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The anti-dyskinetic effect of dopamine receptor blockade is enhanced in parkinsonian rats following dopamine neuron transplantation

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Abstract

Graft-induced dyskinesia (GID) is a serious complication induced by dopamine (DA) cell transplantation in parkinsonian patients. We have recently shown that DA D_2 receptor blockade produces sticking blockade of dyskinesia induced by amphetamine in grafted 6-OHDA-lesioned rats, a model of GID.

This study was designed to investigate whether blockade of DA D_1 receptors could produce similar outcome, and to see whether the effect of these treatments in grafted rats was specific for dyskinesia induced by amphetamine, or could also influence L-DOPA-induced dyskinesia (LID). L-DOPA-primed rats received transplants of fetal DA neurons into the DA-denervated striatum. Beginning at 20 weeks after transplantation rats were subjected to pharmacological treatments with either L-DOPA (6 mg/kg) or amphetamine (1.5 mg/kg) alone, or in combination with the D_1 receptor antagonist SCH23390, the D_2 receptor antagonist eticlopride, and the 5-HT_{1A} agonist/D_2 receptor antagonist buspirone. Grafted rats developed severe GID, while LID was reduced. Both eticlopride and SCH23390 produced near-complete suppression of GID already at very low doses (0.015 and 0.1 mg/kg, respectively). Buspirone induced similar suppression at a dose as low as 0.3 mg/kg, which is far lower than the dose known to affect LID in non-grafted dyskinetic rats. In agreement with our previous results, the effect of buspirone was independent from 5-HT_{1A} receptor activation, as it was not counteracted by the selective 5-HT_{1A} antagonist WAY100635, but likely due to D_2 receptor blockade. Most interestingly, the same doses of eticlopride, SCH23390 and buspirone were found to suppress LID in grafted but not in control dyskinetic rats. Taken together, these data demonstrate that the DA cell grafts strikingly exacerbate the effect of DA D_1 and D_2

receptor blockade against both GID and LID, and suggest that the anti-GID effect of buspirone seen in patients may also be due to blockade of DA D_2 receptors.

Key words (up to 10): Graft-induced dyskinesia; L-DOPA-induced dyskinesia; Parkinson's disease; Dopamine D_1 receptor; Dopamine D_2 receptor; Eticlopride; SCH23390; Buspirone; cell transplantation;

Introduction

Administration of L-DOPA remains the most effective treatment for Parkinson's disease (PD). However, the appearance of dyskinesia during the progression of the disease has prompted researchers to investigate alternative approaches to treat this debilitating condition. A number of pre-clinical studies demonstrated that transplantation of ventral mesencephalic (VM) cells into the host striatum generates fully mature dopamine (DA) neurons and provides significant restoration of motor functions in animal models (Bjorklund, 1992; Redmond et al., 2008; Winkler et al., 2000). Based on these promising experimental results, clinical investigations have been performed in advanced PD patients using tissue from aborted foetuses (Freed et al., 1992; Lindvall et al., 1994; Lindvall et al., 1992). While open-label trials have provided promising, albeit highly variable, results, double-blind studies have been largely disappointing (Freed et al., 2001; Olanow et al., 2003). Nevertheless, some of the transplanted patients have greatly benefited from cell grafting, such that they could reduce, or even suspend, L-DOPA treatment, providing prove-of-concept that this approach can yield significant and long-lasting amelioration of motor function. Moreover, recent post-mortem and positron emission tomography studies have shown that a significant number of grafted DA cells survived in the host caudate/putamen up to sixteen years after transplantation (Li et al., 2010; Mendez et al., 2008; Politis et al., 2011; Politis et al., 2010). Most probably, the lack of standardization in the surgical procedures, tissue preparation and patients selection, as well as the presence or absence of post-surgery immunosuppression have contributed to the variability of the results and the negative outcome of the double-blind trials (Barker et al., 2013). In fact, a new clinical study has recently been funded by the EU FP7 program. The

goal of this project is to optimize the procedure for DA cell transplantation using embryonic tissue, with the intent to improve the overall efficacy and reproducibility, and pave the way for future stem cell therapies (Barker et al., 2013).

One complication that has hampered further exploration of this therapeutic approach is the appearance of *off*-state dyskinesias, which are independent from L-DOPA administration, in a subset of grafted patient (Freed et al., 2001; Hagell et al., 2002; Olanow et al., 2003). Recent animal and human studies have suggested that inclusion of serotonergic neurons in the graft may contribute to the appearance of these so-called graft-induced dyskinesias (GID). In fact, the embryonic VM tissue used for transplantation is known to contain a variable number of serotonin (5-HT) neurons, depending on the landmarks used for dissection of the fetal tissue (Carlsson et al., 2007). In agreement, Mendez et al. (2008) have reported that the VM grafts contain large numbers of 5-HT neurons, as studied post-mortem. Moreover, positron emission tomography (PET) imaging studies have revealed the presence of an intense 5-HT hyperinnervation in the striatum of grafted patients showing GID, and administration of buspirone, a 5-HT_{1A} receptor agonist able to dampen activity of serotonin neurons, has been shown to significantly reduce GID in these patients (Politis et al., 2011; Politis et al., 2010).

