

Platelet-derived growth factor-BB has neurorestorative effects and modulates the pericyte response in a partial 6-hydroxydopamine lesion mouse model of Parkinson's disease

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ARTICLE INFO

Article history:

Received 21 April 2016

Revised 24 May 2016

Accepted 5 June 2016

Available online 7 June 2016

Keywords:

Parkinson's disease

6-Hydroxydopamine

Platelet-derived growth factor-BB

Pericyte

Regulator of G protein signalling 5

Neurorestoration

ABSTRACT

Parkinson's disease (PD) is a neurodegenerative disease where the degeneration of the nigrostriatal pathway leads to specific motor deficits. There is an unmet medical need for regenerative treatments that stop or reverse disease progression. Several growth factors have been investigated in clinical trials to restore the dopaminergic nigrostriatal pathway damaged in PD. Platelet-derived growth factor-BB (PDGF-BB), a molecule that recruits pericytes to stabilize microvessels, was recently investigated in a phase-1 clinical trial, showing a dose-dependent increase in dopamine transporter binding in the putamen of PD patients. Interestingly, evidence is accumulating that PD is paralleled by microvascular changes, however, whether PDGF-BB modifies pericytes in PD is not known.

Using a pericyte reporter mouse strain, we investigate the functional and restorative effect of PDGF-BB in a partial 6-hydroxydopamine medial forebrain bundle lesion mouse model of PD, and whether this restorative effect is accompanied by changes in pericyte features.

We demonstrate that a 2-week treatment with PDGF-BB leads to behavioural recovery using several behavioural tests, and partially restores the nigrostriatal pathway. Interestingly, we find that pericytes are activated in the striatum of PD lesioned mice and that these changes are reversed by PDGF-BB treatment.

The modulation of brain pericytes may contribute to the PDGF-BB-induced neurorestorative effects, PDGF-BB allowing for vascular stabilization in PD. Pericytes might be a new cell target of interest for future regenerative therapies.

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1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder, with a progressive loss of the dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) and an associated loss of DA striatal fibres (de Rijk et al., 1997). The lack of dopamine in the striatum is associated with typical motor symptoms such as bradykinesia, rigidity, tremor and balance problems (Fearnley and Lees, 1991).

There is an unmet clinical need for effective and long-lasting neurorestorative strategies to prevent further degeneration of nigrostriatal neurons and axons, and to slow disease progression.

Evidence is accumulating that vascular alterations, in particular angiogenesis, may be a possible mechanism involved in the pathogenesis of PD (Faucheux et al., 1999; Janelidze et al., 2015; Wada et al., 2006; Yasuda et al., 2007). Pericytes are one of the key players in angiogenesis and blood-brain-barrier integrity (Armulik et al., 2010). They also contribute to local homeostasis, secrete growth factors (Shimizu et al., 2012) and inflammatory molecules (Kovac et al., 2011) and are multipotent cells (Dore-Duffy, 2008; Ozen et al., 2012; Ozen et al., 2014; Paul et al., 2012). Importantly, cerebral pericytes express platelet-derived growth factor (PDGF) receptor β (PDGFR β) (Winkler et al., 2010), and are recruited by PDGF-BB-secreting endothelial cells to stabilize newly formed blood vessels (Armulik et al., 2005). PDGF-BB is an endogenous growth factor that not only plays a role in angiogenesis, but also has neuroprotective effects on DA neurons in vitro (Nikkhah et al., 1993; Pietz et al., 1996), and has been shown to restore the nigrostriatal pathway in different partial lesion PD models in vivo (Zachrisson et al., 2011). Based on these findings, PDGF-BB has entered

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clinical trials and safety and tolerability of PDGF-BB have recently been evaluated in PD patients (Paul et al., 2015). However, it is currently unclear whether PDGF-BB can induce long-lasting improvement of symptoms and whether it alters features of pericytes.

Neuroprotective and neurorestorative interventions in PD require models that resemble earlier stages of PD, where some DA cells and fibres still remain. We therefore use a partial lesion PD model in pericyte-reporter mice (Boix et al., 2015). This model allows for a quantifiable behavioural and histological readout in order to study the effect of intracerebroventricular (i.c.v.) administration of PDGF-BB on motor symptoms. 6-hydroxydopamine (6-OHDA) was injected into the medial forebrain bundle (MFB) to avoid injection of the toxin in target regions for neuroprotection and vascular changes, such as the striatum.

We examine if behavioural recovery is associated with nigrostriatal DA pathway restoration in this model immediately and 2 months after treatment. Further, we specifically examine whether a partial 6-OHDA lesion is accompanied by pericyte alterations and whether these changes are affected by PDGF-BB.

2. Material and methods

2.1. Animals

We used 45 male *rgs5^{GFP/+}* knock-out/knock-in mice (C57Bl/6 background) (Nisancioglu et al., 2008). In this reporter mouse, the *Green fluorescent protein (GFP)* gene is expressed in the cytoplasm under the pericyte-specific *Regulator of G protein signalling 5 (RGS5)* promoter, making it possible to track pericytes. Mice have a normal phenotype. Animals were housed in a 12 h/12 h light/dark cycle with access to food and water ad libitum. All experiments were carried out in accordance with the European Directive 2010/63/EU guidelines, and approved by the Ethical Committee at Lund University.

2.2. Partial 6-hydroxydopamine lesion model

A partial MFB 6-OHDA lesion was performed as described previously (Boix et al., 2015). At the day of surgery, animals weighed 25–30 g. Animals were anesthetized with 5% isoflurane (IsoFlo vet, Apoteksbolaget, Sweden), diluted in a 2:1 oxygen:nitrous oxide mixture for 2 min and placed on the stereotaxic frame. Mice received 2% isoflurane throughout the entire surgery (ca. 30 min). 100 μ L Marcain (0.25 mg/mL, Apoteksbolaget, Sweden) was injected subcutaneously (s.c.) on top of the skull as a local analgesia, and a skin cut <1 cm was performed to expose the skull. 6-OHDA (Sigma) was dissolved in 0.9% NaCl and 0.02% ascorbic acid to reach a final concentration of 1 μ g/ μ L, and the solution was used within the next 3 h. 6-OHDA was injected into the right MFB at the following coordinates from the bregma: antero-posterior (A/P) = −1.2; medio-lateral (M/L) = −1.3; and dorso-ventral (D/V) = −4.75 (according to the dura mater), using a 10 μ L-Hamilton syringe connected to a glass capillary (tip diameter ca. 50 μ m), with a flow rate of 0.5 μ L/min (final volume = 1 μ L). Sham-lesioned animals received only 1 μ L of 0.9% NaCl with 0.02% ascorbic acid. Sham-lesioned animals are referred to as controls hereafter. The glass capillary was left in place for the following 5 min to avoid back-flow, before being slowly retracted. The skin was sutured, animals received 0.9% NaCl s.c. (1 mL) and daily care the first week with food soaked in 10% sucrose and high calorie jelly food (DietGel Boost, Clear H2O Co.). Animal weight loss was monitored not to be > 15% of the initial weight.

2.3. Behavioural tests

Three weeks after the 6-OHDA lesion, animals underwent a battery of behavioural tests to assess the degree of the unilateral nigrostriatal lesion. Mice with a partial lesion ($n = 38$ out of $n = 45$) were selected as previously described (Boix et al., 2015). Briefly, we have previously

shown that certain behavioural tests can reliably predict the degree of cell loss in MFB lesioned mice, whereby the stepping test and the amphetamine-induced rotation test are the best predictors (for details see Boix et al., 2015). Four animals had a total lesion, one only a mild lesion and two animals died before the end of the experiment.

For all tests, animals were habituated to the room at least 2 h before the test. The same behavioural tests were then performed 13–15 weeks after the 6-OHDA lesion (Fig. 1).

