Total Cell Number and Viability of NT2 Cells

Because the therapeutic dose of lithium treatment ranges between 0.5 mM and 1.5 mM, we used the dos-

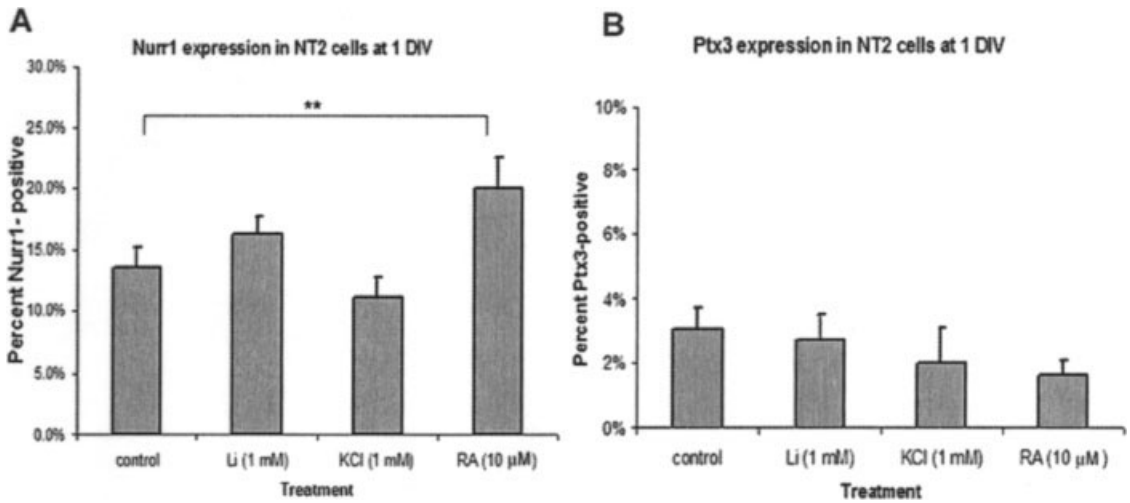

Fig. 2. Percentage of Nurr1- and Ptx3-positive NT2 cells after 1 day of lithium or retinoic acid treatment. **A:** About 12% of NT2 cells endogenously express Nurr1. Exposure to Li (1.0 mM) did not increase the Nurr1 expression, whereas RA treatment (10 μM) increased Nurr1 expression to 20%. **B:** About 3% of NT2 cells express Ptx3 after 1 DIV, and none of the treatments increased Ptx3 expression in NT2 cells. $^{**}p < 0.05$.

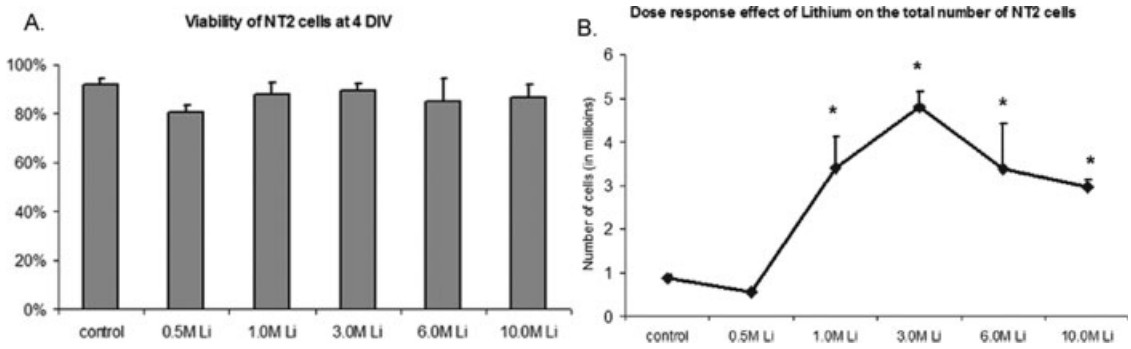

Fig. 3. Influence of lithium dosage on the proliferation and viability of NT2 cells after 4 DIV. **A:** Li treatment between 0.5 and 10 mM had no effect on the viability of NT2 cells. **B:** The total number of cells was significantly greater when NT2 cells were exposed to between 1.0 and 10 mM of Li treatment, with 3.0 mM containing the most NT2 cells. $^{*}p < 0.01$.

age of 1.0 mM for the remaining experiments. We next determined the influence of 1.0 mM of lithium treatment on the viability and proliferation of NT2 cells. NT2 cells are known to divide rapidly in culture and have to be passaged after 4 DIV. Because we wanted to determine lithium's effect without passaging the cells, we focused on 1 and 4 DIV.