Abnormal movements phenotypically similar to L-DOPA-induced dyskinesia (LID) can be seen in VM grafted rats only after administration of amphetamine (Carlsson et al., 2006; Lane et al., 2006), which is known to evoke massive DA release from grafted DA neurons (Zetterstrom et al., 1986). Thus, amphetamine-induced dyskinesia in grafted rats has become a widely used and reproducible model of GID (Carlsson et al., 2007; Garcia

et al., 2011; Lane et al., 2009a; Lane et al., 2008; Lane et al., 2009b). Using this model, we have recently shown that buspirone can produce suppression of GID in grafted rats, as seen in patients; however, we have demonstrated that this effect is independent from activation of 5-HT_{1A} receptors on serotonergic neurons, but conceivably due to blockade of DA D_2 receptors, for which buspirone is known to be a weak antagonist (Eison and Temple, 1986; McMillen et al., 1983; Rijnders and Slangen, 1993; Scuvee-Moreau et al., 1987). Indeed, buspirone could fully suppress GID even when the intrinsic serotonergic innervation was removed by a lesion with a selective toxin, and its effect was mimicked by the administration of a low dose of the selective DA D_2 receptor antagonist eticlopride (Shin et al., 2012).

The present study was designed to investigate whether the striking effect induced by DA receptor blockade in suppressing amphetamine-induced dyskinesia in grafted rats is restricted to DA D_2 receptors, or extends to DA D_1 receptors as well. Moreover, DA D_1 and D_2 receptor antagonists were also tested against LID in grafted and control dyskinetic rats to investigate whether the anti-dyskinetic effect was specific for dyskinesia induced by amphetamine, or also by L-DOPA, possibly revealing a general exacerbation of the anti-dyskinetic effect of DA receptor blockade induced by the graft.

Materials and methods

Animals

Adult female Sprague Dawley rats (225–250 g at the start of the experiment, Charles River, Sweden) were used in the present study and housed on a 12 h light/dark cycle (light on 7:00 – 19:00) with free access to food and water. All animal works were performed in accordance with regulations set by Swedish legislation 1988:543 and EU-directive 2010/63.

Drugs

All the drugs were diluted in 0.9% sterile saline and injected *s.c.* unless otherwise stated. 3-Chloro-5-ethyl-*N*-[[(2*S*)-1-ethyl-2-pyrrolidinyl)methyl]-6-hydroxy-2-methoxy-benzamide hydrochloride (Eticlopride, 0.015 and 0.03 mg/kg); (*R*)-(+)-7-Chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrochloride (SCH23390, 0.1 mg/kg, *i.p.*); 8-[4-[4-(2-Pyrimidinyl)-1-pipirazin yl]butyl]-8-azaspiro[4,5]decane-7,9-dione hydrochloride (Buspirone, 0.3 and 1 mg/kg); (*S*)-*N*-*tert*-Butyl-3-(4-(2-methoxyphenyl)-piperazin-1-yl)-2-phenylpropanamide dihydrochloride (WAY-100135, 0.4 mg/kg); 4-[2-(Dipropylamino)ethyl]-1,3-dihydro-2*H*-indol-2-one hydrochloride (Ropinirole, 0.2 mg/kg, *i.p.*); and (±)-6-Chloro-2,3,4,5-tetrahydro-1-phenyl-1*H*-3-benzazepine hydrobromide (SKF81297, 0.5 mg/kg) were purchased from Tocris Bioscience, UK. DL-Serine 2-(2,3,4-trihydroxybenzyl)hydrazide hydrochloride (Benserazide, 10 mg/kg) and 2,4,5-Trihydroxyphenethylamine hydrochloride (6-OHDA, 3.5 µg/µl free base in 0.02% L-ascorbic acid in 0.9% saline, into the medial forebrain bundle (MFB)) were purchased from Sigma-Aldrich, Sweden. L-3,4-

Dihydroxyphenylalanine methyl ester hydrochloride (L-DOPA, 6 or 12 mg/kg) and D-Amphetamine Sulphate (1.5 mg/kg, *i.p.*) were purchased from Research Organics, Cleveland, OH and Apoteksbolaget, Sweden, respectively.