2.3.1. Corridor test

For body lateralization assessment, animals performed the corridor test, adapted for mice (Grealish et al., 2010). Briefly, animals were food-restricted and kept at 85% of their initial body weight for the entire test time (7 days). They were first habituated to the corridor (60 cm-long, 4 cm-wide and 15 cm-high) with sugar pellets randomly scattered on the floor, 10 min per day, for the first 2 days. From day 3 to day 7, animals were habituated to an empty corridor for 5 min, and then placed in a corridor presenting 10 pots on each side, spaced by 5 cm and containing 2 to 3 sugar pellets in each. Contralateral and ipsilateral retrievals were scored until either 20 retrievals were counted or 5 min elapsed.

2.3.2. Stepping test

To assess forelimb akinesia, the stepping test was used as previously described for mice (Blume et al., 2009). Mice were lifted gently from their tail and pulled backward in a 50 cm-long, 10 cm-wide corridor, within 10 s. The test was repeated 3 to 5 times per animal, and the whole experiment was videotaped and analysed using a slow motion video player (VLC software). Contralateral and ipsilateral forelimb adjusting steps were quantified.

2.3.3. Cylinder test

Mice performed the cylinder test to evaluate spontaneous forelimb lateralization (Schallert et al., 2000). Animals were placed in a glass cylinder (19 cm-diameter, 20 cm-high) with mirrors behind to allow a 360° vision. Mice were left to explore for 3 min, and the whole experiment was videotaped and analysed using a slow motion video player (VLC software). Contralateral and ipsilateral wall touches (contact with full digits) were quantified.

2.3.4. Drug-induced rotation tests

To assess rotational asymmetry, animals were tested with apomorphine and amphetamine (Ungerstedt and Arbuthnott, 1970). Mice were placed in an automated rotometer apparatus (Omnitech electronics) consisting of a glass bowl (50 cm-diameter) covered with bedding, where mice were harnessed and left to habituate for 15 min. For apomorphine-induced rotation test, animals were primed 4 and 2 days before the test with a s.c. injection of apomorphine (Sigma) at a concentration of 0.1 mg/kg, dissolved in 0.9% NaCl and 0.02% ascorbic acid. They received the same dose on the test day. Contralateral and ipsilateral turns were recorded for 40 min, and the net contralateral turns were calculated. At least 3 days after, animals were placed in the

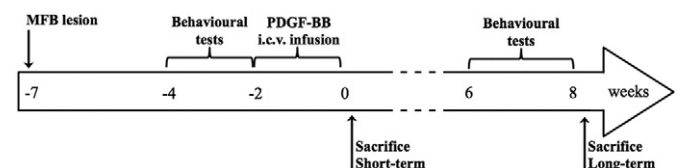


Fig. 1. Design of the experiment. Animals were lesioned in the MFB with 6-OHDA or saline (controls) and allowed to recover for the next 3 weeks. Mice then underwent a battery of behavioural tests to select partially lesioned mice, before receiving either PDGF-BB or vehicle treatment into the lateral ventricle for 2 weeks. Once the mini-pump was removed, animals were either sacrificed immediately after treatment, or kept for 6 more weeks, where behavioural tests were performed 6 to 8 weeks after the treatment, before mice were sacrificed. MFB: medial forebrain bundle; i.c.v.: intracerebroventricular.

apparatus as above and were injected intraperitoneally (i.p.) with 5 mg/kg *D*-amphetamine (Sigma) dissolved in 0.9% NaCl and 0.02% ascorbic acid (injected volume = 200 μ L). The contralateral and ipsilateral turns were recorded for 40 min, and the net ipsilateral turns were evaluated.

2.4. PDGF-BB mini-osmotic pump implantation

5 weeks post lesion (Fig. 1), a mini-osmotic pump (Alzet, model 1004) was implanted. For this, animal anaesthesia and local analgesia, placement on the stereotaxic frame, and skin cut were performed similarly as described above (see *Partial 6-hydroxydopamine lesion model*). The catheter was implanted into the right lateral ventricle ($A/P = -0.5$; $M/L = -1$ and $D/V = -2$ according to the dura mater), attached with glue (loctite 454 glue; Alzet) and linked to the mini-osmotic pump by a ca. 3 cm-long plastic tube (brain infusion kit; Alzet). The mini-osmotic pump was inserted s.c. in the back of the animal (pump flow rate: 0.11 μ L/h) and contained human recombinant PDGF-BB (R&D System) diluted in artificial cerebrospinal fluid (aCSF; 148 mM NaCl, 3 mM KCl, 1.4 mM anhydrous CaCl_2 , 0.8 mM MgCl_2 , 1.5 mM Na_2HPO_4 , 0.2 mM NaH_2PO_4) and supplemented with mouse serum albumin (100 μ g/mL; Sigma) and gentamicin (50 μ g/mL; Life Technologies) in order to reach a delivery rate of 36 ng of PDGF-BB per day. Animals were randomly assigned to either vehicle-infused sham-lesioned group (controls, $n = 14$), vehicle-infused 6-OHDA-lesioned group ($n = 12$), or PDGF-BB-infused 6-OHDA-lesioned group ($n = 12$). Mice were treated for 14 days with PDGF-BB or vehicle solution. The vehicle solution contained aCSF, mouse serum albumin and gentamicin at the same concentrations. The mini-osmotic pump was then removed after 14 days. A skin cut <1 cm was done on the back of the animal for the mini-osmotic pump to be gently pulled out, and the tube was cut and clogged with glue, before suturing the animal. Animals were randomly selected for short term or long-term survival and brains processed for histology ($n = 26$) or protein analysis ($n = 12$).

2.5. Tissue processing

Animals were sacrificed either within 12 h after (referred later as “shortly after”) or 8 weeks after PDGF-BB or vehicle treatment (Fig. 1).

For immunohistochemistry, animals (vehicle-treated sham group: $n = 10$; vehicle-treated lesioned group: $n = 8$; PDGF-BB-treated lesioned group: $n = 8$) were deeply anesthetized with an i.p. injection of a lethal dose of sodium pentobarbital (Apoteksbolaget, Sweden), and were transcardially perfused with first 0.9% NaCl for 5 min, and then by cold 4% paraformaldehyde (PFA) for 5 min. Brains were removed and PFA post-fixed for 24 h, before incubating them in 30% sucrose for several days. They were placed on a frozen microtome and coronal sections were cut at 30 μ m-thick, in a 1:10 series. Sections were kept in an anti-freeze solution and stored at 4 °C until further analysis.

For western blot analysis, mice (all different groups; $n = 4$) were sacrificed by cervical dislocation, brains were removed and cut on ice in a 1 mm-thick coronal sections. The striatum was carefully delineated using the corpus callosum and the lateral ventricles as reference points, taken out and frozen in liquid nitrogen. The tissue was kept in -80 °C freezer.

2.6. Immunohistochemistry

Sections were washed 3 times in phosphate buffer saline (PBS) and, for brightfield staining, endogenous peroxidase was quenched with 3% H_2O_2 and 10% methanol diluted in PBS. For PDGFR β , a heat-induced epitope retrieval was performed by incubating the sections in citrate buffer at 80 °C for 30 min, before letting them cool down at room temperature (RT) for 15 min. Sections were then incubated first with a blocking solution containing 5% normal goat serum (NGS) diluted in 1% TritonX-100/

PBS (PBSTX) for 30 min at RT, and then with primary antibodies diluted with 3% NGS in PBSTX, overnight at RT: rabbit anti-Tyrosine hydroxylase (TH; 1:1000; Millipore), chicken anti-GFP (1:5000; Abcam), rabbit anti-human PDGFR β (1:200; Cell Signalling), rat anti-mouse CD13 (1:100; AbDSerotec), rabbit anti-Chondroitin sulfate proteoglycan NG2 (NG2; 1:200; Millipore) and rabbit anti-Glucose transporter 1 (GLUT1; 1:400; Abcam).

