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Research paper

Roscovitine, an experimental CDK5 inhibitor, causes delayed suppression of microglial, but not astroglial recruitment around intracerebral dopaminergic grafts

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ABSTRACT

Inhibitors of cell cycle proteins are known to reduce glial activation and to be neuroprotective in a number of settings. In the context of intracerebral grafting, glial activation is documented to correlate with graft rejection. However, the effects of modification of glial reactivity following grafting in the CNS are poorly understood. Moreover, it is not completely clear if the glial cells themselves trigger the rejection process, or are they secondarily activated.

The present study investigated the effect of microglial inhibition by the cyclin-dependant kinase 5 (CDK5) inhibitor roscovitine following intracerebral transplantation in the rodent model of Parkinson's disease. Single cell suspension of rat E14 ventral mesencephalic tissue was transplanted to the dopamine-depleted striatum of unilaterally 6-hydroxydopamine (6-OHDA) lesioned male Sprague-Dawley rats. Experimental animals received injections of roscovitine (20 mg/kg) or a vehicle solution three times following the procedure. Immunohistochemistry was carried out on Day 7 and Day 28 to quantitatively describe the glial reaction adjacent to grafts.

The data confirm that systemic roscovitine treatment significantly reduced microglial recruitment adjacent to the grafts on Day 28, without exhibiting significant effects on astroglia. However, this was not found to correlate with elevated numbers of neurons in the grafts. Moreover, microglial reaction surrounding grafts was less pronounced compared to control animals, subjected to the mechanical influence only, even without roscovitine treatment.

Our results are the first to show the effect of cell cycle inhibition in the context of neuronal transplantation. The findings suggest that microglial activation around intracerebral grafts can be modified pharmacologically. However, the results do not confirm direct neuroprotective effects of cell cycle inhibition after intracerebral transplantation. Reducing microglial recruitment around grafts could be beneficial by reducing inflammation-related degenerative processes. Sparing astrocytes in the same time provides transplanted cells with essential trophics and support. We consider microglial inhibition to be a possible approach for reducing later graft-related complications.

1. Introduction

Ever since the first experiments with neural transplantation, the graft-host interface has been recognized as a site of important events of interaction between transplanted tissue and recipient brain (Saltykow, 1905). Introduction of cells into the CNS causes a pronounced activation of glial cells of the host, forming a glial scar around the graft. This scar tissue is both a barrier for outgrowing axons, while simultaneously being an active participant in graft integration (Barker et al., 1996).

With dopamine replacement strategy in mind, transplantation of

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foetal mesencephalic tissue in the standard unilateral model of Parkinson's disease (PD) has been shown to cause massive activation of microglial cells, both in the graft core, as well as along its borders (Shinoda et al., 1996). This activation is not transient, but is rather sustained for a significant time following transplantation (Kordower et al., 1997). The functional meaning of microglial activation is intimately connected with the process of graft rejection (Borlongan et al., 1996a). Recruitment of activated microglia is more pronounced in the absence of post-transplantational immunosuppression, which highlights the role of microgliocytes as immunocompetent cells of the brain (Kordower et al., 1997; Olanow et al., 2003). Moreover, concentration of activated microglia around grafts is associated with poorer functional outcome (Winkler et al., 2005).

Glial reaction following transplantation to the CNS can be viewed as triggered by two factors – the tissue trauma and the immunogenicity of the transplanted cells. Experiments with the microtransplantational technique show reverse correlation between the degree of tissue trauma and the number of integrated cells of the graft (Nikkhah et al., 1994a, b). At the same time, greater traumatic influence causes more pronounced gliosis. However, it is not completely clear what is the mechanism in which a massive glial reaction secondary to tissue trauma can influence the transplanted cells. The microglial/macrophageal reaction, which is the quickest following disruption of the integrity of the nervous tissue (Roth et al., 2014; Corps et al., 2015), can be discussed both as being an adaptive mechanism, as well as a proinflammatory factor, leading to poorer neurological outcome. Its suppression can be a promising strategy in reducing neuronal damage following trauma (De Rivero Vaccari et al., 2009; Kim et al., 2012; Wang et al., 2014). Up till now, modification of glial reactivity has not been tested in the context of intracerebral transplantation.

The expression of cell cycle proteins is increased in different cell populations of the CNS as a response to injury. In post-mitotic cells (neurons) they lead to apoptosis, whereas in mitotically active cells (glia) they trigger proliferation (Cernak et al., 2005). Inhibitors of the cell cycle have been therefore shown to act as neuroprotectors, while in the same time reducing glial scarring (Tian et al., 2006; Tian et al., 2007). Regarding neuronal transplantation, pre-grafting treatment of the cell suspension with cell cycle inhibitors has led to increased number of integrated neurons in the graft (Zawada et al., 2001). However, cell cycle inhibition has not yet been tested as a systemic treatment following neural transplantation.

Cyclin-dependant kinase 5 (CDK5) is one of the small serine/threonine cyclin-dependent kinases (CDK), active exclusively in the nervous system (Tsai et al., 1993). It is known to be involved in neuronal apoptosis triggered by DNA damage (Basu and Tu, 2005; Lee and Kim, 2007). Activation of CDK5-dependent pathways may ultimately lead to neuronal death via caspase activation (Cheung and Ip, 2004). CDK5 is particularly active in dopaminergic neurons, when they are exposed to neurotoxins in models of PD (Henchcliffe and Burke, 1997; Neystat et al., 2001), due to the CDK5-dependent activation of inflammasomes (Zhang et al., 2016).

Roscovitine, an inhibitor of CDK5, is a powerful neuroprotector after trauma and ischemia of the CNS (Hilton et al., 2008; Menn et al., 2010). Its effects are based both on anti-apoptotic as well as on microglia-inhibiting properties. It this way, it could interact both with grafted neurons, as well as modify the conditions of the host brain. However, no data exists regarding the effect of this CDK inhibitor on grafted dopaminergic neurons in vivo. Roscovitine has also been reported to increase dopaminergic transmission by acting on the dopamine transporter in a CDK5-independent manner (Price et al., 2009), making it an even more interesting candidate for testing the effect of cell cycle inhibition following cell transplantation in the PD model.

2. Aim

The aim of the present study is to demonstrate the effects of the systemic administration of the cell cycle inhibitor roscovitine following intracerebral transplantation of E14 ventral mesencephalic tissue in a rodent model of PD. The known ability of roscovitine to both inhibit microglia as well to provide neuroprotection could lead to less microglia being recruited around grafts and/or more dopaminergic cells being integrated with the host brain. Furthermore, dopamine-related effects of roscovitine could contribute to the functionality of the grafts. Additionally, we aim to describe the relative role of transplantation and mechanical trauma in the development of the post-grafting gliosis. This would shed light on the mechanisms of microglial activation following intracerebral transplantation and explore a novel potential neuroprotective strategy to be used for enhancing the transplantation results.