When we examined the viability by using FDA to stain living cells green and PI, which labels dead cells red (Fig. 4A), the overall ANOVA revealed significant differences among the three groups, Li-treated, KCl-treated, and control NT2 cells (Fig. 4B; $F_{2,59} = 20.4$, $p < 0.0001$). The Li-treated NT2 cells had significantly more viable cells compared with the control NT2 cells ($w = 35.6$, $p < 0.01$) and the KCl-treated NT2 cells ($w = 24.7$, $p < 0.01$). There was no difference in the number of viable cells between the untreated NT2 cells and the NT2 cells treated with KCl ($w = 0.99$, $p > 0.05$). Even though the viability of the Li-treated NT2 cells was significantly greater than observed in the control groups, there was no difference in the total number of cells after 1 DIV (Fig. 4C; $F_{2,59} = 0.50$, $p = 0.61$).

Next we examined the total number and the viability of NT2 cells after 4 days of Li treatment. There was no difference in the total number of viable cells between the untreated and the Li-treated NT2 cells ($F_{1,5} = 0.43$, $p = 0.55$), both cultures being over 90% viable (Fig. 4D). However, there was a significant increase in the total number of cells in the Li-treated group (Fig. 4E; $F_{1,5} = 14.3$, $p = 0.02$) at 4 DIV. There