Experimental design

All rats received injections of 6-OHDA unilaterally into the MFB (detailed below). Three weeks after surgery animals were injected with 2.5 mg/kg of amphetamine and the rotational behavior was measured by an automated system. Only animals exhibiting at least 3 turns/min were recruited into the study (Tronci et al., 2012). Starting a week later, L-DOPA and benserazide were injected daily for 3 weeks to establish stable LID, as measured with the abnormal involuntary movement scale (AIMs). Dyskinetic rats (total AIMs score ≥30) were split into 2 groups to receive a suspension of fetal VM cells into the lesioned striatum, or saline as control. L-DOPA injection was resumed 2 weeks post-grafting twice weekly (Lee et al., 2000). From 20 weeks post-grafting pharmacological tests were carried out including LID, GID, and DA agonists-induced dyskinesia. For GID tests, grafted rats were allocated into two groups, with equal baseline AIMs score, to receive either amphetamine only, or amphetamine plus the selected compound. For LID tests, both grafted and control rats were allocated into two groups (4 subgroups in total), with equal AIMs scores, to receive either L-DOPA only, or L-DOPA plus the selected compound. A minimum of a 3-day washout was allowed between drug tests. New baselines were taken during the course of the study to make sure of the stability of the AIMs score, both for GID and LID. Trans-cardial perfusion with 4% paraformaldehyde was performed at the end of the pharmacological studies.

Lesion surgery

Stereotaxic surgery was performed under general anesthesia, induced by *i.p.* injection (1.4 – 1.6 ml) of a 20:1 mixture of Fentanyl and Dormitor® (Apoteksbolaget, Sweden). 14 µg of 6-OHDA (3.5 µg/µl free base in 0.02% L-ascorbic acid in 0.9% sterile saline) were injected into the MFB (AP = -4.4 mm from bregma; ML = -1.2 mm from bregma; DV = -7.8 from the dura surface; Tooth bar = -2.4 mm) using a stereotaxic frame (Stoelting, Wood Dale, IL). 4 µl were injected over 4 min and the Hamilton syringe was kept in place for an additional 3 min before retracted slowly. Antisedan® (0.28 mg/kg, *s.c.*, Apoteksbolaget, Sweden) was injected to reverse sedative effects of anesthetics and Temgesic® (0.04 mg/kg, *s.c.*, Apoteksbolaget, Sweden) to relieve pain after the surgery.

Cell preparation and Transplantation surgery

The conventional VM cut was made from E14 rat embryonic brain, as described previously (Carlsson et al., 2007; Kirik et al., 2001; Nikkhah et al., 1994; Winkler et al., 1999). In this dissection, the caudal cut of VM was made slightly caudal of the isthmus to include the rostral part of the pontine raphe region.

Dissected tissues were incubated in Dulbecco's modified eagle medium (DMEM, Invitrogen, Sweden) containing 0.1% trypsin (Sigma-Aldrich, Sweden) and 0.05% DNase (Sigma-Aldrich, Sweden) for 20 min at 37 °C and then mechanically dissociated into a single cell suspension. After centrifugation (500*g*, 5 min, 4 °C), viable cell numbers were estimated with trypan blue staining (Sigma-Aldrich, Sweden) and cells were re-suspended in DMEM/DNase at a concentration of 130,000 cells/µl. Prepared cells were kept at room temperature until grafted.

Transplantation surgery was performed under general anesthesia, as used for 6-OHDA lesion surgery. 130,000 cells were grafted into each lesioned striatum at the following stereotaxic coordinates: AP = +0.2 mm from bregma; ML = -3.5 mm from bregma; DV = -5.0 and -4.0 mm from the dura surface; Tooth bar = 0.0. To give less damage to the brain and donor cells, a glass capillary (outer diameter 60 – 80 μm) was fitted onto the needle of a Hamilton syringe (Nikkhah et al., 1994). Half of the cell suspension was injected at each of the two sites of deposit over 1 min, with 1 min waiting time before the second deposit. Left-over cells were subjected to estimation of cell viability using trypan blue exclusion, which was > 95%.

Abnormal Involuntary Movements tests

L-DOPA-induced dyskinesia

In order to induce stable AIMs, L-DOPA (6 mg/kg) together with the peripheral DOPA-decarboxylase inhibitor, benserazide (10 mg/kg), was dissolved in 0.9% sterile saline and injected (*s.c.*) daily for 3 weeks. AIMs were evaluated according to the rat dyskinesia scale described in detail previously (Lee et al., 2000; Winkler et al., 2002). Briefly, the animals were placed individually in transparent plastic cages without bedding material and scored every 20 min following the injection of L-DOPA until no AIMs were observed. The severity of the dyskinetic movements were scored as follows: 0, absent; 1, occasional (i.e. present less than 50% of the observation time); 2, frequent (i.e. present more than 50% of the observation time); 3, continuous but interrupted by strong sensory stimuli; 4, continuous and not interrupted by strong sensory stimuli. Scores were given in four subtypes of AIMs according to their topographic distribution as forelimb, orolingual,

axial and locomotor behaviors. The forelimb and orolingual dyskinesia are predominantly seen as hyperkinesia whereas the axial dyskinesia is often of a dystonic type. Enhanced manifestations of normal behaviors such as grooming, gnawing, rearing and sniffing were not included in the rating. Total AIMs score was calculated by combining forelimb, orolingual and axial dyskinesia scores.

