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Transforming Growth Factor β Cooperates with Persephin for Dopaminergic Phenotype Induction

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Key Words. Neurotrophins • Cell fate • Stem cells • Transforming growth factor β • Neuronal differentiation

ABSTRACT

The aim of the present study was to investigate the putative cooperative effects of transforming growth factor β (TGF-β) and glial cell line-derived neurotrophic factor (GDNF) family ligands in the differentiation of midbrain progenitors toward a dopaminergic phenotype. Therefore, a mouse midbrain embryonic day (E) 12 neurospheres culture was used as an experimental model. We show that neurturin and persephin (PSPN), but not GDNF, are capable of transient induction of dopaminergic neurons in vitro. This process, however, requires the presence of endogenous TGF-β. In contrast, after 8 days in vitro GDNF rescued the TGF-β neutralization-dependent loss of the TH-positive cells. In vivo, at E14.5, no apparent phenotype concerning dopaminergic neurons was observed in *Tgf-β2*$^{-/-}$/*gdnf*$^{-/-}$ double mutant mice. In vitro, combined TGF-β/PSPN treatment achieved a yield of approximately 20% TH-positive cells that were less vulnerable against 1-methyl-4-phenyl pyridinium ion toxicity. The underlying TGF-β/PSPN differentiation signaling is receptor-mediated, involving p38 mitogen-activated protein kinase and phosphatidylinositol 3-kinase pathways. These results indicate that phenotype induction and survival of fully differentiated neurons are accomplished through distinct pathways and individual factor requirement. TGF-β is required for the induction of dopaminergic neurons, whereas GDNF is required for regulating and/or maintaining a differentiated neuronal phenotype. Moreover, this study suggests that the combination of TGF-β with PSPN is a potent inductive cocktail for the generation of dopaminergic neurons that should be considered in tissue engineering and cell replacement therapies for Parkinson's disease. STEM CELLS *2008;26:1683–1694*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Stem cells have been considered as potent candidates in cell replacement therapies for neurodegenerative disorders, such as Parkinson's disease [1]. Consequently, the signals and molecular mechanisms that mediate and regulate the differentiation of stem/progenitor cells into dopaminergic neurons have been extensively investigated. Thus, many studies have focused on elucidating the transcriptional code specific for the induction and specification of mesencephalic dopaminergic neurons. Analysis of deficient mice has identified transcription factors, such as Nurr1, Pitx3, En1, Lmx1b, and Lmx1a, as key components in the model of dopaminergic neuron development [2–7]. Together with the signaling molecules sonic hedgehog and fibroblast growth factor (FGF) 8, the role of transforming growth factor β (TGF-β) has also been highlighted [8–12].

Glial cell line-derived neurotrophic factor (GDNF), neurturin (NRTN), artemin (ARTN), and persephin (PSPN), also named the GDNF family ligands (GFLs), are distant members of the TGF-β superfamily. GFLs signal through a multicomponent receptor system comprised of a high-affinity binding component, a GPI-linked GDNF family receptor (GFR) α subunit, and a common signaling component, the transmembrane tyrosine kinase Ret (for review, see [13]). GFLs differentially promote survival and regulate differentiation in peripheral and central neuronal populations. Because of their survival-supporting effect on midbrain dopaminergic neurons, GFLs have been investigated in several animal models of Parkinson's disease. It appears that, besides the well-documented neuroprotective effects of GDNF on midbrain dopaminergic neurons in vitro and in vivo, NRTN prevented degeneration of mature dopaminergic neurons in the 6-hydroxydopamine model [14–16]. Moreover, ARTN and PSPN have been shown to have effects equivalent to those of GDNF and NRTN and prevented the loss of dopaminergic neurons in lesion models of Parkinson's disease as well [17, 18].

Author contributions: E.R.: conception and design, financial support, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; O.O.: collection and assembly of data, data analysis and interpretation; B.R., S.Hee., and S.Hei.: collection and assembly of data; M.W.: collection and assembly of data, data analysis and interpretation; K.K.: conception and design, financial support, data analysis and interpretation, manuscript writing, final approval of manuscript.

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During the last decade, an increasing number of studies have provided convincing evidence that multiple ways of interactions between members of the superfamily, as well as between families of growth factors and neurotrophins, may occur, by means of receptor cross-talk, cooperativity, or dependence of one factor on the presence of a cofactor. Although TGF-β, GDNF, NRTN, ARTN, and PSPN themselves are able to support survival of mesencephalic dopaminergic neurons, GDNF requires TGF-β to exert its entire neurotrophic potential in vitro and in vivo [19, 20]. TGF-β is apparently required for recruitment of the GFRα1 receptor in the plasma membrane [21]. Moreover, although NRTN, ARTN, and PSPN promote the morphological differentiation of mesencephalic dopaminergic neurons [22], midbrain neural progenitors require TGF-βs as an inductive signal to acquire a dopaminergic cell fate [12]. In the developing midbrain GDNF, NRTN, ARTN, and PSPN are spatially and temporally differentially expressed. Whereas no GDNF expression could be detected in the ventral midbrain during mouse embryonic development, NRTN expression was found at embryonic days (E) 10–14 but was absent at E16 and E18 [23]. Moreover, although in the rat ventral mesencephalon PSPN mRNA was highly expressed prenatally [24], the ventral mesencephalon of E14 rats lacks ARTN mRNA expression [25].

Surprisingly, *gdnf*$^{-/-}$ [26–28], *nrtn*$^{-/-}$ [29], *artn*$^{-/-}$ [30], and *pspn*$^{-/-}$ [31] mice lack severe deficits in dopaminergic neurons. In contrast, in *Tgf-β2*$^{-/-}$/*Tgf-β3*$^{+/-}$ and in *Tgf-β2*$^{+/-}$/*Tgf-β3*$^{-/-}$ mice the number of TH-immunopositive cells at E14.5 was significantly reduced compared with wild-type mice [12].

In the present study we investigated putative cooperative effects of TGF-β with GFLs on midbrain progenitor cell differentiation toward dopaminergic cell fate. The results demonstrate that phenotype induction and survival of fully differentiated neurons are accomplished through distinct pathways and requirements for individual factors. TGF-β is required for the induction of dopaminergic neurons, whereas GDNF is required for regulating and/or maintaining a differentiated neuronal phenotype. Moreover, this study suggests that TGF-β combined with PSPN is a potent inductive cocktail for the generation of dopaminergic neurons that should be considered in tissue engineering.

Materials and Methods

TGF-β, GDNF, NRTN, PSPN, and FGF2 were obtained from tebu-bio (Offenbach, Germany, http://www.tebu-bio.com). Lyophilized factors were resuspended in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, http://www.invitrogen.com) with 0.25% bovine serum albumin (BSA) (Sigma-Aldrich, Taufkirchen, Germany, http://www.sigmaaldrich.com), 100 U/ml penicillin, 0.5 μg/ml streptomycin, and 100 μg/ml neomycin (all from Gibco) to give a final concentration of 1 μg/ml and stored in aliquots of 50–100 μl at −70°C until use. Lyophilized TGF-β1 was resuspended in 1 mM HCl containing 0.25% BSA to give a final concentration of 0.5 μg/ml and stored in aliquots of 50 μl at −70°C until use. The neutralizing monoclonal mouse anti-TGF-β antibody (MAB1835) recognizing all three isoforms was obtained from R&D Systems Inc. (Wiesbaden, Germany, http://www.rndsystems.com). Activity of this antibody was determined using the Mv1Lu mink lung epithelial cell system [32, 33]. SB431542 was purchased from Tocris Bioscience (Bristol, U.K., http://www.tocris.com), SB203580 and LY294002 were obtained from Calbiochem (Schwalbach am Taunus, Germany, http://www.emdbiosciences.com). 1-Methyl-4-phenyl pyridinium ion (MPP^+) was purchased from Sigma-Aldrich.

Dot Blot

An aliquot of 40 ng of TGF-β1, GDNF, NRTN, or PSPN was loaded onto a nitrocellulose membrane. The membrane was blocked with 3% low-fat milk powder in Tris-Tween 20-buffered saline (TTBS) and incubated with primary anti-TGF-β1,2,3 antibody (1:200) overnight at 4°C. The following day the membrane was incubated with anti-mouse IgG coupled to peroxidase (1:10,000) and was developed using the Amersham enhanced chemiluminescence detection system. Signals were visualized on x-ray film.

Cell Culture of E12 Ventral Mesencephalon

Ventral mesencephalon from mouse E12 was isolated as described earlier [11, 12, 34]. Briefly, pregnant NMRI mice were sacrificed by cervical dislocation, and E12 embryos were collected in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (Sigma-Aldrich). The day of vaginal plug identification was designated E1. Ventral mesencephalon was dissected, freed from meninges, and incubated in 0.15% trypsin for 15 minutes at 37°C. Small pieces of ventral mesencephalon were subsequently dissociated by gentle trituration using fire-polished Pasteur pipettes. Dissociated cells were resuspended in high-glucose DMEM-Ham's F-12 medium supplemented with 0.25% BSA, N1 additives, and 100 U/ml penicillin, 0.5 μg/ml streptomycin, and 100 μg/ml neomycin (Gibco). Cells in suspension were plated in noncoated culture dishes and cultured in the presence of 20 ng/ml FGF2. After 3 days in culture, formation of neurospheres was evident. Cells were allowed to expand for 7 days before the neurospheres were dissociated, and cells were plated onto polyornithin- and laminin-coated 12 mm^2 glass coverslips in 24-well plates at a density of 200,000 cells/coverslip. FGF2 was removed from the expansion medium, and cells were incubated in serum-free medium in a 95% air/5% CO_2 atmosphere at 37°C.