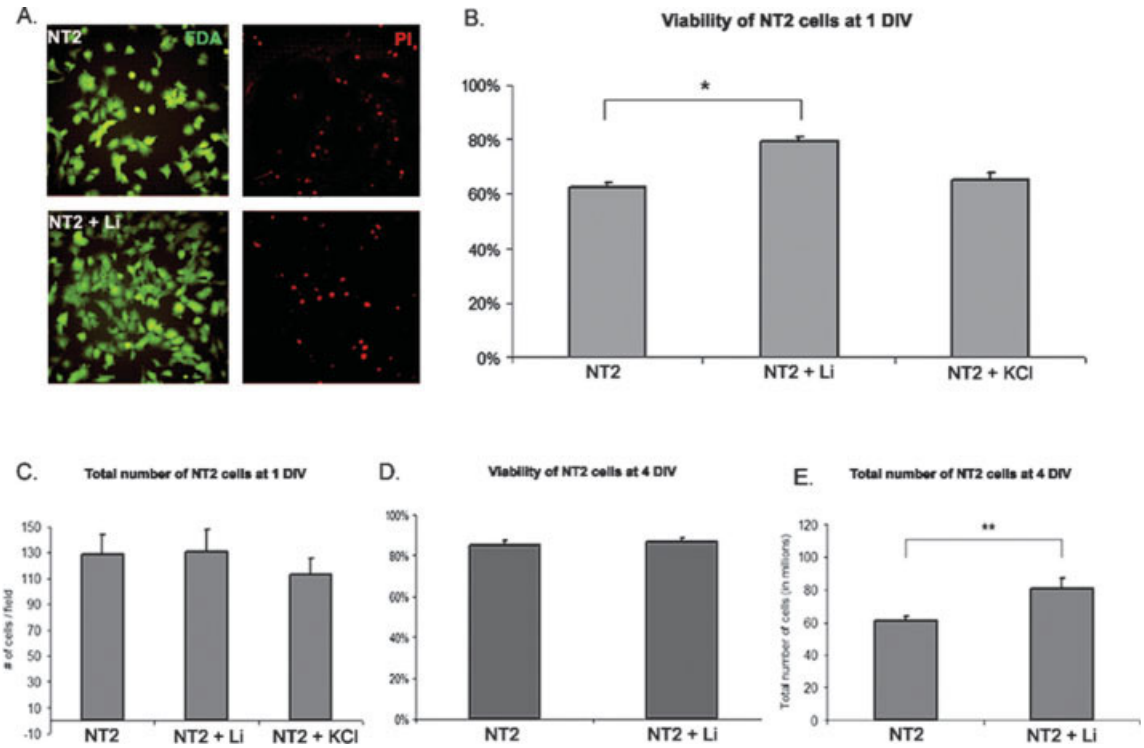

Fig. 4. Influence of lithium treatment on the viability and total cell number in NT2 cultures. **A,B:** The viability increased when NT2 cells were exposed to 1 day of Li as assessed by fluorescein diacetate (FDA) to stain living cells green and propidium iodide (PI), which stains dead cell red. **C:** Total NT2 cell number did not increase after 1 DIV. **D:** After 4 DIV, the viability of NT2 cells was above 80% in the Li-treated cultures, and the untreated NT2 cells increased from 60% at 1 DIV to above 80% at 4 DIV as well. **E:** After 4 DIV, the total cell number in the Li-treated cultures increased by approximately 20%. $^{*}p < 0.01$, $^{**}p < 0.05$.

was an average of 80 million cells in the Li-treated groups compared with 60 million in the untreated NT2 cells at 4 DIV ($w = 13.2$, $p < 0.05$).

Does Lithium Increase Cell Number by Increasing Proliferation or Increasing Antiapoptotic Signals in NT2 Cells

For the number of cells in culture after 4 DIV to increase, either the NT2 cells must be proliferating or there must be decreased cell death, or both. We first examined proliferation in these cultures by using immunocytochemical techniques. The Ki67 antigen is a marker for proliferating cells in all active phases of the cell cycle (G1, S, G2, and M phase) and is absent in resting (G0) cells. By using immunolabeling for Ki67 and counterstaining with DAPI (Fig. 5A, blue), we observed an increase in Ki67-immunolabeled proliferative cells in the Li-treated cultures (Fig. 5B, red). Nearly all the NT2 cells treated with Li were positive for Ki67 compared with only about half of the untreated and 1 mM KCl-treated NT2 cells (Fig. 5A). ANOVA revealed a significant difference between the treatment groups of NT2 cells (Fig. 5B; $F_{2,59} = 59.8$, $p < 0.0001$). Li-treated NT2 cells had significantly more Ki67-positive cells compared with the untreated NT2 cells ($w = 100.8$, $p < 0.01$) and KCl-treated NT2 cells ($w = 76.8$, $p < 0.01$). There was no difference in the number of Ki67-positive cells in the untreated and KCl-treated NT2 cells ($w = 1.63$, $p > 0.05$). Therefore, after only 1 day of Li treatment, there were significantly more NT2 proliferating cells than in the untreated group.

We also wanted to determine whether the increase in total cell number of NT2 cells with Li treatment might involve an increase of antiapoptotic protein B-cell lymphoma protein-2 (Bcl-2). We previously determined that 4 weeks of RA treatment increased the Bcl-2 protein in the neuronal hNT population. However, our ANOVA revealed no difference in the relative amount of Bcl-2 protein in the untreated and Li-treated NT2 cells at 4 DIV (Fig. 5D; $F_{1,7} = 0.35$, $p = 0.57$).

Lithium's Effect on the Expression of Dopaminergic Transcription Factors in NT2 Cells at 4 DIV

In this study, we used Western blot analysis to determine whether lithium treatment induced expression of En-1 (55 kDA), Ptx3 (35 kDA), and Nurr1 (70 kDA; Fig. 6A,C,E) in the NT2 cells. All three were expressed in the untreated NT2 population. When NT2 cells were exposed to 4 days of 1 mM of Li treatment, there was no change in the relative amount of En-1 (Fig. 6B; $F_{1,11} = 1.2$, $p = 0.31$) or Nurr1 (Fig. 6F; $F_{1,5} = 0.6$,