Amphetamine-induced dyskinesia

In order to evaluate graft-induced dyskinesia, amphetamine (1.5 mg/kg in 0.9% sterile saline) was administered *i.p*. and AIMs were monitored every 20 min following the drug injection, until the signs of AIMs had subsided, using the same rating scale as for LID (Carlsson et al., 2006). 72 hr of washout between the two tests was allowed to avoid possible carry-over effects.

Ropinirole/SKF81297-induced dyskinesia

To investigate DA D_2 and D_1 receptor supersensitivity, ropinirole (0.2 mg/kg in 0.9% sterile saline, *i.p.*) and SKF81297 (0.5 mg/kg in 0.9% sterile saline, *s.c.*) was administered and AIMs were scored using the same scale as for LID. Rats were observed every 10 min for ropinirole and 20 min for SKF81297 following the drug injection until no sign of AIMs was seen.

Histological analysis

Perfusion

At the end of the experiment animals received *i.p.* injection of sodium pentobarbitone (60 mg/kg, Apoteksbolget, Sweden) and were trans-cardially perfused with 100 ml of 0.9%

saline followed by 250 ml of ice-cold paraformaldehyde (4% in phosphate buffered saline, Sigma-Aldrich, Sweden). The brains were removed and post-fixed for 2 hr in the same fixative before cryo-protection in 25% sucrose in phosphate buffered saline overnight.

Immunohistochemistry

Cryo-protected brains were cut coronally at 40 µm thickness in 6 series using a freezing slide-microtome (Leica) and free-floating sections were quenched for 15 min with 3% H_2O_2 and 10% methanol in potassium-phosphate buffered saline (KPBS). Sections were incubated with blocking solution (5% normal goat serum and 0.25% tritonX-100 in KPBS) for 1 hr followed by primary antibodies (rabbit anti-tyrosine hydroxylase (TH), AB152, Chemicon, 1:1000) in blocking solution overnight at room temperature. Next day, incubation in blocking solution with secondary antibodies (goat-anti rabbit, BA 1000, Vector Laboratories, 1:200) for 1 hr at room temperature was followed by streptavidin-biotin complex solution (ABC Elite, Vector Laboratories) for 1 hr. The visualization of the primary and secondary antibodies was carried out by peroxidase (0.01% H_2O_2) driven precipitation of di-amino-benzidine. The sections were mounted onto subbed slides and air-dried overnight before being dehydrated and coverslipped using DePeX mountant (BDH Chemicals, UK).

Cell counting

Due to their small size and irregular shapes of the grafts, stereological counting procedure was avoided. Instead, all the TH^+ cells were counted on every 6^{th} coronal section of the

grafts as reported previously (Grealish et al., 2010; Shin et al., 2012). Total numbers of cells positive for TH were estimated by correcting for double counting caused by cells spanning more than one section (Abercrombie, 1946).

Statistical analysis

All data are expressed as mean ± the standard error of the mean. Statistical significance was set at $p<0.05$. When comparing two treatments on two groups, two-way ANOVA test with Bonferroni post hoc test were used (Fig. 3 and Fig 5B). Otherwise two-tailed unpaired t test was used to find statistical differences between two groups. Moreover, non-parametric test (Mann-Whitney test) was applied to confirm the statistical significance. All statistics in this study were performed using Prism 5 for Mac OS X, version 5.0c (GraphPad Software, Inc.).

Results

6-OHDA-lesioned rats were primed with daily injections of L-DOPA (6 mg/kg, plus benserazide, *s.c.*) until a stable expression of LID was achieved, and then split into 2 groups (with equal AIMs) to be subjected to a striatal injection of either VM cells (DA group, n=16), or saline as control (n=11).

Effect of VM graft on LID and GID

Grafted and control rats were injected with L-DOPA twice weekly during maturation of the graft. Twenty weeks after grafting animals were scored for LID. As shown in Fig. 1A, grafted rats had significantly lower LID (about 33% less) compared to controls. Animals were then tested for GID after amphetamine administration; as shown in Fig. 1B, grafted rats presented severe dyskinesia (the average of three tests is presented), while no single involuntary movement was induced in control rats.

These data suggest that a significant number of DA cells survived in the host striatum of grafted animals, providing a buffering system for the exogenously administered L-DOPA, as well as a significant releasable pool of DA upon amphetamine administration.

Effect of DA D_1 and D_2 receptor blockade on GID

We have recently shown that blockade of DA D_2 receptor by the highly selective D_2 receptor antagonist eticlopride suppressed GID at doses that were ineffective against LID in control rats (Shin et al., 2012). Therefore, we first tested whether similar effect could

be reproduced in this study, where animals were significantly more dyskinetic upon amphetamine administration than the ones employed in the former investigation (average GID score 49 vs 22 for the present and previous study, respectively). The same dose of buspirone (0.03 mg/kg *s.c.*) used in Shin et al., 2012 was initially tested in this group of rats, and completely suppressed GID without any sign of motor suppression (data not shown), as previously reported. Moreover, as shown in Fig. 2A, a single injection of eticlopride at a dose as low as 0.015 mg/kg produced near-maximal suppression of GID. We therefore investigated whether similar effect could be produced by DA D_1 receptor blockade. Thus, the selective DA D_1 receptor antagonist SCH23390 was given at 0.1 mg/kg dose, which was found not to affect LID in control rats in preliminary tests (not shown). Similar to eticlopride, SCH23390 was found to produce an almost complete suppression of GID (Fig. 2B).