3. Materials and methods

3.1. Experimental animals

Adult male Sprague-Dawley rats (body weight 300–350 g) were used (Charles River Laboratories, Germany). Throughout the experiment they were kept under standard conditions (temperature 22 ± 2 °C and 12 h/12 h light/dark cycle) with food and water available ad libitum. All animal handling was done in strict adherence to governmental (Directive 2010/63/EU of the European Parliament and of the Council of September 22, 2010) and institutional (TVA G-10/110 of the Veterinary board for animal research of the University of Freiburg) animal care regulations.

For the surgical procedures, the animals were anaesthetized with 4% isoflurane (Forene, Abbot, Germany), using O_2 as the carrier gas, in an air-filled induction chamber. Throughout the surgery, anaesthesia was maintained with a gas mask on the nose of the animal at about 2% isoflurane. The head of the animals was shaved and treated with povidone iodine, the eyes were protected with eye ointment. Subsequently, the animals were fixed in a standard stereotactic frame (Stoelting, Germany) and a sagittal incision through soft tissues was made to expose the bregma. All subsequent steps were performed under a stereomicroscope. The craniotomies were made using a high-speed drill. After surgery, the extracranial soft tissues were closed with sterile clips. The animals were given post-surgical care involving intraperitoneal saline administration for hydration as well as providing soaked chaw in the postoperative days.

3.2. 6-OHDA lesion

The experimental animals were subjected to a unilateral lesion of the nigrostriatal pathway (Ungerstedt, 1968; Furlanetti et al., 2015) by injecting 3.6% 6-OHDA solution stabilized with 0,2 mg/ml ascorbic acid into the right medial forebrain bundle. Two deposits were made, as follows: 2,5 µl at AP: –4,4, L: –1,2, DV –7,8 with the toothbar sat at − 2,3, and 3 µl at AP: –4,0, L: –0,8, DV –8,0 with the toothbar at + 3,4 (coordinates in mm after Paxinos and Watson, 1997). Effect of the lesion was confirmed 28 days hereafter via amphetamine-induced rotational behaviour testing. Only animals exhibiting > 7 rotations ipsilateral to the lesion were included in the experiment, since such a result would correlate with almost complete depletion of striatal dopamine (Nikkhah et al., 1993).

3.3. Transplantation procedure

For transplantation, a suspension of E14 Sprague-Dawley rat fetal ventral mesencephalic tissue, was used. After terminal anaesthesia of timely-pregnant females, the embryos were removed together with the uterus. Tissue from the ventral mesencephalon of each embryo was dissected and processed (Pruszak et al., 2009) and a single-cell suspension was prepared as described previously (García et al., 2011). After confirmation of the viability of the neuroblasts with Trypan blue vital stain, the concentration of the suspension was adjusted to

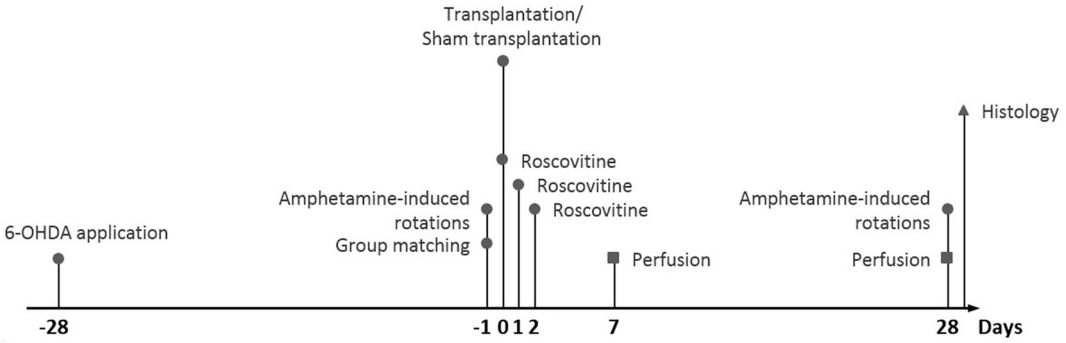

Fig. 1. Timeline of the experiment.

100,000 cells/μl, of which we accept about 10% to be dopaminergic neurons (Nikkhah et al., 1994a). The transplantation procedure itself was performed with a Hamilton syringe equipped with a 26G steel cannula. Two deposits of the suspension (100,000 cells each) were made into each animal's right striatum in the following stereotaxic coordinates: AP: +0,2, L: –3,5, DV: –5,0 and −4,0, respectively (coordinates in mm according to Paxinos and Watson, 1997). After injection, the cannula was held in place for 5 min, before being slowly retracted. Sham-transplanted animals were subjected to an impact with the same steel cannula at the same coordinates, retaining it for the same time, but without injecting anything.

3.4. Experimental design

The timeline of the experiment is outlined on Fig. 1. The experimental groups used are summarized in Table 1. Each group consisted of 8 animals. Each transplantation group has a sham group as a morphological control. This aims to assess the relative role of transplantation and mechanical trauma in eliciting gliosis and to highlight any possible differences of action of roscovitine in the presence/absence of transplanted cells.

The abbreviations from Table 1 are used for the presentation of the data in the bar graphs as well.

All animals sacrificed on Day 28 were additionally tested with amphetamine-induced rotations immediately before perfusion.

3.5. Roscovitine administration

Roscovitine (Meijer Laboratories, Roscoff, France) was initially solubilized in dimethyl sulfoxide (DMSO) (Sigma, USA), aliquoted and stored at −20 °C until administration. Before injection, the roscovitine solution was diluted with PEG300 and physiological saline in 5/45/45 (V/V/V) relation. The dosage of roscovitine was 20 mg/kg. The vehicle solution contained the same components, but without roscovitine. Animals were injected subcutaneously at the time of transplantation, as well as 24 and 48 h following surgery. Subcutaneous route was chosen due to the relatively short plasma half-life (< 30 min) of roscovitine after single bolus administration (Vita et al., 2005) and the good neuroprotective results obtained with this way of administration (Menn et al., 2010). Roscovitine is known to reach concentrations up to 30% of plasma concentrations in brain tissue (Vita et al., 2005). The dose regime was developed mainly due to the intensive cell death in the first 1–4 days after transplantation (Brundin et al., 2000).

Table 1
Experimental groups used in the design of the study. The group names are used throughout the text and figures. Tx = transplantation; Sham = sham-transplantation; Rosco = roscovitine; D7 = Day 7; D28 = Day 28.

