

<sup>1</sup>Department of Clinical and Molecular Biomedicine, Pharmacology Section, University of Catania Medical School, Viale A. Doria 95125 Catania, Italy.; <sup>2</sup>OASI Institute for Research and Care on Mental Retardation and Brain Aging, Neuropharmacology Section; V. Conte Ruggero 73, 94018Troina (EN), Italy; <sup>3</sup>Department of Physiological Sciences, University of Catania Medical School, Viale A. Doria 95125 Catania, Italy; <sup>4</sup>Department of Clinical Neurosciences, Cambridge Centre for Brain Repair and Cambridge Stem Cell Initiative, Univ. of Cambridge, ED Adrian Building, Forvie Site, Robinson Way, Cambridge CB2 0PY, UK.

Contact: Bianca Marchetti, PhD; Professor of Clinical Pharmacology, Dept. of Clinical and Molecular Biomedicine, Pharmacology Section, University of Catania, Viale A. Doria 95125 Catania, Italy; email: biancamarchetti@libero.it; The authors declare no conflict of interest.

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## Wnt/ $\beta$ -Catenin Signaling is Required to Rescue Midbrain Dopaminergic Progenitors and Promote Neurorepair in Ageing Mouse Model of Parkinson's Disease

FRANCESCA L'EPISCOPO<sup>1,2</sup>, CATALDO TIROLO<sup>2</sup>, NUNZIO TESTA<sup>2</sup>,  
SALVATORE CANIGLIA<sup>2</sup>, MARIA CONCETTA MORALE<sup>2</sup>, MARIA  
FRANCESCA SERAPIDE<sup>3</sup>, STEFANO PLUCHINO<sup>4</sup> & BIANCA  
MARCHETTI<sup>1,2</sup>

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### SUMMARY

Wnt/ $\beta$ -catenin signaling is required for specification and neurogenesis of midbrain dopaminergic (mDA) neurons, the pivotal neuronal population that degenerates in Parkinson's disease (PD) and in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD. Wnt/ $\beta$ -catenin signaling plays a vital role in adult neurogenesis but whether it might engage DA neurogenesis/neurorepair in the affected PD brain is yet unresolved. Recently, the adult midbrain aqueduct periventricular regions (Aq-PVRs) were shown to harbor neural stem/progenitor cells (mNPCs) with DA potential *in vitro*, but restrictive mechanisms *in vivo* are believed to limit their DA regenerative capacity. Using *in vitro* mNPC culture systems we herein demonstrate that aging is one most critical factor restricting mNPC neurogenic potential via dysregulation of Wnt/ $\beta$ -catenin signaling. Coculture paradigms between young/aged (Y/A) mNPCs and Y/A astrocytes identified glial age and a decline of glial-derived factors including Wnts as key determinants of impaired neurogenic potential, whereas Wnt activation regimens efficiently reversed the diminished proliferative, neuronal and DA differentiation potential of A-mNPCs. Next, *in vivo* studies in wild (Wt) and transgenic  $\beta$ -catenin reporter mice uncovered Wnt/ $\beta$ -catenin signaling activation and remarkable astrocyte remodeling of Aq-PVR in response to MPTP-induced DA neuron death. Spatio-temporal analyses unveiled  $\beta$ -catenin signaling in predopaminergic (Nurr1<sup>+</sup>/TH<sup>+</sup>) and imperiled or rescuing DAT<sup>+</sup> neurons during MPTP-induced DA neuron injury and self-repair. Aging inhibited Wnt signaling, whereas  $\beta$ -catenin activation *in situ* with a specific GSK-3 $\beta$  antagonist promoted a significant degree of DA neurorestoration associated with reversal of motor deficit, with implications for neurorestorative approaches in PD. STEM CELLS 2014; 00:000–000

### INTRODUCTION

Parkinson's disease (PD) is a common, age-dependent neurodegenerative movement disorder characterized by the progressive loss of midbrain dopaminergic (mDA) neurons of the substantia nigra pars compacta (SNpc)

and their projections into the corpus striatum leading to substantial decreases in dopamine levels [1]. Current pharmacological treatments for PD are comprised mainly of dopamine replacement for symptom alleviation, but lack of effectiveness in preventing clinical

progression, therefore neuroprotective therapies are actively investigated [2].

Recently, stem cell-based replacement therapies have emerged as potential strategies [3-5] and the molecular mechanisms that govern neurogenesis and differentiation of mDA neurons have attracted intense investigations for the identification of intrinsic DA neuron determinants. Particularly, *Wingless-type MMTV integration site (Wnt)* pathway is recognized as one central player in mDA neurogenesis [6-14].

Besides its recognized role in regulating multiple processes in developing brain, accumulating evidence points to Wnt signaling as vital regulator of adult neuron maintenance, while Wnt dysregulation has been linked to neuron demise in major neurodegenerative disorders, including PD [15-25].

Aging, the strongest risk factor for PD, and the 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine (MPTP) mouse model of PD are associated with a gradual decline of the capacity of mDA neurons to recover upon injury [26-28]. This is at least in part due to impaired neurogenic potential [29-33].

Neural progenitors in neurogenic areas such as the subventricular zone of the lateral walls of the lateral ventricles (SVZ) and the subgranular zone (SGZ) of the hippocampus are in intimate contact with astrocytes which helps to generate an instructive "niche" that promotes neurogenesis [34-37, and Refs herein]. Notably, astrocyte-derived Wnts and Wnt/ $\beta$ -catenin signaling activation contribute to the regulation of adult neurogenesis [22, 38-40], but with age, a dysfunctional Wnt/ $\beta$ -catenin signaling impairs both hippocampus SGZ [41,42] and SVZ [33] niches.

The occurrence of neurogenesis in the adult SNpc still remains controversial [29, 32, 43, 44]. However, the midbrain-aqueduct periventricular regions (Aq-PVRs) harbor multipotent clonogenic neural stem/progenitor cells (mNPCs) with an exclusive nigral DA differentiation potential [45-47], that might provide DA neurons that ultimately migrate to reach the SN [44, 47,48,49]. However, strong cell-intrinsic and extrinsic restrictive factors *in vivo*, are believed to limit their neurogenic and DA regenerative capacities.

In the light of our recent studies indicating a potential role for astrocyte-derived factors and Wnt/ $\beta$ -catenin signaling in promoting neurogenesis and DA neurogenesis from adult mNPCs [20], we herein investigated the potential of Wnt/ $\beta$ -catenin signaling activation for endogenous mDA cell replacement or repair in the MPTP mouse model of PD, and started addressing the following questions: a. which factors/mechanisms regulate the behavior of these Aq-PVRs midbrain progenitors and/or the permissiveness of the Aq microenvironment in promoting neurogenesis and DA neurogenesis during the process of aging and MPTP-induced nigrostriatal injury, *in vitro* ?. b. are Wnt/ $\beta$ -catenin-responsive progenitors present in periaqueductal regions *in vivo*, and if so, will they respond to MPTP-induced DA neuron injury ?. c. is it possible to activate

these progenitors to rescue dopaminergic plasticity in aged-MPTP mice ?. We herein identify the midbrain Aq-PVR as novel Wnt responsive region and present evidences suggesting the potential for mDA neuron restoration by activating Wnt/ $\beta$ -catenin signaling in endogenous sources, with therapeutical implications for PD.

## EXPERIMENTAL PROCEDURES

### Mice and treatments

Young (2-5 month-old) and aged (9-12 month-old) male C57BL and BATGAL [50] reporter mice were used. Transgenic BATGAL mice express nuclear beta-galactosidase under the control of the beta-catenin-activated transgene (BAT) promoter [50]. The BATGAL reporter mice used in this study were purchased from the Jackson laboratory (Tg(Bat-lac-Z)<sup>3</sup> Picc/Tg(Bat-lacZ)<sup>3</sup>Picc) on a C57Bl background. Mice were maintained in standard laboratory conditions and all studies carried out in strict accordance with the the Guide for the Care and Use of Laboratory Animals (NIH), approved by the Institutional Animal Care and Use Committee guidelines. All surgeries were performed under anesthesia. The MPTP-based mouse model of PD was used [33, 51]. At the indicated days post-MPTP treatment (dpt), mice were given bromodeoxyuridine (BrdU, 50 mg kg<sup>-1</sup>, injected four times, 2 hrs apart) and killed 2 h after the last injection [22, 33].

### Neural stem/precursor cells

For isolation and processing of stem/neuroprogenitors from midbrain-Aq-PVRs (mNPCs) of young and older mice we used the neurosphere culturing techniques similar to those used for propagation of NPCs derived from the SVZ, as previously reported [20,22,33,52] and detailed in Supplemental informations. Neurospheres at passage number 3-4 and 8-10 were used in all *in vitro* experiments. For proliferation and differentiation studies, the neurospheres were mechanically dissociated into single-cells and plated at a final density of  $1 \times 10^5$  cells/cm on poly-D-lysine coated 24-well plates. Proliferation was studied after exposure to proliferative medium for 3 DIV, by addition of the nucleotide analogue BrdU (5  $\mu$ M) at 2 DIV and the cells fixed after 24 h at 3 DIV. The differentiation of mNPCs was initiated by removal of mitogens and plating the cells on PDL (monotypic cultures), or onto astrocyte monolayers (co-cultures) in the absence or the presence of the indicated treatments, as described. mNPCs were left to differentiate for 10 DIV.

### Glial cell cultures, NPC-glial co-cultures and cell treatments

Purified astrocyte cell cultures obtained from postnatal days 2 (P2) and older (2-24 M) mouse midbrain-Aq, region were used for co-culture paradigms with Y/A-NPCs, either untreated (PBS) or treated [20,22,33]. For DA differentiation, mNPCs were grown alone, or layered on

the top of astrocytes. The differentiation medium contained 2.5% FCS instead of BSA. After 3 DIV, the growth medium was changed and replaced with fresh differentiation medium (N2 medium without serum, containing 1 mg/ml BSA and 200  $\mu$ M ascorbic acid). For pharmacological activation of *Wnt/ $\beta$ -catenin* signaling we used the specific GSK3 $\beta$  inhibitor, AR-AO14418 [N-(4-methoxybenzyl)-N'-(5-nitro-1,3-thiazol-2-yl)urea] (AR, 5  $\mu$ M), or the Wnt ligand, Wnt1 (100 ng/ml) [20-22]. For Wnt antagonism we used Dickkopf-1 (Dkk-1, 100 ng/ml, R&D Systems, MN, USA), or Frizzled-1-cysteine rich domain (Fzd-A, 200 ng/ml, R&D Systems) [20-22].

### Immunocytochemistry

Cell cultures were fixed in 4% paraformaldehyde in PBS or with paraformaldehyde/PBS followed by ice-cold acidic ethanol and HCL for BrdU staining [20, 22, 33]. Analyses performed using a confocal laser microscope and computer assisted image analysis (Leica). Quantification of the amount of cells expressing a given marker or marker combinations was determined relative to the total number of DAPI-labeled nuclei or Tuj1<sup>+</sup> cells using the Leica lite Software and three-dimensional overlay to avoid false-positive/negative overlay and double counting.

Caspase-3 activity was evaluated as a marker of cell death [19,20], using the fluorogenic substrate DEVD-AFC (Ac-Asp-Glu-Val-Asp (DEVD)-pNA (Upstate Biotechnology). Samples were analyzed in a plate reader at 405 nm and enzymatic activity is expressed as arbitrary fluorescent units [20,21,33].

### Immunohistochemistry and cell counting

Serial coronal sections (14  $\mu$ m-thick), encompassing the striatum (Bregma 1.54 to bregma -0.46) and the SNpc-Aq regions (Bregma -2.92 to bregma -4.84 mm) according to *Franklin and Paxinos* [53] were collected, mounted on poly-L-lysine-coated slides and pre-absorbed primary antibodies reported in Supplemental Table 1. Quantification of the amounts of cells expressing a given marker or marker combination, in any given experiment was determined as above [20, 22, 33]. For semiquantitative analyses, cells (~ 100) were counted within the complete region of interest using the Leica lite Software and three-dimensional overlay as above, in sections from five different animals, and each region quantified in at least five separately stained coronal slices. All cells (DAPI-stained nuclei) within the first 300  $\mu$ m of the Aq round were counted.

### RNA extraction, reverse transcription and real-time PCR

Tissue/cell samples were processed for total RNA isolation as described in full details [20, 22, 33] using Taqman Assay Reagents (Applied Biosystems), qt-RT-PCR performed according to manufacturers protocol. The assay IDs are reported in Supplemental Table 2. Results are expressed as arbitrary units (AU).

### Western blot Analysis

Protein extracts prepared from tissue/cells isolated from saline or MPTP mice (n = 6-8 mice/age-group/tp) and from cell cultures (run in triplicates) within the different experimental groups [20, 22, 33]. Primary antibodies sources and dilutions used are detailed in Supplemental Table 1. Bands from the Western blots were densitometrically visualized, the signals quantified on X-ray films using, the data subjected to statistical analysis of variance.

### Effect of manipulation of *Wnt/ $\beta$ -catenin* signaling, *in vivo*

Pharmacological activation of Wnt/ $\beta$ -catenin signaling was carried out with AR [18-20]. Mice anesthetized with chloral hydrate (600 mg/kg) were positioned in a stereotaxic apparatus. The following stereotaxic coordinates were used : AP= 3.5 mm posterior from the bregma, L= 0.2 mm lateral to the midline [53]. The needle was placed at a distance of 2.8 mm from the dura [53]. Groups of mice received unilateral infusion of either the Wnt/ $\beta$ -catenin signaling activator AR or the vehicle (PBS), which served as control, into the Aq-PVR. Mice were let to recover for 24 h, then treated with MPTP. At different time-intervals, part of the mice were subjected to the Rotarod analysis for motor coordination. On the day of sacrifice mice were given BrdU and killed 2 h after the last injection.