These results suggest that dyskinesia induced by amphetamine can be efficiently suppressed by doses of both DA D_1 and D_2 receptor antagonists that are known not to affect LID in control rats.

Effect of DA D_1 and D_2 receptor blockade on LID in grafted and control rats

As the GID seen in the amphetamine-treated grafted rats appeared to be strikingly sensitive to D_1 and D_2 receptor blockade, we next investigated whether this effect was specific for dyskinesia induced by amphetamine, or rather, could have been due to an increased response of the host striatum to DA receptor blockade, due to the presence of the graft. If so, a similar inhibitory effect against LID would be obtained with either

eticlopride or SCH23390 in the grafted rats, but not in the non-grafted controls. In fact, eticlopride and SCH23390 produced near complete suppression of LID in grafted rats but were ineffective in control animals (Fig. 3A and B). The same suppression was seen in grafted animals even after doubling the L-DOPA dose (12 mg/kg), which produced a degree of LID similar to the one seen after 6 mg/kg in control rats (Fig. 4A and B).

These results demonstrate that increased anti-LID effect of D_1 and D_2 receptor antagonists is only seen in the transplanted animals but not in controls.

Effect of buspirone on LID and GID in grafted and control rats

Politis and co-workers have demonstrated that the partial 5-HT_{1A} agonist buspirone can produce significant dampening of GID in transplanted patients, suggesting that 5-HT neurons may be implicated in the aetiology of GID, as previously demonstrated for LID. In our recent study (Shin et al., 2012), we have shown that buspirone induced complete suppression of GID also in the rat model. However, we demonstrated that this effect was not due to 5-HT_{1A} receptor activation, as neither was prevented by removal of 5-HT innervation, nor by pre-treatment with the selective 5-HT_{1A} antagonist WAY100635. As buspirone has a low antagonistic affinity (under physiological conditions) for the DA D_2 receptor, and its effect was mimicked by eticlopride, we have suggested the anti-GID effect of buspirone could, at least in part, be due to blockade of DA D_2 receptors (Shin et al., 2012).

To investigate whether the anti-dyskinetic effect of a low dose of buspirone is restricted to GID, or may also affect LID in grafted rats, transplanted and control animals were

injected with buspirone after treatment with either amphetamine or L-DOPA. Buspirone 1 mg/kg produced complete suppression of GID (data not shown) without inducing any sign of motor immobility, as seen previously (Shin et al., 2012). Moreover, significant antidyskinetic effect was also seen for a dose of buspirone as low as 0.3 mg/kg (Fig. 5A). Interestingly, as seen with D_1 and D_2 receptor antagonists, buspirone (0.3 mg/kg) could significantly affect LID in grafted but not in control rats (Fig. 5B). The anti-GID and anti-LID effect of buspirone was not antagonized by pre-treatment with the selective 5-HT_{1A} antagonist WAY100635 (not shown).

Taken together, these results suggest that the anti-dyskinetic effect of a low dose of buspirone against GID and LID in grafted rats is not due to 5-HT_{1A} receptor activation but likely due to DA D_2 receptor blockade.

Effect of DA D_1 and D_2 receptor activation in grafted and control rats

In order to investigate the sensitization state of the DA D_1 and D_2 receptors, grafted and control rats were challenged with the D_1 and D_2 receptor agonist ropinirole (0.2 mg/kg i.p.), and SKF81297 (0.5 mg/kg s.c.), respectively. As already seen with L-DOPA, both ropinirole and SKF81297 produced significantly less dyskinesia in grafted compared to control rats (Fig. 6A and B).

These results suggest that the DA grafts partially normalize the sensitization state of the DA receptors in the host striatum.

Histological analysis of graft survival

After pharmacological manipulations, rats were allowed to have a washout period and trans-cardially perfused with a fixative. Fixed and cryo-protected brains were cut and subjected to TH immunohistochemistry. TH^+ cells were counted and adjusted with Abercrombie method. Total estimation of TH^+ cells in the grafts is 2451±477. This number is higher than what found previously (Shin et al., 2012); this may explain the more severe GID shown by the animals of this study compared to the previous one.