Group	Transplantation	Roscovitine	Survival
Tx Rosco D7	yes	yes	7 days
Sham Rosco D7	sham	yes	7 days
Tx Vehicle D7	yes	no	7 days
Sham Vehicle D7	sham	no	7 days
Tx Rosco D28	yes	yes	28 days
Sham Rosco D28	sham	yes	28 days
Tx Vehicle D28	yes	no	28 days
Sham Vehicle D28	sham	no	28 days

3.6. Immunohistochemistry

Following terminal anaesthesia with ketamine (150 mg/kg) and xylazine (10 mg/kg), the animals were transcardially perfused initially with 300 ml ice-cold phosphate-buffered saline (PBS), followed by 300 ml ice-cold 4% paraformaldehyde in PBS. The brains were removed and postfixed in the same fixative overnight. After cryoprotection with 20% sucrose the brains were cut in the frontal plane into 40 μm thick sections on a freezing microtome. The resulting serial free-floating sections were processed for immunohistochemistry following a standard protocol. The primary antibodies used were against tyrosine hydroxylase (TH) in a dilution 1:2500 (mouse anti-TH; T1299, Sigma, Germany), against ionized calcium-binding adapter molecule 1 (Iba1) in 1:700 dilution (rabbit anti-Iba1, 019-19,741, Wako, Japan) and against glial fibrilary acidic protein (GFAP) in 1:800 dilution (rabbit anti-GFAP, ab7779, Abcam, United Kingdom). The reaction was visualized with Vectastain's ABC kit with 3,3-diaminobenzydine (DAB) as a chromogen used per manufacturer's specifications. Great care was taken to perform all reactions in a highly standardized manner, performing the stainings in batches with precise and constant incubation time with all reagents. Optimal color development time was estimated under visual control, and then applied to all sections stained in the batch. Those measures enabled the subsequent quantitative analysis. Stained sections were mounted on glass slides, dehydrated, cleared in xylene and coverslipped with Histofluid.

3.7. Image acquisition and analysis

Brightfield images were obtained using a Nikon Eclipse 80i microscope equipped with a DMX 1200c camera (Nikon Instruments Europe, Netherlands) using constant settings for light, exposure and gamma correction. Image analysis was carried out using NIS Elements AR v.2.30 (Nikon Instruments Europe, Netherlands) and ImageJ (National Institute of Health, USA). For the TH stained sections, all of the visible TH+ cell bodies on each 6th section were manually counted under 40× magnification. The total number of TH+ cells per graft was calculated therefrom using Abercrombie's formula (Abercrombie, 1946). Moreover, the optical density of TH+ fibers as a quantitative measure of the degree of reinnervation by the grafts was established on Day 28 after transplantation. It was expressed as the percentage of the mean gray value of the transplanted striatum of the mean gray value of the contralateral, intact one. For the Iba1- and GFAP-stained sections, cell

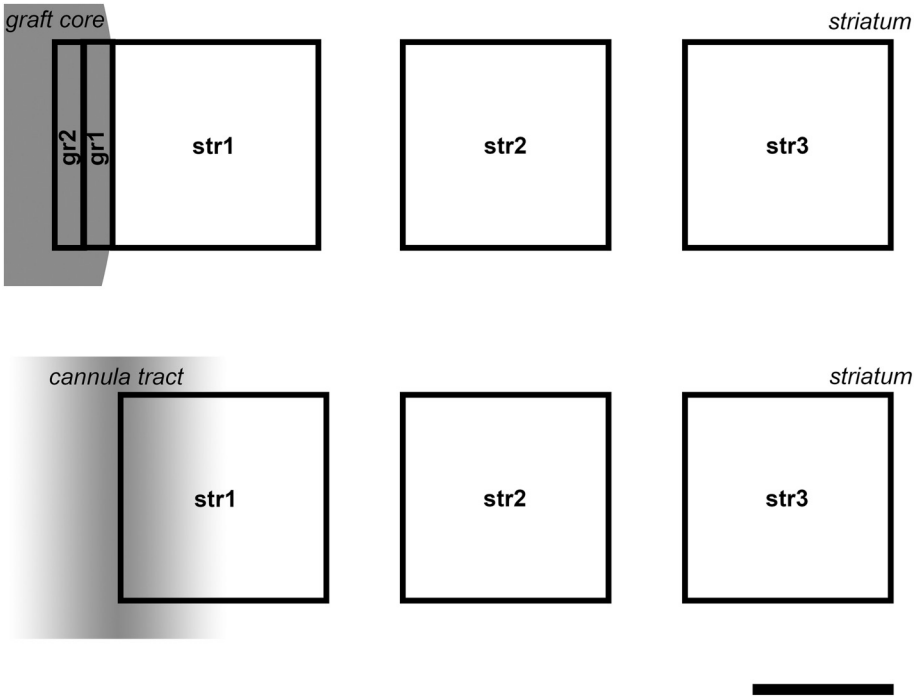

Fig. 2. Zones of interest in transplanted (above) and sham-transplanted (below) animals. For details regarding the sampling procedure, refer to Tomov et al., 2018. Scale bar = 100 μm.

density as well as immunopositive area fraction was estimated in two zones inside the graft core (for transplanted animals only), as well as in three zones in the tissue of the host striatum (as shown on Fig. 2). The used methodology was described in detail previously (Tomov et al., 2018).

3.8. Statistical analysis

Raw data was analyzed in GraphPad Prism 6 for Windows (GraphPad Software, Inc., USA) applying one-way analysis of variance (one-way ANOVA) and Tukey-Kramer's post-hoc test for multiple comparisons. *P*-values $< .05$ were accepted to be statistically significant. For the graphical representations all experimental data is expressed as mean values ± S.E.M.

4. Results

Perykaria of intensively stained TH+ cells were observed in all grafts (Fig. 3, A and B). They were gathered in clusters, which were either associated with the graft-host interface, or found deep inside the graft core. Occasionally some cells were seen at a distance from the graft in the tissue of the host striatum. The appearance of the TH+ neurons considerably changed from Day 7 to Day 28. The outlines of the cell bodies became sharper, and the emerging processes became more clearly visible on Day 28 (Fig. 3B). Administration of roscovitine was not associated with any alterations of the morphological characteristics of TH+ neurons in the grafts.

Quantitative analysis of TH immunoreactivity failed to demonstrate significant differences in the number of TH+ neurons between groups treated and untreated with roscovitine (747 ± 239.8 vs. 1133 ± 472.1 for Day 7, $p > .05$; 984.5 ± 312.1 vs. 661 ± 241.1 for Day 28, $p > .05$). The numbers of integrated dopaminergic neurons in the grafts was not significantly different in the two time points examined either. (Fig. 4). The degree of reinnervation by the grafts on Day 28 is not affected by roscovitine administration (14.72 ± 3.286% vs. 11.96 ± 3.970%, $p > .05$) (Fig. 5). The functionality of grafts was evident by the change of the amphetamine-induced rotational behavior, compared to the sham-transplanted animals (Fig. 5). However, no functional effect of roscovitine treatment was observed (3.743 ± 2.02 vs. 4.045 ± 1.613 ipsilateral turns per minute, $p > .05$).