### Dopaminergic endpoints in striatum and ventral midbrain

The principal DA endpoints at SNpc and striatal levels were investigated before and at the indicated time-intervals upon MPTP administration. Briefly, specific high-affinity neuronal dopamine uptake in striatum expressed as femtomoles of dopamine uptake per microgram of protein minus the femtomoles of mazindol uptake [20] was determined before and 21 and 45 dpt. Values are represented as % changes in dopamine uptake vs. control. Striatal dopamine (DA) was determined by HPLC as detailed elsewhere [56] in striata of both sides. The loss of TH<sup>+</sup> SNpc neurons was determined by serial section analysis of the total number of TH<sup>+</sup> cells counted through the entire rostro-caudal (RC) axis of the murine SNpc [53], and counting performed as detailed [20,21] either before and 1-3, 7-14, 21-30 and 45-65 d after MPTP. DAT- or TH- fluorescence intensity (FI) in striatum was assessed at the same time-points in n = 3 coronal sections, in n= 6 mice/group/time-point, and changes in average FI (mean $\pm$  SD) expressed as percentage (%) of saline-injected controls [20].

For the analysis of motor coordination, an accelerating rotarod (five-lane accelerating rotarod; Ugo Basile, Comerio, Italy) was used [20]. Mice have to keep their balance on a horizontal rotating rod (diameter, 3 cm) and rotation speed was increased every 30 sec by 4 rpm. Five mice were tested at the same time, separated by large disks. A trial starts when the mouse is placed

on rotating rod, and it stops when the mouse falls down or when 5 min are completed. Falling down activates a switch that automatically stops a timer. The testing day, each mouse is submitted to 5 trials with an intertrial interval of 30 min. Mice housed five per cage were acclimated to a 12h shift in light/dark cycle so that the exercise occurred during the animals normal wake period. Saline- and MPTP-treated mice undergoing PBS or AR infusions as described were assessed for their Rotarod performance at -7 d and +3, +7, +14, +28, +35 and +45 post-MPTP (10/experimental group).

### Data Analysis

Statistical significance between means  $\pm$  SEM was analyzed by a two-way analysis of variance (ANOVA), and Student's *t*-test for paired or unpaired data. Experimental series performed on different days were compared by the Student-Newman-Keuls *t*-test. A value of  $p < 0.05$  was considered to be statistically significant.

## RESULTS

### Age and MPTP impair stem/neuroprogenitors from midbrain Aq-PVRs (mNPCs)

#### The *in vitro* model

Stem/neuroprogenitors from adult mouse midbrain caudal ventricular regions (mNPCs) (Fig.1, A) express proliferation (bromodeoxyuridine, BrdU), precursor (nestin, Musashi1), proneural (oligodendrocyte transcription factor 2, Olig2; neurogenin2, Ngn2), and astrocyte cell markers (glial fibrillary acid protein, GFAP) during *in vitro* clonal expansion (Fig 1, B, C), both at a protein and/or mRNA levels, confirming previous studies including our own [20,45,46]. The high expression of the midbrain marker engrailed 2 (*En2*) as opposed to very low forebrain (*Dlx2*) and hindbrain (*Hoxd3*) markers, supported their midbrain phenotype. The proliferative, differentiation and survival properties of mNPCs from 2-5 (young, Y) and 9-12 month-(M)-old (aged, A) mice were then assessed in basal conditions and during MPTP-induced injury and recovery (Fig. 1, D). Y-mNPCs of 3-4 passages grown in proliferative medium for 3 days *in vitro* (DIV) express nestin, of which a certain percentage incorporate BrdU (Fig. 1, E, G). When shifted in differentiation medium and stained after 10 DIV, a certain proportion of cells express the neuronal marker Tuj1 (Fig 1, E, H), thereby proving their NPC behavior. By contrast, A-mNPCs show significantly decreased percentages of BrdU<sup>+</sup> and Tuj1<sup>+</sup> cells (Fig. 1, F, G, H), indicating that with age, mNPC proliferative and neuron differentiation potential are impaired.

In Y-mNPCs isolated 7 d post-MPTP treatment, both proliferation and neuron differentiation were only transiently decreased (Fig.1, E, G, H), to return back to pre-MPTP levels by 45 dpt (Fig.1, E, G, H). In A-mNPCs, BrdU<sup>+</sup> and Tuj1<sup>+</sup> cells at both 7 and 45 dpt were significantly ( $p < 0.05$ ) reduced as compared to Y-mNPC

counterparts (Fig.1, F, G, H). Of note, MPTP further reduced neuron differentiation potential of A-mNPC at 7 dpt with respect to their saline-treated counterparts, with a return back to aged control levels by 45 dpt (Fig. 1, H). On the other hand, MPTP did not change the already low levels of A-mNPCs proliferation at both time-points (Fig. 1, G).

Using the fluorogenic substrate DEVD-AFC to measure Caspase3-like activity as death marker, we found increased DEVD-signal in mNPC isolated from A-MPTP mice as compared to Y-counterparts (Fig.1, I), indicating reduced mNPC survival capacity.

When Y/A-mNPCs were expanded for 8-10 passages in the presence of FGF-II and EGF and neuron differentiation potential studied after withdrawal of growth factors, addition of serum and N2 supplements for 10 DIV, we found that Y- and A-mNPCs expressed nestin, Tuj1, and GFAP, however in A-mNPC cultures, fewer Tuj1<sup>+</sup> cells were counted (Fig.1, F, J) as compared to Y-mNPCs (Fig.1, E, J) with a large proportion of GFAP<sup>+</sup> astrocytes (Fig.1, F). Altogether, aging and MPTP impair mNPCs plasticity with no recovery after *in vitro* expansion which correlate with the failure of TH<sup>+</sup> neuron to recover from MPTP insult.

### Aging inhibits canonical Wnt/ $\beta$ -catenin signaling in mNPCs : effect of glial age

Using qRT-PCR and specific primers for  $\beta$ -catenin, the hallmark of "canonical" Wnt/ $\beta$ -catenin pathway [54], we found that Y-mNPC express moderately high  $\beta$ -catenin both at a mRNA and protein levels, while these levels are sharply reduced in A-mNPCs (Fig. 2, A, C). In mNPCs isolated 45 d post-MPTP,  $\beta$ -catenin mRNA and protein levels were still decreased in A-mNPC as opposed to younger counterparts showing a significant up-regulation of  $\beta$ -catenin (Fig. 2, B, C). Additionally, *CyclinD1*, a  $\beta$ -catenin target gene regulating the progression of the G1 to the S phase of the cell cycle, was reduced in A- as compared to Y-mNPCs counterparts, both after saline or MPTP exposure (Fig. 2, A-B), thus supporting reduced canonical Wnt/ $\beta$ -catenin signaling. In keeping with these findings, *Axin2*, a Wnt target gene regarded as one most reliable endogenous index for measuring Wnt/ $\beta$ -catenin pathway activation [55], was significantly decreased in A- as compared to Y-mNPCs, with or without MPTP treatment (Fig. 2, A-B). Next, the expression of glycogen synthase kinase-3 $\beta$  (*GSK-3 $\beta$* ) which is part of a destruction complex leading to  $\beta$ -catenin ubiquitination and degradation by the proteasome [54] was significantly increased both at mRNA and protein levels in A- compared to Y-mNPCs (Fig. 2, A-C), thus indicating that *GSK-3 $\beta$*  up-regulation may play a role in the depletion of  $\beta$ -catenin observed in A-mNPCs. Likewise, using immunocytochemistry, decreased  $\beta$ -catenin-IF in A- as compared to Y-mNPC cultures was observed (Supplementary Figure 2, A-B) whereas exposure to Wnt1 (100 ng/ml) sharply increased BrdU-,  $\beta$ -catenin- and Tuj1-IF cell formation in both Y- and A-

mNPC, albeit this effect was stronger in younger counterparts (Supplementary Figure 2, C-H)

Astrocytes within the adult neurogenic niches produce Wnts that act in a paracrine manner on neuronal progenitors to induce expansion and/or neuron differentiation [8, 20, 22, 38-40]. Given that our previous study documented robust proneurogenic abilities of midbrain astrocytes [20], we used different co-culture paradigms between young/old Aq-PVR astrocytes and Y/A- mNPC (Fig. 2, D), in the absence or the presence of Wnt/ $\beta$ -catenin activators/inhibitors to address the effect of glial age and glial-derived Wnt. In accord with previous studies [8, 20], co-culture of Y-mNPCs with purified astrocytes markedly stimulated neurogenesis. Here we further show that this process depends on age, with 2d- and 2M (Fig. 2, E, M)-old astrocytes sharply increasing the proportion of BrdU<sup>+</sup> and Map2a<sup>+</sup> out of the Dapi<sup>+</sup> nuclei, compared to mNPC exposed to a non-specific cell type (NS), whereas exposure to 10 or 24 M-old astrocytes reversed astrocytes pro-neurogenic abilities (Fig. 2, F, M). On the other hand, A-mNPCs layered on the top of astrocyte monolayers of 2d- and 2M-old, showed a remarkable increase Map2a<sup>+</sup> cell number as compared to A-mNPC grown alone (Fig. 2, I, N), indicating that while glial is a limiting factor A-mNPCs still retain their neurogenic potential if adequately stimulated. Reciprocally, when A-mNPCs were exposed to 10M- (Fig. 2, J) or 24 M-old (Fig. 2, N) astrocytes a further inhibition of proliferative potential and the formation of Map2a<sup>+</sup> cells were observed, suggesting a synergy between astrocyte age and mNPC age in promoting neurogenic impairment.

Astrocyte-derived Wnts decline with age within the midbrain [20], the hippocampus [41] and the SVZ [33]. Here again, Wnt1 mRNA measured in Y-astrocytes (A.U= 0.6  $\pm$  0.05) decreased to almost undetectable levels in 10-24 M astrocytes of the midbrain-Aq region. Then, to activate  $\beta$ -catenin signaling in aged cocultures, we used a specific antagonist of GSK-3 $\beta$ , AR-AO14418 (AR) that stabilizes  $\beta$ -catenin in adult NPCs [20, 22, 57] and observed that aged-astrocyte-induced decreased neuron production was efficiently counteracted in both Y- and A-mNPC co-cultures (Fig. 2, O). Conversely, using the Wnt/ $\beta$ -catenin antagonists, Dkkopf-1 (Dkk1, 100 ng/ml), or Frizzled-1-cysteine rich domain (Fzd-A, 200 ng/ml), that binds to the Wnt ligand preventing its interaction with the Fzd receptor, we found a significant reversal of young astrocyte proneurogenic capacities, thereby supporting Wnt/ $\beta$ -catenin signaling interdiacy (Fig. 2, O).

In the study of Hermann et al [45], using a multistep differentiation protocol in co-culture conditions with PA6 stromal cells, a small number of mNPCs acquired morphological and functional properties of mDA neurons in culture [45]. In our previous studies [20], we showed that co-culture with astrocytes or activated astrocytes (that received a pre-treatment with the chemokines CCL3, CXCL11 or CXCL10 for 24 h) did stimulate tyrosine hydroxylase-positive (TH<sup>+</sup>) neuron

formation. We then exposed both Y/A-mNPCs to astrocytes or activated astrocytes as described [20] and observed, significantly higher numbers of Map2a<sup>+</sup> neuron formation (Fig. 2, G, K), of which a certain percentage of TH<sup>+</sup> out of the Tuj1<sup>+</sup> cells was counted (Fig. 2, H, L, P). We next verified the role of astrocyte-derived Wnt with Dkk1, and found a significant inhibition in the number of TH<sup>+</sup> cells (Fig. 2, P), whereas the GSK-3 $\beta$  antagonist, AR, sharply increased TH<sup>+</sup> neuron production (Fig. 2, P), thus supporting a permissive role of astroglial-derived factors together with canonical Wnt ligands in TH<sup>+</sup> neuron formation.

Together, Wnt/ $\beta$ -catenin signaling is dysfunctional in aged mNPC. Astrocytes with age loose proneurogenic and DA differentiation abilities via decline of astrocyte-derived factors including Wnts. Additionally, astrocyte-coculture paradigms coupled to Wnt-activation regimens can rescue these progenitors and promote TH<sup>+</sup> neuron formation.