Discussion

In this study we demonstrate for the first time that fetal VM grafts induce a striking enhancement of the anti-dyskinetic effect induced by DA D_1 and D_2 receptor blockade, as seen in either L-DOPA- or amphetamine-treated 6-OHDA-lesioned rats. Indeed, this effect was observed at doses of the DA D_1 receptor antagonist SCH23390 and DA D_2 receptor antagonist eticlopride that were far lower than the ones required to affect LID in control (non-grafted) L-DOPA-primed rats. Interestingly, a similar effect on LID and GID was seen in transplanted rats after administration of buspirone at a low dose (0.3 mg/kg). Buspirone is known to be a partial 5-HT_{1A} receptor agonist, with lower affinity for the DA D_2 receptor, at least under physiological conditions. Previous studies have shown the ability of buspirone to reduce LID in 6-OHDA lesioned rats, albeit at a dose range of 2-4 mg/kg (Dekundy et al., 2007), which is in agreement with the increasing body of evidence pointing to a key role of 5-HT neurons in the induction of LID. In fact, 5-HT neurons convert exogenously administered L-DOPA to DA and mediate its un-

regulated synaptic release, contributing to pulsatile stimulation of striatal DA receptors (Carta et al., 2007). However, we have recently demonstrated (Shin et al., 2012), and confirmed here, that the anti-dyskinetic effect of buspirone in grafted rats is not due to activation of 5-HT_{1A} receptors, as neither is abolished by toxin lesion of 5-HT neurons, nor by administration of the selective 5-HT_{1A} antagonist WAY100635. This may have important clinical implications, as buspirone has recently been shown to produce significant reduction of GID in transplanted PD patients (Politis et al., 2010). Given that grafted patients showing severe GID were also found to present a pronounced striatal serotonergic hyperinnervation in the grafted putamen, as revealed by PET scanning, Politis and co-workers suggested that the anti-GID effect of buspirone in their patients was likely due to dampening of 5-HT neuron activity (Politis et al., 2010). By contrast, our experimental data suggest that the potent anti-dyskinetic effect produced by buspirone after either amphetamine or L-DOPA administration in grafted rats, may be due to its antagonistic activity on DA D_2 receptors. Thus, grafted rats appeared to become more sensitive to the antagonistic activity of buspirone on DA D_2 receptors, as suggested by the test with eticlopride. However, the data of the present study do not exclude a possible contribution of the 5-HT system in the appearance of GID. In fact, we have previously shown that the 5-HT releaser fenfluramine worsened amphetamine-induced dyskinesia in grafted rats, while combination of highly selective 5-HT_{1A} and 5-HT_{1B} receptor agonists (known to dampen activity of serotonin neurons) produced a significant reduction (Shin et al., 2012). Nevertheless, our results reveal an intriguing alteration in the response of grafted rats to DA receptor blockade.

Clinical investigations are required to understand if these results are predictive of a similar outcome in grafted patients. In this regard, it is interesting to note that the effect of buspirone against GID, as seen in transplanted patients, was more pronounced than what previously reported for LID in non-grafted PD patients. In this case buspirone has been shown to be either marginally efficacious, or not effective at all (Bonifati et al., 1994; Kleedorfer et al., 1991). If the anti-GID effect of buspirone in patients is also due to DA D_2 receptor blockade, low doses of selective DA receptor antagonists should produce similar results, and provide suppression of GID, at doses that may be significantly lower than the ones needed to affect LID in non-grafted patients.

While intriguing, the exacerbated effect of DA receptor blockade appears paradoxical; in fact, sensitivity of D_1 and D_2 receptors seems partially normalized by the graft, as revealed by the reduction of LID, and the lower dyskinesia induced by direct D_1 and D_2 receptor agonists in grafted- compared to non-grafted control rats. We hypothesize that DA receptor blockade may unmask compensatory or maladaptive mechanisms that develop in the host striatum during chronic exposure to graft-derived DA. Such alterations may involve altered expression of DA receptors at synaptic membranes and/or modification of DA receptor signalling cascade. In fact, reduction in the number or function of synaptic DA receptors may explain both the reduced LID, and the increased antidyskinetic effect of DA receptor antagonists, as fewer (or less functional) receptors may be easier to inhibit. However, further work is required to verify this hypothesis.

A better understanding of the long-term effects induced by DA cell transplantation in the host parkinsonian brain is instrumental to clarify the mechanisms underlying the

appearance of GID in grafted patients, and to allow further clinical investigation of cell-therapy for the treatment of PD.

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Figure legends

Fig. 1. L-DOPA-induced and amphetamine-induced dyskinesia in grafted and control rats. (A) 20 weeks after the transplantation, grafted rats showed significantly less AIMs than control rats when treated with 6 mg/kg L-DOPA (plus benserazide) (Two-tailed unpaired t test, $p<0.001$; Mann-Whitney test, $p<0.001$). (B) Upon *i.p.* injection of amphetamine (1.5 mg/kg) all of the grafted rats showed marked AIMs, while none of the control animals showed any signs of AIMs.

