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Dopamine D1 receptor activation improves adult hippocampal neurogenesis and exerts anxiolytic and antidepressant-like effect via activation of Wnt/β-catenin pathways in rat model of Parkinson's disease

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ABSTRACT

Parkinson's disease (PD) is primarily characterized by midbrain dopamine depletion. Dopamine acts through dopamine receptors (D1 to D5) to regulate locomotion, motivation, pleasure, attention, cognitive functions and formation of newborn neurons, all of which are likely to be impaired in PD. Reduced hippocampal neurogenesis associated with dopamine depletion has been demonstrated in patients with PD. However, the precise mechanism to regulate multiple steps of adult hippocampal neurogenesis by dopamine receptor(s) is still unknown. In this study, we tested whether pharmacological agonism and antagonism of dopamine D1 and D2 receptor regulate nonmotor symptoms, neural stem cell (NSC) proliferation and fate specification and explored the cellular mechanism(s) underlying dopamine receptor (D1 and D2) mediated adult hippocampal neurogenesis in rat model of PD-like phenotypes. We found that single unilateral intra-medial forebrain bundle administration of 6-hydroxydopamine (6-OHDA) reduced D1 receptor level in the hippocampus. Pharmacological agonism of D1 receptor exerts anxiolytic and antidepressant-like effects as well as enhanced NSC proliferation, long-term survival and neuronal differentiation by positively regulating Wnt/β-catenin signaling pathway in hippocampus in PD rats. shRNA lentivirus mediated knockdown of Axin-2, a negative regulator of Wnt/β-catenin signaling potentially attenuated D1 receptor antagonist induced anxiety and depression-like phenotypes and impairment in adult hippocampal neurogenesis in PD rats. Our results suggest that improved nonmotor symptoms and hippocampal neurogenesis in PD rats controlled by D1-like receptors that involve the activation of Wnt/β-catenin signaling.

1. Introduction

The formation of newborn neurons from neural stem cells (NSCs) is termed as neurogenesis. This process occurs throughout life in two well-known neurogenic regions, subventricular zone (SVZ) of the lateral ventricle (LV) and dentate gyrus (DG) of hippocampus (Gage, 2000; Riquelme et al., 2008). Neurogenesis is a very finely tuned process encompassing NSC proliferation, differentiation, migration, maturation and integration of newborn mature neurons into the existing neuronal circuitry (Gage, 2000). Hippocampal neurogenesis has been shown to regulate contextual fear conditioning, pattern separation, memory formation/consolidation and neuropsychiatric disorders such as anxiety, depression, schizophrenia and bipolar disorder (Besnard and Sahay, 2016; Ming and Song, 2011; Sahay et al., 2011). Studies have shown that enriched environment, stress, maternal isolation, neurotrophic factors, running and neurotransmitters such as serotonin, glutamate and dopamine modulate neurogenesis. Reduced formation of newborn neurons or increased death rate of existing neurons is a common feature of neurodegenerative diseases, including Huntington disease (HD), Alzheimer's disease (AD) and Parkinson's disease (PD) (Ming and Song, 2011; Mishra et al., 2018; Schwab et al., 2017; Singh et al., 2017b).

PD is characterized by selective dopaminergic (DAergic) neuronal degeneration in nigrostriatal pathway that results in the loss of dopamine neurotransmitter (Chaudhuri et al., 2006). Apart from typical motor symptoms (resting tremors, bradykinesia and rigidity), nonmotor symptoms such as cognitive impairment (attention deficits, memory difficulties, dementia), sleep difficulty and psychiatric disorders (anxiety, depression and psychosis) result from impairment in mesocorticolimbic pathway (Chaudhuri et al., 2006; Zeng et al., 2018). DAergic fibers originating from ventral tegmental area (VTA) and

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substantia nigra pars compacta (SNpc) directly innervate hippocampus and cortex, suggesting a functional and an anatomical link between nigrostriatal DAergic neurons and hippocampal dependent functions (Alexander, 2004; Barzilai and Melamed, 2003). Dopamine neurotransmitter exerts its functions by acting on DAergic receptors, which are classified as D1-like receptor (D1 and D5) and D2-like receptors (D2, D2 and D4) and the association between D1 and D2-like receptors has been widely investigated in cognitive and psychiatric behaviour (Cools and Van Rossum, 1976; Ellenbroek et al., 2014). Genetic deletion of dopamine D1 receptor attenuates the reinforcing properties of reward stimuli and induces spatial memory deficits in adult mice (El-Ghundi et al., 1999; Holmes et al., 2004). Pharmacological treatment of D1 receptor antagonist SCH-39166 significantly reduce negative symptoms of schizophrenia without affecting positive symptoms and general psychopathology score in human patients (Den Boer et al., 1995), whereas the same antagonist treatment potentially attenuate cocaine induced euphoric effect in cocaine addicts (Romach et al., 1999). A study showed that cannabinoid CB1 receptor agonist induced anxiolytic effect in rat amygdala is mediated by dopamine D1 and D2 receptors (Zarrindast et al., 2011). Similarly, D2 receptor agonist quinpirole induced behavioural depression is potentially reversed by the D1 receptor agonist SSC23390, suggesting that anxiety and depression-like phenotypes could be due to the deprivation of dopamine at postsynaptic D1 receptor (Jackson et al., 1989). D2 receptor knockout mice also showed increased anxiety-like behaviour in response to chronic stress (Sim et al., 2013). Further, the age-associated reduction in D2 receptor in cortex and hippocampus has been demonstrated in normal healthy individuals and patients with advanced PD (Kaasinen et al., 2000a, 2000b).

Neurogenesis involves the proliferation, differentiation and self-renewal of NSCs and is regulated by intracellular signaling pathways, including Wnt/β-catenin signaling pathway (Chavali et al., 2018; Faigle and Song, 2013). In the absence of Wnt ligands, a cytoplasmic complex composed of Axin-2/GSK-3β/APC (β-catenin destruction complex) directly phosphorylate β-catenin and induce proteasomal degradation of phosphorylated β-catenin (Qu et al., 2017). This low level of cytoplasmic β-catenin lead to reduced nuclear translocation of β-catenin (Qu et al., 2017). In the presence of Wnt ligands, the destruction complex Axin-2/GSK-3β/APC gets disrupted that lead to accumulation of cytoplasmic β-catenin and results in increased translocation of β-catenin into nucleus. Wnt/β-catenin signaling promotes self-renewal, proliferation and differentiation of cortical and hippocampal NSC in a stage specific manner (Hirabayashi et al., 2004; Lie et al., 2005; Munji et al., 2011). It has been demonstrated that adult hippocampal derived NSC express the components of Wnt signaling (Lie et al., 2005), suggesting that Wnt signaling remains active in the neurogenic niches. Wnt-3a activation is necessary and sufficient to induce adult hippocampal NSC proliferation and neuronal differentiation and for the contextual fear memory acquisition and consolidation (Lie et al., 2005; Xu et al., 2015). Interestingly, it has been identified that dopamine D2 receptor directly interacts with β-catenin and inhibit Lef-1 dependent transcriptional activity (Min et al., 2011), suggesting a novel role of dopamine receptor in Wnt regulation. The association between D2 receptor and Wnt signaling was further confirmed by a study, which showed that antipsychotic drugs activated Wnt/β-catenin signaling in *in-vitro* and *in-vivo* experimental settings via regulating Dvl-3 and dopamine D2 receptor (Sutton et al., 2007). Treatment with Wnt-5a enhanced neurite outgrowth and development of dopamine neurons in wild type mice, but not in D2 receptor null mice because D2 receptor showed interaction with Wnt-5a (Yoon et al., 2011). In support of these findings, it has been identified that activation of Wnt proteins and inhibition of GSK-3β is essentially required for the antidepressant action of fluoxetine and ketamine (Beurel et al., 2011; Zhou et al., 2016). However, the precise mechanism of dopamine receptor mediated control of adult hippocampal neurogenesis in neurodegenerative model is not fully explored. In the present study, we showed that D1 receptor stimulation increased adult hippocampal neurogenesis and exert antidepressant anxiolytic effects via activation of Wnt/β-catenin signaling pathway in 6-hydroxydopamine (6-OHDA)-induced rat model of PD-like phenotypes.

2. Material and methods

2.1. Animals

Adult male Sprague Dawley (SD) rats, weighing 200–250 gm, were procured from National Laboratory Animal Center (NLAC) of the Central Drug Research Institute, Lucknow, India. Animals were housed on a 12 h light/dark cycle at 23 ± 1 °C temperature with *ad libitum* access to food and water. Animals were habituated for 5 days in housing condition prior to starting the experiments. All animal protocols and experimental procedures were carried out in accordance with our Institutional Animals Ethics Committee (IAEC) following the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiment on Animals), which complies with international norms of INSA (Indian National Science Academy).

2.2. 6-OHDA lesioning

6-OHDA surgical procedure was performed as described in our previous studies (Singh et al., 2017a, 2017b). Desipramine (25 mg/kg/i.p) (Sigma Chemicals, St. Louis, MO, USA), a noradrenergic reuptake inhibitor was injected 30 min prior to 6-OHDA injection to protect noradrenergic neurons from damage. After desipramine injection, rats were anaesthetized with sodium pentobarbital (40 mg/kg, i.p). 6-OHDA hydrobromide (Total 16 μg (8 μg/μl) dissolved in 0.02% ascorbic acid) was unilaterally injected into the right medial forebrain bundle (MFB) (coordinates related to Bregma, AP = - 4.4, ML = 1.2, DV = 7.8 mm) using a 30-gauge Hamilton syringe (Hamilton Company, Switzerland) set at a rate of 0.5 μl/min. The needle was left on the same place for another 5 min to allow the diffusion of 6-OHDA.

2.3. Lentivirus administration

To study the role of Wnt/β signaling in adult hippocampal neurogenesis and behavioural functions in rat model of PD-like phenotypes, we stereotaxically injected Axin-2 shRNA lentivirus particles into hippocampus. Ready to use pLKO.1-puro-CMV-tGFP Mission® lentivirus particles were purchased from Sigma Aldrich (St. Louis, USA). The Axin-2 sequence targeting knockdown is 5′-AAAGGGAAATTACAGGT ATTA-3′ and nontarget or scrambled (sc) shRNA (Sigma Aldrich, St. Louis, USA) was used as a control. Rats were anaesthetized with sodium pentobarbital (40 mg/kg, i.p) and green fluorescent protein (GFP) tagged Axin-2 shRNA lentivirus particles (2.1×10^7 TU/ml) were injected (2ul) into 6-OHDA + SKF-38393 + SCH-23390 treated rats in intrahippocampal region (coordinates related to Bregma, AP = −3.2, ML = ± 1.2, DV = −4.1 mm) using a 30-gauge Hamilton syringe (Hamilton Company, Switzerland) set at a rate of 0.5 μl/min. Equal volume of pLKO.1-puro-CMV-tGFP scrambled shRNA lentivirus particles (sc shRNA, 1.3×10^7 TU/ml) were injected into hippocampus in control and 6-OHDA rats. At the end of injection, needle was left at the same location for over 5 min to allow the diffusion before being slowly retracted.

2.4. Drug administration: animal was randomly divided into following groups

2.4.1. Experimental setup-1

2.4.1.1. Vehicle control group. Vehicle control group received stereotaxic infusion of saline (2 μl) into right MFB.

2.4.1.2. 6-OHDA lesioned group. Animal received 2 μl of 6-OHDA (8 μg/

μl) dissolved in saline containing 0.02% ascorbic acid into right medial forebrain bundle (MFB).

2.4.1.3. 6-OHDA + SKF-38393 (D1 receptor agonist). 6-OHDA treated animal received SKF-38393 (10 mg/kg, i.p) (Neisewander et al., 1995; Walters and Howard, 1990) starting 3 day prior to 6-OHDA lesioning and continued for another 21 days.

2.4.1.4. 6-OHDA + SKF-38393 + SCH-23390 (D1 antagonist). 6-OHDA + SKF-38393 treated rats were co-administered with SCH-23390 (0.5 mg/kg/i.p) (Walters and Howard, 1990) after 20 min of SKF-38393 injection.

2.4.1.5. 6-OHDA + bromocriptine (D2 agonist). Bromocriptine, a D2-family receptor agonist was injected intraperitoneally (2 mg/kg, i.p) (Izquierdo-Claros et al., 2000) starting 3 days prior to 6-OHDA injection and continued for another 21 days.

2.4.1.6. 6-OHDA + bromocriptine + raclopride (D2 antagonist). 6-OHDA + Bromocriptine treated rats were co-administered with raclopride (5 mg/kg/i.p) (Izquierdo-Claros et al., 2000). Bromocriptine was injected 20 min prior to the injection of raclopride in 6-OHDA lesioned rat in a similar time schedule.

2.4.1.7. 6-OHDA + L-DOPA. L-DOPA (25 mg/kg, i.p.) (Lindgren et al., 2007) was administered 30 min after benserazide (15 mg/kg, i.p.) (Xie et al., 2014). L-DOPA treatment started 3 days prior to 6-OHDA lesioning and continued for another 21 days.

2.4.2. Experimental setup-2

2.4.2.1. scshRNA group (scrambled/non-targeted shRNA). Control rats received stereotaxic infusion of 2 μl of scshRNA lentivirus particles into hippocampus.

2.4.2.2. 6-OHDA + scshRNA lesioned group. Received 2 μl of 6-OHDA (8 μg/μl in normal saline) into right MFB and also received 2 μl of scshRNA lentivirus particles in hippocampus at the same time.

2.4.2.3. 6-OHDA + 6-OHDA + Axin-2shRNA. Received 2 μl of 6-OHDA (8 μg/μl in normal saline) into right MFB and also received 2 μl of green fluorescent protein (GFP) tagged Axin-2 shRNA lentivirus particles (2.1×10^7 TU/ml) into right hippocampus.

2.4.2.4. 6-OHDA + scshRNA + SKF-38393. 6-OHDA + scshRNA treated animal received SKF-38393 (10 mg/kg, i.p), starting 3 days prior to 6-OHDA lesioning and continued for another 21 days.

2.4.2.5. 6-OHDA + scshRNA + SKF-38393 + SCH-23390. 6-OHDA + scshRNA treated rats received SKF-38393 (10 mg/kg, i.p) injection. After 20 min, rats were co-administered with SCH-23390 (0.5 mg/kg/i. p). The treatment of SKF and SCH was started 3 days prior to 6-OHDA injection and continued for another 21 days (from the day of 6-OHDA injection).