### MPTP injury promotes GFAP and $\beta$ -catenin remodeling in Aq-PVRs: effect of age

#### The in vivo model

Next, we addressed the role of Wnt/ $\beta$ -catenin signaling *in vivo*. First, using fluorescence immunohistochemistry coupled to confocal laser microscopy applied to coronal midbrain sections we identified  $\beta$ -catenin-IF cells in midbrain Aq-PVRs of both Y- (Fig. 3 A1-4, E) and A- (C1-C4, E) saline-treated mice, albeit they were reduced with age.  $\beta$ -catenin-IF signal was localized in the ventricular lining and the Aq round. Bright GFAP<sup>+</sup> cells were seen bordering the ventricular wall all along the Aq-PVRs. Upon MPTP-induced DA neuron injury time-course analyses uncovered a remarkable increase  $\beta$ -catenin<sup>+</sup> cells by 1 d post-lesion (Fig. 3, B1, B2, E), whereas A-mice showed only a slight increase of  $\beta$ -catenin<sup>+</sup> cells (Fig. 3, D1-D4, E), suggesting an impairment of the aged Aq-PVR  $\beta$ -catenin response to injury. In Y-MPTP mice,  $\beta$ -catenin<sup>+</sup> cells were delineated by a strongly immunolabeled and thick GFAP<sup>+</sup> astrocyte cell layer (Fig. 3, B2-B4), while A-mice showed smaller increases in both  $\beta$ -catenin<sup>+</sup>- and GFAP<sup>+</sup> cells (Fig. 3, D2-D4). By 3 dpt, GFAP<sup>+</sup> astrocytes were seen almost segregating the  $\beta$ -catenin<sup>+</sup> cell domain (Fig. 2, B3, B4) in young mice, showing elongated cell bodies and long processes, coursing together/intermingled with  $\beta$ -catenin<sup>+</sup> cells (Fig. 3, B4). Especially, a remarkable increase of polysialylated neural cell adhesion molecule, PSA-Ncam<sup>+</sup> cells within the  $\beta$ -catenin<sup>+</sup> domain was observed (Supplementary Figure 3), suggesting stem/neuroprogenitor activation early upon DA neuron injury. By 7 post-injury,  $\beta$ -catenin<sup>+</sup> cells were downregulated, to recover over pre-MPTP levels by 21 dpt, indicating that changes in Aq microenvironment might influence cell survival or migration. In stark contrast, in A-mice, after a slight increase of  $\beta$ -catenin<sup>+</sup> cells 1-3 dpt, a significant reduction was observed at all times studied

(Fig. 3, D1-D4, E). By applying q-RT-PCR to freshly isolated midbrain periaqueductal tissues (Fig. 3, F) we found that *Wnt1* and  *$\beta$ -catenin* mRNAs were significantly increased in young mice 1 d after MPTP, while by 7 d a down regulation of *Wnt1* and  *$\beta$ -catenin* transcripts occurred to recover back by 21 dpt on (Fig. 3, F), whereas in older counterparts *Wnt1* and  *$\beta$ -catenin* mRNAs were reduced at all time-studied, in the face of up-regulated GSK-3 $\beta$  transcript levels (Fig. 3, F), thereby denoting failure of *Wnt1*, to be induced in response to DA neuron injury in A-mice.

### MPTP injury promotes $\beta$ -catenin signaling in Aq-PVR niche of young BATGAL reporters

With LacZ driven by a *siamois* promoter and seven T-cell factor/lymphoid enhancer factor (TCF/LEF) binding sites, transgenic (Tg) BATGAL mice allow the visualization of cells with transcriptionally active  $\beta$ -catenin signaling through the expression of Xgal and  $\beta$ -galactosidase ( $\beta$ -gal) the protein product of the *LacZ* transgene [50]. Wt and Tg mice were treated with either saline or MPTP and midbrain sections were stained with Xgal and anti- $\beta$ -gal antibodies (Abs). Confirming previous studies [38, 39], BATGAL<sup>+</sup> cells were found in SVZ and hippocampal SGZ of Tg (not shown) but not Wt controls. Within the Aq-PVRs of young saline-treated young reporters (at Bregma - 3.08 to -3.64), we identified Xgal<sup>+</sup> nuclei as opposed to Wt controls, where no Xgal staining was detected (Fig. 4 A1-2). As soon as after 1 day post MPTP-injury, Xgal staining sharply increased in the Aq round and the periaqueductal area (A3-A4).  $\beta$ -gal staining colocalized with dapi counterstained nuclei (B1-B2), occupying the domain of the  $\beta$ -catenin<sup>+</sup> cells, with no colocalization of  $\beta$ -gal with GFAP<sup>+</sup> cells (B1-2). Here again, MPTP-induced a profound remodelling of Aq architecture, with GFAP<sup>+</sup> astrocytes emanating long processes, as they were “embrassing”  $\beta$ -gal<sup>+</sup> nuclei (Fig. 4, B1-2). Using the *Axin2-LacZ* mouse strain [55, 58] (Tg Axin, Fig. 4, B3) where the Wnt target gene *Axin2* is mutated by insertion of LacZ, MPTP induced a comparable increase in  $\beta$ -Gal<sup>+</sup> nuclei and GFAP activation, mirroring BATGAL cell activation, thus corroborating endogenous Wnt/ $\beta$ -catenin signaling activation in Aq progenitors in response to MPTP-induced DA neuron death in the two reporter lines. Increased  $\beta$ -catenin signaling of BATGAL mice correlated with  *$\beta$ -catenin* and *Wnt1* mRNAs expression, confirming Wnt/ $\beta$ -catenin activation observed both *in vitro* and *in vivo* (see Figs 2-3). On the other hand, when BrdU was injected on the day of sacrifice and mice killed after 2 h, we did not observe BrdU-expressing nuclei, possibly indicating that these  $\beta$ -gal<sup>+</sup> cells may represent postmitotic precursors [46, 59, 60].

During development, astrocyte-derived Wnts and  $\beta$ -catenin control DA neurogenesis by maintaining the integrity of the neurogenic niche and promoting the progression from Nurr1<sup>+</sup>/TH<sup>-</sup> post-mitotic progenitors to Nurr1<sup>+</sup>/TH<sup>+</sup> neurons with a pivotal role exerted by Wnt1 [6-10, 61-63]. In BATGAL mice, *Nurr1/NR4A2* transcript

levels were sharply increased (Fig. 4, D). Interestingly, waves of Nurr1<sup>+</sup>/TH<sup>-</sup> cells were seen as they were migrating off the aqueductal region (Fig. 4, C1-C2). Tracking X-Gal/ $\beta$ -gal spatiotemporally, we found maximal X-Gal/ $\beta$ -gal in Aq-PVR region at the peak of DA neurodegeneration (i.e.: 1-3 dpt, Fig. 4, F). Later on, Xgal stained nuclei and  $\beta$ -gal<sup>+</sup>/Nurr1<sup>+</sup> neurons emanated from the ventral midline (Fig. 4, C3, C4, E, F). Together, Wnt/ $\beta$ -catenin signaling is dynamically regulated in Aq-PVR in young reporters in response to mDA neuron death. In the studied conditions,  $\beta$ -catenin signaling is not associated to proliferation but is spatiotemporally activated in postmitotic Nurr1<sup>+</sup>/TH<sup>-</sup> neurons.

### MPTP-induced neuron injury/repair activates Wnt/ $\beta$ -catenin signaling outside the Aq-PVR niche

The question, then, arises as to whether/how activation of  $\beta$ -catenin signaling in young mice might have functional consequences for DA neuron injury and repair after MPTP exposure. In Wt mice (Fig. 5, A1), bright DAT<sup>+</sup> neurons of the SNpc (A9) and the ventral tegmental area (VTA, A10) can be observed [48]. DAT<sup>+</sup> positive neurons are also seen merging from the Aq (see inset in A1) and GFAP<sup>+</sup> astrocytes are clearly visualized both within the Aq-PVRs and SNpc (Fig. 5, A1). In Tg saline-injected mice, discrete  $\beta$ -gal staining was observed in the SNpc (Fig. 5, A2). Immunofluorescence coupled to confocal laser microscopy of dual staining with  $\beta$ -gal and DAT, followed by quantitative analysis of the percentage of DAT<sup>+</sup>/ $\beta$ -gal<sup>+</sup> over the total number of DAT<sup>+</sup> in SNpc, indicated  $\beta$ -catenin signaling in approximately 6% of mature DAT<sup>+</sup> neurons (Fig. 5, A2, A3, D1), likely involved in the maintenance of adult SNpc DA neuron survival.

In Wt mice, MPTP-induced an approximate 60% loss of SNpc TH<sup>+</sup> neurons by 7dpt, associated by an almost complete disappearance of TH<sup>+</sup> neurons along the midline of the ventral tegmentum, and a sharp increase of GFAP<sup>+</sup> astrocytes in both Aq-PVRs and SNpc levels (Fig. 5, B1 and S Table 3). In Tg mice, tracking Xgal/ $\beta$ -gal in SNpc as a function of time and DA degeneration showed numerous Xgal<sup>+</sup> nuclei by 7 dpt (Fig. 5, B2-3). Dual staining with DAT and  $\beta$ -Gal (Fig. 5, B4) showed active  $\beta$ -catenin signaling in a certain proportion of DAT<sup>+</sup> neurons, but no colocalization between GFAP and  $\beta$ -gal (Fig. 5, B5). Spatio-temporal analyses carried out during the active degeneration phase (1-3 dpt), the stabilization of the lesion (7-14 dpt) and the recovery phase (>21 dpt), indicated a significant increase of  $\beta$ -catenin signaling 7-45 dpt (Fig. 5, D1), thus suggesting increased  $\beta$ -catenin signaling in DAT<sup>+</sup> neurons surviving and recovering from MPTP insult.

In Wt mice, with time, a slow nigrostriatal DA neuron recovery is observed between 45-65 dpt [20], as reflected by the significant increase of DA cell body numbers in SNpc, of DAT-IF fibers in striatum, and of DAT<sup>+</sup>/Nurr1<sup>+</sup> in SNpc (S Table 3). By 65 dpt, an intense

trafficking of bright TH<sup>+</sup> neurons and GFAP<sup>+</sup> astrocytes were still seen coursing from the Aq and midline as they were migrating to reach the VTA and SNpc DA subfields (Figs. 5 C1, Fig. 6, B1-B9). In Tg mice, the recovery of nigral cell bodies started already from 21-30 dpt on, as reflected by the significant increase in percentages of DAT<sup>+</sup> neurons from almost 38% (at 7-14 dpt) to approximately 58% (at 21-30 dpt) (Fig. 5, D1). Likewise, Nurr1<sup>+</sup>/DAT<sup>+</sup> neurons increased from almost 28% (7-14 dpt) to 60% (21-30 dpt) (Fig. 5 D2).

Dual staining with  $\beta$ -gal and DAT showed a remarkable increase of DAT<sup>+</sup> neurons, of which a significant percentage co-expressed  $\beta$ -gal (Fig. 5, C2, C3 D1, arrowhead), while other  $\beta$ -gal<sup>+</sup> cells were DAT<sup>-</sup> (Fig. 5, D1). Analyses of the percentages of DAT<sup>+</sup>/ $\beta$ -gal<sup>+</sup> as above indicated that at both 21-30 and 45-65 dpt,  $\beta$ -catenin signaling occurred in > 50% of repairing DA neurons (Fig. 5, D1). Coupled to the quantitative analysis of Nurr1<sup>+</sup>/ $\beta$ -gal<sup>+</sup> and Nurr1<sup>+</sup>/DAT<sup>+</sup> cells (Fig. 5, D2), these results suggest ongoing  $\beta$ -catenin signaling activation both in Nurr1<sup>+</sup>/DAT<sup>-</sup> predopaminergic and recovering DAT<sup>+</sup> neurons during the neurorescue process (Fig. 5, D1-D2, E).

### **Aging-induced failure to activate Wnt/ $\beta$ -catenin signaling: effect of direct $\beta$ -catenin activation *in situ* in nigrostriatal DA recovery**

In aged mice, after MPTP exposure, Xgal/ $\beta$ -gal staining as well as mRNAs for *Wnt1*,  *$\beta$ -catenin* and *Nurr1* were all significantly reduced (Fig. 7, A1-4), in the face of exacerbated *GSK-3 $\beta$*  transcript levels (Fig. 7, C3), supporting dysregulated *Wnt1* and  $\beta$ -catenin expression with age. The effect of intracerebral infusion of the *GSK-3 $\beta$*  antagonist, AR or PBS, in midbrain Aq-PVR 24 h before MPTP was next addressed. AR infusion in Aq-PVRs resulted in  $\beta$ -catenin signaling activation as revealed by a remarkable increase of  $\beta$ -gal<sup>+</sup> (Fig. 7, A2-A3) nuclei and robust BrdU incorporation (Fig. 7, B2, B3) in the Aq round 2 post-MPTP. Strongly immunolabeled GFAP<sup>+</sup> astrocytes and  $\beta$ -catenin<sup>+</sup> cells were observed (Fig. 7, B1-B4) all around the Aq, of which a certain number co-expressed BrdU (Fig. 7, B1-B4, A3, inset), indicating the ability of direct *GSK-3 $\beta$*  antagonism to promote proliferation and to induce Aq remodeling as observed in young mice.

At midbrain level, in Wt aged mice, upon MPTP treatment, DA cell body numbers, striatal DAT innervation and DAT<sup>+</sup>/Nurr1<sup>+</sup> cell numbers were all significantly reduced with no recovery until 45 dpt as compared to younger counterparts (Supplementary Table 3). In Tg mice, spatio-temporal analyses in mid-brain sections of PBS/AR-infused mice were carried out as above to monitor nigrostriatal recovery upon MPTP (Fig. 7 C1-C6 and Supplementary Figure 7). Hence, dual staining of midbrain sections with DAT and Nurr1, and time-course analysis of the percentages of DAT<sup>+</sup>/Nurr1<sup>+</sup> showed that almost 65% of DAT<sup>+</sup> neurons co-express Nurr1, whereas AR infusion increased this percentage

to almost 85% (Fig. 7, C1,C4). MPTP induced a sharp decrease of DAT<sup>+</sup>/Nurr1<sup>+</sup> neurons for all the experimental period (Fig. 7, C2, C4), whereas AR infusion induced a less severe decrease of DAT<sup>+</sup>/Nurr1<sup>+</sup> neurons 2 dpt, and with time, AR-infused mice did show progressive increases of SNpc Nurr1<sup>+</sup>/DAT<sup>+</sup> neurons (Fig. 7, C4). Likewise,  $\beta$ -gal signal increased with time in DAT<sup>+</sup> neurons (Fig. 7, C5-7), and by 45 dpt, the percentages of DAT<sup>+</sup>/Nurr1<sup>+</sup> and DAT<sup>+</sup>/ $\beta$ -gal<sup>+</sup> neurons were comparable to younger counterparts, suggesting that activation of  $\beta$ -catenin signaling in midbrain Aq niche can promote neurorecovery. At striatal level, the markers of DA functionality were significantly increased between 21-45 dpt in AR compared to PBS mice (Suppl Fig. 7A-C). Additionally, motor behavior analyses with the Rotarod, indicated that motor coordination deficit of aged MPTP mice was reversed by AR infusion, whereas PBS-infused MPTP mice failed to recover from the motor deficit (Supplementary Fig. 7D), supporting the requirement of  $\beta$ -catenin signaling activation to promote nigrostriatal recovery of aged mice.