For brightfield staining, sections were washed 3 times 10 min and incubated with the biotinylated goat anti-rabbit secondary antibody (1:200; Vector Laboratories), in 3% NGS diluted in PBSTX, for 2 h at RT. They were rinsed 3 times 10 min and secondary antibody signal was enhanced with an avidin-biotin solution (Vectastain Elite ABC kit; Vector Laboratories), 1 h at RT. Sections were washed as above and the antigen was revealed using the chromogen 3,3'-diaminobenzidine (DAB Peroxidase Substrate Kit; Vector Laboratories). Sections were washed, mounted and left to dry overnight at RT. They were dehydrated in gradual ethanol concentrations, incubated 2×5 min in Xylene and coverslipped using DPX mounting medium.

For immunofluorescence, sections were washed 3 times 10 min after the primary antibodies and incubated with the corresponding secondary antibodies diluted in 3% normal donkey serum in PBSTX, for 1 h at RT: CY3-conjugated donkey anti-rat IgG, CY3-conjugated donkey anti-rabbit IgG, AlexaFluor 647-conjugated donkey anti-rabbit IgG and CY5-conjugated donkey anti-rat IgG (1:500; Jackson ImmunoResearch). For GFP staining, sections were incubated with a biotinylated goat anti-chicken secondary antibody (1:200; Vector Laboratories) for 2 h at RT, washed 3 times 10 min and then incubated with AlexaFluor 488-conjugated Streptavidin (1:2000; Life Technologies) for 2 h at 4 °C. Sections were washed as above and incubated with DAPI (1:1000 diluted in PBS) for 10 min at RT, rinsed 3 times 10 min, mounted and coverslipped.

2.7. TH⁺ nigral cell quantification

To estimate the TH⁺ cell number in the SNpc, unbiased stereology counting was performed using the optical fractionator probe (Stereo Investigator software). Sections were placed on a Nikon 80i microscope presenting X-Y motorized stage and a Z-axis motor (Leica). SNpc area was delineated at magnification $5\times$ and TH⁺ cells were counted at 100 X. TH⁺ cells were counted only when the cell soma was inside the stereology box or touching the including line. 4 to 6 SNpc sections were used per brain, on average, in a 1:5 series, and the sampling interval was adjusted to have a Gundersen coefficient of error < 10% (average = 7%). TH⁺ cell estimation was calculated according to the following formula:

$$N = \sum Q \times \frac{1}{asf} \times \frac{1}{hsf} \times \frac{1}{ssf}$$

where $\sum Q$ is the total cell count, *asf* is the area sampling fraction *hsf* is the height sampling fraction and *ssf* is the section sampling fraction (West, 1999).

2.8. TH⁺ striatal fibre quantification

TH⁺ striatal fibre density was estimated with densitometry. For this, 4 high-resolution pictures of the striatum were taken per brain, at magnification $10\times$, using a brightfield Nikon microscope, spanning from +1.18 to -0.1 according to bregma. The striatum was divided into dorsal and ventral equal-halves according to the dorso-ventral axis (including the nucleus accumbens), delineated and the optical density was calculated using the ImageJ software (National Health Institute, USA). The measured values were then corrected for the background staining by subtracting optical density measure of the corpus callosum, for each picture.

2.9. Pericyte quantification

For pericyte counts, sections were analysed with a brightfield microscope for PDGFR β ⁺ cell counts, and a Zeiss LMS510 confocal microscope (Carl Zeiss), using 488, 594 and 647 nm excitation wavelengths, for GFP⁺, CD13⁺ and/or NG2⁺ cells. Two to 3 sections for the striatum (from +0.98 to −0.02 according to bregma) and 1 to 2 sections for the SN (from −3.08 to −3.52 according to bregma) were analysed per animal. For the dorso-lateral and the ventro-medial striatum, 4 pictures of the ipsilateral side were taken at 40 $\times$ and CD13⁺, NG2⁺ and/or GFP⁺ pericyte cell somas were counted. For PDGFR β ⁺ cells, a 20 $\times$ magnification picture was taken for both ipsilateral striatum regions and the SN. All cell counts are expressed as percentage of the control. The area covered by GLUT1⁺ or CD13⁺ cells was estimated with the ImageJ software (National Health Institute, USA), where 20 $\times$ magnification pictures were converted into grayscale pictures, and the threshold tool

was used to produce a binary image. The total vascular area (GLUT1 staining) was expressed as percentage of the total area. The coverage of CD13⁺ pericytes was expressed as percentage of the total GLUT1⁺ vascular area.

2.10. Protein extraction and western blot

Proteins were precipitated incubating the first flowthrough of the RNeasy mini RNA extraction kit (Quiagen) accordingly to the manufacturer instructions with acetone 3:1 v/v for 4 h at −20 °C, before centrifuging at 15,000 rcf (15 min at 4 °C), prior to collect the protein pellet.

Protein pellets were resuspended in RIPA buffer (Thermo scientific) supplemented with protease inhibitor (Thermo scientific) and sonicated 5 $\times$ 10 s at 4 °C. Protein concentrations were evaluated with BCA kit (Pierce), and 5 μ g of each sample were incubated in Laemmli buffer