2.4.2.6. 6-OHDA + SKF-38393 + SCH-23390 + Axin-2 shRNA group. 6-OHDA + SKF + SCH treated rats simultaneously received Axin-2 shRNA lentivirus particles (2 μl) into right hippocampus and were allowed to survive for 21 days.

2.5. BrdU administration

Bromodeoxyuridine (BrdU) is a thymidine analogue that binds with DNA in S-phase of the cell cycle, hence used as a marker for cell proliferation. Therefore, BrdU was used to determine the effect of D1 receptor, D2 receptor and Axin-2 shRNA on proliferation and differentiation of NSCs in hippocampus. From the day of 6-OHDA surgery, animals received daily injection of BrdU (50 mg/kg, i.p, Sigma Aldrich, St. Louis, USA) for three consecutive days and sacrificed on day 21 post-6-OHDA surgery.

2.6. Gene expression analysis (qRT-PCR)

Total RNA from hippocampus was isolated using Trizol reagent (Invitrogen, USA) and RNase-free DNase (Ambion, USA) was added to remove the contamination of genomic DNA as described in our previous publication (Singh et al., 2017b). In brief RNA pellets were dissolved in 20 μl of DEPC-treated water (Thermo Fisher scientific, USA) and RNA concentration was determined spectrophotometrically by Nanodrop (Thermo scientific, USA). 2 μg of total RNA was reverse transcribed using the high capacity cDNA reverse transcription kit (Applied Biosystems Invitrogen, USA) following the manufacturer's instructions. Total 50 ng/ul of reverse transcribed product was used for qRT-PCR reaction. Target gene expression and quantification was performed in duplicate using Quantstudio 12 K Flex qRT-PCR machine (Applied Biosystems, Foster City, CA, USA) and SYBR Green chemistry (Thermo scientific, USA). Relative gene expression analysis was performed using the relative cycle threshold method, normalized to β-actin expression, and fold change was calculated relative to the control. The following primers were used in this study Ngn2 forward (For), CCAACTCCACG TCCCCATAC, (reverse) Rev, CAGGTGAGGTGCATAACGGT; MASH1, (For) TCCGGTTTCGTCCTACTCCT, (Rev) CATTCCCAGTAGGGCCTGTC; Axin-2; For, TAGGCGGAATGAAGATGGGC, Rev, GTCCGGAAGAGGTA TGCACC; Wnt3a For, GTCGGGTTCTTCTCTGGTCC, Rev, CTGGGCATG ATCTCCACGTA; Wnt5a For, AAGCTAATTCTTGGTGGTCCCT, Rev, TGTCCTTGAGAAAGTCCCGC; Cyclin D1 For, TTCTTGTCTCTGGAGCC CCT, Rev, ACAACTTCTCGGCAGTCAGG; Nestin For, TCCCAACTGGTC GGTCAATG, Rev, CCGGTTTCCAGTTTGATCGC; Lef1 For, GTTCAGGC AAGCCTACCCAT, Rev, TTCACGTGCATTAGGTCGCT; Neuro For, D1 ATAGAGACACTGCGCTTGGC, Rev GCTGGGACAAACCTTTGCAG β-actin (For) GAGTACAACCTTCTTGCAGCTC, (Rev) CATACCCACCATC ACACCCTG.

2.7. Immunoblotting

Brain tissue was homogenized in NIPER reagent (Pierce/Thermo Fisher Scientific, USA) containing protease and phosphatase inhibitor (Peirce/Thermo Fischer, USA) according to manufactured instruction to extract nuclear and cytosolic fraction of protein. Total protein concentration was measured using BCA protein assay kit (Pierce/Thermo Fisher, USA). Protein sample were boiled at 95 °C for 5 min in Laemmli's sample buffer and separated on 10% SDS-PAGE gel. After electrophoresis separated protein was transferred on to the PVDF (polyvinyl difluoride) membrane and blocked with 5% BSA in TBST for 2 h at room temperature. Membrane was incubated with primary antibodies for overnight at 4 °C. The primary antibodies used in this study were mouse anti- GSK-3β (1:1000, Thermo Scientific, IL, USA), mouse anti-pTyr216-GSK-3β (1:1000, Millipore, Temecula, CA, USA), rabbit anti-p-β-catenin (1:500, Thermo Scientific, USA), mouse anti- APC (1:1000, Abcam, Cambridge, UK), rabbit anti- Axin-2 (1:1000, Abcam, Cambridge, UK), rabbit anti- Lef-1 (1:500, Merck Millipore, CA, USA), rabbit anti- β-catenin (1:5000, Abcam, Cambridge, UK), mouse anti- Wnt-3a (1:1000, Merck Millipore, USA), mouse anti- Wnt-5a (1:1000, Abcam), rabbit anti-histone H3 (1:500, Sigma), mouse anti- β-actin (1:2000, Sigma). After incubation in primary antibodies membrane was washed three times with TBST and incubated for 2 h at room temperature with horseradish peroxidase (HRP) conjugated secondary antibody: goat anti-rabbit IgG or rabbit anti-mouse IgG (1:3000, Sigma Aldrich, St Louis, USA). Immunoreactivity proteins were visualized using enhanced chemiluminescent (ECL) substrate kit (Thermo Pierce, USA) and protein band intensity was quantified by myImage analysis software (Thermo scientific, USA).

2.8. Immunohistochemistry (IHC)

IHC was performed as described in our previous publication (Singh et al., 2017a, 2017b, 2018a). Animals were deeply anesthetized with pentobarbital sodium (100 mg/kg, ip) and transcardially perfused with 0.9% normal saline followed by 4% ice-cold PFA (paraformaldehyde) in phosphate buffer saline (PBS), overnight at 4 °C. Brains were cryoprotected in increasing concentrations (10%, 20%, 30%) of sucrose at 4 °C. Serial free-floating coronal sections (30 μm thick) were cut using cryostat (Thermo Scientific, USA) and every 5th serial section encompassing hippocampus was collected for immunohistochemical analysis. Sections were permeabilized with TBS containing 0.2% Triton X-100 (TBST) for 30 min at room temperature and blocked with 5% BSA in TBST for 2 h at room temperature followed by incubation in primary antibody: rabbit anti-NeuN antibody (1:1000, Millipore, USA), rabbit anti-Nestin antibody (1:200, Sigma Aldrich, USA), rabbit anti-Ki-67 antibody (1:100, Millipore, USA). For bromodeoxyuridine (BrdU) staining, permeabilized section were incubated in 1N−HCl for 10 min at 4 °C followed by 2N−HCl at 37 °C for 30 min to denature the DNA. After HCl treatment, sections were incubated in borate buffer (0.1 M, pH-8.5) to neutralize the acidic medium. Sections were blocked with 5% BSA in TBST, washed with TBS and subsequently incubated with mouse anti-BrdU antibody (1:200, Millipore, USA). After incubation in primary antibodies sections were washed three times with TBS and incubated in dark with Alexa Fluor- 488/594 conjugated secondary antibodies (1:1000, Molecular Probes, Eugene, USA) for 2 h at room temperature. The section was mounted on glass slides with Fluoroshield DAPI mounting medium (Sigma Aldrich, USA) and analyzed by Leica inverted fluorescent microscope equipped with digital CCD camera (Leica, Watzlar, Germany) using 10x or 20x objective. Immunopositive cells were quantified/analyzed with image J software (NIH) according to the method published in our previous article (Singh et al., 2016).

2.9. Behavioural tests

2.9.1. Open field test

We performed open field test on day 21 post-6-OHDA injection using Optovarimax (Columbus Instruments, USA) to examine rearing activity as a measure of anxiety-related behaviour. Each open-field cage was divided into two zones; outer zone and inner/central zone. In particular, Cage was divided into 16 squares and the central 4 squares were considered as the inner zone. Animals were individually placed in the center of the cage and habituated for a period of 10 min before starting the experiment. Exploratory behaviour in terms of rearing counts was monitored in the central zone for a period of 30 min. Before introducing next animals, open field arena was swabbed with 10% alcohol to avoid the mixing of odor due to previous animals.

2.9.2. Forced swim test

Forced swim test was performed for the assessment of learned helplessness or depression-like behaviour. Rats are placed into cylindrical tank (height 45 cm &diameter 18 cm) filled with 25 ± 1 °C water to a depth of 25 cm. Animal testing was divided into two swimming session; first session was termed as training/acclimatization session in which the animals were forced to swim for a period of 15 min. Animals were pretested on day 20 post-6-OHDA injection. After 24 h, animals were tested under the same condition (test session) for a period of 5 min and the immobility time was recorded. After each session, animals were taken out of tank and allowed to dry under lamp before placing them in their cage. Immobility time is defined as lack of motion of the whole body, where there is no struggling and lack of movement of three out of four paws or minimal movement to keep the head above water.

2.9.3. Light and dark test

Light and dark test was performed on day 21 post-6-OHDA injection to analyze the anxiety-like phenotype in rats. The apparatus consists of a cage (50 × 25 × 30 $cm^{3)}$ divided into two sections of unequal size by opaque wall with small open door (7 cm × 7 cm). One chamber was opened and white colored whereas the other one was closed and dark. Animals were individually placed into the center of light (opened) chamber and allowed to move freely for a period of 300 s to acclimatize in the environment. After 300 s, time spend in each chamber was recorded by an individual blind to the experimental conditions and results. Before testing the animals as well as placing the next animal both chambers were cleaned thoroughly with 10% ethanol.

2.9.4. Social interaction test

Social interaction behaviour of rats was tested according to Crawley's methods (Crawley, 2007). The test was performed on day 21 post-6-OHDA injection in three chambered transparent plexiglass rectangular box (L 112 cm, W 56 cm, H 40 cm) and dividing wall had small retractable doorways allowing free access into all three chambers. The test procedure was divided into two phases; in the habituation phase, the experimental rat was placed into the middle chamber to explore the apparatus (three chambers) for a period of 10 min. During the test/sociability phase, an age/weight matched rat unfamiliar to experimental rat was introduced in inverted wire cup in unsocial chamber side. The experimental rat was allowed to explore the arena and to interact with conspecific (unfamiliar) rat for a period of 10 min and the time spent in active interaction in close proximity by experimental rat was scored by two observer blinds to experimental groups. The arena was swabbed with 10% alcohol before placing next animals.

2.9.5. Rotational behaviour

To assess the effect of dopamine receptor agonist/antagonist on neural degeneration and repair and motor asymmetry, rats were challenged with Amphetamine (5 mg/kg, i.p.) at 2 weeks post-6-OHDA lesioning. The rotational behaviour testing was started after 30 min of amphetamine injection and monitored for a period of 30 min following our earlier published method (Singh et al., 2016). Results are expressed as net ipsilateral rotations per 30 min (net ipsilateral rotations = ipsilateral rotations in 30 min − contralateral rotations in 30 min).

2.10. Statistical analysis

All data are expressed as means ± standard error of mean (SEM), and difference between the groups was determined using one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test. All statistical analysis was performed by GraphPad Prism software 5.00 (San Diego, CA, USA), and all graphs were made by the same software. The difference between the groups was considered to be statistically significant when “P” values were less than 0.05.

3. Results

3.1. Basal level of dopamine receptors in the hippocampus in 6-OHDA lesioned rats

To investigate the effect of 6-OHDA on dopamine receptor expression, we measured the levels of dopamine D1 and D2 receptors in hippocampus (Fig. 1A). The level of D1 receptor was significantly (Fig. 1B, $P < 0.001$) down-regulated in the hippocampus of 6-OHDA lesioned rats as compared to control rats. In contrast, the level of D2 receptor was unaltered (Fig. 1C, $P > 0.05$) in the hippocampus of 6-OHDA lesioned rats, when compared with control rats.

3.2. D1 receptor agonism attenuates 6-OHDA induced anxiety and depression-like behaviour in adult rats

To understand the role of dopamine receptors in anxiety and social behaviour, rats were treated with agonist and antagonist of dopamine

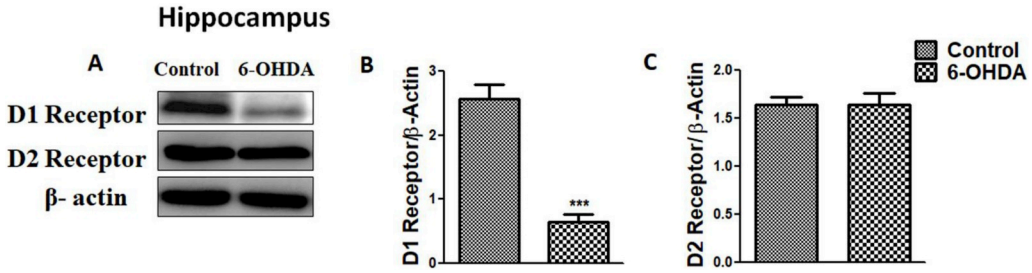

Fig. 1. Intra-MFB administration of 6-OHDA reduces D1 receptor expression in hippocampus of adult rat. (A) Representative Immunoblots show the expression of D1 receptor and D2 receptor in the hippocampus. Bar graphs show the quantification of **(B)** D1 receptor, **(C)** D2 receptor relative protein density in the hippocampus. The protein density of D1 and D2 receptor was normalized with β-actin. Data are expressed as mean ± SEM of n = 5 rats/group. Data were analyzed by student t - test (***P < 0.001) * = Control vs 6-OHDA.