## **DISCUSSION**

Using *in vitro* cell culture systems and *in vivo* models applied to  $\beta$ -catenin reporters, this study uncovered the adult rodent midbrain Aq-ventricular region as a novel Wnt-responsive niche. MPTP-induced DA neuron death promoted a remarkable astrocyte-dependent remodeling and Wnt/ $\beta$ -catenin signaling activation in Nurr1<sup>+</sup> post-mitotic DA precursors, in surviving and repairing SNpc-DA neurons, a process correlated with a robust time-dependent DA neurorescue. By contrast, the changing properties of midbrain-Aq microenvironment with age impact in DA neurogenic potential of Aq-mNPCs via loss of astrocytic *Wnt1* and failure of Wnt/ $\beta$ -catenin signaling activation both inside and outside the niche, in turn associated to failure to recover from MPTP insult. Importantly, aged mNPCs still retain their neurogenic and DA differentiation potential when Wnt/ $\beta$ -catenin signaling is restored via "astrocyte rejuvenation"-induced *Wnt1* expression or under Wnt/ $\beta$ -catenin activation regimens, such as *GSK-3 $\beta$*  antagonism, leading to DA neuron formation. Together, these findings suggest that disruption of a key neurodevelopmental signaling pathway with age may predispose to loss of mDA plasticity via inhibition of Wnt/ $\beta$ -catenin signaling as a prelude for PD development and vulnerability. These results may indicate the potential to restore mDA neuron functionality by activating Wnt/ $\beta$ -catenin signaling in endogenous Wnt-responsive sources, through either pharmacological/cellular approaches aimed at activating/recruiting endogenous progenitors and rescuing the imperiled/diseased DA neurons [44,47].

A lack of appropriate niche environmental signals is recognized to restrict the neurogenic potential of multipotent progenitors isolated from PD-related brain regions [29,32,43-46,64]. Studying the PVRs throughout

the whole ventricular axis of the young intact mouse brain, Hermann et al. [46] reported that proliferative cell populations are restricted to the PVR-LV. However, in Aq-PVRs, the PSA-Ncam<sup>+</sup>-like cells observed *in vivo* strongly correlated with the number of neurosphere-forming cells isolated, *in vitro*, suggesting that a quiescent subtype of PSA-Ncam<sup>+</sup> cells might be the source of mNPCs endowed with neurogenic and DA differentiation potential [45,46]. Here, the profound morphological remodeling of the Aq-PVRs and  $\beta$ -catenin signaling activation upon DA neuron injury may suggest a high degree of plasticity of this caudal midbrain ventricular region upon lesion of DA nigrostriatal system, while loss of plasticity was observed with age. Interestingly, such up-regulated Wnt/ $\beta$ -catenin response in Aq-PVRs, close to the SNpc DA cell bodies, as opposed to the transient  $\beta$ -catenin down-regulation observed in SVZ [22, 39] adjacent to striatal DA terminals [29, 30, 31], denotes a region-specific response to DA neurodegeneration. The positive correlation between NPC proliferation and  $\beta$ -catenin signaling in the SVZ [22,39,58] as opposed to the Aq-PVRs [45,59], may be linked to factors/mechanisms restraining the proliferative potential within the Aq-PVRs, *in vivo*, as indicated by the robust proliferation observed after growth factor [65] or herein with GSK-3 $\beta$  antagonist infusion. Within the SVZ, the intimate contact of NPCs with surrounding glia [37 and Refs herein], coupled to the vast array of growth/neurotrophic factors, neurotransmitters (in particular dopamine), morphogens, cytokines and Wnt/ $\beta$ -catenin signaling components, contribute to the NPC homeostatic regulation via complex cell-cell interactions and signaling cascades [22, 29-33, 37, 66-69], suggesting that similar mechanisms may be at play here. Different lines of evidences point to age-dependent dysregulation of Wnt signaling as causal factor in aging-induced impairment of both SVZ and SGZ niches. In the SVZ, aging and MPTP antagonize Wnt/ $\beta$ -catenin signaling leading to neurogenic impairment via crosstalk with inflammatory pathways at least in part mediated by up-regulation of microglial proinflammatory mediator-induced downregulation of Wnt/ $\beta$ -catenin signaling [22,33,68,69], with potential implications for mNPC neurogenic impairment herein observed [68, 69]. In SGZ, decreased Wnt3 release from aged hippocampal astrocytes regulate the age-associated decline of adult neurogenesis [41]. Importantly, the endogenous Wnt antagonist Dkk1 increases with age, resulting in the suppression of adult neurogenesis and proliferation, whereas Dkk1 knockout mice show increased Wnt signaling, leading to enhanced neurogenesis and improved spatial memory [42]. Our findings add to this scenario the midbrain ventricular region and both pre-DA and DA neurons as candidate targets that respond to SNpc lesion with an intrinsic repair program against injury [57, 60, 68, 69]. In this connection, the activation of astrocyte compartment and increased Wnt/ $\beta$ -catenin signaling herein observed in Nurr1<sup>+</sup>/TH<sup>-</sup> precursors of Aq-PVRs appears

of specific interest in the light of the pivotal roles of Wnt1 and  $\beta$ -catenin in the development of these neurons [see 6-10, 13].  $\beta$ -catenin maintains adherent junctions and cell polarity of progenitor cells, as well as the integrity of radial glia which provide scaffolds for newly generated DA neurons to migrate on towards their final destinations [61,62,70]. Radial glia-like progenitors express Wnt1 [68], while deletion of Wnt1 induces a severe loss of radial glia-like cells and DA neurons [6, 7, 10, 13, 61,62]. Wnt signaling via  $\beta$ -catenin promotes the differentiation of Nurr1<sup>+</sup>/TH<sup>-</sup> DA precursors. Then, when the degradation of  $\beta$ -catenin is inhibited with specific GSK-3 $\beta$  antagonists, the size of DA neurons increases, through conversion of precursors expressing Nurr1 into TH<sup>+</sup> [7]. By contrast, removal of  $\beta$ -catenin in DA progenitors reduces the progression from committed DA progenitors to DA neuron [6,7,10, 61,62]. Of specific mention, the Wnt/ $\beta$ -catenin-dependent [63] Nurr1 expression is required for the maintenance, the survival and protection of adult mDA neurons [71]. On the other hand, active GSK-3 $\beta$  overexpression is critically involved in neuronal death and depletion of the neurogenic niches, whereas GSK-3 $\beta$  inhibitors can in part mitigate these effects [20, 21,72-75]. Here, with age, astrocyte dysfunction with loss of endogenous Wnts lead to up-regulated GSK-3 $\beta$  levels, engendering a. disruption of Aq-PVR architecture and function with failure of Wnt/ $\beta$ -catenin/Nurr1 signaling activation and neurogenic impairment; b. increased vulnerability of mDA neurons likely responsible for reduced of both DA survival and repair.

The observation that  $\beta$ -catenin signaling is active in discrete DA neuronal populations of the intact brain is of interest in the light of its recognized role in neuronal synapse regulation and remodeling as well as in DA neuron maintenance and neuroprotection, as part of a neuron-astrocyte crosstalk (17, 20-22, 25, 68, 69). Here, stabilization of  $\beta$ -catenin reinstated nigrostriatal plasticity and motor coordination in aged MPTP, whereas ablation of  $\beta$ -catenin in DA neurons alters motor and reward-associated memories and affect striatal mRNA levels for several markers known to regulate synaptic plasticity and DA neurotransmission [76].

In conclusion, coupled to the increasing evidences on the key role of Wnt/ $\beta$ -catenin signaling cascades in neurodevelopment, neurodegeneration and regeneration of DA neurons [see 18], developing targeted *in situ* pharmacological interventions/cell manipulations that boost the inherent DA regenerative potential may have implications for restorative and regenerative approaches in PD [3,4,9,12,13,68,69,77-83].

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## AUTHOR CONTRIBUTIONS

B.M.: Conception and design, Data analysis and interpretation, Manuscript writing, Final approval manuscript; S.P.: Conception and design, data analysis and interpretation; F.L.: collection and assembly of data; C.T.: collection and assembly of data; N.T.: collection and assembly of data; S.C.: collection and assembly of data; M.C.M.: collection and assembly of data; M.F.S.: collection and assembly of data

## REFERENCES

- Di Monte DA, Langston J.W. Idiopathic and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced Parkinsonism. In: "Neuroglia", Kettenmann, H. and Ransom, B.R. (eds), Oxford University Press 1995; Chapter 65: 989-997.
- Olanow CV, Schapira AH. Therapeutic prospects for Parkinson disease. *Ann Neurol* 2013; 74:337-47.
- Politis M, and Lindvall O. Clinical application of stem cell therapy in Parkinson's disease. *BMC Medicine* 2012; 10:1
- Herrmann A, Storch A. Induced neural stem cells (iNSCs) in neurodegenerative diseases. *J Neural Transm.* 2013;120 Suppl 1:S19-25.
- Ali, F., Stott, S.R, Barker, R.A. Stem cells and the treatment of Parkinson's disease. *Exp Neurol.* 2013, pii: S0014-4886(13)00004-6.
- Prakash N, Wurst W. Wnt1-regulated genetic networks in midbrain dopaminergic neuron development. In "Wnt signaling cascades in neurodevelopment, neurodegeneration and regeneration", *Journal of Mol Cell Biol* 2014, Special Issue, Vol 6 (1): 34-41
- Castelo-Branco G, Rawal N, Arenas, E. GSK-3 $\beta$  inhibition/ $\beta$ -catenin stabilization in ventral midbrain precursors increases differentiation into dopamine neurons. *J of Cell Sci* 2004; 11:5731-37.
- Castelo-Branco G, Sousa KM, Bryja V, et al. Ventral midbrain glia express region-specific transcription factors and regulate dopaminergic neurogenesis through Wnt-5a secretion. *Mol Cell Neurosci* 2006; 31:251-62.
- Parish CL, Lachlan H, Thompson LH. Modulating Wnt signaling to improve cell replacement therapy for Parkinson's disease. In "Wnt signaling cascades in neurodevelopment, neurodegeneration and regeneration", *Journal of Mol Cell Biol* 2014, Special Issue, Vol 6(1): 54-63
- Joksimovic M, Awatramani R. Wnt/ $\beta$ -catenin signaling in midbrain dopaminergic neuron specification and neurogenesis. In "Wnt signaling cascades in neurodevelopment, neurodegeneration and regeneration", *Journal of Mol Cell Biol* 2014, Special Issue, Vol 6(1): 27-33.
- Kriks S, Shim JW, Piao J, et al. Dopamine neurons derived from human ES cells efficiently engraft in animal models of Parkinson's disease. *Nature* 2011; 480:547-51.
- Kirkeby A, Grealish S, Wolf DA, et al. Generation of regionally specified neural progenitors and functional neurons from human embryonic stem cells under defined conditions. *Cell Rep* 2012; 1:703-14.
- Arenas E. Wnt signaling in midbrain dopamine neuron development and Regenerative medicine for Parkinson's disease. In "Wnt signaling cascades in neurodevelopment, neurodegeneration and regeneration", *Journal of Mol Cell Biol* 2014, Special Issue, Vol 6(1): 42-53.
- Blakely BD, Bye CR, Fernando CV, et al. Ryk, a receptor regulating Wnt5a-mediated neurogenesis and axon morphogenesis of ventral midbrain dopaminergic neurons. *Stem Cells Dev* 2013; 22: 2132-44.
- Clevers H, Nusse R. Wnt/ $\beta$ -catenin signaling and disease. *Cell* 2012; 149:1192-205.
- Inestrosa NC, Varela-Nallar L. Wnt Signalling in the Nervous System and in Alzheimer's Disease. In "Wnt signaling cascades in neurodevelopment, neurodegeneration and regeneration", *Journal of Mol Cell Biol* 2014, Special Issue, Vol 6(1): 64-74.
- Purro SA, Galli S, Salinas PC. Dysfunction of Wnt signaling and synaptic disassembly in neurodegenerative diseases. In "Wnt signaling cascades in neurodevelopment, neurodegeneration and regeneration", *Journal of Mol Cell Biol* 2014, Special Issue, Vol 6(1): 75-80.
- Harvey K, Marchetti B. Regulating Wnt signalling, a strategy to prevent neurodegeneration and induce regeneration. In "Wnt signaling cascades in neurodevelopment, neurodegeneration and regeneration", *Journal of Mol Cell Biol* 2014, Special Issue, Vol 6(1):1-2.
- Berwick DC, Harvey K. The regulation and deregulation of Wnt signalling by PARK genes in health and disease. In "Wnt signaling cascades in neurodevelopment, neurodegeneration and regeneration", *Journal of Mol Cell Biol* 2014, Special Issue, Vol 6(1): 3-12.
- L'Episcopo F, Tirolo C, Testa N, et al. Reactive astrocytes and Wnt/ $\beta$ -catenin signaling link nigrostriatal injury to repair in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of Parkinson's disease. *Neurobiol of Dis* 2011a; 41:508-27.
- L'Episcopo F, Serapide MF, Tirolo C, et al. A Wnt1 regulated Frizzled-1/ $\beta$ -catenin signaling pathway as a candidate regulatory circuit controlling mesencephalic dopaminergic neuron-astrocyte crosstalk: Therapeutical relevance for neuron survival and neuroprotection. *Mol Neurodegen* 2011b; 13:6-49.
- L'Episcopo F, Tirolo C, Testa N, et al. Plasticity of subventricular zone neuroprogenitors in MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) mouse model of Parkinson's disease involves crosstalk between inflammatory and Wnt/ $\beta$ -catenin signaling pathways: functional consequences for neuroprotection and repair. *J Neurosci* 2012; 32: 2062-85.
- Berwick DC, Harvey K. LRRK2 functions as a Wnt signaling scaffold, bridging cytosolic proteins and membrane-localized LRP6. *H Mol Genet* 2012; 21: 4966-79.
- Gollamudi S, Johri A, Calingasan NY, et al. Concordant signaling pathways produced by pesticide exposure in mice correspond to pathways identified in human Parkinson's disease. *PLoS One* 2012; 7:e36191.
- Marchetti B, L'Episcopo F, Morale MC, et al. Uncovering novel actors in astrocyte-neuron crosstalk in Parkinson's disease: the Wnt/ $\beta$ -catenin signaling cascade as the common final pathway for neuroprotection and self-repair. *Eur J Neurosci* 2013; 37:1550-63.
- Bezard E, Gross CE. Compensatory mechanisms in experimental and human parkinsonism: towards a dynamic approach. *Prog Neurobiol* 1998; 55:93-116.
- Hornykiewicz O. Parkinson's disease and the adaptive capacity of the nigrostriatal dopamine system: Possible neurochemical mechanisms. In: *Advances in neurology* 1993; Vol 60.
- Collier TJ, Lipton J, Daley BF, et al. Aging-related changes in the nigrostriatal dopamine system and the response to MPTP in nonhuman primates: diminished compensatory mechanisms as a prelude to parkinsonism. *Neurobiol Dis* 2007; 26:56-65.
- Borta A, Holinger GU. Dopamine and adult neurogenesis. *J Neurochem* 2007; 100:587-95.
- O'Keeffe GO, Barker RA, Caldwell MA. Dopaminergic modulation of neurogenesis in the subventricular zone of the adult brain. *Cell Cycle* 2009; 8:18.
- Winner B, Desplats P, Hagl C, et al. Dopamine receptor activation promotes adult neurogenesis in an acute Parkinson model. *Exp Neurol* 2009a; 219: 543-52.
- Winner B, Vogt-Weisenhorn DM, Chichung D, et al. Cellular Repair Strategies in