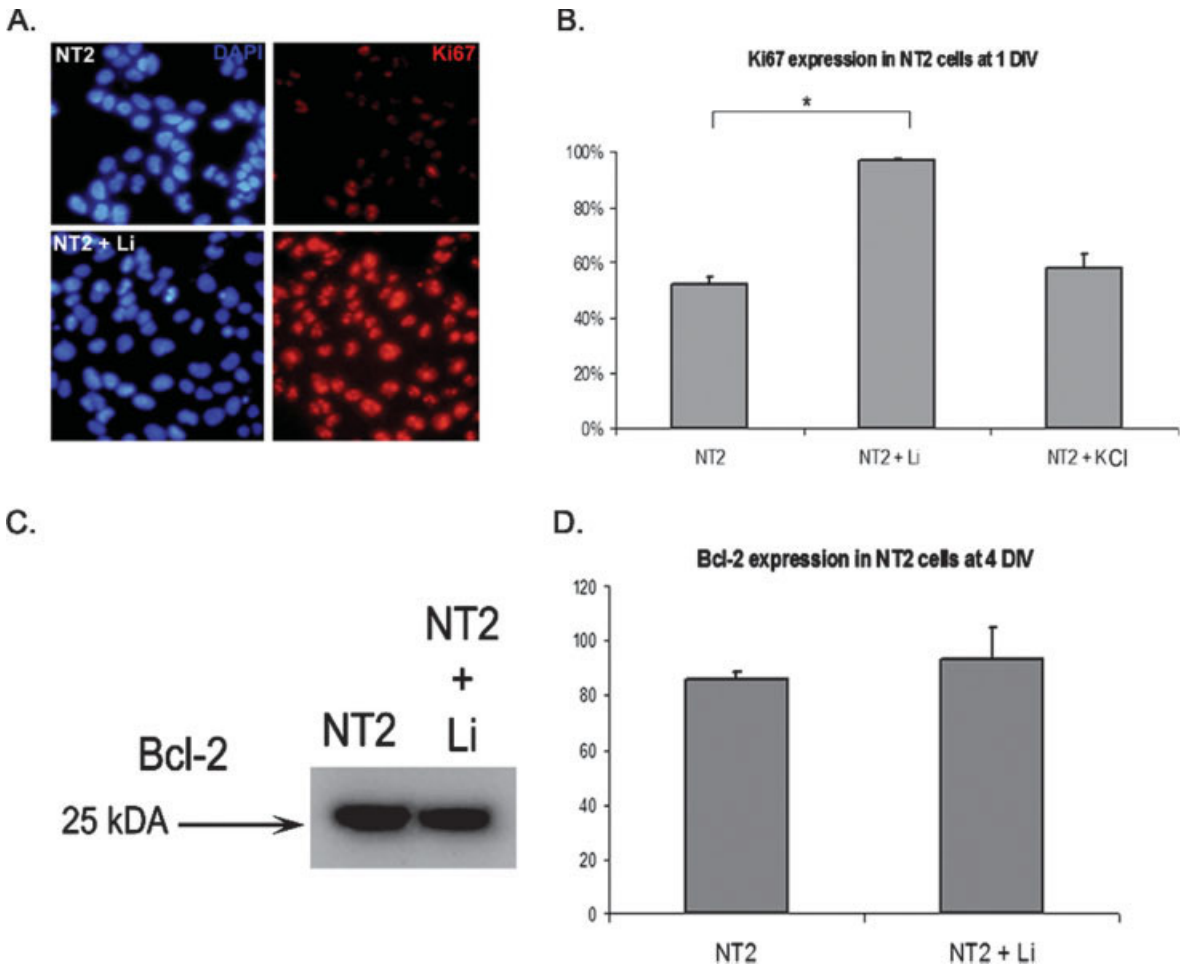

Fig. 5. Lithium may increase cell number through increased proliferation or increased antiapoptotic signals. **A:** NT2 cells were stained with a marker labeling proliferating cells, Ki67 (red), and their nuclei visualized with DAPI (blue). **B:** When the number of Ki67-positive cells was quantified after 1 DIV, about 50% of untreated NT2 cells were proliferating, whereas nearly 99% of Li-treated NT2 cells were Ki67 positive (red). A 25-kDA band for Bcl-2 was observed in NT2 cells (**C**); however, 4 days of Li (1.0 mM) did not increase the expression of Bcl-2 (**D**). *$p < 0.01$.