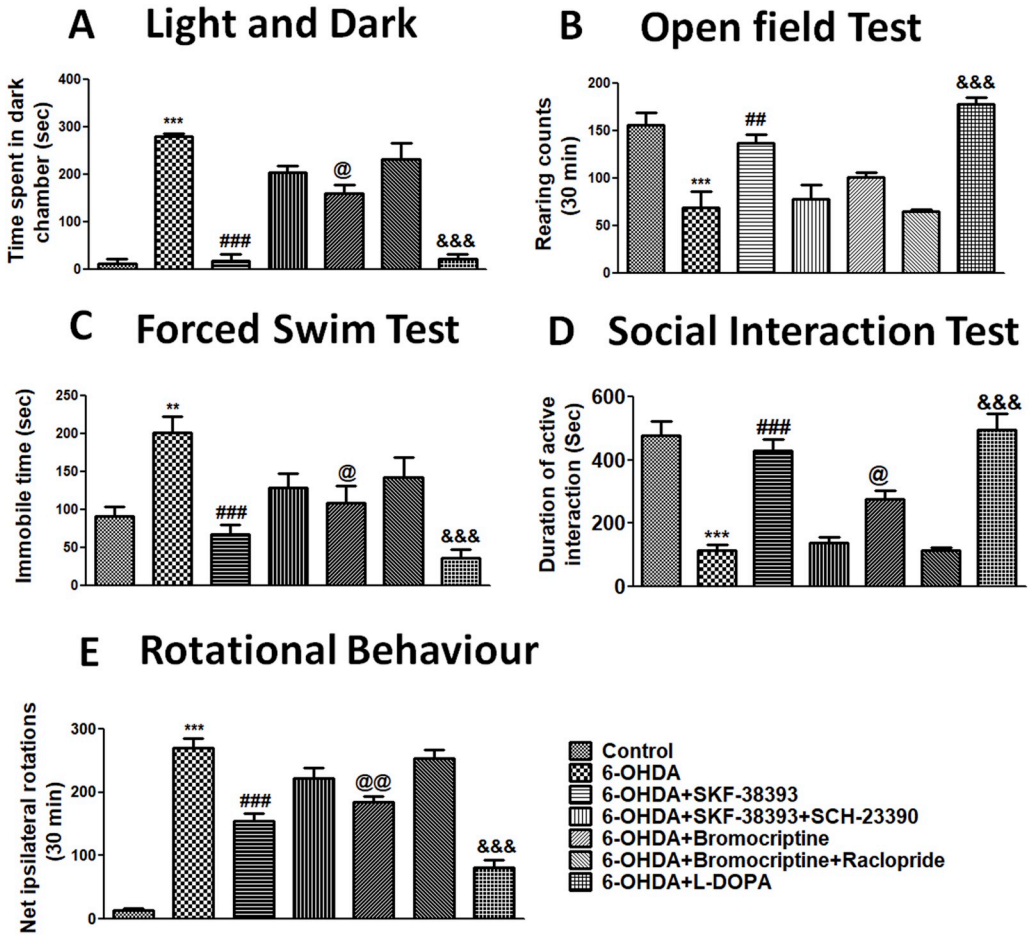

Fig. 2. Selective stimulation of D1 receptor decreases depression and anxiety-like phenotypes in rat model of PD-like phenotype. Figure A–E shows the effect of D1 and D2 receptor agonist and antagonist in behavioural function of 6-OHDA lesioned rats. **(A)** Bar graph shows the time spent in a dark chamber. **(B)** Bar graph shows the rearing activity counts in an open-field arena over 30 min periods. **(C)** Bar graph shows the duration of animal immobility in the FST. **(D)** Bar graph shows the time spent by the experimental rat in active interactions with conspecific rat during social interaction test. **(E)** Bar graph shows the amphetamine (5 mg/kg) induced net ipsilateral rotations. Data are expressed as mean ± SEM of n = 8 rats/group. Data were analyzed by One-way ANOVA followed by Bonferroni's Multiple Comparison Test (*P < 0.05, **P < 0.01, ***P < 0.001, $^{\#}$P < 0.05, $^{\#\#}$P < 0.01, $^{\#\#\#}$P < 0.001 $^{@}$P < 0.05, $^{@@}$P < 0.01, $^{@@@}$P < 0.001, $^{\&}$P < 0.05, $^{\&\&}$P < 0.01, $^{\&\&\&}$P < 0.001) * = Control vs 6-OHDA, # = 6-OHDA vs 6-OHDA + SKF-38393, @ = 6-OHDA vs 6-OHDA + Bromocriptine, & = 6-OHDA vs 6-OHDA + L-DOPA.

D1 and D2 receptors. We found that 6-OHDA lesioned rats showed significantly more time in the dark chamber (Fig. 2A, P < 0.001), reduced rearing counts in an open arena (Fig. 2B, P < 0.001), increased immobility in the FST (Fig. 2C, P < 0.01), reduced time spent by experimental rat in active interaction with conspecific rat in SIT (Fig. 2D, P < 0.001) and increased net ipsilateral; rotations (Fig. 2E, P < 0.001) as compared to the control group. Interestingly, D1 receptor agonist SKF-38393 significantly reduced the time spent in the dark chamber (Fig. 2A, P < 0.001), improved the rearing counts (Fig. 2B, P < 0.01), reduced immobility time (Fig. 2C, P < 0.001), increased time spent by experimental rat in active interaction with conspecific rat in SIT (Fig. 2D, P < 0.001) and decreased net ipsilateral; rotations (Fig. 2E, P < 0.001) as compared to 6-OHDA lesioned group. The effect of D1 agonist behavioural parameters was blocked by cotreatment with D1 antagonist SCH-23390 in 6-OHDA lesioned rats. Similarly, D2 receptor agonist Bromocriptine significantly reduced the time spent in the dark chamber (Fig. 1A, P < 0.05), reduced immobility time (Fig. 2C, P < 0.05), increased time spent by experimental rat in active interaction with conspecific rat in SIT (Fig. 2D, P < 0.05) and decreased net ipsilateral; rotations (Fig. 2E, P < 0.01) as compared to 6-OHDA lesioned group. However, D2 receptor agonist Bromocriptine or D2 antagonist raclopride treated 6-OHDA lesioned rats did not show any alteration in rearing counts (Fig. 2B, P > 0.05), when compared with 6-OHDA lesioned rats. Interestingly, the effect of D2 agonist on behavioural components was blocked by cotreatment with D2 antagonist Raclopride in 6-OHDA lesioned rats, indicating that raclopride antagonize the effect of the D2 agonist. Likewise, L-DOPA treatment in 6-OHDA lesioned rats significantly decreased the time spent in the dark chamber (Fig. 2A, P < 0.001), enhanced the rearing counts (Fig. 2B, P < 0.001), decreased immobility (Fig. 2C,

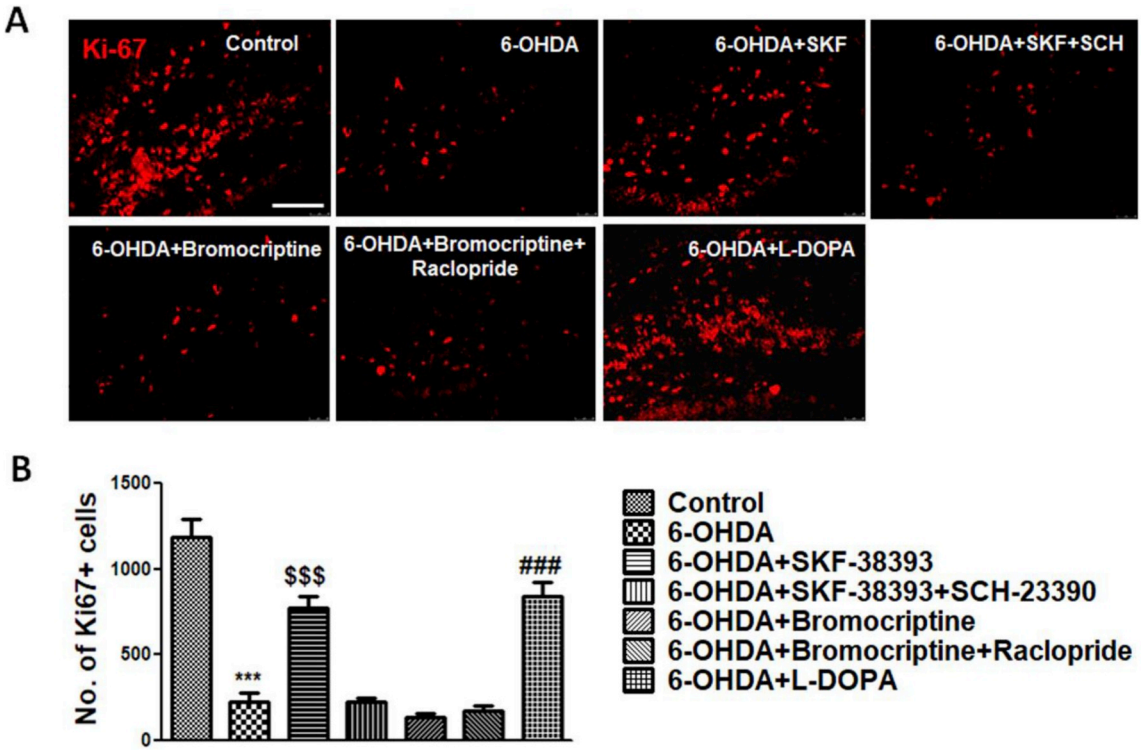

Fig. 3. D1 receptor stimulation attenuates 6-OHDA induced loss in NSC proliferation in hippocampal DG. (A) Representative photomicrographs show immunostaining of Ki-67 in the hippocampal DG. Scale bar: 50 μm. **(B)** Bar graph shows the quantification of number of Ki-67$^+$ cell in the hippocampal DG. Data are expressed as mean ± SEM of n = 4 rats/group. Data were analyzed by One-way ANOVA followed by Bonferroni's Multiple Comparison Test (*P < 0.05, **P < 0.01, ***P < 0.001, $^{\$}$P < 0.05, $^{\$\$}$P < 0.01, $^{\$\$\$}$P < 0.001, $^{\#}$P < 0.05, $^{\#\#}$P < 0.01, $^{\#\#\#}$P < 0.001) * = Control vs 6-OHDA, $ = 6-OHDA vs 6-OHDA + SKF-38393, # = 6-OHDA vs 6-OHDA + L-DOPA.

P < 0.001), increased sociability (Fig. 2D, P < 0.001) and decreased net ipsilateral; rotations (Fig. 2E, P < 0.001) as compared to 6-OHDA lesioned rats.

3.3. D1 receptor agonism enhances proliferation and long-term survival of NSCs in hippocampus in rat model of PD-like phenotypes

In order to investigate the effect of dopamine receptor signaling on neural progenitor cell (NPC) proliferation, we performed immunostaining of Ki-67 in the hippocampus on day 2 after 6-OHDA injection (Fig. 3A). We found significantly (Fig. 3B, P < 0.001) less Ki-67$^+$ cells in hippocampal DG in 6-OHDA lesioned rats as compared to the control rats, Whereas, significantly (Fig. 3B, P < 0.001) higher number of Ki-67$^+$ cells in hippocampal DG were observed following the treatment of SKF-38393 when compared with 6-OHDA lesioned rats. SCH-23390 treatment in 6-OHDA lesioned rats attenuated the effect of SKF-38393 on Ki-67$^+$ cells in the hippocampal DG. Interestingly, Ki-67$^+$ cell number was not significantly (Fig. 3B, P > 0.05) altered following the treatment of Bromocriptine or cotreatment with raclopride in 6-OHDA lesioned rats when compared with 6-OHDA lesioned rats. In contrast, L-DOPA treatment in 6-OHDA lesioned rats significantly (Fig. 3B, P < 0.001) increased the number of Ki-67$^+$ cells in the hippocampal DG as compared to 6-OHDA lesioned rats, indicating that D1 agonism enhances the proliferation of NPCs in rat model of PD-like phenotypes.

Next, we performed double immunolabeling of BrdU and nestin in the hippocampus on day 21 post-6-OHDA injection to analyze long-term survival and proliferation of NSCs (Fig. 4A). We observed that the number of BrdU$^+$ (Fig. 4B, P < 0.001), nestin$^+$ (Fig. 4C, P < 0.001) and BrdU$^+$/nestin$^+$ (Fig. 4D, P < 0.001) cells were significantly reduced in 6-OHDA lesioned rats as compared to control rats. SKF-38393 treatment in 6-OHDA lesioned rats significantly increased the number of BrdU$^+$ (Fig. 4B, P < 0.001), nestin$^+$ (Fig. 4C, P < 0.05) and BrdU$^+$/nestin$^+$ (Fig. 4D, P < 0.05) cells in the hippocampal DG as compared to 6-OHDA lesioned rats. Interestingly, this effect was attenuated by cotreatment with SCH-23390 in 6-OHDA lesioned rats. The number of BrdU$^+$ (Fig. 4B, P > 0.05), nestin$^+$ (Fig. 4C, P > 0.05) and BrdU$^+$/nestin$^+$ (Fig. 4D, P > 0.05) cells were not significantly altered in 6-OHDA lesioned rats following the treatment of Bromocriptine or cotreatment with raclopride when compared with 6-OHDA lesioned rats. However, L-DOPA treatment in 6-OHDA lesioned rats significantly increased the number of BrdU$^+$ (Fig. 4B, P < 0.001), nestin$^+$ (Fig. 4C, P < 0.001) and BrdU$^+$/nestin$^+$ cells (Fig. 4D, P < 0.001) in the hippocampal DG as compared to 6-OHDA lesioned rats, indicating that D1 agonist increase long-term survival (BrdU$^+$) and proliferation (BrdU$^+$/nestin$^+$) of NSCs in hippocampus in rat model of PD-like phenotypes.