Parkinson's Disease. *The Adv Neurol Disord* 2009; 2:51-60.

33 L'Episcopo F, Tirolo C, Testa N, et al. Aging-induced Nrf2-ARE pathway disruption in the subventricular zone (SVZ) drives neurogenic impairment in parkinsonian mice via PI3K-Wnt/ $\beta$ -catenin dysregulation. *J Neurosci* 2013; 33:1462-85.

34 Alvarez-Builla A, Garcia-Verdugo JM, Tramontin AD. A unified hypothesis on the lineage of neural stem cells. *Nat Rev Neurosci* 2001; 2:2287-93.

35 Song H, Stevens CF, Gage FH. Astroglia induce neurogenesis from adult neural stem cells. *Nature* 2002; 417:39-44.

36 Lim DA, Alvarez-Builla A. Interaction between astrocytes and adult subventricular zone precursors stimulates neurogenesis. *PNAS USA* 1999; 96:7526-31.

37 Fuentealba LC, Obernier K, Alvarez-Builla A. Adult neural stem cells bridge their niche. *Cell Stem Cell* 2012; 10:698-708.

38 Lie DC, Colamarino SA, Song HG, et al. Wnt signaling regulates adult hippocampal neurogenesis. *Nature* 2005; 437:1370-75.

39 Adachi K, Mirzadeh Z, Sakaguchi M, et al.  $\beta$ -catenin signaling promotes proliferation of progenitor cells in the adult mouse subventricular zone. *Stem Cells* 2007; 25:2827-36.

40 Kuwabara T, Hsieh J, Muotri A, et al. (2009) Wnt-mediated activation of NeuroD1 and retro-elements during adult neurogenesis. *Nat Neurosci* 12,1097-1105.

41 Okamoto M, Inoue K, Iwamura H, et al. Reduction in paracrine Wnt3 factors during aging causes impaired adult neurogenesis. *FASEB J* 2011; 25:3570-82.

42 Seib DRM, Corsini NS, Ellwanger K, et al. Loss of Dickkopf-1 restores neurogenesis in old age and counteracts cognitive decline. *Cell Stem Cell* 2013; 12:204-14.

43 Lie DC, Dzieczapolski G, Willhoite AR, et al. The adult substantia nigra contains progenitor cells with neurogenic potential. *J Neurosci* 2002; 22:6639-49.

44 Hermann A, Storch A. Endogenous regeneration in Parkinson's disease: Do we need orthotopic dopaminergic neurogenesis? *Stem Cells* 2008; 26:2749-52.

45 Hermann A, Maisel M, Wegner F, et al. Multipotent neural stem cells from the adult tegmentum with dopaminergic potential develop essential properties of functional neurons. *Stem Cells* 2006; 24: 949-64.

46 Hermann A, Suess C, Fauser M, et al. Rostro-caudal loss of cellular diversity within the periventricular regions of the ventricular system. *Stem Cells* 2009; 27:928-41.

47 Meyer AK, Maisel M, Hermann A, et al. Restorative approaches in Parkinson's Disease: Which cell type wins the race? *J of Neurol Sci* 2010; 289:93-103.

48 Hokfelt T, Martensson R, Bjorklund A, et al. Distributional maps of tyrosine-hydroxylase-immunoreactive neurons in the rat brain. In: Bjorklund A, Hokfelt T, (eds). Classical transmitters in the CNS, part I, Elsevier, Amsterdam. *Handbook of chemical neuroanatomy* 1984; Vol 2: 277- 379.

49 Kawano H, Ohyama K, Kawamura K, et al. Migration of dopaminergic neurons in the embryonic mesencephalon of mice. *Brain Res Dev Brain Res* 1995; 86:101-13.

50 Maretto S, Cordenonsi M, Dupont S, et al. Mapping Wnt/ $\beta$ -catenin signaling during mouse development and in colorectal tumors. *PNAS* 2003; 100:3299-3304.

51 Jackson-Lewis V, Przedborski S. Protocol for the MPTP model of Parkinson's disease. *Nat Prot* 2007; 2:141-51.

52 Pluchino S, Zanotti L, Rossi B, et al. Neurosphere-derived multipotent precursors promote neuroprotection by an immunomodulatory mechanism. *Nature* 2005; 436:266-71.

53 Franklin KBJ, Paxinos G. *The mouse brain in stereotaxic coordinates*. (Academic Press Inc) 1997.

54 Gordon MD, Nusse R. Wnt signaling: Multiple pathways, multiple receptors, and multiple transcription factors. *J Biol Chem* 2006; 281:22429-33.

55 Lustig B, Jerchow, B, Sachs, M, et al. Negative feedback loop of Wnt signaling through upregulation of conductin/axin2 in colorectal and liver tumors. *Mol Cell Biol* 2002; 22:1184-93.

56 Morale MC, Serra PA, Delogu MR, Migheli R, Rocchitta G, Tirolo C, Caniglia S, Testa N, L'Episcopo F, Gennuso F, Scotto GM, Barden N, Miele E, Desole MS, Marchetti B. Glucocorticoid receptor deficiency increases vulnerability of the nigrostriatal dopaminergic system: critical role of glial nitric oxide. *FASEB J*. 2004 Jan;18(1):164-6. Epub 2003 Nov 20.

57 Osakada F, Ooto S, Akagi T, et al. Wnt signaling promotes regeneration in the retina of adult mammals. *J of Neurosci* 2007; 27:4210-19.

58 Kalani MHS, Chschier SH, Cord BJ, et al. Wnt-mediated self-renewal of neural stem/progenitor cells. *PNAS* 2008; 105:16070-75.

59 Yoshimi K, Ren YR, Seki T, et al. Possibility for neurogenesis in substantia nigra of parkinsonian brain. *Ann Neurol* 2005; 58:31-40.

60 White BD, Nathe RJ, Maris DO, et al.  $\beta$ -Catenin signaling increases in proliferating NG2+ progenitors and astrocytes during post-traumatic gliogenesis in the adult brain. *Stem Cell* 2010; 28:297-307.

61 Tang M, Miyamoto, Y, Huang EJ. Multiple roles of  $\beta$ -catenin in controlling the neurogenic niche for midbrain dopamine neurons. *Develop* 2009; 136:2027-38.

62 Tang M, Villaescusa JC, Luo SX, et al. Interactions of Wnt/ $\beta$ -catenin signaling and sonic hedgehog regulate the neurogenesis of ventral midbrain dopamine neurons. *J Neurosci* 2010; 7:9280-91.

63 Kitagua H, Ray WJ, Glantsching H, et al. A regulatory circuit mediating convergence between Nurr1 transcriptional regulation and Wnt signalling. *Mol Cell Biol* 2007; 27:7486-96.

64 Wang S, Okun MS, Suslov O, et al. Neurogenic potential of progenitor cells isolated from postmortem human Parkinsonian brains. *Brain Res* 2012; 1464:61-72.

65 Martens DJ, Seaberg RM, van der Kooy D. In vivo infusions of exogenous growth factors into the fourth ventricle of the adult mouse brain increase the proliferation of

neural progenitors around the fourth ventricle and the central canal of the spinal cord. *Eur J Neurosci* 2002; 16:1045-57.

66 Pluchino S, Muzio L, Imitola J, et al. Persistent inflammation alters the function of the endogenous brain stem cell compartment. *Brain* 2008; 131:2564-78.

67 Ekdahl CT, Kokaia Z, Lindvall O. Brain inflammation and adult neurogenesis: the dual role of microglia. *Neuroscience* 2009; 158:1021-29.

68 Marchetti B, Pluchino S. Wnt your brain be inflamed? Yes, it Wnt! *Trends Mol Med* 2013; 19:144-56.

69 L'Episcopo F, Tirolo C, Caniglia S, et al. Targeting Wnt signaling at the neuroimmune interface in dopaminergic neuroprotection/repair in Parkinson's disease. In "Wnt signaling cascades in neurodevelopment, neurodegeneration and regeneration", *Journal of Mol Cell Biol* 2014, Special Issue, Vol 6(1):13-26.

70 Bonilla S, Hall AC, Pinto L, et al. Identification of Midbrain Floor Plate Radial Glia-Like Cells as Dopaminergic Progenitors. *Glia* 2008; 56:809-20.

71 Kadkhodaei B, Ito T, Joodmardi E, et al. Nurr1 is required for maintenance of maturing and adult midbrain dopamine neurons. *J Neurosci* 2009; 29:15923-32.

72 Duka T, Duka V, Joyce JN, et al.  $\alpha$ -Synuclein contributes to GSK-3 $\beta$ -catalyzed Tau phosphorylation in Parkinson's disease models. *FASEB J* 2009; 23:2820-30.

73 Petit-Paitel A, Brau F, Cazareth J, et al. Involvement of cytosolic and mitochondrial GSK-3 $\beta$  in mitochondrial dysfunction and neuronal cell death of MPTP/Mpp+ treated neurons. *Plos One* 2009; 4:e5491.

74 Wang W, Yang Y, Ying C, et al. Inhibition of glycogen synthase kinase-3 $\beta$  protects dopaminergic neurons from MPTP toxicity. *Neuropharmacol* 2007; 52:1678-84.

75 Sirerol-Piquer M, Gomez-Ramos P, Hernández F, et al. GSK3 $\beta$  overexpression induces neuronal death and a depletion of the neurogenic niches in the dentate gyrus. *Hippocampus* 2011; 21:910-22.

76 Diaz-Ruiz O, Zhang YJ, Shan F, et al. Attenuated response to methamphetamine sensitization and deficits in motor learning and memory after selective deletion of  $\beta$ -catenin in dopamine neurons. *Learn Mem* 2012; 19:341-50.

77 Klaisle P, Lesemann A, Huehnchen P, et al. Physical activity and environmental enrichment regulate the generation of neural precursors in the adult mouse substantia nigra in a dopamine-dependent manner. *BMC Neurosci* 2012; 13:132.

78 Esfandiari F, Fathi A, Gourabi H, et al. Glycogen synthase kinase-3 inhibition promotes proliferation and neuronal differentiation of human-induced pluripotent stem cell-derived neural progenitors. *Stem Cells Dev* 2012; 21:3233-43.

79 Kokaia Z, Martino G, Schwartz M, Lindvall O. (2012) Cross-talk between neural stem cells and immune cells: the key to better brain repair? *Nat Neurosci* 15, 1078-87.

80 Srikanth M, and Kessler JA. Nanotechnology-novel therapeutics for CNS disorders. *Nat Rev. Neurol* 2012; 8:307-18.

81 Sundberg M, Bogetofte H, Lawson T, et al. Improved cell therapy protocols for Parkinson's disease based on differentiation efficiency and safety of hESC, hiPSC-, and non-human primate iPSC-derived dopaminergic neurons. *Stem Cells* 2013; 31:1548-62.