$p = 0.48$) protein. However, there was a significant decrease in the concentration of Ptx3 protein in NT2 cells after 4 days of Li treatment (Fig. 6D; $F_{1,7} = 6.2$, $p = 0.047$).

Lithium's Influences on the GSK3β/β-Catenin Pathway in NT2 Cells

Because lithium inhibits the activity of GSK3β, an important enzyme in the Wnt pathway, we examined the affect of lithium treatment on the expression of this enzyme and β-catenin, which activates transcription of Wnt target genes. Western blot analysis revealed bands for GSK3β (50 kDA) and β-catenin (90 kDA) in the NT2 cells (Fig. 7A,C). Four days of Li treatment did not inhibit the expression of GSK3β (Fig. 7B; 20 $F_{1,11} = .00$, $P = 0.99$) or increase the β-catenin (Fig. 7D; $F_{1,15} = 0.30$, $P = 0.59$) protein in NT2 cells.

DISCUSSION

Our Western blot analysis revealed that NT2 cells express two transcription factors, En-1 and Ptx3, critical for the proper development of midbrain DA neurons. The 3–5 weeks of RA treatment (10 μM) generated neurons were negative for both En-1 and Ptx3, suggesting that RA exposure does not drive NT2 cells down the DA pathway. In addition, exposing NT2 cells to Li treatment for up to 4 days (1 mM) also did not influence En-1, Ptx3, or Nurr1 expression; however, Li treatment did significantly increase viability and proliferation of this cell line.

Development of Midbrain DA Neurons

The transcription factors En-1 and Ptx3 as well as Nurr1 are critical for the proper development of midbrain DA neurons (Zetterstrom et al., 1996; Baffi et al., 1999; Simon et al., 2001, 2003; Smidt et al., 2004a,b).

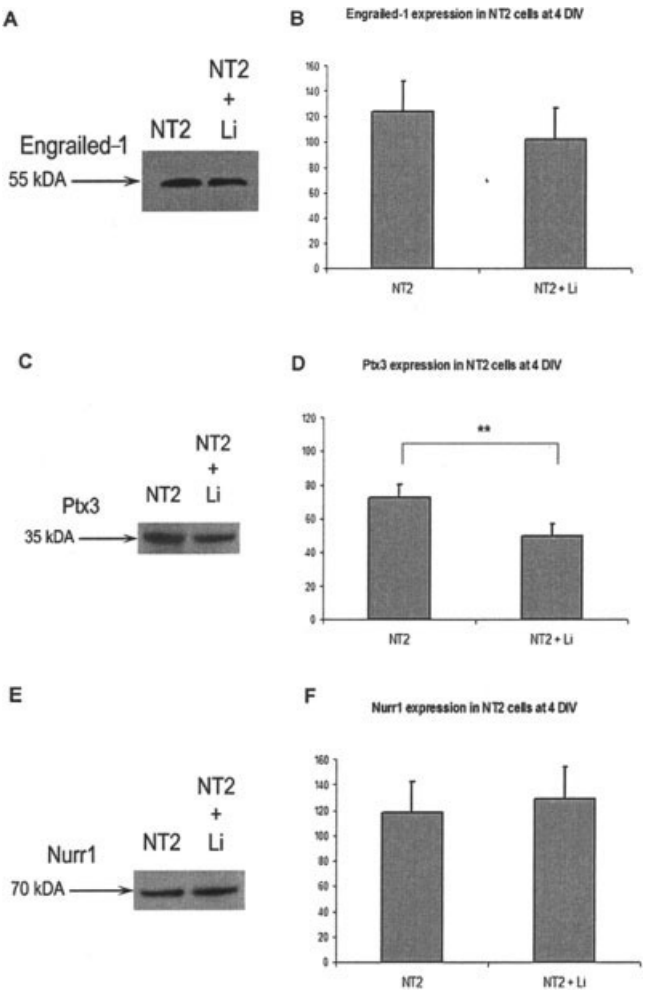

Fig. 6. Expression of essential dopaminergic transcription factors in NT2 cells after lithium treatment. Four days of Li treatment (1.0 mM) did not increase the expression of En-1 (**A,B**) or Nurr1 (**E,F**) in NT2 cells. Furthermore, Li-treated cells showed a decrease in relative Ptx3 protein (**C,D**). $^{**}p < 0.05$.