Fig. 2. Effect of low doses of DA D_2 and D_1 receptor antagonists on amphetamine-induced dyskinesia. Eticlopride (0.015 mg/kg) (A) and SCH23390 (0.1 mg/kg) (B) significantly decreased amphetamine-induced dyskinesia in grafted rats (Two-tailed unpaired t test, $p<0.001$ for both tests; Mann-Whitney test for eticlopride, $p<0.01$ and for SCH23390, $p<0.001$).

Fig. 3. Effect of low doses of DA D_2 and D_1 receptor antagonists on L-DOPA (6 mg/kg)-induced dyskinesia in control and grafted rats. Eticlopride (0.015 mg/kg) (A) and SCH23390 (0.1 mg/kg) (B) significantly diminished L-DOPA-induced dyskinesia in grafted rats, whereas the same doses were not able to affect AIMs in control animals (Two-way ANOVA and Bonferroni post hoc tests were used. For Eticlopride, Group: $F_{1,20}$=145.4, $p<0.0001$; Treatment: $F_{1,20}$=34.4, $p<0.0001$; GroupxTreatment: $F_{1,20}$=8.0, $p<0.05$. ***, $p<0.001$ compared to LD in grafted rats; Mann-Whitney test for control group, weak significance was found ($p=0.03$). For grafted group, $p<0.01$; for SCH23390, Group: $F_{1,20}$=59.4, $p<0.0001$; Treatment: $F_{1,20}$=9.0, $p<0.01$; GroupxTreatment: $F_{1,20}$=6.3, $p<0.05$. ***, $p<0.001$ compared to LD in grafted rats; Mann-Whitney test for control group, $p>0.05$ and for grafted group, $p<0.001$).

Fig. 4. Effect of low doses of DA D_2 and D_1 receptor antagonists on L-DOPA (12 mg/kg)-induced dyskinesia in grafted rats. Eticlopride (0.015 mg/kg) (A) and SCH23390 (0.1 mg/kg) (B) could still significantly diminish AIMs in grafted rats treated with 12 mg/kg L-DOPA (Two-tailed unpaired t test, $p<0.001$ for both tests; Mann-Whitney test for eticlopride, $p<0.01$ and for SCH23390, $p<0.001$).

Fig. 5. Effect of a low dose of buspirone on amphetamine and L-DOPA-induced dyskinesia. Buspirone (0.3 mg/kg) significantly reduced AIMs evoked by both amphetamine (A) (Two-tailed unpaired t test, $p<0.001$, Mann-Whitney test, $p<0.01$) and L-DOPA (B) in grafted rats but not in controls (Two-way ANOVA and Bonferroni post hoc tests were used. Group: $F_{1,20}$=167.6, $p<0.0001$; Treatment: $F_{1,20}$=18.4, $p<0.001$; GroupxTreatment: $F_{1,20}$=1.7, $p>0.05$. ***, $p<0.001$ compared to LD in grafted rats; Mann-Whitney test for control group, $p>0.05$ and for grafted group, $p<0.01$).

Fig. 6. DA D_2 and D_1 receptor agonist-induced dyskinesia. Ropinirole (0.2 mg/kg) (A) and SKF81297 (0.5 mg/kg) (B) evoked significantly less dyskinesia in grafted rats compared to controls (Two-tailed unpaired t test, $p<0.01$ for both tests; Mann-Whitney test for ropinirole, $p<0.001$ and for SKF81297, $p<0.01$).

Fig. 7. Graft histology. A representative TH staining showing a VM graft at two different magnifications. The number shown is the mean number of cells in the graft ± SEM.