Iba1+ microglial cells were observed throughout the brain tissue. Their presence however was most prominent along the graft-host interface. On Day 7, ameboid microgliocytes were seen engulfing the grafts almost completely (Fig. 3C). Moreover, clusters of ameboid microglia were seen in the tissue of the striatum, adherent to the graft-host interface. The localization of those microglial clusters often coincided with the localization of TH+ neuronal ones. The immediate proximity of the grafts was markedly populated by large microglial cells with juicy processes, contrasting with the typical resident microglia of the striatum. Further away from the graft, those prominent microgliocytes were still seen, albeit in lesser numbers, intermingling with the normally ramified resident cells. The graft cores themselves, despite not being intensively Iba1+ were also populated by mostly ameboid microglia. In the periphery of the graft, microglial cells from the prominent clusters were seen crossing the graft-host interface and invading the graft tissue.

On Day 28, the grafts were still surrounded by a prominent microglial reaction, and clusters of microgliocytes were still present along the graft-host interface (Fig. 3D). The microglial cells were large, with prominent short and thick processes, occasionally ameboid. However, the sections of the graft-host interface not characterized by the presence of clustered microglia were rather unremarkable surrounded by microglia with quiescent appearance. The graft cores were uniformly populated by ramified microgliocytes.

The cannula tract through the striatum of the sham-transplanted animals was visible as a band of densely gathered microglial cells with large bodies and thick processes, without much evolution in time (Fig. 10A). On Day 7, occasional ameboid cells were visible among the ramified ones. On Day 28, virtually all cells were ramified, however they retained their thick processes.

The quantitative assessment of the microglial reaction surrounding the grafts revealed that roscovitine administration causes a suppression of microglial recruitment. This was evident by the significantly attenuated values of both immunopositive area fraction and cell density,

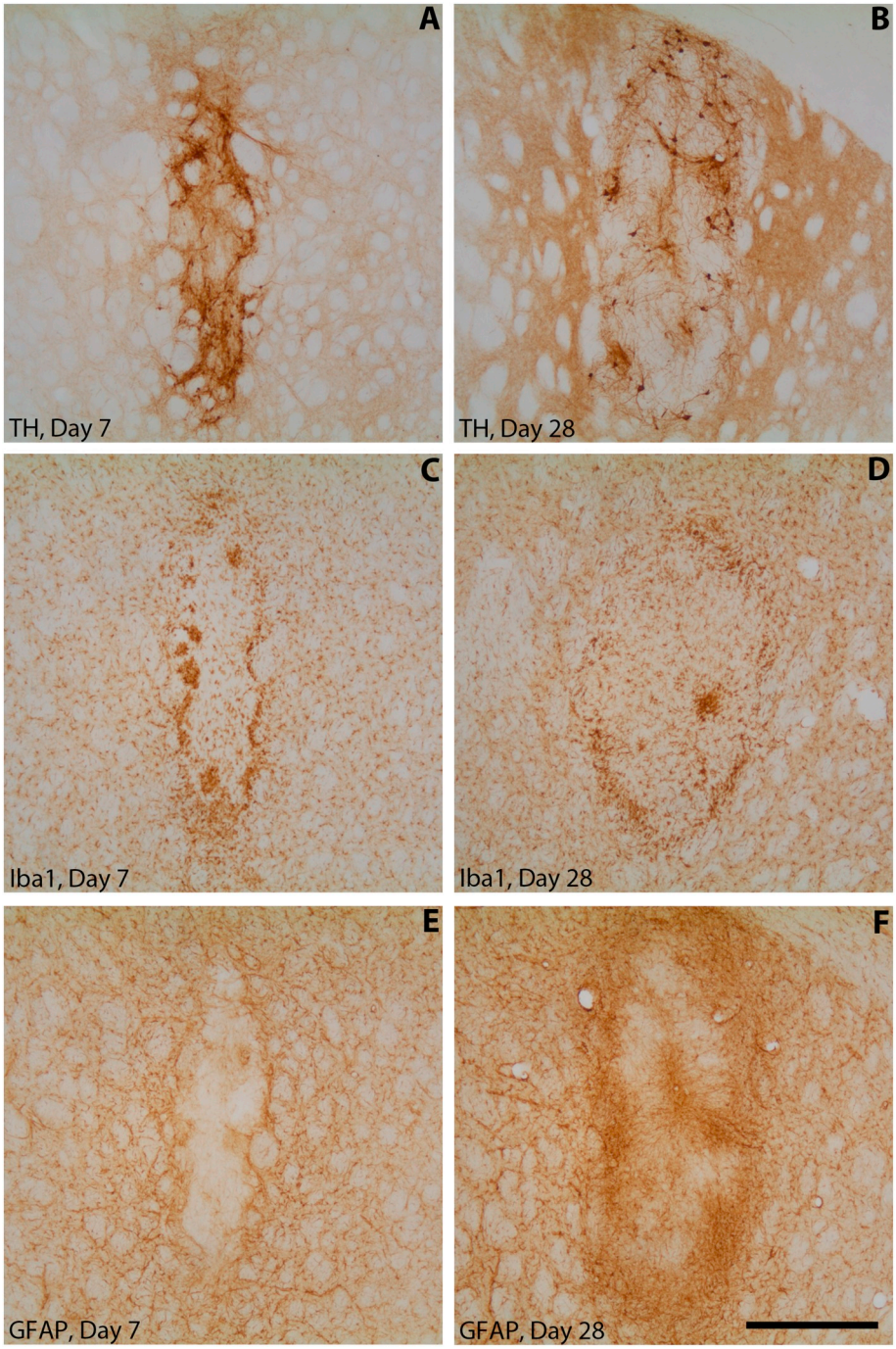

Fig. 3. Immunohistochemical staining of intracerebral grafts on Day 7 (A, C, and E) and Day 28 (B, D, and F). Note the presence of clusters of ameboid microglia along the graft-host interface (C and D). Scale bar = 500 μm.

associated with treatment with roscovitine. (Fig. 6A and B). The Iba1 immunopositive area fraction on Day 28 showed pronounced differences between treated and untreated animals. Roscovitine administration was associated with less Iba1 immunoreactivity, both in the cores of the grafts, as well as in the host striatum (0.2392 ± 0.02324 vs. 0.4276 ± 0.03023, $p < .001$ for gr1; 0.2571 ± 0.01969 vs. 0.4512 ± 0.03025, $p < .0001$ for gr2; 0.3212 ± 0.02186 vs. 0.5043 ± 0.03198, $p < .001$ for str1). Moreover, on Day 28, less cells were counted in the zone of the host striatum, intimately adherent to the grafts in the animals receiving treatment with roscovitine, compared to the identical zone in the animals receiving the vehicle solution only (1440 ± 57.8 vs. 1988 ± 101.6 cells/mm^2, $p < .001$ for str1) (Fig. 6B). No significant differences in the values of either parameter were present on Day 7.