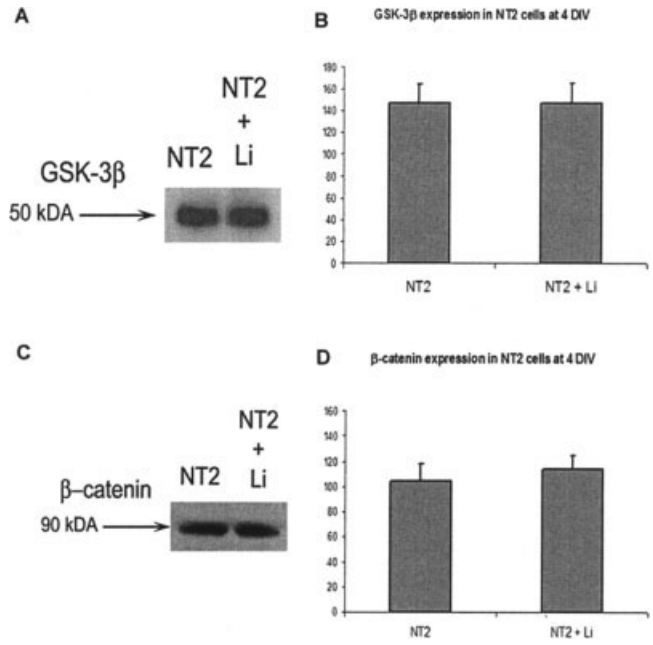

Fig. 7. Western blot analysis of the GSK3β/β-catenin pathway in NT2 cells. Four days of Li treatment (1.0 mM) did not inhibit the GSK3β (**A,B**) in NT2 cells, so no accumulation of β-catenin (**C,D**) was observed.

In addition, all three transcription factors are also expressed into adulthood, suggesting their importance in maintaining the DA phenotype (Saucedo-Cardenas and Conneely, 1996; Zetterstrom et al., 1996; Smidt et al., 1997). Furthermore, mutations in any of these three genes have been implicated in various neurological diseases, including Parkinson's disease (PD; Xiao et al., 1996; Kruger et al., 1999a,b; Piccini et al., 1999; Oliveri et al., 2000; Ramsden et al., 2001; Xu et al., 2002; Carmine et al., 2003; Klein et al., 2003; Le et al., 2003; Zimprich et al., 2003, 2004; Huang et al., 2004). For instance, reduced expression of Nurr1 increased the vulnerability of midbrain DA neurons to 1-methyl-4-phenyl-1,2,3,6-tatrahydropyridine (MPTP)-induced injury, which is used as a model for PD (Le et al., 1999). Moreover, a polymorphism in the Nurr1 intron was found in patients with PD (Xu et al., 2002). In addition, two heterozygous mutations on Nurr1 were revealed in 10 of 107 individuals with familial PD (Le et al., 2003). Finally, genetic mutations in α-synuclein have been implicated in PD and En-1 and En-2 have been shown to regulate α-synuclein (Polymeropoulos et al., 1997; Simon et al., 2001).

RA's Influence on the Dopaminergic Potential

All three critical transcription factors, En-1, Nurr1, and Ptx3, must be present in the neuron to be considered a true midbrain DA neuron. The fact that RA increases the expression of Nurr1 and not Ptx3 or En-1 suggests that it does not drive the NT2 cells toward the DA phenotype. Multiple pathways are activated in parallel for the proper development of VM cells. The earliest known transcription factor expressed in proliferating VM precursors cells, Lmx1b, is known to act on the Wnt pathway as well as to increase the expression of Ptx3 (for review see Burbach et al., 2003). The Wnts are highly conserved glycoproteins important for development (McMahon and Bradley, 1990; Thomas and Capecchi, 1990; Nusse, 1992; Nusse and Varmus, 1992; Salinas and Nusse, 1992; Parr et al., 1993; Fritzsch et al., 1995; Danielian and McMahon, 1996). Three specific Wnt proteins, Wnt1, Wnt3a, Wnt5a, have been implicated in the development of midbrain DA neurons and were shown to influence the expression of En-1, Ptx3, and Nurr1 (Castelo-Branco et al., 2003). Wnt3a promoted the proliferation of precursor cells expressing Nurr1 but did not increase the number of TH-positive