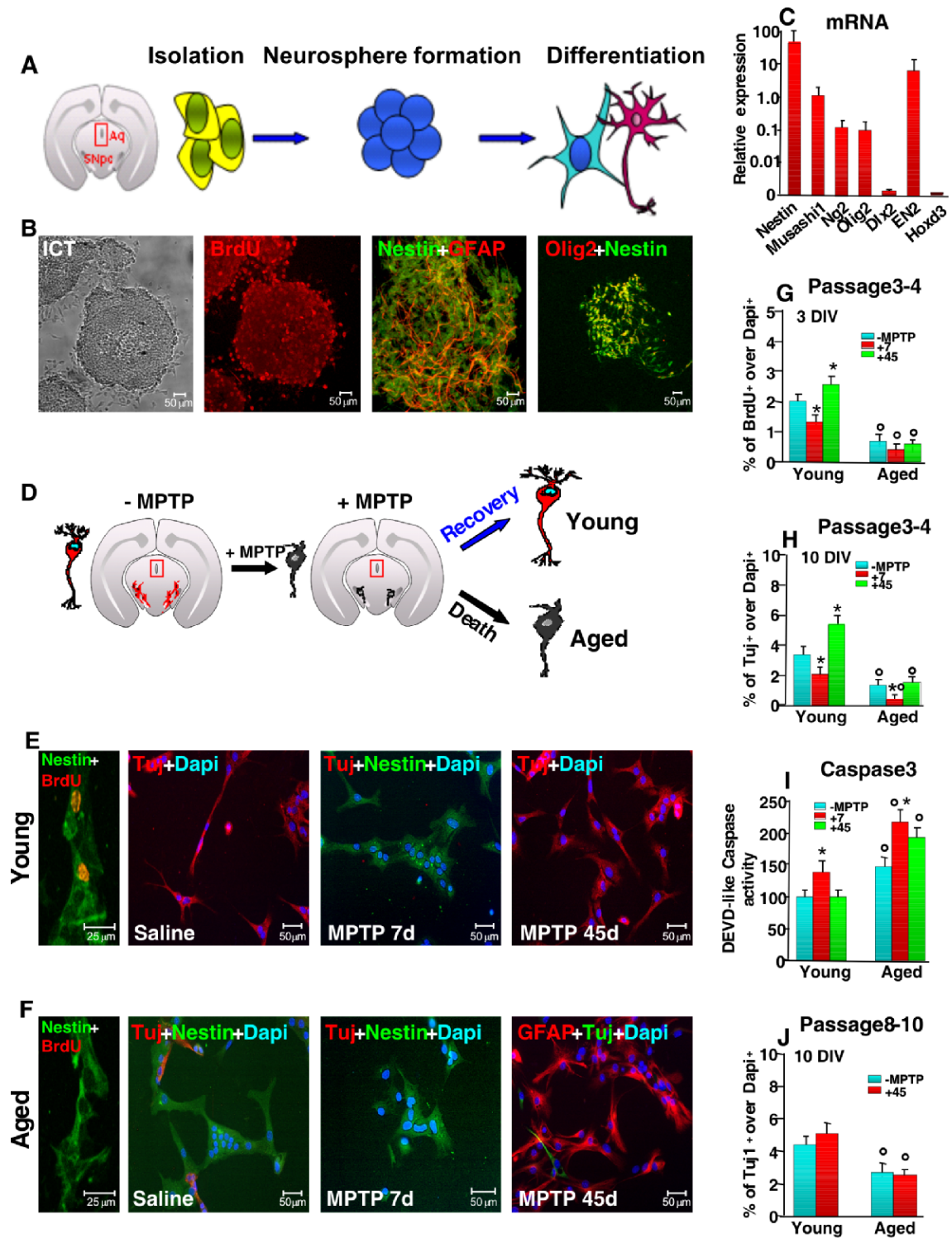
82 Lian X, Zhang J, Azarin SM, Zhu K, Hazeltine LB, Bao X, Hsiao C, Kamp TJ, Palecek SP. Directed cardiomyocyte differentiation from human pluripotent stem cells by modulating Wnt/ $\beta$ -catenin signaling under fully defined conditions. *Nat Protoc.* 2013 Jan;8(1):162-75.

83 Zhao, W.N., Cheng, C., Theriault, K.M., Sheridan, S.D., Tsai, L.H., Haggarty, S.J. (2012). A high-throughput screen for Wnt/ $\beta$ -catenin signaling pathway modulators in human iPSC-derived neural progenitors. *J Biomol Screen.* 17,1252-63.

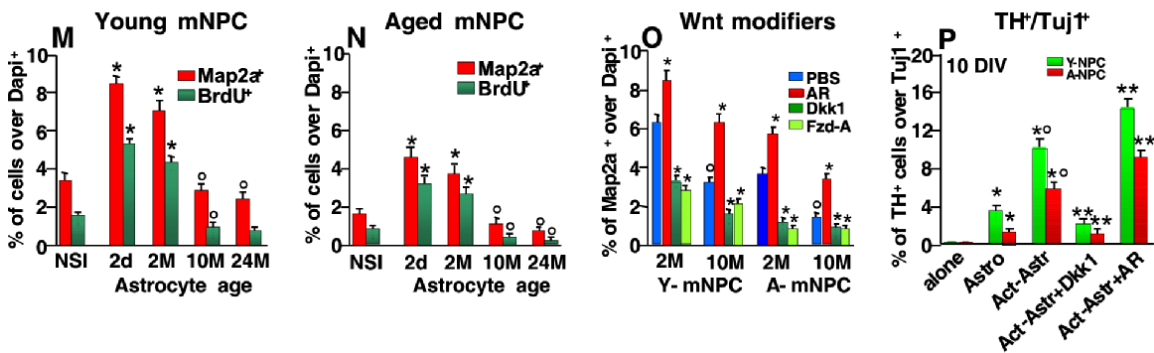
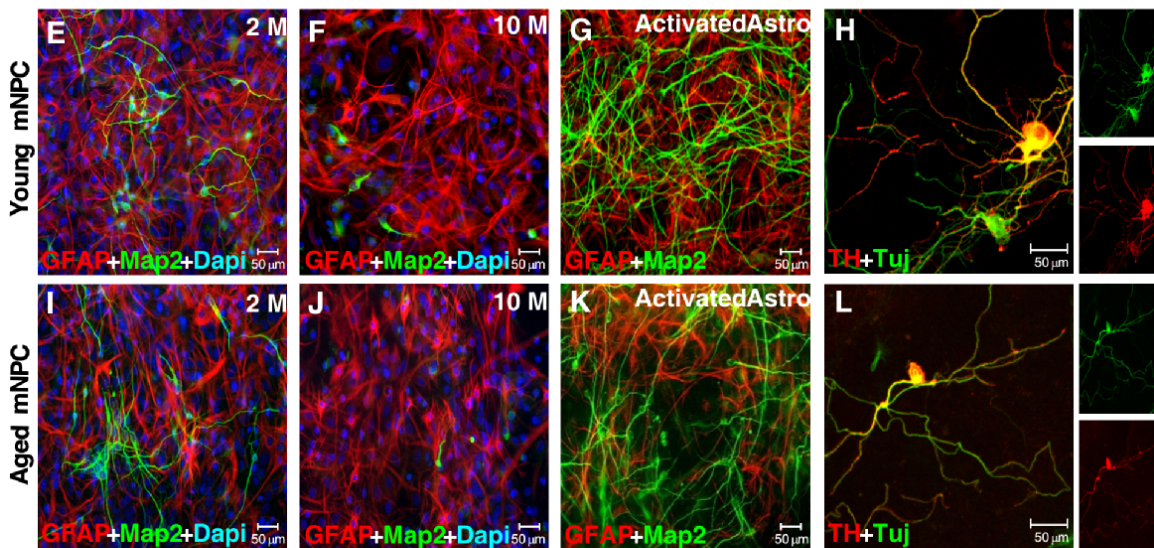
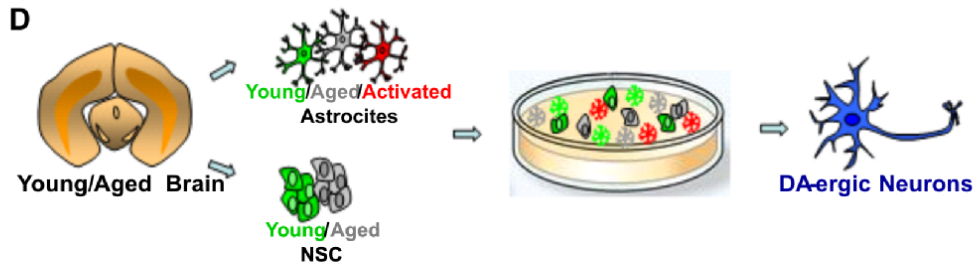
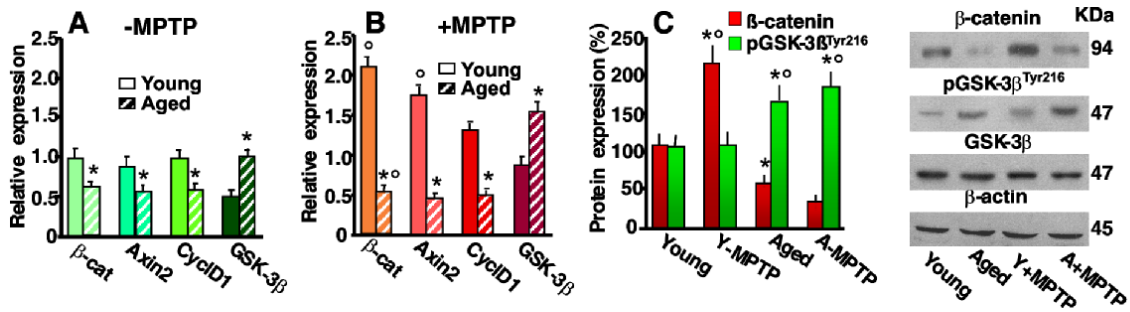


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**Figure 1.** The neurogenic potential of mNPCs decreases with age and fails to recover upon MPTP exposure. **A:** scheme of stem/neuroprogenitor isolation from midbrain periaqueductal region. SNpc: substantia nigra pars compacta. **B:** ICT of neurospheres stained with BrdU (red), and dual staining with GFAP (red)/nestin (green), and nestin (green)/Olig2 (red). **C:** Stem and proneural gene marker expression as determined by quantitative Real time PCR (qt-RT-PCR) show high mRNA levels (arbitrary units, AU) of the midbrain marker, *Engrailed 2 (EN2)*. The means  $\pm$  SEM of three individual determinations is shown. **D:** scheme of MPTP-induced DA neuron death and recovery in young (Y) as opposed to aged (A) mice. **E-F:** Representative immunocytochemical images (scale bar length: 50  $\mu$ m) comparing Y- (E) and A- (F) mNPCs from saline and 7 and 45 days post-MPTP (dpt). Dual labelling with nestin (green) and BrdU (red) or nestin (green) and Tuj1 (red) counterstained with Dapi (blue) show reduced BrdU<sup>+</sup> and Tuj1<sup>+</sup> cell formation with age (F). Dual staining with Tuj1 (green) and GFAP (red) show increased astrocyte formation in aged neurospheres (F). **G-H :** proliferation (G) as assessed by BrdU incorporation, neuron differentiation, as assessed by Tuj1, at 3-4 passages (I) and 8-10 passages (J). **I:** Comparison of cell death as assessed by Caspase3-like activity using the fluorogenic substrate DEVD as a function of age and MPTP. Differences analyzed by ANOVA followed by Newman-Keuls test. \*  $p < 0.05$  vs -MPTP;  $^{\circ}p < 0.05$  vs young, within each respective group. The means  $\pm$  SEM from three individual experiments is shown.