One, 4, and 7 days after plating (day in vitro [DIV] 1, 4, and 7) factors were applied at a final volume of 750 μl of medium at the following concentrations: 1 ng/ml TGF-β1, 10 μg/ml anti-TGF-β antibody, 10 ng/ml GDNF, 10 ng/ml NRTN, and 10 ng/ml PSPN. LY294002, SB431542, and SB203580 were applied at 20 nM, 10 μM, and 20 μM, respectively. At DIV3, DIV5, and DIV8 cells were fixed and processed for immunocytochemistry.

Immunocytochemistry

Immunocytochemistry on cultured cells was performed essentially as described earlier [12, 19]. Cultures were fixed in 4% paraformaldehyde for 30 minutes at room temperature, permeabilized with acetone for 10 minutes at −20°C, and blocked with 10% normal goat serum (NGS) in phosphate-buffered saline (PBS). Subsequently cells were incubated overnight at 4°C with primary antibodies diluted in PBS containing 10% NGS and 0.1% Triton X-100. Rabbit polyclonal anti-TH (1:1,000) (Chemicon, Hofheim, Germany, http://www.chemicon.com) and anti-Pitx3 (1:400) (Chemicon), mouse monoclonal anti-Nurr1 (1:200) (Santa Cruz Biotechnology; Santa Cruz, CA, http://www.scbt.com), anti-β-III-tubulin (1:25) (Developmental Studies Hybridoma Bank, Iowa City, IA, http://www.dshb.biology.uiowa.edu), and anti-GABA (1:50) (Sigma-Aldrich) and goat polyclonal anti-Pitx3 (1:50) (Santa Cruz Biotechnology) were used as primary antibodies. Donkey anti-rabbit, anti-mouse, or anti-goat IgGs coupled to either indocarbocyanin (CY3) or to fluorescein isothiocyanate (FITC) were used as secondary antibodies. Cells were viewed with a fluorescence microscope (Carl Zeiss, Jena, Germany, http://www.zeiss.com).

In control experiments performed by omitting the primary antibody, immunostaining was abolished. In addition, the specificity of Pitx3 labeling was assessed by preincubating the goat polyclonal antibody (1:50) with the respective blocking peptide (1:10) (Santa Cruz Biotechnology).

For immunodetection of c-Ret, GFRα1, GFRα2, and GFRα4, the following protocol was applied: fixed cells were incubated with 1% SDS diluted in PBS for 5 minutes and subsequently washed with PBS. After blocking with 1% BSA diluted in PBS for 15 minutes, cells were incubated with primary antibodies diluted in PBS containing either 10% NGS (for c-Ret and GFRα2) or 10% horse serum (for GFRα1 and GFRα4) and 0.1% Triton X-100 overnight at 4°C. Rabbit polyclonal anti-c-Ret (1:25) and anti-

GFRα2 (1:25) (Santa Cruz Biotechnolgy), and goat polyclonal anti-GFRα1 (1:25) and -GFRα4 (1:25) (Santa Cruz Biotechnology) were used as primary antibodies. Donkey anti-rabbit or anti-goat IgGs coupled to CY3 were used as secondary antibodies.

Reverse Transcriptase-Polymerase Chain Reaction

Total RNA was isolated from mouse ventral mesencephalic primary dissected tissue, using the Qiagen RNeasy kit and following the manufacturer's instructions (Qiagen, Hilden, Germany, http://www.qiagen.com). Total RNA (1.0 μg) was reverse transcribed from an oligo(dT) primer using a Omniscript kit (Qiagen). Samples (3 μl) of the reverse transcriptase (RT) reaction were used in a polymerase chain reaction (PCR) containing 2.5 U of *Taq* DNA polymerase, 0.2 μM concentrations of specific primers, 1× PCR buffer (Qiagen), 200 μM deoxynucleoside triphosphates, and 2 mM $MgCl_2$. For amplification of cDNA encoding receptors of the GDNF family members the following forward (F) and reverse (R) primer sequences were used:

C-Ret F: 5′-TGGCACACCTCTGCTCTATG-3′ (corresponding to nucleotides [nt] 749–768; GenBank Accession number NM 001080780)
C-Ret R: 5′-TGTTCCCAGGAACTGTGGTC-3′ (corresponding to nt 934-915; GenBank accession number NM 001080780)
GFRα1 F: 5′-GGGCACATGGAAGTTTCTTT-3′ (corresponding to nt 2,715-2,734; GenBank accession number NM 010279.2)
GFRα1 R: 5′-TATCAGGAGACCGTGCACCA-3′ (corresponding to nt 3,170-3,151; GenBank accession number: NM 010279.2)

for full-length GFRα2 (GenBank accession number NM 008115.2):

GFRα2 F: 5′-CCACCATGATCTTGGCAAACGCCTTC-3′
GFRα2 R: 5′-CTAGGCCAAGGTCACCATCAG-3′

for full-length GFRα4:

GFRα4 F: 5′-CCACCATGGCCCACTGCATGGAGTC-3′
GFRα4 R: 5′-TTCAGCTCAGTGAGCAGTCATCG-3′

For detection of cDNAs encoding the c-Ret and GFRα1 proteins, the protocol was denaturation at 95°C for 15 minutes, for the optimum number of cycles (depending on primer pair) of PCR amplification under the following conditions: denaturation at 94°C for 1 minutes, annealing at the appropriate temperature (depending on primer pair) for 1minute, and elongation at 72°C for 1 minutes. Final extension at 72°C for 10 minutes was terminated by rapid cooling at 4°C.

For amplification of GFRα2 and GFRα4, the following protocol was used. After denaturation at 95°C for 2 minutes, nine cycles were performed at 95°C for 40 seconds, 55°C for 40 seconds, and 72°C for 1 minute 20 seconds, followed by 26 cycles at 95°C for 40 minutes, 55°C for 40 minutes, and 72°C for 1.20 minutes, increasing by 5 seconds for each cycle. Final extension at 72°C for 7 minutes was terminated by rapid cooling at 4°C.

PCR products were analyzed by agarose gel electrophoresis. The size of the reaction products was determined after ethidium bromide staining.

Immunoblotting

Cells were harvested in 10 ml of ice-cold homogenizing buffer containing 280 mM mannitol, 10 mM HEPES, 10 mM KCl, 1 mM $MgCl_2$, adjusted to pH 7.0, and a protease inhibitor "cocktail" (10 μM leupeptin, 2 mM benzamidine, and 0.1 mM Pefabloc SC), scraped off the culture flasks with a rubber policeman, pelleted by centrifugation at 250*g* for 5 min, and resuspended in homogenization buffer. Homogenization was performed by sonication. Protein concentration was determined according to Bradford [35]. Electrophoresis and blot procedures were performed as described [36]. Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Blots were blocked with 3% nonfat dry milk in TTBS for 8 hours and incubated with primary antibody overnight at a dilution of 1:500 for phosphorylated extracellular signal-regulated kinase (pERK) (rabbit polyclonal, obtained from Santa Cruz Biotechnology), phosphorylated Akt (pAkt) (rabbit polyclonal purchased from Cell Signaling Technology, Beverly, MA, http://www.cellsignal.com; New England Biolabs, Frankfurt, Germany, http://www.neb.com), and anti-GFRα4 (goat polyclonal; Santa Cruz Biotechnology) and 1:10,000 for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (mouse monoclonal purchased from Abcam, Cambridge, U.K., http://www.abcam.com), respectively. After incubation with goat anti-mouse, goat anti-rabbit, or donkey anti-goat IgG coupled to horseradish peroxidase secondary antibodies (1:10,000 dilution), blots were developed in enhanced chemiluminescence reagents, and signals were visualized on x-ray film. Subsequently, films were scanned using a flat-bed scanner and the signal ratios pERK/GAPDH, pAkt/GAPDH, and GFRα4/GAPDH were quantified densitometrically. Differences in signal ratios were tested for significance using Student's *t* test. Results with levels of $p < .05$ were considered significant.

Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling Assay

Dissociated mouse E12 ventral mesencephalic neurospheres were plated onto 12 mm^2 coverslips at a density of 200,000 cells/well. Cultures were treated with TGF-β alone or in combination with PSPN at DIV1. At DIV2 and DIV3 cells were fixed in 4% paraformaldehyde and subsequently washed. For detection of apoptotic cells, a terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay kit from Roche Diagnostics (Basel, Switzerland, http://www.roche-applied-science.com) was used following the manufacturer's instructions. Apoptotic cells were then visualized by fluorescence microscopy.