neurons. Wnt1 and Wnt5a increased the number of TH-positive neurons. Wnt1 predominantly increased the proliferation of Nurr1 precursors, whereas Wnt5a increased the proportion of Nurr1-positive precursors that acquired the DA phenotype and up-regulated Ptx3. Recently, the expression of various Wnts in NT2 cells after 72 hr of RA treatment was reviewed (Katoh, 2002a). RA exposure decreased Wnt3a and slightly down-regulated Wnt5a (Katoh, 2002b; Saitoh and Katoh, 2002). Our observations are consistent with those of Mena and colleagues (1994), who showed that RA suppresses the DA phenotype and induces choline acetyltransferase activity in fetal rat midbrain neurons and a human neuroblastoma cell line.

Lithium's Effect on the NT2 Cell Line

En-1, Ptx3, and Nurr1 are critical transcription factors for the development of the DA phenotype in nigral neurons. Li treatment of the NT2 cells had no effect of En-1 or Nurr1 expression, but it decreased Ptx3 expression. All of these transcription factors can be influenced by the Wnt signaling pathways. Insofar as two of the three transcription factors did not change after 4 days of 1.0 mM Li treatment, the observation that GSK3β activity and β-catenin activity did not change either might not be too surprising, even though lithium does inhibits GSK3β activity.

It might be necessary to treat NT2 cells for over 4 days to see a decrease in GSK3β. In patients treated for manic depressive disorder, it can take up to 2 weeks for lithium to induce its therapeutic effects (Jacobson, 2001). The observation that Ptx3 activity decreased while GSK3β activity did not change could be interpreted to mean that lithium's effects are not mediated through the GSK3β/β-catenin signaling pathway, although it may still be modulating Wnt signaling; for example, the ability of Wnt-11 to induce cardiogenesis involves the protein kinase C (PKC) signaling cascade (Pandur et al., 2002; Koyanagi et al., 2005). Furthermore, Wnt-11 modulates cell proliferation through the PKC-calcium signaling pathways (Ouko et al., 2004); cellular proliferation is altered by our Li treatments. Wnt-11 is found in the NT2 cells prior to RA induction (Katoh, 2002a).

Li treatment did increase the viability and proliferation of the NT2 cells. After 24 hr of Li treatment, Li-treated NT2 cells were more viable compared with the untreated NT2 cells as assessed by FDA/PI staining. In addition, untreated NT2 cultures had fewer proliferating cells than Li-treated NT2 cultures as determined with Ki67 immunolabeling; this resulted in significantly more cells in Li-treated cultures at 4 days. The viability remained above 80% in the Li-treated group, whereas untreated NT2 cells increased from 65% at 1 DIV to 80% by 4 DIV. Once again, as was observed in the dose-response curve, there was about a 20% increase in total cell number after 4 days of Li treatment.

Although increased immunostaining for Ki67 suggests an increase in proliferation, the increase in cell number could also be a function of a decrease in cell death. To address this issue, we examined the effect of Li treatment on an antiapoptotic marker, B-cell lymphoma protein-2 (Bcl-2). In vivo Li treatment was shown to increase the expression of Bcl-2 by inhibiting GSK3β (Manji et al., 2000b). We showed that Bcl-2 was not enhanced after 4 days of Li treatment, as we suspected, in that it involves the GSK3β pathway. We can conclude that the increase in total cell number by Li treatment was not due to an increase in the survival-promoting antiapoptotic protein Bcl-2 at the examined time point. However, long-term exposure (>7 DIV) might be necessary to observe an up-regulation of antiapoptotic markers. For example, it took 7 days of Li treatment to protect cultured rat cerebellar, cerebral cortical, and hippocampal neurons against glutamate-induced excitotoxicity by the up-regulation of antiapoptotic markers (Nonaka et al., 1998; Grimes and Jope, 2001). Alternatively, Bcl-2 may be an early signal that initiated antiapoptotic pathways but was no longer elevated at 4 DIV. While not directly measuring markers of apoptosis, the FDA/PI data at 1 DIV suggested that there was less cell death underway in the Li-treated cultures. Li might also directly inhibit apoptotic pathways, such as those mediated through Bax signaling (Somervaille et al., 2001).