3.4. D1 receptor agonism enhances the adult hippocampal neurogenesis in rat model of PD-like phenotypes

Further, to determine the effect of dopamine receptor modulation on the birth of newborn neurons in rat model of PD-like phenotypes, we performed double immunolabeling of NeuN and BrdU in the hippocampus (Fig. 5A). The number of NeuN$^+$/BrdU$^+$ cells was significantly (Fig. 5B, P < 0.001) decreased in hippocampal DG in 6-OHDA lesioned rats as compared to control rats. Interestingly, the number of NeuN$^+$/BrdU$^+$ cell was significantly (Fig. 5B, P < 0.05) increased in hippocampal DG in 6-OHDA lesioned rats following treatment of SKF-38393 when compared with 6-OHDA lesioned group. In contrast, SCH-23390 cotreatment attenuated this effect in NeuN$^+$/BrdU$^+$ cells in 6-OHDA lesioned rats. Similarly, Bromocriptine or raclopride treatment in 6-OHDA lesioned rats did not affect (Fig. 5B, P > 0.05) the number of NeuN$^+$/BrdU$^+$ cells in hippocampal DG when compared with 6-OHDA

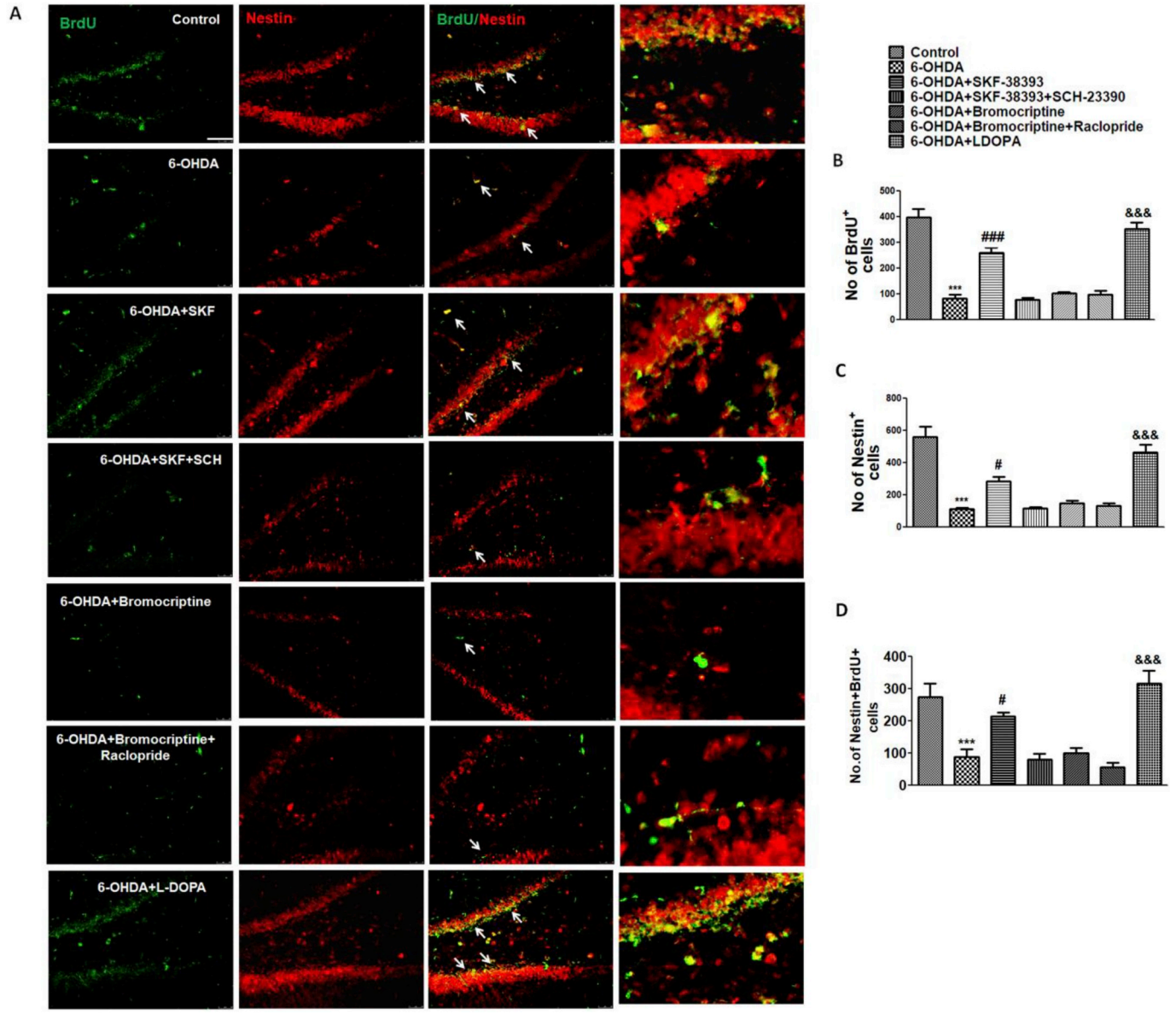

Fig. 4. D1 receptor stimulation promotes long term survival and proliferation of NSC in the hippocampal DG in rat model of PD like phenotypes. (A) Representative photomicrograph shows immunostaining of BrdU (a cell Proliferation marker; green) and Nestin (a marker of NSC; red) in the hippocampal dentate gyrus (DG). Scale bar: 50 μm. White arrows indicate the colocalized cells ($Nestin^+/BrdU^+$) in yellow colour. Fourth panel (right) shows the magnified image of colocalization (BrdU and Nestin). **(B)** Bar graph shows the quantification of $BrdU^+$ cells in hippocampal DG region. **(C)** Bar graph shows the quantification of $Nestin^+$ cells in hippocampal DG region. **(D)** Bar graph shows the quantification of colocalized $Nestin^+/BrdU^+$ cells in the hippocampal DG region. Data are expressed as mean ± SEM of n = 4 rats/group. Data were analyzed by One-way ANOVA followed by Bonferroni's Multiple Comparison Test ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, $^{\&}P < 0.05$, $^{\&\&}P < 0.01$, $^{\&\&\&}P < 0.001$) * = Control vs 6-OHDA, # = 6-OHDA vs 6-OHDA + SKF-38393, & = 6-OHDA vs 6-OHDA + L-DOPA.

lesioned rats. We observed significantly (Fig. 5B, $P < 0.001$) increased number of $NeuN^+/BrdU^+$ cells in the hippocampus of 6-OHDA lesioned rats following the treatment of L-DOPA as compared to 6-OHDA lesioned rats, suggesting that D1 receptor signaling is preferentially involved in the regulation of hippocampal neurogenesis in rat model of PD-like phenotypes. In order to determine whether dopamine receptor modulation affects the expression of proneural genes in rat model of PD-like phenotypes, we performed qRT-PCR to analyze the mRNA expression of proneural genes in hippocampus (Fig. 5C). The mRNA expression of pro-neural genes, such as NeuroD1 (Fig. 5C, $P < 0.001$), Ngn2 (Fig. 5C, $P < 0.001$), DCX (Fig. 5C, $P < 0.001$), Nestin (Fig. 5C, $P < 0.001$) and Mash1 (Fig. 5C, $P < 0.001$) was significantly down-regulated in the hippocampus in 6-OHDA lesioned rats as compared to control rats. SKF-38393 treatment in 6-OHDA lesioned rats significantly up-regulated the mRNA expression of Ngn2 (Fig. 5C, $P < 0.001$), NeuroD1 (Fig. 5C, $P < 0.001$), DCX (Fig. 5C, $P < 0.001$), Nestin (Fig. 5C, $P < 0.001$) and Mash1 (Fig. 5C, $P < 0.001$) which was attenuated by cotreatment of SCH-23390 in 6-OHDA lesioned rats. The mRNA expression of all these pro-neural genes was not significantly altered (Fig. 5C, $P > 0.05$) in the hippocampus of 6-OHDA lesioned rats following the treatment of Bromocriptine or D2 antagonist raclopride. Whereas, L-DOPA treatment in 6-OHDA lesioned rats significantly up-regulated the mRNA expression of Ngn2 (Fig. 5C, $P < 0.001$), NeuroD1 (Fig. 5C, $P < 0.001$), DCX (Fig. 5C, $P < 0.001$), Nestin (Fig. 5C, $P < 0.001$) and Mash1 (Fig. 5C, $P < 0.001$) in hippocampus as compared to 6-OHDA lesioned rats, indicating that D1 agonist induced adult hippocampal neurogenesis may be the cause of enhanced levels of proneural genes in rat model of PD-like phenotypes.

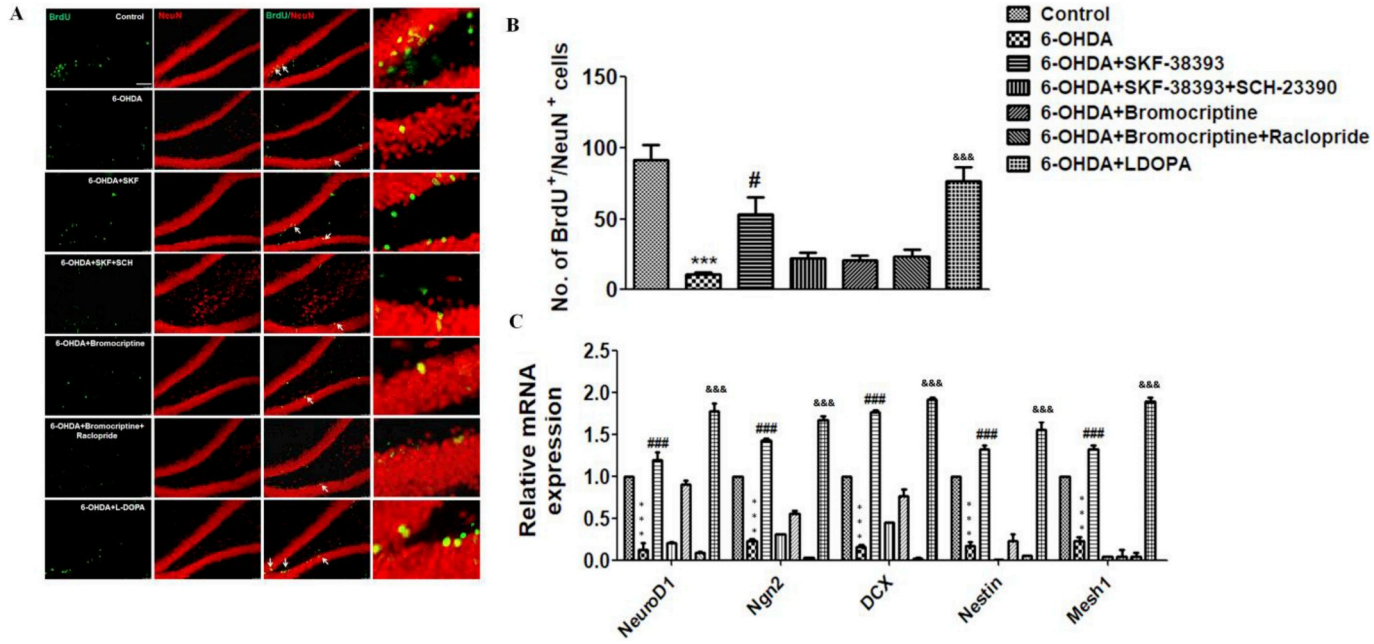

Fig. 5. D1 receptor stimulation improves neuronal differentiation of NSC in hippocampal DG in rat model of PD like phenotypes. (A) Representative photomicrograph shows immunostaining of BrdU (a cell proliferation marker; green) and NeuN (a marker of mature neurons; red) in hippocampal DG. Scale bar: 50 µm. White arrows indicate the newly formed mature neuronal cells ($NeuN^+/BrdU^+$) in yellow colour. Fourth panel (right) shows the magnified image of colocalization (BrdU and NeuN) **(B)** Bar graph shows the quantification of $NeuN^+/BrdU^+$ cells in the hippocampal DG. **(C)** Bar graph shows the gene expression analysis of proneural genes, NeuroD1, Ngn 2, DCX, Nestin and Mash 1 in the hippocampus region. β-actin was used as a housekeeping gene for the normalization of target gene expression. Data were analyzed by one-way and Two-way ANOVA followed by Bonferroni's Multiple Comparison test and Bonferroni post hoc test ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, $^{\&}P < 0.05$, $^{\&\&}P < 0.01$, $^{\&\&\&}P < 0.001$) * = Control vs 6-OHDA, # = 6-OHDA vs 6-OHDA + SKF-38393, & = 6-OHDA vs 6-OHDA + L-DOPA.

3.5. D1 agonist positively regulate Wnt/β-catenin signaling in the hippocampus of rat model of PD-like phenotypes

In order to determine the possible mechanism by which dopamine receptor modulation regulate behavioural functions and adult hippocampal neurogenesis, we analyzed the mRNA expression of Wnt/β-catenin signaling pathway genes in the hippocampus (Fig. 6A). The mRNA expression of Wnt-3a (Fig. 6A, $P < 0.001$), Lef-1 (Fig. 6A, $P < 0.001$), β-catenin (Fig. 6A, $P < 0.001$), and CyclinD1 (Fig. 6A, $P < 0.001$) was significantly down-regulated, whereas Axin-2 (Fig. 6A, $P < 0.001$) was found to be significantly up-regulated in the hippocampus in 6-OHDA lesioned rats as compared to control rats. We observed that mRNA expression of Wnt-3a (Fig. 6A, $P < 0.001$), Lef-1 (Fig. 6A, $P < 0.001$), β-catenin (Fig. 6A, $P < 0.001$), and CyclinD1 (Fig. 6A, $P < 0.001$) was up-regulated and Axin-2 (Fig. 6A, $P < 0.001$) was significantly down-regulated in the hippocampus of 6-OHDA lesioned rats following the treatment of SKF-38393 when compared with 6-OHDA lesioned rats. However, this effect of SKF-38393 on mRNA expression was attenuated by cotreatment with SCH-23390 in 6-OHDA lesioned rats. The mRNA expression of all the above-mentioned

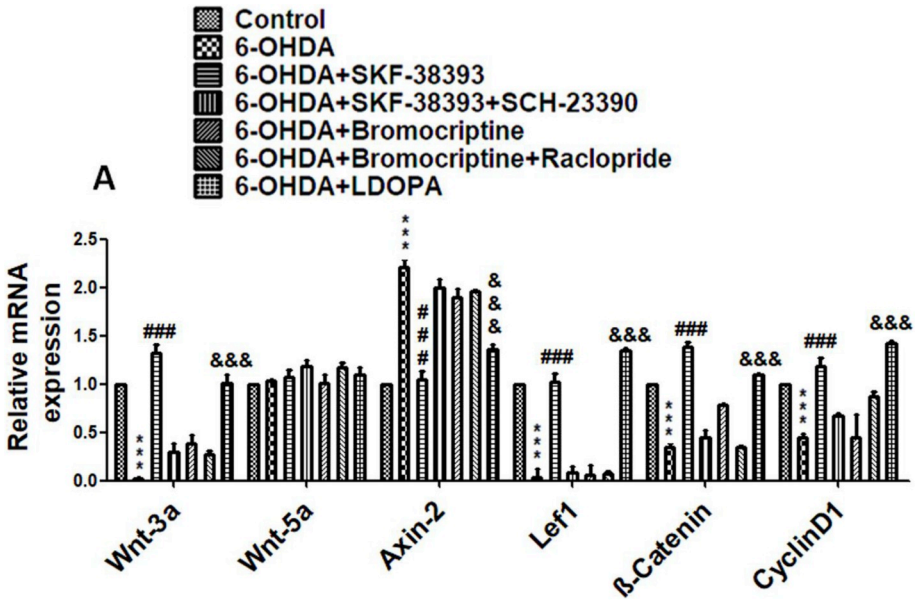

Fig. 6. D1 receptor stimulation regulates Wnt/β-catenin target genes expression in hippocampus in rat model of PD-like phenotypes. Bar graph shows the gene expression analysis of Wnt/β-catenin signaling genes; Wnt-3a, Wnt-5a, Axin-2, β-catenin, Lef-1 and cyclinD1 in **(A)** hippocampus region. β-actin was used as a housekeeping gene for normalization of target gene expression. Data are expressed as mean ± SEM of n = 4 rats/group. Data were analyzed by Two-way ANOVA followed by Bonferroni post hoc test ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, $^{\&}P < 0.05$, $^{\&\&}P < 0.01$, $^{\&\&\&}P < 0.001$) * = Control vs 6-OHDA, # = 6-OHDA vs 6-OHDA + SKF-38393, & = 6-OHDA vs 6-OHDA + L-DOPA.