5-Bromo-2′-Deoxyuridine Incorporation

Dissociated mouse E12 ventral mesencephalic neurospheres were plated onto 12 mm^2 coverslips at a density of 200,000 cells/well. For 5-bromo-2′-deoxyuridine (BrdU) labeling and detection, a detection kit from Boehringer/Mannheim (Mannheim, Germany, http://www.boehringer.com) was used. In brief, dissociated cells were incubated at DIV1, DIV2, and DIV3 with BrdU for 1 h in a 95% air/5% CO_2 atmosphere at 37°C, fixed with 70% ethanol in 50 mM glycine buffer (pH 2.0) for 20 minutes at −20°C, and incubated with anti-BrdU antibody for 30 minutes at 37°C. Subsequently, coverslips were washed with buffer, incubated with mouse IgG coupled to FITC for 30 minutes at 37°C, washed again with buffer, and mounted with Vectashield (Vector Laboratories, Burlingame, CA, http://www.vectorlabs.com). Finally, coverslips were viewed with an epifluorescence microscope.

Treatment of the Cultures with MPP^+

MPP^+ at a final concentration of 2 μM was added at DIV5 in dissociated neurospheres without treatment (controls) and in dissociated neurospheres treated with TGF-β in combination with PSPN at DIV1 and DIV4. One and 3 days after toxin application, that is, DIV6 and DIV8 after plating of dissociated neurospheres, respectively, controls and treated cells were fixed in 4% paraformaldehyde and processed for immunocytochemistry, as described above. For comparison between controls and treated cells, the cell number of TH-immunopositive cells at the day of MPP^+ application, that is, at DIV5 after plating of dissociated neurospheres, was set to 100%. Statistical analysis was performed as described below.

Animals

Tgf-$\beta 2^{+/-}$ heterozygous mice were offspring from breeding pairs kindly provided by T. Doetschman, University of Cincinnati (Cincinnati, OH). The generation of these strains has been described elsewhere [37, 38]. *gdnf*$^{+/-}$ heterozygous mice were kindly provided by M. Saarma, Institute for Biotechnology, University of Helsinki (Helsinki, Finland). *Tgf-*$\beta 2^{+/-}$*/gdnf*$^{-/-}$, *Tgf-*$\beta 2^{-/-}$*/gdnf*$^{+/-}$, and double mutant *Tgf-*$\beta 2^{-/-}$*/gdnf*$^{-/-}$ mice were generated by cross-breading the two heterozygous strains and setting up matings between these double heterozygous mice (*Tgf-*$\beta 2^{+/-}$*gdnf*$^{+/-}$ × *Tgf-*$\beta 2^{+/-}$*gdnf*$^{+/-}$). For morphological comparison *Tgf-*$\beta 2^{+/+}$*gdnf*$^{+/+}$ littermates were used as wild-type controls. The morning of the day on which a vaginal plug was detected in mating was designated

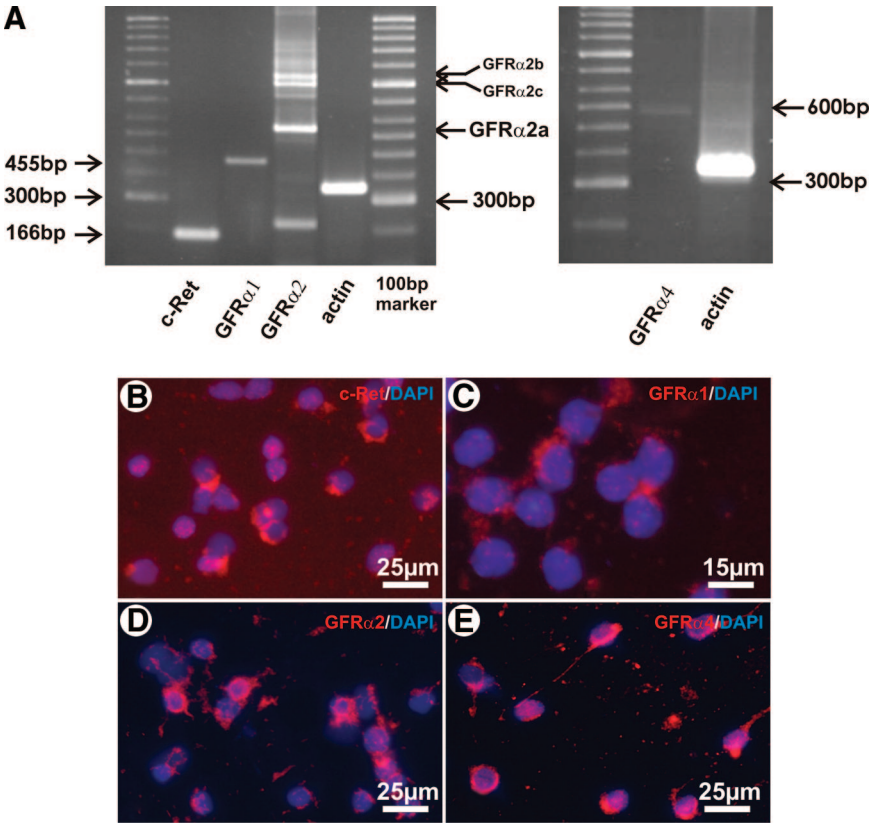

Figure 1. Expression of GFL receptors. **(A):** Neurospheres from mouse embryonic day 12 ventral mesencephalon were processed for reverse transcriptase-polymerase chain reaction using specific primers. The receptors involved in signaling of glial cell line-derived neurotrophic factor family ligands, c-Ret, GFRα1, and GFRα2 are expressed in ventral mesencephalic neurospheres. GFRα2 splice isoforms GFRα2a, GFRα2b, and GFRα2c are also detectable. In contrast, GFRα4 expression is weak. **(B–E):** Protein abundance for c-Ret **(B)**, GFRα1 **(C)**, GFRα2 **(D)** and GFRα4 **(E)** in dissociated neurospheres using immunofluorescence light microscopy. Cell nuclei were stained with DAPI (blue). Abbreviations: bp, base pairs; DAPI, 4,6-diamidino-2-phenylindole; GFR, GDNF family receptor.

gestation day 0.5. Analysis was performed on 14.5-day-old embryos. Heads were fixed in Bouin's fixative (75% picric acid, 20% formaldehyde, and 5% glacial acetic acid) for several hours, dehydrated in a graded series of ethanol, and embedded in paraffin wax. Paraffin blocks were cut into 10-μm serial sections.

Immunohistochemistry

Paraffin sections (10 μm) were deparaffinized and heated for 5 minutes in citrate buffer (pH 6.0) in a microwave oven at 600 W to improve antigen retrieval. After blockade of endogenous peroxidase activity by 5 minutes of treatment with 3% H_2O_2 in H_2O, sections were preblocked with a Vector blocking kit (Linaris GmbH, Bettinen, Germany, http://www.linaris.com) to avoid nonspecific binding of the biotin/avidin system used for immunodetection (see below). Immunostaining was performed using a specific monoclonal mouse anti-TH antibody at a dilution of 1:100 following the manufacturer's instructions for the Vector M.O.M. peroxidase immunodetection kit (Linaris GmbH). After a 1-hour incubation at room temperature the reaction was visualized by a nickel-intensified 3,3′-diaminobenzidine (Kem-En-Tec Diagnostics A/S, Taastrup, Denmark, http://www.kem-en-tec.com) reaction. For controls, PBS was substituted for the primary antisera to test for nonspecific labeling. No specific cellular staining was observed when the primary antiserum was omitted.

Numbers of TH-labeled neurons on the complete series of 10 μm transverse sections were counted. A neuron was designated as TH-positive if it showed a darkly labeled cytoplasm and a clearly visible, unstained nucleus. Only cells fulfilling these criteria were included in the cell counts. To avoid double counting the same cell on two sequential sections, only every fifth section was counted.

Statistics

Data are presented as the mean ± SEM. Statistical analysis was performed using the Student's double *t* test when one treated group was compared with the control. For multiple comparisons between treated groups statistical differences were compared using a one-way analysis of variance and Dunnett's post hoc test for multiple comparisons. Differences were considered statistically significant at $p < .05$, $p < .01$, and $p < .001$.

RESULTS

C-Ret, GFRα1, GFRα2, and GFRα4 Are Expressed in Neurospheres from Mouse E12 Ventral Midbrain

GFLs commonly bind to their preferential receptor and subsequently activate Ret transmembrane receptor kinase [13]. Figure 1 illustrates expression of the receptors c-Ret, GFRα1, GFRα2, and GFRα4 in neurospheres generated from mouse E12 ventral mesencephalon by RT-PCR using specific primers. Transcripts of the expected size of ~166 and ~455 base pairs (bp) for c-Ret (lane two) and GFRα1 (lane 3), respectively, were detected. However, when we amplified GFRα2 (lane 4) from ventral mesencephalic neurospheres, cDNA fragments at ~650, ~900, and ~1000 bp were detected, presumably corresponding to the GFRα2 splice isoforms GFRα2a (638 bp), GFRα2c (993 bp), and GFRα2b (1,077bp), respectively. In addition, a band at ~200 bp could also be observed, the nature of which is not clear yet. The GFRα4 amplification product was weak, but nevertheless detectable at ~600 bp (lane 8). In the absence of RT enzyme, no bands were visible (data not shown).