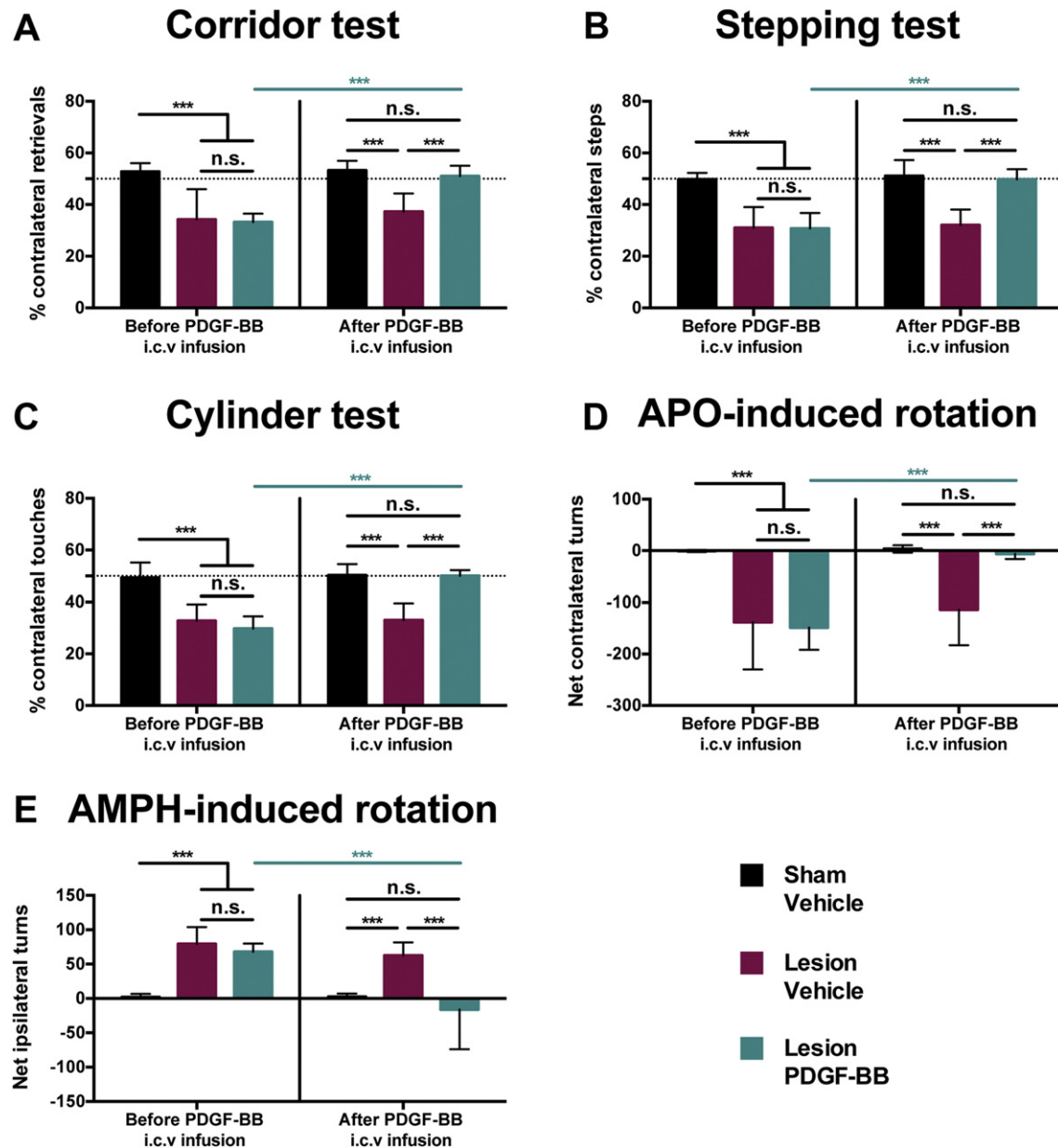


Fig. 2. PDGF-BB induces behavioural recovery. Lesioned animals treated with PDGF-BB showed behavioural recovery in the corridor (A), stepping (B), cylinder (C), apomorphine-induced rotation (D) and amphetamine-induced rotation (E) test compared with 6-OHDA-lesioned mice treated with vehicle. All 6-OHDA-lesioned animals significantly improved their behaviour compared to baseline after PDGF-BB treatment. One-way ANOVA with Tukey post-hoc (black asterisks) or paired *t*-test (blue asterisks), ****p* < 0.001, n.s.: non-significant. Control group: *n* = 10; vehicle-treated lesioned group: *n* = 9; PDGF-BB-treated lesioned group: *n* = 8. MFB: medial forebrain bundle; i.c.v.: intra-cerebroventricular; APO: apomorphine; AMPH: amphetamine.

(5 min at 100 °C). For Western blot analysis, samples were resolved by SDS-PAGE and analysed using rabbit anti-NG2 antibody (1:1000, Millipore), chicken anti-GFP (1:5000, Abcam), rabbit anti-TH (1:1000, Millipore), and anti- β -actin-HRP antibody (1:10,000, Sigma). Images were acquired using ChemiDoc MP system (Biorad).

2.11. Statistics

Data were analysed using Graph Pad Prism Software, and are expressed as mean $\pm$ standard deviation. Multiple group comparison was performed using a one-way ANOVA followed by a Tukey post hoc test. For comparison of behavioural scores before and after PDGF-BB treatment, a paired *t*-test was used. Comparisons were considered significant with a *p*-value <0.05.

3. Results

3.1. PDGF-BB restores behavioural deficits

We first investigated whether PDGF-BB restores the behavioural impairment observed in a unilateral partial 6-OHDA lesion. For this, behaviour was tested before and 6–8 weeks after the treatment (Fig. 2).

Before treatment, all selected 6-OHDA-lesioned animals had a significant behavioural impairment within the cut-off values that were

previously described for predicting a partial MFB lesion (Boix et al., 2015) (Fig. 2A–E). Partially lesioned animals were then randomly divided to receive either vehicle or PDGF-BB treatment.

Interestingly, PDGF-BB-treated animals displayed behavioural recovery and presented behavioural scores similar to controls, but significantly different from the vehicle-treated 6-OHDA-lesioned group in both non-drug-induced and drug-induced behavioural tests. In contrast, vehicle-treated 6-OHDA-lesioned animals did not show any significant behavioural recovery compared to baseline. These results suggest that a 2 week i.c.v. administration of PDGF-BB restored the behavioural impairment observed in a unilateral 6-OHDA partial lesion model of PD, including both non-pharmacological and pharmacological behavioural tests.

3.2. PDGF-BB partially restores the nigrostriatal DA system

Next we examined whether the observed behavioural recovery was associated with a restoration of the nigrostriatal DA system (Fig. 3).

Shortly after the end of treatment, the striatal TH⁺ fibre density was significantly lower in vehicle-treated 6-OHDA-lesioned animals in both the dorsal and ventral striatum, compared to controls (Fig. 3A and B, respectively). Similarly, the TH⁺ fibre density was lower in PDGF-BB-treated 6-OHDA-lesioned animals, both in the dorsal and ventral striatum, but this was not significantly different from controls or vehicle-treated 6-OHDA-lesioned mice. The loss of TH⁺ fibres was associated