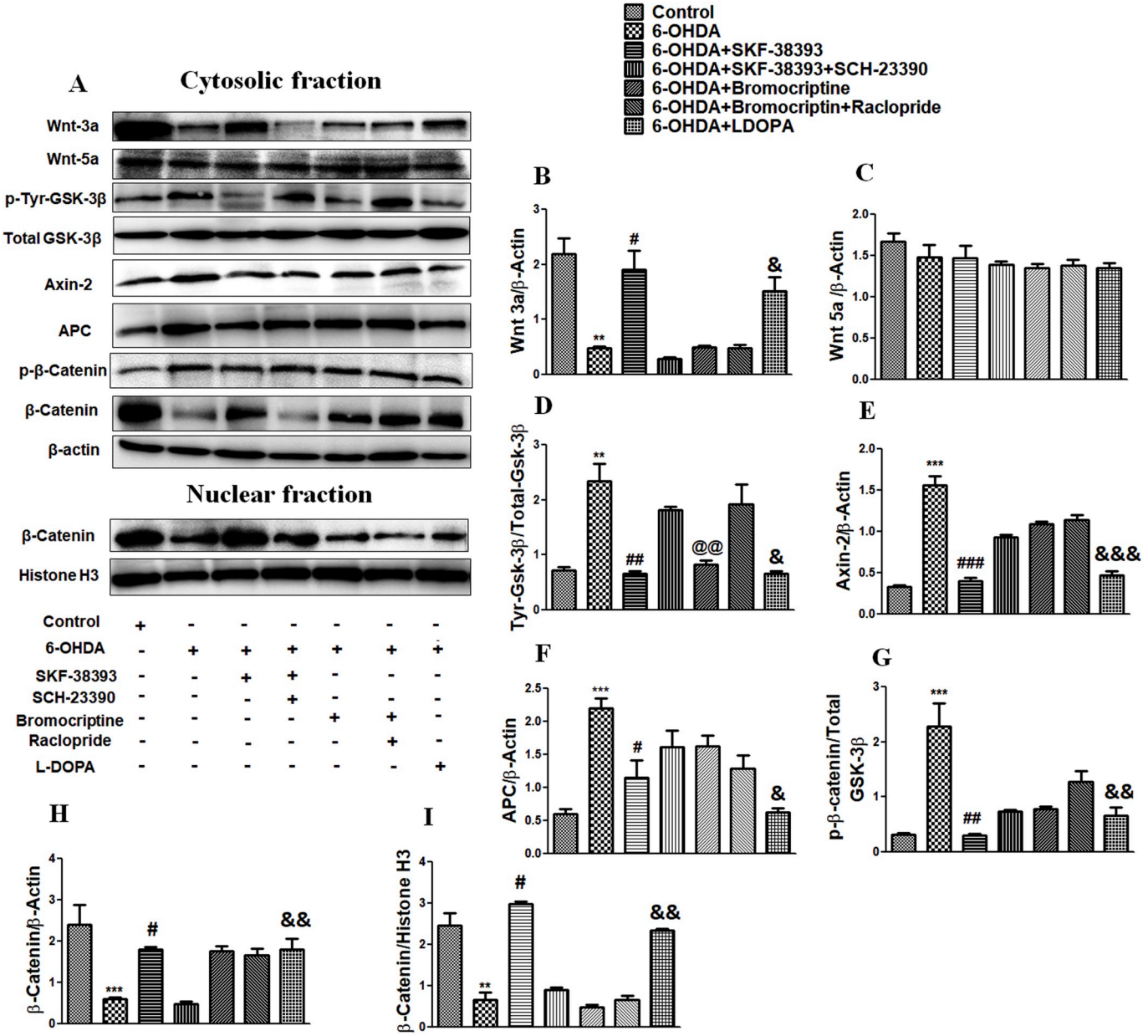

Fig. 7. D1 stimulation induces the activation of Wnt/β-catenin signaling in hippocampus in rat model of PD-like phenotypes. (A) Representative Immunoblots show the expression of Wnt/β-catenin pathway proteins in hippocampus. Bar graphs show the quantification of **(B)** Wnt-3a, **(C)** Wnt-5a, **(D)** p-Tyr216GSK-3β, **(E)** Axin-2, **(F)** APC, **(G)** phosphorylated-β-catenin and **(H)** nonphosphorylated-β-catenin relative protein density in the cytosolic fraction of hippocampus. **(I)** Bar graph shows the quantification of the relative protein density of β-catenin in nuclear fraction. The protein density of p-Tyr216-GSK-3β was normalized with total GSK-3β protein density and the density of Wnt-3a, Wnt-5a, Axin-2, APC and β-catenin was normalized with β-actin in cytosolic fraction. Phosphorylated-β-catenin level was normalized with nonphosphorylated-β-catenin in cytosolic fraction. The protein density of β-catenin was normalized with Histone H3 protein density in nuclear fraction. Data were analyzed by One-way ANOVA followed by Bonferroni's Multiple Comparison Test ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, $^{@}P < 0.05$, $^{@@}P < 0.01$, $^{@@@}P < 0.001$, $^{\&}P < 0.05$, $^{\&\&}P < 0.01$, $^{\&\&\&}P < 0.001$) * = Control vs 6-OHDA, # = 6-OHDA vs 6-OHDA + SKF-38393, @ = 6-OHDA vs 6-OHDA + Bromocriptine, & = 6-OHDA vs 6-OHDA + L-DOPA.

genes remained unaltered following the treatment of Bromocriptine or cotreatment with D2 antagonist in 6-OHDA lesioned rats. Interestingly, L-DOPA treatment in 6-OHDA lesioned rats significantly up-regulated the mRNA expression of Wnt-3a (Fig. 6A, $P < 0.001$), Lef-1 (Fig. 6A, $P < 0.001$), β-catenin (Fig. 6A, $P < 0.001$), and CyclinD1 (Fig. 6A, $P < 0.001$) and down-regulated the expression of Axin-2 (Fig. 6A, $P < 0.001$) when compared with 6-OHDA lesioned group.

Further, we performed Western blot analysis to quantify protein levels of different intermediate candidate of Wnt/β-catenin signaling pathway (Fig. 7A). We found that Wnt-5a (Fig. 7C, $P > 0.05$) level remained unaltered in all treatment groups. The level of Wnt-3a was (Fig. 7B, $P < 0.01$) reduced and the level of p-Tyr216 GSK-3β (Fig. 7D, $P < 0.01$) Axin-2 (Fig. 7E, $P < 0.001$), APC (Fig. 7F, $P < 0.001$) and p-β-catenin (Fig. 7G, $P < 0.001$) was significantly increased in the cytosolic fraction of hippocampus of 6-OHDA lesioned rats as compared to control rats. We also observed that the levels of nonphosphorylated β-catenin was reduced in cytosolic (Fig. 7H, $P < 0.001$) and nuclear fractions (Fig. 7I, $P < 0.01$) of hippocampus in 6-OHDA lesioned rats as compared to control rats. We found increased levels of Wnt-3a (Fig. 7B, $P < 0.05$) and reduced levels of p-Tyr216 GSK-3β (Fig. 7D, $P < 0.05$), Axin-2 (Fig. 7E, $P < 0.001$), APC (Fig. 7F, $P < 0.05$) and p-β-catenin protein (Fig. 7G, $P < 0.01$) in cytosolic fraction of

hippocampus in 6-OHDA lesioned rats following the treatment of SKF-38393 when compared with 6-OHDA lesioned group. SKF-38393 treatment in 6-OHDA lesioned rats significantly increased the level of nonphosphorylated β-catenin in cytosolic (Fig. 7H, $P < 0.05$) and nuclear fractions (Fig. 7I, $P < 0.05$) of hippocampus as compared to 6-OHDA lesioned rats, indicating that D1 agonism enhances the nuclear translocation of β-catenin in PD rats. However, SCH-23390 treatment attenuated the effect of D1 agonist SKF-38393 on Wnt pathway in 6-OHDA lesioned rats. The levels of Wnt/β-catenin pathway related proteins remained unaltered in 6-OHDA lesioned rats following the treatment of bromocriptine or raclopride when compared with 6-OHDA lesioned group. Interestingly, L-DOPA treatment in 6-OHDA lesioned rats significantly increased Wnt-3a (Fig. 7B, $P < 0.05$) level and reduced p-Tyr216 GSK-3β (Fig. 7D, $P < 0.05$), Axin-2 (Fig. 7E, $P < 0.001$), APC (Fig. 7F, $P < 0.05$), and p-β-catenin (Fig. 7G, $P < 0.01$) protein levels in the cytosolic fraction of hippocampus when compared with 6-OHDA lesioned group. We also found that L-DOPA treatment in 6-OHDA lesioned rats significantly increased the levels of nonphosphorylated β-catenin in cytosolic (Fig. 7H, $P < 0.01$), and nuclear fractions (Fig. 7I, $P < 0.01$) of the hippocampus when compared with 6-OHDA lesioned group.

3.6. Axin-2 knockdown mediated activation of Wnt/β-catenin signaling improves hippocampal neurogenesis and behavioural functions in D1 antagonist treated PD rats

In order to investigate whether D1 receptor mediated effects in rat model of PD-like phenotypes involves Wnt/β-catenin signaling, we genetically activated β-catenin signaling using shRNA against Axin-2 (Fig. 8A). Wnt signaling regulatory step involves the phosphorylation, ubiquitination and degradation of downstream effector protein, β-catenin by cytoplasmic degradation complex that consists of Axin-2, a central scaffold protein and three other components, APC, GSK-3β and casein kinase-1. Therefore, we stereotaxically injected Axin-2 shRNA into ipsilateral hippocampus and animals were sacrificed after 21 days. We also evaluated the effect of Axin-2 shRNA on key intermediate of canonical Wnt signaling in control rats (Fig-S1A). We found that Axin-2 shRNA markedly reduced of pTyr-GSK-3β (Fig. S1B, $P < 0.01$), Axin-2 (Fig. S1C, $P < 0.01$), APC (Fig. S1D, $P < 0.05$), p-β-catenin (Fig. S1E, $P < 0.01$), and increased β-catenin protein (Fig. S1F, $P < 0.05$) levels in control Axin-2 shRNA treated rats as compared to scshRNA treated rats (Fig- S1). We found that cotreatment of Axin-2 shRNA with SCH-23390 significantly reduced p-Tyr216 GSK-3β (Fig. 8D, $P < 0.01$), Axin-2 (Fig. 8E, $P < 0.05$) APC (Fig. 8F, $P < 0.05$) and p-β-catenin (Fig. 8G, $P < 0.01$) levels and increased Wnt-3a (Fig. 8B, $P < 0.01$) in cytosolic fraction of hippocampus in 6-OHDA lesioned rats as compared to D1 antagonist treated 6-OHDA lesioned rats and also increased the levels of nonphosphorylated β-catenin in cytosolic (Fig. 8H, $P < 0.01$) and nuclear fractions (Fig. 8I, $P < 0.05$) of the hippocampus.

Next, we examined the effect of Wnt/β-catenin activation on NSC proliferation (Fig. 9A), long term survival (Fig. 10A) and neuronal differentiation (Fig. 11A) in SCH-23390 treated 6-OHDA lesioned rats. We found that the number of Ki-67$^+$ (Fig. 9B, $P < 0.001$) and BrdU$^+$/nestin$^+$ (Fig. 10B, $P < 0.001$) cells were significantly increased in Axin-2 shRNA treated and D1 receptor agonist SKF-38393 treated 6-OHDA lesioned rat as compared to D1 antagonist treated 6-OHDA lesioned rats. Interestingly, the number of Ki-67$^+$ (Fig. 9B, $P < 0.001$) and BrdU$^+$/nestin$^+$ (Fig. 10B, $P < 0.001$) cells were significantly increased in SKF + SCH treated 6-OHDA lesioned rats following cotreatment with Axin-2 shRNA as compared to SKF + SCH treated 6-OHDA lesioned rats.

Next, we investigated a role of Wnt/β-catenin signaling activation in neuronal differentiation. We found that the number of BrdU$^+$/NeuN$^+$ (Fig. 11B, $P < 0.001$) cells were significantly increased in Axin-2 shRNA treated and D1 receptor agonist SKF-38393 treated 6-OHDA lesioned rat as compared to D1 antagonist treated 6-OHDA lesioned rats. Axin-2 shRNA cotreatment in SKF + SCH treated 6-OHDA lesioned rats also significantly increased the number of BrdU$^+$/NeuN$^+$ (Fig. 11B, $P < 0.001$) cells in the hippocampal DG as compared to SKF + SCH treated 6-OHDA lesioned rats., suggesting that the activation of Wnt/β-catenin signaling potentially enhances NSC proliferation, long term survival and neuronal differentiation in 6-OHDA lesioned rats by attenuating the effect of D1 antagonist.

Further, to determine whether D1 receptor mediated behavioural response is regulated by Wnt/β-catenin signaling. We showed that the behavioural response of D1 agonist SKF-38393 was attenuated by co-treatment with D1 antagonist SCH-23390 in 6-OHDA lesioned as compared to 6-OHDA lesioned rats (Fig. 2A–E). Interestingly, Axin-2 shRNA treatment in 6-OHDA lesioned rats significantly reduced the time spent in dark chamber (Fig. 12A, $P < 0.001$), increased rearing count (Fig. 12B, $P < 0.01$), decreased immobility (Fig. 12C, $P < 0.001$), increased social interaction (Fig. 12D, $P < 0.001$) and reduced ipsilateral rotations (Fig. 12E, $P < 0.001$) when compared with scshRN treated6-OHDA lesioned rats. Interestingly, cotreatment with Axin-2 shRNA in SKF + SCH treated 6-OHDA lesioned rats significantly reduced the time spent in dark chamber (Fig. 12A, $P < 0.001$), increased rearing count (Fig. 12B, $P < 0.01$), decreased immobility (Fig. 12C, $P < 0.001$), increased social interaction (Fig. 12D, $P < 0.01$) and reduced ipsilateral rotations (Fig. 12E, $P < 0.001$) as compared to SKF + SCH + scshRNA treated 6-OHDA lesioned rats, suggesting that D1 receptor mediated improvement in behavioural functions at least in part promoted by the activation of Wnt signaling in rat model of PD-like phenotypes.