Protein abundance for c-Ret, GFRα1, GFRα2, and GFRα4 in the cellular system was further confirmed by immunofluorescence. As shown in Figure 1B–1E, immunoreactivity of moderate to strong intensity was detected for all receptors in dissociated neurospheres.

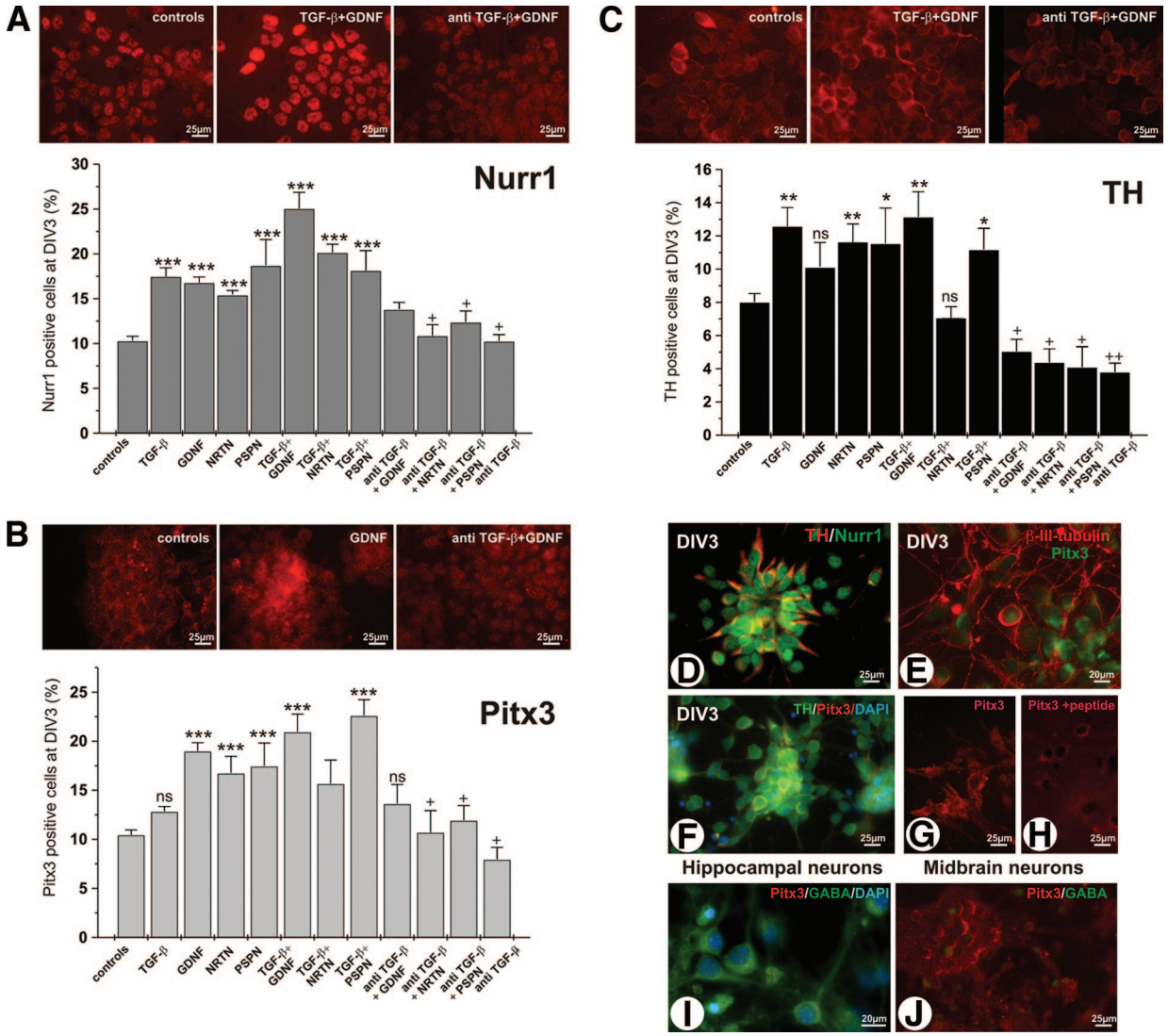

Figure 2. Differentiation of neurospheres derived from mouse embryonic day 12 ventral midbrain after factor treatment at DIV1. Protein abundance of dopaminergic phenotype markers Nurr1 **(A)**, Pitx3 **(B)**, and TH **(C)** was assessed by immunofluorescence light microscopy at DIV3. Quantification: TGF-β treatment of the cultures significantly increased the number of Nurr1- and TH-positive neurons compared with untreated controls. GDNF treatment of the neurospheres increased the number of Nurr1- and Pitx3- but not TH-immunoreactive cells compared with the controls. Treatment of the cultures with NRTN or PSPN significantly increased the numbers of Nurr1-, Pitx3-, and TH-immunopositive cells compared with the untreated controls. Treatment of the cells with TGF-β in combination with GDNF but not with NRTN or PSPN resulted in an additional increase in the number of Nurr1- and TH-positive cells compared with single factor treatment. Neutralization of endogenous TGF-β in the presence of exogenous GDNF decreased the number of Nurr1- and TH-positive cells compared with TGF-β treatment alone, whereas neutralization of endogenous TGF-β in the presence of exogenous NRTN significantly decreased the number of Nurr1- and TH- but not Pitx3-positive cells compared with NRTN treatment alone. Data are given as mean $\pm$ SEM ($n = 3$); p values derived from Student's t-test are as follows: ***, $p < .001$, **, $p < .01$, and *, $p < .05$ for increased numbers of immunoreactive cells after factor treatment compared with the untreated controls; and +++, $p < .001$, ++, $p < .01$, and +, $p < .05$ derived from one-way analysis of variance and Dunnett's post hoc test for decreased numbers of immunoreactive cells compared with single factor treatment; ns, not significant. **(D):** Double immunofluorescence for TH (red) and Nurr1 (green) showed partial colocalization of the proteins. **(E):** Double immunofluorescence for the neuronal marker β-III-tubulin (red) and Pitx3 (green) revealed colocalization of the proteins in some but not all neurons. **(F):** Colocalization of TH (green) and Pitx3 (red) at DIV3. **(G):** Pitx3 labeling in dissociated and plated neurospheres. **(H):** Pitx3 labeling was absent when the primary antibody had been preincubated with the blocking peptide. **(I):** Double immunofluorescence for GABA (green) and Pitx3 (red) in primary hippocampal cultures shows absence of Pitx3 protein abundance in these cells. **(J):** In contrast, dissociated and plated neurospheres revealed Pitx3 (red) but not GABA (green) immunoreactivity. Abbreviations: DAPI, 4,6-diamidino-2-phenylindole; DIV, day in vitro; GDNF, glial cell line-derived neurotrophic factor; NRTN, neurturin; PSPN, persephin; TGF-β, transforming growth factor β; TH, tyrosine hydroxylase.

Members of the GDNF Family May Trigger Differentiation of Midbrain Progenitors Toward Dopaminergic Phenotype, but They Require TGF-β

The transcription factors Nurr1 and Pitx3 have been established as early markers during the process of the differentiation of midbrain progenitors toward dopaminergic cell fate [2–7]. Therefore, we first examined the effects of combined TGF-β/GFL treatment of dissociated neurospheres on the immunoreactivity for these factors. Figure 2A shows that treatment of the cultures with TGF-β, GDNF, NRTN, or PSPN at DIV1 significantly increased the number of Nurr1-immunopositive cells at DIV3. Moreover, treatment of the cells with TGF-β in combi-

nation with GDNF, but not with NRTN or PSPN, resulted in an additional, significant increase of Nurr1-positive cells compared with single factor treatment. Neutralization of endogenous TGF-β in the presence of GDNF significantly decreased the number of Nurr1-immunoreactive cells compared with single TGF-β or GDNF treatment, but still the number of Nurr1-immunopositive cells was significantly increased compared with untreated controls. In contrast, neutralization of endogenous TGF-β in the presence of NRTN or PSPN significantly decreased the number of Nurr1-immunoreactive cells compared with single TGF-β or GFL treatment at the level of the untreated controls.

Factor treatment of neurospheres revealed differential effects on the early dopaminergic marker Pitx3. As illustrated in Figure 2B, GDNF, NRTN, and PSPN, but not TGF-β, significantly increased the number of Pitx3-positive cells compared with the controls. However, combined treatment of the cultures with TGF-β and GFLs did not further increase the number of Pitx3-immunoractive cells compared with single factor treatment. Interestingly, neutralization of endogenous TGF-β significantly reduced the number of Pitx3-positive cells compared with single factor treatment, at the level of the untreated controls.