Comparing the same two parameters of the microglial reaction for the sham-transplanted animals, a significant difference of the Iba1+ cell density only, and not of the Iba1+ immunoreactive area, was evident (Fig. 6C and D). It was observed closest to the cannula track, and what's more on Day 7 (3398 ± 90.98 vs. 4328 ± 96.44 cells/mm^2, $p < .0001$ for str1). On Day 28 treated and untreated animals did not show any significant differences in microglial reaction surrounding the cannula tract. Regardless of treatment with roscovitine, microglial reaction subsided from Day 7 to Day 28, both in transplanted and in sham-transplanted animals.

Furthermore, the microglial recruitment around grafts and around cannula tracts was different on each of the time points examined. Iba+

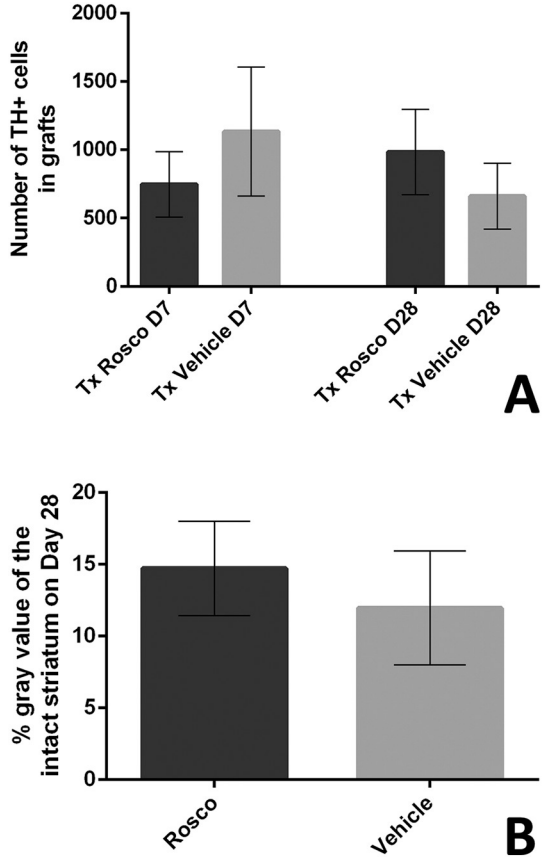

Fig. 4. Number of dopaminergic cells in the grafts (A) and reinnervation of the lesioned striatum, expressed as percentage of the gray value of the intact (left) striatum (B).

immunoreactive area and cell density was significantly reduced in the transplanted animals, when compared to the sham-transplanted ones, both on Day 7, as well as on Day 28 (Fig. 7).

GFAP+ astrocytes around the grafts were seen to form an almost uninterrupted envelope around the graft on Day 7 (Fig. 3E). The astrocytes were densely crowded along the graft-host interface, and less crowded further away from it in the host striatum. Their bodies were quite prominent and the processes – thick. In the same time point, the graft cores remained almost devoid of astrocytic bodies, however pierced by multiple, extremely fine astrocytic processes. On Day 28, the envelope around the grafts changed to a more prominent appearance (Fig. 3F). Astrocytes surrounding the grafts were dark, and with many thin processes. Most of the processes were directed radially to the middle of the graft, but some were seen interweaving, thus forming a strongly GFAP+ zone covering, and to some extent obscuring the graft-host interface, while also irradiating towards the tissue of the striatum. The graft cores were characterized by the presence of an intensively stained periphery, containing mostly processes, penetrating from outside the graft. A characteristic finding was the presence of blood vessels, both in the graft cores and passing through the graft-host interface, tightly cuffed by astrocytes. Roscovitine did not change the appearance of the reactive astrocytes surrounding the grafts.

When comparing the astroglia of transplanted and sham-transplanted striata, a striking difference could be observed. While the astrocytes formed an envelope with intricate architecture around the grafts, that was not the case with the cannula tracts. The cannula tract was visualized as a strip of significant width, filled with densely crowded cells with dark bodies and thick, but not excessively long processes (Fig. 10B). Even on careful observation the appearance of the cannula tract did not change from Day 7 to Day 28.

As far as the astroglial reaction around grafts and cannula tracts is concerned, no significant effect of roscovitine could be demonstrated (Figs. 8 and 9). Neither the morphology nor the quantitative parameters of the astrocytes around the zones of influence were influenced by the cell cycle inhibitor. The only association with the administration of roscovitine was, surprisingly, the presence of more astrocytes in the vicinity of the cannula tract through the striatum of sham-transplanted animals on Day 7 (1621 ± 175.1 vs. 1207 ± 24.79 cells/mm2, $p < .0001$) (Fig. 8D). Comparing astrogliosis in the striatum around grafts and cannula tracts on each of the time points, no significant difference could be demonstrated (Fig. 9). The intensive development of an astroglial envelope could therefore be traced back only to the periphery of the grafts, and not to the striatum.

5. Discussion

The present study is the first one to examine the effects of the cell cycle inhibitior roscovitine following intracerebral transplantation of cells. Despite the neuroprotective effects of roscovitine were proven in a number of other experiments (Kabadi and Faden, 2014; Rousselet et al., 2017), we did not establish its effects on TH+ neurons following grafting. This observation could be traced back to a number of reasons. First, the dopaminergic neurons in the cell suspension, which undergo apoptosis, may not be salvaged by CDK5 inhibition. The degeneration of those neurons can happen caspase-independently (Di Giovanni et al., 2005) and thus CDK inhibition would not have an effect on the process. The enzyme activity of CDK5 could as well also be promoting neuronal survival (Cheung and Ip, 2004; Li et al., 2002; Zheng et al., 2007). The exact biochemical pathways activated in apoptotic neurons post grafting, are yet unknown. Second, roscovitine might not have been able to penetrate the tissue of the grafts, due to the not yet established