4. Discussion

The present study demonstrates that a single unilateral intra-MFB administration of 6-OHDA down-regulate dopamine D1 receptor levels in ipsilateral hippocampus. 6-OHDA induced decline in D1 receptor signaling impairs NSC proliferation and neuronal differentiation in hippocampal DG leading to anxiety and depression-like phenotypes. D1 receptor agonism induced adult hippocampal neurogenesis associated with reduced anxiety and depression-like behaviour in 6-OHDA induced rat model of PD-like phenotypes. D1 receptor mediated effects on neurogenesis and behavioural functions involves the activation of Wnt/β-catenin signaling in the hippocampus.

Compelling experimental evidence supports the role of dopamine system in regulating neurogenesis in SVZ and hippocampus (Chiu et al., 2014; O'Keeffe et al., 2009; Takamura et al., 2014; Winner et al., 2009; Zhang et al., 2016), but the underlying mechanism of dopamine mediated control of adult hippocampal neurogenesis is still unknown. VTA and SNpc DAergic axons directly innervate SVZ and hippocampal DG and SGZ, suggesting a functional and an anatomical association between dopamine system and neurogenic regions (Hoglinger et al., 2014). In our study, single intra-MFB administration of 6-OHDA reduced D1 receptor levels associated with decreased proliferating Ki-67$^+$ NPC and BrdU$^+$/nestin$^+$ NSCs in the hippocampal DG. Additionally, long-term survival of NPCs (BrdU$^+$) was also reduced in the DG following 6-OHDA injection. SKF-38393 and L-DOPA treatment significantly increased the long-term survival and proliferation of NSCs in the hippocampus in parkinsonian rats. Interestingly, the effect of D1 agonist on NSCs was attenuated following the treatment with D1 antagonist SCH-23390 in parkinsonian rats. However, D2 agonist Bromocriptine and antagonist raclopride were not able to affect long-term survival and proliferation of NSCs in parkinsonian rats, suggesting a positive and a preferential effect of D1 receptor stimulation on NSCs dynamics in PD rats. We have previously shown that 6-OHDA treatment reduced the expression of D1 receptor in hippocampus and prefrontal region (Singh et al., 2018b). Further, Blunt et al. (1992), also showed reduction in D1 receptor density in the ipsilateral striatum following 6-OHDA injection, while at the same time observed increased D2 receptor density (Blunt et al., 1992). This suggests that 6-OHDA regulates the

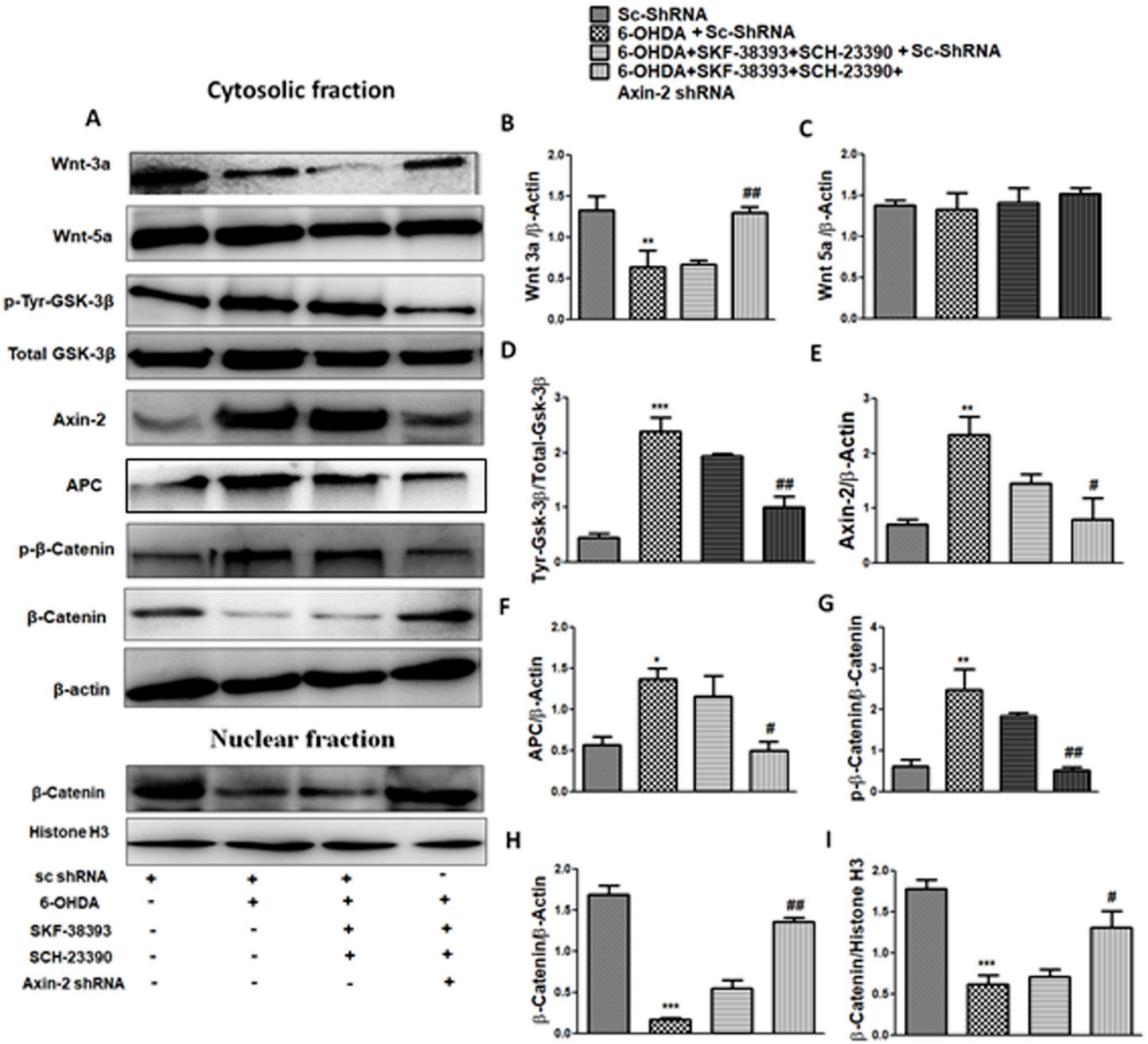

Fig. 8. Axin-2- shRNA activates Wnt/β-catenin signaling in D1 receptor antagonist SCH-23390 treated 6-OHDA lesioned rat. (A) Representative Immunoblots show the expression of Wnt-3a, Wnt-5a, p-Tyr216 GSK-3β, nonphosphorylated (total) GSK-3β, Axin2, APC, p-β-catenin and β-catenin in cytosolic fraction and β-catenin in nuclear fraction. Bar graphs show the quantification of the relative protein density of **(B)** Wnt-3a, **(C)** Wnt-5a**, (D)** p-Tyr216 GSK-3β **(E)** Axin-2, **(F)** APC **(G)** p-β-catenin and **(H)** β-catenin in cytosolic fraction. **(I)** Bar graph shows the quantification of the relative protein density of β-catenin in nuclear fraction. The protein density of p-Tyr216-GSK-3β was normalized with total GSK-3β protein density and the density of other proteins was normalized with β-actin in cytosolic fraction. Phosphorylated-β-catenin level was normalized with nonphosphorylated-β-catenin in cytosolic fraction. The protein density of β-catenin was normalized with Histone H3 protein density in nuclear fraction. Data are expressed as mean ± SEM of n = 5 rats/group. Data were analyzed by One-way ANOVA followed by Bonferroni's Multiple Comparison Test (*P < 0.05, **P < 0.01, ***P < 0.001, $^{\$}$P < 0.05, $^{\$\$}$P < 0.01, $^{\$\$\$}$P < 0.001) * = Control vs 6-OHDA, $ = 6-OHDA vs 6-OHDA + SKF-38393 + SCH-23390 + Axin-2 shRNA.

expression of dopamine receptor regionally through unknown mechanism. This is an area of further investigation that how dopamine receptor expression regulates regionally in the brain. For now, our results are not sufficient to define the exact mechanism of dopamine expression regulation. On the other hand, we showed the reduction in newborn neuron formation in the hippocampus and reduced D1 receptor density on mature neurons associated with neurodegeneration in the hippocampus (Singh et al., 2018b). Therefore, the reduction in D1 receptor could be an additive effect contributed by NPCs depletion. Additionally, our experimental data showed a single time point evaluation of biochemical and behavioural parameters in 6-OHDA and D1/D2 pharmacological agent treated rats. The D1 mediated effect could be associated with the differential sensitivity of dopamine receptor. Secondly, at the time of experimental evaluation, the obtained results are associated with increased D1 expression; therefore, our data cannot differentiate the potency between the receptors.

D2 receptor agonist Pramipexole (PPX) has been reported to increase proliferating cells in the hippocampus of adult naive mice (Salvi et al., 2016) and in SVZ of naïve rats, while having no effect on proliferation in the hippocampus of 6-OHDA injected rats (Winner et al., 2009). This discrepancy could be due to different species and the toxin dose used in these studies. Dopamine promote proliferation of adult rat

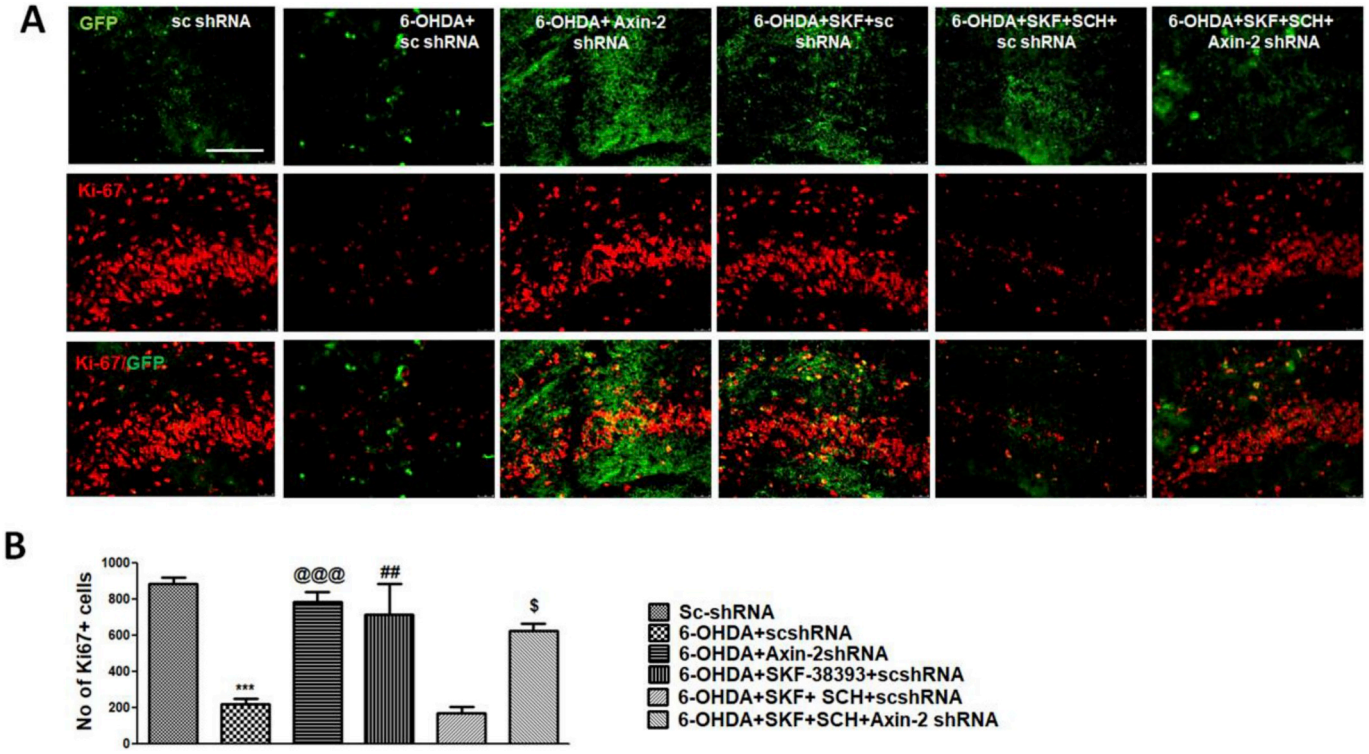

Fig. 9. Wnt/β-Catenin signaling activation attenuates D1 antagonist SCH-23390 induced reduction in NSC proliferation in hippocampal DG. (A) Representative photomicrographs show immunostaining of green fluorescent protein (GFP, green) and Ki-67 (red) in the hippocampal DG. Scale bar: 50 μm. **(B)** Bar graph shows the quantification of number of Ki-67$^+$ cell in the hippocampal DG number of Ki-67$^+$ cells in the hippocampal DG region Data are expressed as mean ± SEM of n = 4 rats/group. Data were analyzed by one-way ANOVA followed by Bonferroni's Multiple Comparison Test (*P < 0.05, **P < 0.01, ***P < 0.001,$^{@}$P < 0.05,$^{@@}$P < 0.01,$^{@@@}$P < 0.00,$^{\#}$P < 0.05,$^{\#\#}$P < 0.01,$^{\#\#\#}$P < 0.001,$^{\$}$P < 0.05, $^{\$\$}$P < 0.01, $^{\$\$\$}$P < 0.001) * = scshRNA vs 6-OHDA, @ = 6-OHDA vs 6-OHDA + Axin-2shRNA, # = 6-OHDA + scshRNA + SKF-38393 + SCH-23390 vs 6-OHDA + SKF-38393 + sc shRNA, $ = 6-OHDA vs 6-OHDA + SKF-38393 + SCH-23390 + Axin-2 shRNA.

DG-derived NPCs via stimulation of D1 receptor, whereas D2 receptor stimulation had no effect on proliferation and survival (Takamura et al., 2014). D1 receptor agonist administration, enhanced proliferating BrdU$^+$ cells in a protein kinase-A (PKA) dependent manner in MPTP treated mice (Zhang et al., 2016). Therefore, D1 induced NSC proliferation may lead to increased long-term survival of NPCs in parkinsonian rats.