TGF-β Alone or in Combination with GFLs Promotes Dopaminergic Phenotype Induction

With regard to the mature dopaminergic phenotype, TGF-β, NRTN, and PSPN were capable of significantly increasing TH-immunopositive cells compared with untreated controls, as illustrated in Figure 2C. Treatment of the cells with GDNF did not influence the number of TH-positive cells at DIV3, confirming previous results [34]. Consequently, neutralization of TGF-β in the presence of GDNF significantly reduced the number of TH-immunoreactive cells compared with the controls. However, interestingly, the presence of exogenous TGF-β diminished the NRTN effects in the number of TH-positive neurons at the level of untreated controls. Neutralization of endogenous TGF-β suppressed the effects of NRTN as well as of PSPN, demonstrating the requirement for TGF-β in dopaminergic phenotype induction. Moreover, neutralization of endogenous TGF-β in the absence of exogenous GFLs resulted in a dramatic decrease of TH-immunopositive cells compared with untreated cultures (Fig. 2C).

Despite treatment, generated TH-positive cells were always Nurr1 positive as well. As shown in Figure 2D, using double immunofluorescence, a clear colocalization of TH with Nurr1 could be observed. However, Nurr1-positive cells were not necessarily TH positive. Similarly, some, but not all, β-III-tubulin cells were additionally Pitx3 positive (Fig. 2E). In contrast, at DIV3 all TH-immunoreactive cells were Pitx3 positive as well, as shown in Figure 2F by double immunofluorescence. To ensure specificity of Pitx3 labeling, control experiments were performed. Figure 2G illustrates Pitx3 staining, which is abolished when cells were incubated with the antibody in the presence of the respective blocking peptide (Fig. 2H). Finally, to demonstrate that the generated neurons were indeed of mesencephalic origin and not forebrain neurons, double immunofluorescence with GABA and Pitx3 was performed. Figure 2I and 2J show the distribution patterns of GABA (green) and Pitx3 (red) in primary hippocampal cultures [36] and in cells derived from dissociated neurospheres from mouse E12 ventral mesencephalon, respectively. In primary hippocampal cultures (Fig. 2I) GABA was distributed at the cytoplasm of the cells, the nucleus was devoid of GABA immunoreactivity, and Pitx3 abundance could not be observed. In contrast, in cells derived from dissociated neurospheres from mouse E12 ventral mesencephalon (Fig. 2J) Pitx3 immunoreactivity was clearly present. A faint nonspecific GABA nuclear labeling was present in a few cells.

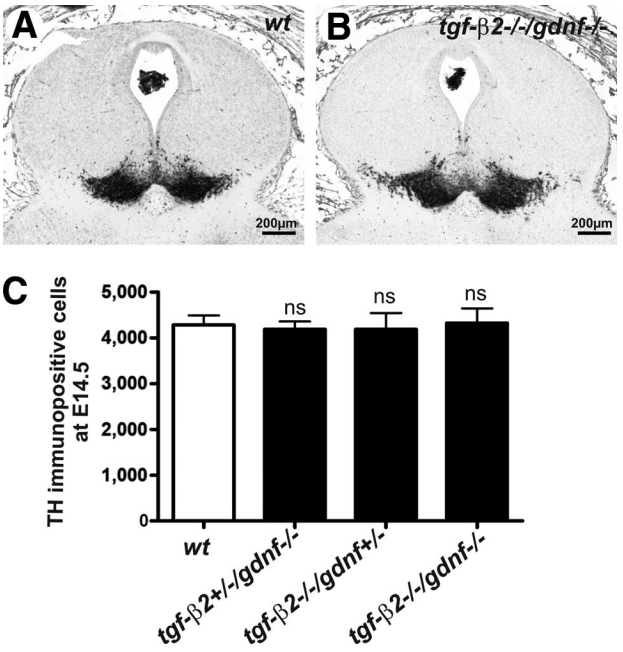

Figure 3. Phenotype analysis of *Tgf-β2*$^{-/-}$*/gdnf*$^{-/-}$ mouse embryos at E14.5. TH immunohistochemistry in midbrain tissue sections of wt **(A)** and *Tgf-β2*$^{-/-}$*/gdnf*$^{-/-}$ **(B)** mouse E14.5 embryos ($n = 3$). **(C)**: Quantification: counting of TH-immunoreactive cells revealed no differences in the numbers of dopaminergic neurons between wt, *Tgf-β2*$^{+/-}$*/gdnf*$^{-/-}$, *Tgf-β2*$^{-/-}$*/gdnf*$^{+/-}$, and double mutant embryos. Abbreviations: E, embryonic day; TH, tyrosine hydroxylase; wt, wild-type.

Tgf-β2$^{-/-}$*/gdnf*$^{-/-}$ Double Mutant Mice Lack Phenotype in Dopaminergic Neurons

The effects of TGF-β and GDNF and their potential interaction in the induction of a dopaminergic phenotype were further studied in vivo. Figure 3A and 3B shows TH immunolabeling, respectively, of fixed mouse E14.5 wild-type (wt) and *Tgf-β2*$^{-/-}$*/gdnf*$^{-/-}$ double mutant embryos by immunohistochemistry. Quantification of cell counts is presented in Figure 3C. The results show no significant differences in the number of dopaminergic neurons in the substantia nigra and ventral tegmental area among wt, *Tgf-β2*$^{+/-}$*/gdnf*$^{-/-}$, *Tgf-β2*$^{-/-}$*/gdnf*$^{+/-}$, or double mutant embryos. Each group consisted of three animals.

TGF-β in Combination with PSPN: A Potential "Dopaminergic Induction Cocktail"

The long-term effects of TGF-β and GFLs on dopaminergic phenotype induction are shown in Figure 4A. At DIV8, that is, after factor treatment of the cultures at DIV1, DIV4, and DIV7, TGF-β alone or in combination with GDNF was still able to significantly increase the number of TH-immunopositive cells. In contrast, treatment of the cells with GDNF, NRTN, or PSPN revealed no significant differences compared with untreated controls. Surprisingly, application of TGF-β in combination with PSPN increased the number of TH-positive cells to approximately 18%, thus revealing significant differences compared with controls and with single TGF-β treatment. These data demonstrate that the combined TGF-β/PSPN treatment not only triggered differentiation of midbrain progenitors toward the dopaminergic phenotype but reached a high yield as well, suggesting that the combination of TGF-β/PSPN may represent the treatment of choice for midbrain dopaminergic neuron induction