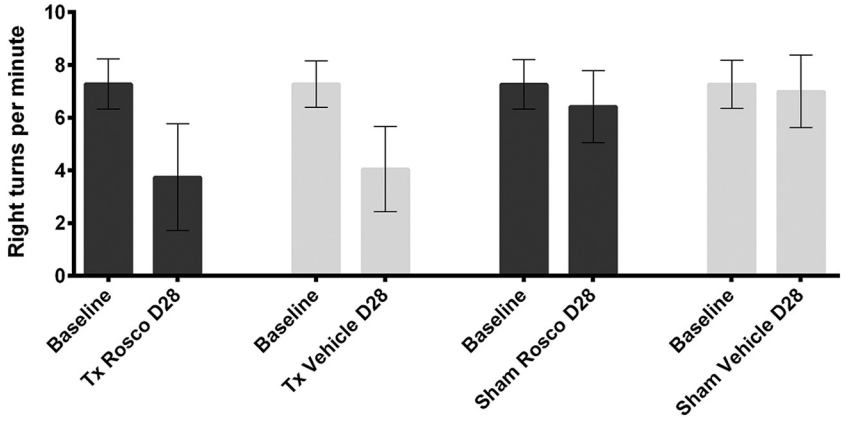

Fig. 5. Changes of rotational behavior before and after surgery.

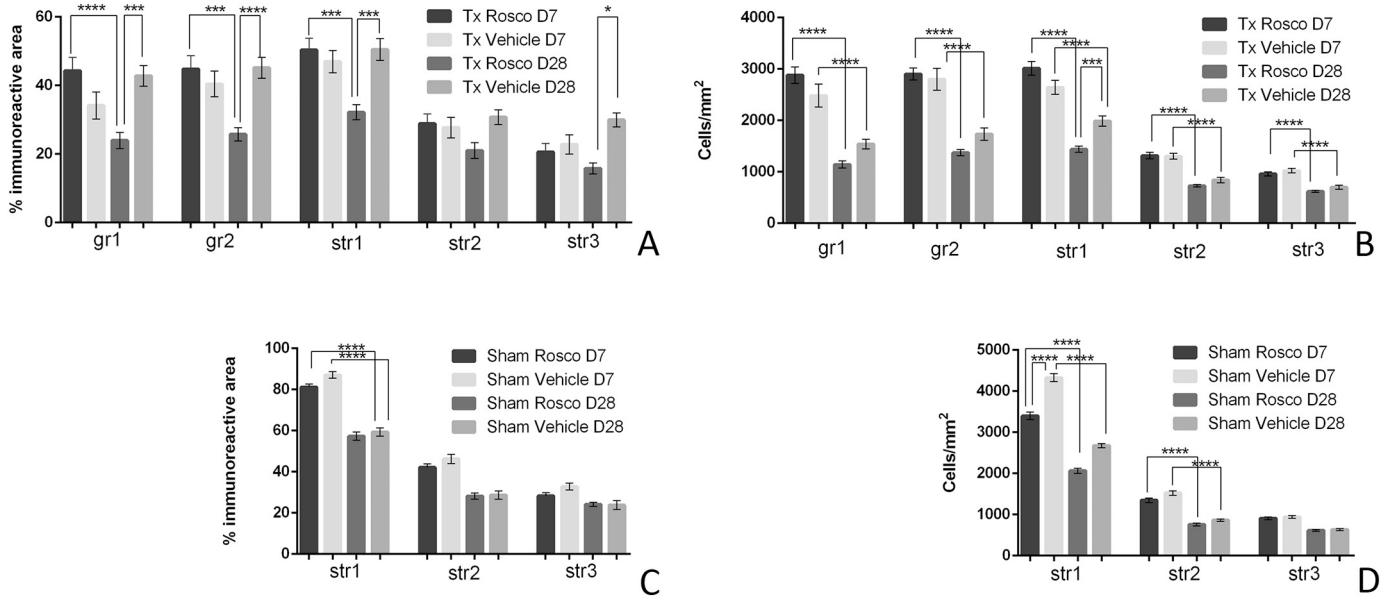

Fig. 6. Dynamics of Iba1+ immunoreactive area (A and C) and cell density (B and D) following transplantation (above) or sham-transplantation (below). **** indicate $p < .0001$, *** – $p < .001$.

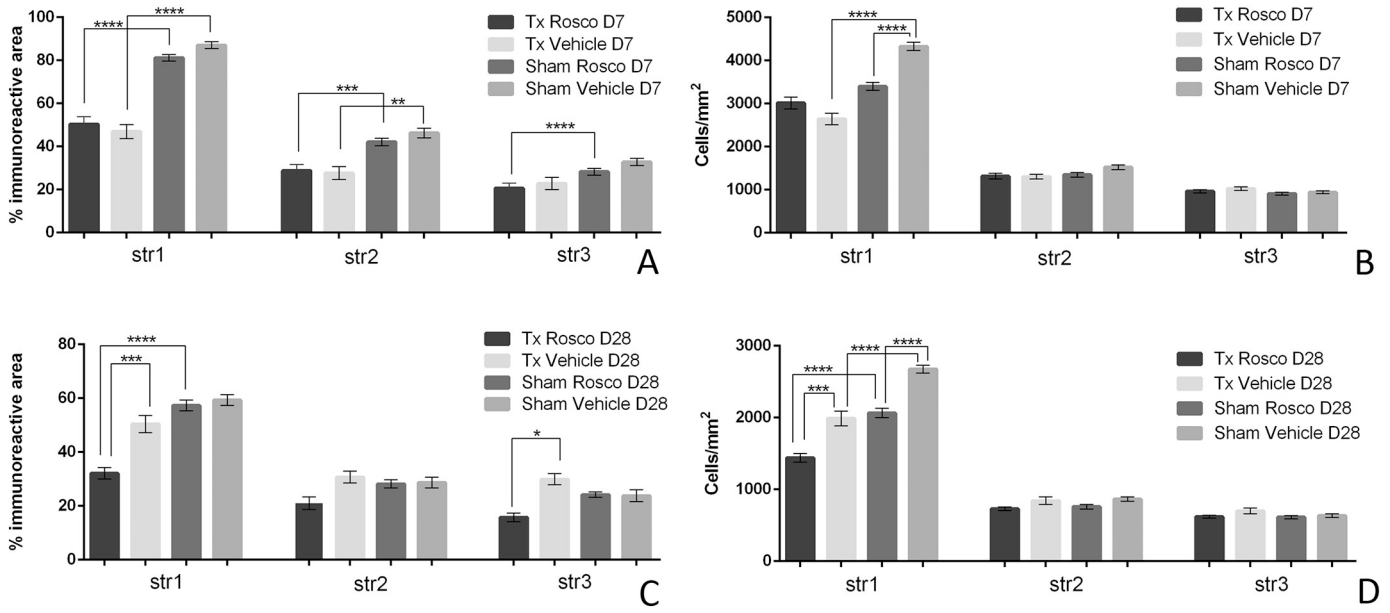

Fig. 7. Quantitative comparison of Iba1 immunoreactivity on Day 7 (A and B) and Day 28 (C and D) for transplanted and sham-transplanted animals. Note the weaker microglial recruitment around grafts, when compared to the cannula tracts in both time points.

microcirculatory bed in the first days following transplantation (Dusart et al., 1989), when it was applied. Third, and most likely, apoptosis of not integrated neurons in the grafts is a mechanism, which cannot be easily stopped by external influence, as shown in other experiments (Marchionini et al., 2004).