Beside analyzing the effect of dopamine receptor stimulation/inhibition on NSC proliferation and survival, we also determined whether these newly generated NSCs were able to differentiate into newborn mature neurons in the hippocampus of parkinsonian rats. We observed that intra-MFB administration of 6-OHDA significantly reduced the number of newborn neurons (BrdU$^+$/NeuN$^+$ cells) in the hippocampus. MPTP treatment has been reported to reduce the number of newborn neurons in the adult mouse hippocampus (Sung, 2015; Zhang et al., 2016). A reduction in NSC pool and neurogenesis has been observed in the SGZ of hippocampus of patients with PD, suggesting a potential effect of dopamine on NSCs (Borta and Hoglinger, 2007; Hoglinger et al., 2004). In support to our study, previous studies have shown that 6-OHDA induced nigral DAergic degeneration and dopamine depletion potentially reduced NSC proliferation and adult neurogenesis in hippocampus and SVZ regions of rats (Salvi et al., 2016; Singh et al., 2017a, 2017b, 2018a), suggesting that 6-OHDA treatment significantly compromised NSC pool in the hippocampus that lead to reduction in neuronal differentiation in adult rats. We observed that the number of newborn neurons in the hippocampal DG was not significantly altered in parkinsonian rats following the treatment with D2 agonist Bromocriptine or D2 antagonist. Interestingly, D1 receptor stimulation by SKF-38393 significantly increased the neuronal differentiation in the hippocampus of parkinsonian rats. We found that the stimulatory effect of D1 agonist on adult hippocampal neurogenesis was reversed following the treatment of D1 antagonist SCH-23390 in parkinsonian rats. Similar to D1 agonist, L-DOPA also enhanced neuronal differentiation in hippocampus of parkinsonian rats, suggesting that dopamine signaling directly regulates adult hippocampal neurogenesis in parkinsonian rats that involves stimulation of D1 receptors. Immunohistochemical, anatomical and gene expression studies demonstrated that D2-like receptors are predominantly expressed on dividing transit-amplifying cells, whereas post-mitotic immature newborn neurons express both D1-like and D2-like dopamine receptors in the SVZ and neurospheres (Coronas et al., 2004; Hoglinger et al., 2004; Kippin et al., 2005). Our findings related to D2 receptor stimulation/inhibition mediated effects on adult neurogenesis are supported by previous studies such as, treatment with L-DOPA or D2 agonist PPX significantly restored adult neurogenesis in hippocampal DG and periglomerular layer of olfactory-bulb in 6-OHDA lesioned rats (Chiu et al., 2015). Another study has reported that PPX significantly enhanced NSC proliferation and adult hippocampal neurogenesis in naïve mice, whereas Ropinirole (D2 agonist) agonist was not able to potentiate the hippocampal neurogenesis (Salvi et al., 2016), suggesting that D2 receptor stimulation mediated enhanced neurogenesis depends upon the potency and selectivity of agonist. In contrast, D2 receptor antagonist haloperidol treatment increased NSC proliferation and their neuronal and glial differentiation in the adult rat brain (Kippin et al., 2005). Our results are further supported by the fact that D2/D3 receptor stimulation was not able to promote proliferation, survival and neurogenesis in murine and human derived NPCs (Milosevic et al., 2007), and the treatment with PPX also did not exerted any effect on NSC proliferation and adult hippocampal neurogenesis in 6-OHDA induced rat model of PD-like phenotypes (Winner et al., 2009). Selective D1, but not D2 receptor

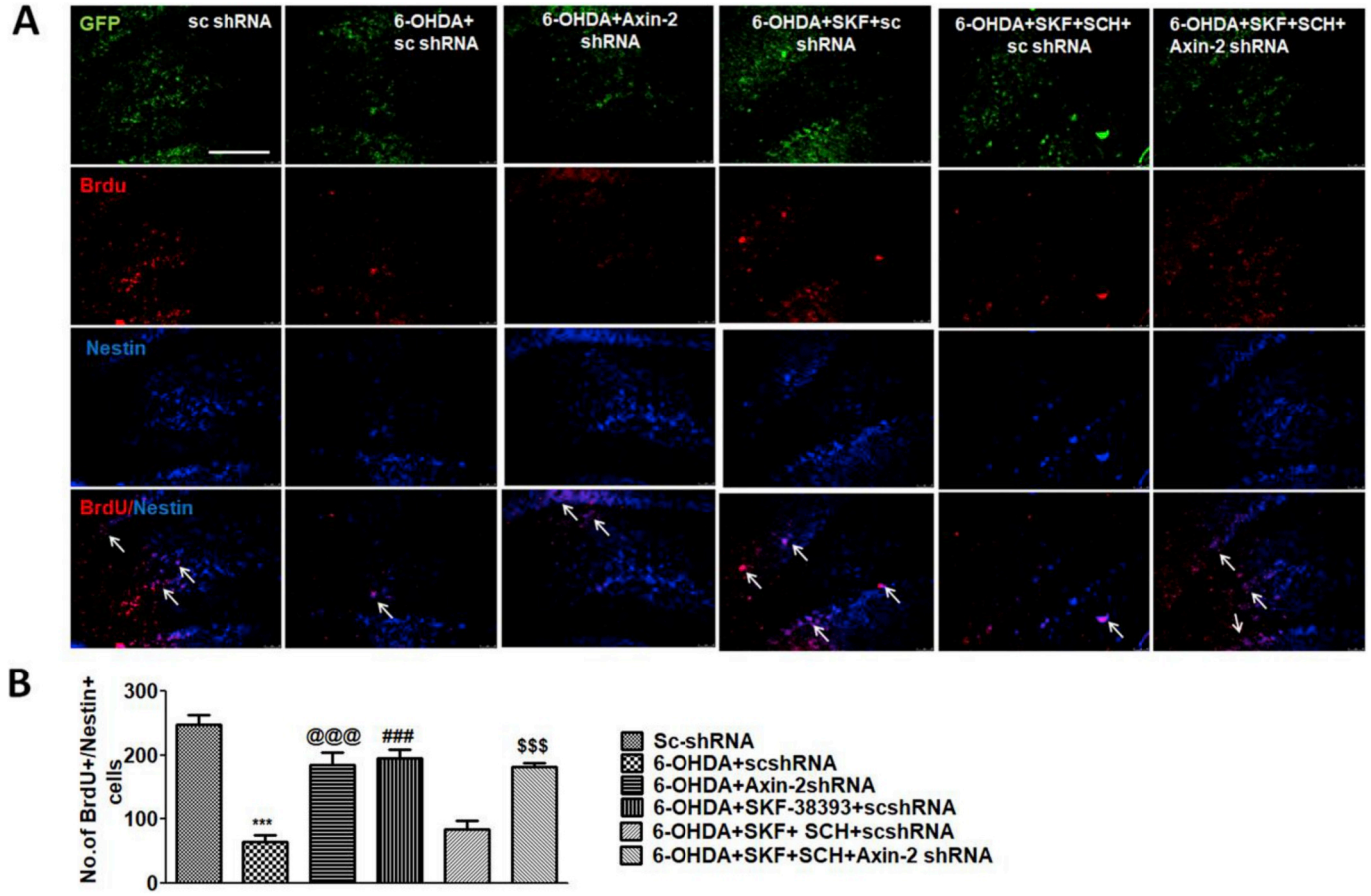

Fig. 10. Wnt/β-Catenin signaling activation attenuates D1 antagonist SCH-23390 induced reduction in long term survival in hippocampal DG. Representative photomicrograph shows immunostaining of green fluorescent protein (GFP, green), BrdU (a cell Proliferation marker; Red) and Nestin (a marker of NSC; Blue) in the **(A)** Hippocampal dentate gyrus (DG). Scale bar: 50 μm. White arrows indicate the colocalized cells ($Nestin^+/BrdU^+$) in pink colour. **(B)** Bar graph shows the quantification of colocalized $Nestin^+/BrdU^+$ cells in the hippocampal DG. Data are expressed as mean ± SEM of n = 4 rats/group. Data are expressed as mean ± SEM of n = 4 rats/group. Data were analyzed by One-way ANOVA followed by Bonferroni's Multiple Comparison Test ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{@}P < 0.05$, $^{@@}P < 0.01$, $^{@@@}P < 0.00$, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, $^{\$}P < 0.05$, $^{\$\$}P < 0.01$, $^{\$\$\$}P < 0.001$) * = scshRNA vs 6-OHDA, @ = 6-OHDA vs 6-OHDA + Axin-2shRNA, # = 6-OHDA + scshRNA + SKF-38393 + SCH-23390 vs 6-OHDA + SKF-38393 + sc shRNA, $ = 6-OHDA vs 6-OHDA + SKF-38393 + SCH-23390 + Axin-2 shRNA.

stimulation was shown to enhance NSC proliferation *in-vitro* and *in-vivo* (Takamura et al., 2014). Moreover, it has been demonstrated that MPTP induced impairment in NSC proliferation and adult neurogenesis in hippocampus requires D1 receptor stimulation (Zhang et al., 2016). L-DOPA induced rotational behaviour is a characteristic feature of dopamine lesioning in one hemisphere due to the imbalance in dopamine content. In contrast, dopamine neuron survival density directly correlates with dopamine content in the SN and striatum regions. Previous studies have shown that adult neurogenesis in the hippocampus, SVZ and SN at least in part under the control of dopamine. In particular, mesencephalic dopamine neuron graft (Clarkson et al., 1998; Grealish et al., 2010) and growth factor (GDNF) promotes dopamine neuron survival that leads to improved motor behavioural response in rodents including reduced rotational frequency. Moreover (Parish et al., 2007), showed functional dopamine neurogenesis in salamander reduced amphetamine induced circling behaviour, whereas AraC treatment (anti-mitotic agent) blocked neurogenesis and enhanced circling behaviour in 6-OHDA treated salamanders, suggesting an effect of dopamine neurogenesis on dopamine imbalance. Further (Chiu et al., 2015), identified that chronic L-DOPA treatment improved neurogenesis in the hippocampus and olfactory bulb as well as promoted nonmotor symptom behavioural recovery in 6-OHDA lesioned rats. Therefore, we conclude that L-DOPA treatment promotes dopamine neuron survival and functional neurogenesis in the SNpc that might be result in reduced amphetamine induced rotational behaviour in 6-OHDA lesioned rats.

NSCs express Wnt signaling components and receptors in hippocampus, which are the critical regulators of NSC self-renewal and fate specification. Wnt signaling remains active in the hippocampus where Wnt-3a is sufficient for induction and regulation of adult hippocampal neurogenesis (Lie et al., 2005), suggesting that Wnt/β-catenin signaling is directly involved in the regulation of hippocampal neurogenesis. Our study demonstrates that single unilateral administration of 6-OHDA significantly down-regulated mRNA and protein levels of Wnt/β-catenin signaling components in the hippocampus of adult rats. Interestingly, D1 agonist SKF-38393 treatment potentially up-regulated Wnt/β-catenin signaling in the hippocampus in parkinsonian rats. The effect of D1 agonist on Wnt/β-catenin signaling was blocked by cotreatment with D1 antagonist SCH-23390, suggesting that D1 receptor agonism positively regulates Wnt/β-catenin signaling in parkinsonian rats. However, in our experimental settings, Bromocriptine or raclopride did not affected Wnt/β-catenin signaling in the hippocampus in parkinsonian rats, indicating that dopamine receptors differentially modulate the Wnt/β-catenin signaling in parkinsonian rats. GSK-3β activation negatively regulate Wnt signaling by inducing proteasomal degradation of β-catenin. Pharmacological inhibition of GSK-3β significantly reduced L-DOPA induced dyskinesia in 6-OHDA lesioned rats, whereas this anti-dyskinetic effect was abolished after the treatment with D1 agonist (Xie et al., 2016). Similarly, SB216763 mediated inhibition of