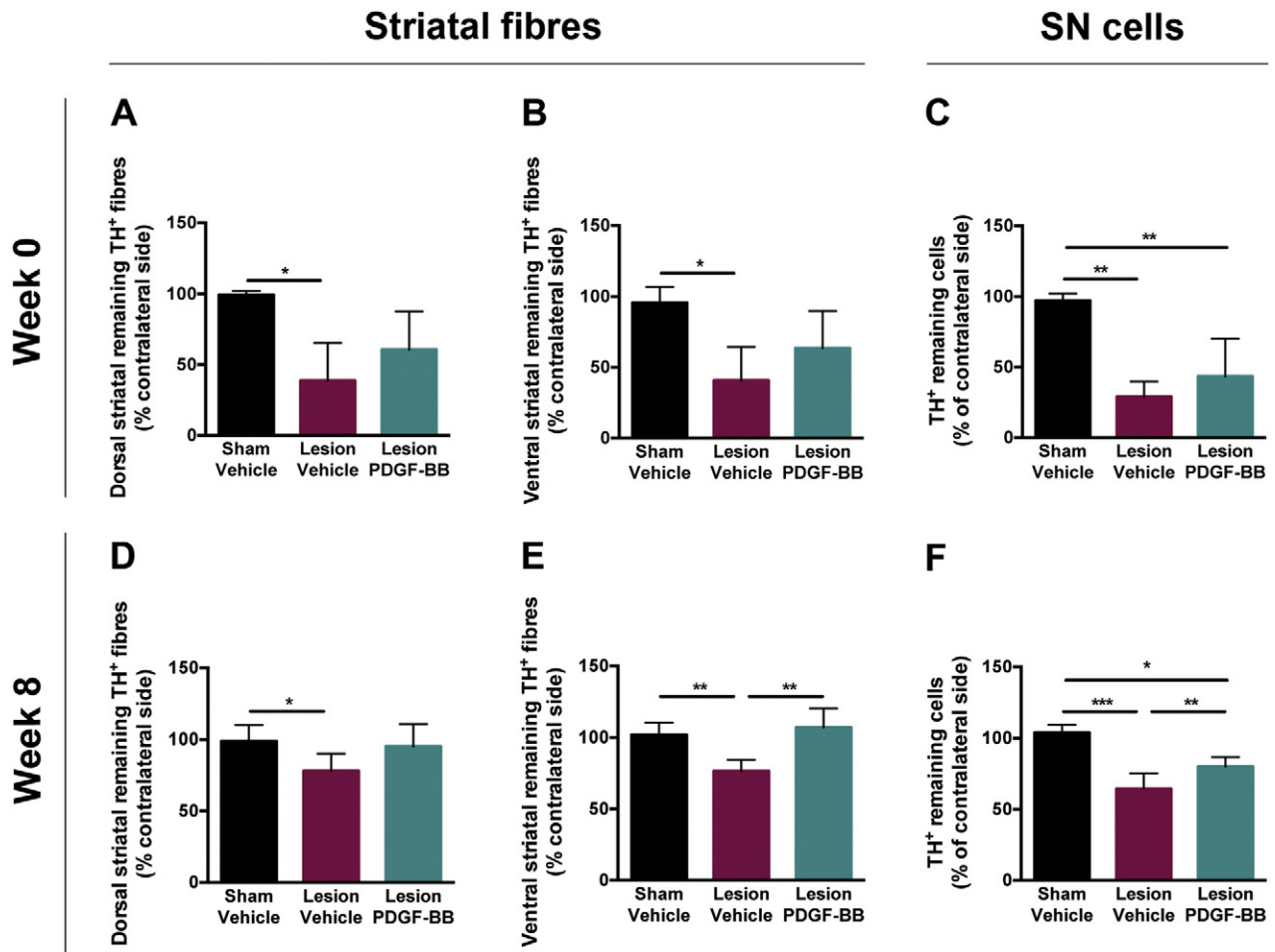


Fig. 3. PDGF-BB partially restores the nigrostriatal DA pathway long-term after treatment. Quantification of TH⁺ fibre density in the dorsal (A) and ventral striatum (B) and remaining TH⁺ cells in the SN (C) shortly after treatment with vehicle or PDGF-BB. Quantification of TH⁺ fibre density in the dorsal (D) and ventral striatum (E) and remaining TH⁺ cells in the SN (F) 8 weeks after treatment with vehicle or PDGF-BB. One-way ANOVA with Tukey post-hoc, **p* < 0.05, ***p* < 0.01, ****p* < 0.001. A–C: Control group: *n* = 4; Vehicle-treated 6-OHDA-lesioned group: *n* = 3; PDGF-BB-treated 6-OHDA-lesioned group: *n* = 4. D–F: Control group: *n* = 6; Vehicle-treated 6-OHDA-lesioned group: *n* = 5; PDGF-BB-treated 6-OHDA-lesioned group: *n* = 4. SN: substantia nigra.