Our data clearly shows that microglial recruitment around grafts is effectively suppressed by roscovitine. This is the first report of the effects of cell cycle inhibition on glial reaction following intracerebral grafting. We confirm the principle microglia-inhibiting effect of roscovitine (Hilton et al., 2008) in yet another situation of microglial activation. Interestingly, we observe a delayed effect of roscovitine on microglia. It seems that in the acute phase of microglial activation, roscovitine does not affect the absolute numbers of microglial cells both around grafts, and around areas of mechanical influence only. The effect of cell cycle inhibition on microglia is demonstrated rather late – on Day 28, confirmatory to the data suggesting that glial reaction to degenerating dopaminergic neurons is delayed (Stott and Barker, 2014).

Furthermore, our data can be interpreted as an evidence for the existence of two mechanisms of microglial recruitment following grafting. The first one is a direct migration of bone-marrow derived cells to the brain parenchyma (Ginhoux and Prinz, 2015), and the second – the proliferation of resident microglial cells. The reported high permeability of graft-associated vasculature for several days after grafting (Akalan and Grady, 1994) might be a prerequisite for extravasation of blood-borne cells, which transform into microglia. However, the contribution of bone marrow-derived cells to the microglial population is relatively small, at least in the normal state. The main pathway of microglial recruitment is the proliferation of the resident pool of microgliocytes (Ginhoux and Prinz, 2015). We believe that the majority of microglial cells surrounding the grafts are a progeny of

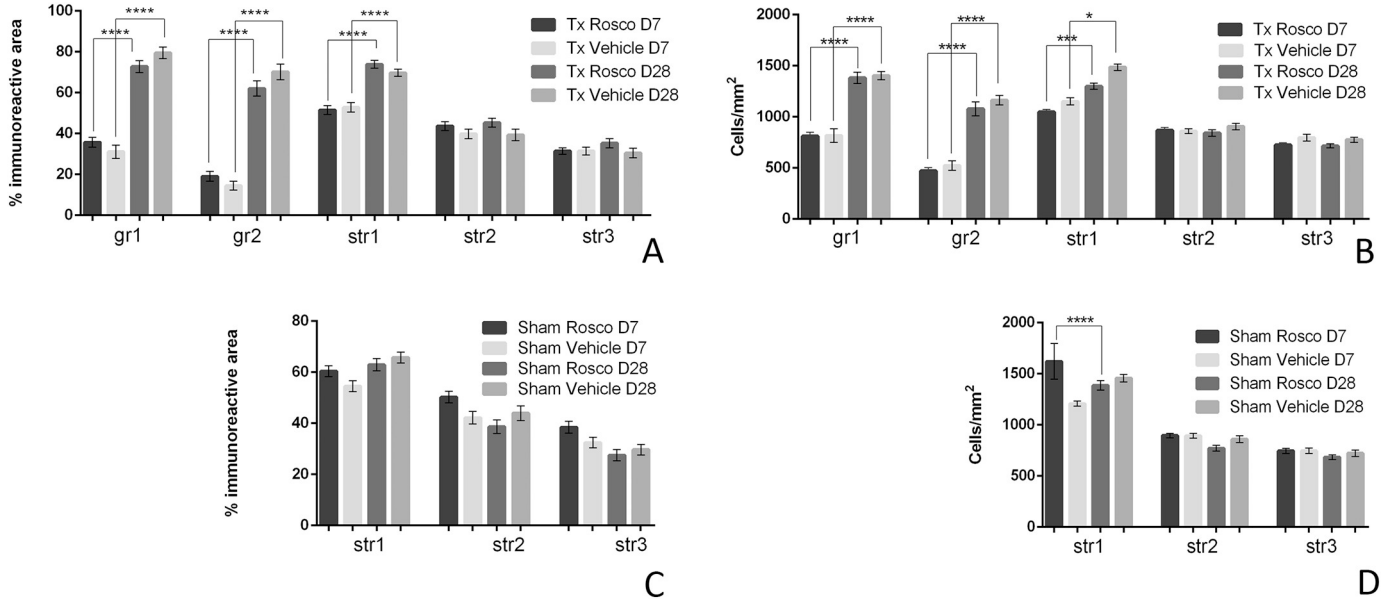

Fig. 8. Dynamics of GFAP+ immunoreactive area (A and C) and cell density (B and D) following transplantation (above) or sham-transplantation (below). * indicates $p < .05$, *** – $p < .001$, **** – $p < .0001$.