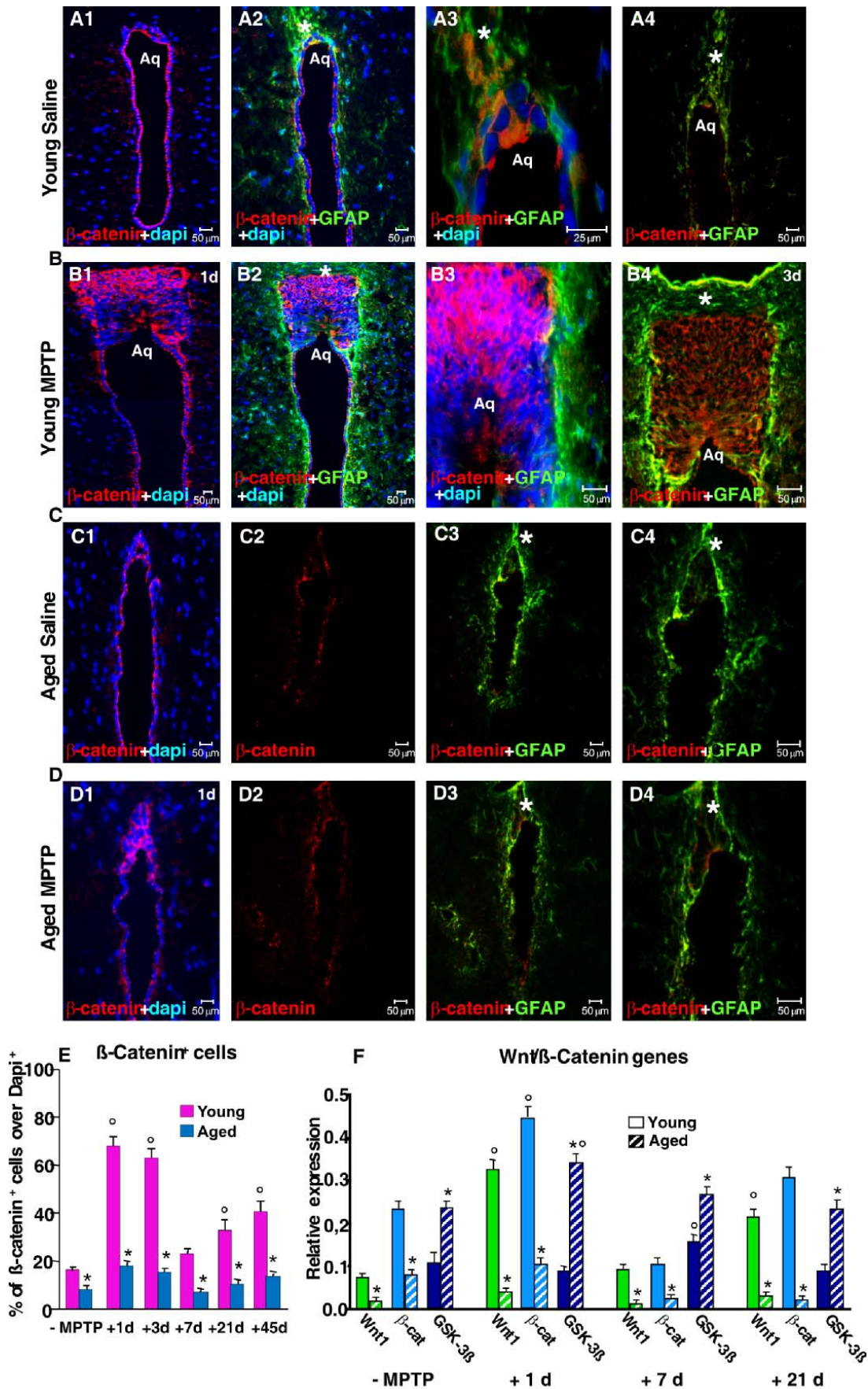


**Figure 2.** Aging inhibits canonical *Wnt/ $\beta$ -catenin* signaling : rescue effect of astrocyte Wnt. **A-B:** Comparison of  $\beta$ -catenin, *Axin2*, *Cyclin D1*, and *GSK-3 $\beta$*  gene expression in Y- and A- mNPCs determined by qt-RT-PCR after saline (-MPTP, A) or 45 dpt (+MPTP, B) in mNPCs of 8-10 passages. Changes in mRNA levels (expressed as AU). The means  $\pm$  SEM of three individual determinations is shown. \* $p < 0.05$  vs young and  $^{\circ}p < 0.05$  vs -MPTP, respectively. **C:** Western blots of  $\beta$ -catenin and active GSK-3 $\beta$  (pGSK-3 $\beta^{\text{Tyr}216}$ ). Densitometric values of  $\beta$ -catenin were normalized to  $\beta$ -actin, and pGSK-3 $\beta^{\text{Tyr}216}$  values with total GSK- $\beta$ , values are expressed as percent changes compared to control (young mNPC). \*  $p < 0.05$  vs young;  $^{\circ}p < 0.05$  vs -MPTP. **D:** scheme of co-culture between young/aged/activated astrocyte and Y/A-mNPCs. **E-L:** dual staining with GFAP (red) and Map2a (green) with Dapi (blue) counterstain comparing Y- (E-H) )/A (I-L) mNPCs co-cultured with 2- (E, I) and 10- (F,G) month-old astro, or activated astro (G, K), respectively. Dual staining with TH (red) and Tuj1 (green) is illustrated in a representative confocal image in Y- (H) and A- (L) mNPC co-cultured with activated astro. **M-P:** percent of BrdU and Map2a expressing cells in cocultures of Y- (M) or A- (N) mNPC cocultured with 2d, 2M, 10M or 24 M astro. The GSK-3 $\beta$  antagonist, AR efficiently counteracted the inhibitory effect of aged astro in both Y/A-NPCs (O). Conversely, Wnt/ $\beta$ -catenin antagonism with Dkkopf-1 (Dkk1), or Frizzled-1-cysteine rich domain (Fzd-A), reversed young astrocyte effects (O). Activated astro promote TH $^{+}$  neuron formation in both Y-and A-mNPCs after 10 DIV in differentiating medium (P). Exposure to AR further increases while Dkk1 inhibits TH $^{+}$  formation. \* $p < 0.05$  vs NSI;  $^{\circ}p < 0.05$  vs 2d and 2M astro in Y-(M) and A-mNPC (N), respectively. \*  $p < 0.05$  vs PBS and  $^{\circ}p < 0.05$  vs 2 M and 10 M astro (O); \*  $p < 0.05$  vs Y-A-NPC cultured alone,  $^{\circ}p < 0.05$  vs Y-/A-NPC with untreated astro;  $p < 0.01$  vs Y-/A-NPCs without Dkk1 or AR (P).

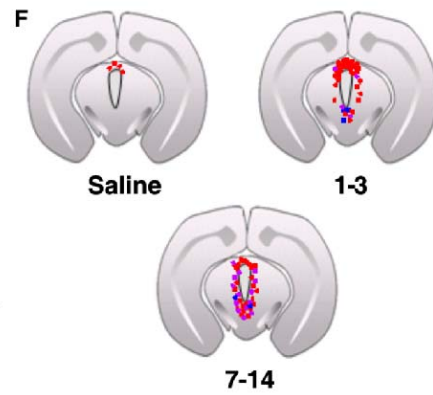
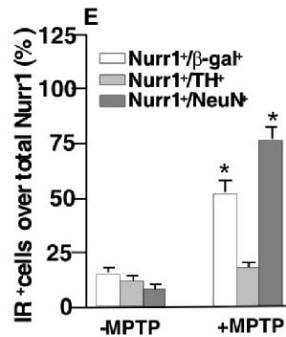
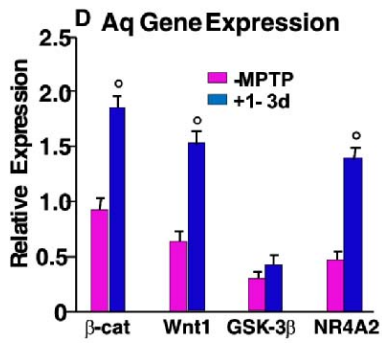
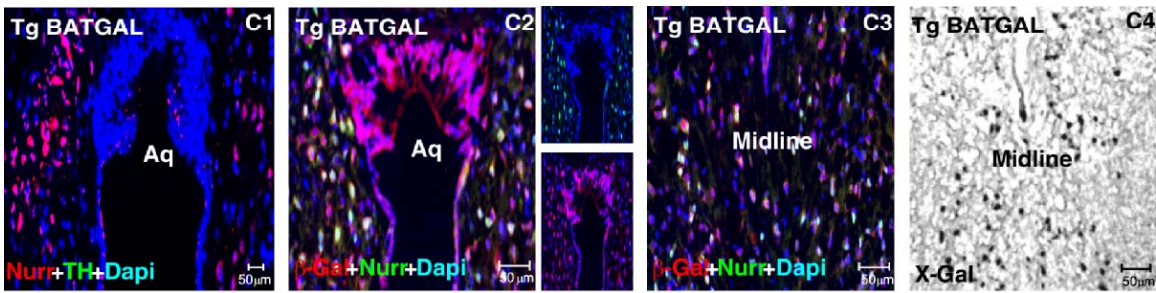
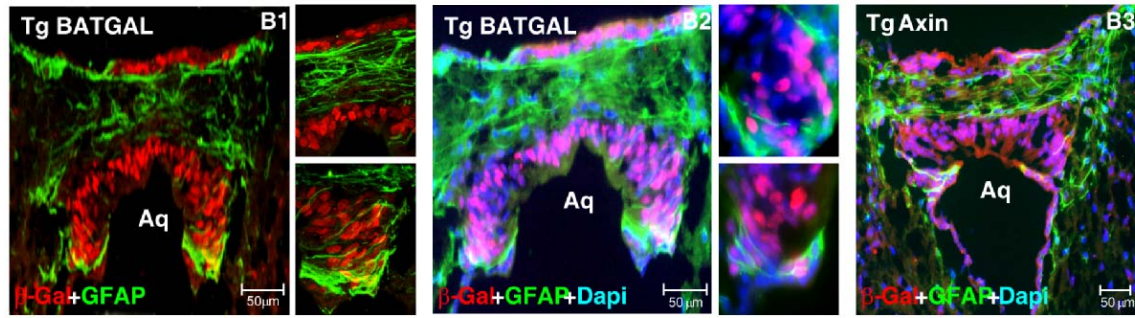
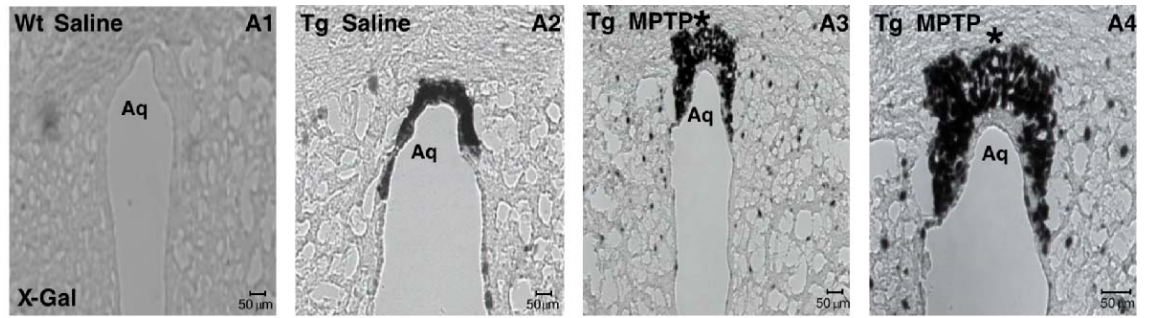


**Figure 3.**  $\beta$ -catenin and Wnt1 expression increase in the midbrain aqueduct periventricular regions (Aq-PVRS): effect of age and MPTP, *in vivo*. Mice treated with saline or MPTP were killed at different time points (6 mice/tp). **A-D:** Representative images showing  $\beta$ -catenin (red) and dual staining with GFAP (green) and dapi counterstained nuclei in young (A-B) and older mice (C-D) in saline and 1-3 dpt. GFAP<sup>+</sup> cells were seen bordering the ventricular wall and in the Aq-tail (white asterisk in A-D). **E:** Temporal changes of  $\beta$ -catenin<sup>+</sup> cells over the dapi+ cell nuclei in young and aged mice Aq after saline and at different tp upon MPTP showing significant increase of  $\beta$ -catenin<sup>+</sup> cell percentages very early (1-3 dpt) after MPTP, as opposed to aged mice. **F:** Changes in mRNA levels in  $\beta$ -catenin, Wnt1 and GSK-3 $\beta$  determined by qt-RT-PCR in acutely isolated samples of midbrain-Aq region (6-8 mice/tp). The means  $\pm$  SEM of three individual determinations is shown. Differences analyzed by ANOVA followed by Newman-Keuls test. \*  $p < 0.05$  vs young; ° $p < 0.05$  vs -MPTP, within each respective group. The means  $\pm$  SEM from three individual experiments is shown.