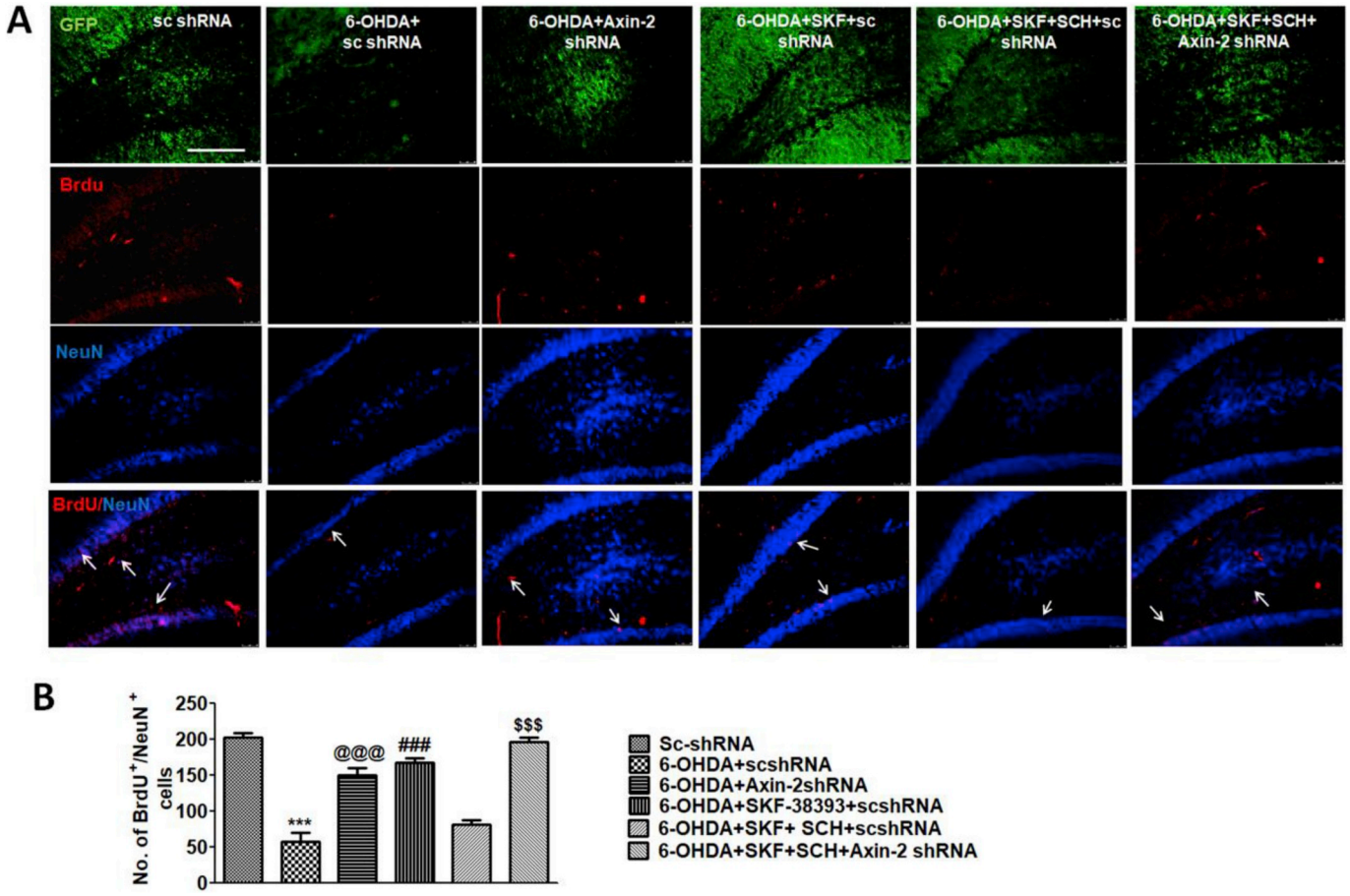

Fig. 11. Wnt/β-Catenin signaling activation attenuates D1 antagonist SCH-23390 induced reduction in neuronal differentiation in hippocampal DG. (A) Representative photomicrograph shows immunostaining of green fluorescent protein (GFP, green), BrdU (a cell Proliferation marker; Red) and NeuN (a marker of mature neuron; Blue) in the hippocampal dentate gyrus (DG). Scale bar: 50 μm. White arrows indicate the colocalized cells ($NeuN^+/BrdU^+$) in pink colour. **(B)** Bar graph shows the quantification of colocalized $NeuN^+/BrdU^+$ cells in the hippocampal DG. Data are expressed as mean ± SEM of n = 4 rats/group. Data are expressed as mean ± SEM of n = 4 rats/group. Data were analyzed by One-way ANOVA followed by Bonferroni's Multiple Comparison Test ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{@}P < 0.05$, $^{@@}P < 0.01$, $^{@@@}P < 0.00$, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, $^{\$}P < 0.05$, $^{\$\$}P < 0.01$, $^{\$\$\$}P < 0.001$) * = scshRNA vs 6-OHDA, @ = 6-OHDA vs 6-OHDA + Axin-2shRNA, # = 6-OHDA + scshRNA + SKF-38393 + SCH-23390 vs 6-OHDA + SKF-38393 + sc shRNA, $ = 6-OHDA vs 6-OHDA + SKF-38393 + SCH-23390 + Axin-2 shRNA.

GSK-3β attenuated D1 receptor agonist SKF-82958 induced hyperactivity in mice (Miller et al., 2010), indicating that Wnt signaling components are involved in dopamine receptor mediated effects. In contrast, few previous reports have shown that anti-psychotic drugs, which acts as antagonist of dopamine D2 receptor, directly enhance the expression of Wnt/β-catenin components such as disheveled-3 (Dvl-3), GSK-3β and β-catenin (Alimohamad et al., 2005; Sutton et al., 2007; Sutton and Rushlow, 2012). These discrepancies in D2 receptor mediated signaling transmission could be due to differential selectivity and affinity of agonist and antagonist, different experimental protocol used, and the analysis performed in different disease condition. Our results are further supported by the fact that overexpression of secreted Wnt antagonist Dickkopf-1 (Dkk-1) significantly reduced the cortico-striatal glutamatergic synapse and dopamine receptor (D1 and D2) clusters with marked impairment in behavioural function in adult mice (Galli et al., 2014). Thus, D1 receptor mediated signaling involves the modulation of Wnt signaling components.

Dopamine D1 and D2-like receptors are widely expressed in hippocampus and play an important role in the regulation of anxiety, depression, contextual fear conditioning, learning and memory (Goldsmith and Joyce, 1994; Hagena and Manahan-Vaughan, 2016; Kemppainen et al., 2003; Sarinana et al., 2014). Unilateral 6-OHDA administration impairs motor behaviour and displayed non-motor behavioural impairments including anxiety, depression and memory impairments (Carvalho et al., 2013; Kaminska et al., 2017). Our recent studies also identified anxiety, depression and cognitive impairments in unilateral 6-OHDA induced rat model of PD-like phenotypes. Our results showed that D1 receptor stimulation significantly reduced 6-OHDA induced anxiety-like phenotypes, whereas the depression-like behaviour in FST was significantly attenuated by D1 or D2 receptor stimulation in parkinsonian rats, suggesting a differential role of dopamine receptor subtypes in cognitive functions in parkinsonian rats. In particular, the performance of animals during behavioural testing also depends on different parameters such as noise, handling, environment and emotional state. Additionally, the behaviour of rats in the open field test mainly determined by two conflicting behaviors: the drive to explore and motivation to avoid potential danger (Bertoglio and Carobrez, 2000). Rats with high levels of emotionality exhibit decreased locomotion and rearing. Thus, the unaltered rearing behaviour observed in the present study may be attributable to reduced emotionality in 6-OHDA lesioned rats compared with control rats. Silva et al. (1997) studied the influence of this physiological state (i.e. lactation) in the elevated plus maze and open field in adult female rats and showed conflicting data. In the open field, total locomotion significantly decreased, and central locomotion (i.e., a parameter related to anxiety) did not differ from virgin rats (Silva et al., 1997). In the light and dark test, D2 agonist treated 6-OHDA lesioned rats displayed a significant reduction in the percentage of time spent on dark chamber

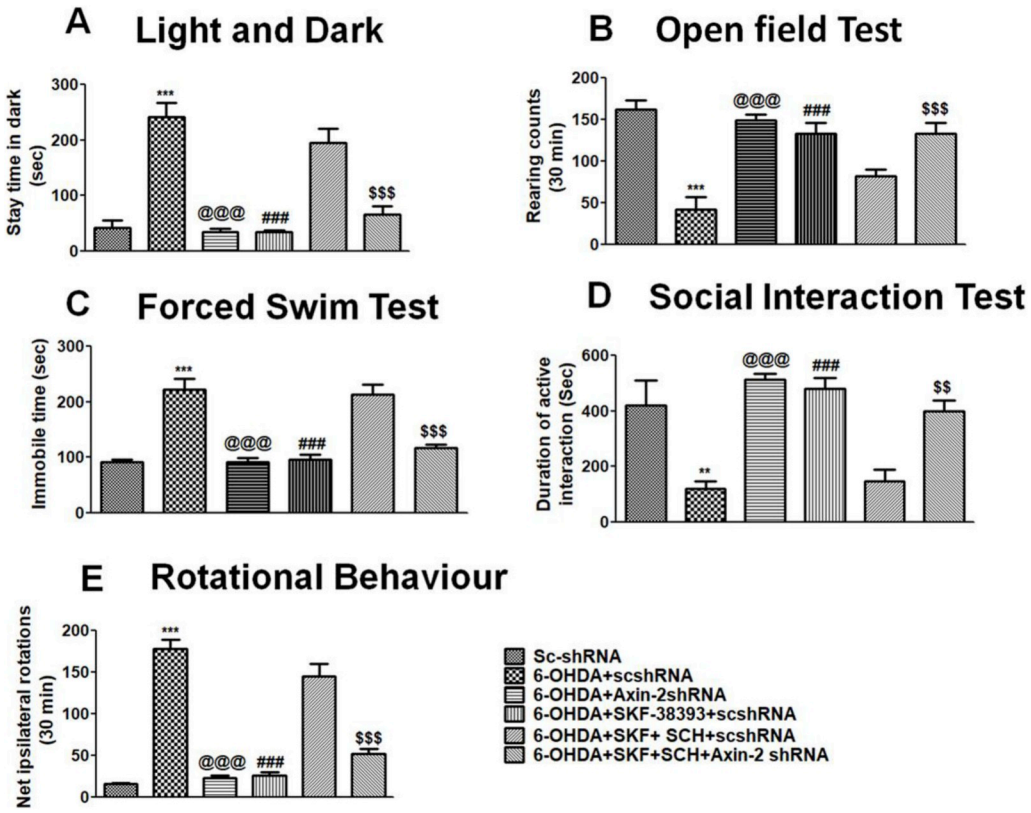

Fig. 12. D1 receptor antagonist induced depression and anxiety like phenotype in rat model of PD-like phenotype via activation of Wnt/β-catenin signaling Light and dark test, forced swim test, Open-field activity and social interaction tests were performed on day 27 and 28 after 6-OHDA injection, respectively. Figure A–D shows the effect of D1 receptor antagonist and D1 receptor antagonist plus Axin-2 shRNA in 6-OHDA lesioned rats. **(A)** Bar graph shows the time spent in a dark chamber. **(B)** Bar graph shows the rearing activity counts in an open-field arena over a 30 min period. **(C)** Bar graph shows the duration (s) of animal immobility in the FST. **(D)** Bar graph shows the time spent (s) by the experimental rat in active interactions with conspecific rat during social interaction test. **(E)** Bar graph shows the amphetamine (5 mg/kg) induced net ipsilateral rotations. Data are expressed as mean ± SEM of n = 5 rats/group. Data are expressed as mean ± SEM of n = 4 rats/group. Data are expressed as mean ± SEM of n = 4 rats/group. Data were analyzed by One-way ANOVA followed by Bonferroni's Multiple Comparison Test (*P < 0.05, **P < 0.01, ***P < 0.001, $^{@}$P < 0.05, $^{@@}$P < 0.01, $^{@@@}$P < 0.00, $^{\#}$P < 0.05, $^{\#\#}$P < 0.01, $^{\#\#\#}$P < 0.001, $^{\$}$P < 0.05, $^{\$\$}$P < 0.01, $^{\$\$\$}$P < 0.001) * = scshRNA vs 6-OHDA, @ = 6-OHDA vs 6-OHDA + Axin-2shRNA, # = 6-OHDA + scshRNA + SKF-38393 + SCH-23390 vs 6-OHDA + SKF-38393 + sc shRNA, $ = 6-OHDA vs 6-OHDA + SKF-38393 + SCH-23390 + Axin-2 shRNA.

and a trend toward a reduction in the percentage of entries into the dark side, suggesting a reduced anxiogenic-like state.

Further, to investigate, whether D1 receptor mediated improvement in behavioural functions requires the activation of Wnt/β-catenin signaling, we stereotaxically injected Axin-2 shRNA lentivirus particles into hippocampus to knockdown Axin-2 expression in D1 antagonist + D1 agonist treated 6-OHDA lesioned rats. Axin-2 is the main component of β-catenin destruction complex and negatively regulates Wnt/β-catenin signaling by reducing the expression of β-catenin (Jho et al., 2002). Axin-2 shRNA treatment significantly reduced the levels of Axin-2 and APC and enhanced β-catenin levels in D1 antagonist + D1 agonist treated 6-OHDA lesioned rats, indicating up-regulation of Wnt/β-catenin signaling. Wnt glycoproteins and their signaling components plays an important role in neural development, synaptogenesis and dendritic development and are directly associated with pathophysiology of neurodegeneration, bipolar disorder, cognitive impairment, anxiety and depression (Ille and Sommer, 2005; Maguschak and Ressler, 2012; Rosso et al., 2005). Several anti-depressants, including citalopram, fluoxetine and venlafaxine have been shown to increase Wnt-2 expression in the hippocampus of rat, indicating that Wnt is a common target of anti-depressants (Okamoto et al., 2010). These studies further support our data that Axin-2 inhibition mediated up-regulation of Wnt/β-catenin signaling potentially reduced time spent in dark chamber, increased rearing activity, reduced immobility time, increased sociability and reduced circling behaviour in D1 antagonist + D1 agonist treated 6-OHDA lesioned rats, suggesting that Wnt signaling activation attenuates the effect of D1 antagonism in 6-OHDA lesioned rats. Interestingly, GSK-3β inhibitor has been shown to exert rapid anti-depressant like activity in FST by enhancing β-catenin levels in mice hippocampus (Kaidanovich-Beilin et al., 2004). Dishevelled-2 (Dvl-2), a downstream positive regulator of Wnt signaling is down-regulated in nucleus accumbens (NAc) of mice susceptible to social defeat stress. Overexpression of dominant negative mutant of DVL-2 in nucleus accumbens (NAc) or pharmacological inhibition of DVL-2 or overexpression of GSK-3β renders mice more susceptible to social defeat stress and promotes depression-like phenotypes. In contrast, overexpression of GSK-3β dominant negative mutant resulted in resilience to social defeat stress, indicating a novel role of Wnt signaling in the regulation of stress and depression (Wilkinson et al., 2011). Therefore, we conclude that Wnt/β-catenin signaling activation improves anxiety and depression-like phenotypes and attenuate the effect of D1 antagonist.

Formation of newborn neurons from NSCs is referred as neurogenesis and several lines of evidence suggest that Wnt/β-catenin signaling and its components are involved in NSC proliferation, self-renewal, differentiation, migration and maturation of newborn neurons (Qu et al., 2010). Newborn neurons in the dentate gyrus (DG) migrate and functionally integrate into the existing hippocampal neuronal circuitry and play an essential role in pattern separation, spatial memory and anxiety and depression (David et al., 2010; Kheirbek et al., 2012). Wnt/β-catenin signaling remains active in the hippocampus where Wnt-3 is necessary and sufficient to induce hippocampal neurogenesis and for contextual fear memory acquisition and consolidation in mice (Lie et al., 2005; Xu et al., 2015), indicating a link among hippocampal neurogenesis, Wnt signaling and hippocampal dependent behavioural functions as observed in our study. Similarly, loss of DKK-1, a negative regulator of Wnt signaling increases NSC proliferation, self-renewal capacity and formation of newborn neurons in the hippocampus and counteracts cognitive decline in adult mice (Seib et al., 2013). Consistent with these findings, we showed that Axin-2 knockdown mediated activation of Wnt/β-catenin signaling attenuated D1 antagonist induced impairment in NSC proliferation and neuronal differentiation in parkinsonian rats. Therefore, Wnt/β-catenin activation induced anxiolytic and anti-depressant effect could be associated with improved

adult hippocampal neurogenesis in D1 antagonist treated parkinsonian rats. Our results are further supported by an earlier report that Wnt/β-catenin effector Lef-1 is required for hypothalamic neuronal differentiation and anxiolytic phenotypes in zebrafish and mice (Xie et al., 2017).

Conflicts of interest

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuint.2018.11.020.

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