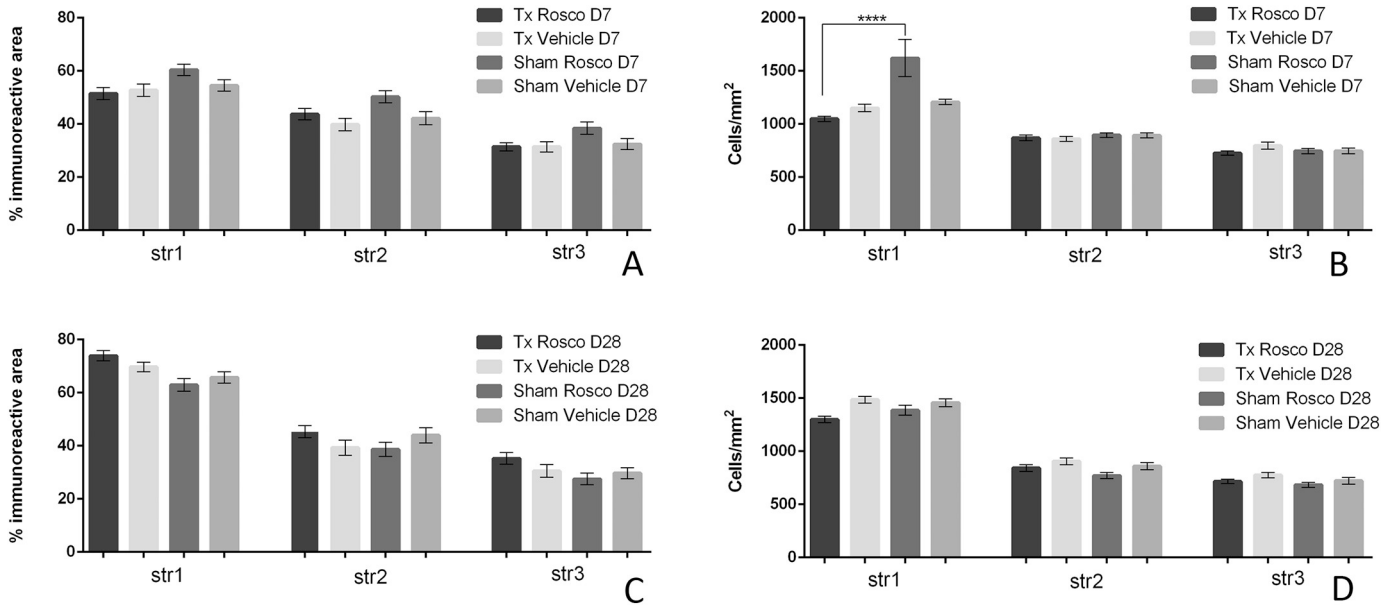

Fig. 9. Quantitative comparison of GFAP immunoreactivity on Day 7 (A and B) and Day 28 (C and D) for transplanted and sham-transplanted animals.

resident cells. Since inhibition of cell cycle proteins can only influence proliferating, and not migrating cells, they would also be the ones affected by roscovitine.

The lack of correlation between microglial suppression and the numbers of integrated dopaminergic cells in the grafts has to be addressed. It was previously suggested that microglia-mediated (inflammatory) neurodegeneration is among the main mechanisms of dopaminergic cell loss in Parkinson's disease (McGeer et al., 1988; Moore et al., 2010). Generally, anti-inflammatory intervention is considered to be neuroprotective in Parkinson's diesease (Tansey and Goldberg, 2010). Therefore, reducing microglia-mediated inflammation is a well-reasoned and justified concept (Liu and Hong, 2003) to be tested following grafting, of course, side by side with other strategies (Borlongan et al., 1999). Since the early days of experimental neural transplantation, it is well known that general immunosuppression can lead to better functional outcome (Borlongan et al., 1996b) following grafting. Co-grafting of dopaminergic cells with Sertoli cells (Sanberg et al., 1997) provides neuroprotection not only by trophic effects, but also by microglial modulation (Sanberg et al., 1996). Roscovitine is neither an anti-inflammatory nor an immunosuppressive agent in the classical sense, and the design of the present study did not rely on similar properties. Roscovitine was rather used as a selective inhibitor of microglia.

Our data does not show a direct relationship between absolute numbers of microglia and of dopamergic neurons after intracerebral transplantation. It is yet to be determined whether anti-inflammatory pharmaceutical intervention would have a positive effect following dopaminergic grafting. Moreover, even if the direct anti-apoptotic properties of roscoivitine could also be beneficial (Cicchetti et al., 2002), we could not demonstrate that. Even though roscovitine administration might not be able to increase the total number of dopaminergic neurons in the grafts, it could be beneficial via other

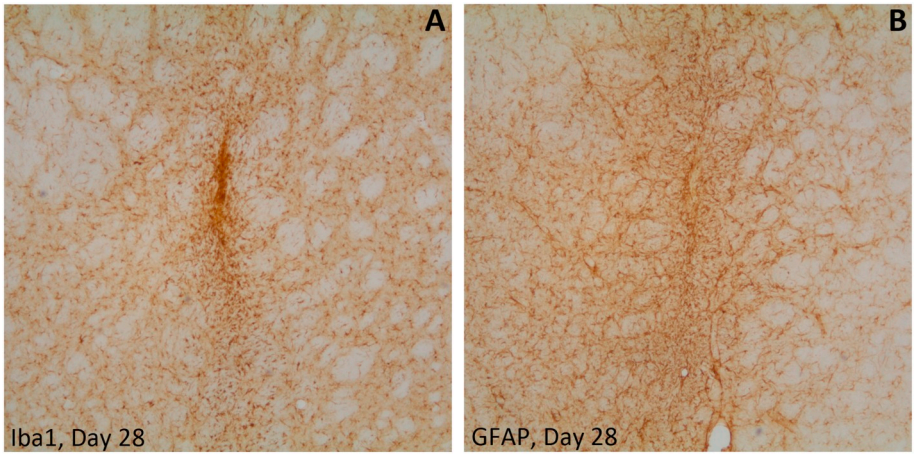

Fig. 10. Iba1- (A) and GFAP-stained (B) cannula tracts trough the striatum of sham-transplanted animals on Day 28. Scale bar = 500 μm.

mechanisms.

Roscovitine administration post-grafting may lead to better long-term functional results. The increased microglial density along the graft-host interface is known to be associated with poorer functional outcome, despite not directly correlating with graft rejection (Winkler et al., 2005). The lack of measurable effect of roscovitine on amphetamine-induced rotational behavior can be explained by the robustness of this test and to the relatively early stage of testing. A longer follow-up with more extensive behavioral experimentation is needed to prove whether roscovitine-caused inhibition of microglia is able to enhance the functionality of grafts.

Roscovitine is known to exert an intensifying effect on dopaminergic transmission, and this effect is not directly related to its CDK5-inhibitory activity (Price et al., 2009). Our data does not suggest an increase in the dopaminergic activity in the roscovitine-treated groups. This can be explained with the short half-life of roscovitine (Vita et al., 2005). The substance would have been entirely eliminated from the organism at the time of our testing.

Unlike other reports (Di Giovanni et al., 2005; Cernak et al., 2005; Hyun et al., 2017), our results suggest that roscovitine administration is not associated with amelioration of astroglial scarring. We attribute this mostly to the strong effect of stimulation of astrocytes by grafted tissue (Tomov et al., 2018). Even when looking at sham-transplanted animals, roscovitine did not have a clearly demonstrable effect on astrocytic reaction. This observation can be traced back to the negligible volume of the injury to the striatum. After retracting of the transplantation instrument, the walls of the cannula tract are approximated, leaving no tissue defect. Bleeding in the cannula tract is also virtually non-existent. Since the glia of the striatum were already hyperreactive following 6-OHDA lesion (He et al., 2001; Henning et al., 2008), we do not consider sham-transplantation to be a major glial activating stimulus per se in this milieu.

6. Conclusion

The present study demonstrated a pronounced effect of inhibition of microglial recruitment by roscovitine around intracerebral grafts in the 6-OHDA model of Parkinson's disease. In the same time, astroglial reaction was not affected by administration of the CDK5 inhibitor. Even though roscovitine did not change the total number of surviving dopaminergic neurons in the grafts, its usage may be beneficial in other ways, through its effect of suppression of neuroinflammation.

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Declaration of interest

None.

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