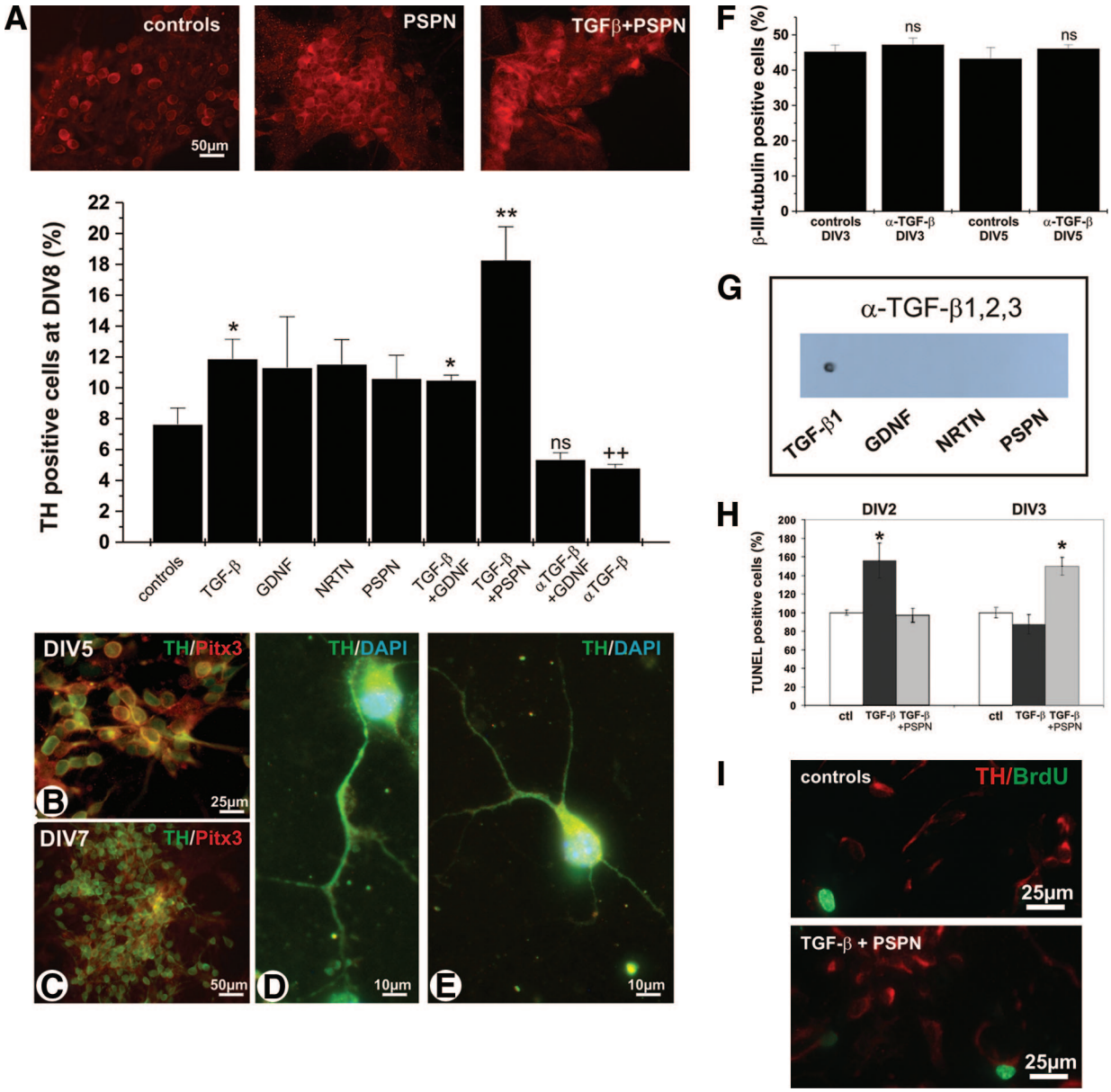

Figure 4. Differentiation potential of neurospheres derived from mouse embryonic day 12 ventral midbrain after factor treatment at DIV1, DIV4, and 7. Protein abundance of TH was assessed by immunofluorescence light microscopy at DIV8 **(A)**. Quantification: TGF-β with or without GDNF treatment of the cultures significantly increased the number of TH-positive neurons compared with the untreated controls. Treatment of the cultures with TGF-β in combination with PSPN significantly increased the number of TH immunoreactive cells compared with the untreated controls and with TGF-β application alone. Neutralization of endogenous TGF-β significantly decreased TH-immunoreactive cells compared with single factor treatment. Data are given as mean ± SEM ($n = 3$); p values derived from Student's t test are as follows: **, $p < .01$ and *, $p < .05$ for increased numbers of immunoreactive cells after factor treatment compared with untreated controls; and ++, $p < .01$ derived from one-way analysis of variance and Dunnett's post hoc test for increased numbers of immunoreactive cells compared with TGF-β treatment alone; ns, not significant. The generated neurons after TGF-β/PSPN treatment revealed at DIV5 **(B)** and DIV7 **(C)** clear colocalization of TH (green) with Pitx3 (red) by double immunofluorescence. **(D, E):** The generated TH-immunoreactive cells after combined TGF-β/PSPN treatment at DIV1, DIV4, and DIV7 reveal typical neuronal morphology. **(F):** The total number of neurons was not affected after treatment of the cells with anti-TGF-β antibody function blocking antibody. The number of β-III-tubulin cells remained unchanged at DIV3 and DIV5 in controls and anti-TGF-β antibody-treated dissociated neurospheres. **(G):** Dot blot analysis demonstrating the specificity of the anti-TGF-β antibody function blocking antibody used. **(H):** Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay revealed an increased number of TUNEL-positive cells at DIV2 after treatment of the cells with TGF-β and at DIV3 after treatment with TGF-β/PSPN. **(I)** Bromodeoxyuridine (BrdU) incorporation assay revealed no colocalization between TH (red) and BrdU (green) in controls or in TGF-β/PSPN-treated cultures. Abbreviations: ctl, control; DAPI, 4,6-diamidino-2-phenylindole; DIV, days in vitro; GDNF, glial cell line-derived neurotrophic factor; NRTN, neurturin; PSPN, persephin; TGF-β, transforming growth factor β; TH, tyrosine hydroxylase.

in vitro. The TH-positive neurons generated after combined TGF-β/PSPN treatment revealed Pitx3 immunoreactivity as well, as shown by examples for DIV5 (Fig. 4B) and DIV7 (Fig. 4C). The morphology of the generated TH-immunoreactive cells after treatment of the cultures with TGF-β and PSPN at DIV1, DIV4, and DIV7 is illustrated in Figure 4D and 4E. At higher magnification, the typical neuronal morphology is demonstrated.

At DIV8, neutralization of endogenous TGF-β in the presence of exogenous GDNF had no effect on the number of

TH-positive neurons compared with the untreated controls, suggesting that GDNF rescued the TGF-β neutralization-dependent decrease of TH-positive cells. However, neutralization of endogenous TGF-β in the absence of exogenous GFLs significantly reduced the number of TH-immunoreactive cells compared with the controls. Reduction of TH-immunoreactive cells after neutralization of endogenous TGF-β was not attributed to reduced total neuronal number after treatment. As shown in Figure 4F, no significant differences in the number of β-III-tubulin cells could be detected in the cultures at DIV3 and DIV5 after treatment with TGF-β-neutralizing antibodies compared with the untreated controls.

To ensure that the neutralizing anti-TGF-β antibody used specifically recognizes TGF-β isoforms and not other growth factors, dot blot analysis was performed. As shown in Figure 4G, the anti-TGF-β antibody cross-reacted with TGF-β1 only and failed to recognize GDNF, NRTN, or PSPN.

Survival Versus Induction

To differentiate whether the observed increase in the TH-immunopositive cells after TGF-β/PSPN treatment was in fact due to dopaminergic phenotype induction rather than to survival-promoting effects or even proliferation after factor treatment, TUNEL and BrdU assays were performed at DIV2 and DIV3 after TGF-β treatment alone or in combination with PSPN.

As illustrated in Figure 4H, at DIV2, the number of TUNEL-positive cells after TGF-β treatment was significantly increased compared with the untreated controls. In contrast, treatment of the cells with TGF-β together with PSPN had no effect on the number of TUNEL-positive cells compared with the controls. This pattern was reversed at DIV3: the number of TUNEL-positive cells after TGF-β treatment was comparable to the controls and significantly increased after application of TGF-β together with PSPN.

To further ensure that the effects of combined TGF-β/PSPN treatment in the number of TH-immunoreactive cells was not due to increased proliferation activity of the cells, BrdU incorporation was performed during differentiation of the dissociated neurospheres, that is, at DIV1, DIV2, and DIV3. Only single, non-neuronal cells were positive for BrdU. Figure 4I illustrates double labeling with TH (red) and BrdU (green) at DIV3. No colocalization between TH and BrdU could be observed, demonstrating that the observed effects after TGF-β/PSPN treatment cannot be attributed to increased proliferation activity.

Signaling Pathway of TGF-β- and PSPN-Induced Dopaminergic Differentiation

Previous observations have documented that TGF-β-induced dopaminergic differentiation is dependent on the TGF-β receptor complex and is mediated via the Smad and p38 mitogen-activated protein kinase (MAPK) pathway [12]. It is also well established that within the components of the ligand/receptor complex for GFLs, Ret acts as the signaling transducer and the GFRαs as the specific ligand binding component [13].

To elaborate on the signaling pathway involved in the combined TGF-β/PSPN-dependent differentiation of mesencephalic progenitors toward a dopaminergic phenotype, cells were treated either with SB431542, an Alk4, Alk5, and Alk7 inhibitor, with SB203580, a potent p38 MAPK pathway inhibitor, or with LY294002, a phosphatidylinositol 3-kinase (PI-3K) inhibitor. The results are presented in Figure 5A. A single application of each inhibitor in the presence of exogenous TGF-β and PSPN significantly decreased the number of TH-positive neurons compared with TGF-β/PSPN treatment, indicating that all pathways are partly required. Moreover, blocking of TGF-β signaling at the receptor level using SB431542 in combination with inhibition of the PI-3K using the inhibitor LY294002 caused a dramatic decrease in the number of TH-immunopositive cells. Taken together, these data suggest that the TGF-β/PSPN effects on differentiation of midbrain progenitors is a TGF-β receptor-mediated process that involves both the Smad, as well as the p38 MAPK pathway, converging with a c-Ret-mediated PI-3K-dependent pathway.

The involvement of p38 MAPK and PI-3K pathways in the induction of dopaminergic neurons after treatment of the cultures with TGF-β in combination with PSPN was further analyzed by Western blot analysis for pERK and pAkt, well-known downstream components of the p38 MAPK and PI-3K pathways, respectively. Figure 5B illustrates the Western blot analysis of dissociated neurosphere culture homogenate from the controls and after treatment with TGF-β and PSPN using anti-GAPDH as a housekeeping gene. pERK and pAkt protein expressions were significantly upregulated in treated cultures compared with the untreated controls (6.6 ± 1.2-fold, **, $p < .01$ and 2.5 ± 0.5-fold, *, $p < .05$ and for pERK and pAkt, respectively).

Upregulation of GFRα2 and GFRα4 Expression after TGF-β/PSPN Treatment

Figure 5C illustrates RT-PCR analysis for c-Ret (lanes 2 and 3), GFRα1 (lanes 4 and 5), and GFRα2 (lanes 6 and 7) transcript expression in controls and after treatment of the cells with TGF-β in combination with PSPN. c-Ret and GFRα1 expression showed no differences between controls and treated cultures. However, in cells that had been treated with TGF-β in combination with PSPN, GFRα2a transcript expression was found to be upregulated compared with the untreated controls (arrow). In addition, as shown in Figure 5D, GFRα4 expression was very weak in controls but was upregulated after TGF-β/PSPN treatment as well. Figure 5D also illustrates the Western blot analysis of the homogenate from the controls and after treatment with TGF-β and PSPN using anti-GAPDH as a housekeeping gene and anti-GFRα4 antibodies. GFRα4 protein abundance was upregulated after treatment (2.16 ± 3.1-fold, *, $p < .05$; $n = 3$).