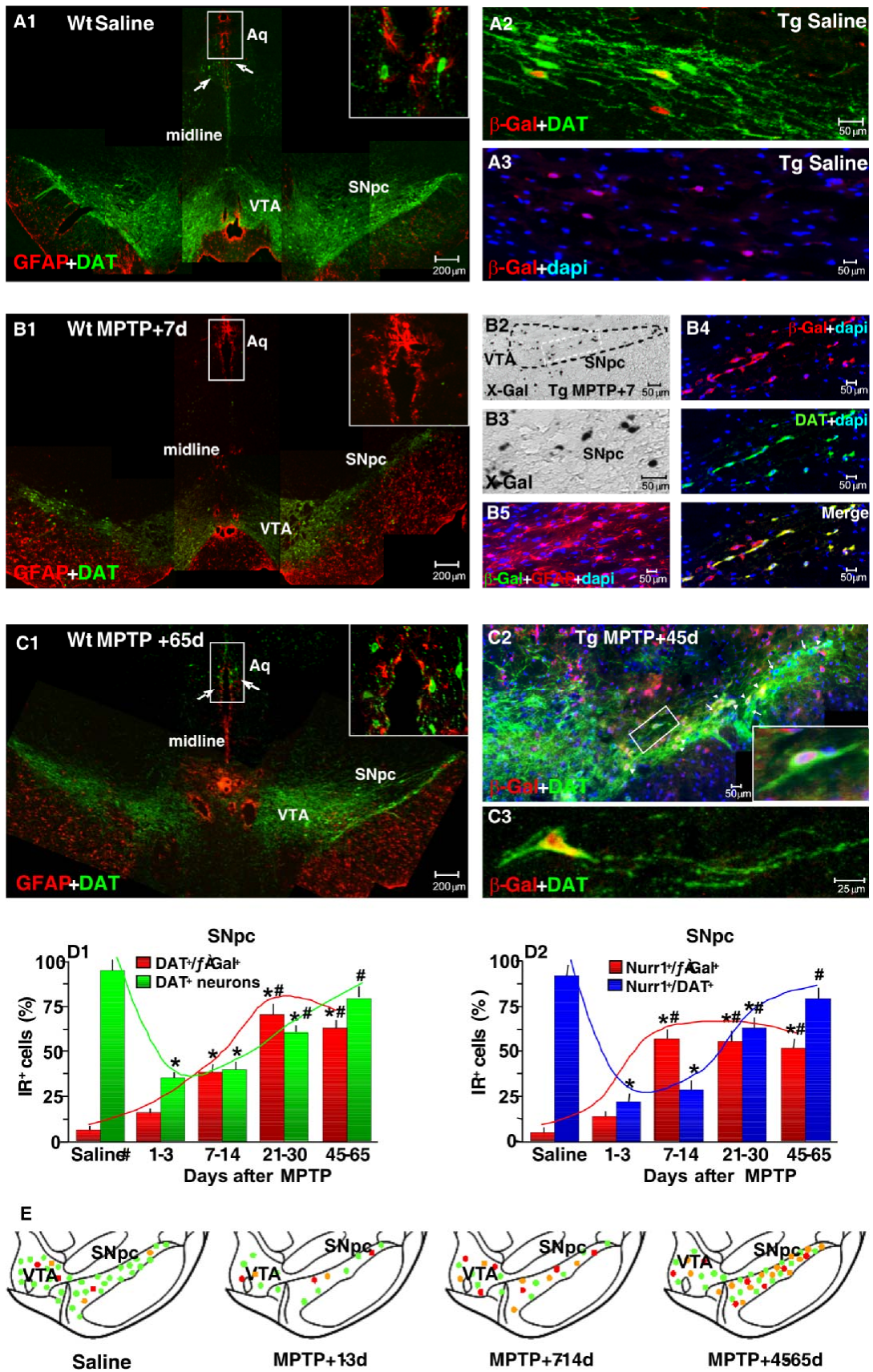




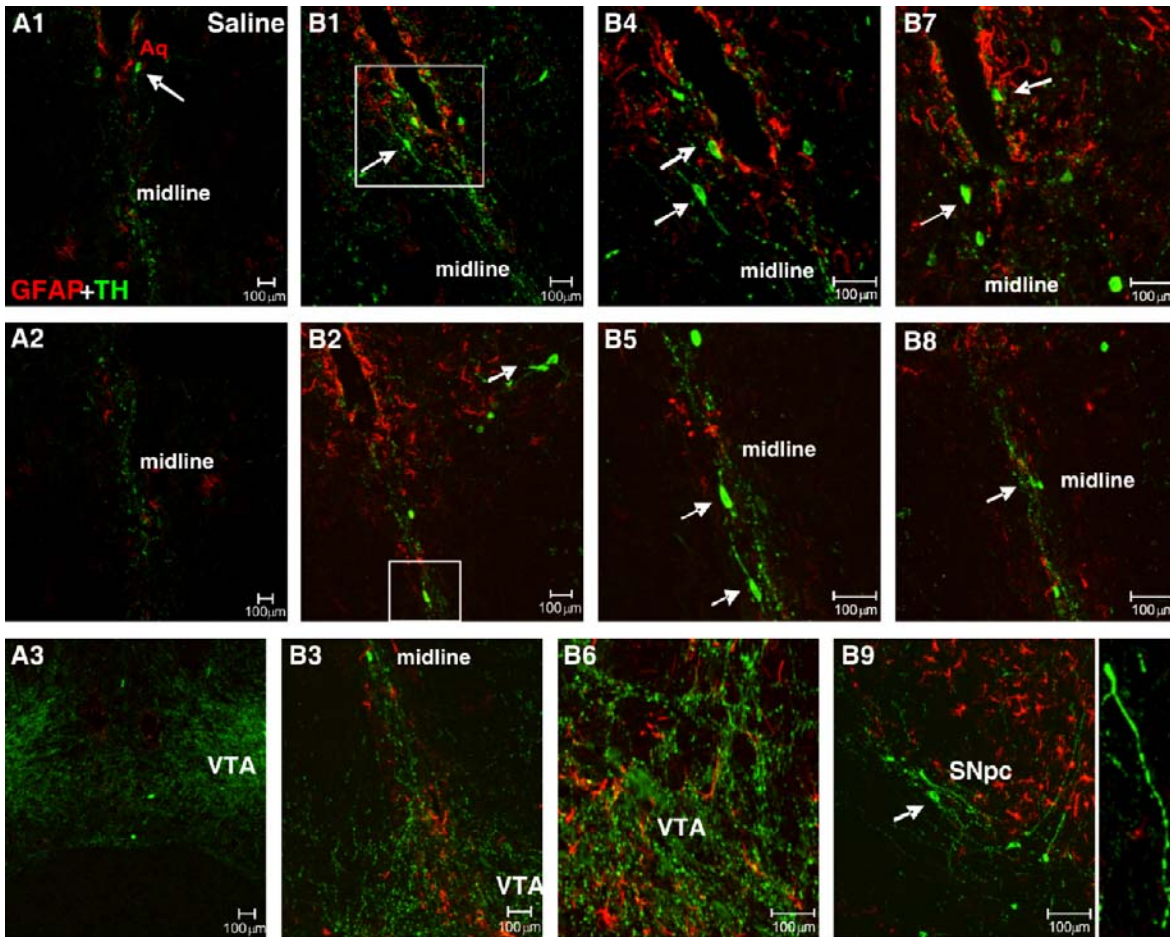
**Figure 4.**  $\beta$ -catenin-signaling in midbrain Aqueduct of young BATGAL mice: effect of MPTP injury. Endogenous  $\beta$ -catenin signaling was identified in saline and upon MPTP injury of young BATGAL transgenic (Tg) and Wt (control), and Axin2-LacZ Tg mice (Tg Axin) reporters, as indicated. **A1-A4:** X-Gal staining in midbrain section of Wt mice show absence of staining (A1), as opposed to Tg mice after saline (A2) or 3 dpt (A2-A3). **B1-B3:** Dual staining with  $\beta$ -galactosidase ( $\beta$ -gal, red) and GFAP (green) shows no co-localization in Aq round 3 dpt (B1-B2). Note the comparable effect of MPTP in Tg Axin reporters (B3). **C1-C4:** In BATGAL mice, numerous Nurr1<sup>+</sup> (red) TH<sup>+</sup> (green) neurons are observed in Aq-PVRs (C1). Dual staining with  $\beta$ -gal (red) and Nurr1 (green) shows co-localization of the two markers (C2,C3). Numerous X-Gal stained nuclei are present in BATGAL reporters upon MPTP. **D:** Quantification of Real time PCR data using specific primers for  $\beta$ -catenin, *Wnt1*, *GSK-3 $\beta$*  and *Nurr1/NR4A2* in acutely isolated samples of mid-brain-Aq region (6 mice/tp). The means  $\pm$  SEM from three individual experiments is shown. \*  $p < 0.05$  vs young;  $^{\circ}p < 0.05$  vs -MPTP, within each respective group. **E:** Quantitative determination of Nurr1<sup>+</sup>/ $\beta$ -gal<sup>+</sup>, Nurr1<sup>+</sup>/TH<sup>+</sup> and Nurr1<sup>+</sup>/NeuN<sup>+</sup> neurons. \*  $p < 0.05$  vs -MPTP by ANOVA. **F:** Scheme of spatiotemporal changes of  $\beta$ -gal<sup>+</sup> (red dots), Nurr1 (blue dots) and marker colocalization (pink dots) upon MPTP.



**Figure 5.**  $\beta$ -catenin signaling in SNpc of BATGAL mice: effect of MPTP-induced DA neuron injury/repair. **A1:** Representative image of a coronal midbrain section from Wt saline mice stained with DAT (green) and GFAP (red) showing DAT<sup>+</sup> neuron in the SNpc, ventral tegmental area (VTA) and Aq-PVR (inset). **A2-A3:** In Tg saline mice, dual staining with  $\beta$ -gal (red) and DAT (green, A2) or with  $\beta$ -gal (red) and dapi (blue) show  $\beta$ -gal-IF signal in DAT<sup>+</sup> neurons of the SNpc. **B1-B5:** MPTP-induced DA neuron loss in Wt mice (B1): DAT<sup>+</sup> neurons (green) are decreased while GFAP<sup>+</sup> astrocytes are increased within the SNpc and Aq-PVR. In Tg mice, a low power-view (B2) and magnification (B3) of a coronal midbrain section show intense X-Gal staining at the SNpc level in DAT<sup>+</sup> neuron domain (boxed). Colocalization of  $\beta$ -gal and DAT in SNpc neurons surviving the insult is shown (B4). Dual staining with GFAP (red) and  $\beta$ -gal (green) shows no co-localization in SNpc (B5). **C1-C3:** Recovery of DAT<sup>+</sup> neurons in Wt (C1) by 65 dpt. DAT<sup>+</sup> neurons are observed in Aq-PVR (inset) and midline down to VTA and SNpc of Wt mice. In Tg mice (C2-C3), a remarkable recovery is observed by 45 dpt when  $\beta$ -gal (red)-IF signal is observed in numerous DAT<sup>+</sup> (green, in C2 and C3) and DAT<sup>-</sup> cells. **D1.** Percentage of DAT<sup>+</sup> neurons in SNpc and percent of DAT<sup>+</sup>/ $\beta$ -gal<sup>+</sup> cells at different time-intervals after MPTP treatment of Tg mice. Green and red lines highlight positive correlation of  $\beta$ -catenin signaling in rescuing/repairing DAT neurons. The means  $\pm$  SEM from 6 mice/group is shown. Differences analyzed by ANOVA followed by Newman-Keuls test as indicated: \*p < 0.05 vs saline; #p < 0.01 vs 1-14 time-point (tp), in each respective group. **D2:** Time-course analysis of percentages of Nurr1<sup>+</sup>/ $\beta$ -gal<sup>+</sup> and percent of Nurr1<sup>+</sup>/DAT<sup>+</sup>. Blue and red lines highlight increased  $\beta$ -gal in Nurr1<sup>+</sup> neurons preceding Nurr1<sup>+</sup>/DAT<sup>+</sup> neuron recovery. The means  $\pm$  SEM determined as above are shown; \*p < 0.05 vs saline; #p < 0.01 vs 1-3 tp for Nurr1<sup>+</sup>/ $\beta$ -gal<sup>+</sup> and vs 7-14tp for Nurr1<sup>+</sup>/DAT<sup>+</sup>. **E:** Spatiotemporal changes of  $\beta$ -gal<sup>+</sup> (red dots), DAT<sup>+</sup> neurons (green dots) and marker colocalization (orange dots) in SNpc upon MPTP.



**Figure 6.** Tyrosine hydroxylase positive neurons ( $\text{TH}^+$ ) and glial fibrillary acid protein positive ( $\text{GFAP}^+$ ) astrocytes in midbrain Aq-PVRs in saline and 65 dpt. Representative confocal images of midbrain coronal sections stained with TH (green) and GFAP (red) from saline- (A1-A4) and MPTP-treated (B1-B9) mice 65 d post injury. In saline control mice, note  $\text{TH}^+$  positive neurons merging from the Aq region (A1), possibly directed to the ventral tegmental area (VTA) (A2-A3). In MPTP mice by 65 post MPTP, reactive  $\text{GFAP}^+$  astrocytes are increased in Aq-PVRs (B1, B2, B4, B7). A certain number of  $\text{TH}^+$  neurons appear to merge from the Aq (B1, B2, B4, B5, B7), seemingly guided by astrocytes, coursing/migrating down to the VTA-SNpc. Note that some  $\text{TH}^+$  neurons have a round-shape morphology (B7), others have elongated cell bodies and bear a long process (B5), while other  $\text{TH}^+$  neuron seemingly navigate in couplets (B2, B8), likely reflecting different stages of maturation. Down to the VTA (B3, B6) and SNpc (B9) note the increased density of  $\text{TH}^+$  neurons intermingled with  $\text{GFAP}^+$  astrocytes (B6). Within the repaired SNpc, bright  $\text{TH}^+$  neurons extending long and thick process (B9) can be observed.



**Figure 7.** Aging-induced inhibition of  $\beta$ -catenin signaling in response to MPTP is restored by direct  $\beta$ -catenin signaling activation *in situ*. Aged mice received an intracerebral infusion of the GSK-3 $\beta$  antagonist, AR, or PBS (A1), the proliferation assessed by BrdU injections. **A2-A3:**  $\beta$ -gal (red) and dapi (blue) counterstaining shows impaired  $\beta$ -catenin signaling in Aq niche of aged MPTP mice infused with PBS (A2), as opposed to AR infused (A3) mice. **A4:** Qrt-RT-PCR data using specific primers for  *$\beta$ -catenin*, *Wnt1*, *GSK-3 $\beta$*  and *Nurr1/NR4A2* in acutely isolated samples of midbrain-Aq region 2 dpt (6 mice/tp). The means  $\pm$  SEM from three individual experiments is shown. Differences analyzed by ANOVA followed by Newman-Keuls test. \*  $p < 0.05$  vs -MPTP;  $^{\circ}p < 0.05$  vs -AR, within each respective group. **B1-B4:** confocal imaging of triple staining with  $\beta$ -catenin, GFAP and BrdU, showing remarkable increase of GFAP $^{+}$  astrocytes and  $\beta$ -catenin $^{+}$  cells, robust proliferation and Aq remodeling after AR (B1-B4). Numerous  $\beta$ -catenin $^{+}$  cells (red) co-express BrdU (green, B2). C2-C5: DA markers in SNpc during injury and recovery. C1-C3: representative confocal image of midbrain section stained with Nurr1 (red) and DAT (green) in aged saline-PBS infused (C1,-AR), 45 d after MPTP (C2,-AR), and +AR infusion (C3). **C4-C6:** temporal analysis of percentages of Nurr1 $^{+}$ /DAT $^{+}$ (C4), of DAT $^{+}$  neurons and of DAT $^{+}$ / $\beta$ -gal $^{+}$  without (C5, -AR) or with AR infusion (C6, +AR). Differences analyzed by ANOVA followed by Newman-Keuls test. \*  $p < 0.05$  vs Saline;  $^{\circ}p < 0.05$  vs -AR ; within each respective time-point and group.