One possible mechanism for Li's action on proliferation is by inhibition of inositol monophosphatase (IMPase) and/or related phosphomonoesterases (Berridge and Irvine, 1989; York et al., 1995). The inhibition of IMPase decreases inositol, which is necessary for the production of various second messengers, such as diacylglycerol (DAG) and inositiol-1,4,5-triphosphate (IP3); these activate PKC and increase intracellular calcium, respectively (Majerus, 1992; Gould and Manji, 2002). Previous research showed that lithium stimulated cell proliferation in some cell types, and this was thought to involve the phosphoinositol pathway (Hori and Oka, 1979; Ptashne et al., 1980; Korycka and Robak, 1991). Recently, it was also demonstrated that Li treatment increased the number of neural progenitor cells both in vitro and in vivo (Hashimoto et al., 2003; Kim et al., 2004). Furthermore, proliferation was also induced by Li in glial cells in the rat pituitary (Levine et al., 2002). NT2 cells have properties similar to those of neural progenitor cells, in that they express various neural antigens, self-replicate, and produce all three neural lineages, neurons, astrocytes, and oligodendrocytes (Andrews et al., 1984; Lee and Andrews, 1986; Pleasure and Lee, 1993; Langlois and Duval, 1997; Bani-Yaghoub et al., 1999; Sandhu et al., 2002). The fact that Li stimulates proliferation in NT2 cells as it does in neural precursors provides yet another example of a similarity between NT2 cells and neural precursors. In addition, increasing cell proliferation may also be important in expanding cell lines, especially cell lines with a low percentage of progenitor cells. Recently, by using DNA microarrays, the Wnt pathway was shown to increase proliferation and cell adhesion of $CD34^+$ thymocytes; CD34 is a marker for hematopoietic progenitor cells (Staal et al., 2004).

Therefore, pretreatment with Li may be an effective protocol for expanding stem cell lines.

CONCLUSIONS

Because the NT2 cells express three transcription factors essential for the development of midbrain DA neurons and have properties similar to those of neural progenitor cells, they are a good source of human cells to use as a model for studying the effect of various pharmacological treatments on proliferation, differentiation, and apoptosis. Different approaches are currently being explored to increase the dopaminergic phenotype in various cell types, such as embryonic stem cells, neural precursors, and mesenchymal cell lines (De Boer et al., 2004). Various TH-inducing agents such as acidic fibroblast growth factor (FGF) and coactivating substances, including 12-O-tetradecanoylphorbol-13-acetate (TPA; PKC activator), 3-isobutyl-1-methylxanthine (IMBX; phosphodiesterase inhibitor), forskolin (PKA activators), and the neurotransmitter DA, can increase TH expression dramatically especially if given in a cocktail 1 week after plating. In NT2 cells, the combination of sonic hedgehog (SHH) and FGF8 increased TH expression by only 2%; however, in conjunction with a cocktail of the known activators listed above, it increased the expression of TH in hNT neurons up to 80% (Iacovitti et al., 2001; Stull and Iacovitti, 2001). Our laboratory has also demonstrated that the TH expression in hNT neurons can be influenced by lithium or by coculturing hNT neurons with Sertoli cells, which are known to secrete several trophic factors (Othberg et al., 1998; Willing et al., 1999; Saporta et al., 2004). It might be necessary to increase the time for which NT2 cells are exposed to lithium to affect the Wnt pathway and eventually induce the expression of TH. Chen and colleagues (1998) reported that chronic Li administration (1.0 mM) resulted in a significant increase in TH levels in the rat frontal cortex, hippocampus, and striatum. However, Li affects multiple pathways, so it will be necessary to identify specifically the pathway by which Li induces TH expression and find more specific agonist/antagonist to produce the desired result.

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