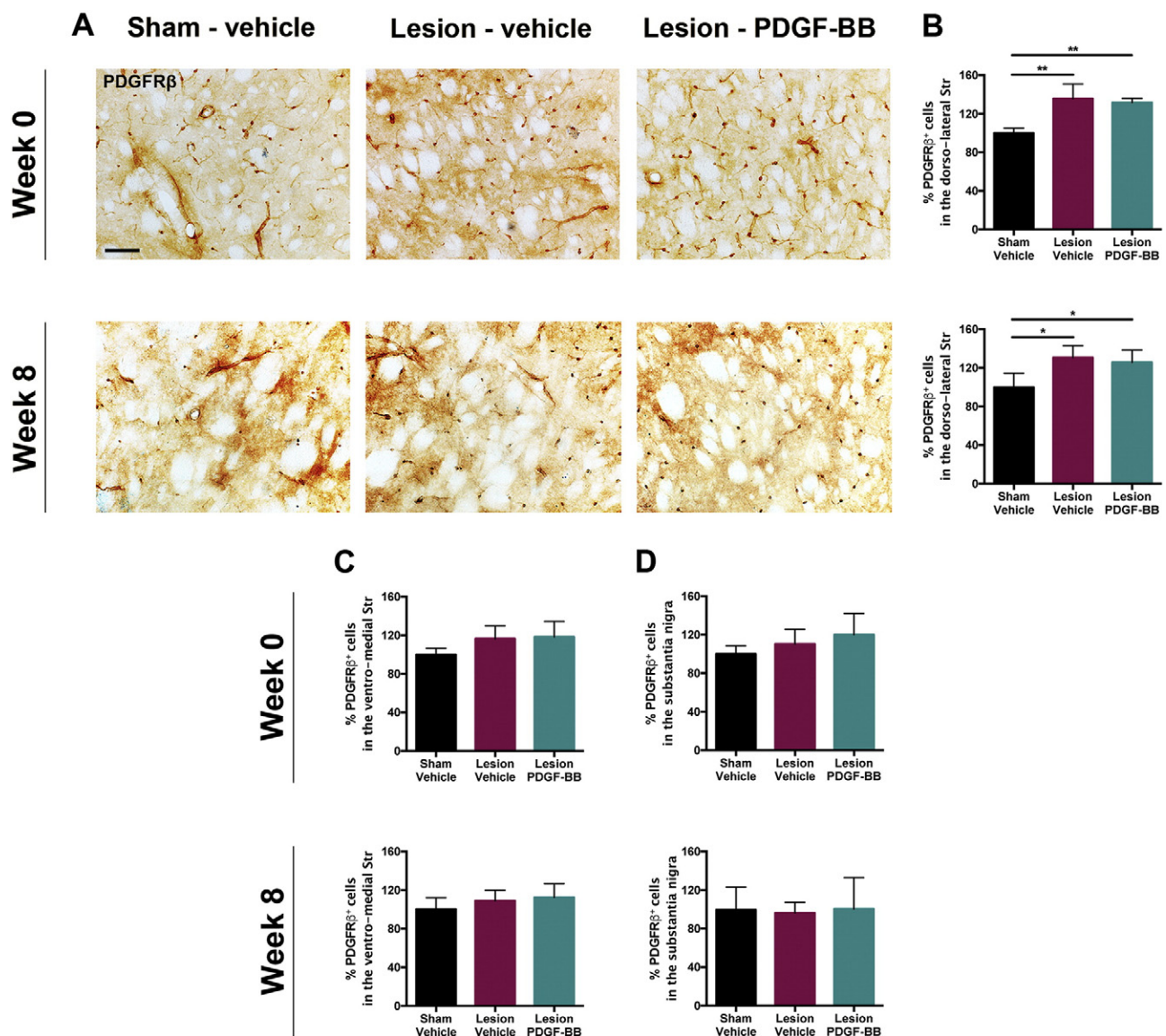


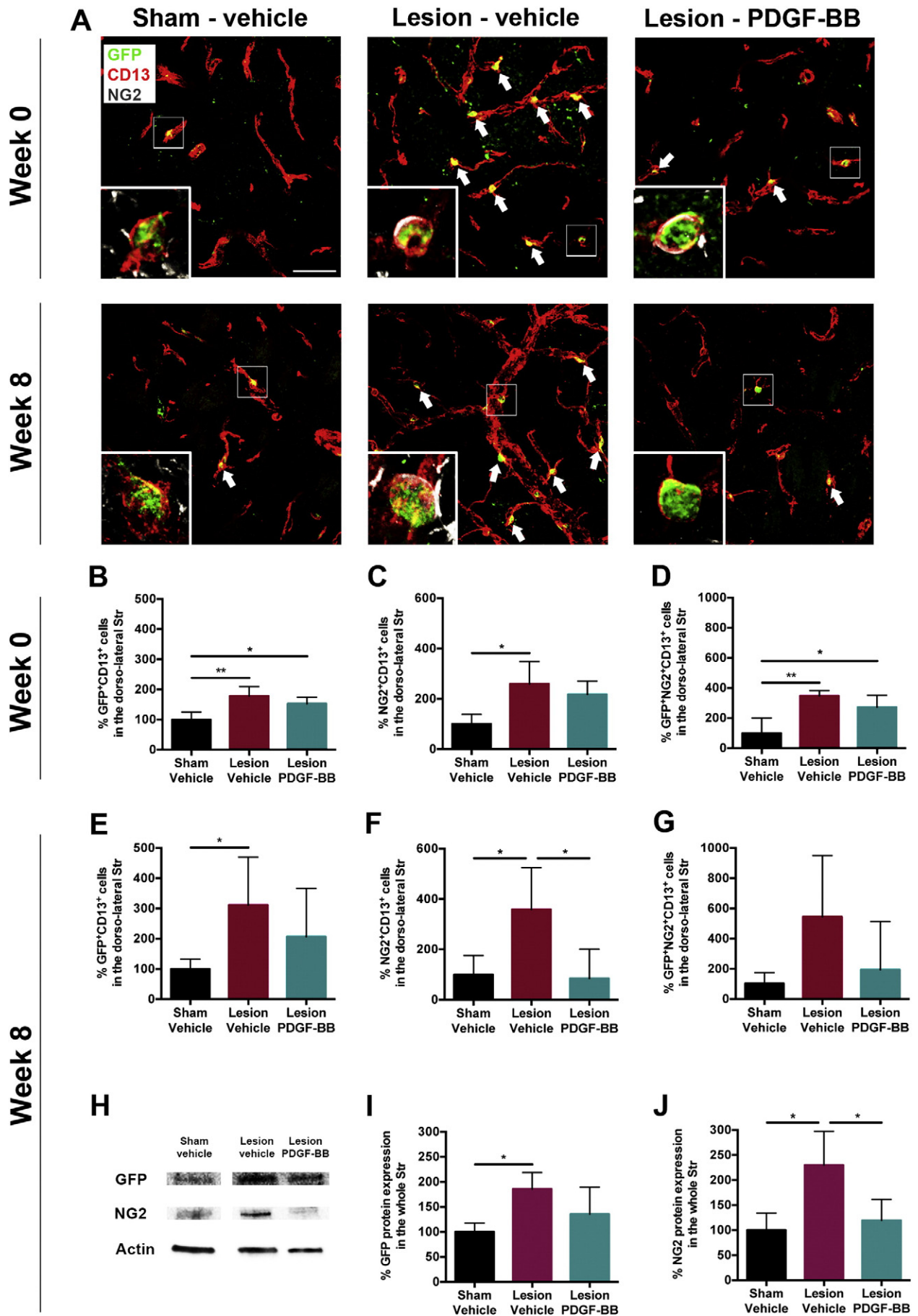
Fig. 4. PDGFR β^+ pericytes are increased in the dorso-lateral striatum in 6-OHDA lesioned mice. Representative pictures of PDGFR β^+ cells in the dorso-lateral striatum (A) immediately (upper panel) or 8 weeks (lower panel) after the end of the treatment. Numbers of PDGFR β^+ pericytes are increased in both vehicle- and PDGF-BB-treated 6-OHDA-lesioned animals (B), immediately (upper panel) or 8 weeks (lower panel) after treatment. No change in PDGFR β^+ pericyte numbers was observed in the ventro-medial striatum (C) or the SN (D), immediately (upper panel) or 8 weeks (lower panel) after treatment. One-way ANOVA with Tukey post-hoc, * $p < 0.05$, ** $p < 0.01$. A-D (upper panel): Control group: $n = 4$; Vehicle-treated 6-OHDA-lesioned group: $n = 3$; PDGF-BB-treated 6-OHDA-lesioned group: $n = 4$. A-D (lower panel): Control group: $n = 6$; Vehicle-treated 6-OHDA-lesioned group: $n = 5$; PDGF-BB-treated 6-OHDA-lesioned group: $n = 4$. Scale bar = 50 μ m.

with a significant loss of SN TH $^+$ cells in the 6-OHDA-lesioned groups compared to controls (Fig. 3C).

Eight weeks after the treatment, vehicle-treated 6-OHDA-lesioned animals had a striatal TH $^+$ fibre density significantly lower than controls both in the dorsal and ventral striatum (Fig. 3D and E, respectively). 6-OHDA-lesioned animals treated with PDGF-BB, in contrast, had a TH $^+$ fibre density in the ventral striatum that was comparable to controls, and significantly higher than in the vehicle-treated 6-OHDA-lesioned group (Fig. 3E). In the vehicle-treated lesioned animals, the number of remaining TH $^+$ cells

in the SN was reduced to $64 \pm 11\%$ compared to controls (Fig. 3F). This is associated with a moderate behavioural impairment, whereby the behavioural scores for these partially lesioned animals were within the cut-off values set for the stepping and amphetamine-induced rotation tests (data not shown), which confirms our initial selection of partial lesion animals based on behavioural cut-off values (Boix et al., 2015). PDGF-BB treatment resulted in a significant increase in TH $^+$ cells in the SNpc compared to vehicle-treated 6-OHDA-lesioned mice and a partial restoration of TH $^+$ cells, when compared to controls at 8 weeks after treatment (Fig. 3F).

Fig. 5. PDGF-BB normalizes pericyte activation in the dorso-lateral striatum long-term after treatment. Representative pictures of GFP $^+$ NG2 $^+$ CD13 $^+$ pericytes in the ipsilateral dorso-lateral striatum (A), immediately (upper panel) or 8 weeks (lower panel) after the end of treatment. Immediately after treatment, GFP $^+$ CD13 $^+$ (B), NG2 $^+$ CD13 $^+$ (C) or GFP $^+$ NG2 $^+$ CD13 $^+$ (D) cells increased in both vehicle- and PDGF-BB-treated 6-OHDA-lesioned groups. Eight weeks after end of treatment, activated pericytes were still increased in vehicle-treated 6-OHDA-lesioned animals when using GFP (E), NG2 (F) or both markers (G), coupled with CD13, which was normalized upon PDGF-BB treatment. Western Blot analysis of the whole ipsilateral striatum (H) showed a similar finding, with an increase in GFP (I) and NG2 (J) protein levels in vehicle-treated 6-OHDA-lesioned mice, which was reversed upon PDGF-BB treatment for NG2. One-way ANOVA with Tukey post-hoc, * $p < 0.05$, ** $p < 0.01$. White rectangles indicate where higher magnification pictures were taken. Arrows indicate GFP $^+$ CD13 $^+$ pericytes. B-D: Control group: $n = 4$; Vehicle-treated 6-OHDA-lesioned group: $n = 3$; PDGF-BB-treated 6-OHDA-lesioned group: $n = 4$. E-G: Control group: $n = 6$; Vehicle-treated 6-OHDA-lesioned group: $n = 5$; PDGF-BB-treated 6-OHDA-lesioned group: $n = 4$. H-J: Control group: $n = 4$; Vehicle-treated 6-OHDA-lesioned group: $n = 4$; PDGF-BB-treated 6-OHDA-lesioned group: $n = 4$. Scale bar = 50 μ m.



These results suggest that PDGF-BB promotes a partial recovery of the TH⁺ nigrostriatal DA pathway that is progressive over time.

3.3. PDGFR β ⁺ pericytes are increased in the dorso-lateral striatum in 6-OHDA-lesioned mice

The main PDGF-BB receptor *in vivo* is PDGFR β , which is highly expressed by pericytes in the brain (Winkler et al., 2010). Therefore, we next examined whether PDGF-BB treatment is associated with any changes in PDGFR β ⁺ pericyte numbers or density.

We found that, shortly after treatment, the number of PDGFR β ⁺ pericytes was significantly increased in the dorso-lateral striatum of 6-OHDA-lesioned animals, irrespective whether animals were vehicle- or PDGF-BB-treated (Fig. 4A and B). This increase in PDGFR β ⁺ pericytes was maintained up to 15 weeks after the 6-OHDA lesion and not affected by PDGF-BB treatment. PDGFR β ⁺ pericyte numbers in the ventro-medial striatum and the SN remained, however, unchanged between groups (Fig. 4C and D).

These findings indicate that PDGFR β ⁺ pericyte numbers increase in response to the partial 6-OHDA lesion specifically in the dorso-lateral striatum.

3.4. PDGF-BB modulates pericyte activation in the dorso-lateral striatum

To further characterize PDGF-BB-mediated changes in pericytes in the partial 6-OHDA lesion mice, we analysed markers specific for activated pericytes including RGS5 and NG2 (Berger et al., 2005; Ozerdem et al., 2001).

We first used immunofluorescence staining for GFP (RGS5) and NG2, in combination with the pericyte marker CD13 (Fig. 5A). Shortly after end of treatment, GFP⁺CD13⁺ cells were increased by 1.5-fold in all 6-OHDA-lesioned mice when compared to controls (Fig. 5B). Similarly, NG2⁺CD13⁺ pericytes were increased by 2.5-fold in both 6-OHDA-lesioned groups compared to controls, which was significant for the vehicle-treated, but not for the PDGF-BB-treated group ($p = 0.054$) (Fig. 5C). The same phenomenon was observed for GFP⁺NG2⁺CD13⁺ triple labelled cells showing a 3 and 2.5 fold increase, respectively, in the vehicle- or PDGF-BB-treated 6-OHDA-lesioned groups (Fig. 5D).