TGF-β/PSPN-Induced Dopaminergic Neurons Are Less Vulnerable to MPP^+ Toxicity

To further characterize the properties of the TGF-β/PSPN-induced dopaminergic neurons, we tested whether these neurons are protected against MPP^+ toxicity. Therefore, TGF-β/PSPN-induced dopaminergic neurons were treated with 2 μM MPP^+ at DIV5. One and 3 days after MPP^+ application, cells were fixed and immunostained for TH. The results are shown in Figure 6. One day after toxin application, the decrease in TH-immunopositive cells between controls and the treated group was comparable. Three days after MPP^+ treatment in controls, the number of TH-immunopositive cells was decreased by 60%. In contrast, in TGF-β/PSPN-treated cells 3 days after MPP^+ the number of immunoreactive cells was decreased by 36%. Statistical analysis revealed significant differences between the decrease in controls and TGF-β/PSPN-generated neurons (**, $p < .01$), demonstrating that the latter neurons are less vulnerable to MPP^+ toxicity.

DISCUSSION

The TGF-β superfamily consists of more than 40 members, including TGF-βs, activin, nodal, the GDNF family, bone morphogenic proteins, and GDF [10, 39]. In our efforts to elucidate

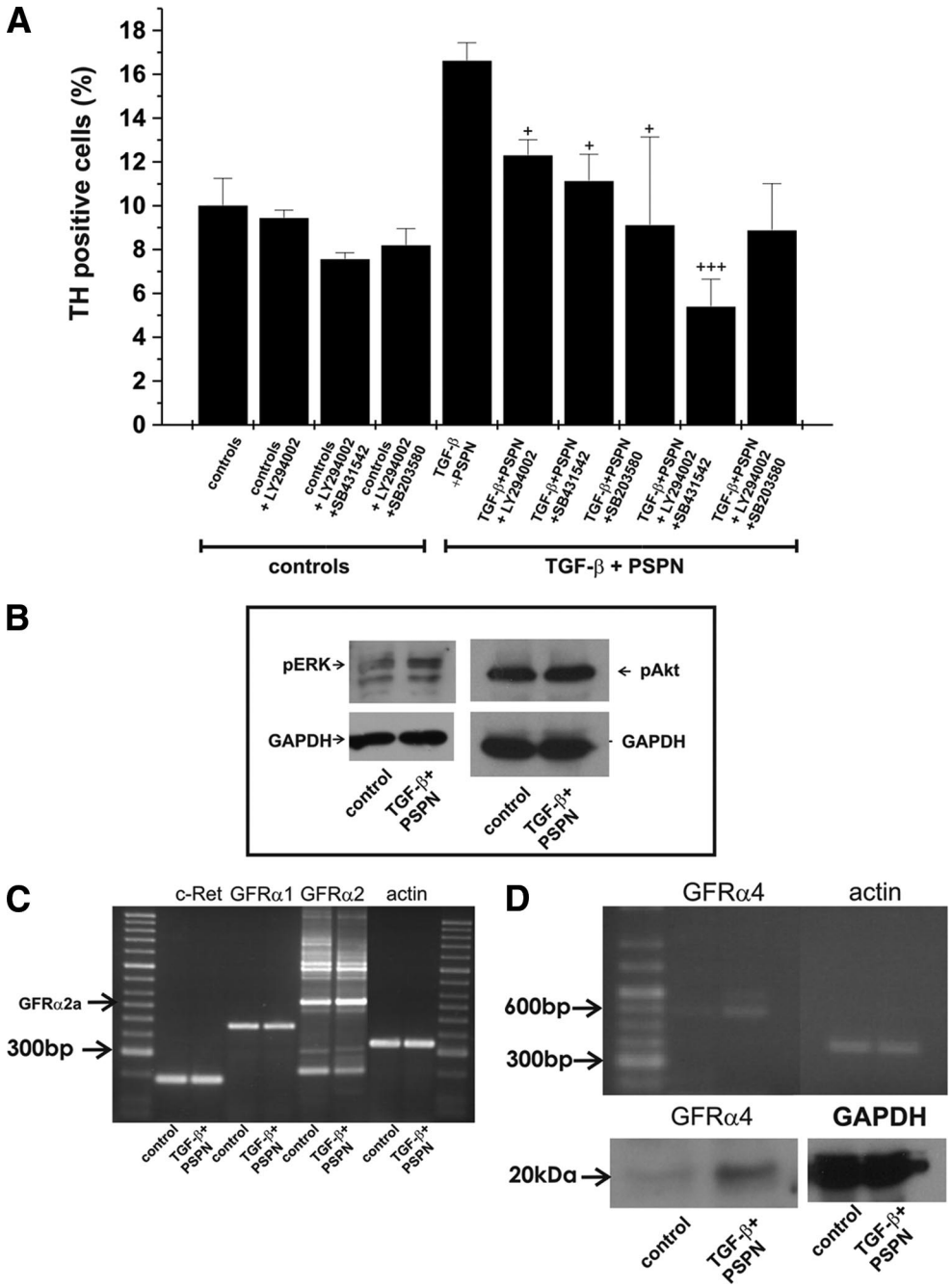

Figure 5. TGF-β/PSPN differentiation signaling pathway. **(A):** The number of TH-immunopositive cells was significantly reduced after treatment of the cultures with exogenous TGF-β/PSPN in the presence of SB431542 (10 μM; ALK4, ALK5, and AL7 inhibitor), SB203580 (20 μM; p38 mitogen-activated protein kinase pathway inhibitor), or LY294002 (20 nM; phosphatidylinositol-3-kinase inhibitor) compared with TGF-β/PSPN treatment. In controls, application of SB431542, SB203580, or LY294002 had no effect on the number of TH-positive cells. Data are given as mean $\pm$ SEM ($n = 3$); +, $p < .05$ and +++, $p < .001$, derived from one-way analysis of variance and Dunnett's post hoc test for decreased numbers of immunoreactive cells, compared with TGF-β/PSPN treatment. **(B):** Western blot analysis for pERK and pAkt in controls and in cultures treated with TGF-β/PSPN. The immunoblots were probed either with monoclonal antibody against GAPDH or with anti-pERK and anti-pAkt antibodies. pERK and pAkt proteins were upregulated in cultures treated with TGF-β/PSPN (**, $p < .01$ and *, $p < .05$ after densitometric analysis of the signal ratio pERK:GAPDH and pAkt:GAPDH and Student's t test). **(C):** Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis for the glial cell line-derived neurotrophic factor family ligand receptors c-Ret, GFRα1, and GFRα2 in controls and after TGF-β/PSPN treatment showed upregulation of GFRα2a after treatment (arrow). **(D):** GFRα4 expression in controls and after treatment of the cultures with TGF-β in combination with PSPN revealed upregulation of the receptor expression by RT-PCR. Western blot analysis (lower row) showed upregulation of GFRα4 protein after TGF-β/PSPN treatment as well. Abbreviations: bp, base pairs; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFR, GDNF family receptor; pAkt, phospho-Akt; pERK, phospho-extracellular signal-regulated kinase; PSPN, persephin; TGF-β, transforming growth factor-β; TH, tyrosine hydroxylase.

the mechanisms that dictate induction and differentiation of mesencephalic dopaminergic neurons, we have previously shown that TGF-βs are required for the induction, differentiation, and survival of midbrain dopaminergic neurons in vivo and

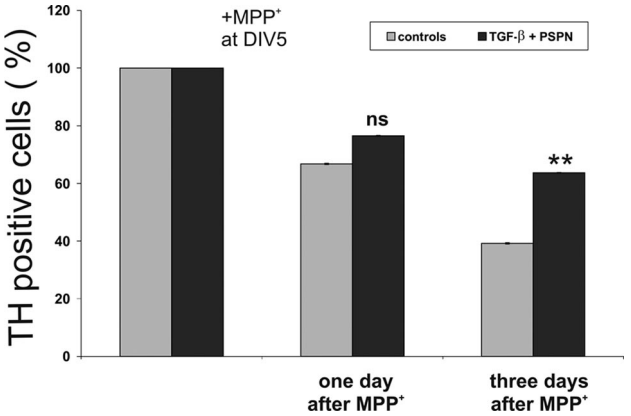

Figure 6. TH immunoreactive cells (%) 1 day and 3 days after MPP^+ application (2 μM) in dissociated neurospheres derived from mouse embryonic day 12 ventral midbrain at DIV5. Three days after MPP^+ treatment, the numbers of TH-immunopositive cells were decreased by 60 and 36% in controls and TGF-β/PSPN-treated cultures, respectively (**, $p < .01$; ns, not significant). Abbreviations: DIV, day in vitro; MPP^+, 1-methyl-4-phenyl pyridinium ion; PSPN, persephin; TGF-β, transforming growth factor-β; TH, tyrosine hydroxylase.

in vitro [8, 9, 12]. TGF-β apparently triggers midbrain progenitors to acquire a dopaminergic phenotype. Although GDNF itself has no differentiation-promoting effects on midbrain dopaminergic precursor cells [34], GDNF and other GFLs, such as NRTN, ARTN, and PSPN, have been identified as potent neurotrophic factors for several neuronal populations, including mesencephalic dopaminergic neurons. In addition, NRTN is an indispensable component in standard protocols that drive differentiation of embryonic stem cells toward dopaminergic neurons, reflecting the beneficial effects of NRTN in this process [40]. Considering this background together with the observation that GDNF requires TGF-β to exert its survival-promoting effects [19–21], the aim of the present study was to investigate putative cooperative effects of TGF-β and GFLs in the differentiation of midbrain progenitors toward dopaminergic neurons. Therefore, the well-characterized neurosphere culture system from mouse E12 ventral mesencephalon has been used as an experimental model in vitro [12, 34].