At 8 weeks after the end of treatment, the number of GFP⁺CD13⁺ pericytes was still significantly higher in vehicle-treated 6-OHDA-lesioned mice versus controls (Fig. 5E). Interestingly, PDGF-BB treatment in 6-OHDA-lesioned mice led to a decrease in the number of GFP⁺CD13⁺ pericytes, even though this did not reach statistical significance. However, PDGF-BB treatment normalized the number of NG2⁺CD13⁺ pericytes 8 weeks post-treatment to levels similar to the control group, whereas NG2-expressing CD13⁺ pericytes were still 3-fold higher in the vehicle-treated 6-OHDA-lesioned group (Fig. 5F). GFP⁺NG2⁺CD13⁺ pericytes were 5-fold increased in the vehicle-treated 6-OHDA-lesioned group, but less in the PDGF-BB-treated 6-OHDA-lesioned group, even though this difference was not significant (Fig. 5G).

Secondly, we quantified the protein expression of GFP and NG2 using western blot in the ipsilateral striatum 8 weeks after the end of the treatment (Fig. 5H), confirming an increase of 2.5 and 3.5 fold for GFP and NG2 protein expression, respectively, in the vehicle-treated 6-OHDA-lesioned group (Fig. 5I and J). Upon PDGF-BB treatment, NG2 protein was significantly decreased to levels similar to the control group, consistent with the immunohistochemical analysis.

In the ventro-medial striatum (Supplementary Fig. 1), pericyte activation was also increased in the 6-OHDA-lesioned groups, irrespective of treatment. However, 8 weeks after treatment, there were no significant differences between groups. Similarly, there were no significant differences in the numbers of activated pericytes between groups in the SN (data not shown).

Taken together, these data indicates that striatal pericytes are activated after a partial 6-OHDA lesion, which can be reversed by PDGF-BB 8 weeks after the end of the treatment.

3.5. PDGF-BB normalizes pericyte coverage, but has no effect on the vascular density in the dorso-lateral striatum

We next investigated whether the pericyte coverage on blood vessels is affected by a partial 6-OHDA lesion or PDGF-BB treatment (Fig. 6).

Shortly after the end of the treatment, CD13⁺ cell numbers, the CD13⁺ pericyte coverage of blood vessel and the total vascular density in the dorso-lateral striatum was similar in all groups (Fig. 6A–D). However, 8 weeks after treatment, the CD13⁺ pericyte coverage had increased significantly in the vehicle-treated 6-OHDA-lesioned group compared to controls, while CD13⁺ cell numbers and the vascular density were unchanged (Fig. 6E–H). Eight weeks after treatment, PDGF-BB had normalized the covered area of CD13⁺ cells to values comparable to controls.

These results indicate that CD13 expression rather than CD13⁺ cell numbers increases significantly in the dorso-lateral striatum, long-term after a partial 6-OHDA lesion, which is normalized upon PDGF-BB treatment.

4. Discussion

Here we demonstrate that a 2-week i.c.v. treatment with PDGF-BB leads to a robust behavioural recovery in a partial 6-OHDA lesion mouse model of PD two months after end of treatment by using several pharmacological and non-pharmacological tests. The behavioural changes are associated with a partial restoration of the nigrostriatal system. Furthermore, we show for the first time that a partial 6-OHDA lesion is paralleled by an increase in pericyte numbers in the striatum, and, in particular, an activation of pericytes. Interestingly, we find that this activated state of pericytes is partially reversed upon PDGF-BB treatment. Thus, PDGF-BB may exert its restorative effect via modulation of pericytes.

Here we extend previous findings (Zachrisson et al., 2011) and show behavioural recovery in response to PDGF-BB i.c.v. treatment not only in the amphetamine-induced rotation test (Zachrisson et al., 2011), but also for the apomorphine-induced rotation test, as well as for three non drug-induced behavioural tests: the cylinder, stepping and corridor test. This demonstrates a clear effect of PDGF-BB that lasts at least 2 months after treatment, which is of relevance for the design of future clinical studies, as it indicates that PDGF-BB may not have to be continuously infused, but can rather be administered at intervals.

PDGF-BB-induced behavioural recovery was associated with a significant increase in striatal TH⁺ fibre density and a partial restoration of TH⁺ cell numbers in the SN, 8 weeks after the end of treatment, confirming previous findings in other rodent PD models (Zachrisson et al., 2011) and in non-human primates (Paul et al., 2013). This partial restoration was sufficient to elicit a complete behavioural recovery after PDGF-BB treatment. The remaining TH⁺ cell loss in PDGF-BB-treated lesioned animals was so mild that the behavioural performance was comparable to controls. In addition, the increase in TH⁺ fibre density in PDGF-BB-treated lesioned animals may also have contributed to the behavioural recovery.

Recently, i.c.v. delivery of PDGF-BB has been investigated in a phase-1 clinical trial in PD patients and a dose-dependent increase in dopamine transporter binding in the putamen was seen, suggesting a possible regenerative effect on striatal DA fibres also in patients (Paul et al., 2015), consistent with our findings.

The increase in TH⁺ cell numbers in the SN is most likely due to an upregulation of TH in the remaining DA cells rather than due to proliferation of TH⁺ cells as neurogenesis in the SN has not been observed (Frielingsdorf et al., 2004). Tyrosine hydroxylase is the rate-limiting enzyme for dopamine synthesis and a marker for DA neurons. It has been

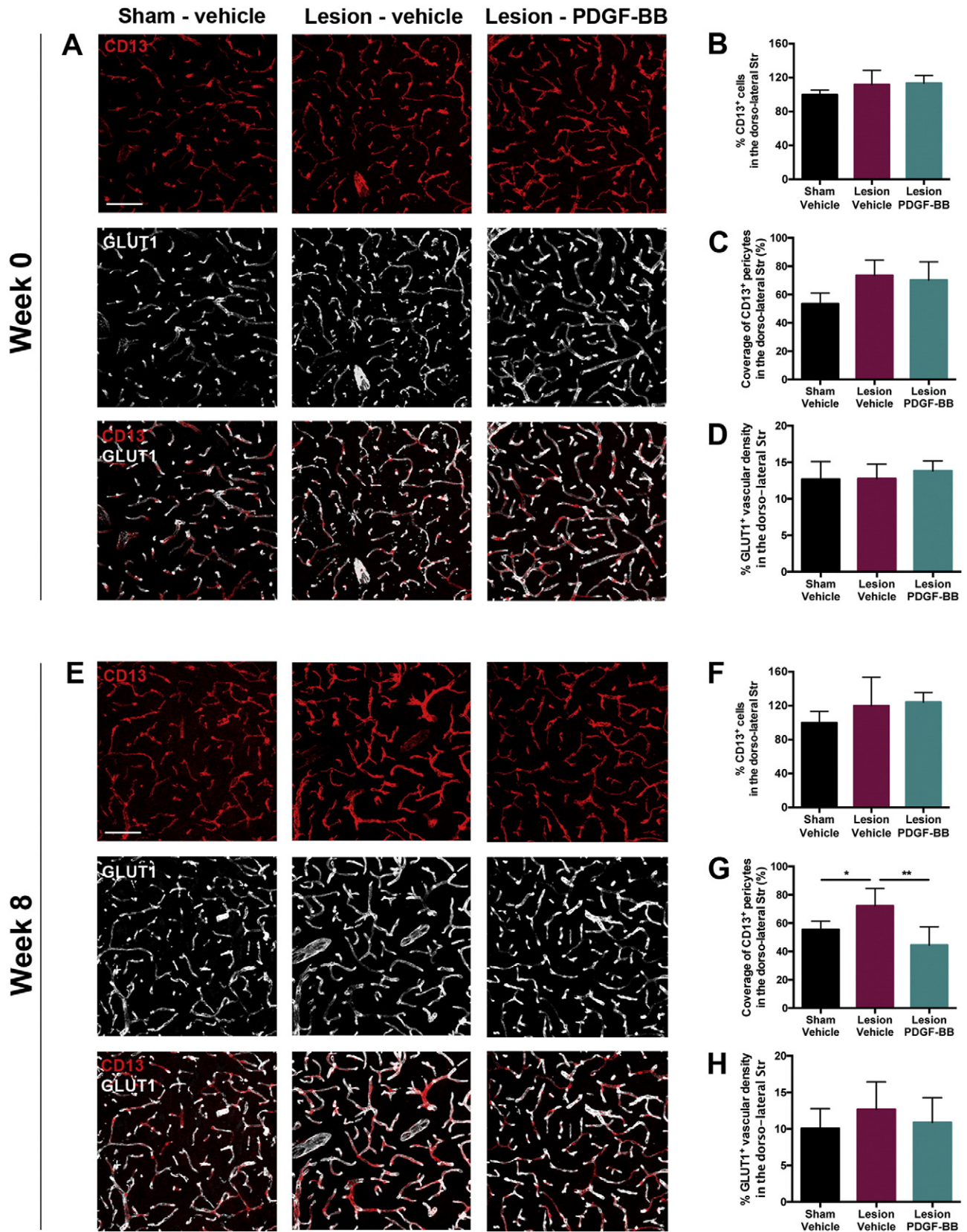


Fig. 6. PDGF-BB reduces CD13⁺ pericyte coverage but did not affect GLUT1⁺ vascular density in the dorso-lateral lesioned striatum. Representative pictures of CD13 and GLUT1 staining in the dorso-lateral striatum ipsilateral to the lesion, shortly after end of treatment (A). No difference was observed between groups for CD13⁺ cell numbers (B), the CD13⁺ pericyte coverage (C) and the vascular density (GLUT1⁺) (D). Representative pictures of CD13 and GLUT1 staining in the dorso-lateral striatum, 8 weeks after end of treatment (E). While the CD13⁺ cell number remained unchanged in all groups (F), CD13⁺ coverage was increased in the vehicle-treated 6-OHDA-lesioned group, but not with PDGF-BB treatment (G). There was no difference in vascular density between groups. One-way ANOVA with Tukey post-hoc, * $p < 0.05$, ** $p < 0.01$. B-D: Control group: $n = 4$; Vehicle-treated 6-OHDA-lesioned group: $n = 3$; PDGF-BB-treated 6-OHDA-lesioned group: $n = 4$. F-H: Control group: $n = 6$; Vehicle-treated 6-OHDA-lesioned group: $n = 5$; PDGF-BB-treated 6-OHDA-lesioned group: $n = 4$. Scale bar: 100 μm .

shown that SN TH⁺ neurons downregulate TH long before cells undergo apoptosis (Bowenkamp et al., 1996; Kordower et al., 2013), indicating that the DA phenotype can be rescued using neurorestorative agents. Thus, PDGF-BB either directly or indirectly promotes dysfunctional DA cells to regain their TH expression and DA neuronal phenotype, and their functionality. This implicates that this treatment may be most beneficial if administered during early or mid stage of the disease, before the majority of cells and fibres are already lost.

The cerebral microvasculature is widely known to undergo structural and functional alterations that may play a role in the pathogenesis and pathophysiology of neurodegenerative disorder like Alzheimer's disease and PD (Farkas et al., 2000; Sagare et al., 2013). Indeed, both in animal models of PD and in post mortem brain sections of PD patients, several vascular alterations have been described (Barcia et al., 2005; Carvey et al., 2005; Chen et al., 2008; Desai Bradaric et al., 2012; Janelidze et al., 2015; Kortekaas et al., 2005; Wada et al., 2006; Yang et al., 2015).

Pericytes are a key component of the neurovascular unit, but phenotypic and morphological changes of pericytes have not been investigated in PD. We describe for the first time an increase in PDGFR β ⁺ pericytes in the dorso-lateral lesioned striatum in a PD mouse model that persists over the time period observed. PDGFR β is the main receptor for PDGF-BB in vivo and is expressed only by pericytes in the brain (Winkler et al., 2010). This increase in PDGFR β ⁺ pericytes may indicate a response to the TH⁺ fibre loss or dopamine depletion that is most pronounced in the dorso-lateral striatum.

Activated pericytes express several typical markers such as RGS5 (Berger et al., 2005) and NG2 (Ozerdem et al., 2001), proteins related to vessel remodeling. Interestingly, we find a clear activation of pericytes in the dorso-lateral striatum after the lesion. Pericyte activation was partially reversed upon PDGF-BB treatment, but persisted in vehicle-treated 6-OHDA-lesioned mice. This effect could be due to a direct mechanism of PDGF-BB on pericytes that leads to vascular stabilization, and thus may decrease the number of activated pericytes. Indeed, PDGF-BB has been shown to negatively regulate RGS5 expression (Cho et al., 2003; Gunaje et al., 2011) and promote pericyte recruitment to stabilize the newly formed blood vessels (Clapp et al., 2009). Therefore, PDGF-BB treatment could mediate a shift between pericyte phenotypes, from an activated to a more resting state. Another more indirect mechanism is also conceivable. It has been shown that dopamine prevents vascular abnormalities in tumours (Chakroborty et al., 2011). The dopamine loss observed in the striatum of 6-OHDA-lesioned animals might therefore lead to an aberrant angiogenic vascular response and activation of pericytes. Restoration of TH⁺ fibres and cells would lead to normalisation of the striatal dopamine level, which in turn would result in normalisation of an overshooting pericyte activation. This would be in agreement with the delayed recovery of TH⁺ fibres and cells after end of treatment.

Consistently, we also observed a significant increase in CD13 expression in 6-OHDA-lesioned animals that was reversed by PDGF-BB. CD13 levels are known to increase upon angiogenesis and inflammation (Di Matteo et al., 2011; Huschak et al., 2003). PDGF-BB may reduce the inflammation in the striatum and CD13 expression. Alternatively, pericytes are known to acquire a different morphology when activated, with a more bulging soma and more stretched processes (Dore-Duffy and Cleary, 2011). This would explain the increase in the CD13⁺ area coverage in 6-OHDA-lesioned mice and the PDGF-BB-induced normalisation solely long after the treatment, as the PDGF-BB effect on pericyte activation is observed only at the long-term time point.

In our study, the vascular density was not affected by the lesion or the PDGF-BB treatment. This may be explained by the fact that our PD model is a partial lesion model and that the nature and extent of vascular changes in PD have been variable (Yang et al., 2015). Also, it remains to be investigated in more detail whether the PDGF-BB-mediated pericyte modulation may differ depending on age or stage of disease.

5. Conclusion

In conclusion, we demonstrated that i.c.v. PDGF-BB infusion promoted robust behavioural recovery persisting for at least 2 months in 5 different behavioural tests, which was associated with a partial restoration of the nigrostriatal pathway. Pericytes are activated in PD in response to the lesion, and this activation is reversed by PDGF-BB treatment. Our data suggest that brain pericytes may play a role in the pathogenesis of PD and could constitute a target for the PDGF-BB-induced neurorestorative mechanism in PD. Future studies will further elucidate the pathophysiological mechanism connecting PD and vascular changes and in particular, the role of pericyte function and dysfunction in PD.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.nbd.2016.06.002>.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgement

We thank Alicja Flasch for excellent technical assistance. This study was supported by grants from the Swedish Medical Research Council (number K2012-61X-22095-01-4), a grant by the European Union (Neural Restoration Trial, NRT; FP7; number 279102), Parkinsonfonden, Aners Stiftelse, Scania University Hospital Donations, Brainfonden and Multipark.

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