GFLs commonly bind to their specific GFRα receptor and subsequently activate Ret transmembrane receptor kinase [13]. The neurospheres express c-Ret, as well as GFRα1, GFRα2, and GFRα4, the preferential receptors for GDNF, NRTN, and PSPN, respectively (Fig. 1), demonstrating that this cell system is suitable for investigating GFL effects. GFRα2 expression has been reported in ventral midbrain of mouse at E10, E12, and E14 [23] but not in prenatal (E14) and postnatal day 7 rat midbrain [41]. During amplification of GFRα2 cDNA additional bands that probably correspond to the GFRα2a, GFRα2b, and GFRα2c splice isoforms were also detected [42]. The band detected during amplification of GFRα4 cDNA probably represents GFRα4 splicing. Alternative splicing of the mouse *gfra4* gene has been shown to give rise to three variants, the putative GPI-anchored, the transmembrane, and the soluble form [43] in a tissue-specific manner. GFRα4 was found to be weakly expressed in the neurospheres (Fig. 1), consistent with previous data showing GFRα4 expression in mouse E12 ventral midbrain and in adult rat midbrain [43, 44]. The GFRα4 fragment observed presumably represents the soluble form of GFRα4. Indeed, immunoblotting of dissociated control neurospheres showed an immunoreactive band at ~20 kDa (Fig. 5D) consistent with the predicted molecular mass of soluble GFRα4. Protein abundance for GFL receptors was further confirmed by immunofluorescence (Fig. 1B–1E). GFRα3 is also expressed in neurospheres (data not shown); however, because ARTN mRNA could not be detected in the ventral midbrain of E14 rats [25], the effects of ARTN in the differentiation potential of the neuropheres has not been considered in the present study. In addition, it should be noted that GFLs can also signal via the neural cell adhesion molecule NCAM [13]. A potential functional implication of this observation in the ventral mesencephalon is not clear yet.

Treatment of dissociated neurospheres with GDNF together with TGF-β showed that GDNF potentiated the TGF-β effects on the number of cells expressing the early dopaminergic marker Nurr1 at DIV3 but was not able to rescue the decreased number of Nurr1-immunoreactive cells after neutralization of endogenous TGF-β (Fig. 2A). Interestingly, GDNF rescued the TGF-β neutralization-dependent loss of the TH-positive cells at DIV8 (Fig. 4A) in vitro. In vivo, no apparent phenotype with regard to dopaminergic neurons could be observed in *Tgf-β2*$^{-/-}$/*gdnf*$^{-/-}$ mice at E14.5 (Fig. 3), matching the data observed in *gdnf*$^{-/-}$ mice, in which only the neurons of the enteric nervous system, but not the midbrain dopaminergic neurons are lost [26–28]. The most likely explanation for these observations is that TGF-β isoforms may compensate for the loss of each other. Indeed, in *Tgf-β2*$^{-/-}$/*Tgf-β3*$^{+/-}$ or *Tgf-β2*$^{+/-}$/*Tgf-β3*$^{-/-}$ mice, the number of mesencephalic TH-positive cells was significantly reduced compared with wild-type mice at E14.5 [12]. Taking the in vitro and in vivo data together, these results also suggest that phenotype induction and survival of fully differentiated neurons are accomplished through distinct pathways and requirements for individual factors. TGF-β is required for the induction of dopaminergic neurons, whereas GDNF is required for differentiated dopaminergic neurons rather than developing ones, suggesting that GDNF may play an essential role in regulating and/or maintaining a differentiated neuronal phenotype. For accomplishing this function, however, GDNF indeed requires TGF-β, as shown in vivo for dopaminergic and sympathetic neurons [20, 45].

Like GDNF, NRTN and PSPN have been shown to exhibit neurotrophic activity on mesencephalic dopaminergic neurons as well. Together with the lack of apparent developmental deficits in dopaminergic neurons in *gdnf*$^{-/-}$ [26–28], *pspn*$^{-/-}$ [31], *nrtn*$^{-/-}$ [29], and *artn*$^{-/-}$ [30] mice, it appears that considerable redundancy of factors with the same actions is present in this central neuronal population. In vitro, treatment of dissociated neurospheres with NRTN or PSPN caused a significant but transient increase in the number of Nurr1-, Pitx3-, and TH-positive cells at DIV3 compared with the untreated controls (Fig. 2). In addition, the effects of individual GFLs were diminished after neutralization of endogenous TGF-β, demonstrating dependence of GFLs on TGF-β. With one exception, the combination of exogenous TGF-β with GFLs had no further beneficial effects on the differentiation of midbrain precursors toward dopaminergic neurons compared with single factor treatment: at DIV8, the combined application of TGF-β with PSPN impressively increased the number of TH-positive cells to approximately 18% (Fig. 4A). The TH-immunopositive neurons generated revealed Pitx3 immunoreactivity, were devoid of GABA expression, and exhibited a mature neuronal morphology (Figs. 2 and 4), suggesting that they are true mesencephalic dopaminergic neurons. A survival-promoting or proliferative effect of combined treatment could be excluded using TUNEL and BrdU assays (Figs. 4H, 4J), suggesting that TGF-β/PSPN has been acting as an inductive signal on precursor cells towards a dopaminergic cell fate.

An open issue concerning ventral mesencephalic precursors remains: does the loss of ability to generate dopaminergic neurons after extensive expansion of ventral mesencephalic precursors and repeated passaging of neurospheres [46, 47] argue

against the presence of true dopaminergic precursors in unpassaged neurospheres, as well as in the mesencephalic neural tube? To answer this question we should consider the fact that neural stem cells in their biological environment in the neural tube during development become more committed [48] with each round of proliferation. In addition, proliferation and commitment of neural stem cells are temporally and spatially strictly regulated. Therefore, a consequence of extensive expansion of precursors and multipassaging of the neurospheres may result in a complete loss of TH-positive cells, reflecting a change in the developmental properties of mesencephalic progenitors.

The effect of TGF-β/PSPN treatment on mesencephalic progenitors toward the dopaminergic cell fate observed in the present study is mediated at least by converging p38 MAPK and PI-3K signaling pathways. Several lines of evidence support this view. Application of SB203580, a specific p38 MAPK inhibitor, or SB431542, a potent ALK4, ALK5, and ALK7 inhibitor, together with LY294002, a PI-3K inhibitor, caused a decrease in the number of TH-positive cells additional to that observed after single inhibitor application (Fig. 5A). Moreover, combined TGF-β/PSPN treatment of the cultures upregulated pERK and pAkt proteins (Fig. 5B). These results are consistent with previous observations documenting that TGF-β-induced dopaminergic differentiation depends on the TGF-β receptor complex and is mediated via the Smad and p38 MAPK pathways [12]. In contrast, the TGF-β/GDNF cooperative effect on the survival of many neuronal subpopulations is PI-3K-independent [21]. These data further support the hypothesis that differentiation and survival of neurons may require the same key players, but distinct underlying mechanisms. The signaling pathways underlying PSPN signaling are poorly understood, but in vitro and in vivo studies have demonstrated that PSPN can promote the survival of many neuronal subpopulation except the peripheral neurons [13, 17, 49], via a PI-3K/AKT pathway. Interestingly, treatment of the cells with TGF-β in combination with PSPN upregulated the expression of GFRα2a and GFRα4 (Fig. 5C, 5D).

Finally, the TGF-β/PSPN-induced TH-positive neurons were less vulnerable against MPP^+ toxicity (Fig. 6). Three days after MPP^+ application about 64% of the generated neurons were still alive compared with 39% of the controls. Intrastriatal grafting of an engineered neural stem cell line to overexpress PSPN in rats promoted survival of dopaminergic neurons, but these cells preferentially differentiated into oligodendrocytes [44]. It would be interesting to show the differentiation potential of neural stem cells after overexpression of PSPN together with TGF-β. The results of the present study suggest that the factor combination TGF-β/PSPN is a potent inductor of midbrain dopaminergic neurons and suggest its application in protocols for the generation of dopaminergic neurons from embryonic stem cells. Moreover, these data provide new insights for tissue engineering using neural stem cells.

ACKNOWLEDGMENTS

This work was funded by the Deutsche Forschungsgemeinschaft (DFG) (Kr1477/10-1 and 10-2 to K.K.), and through the DFG Research Center for Molecular Physiology of the Brain (CMPB, to E.R.). B.R. was funded through Deutscher Akademischer Austausch Dienst. We thank H. Böttcher and E. Gimbel for excellent technical assistance. B.R. is currently affiliated with Faculty of Pharmacy, An-Najah National University, Nablus, Palestine.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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