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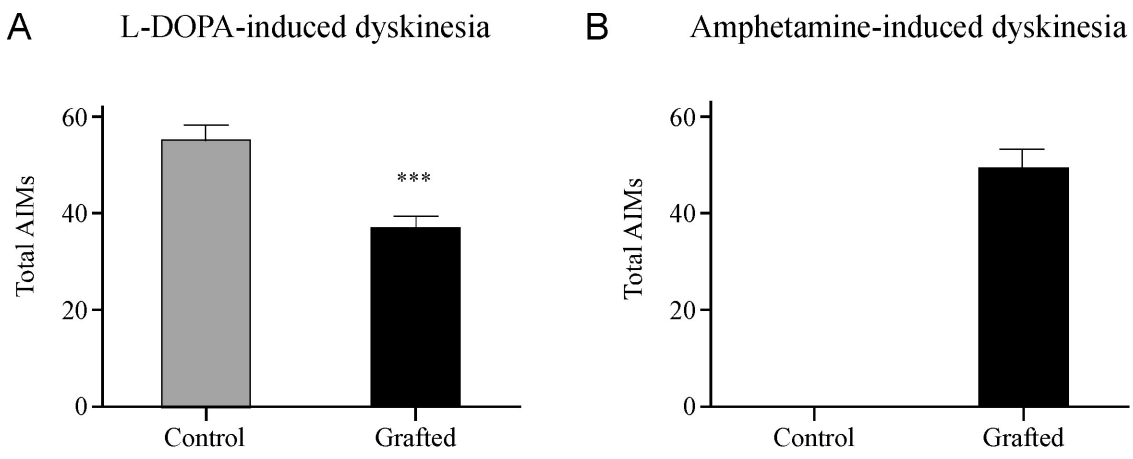
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***
Control
Grafted
B
Amphetamine-induced dyskinesia
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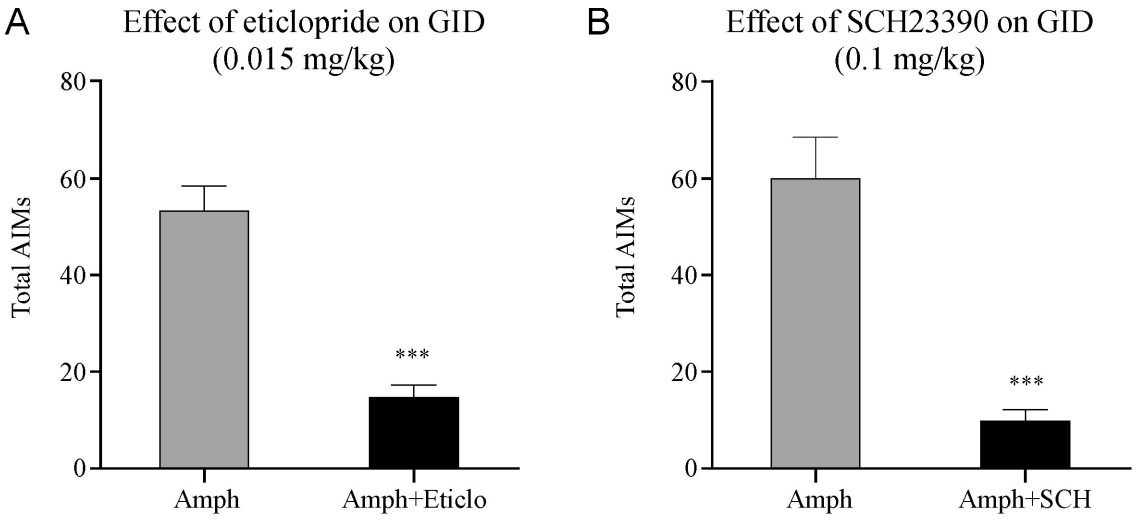
40
20
0
Control
Grafted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40
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***
Amph
Amph+Eticlo
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Effect of SCH23390 on GID
(0.1 mg/kg)
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Amph
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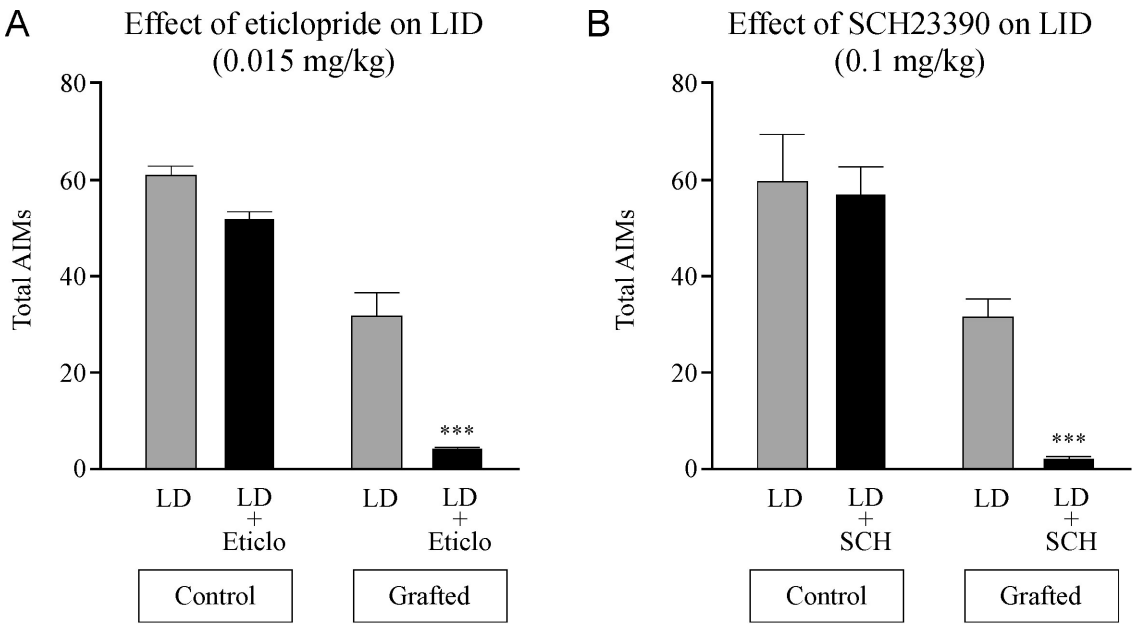
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***
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LD + Eticlo
LD
LD + Eticlo
Control
Grafted
B
Effect of SCH23390 on LID
(0.1 mg/kg)
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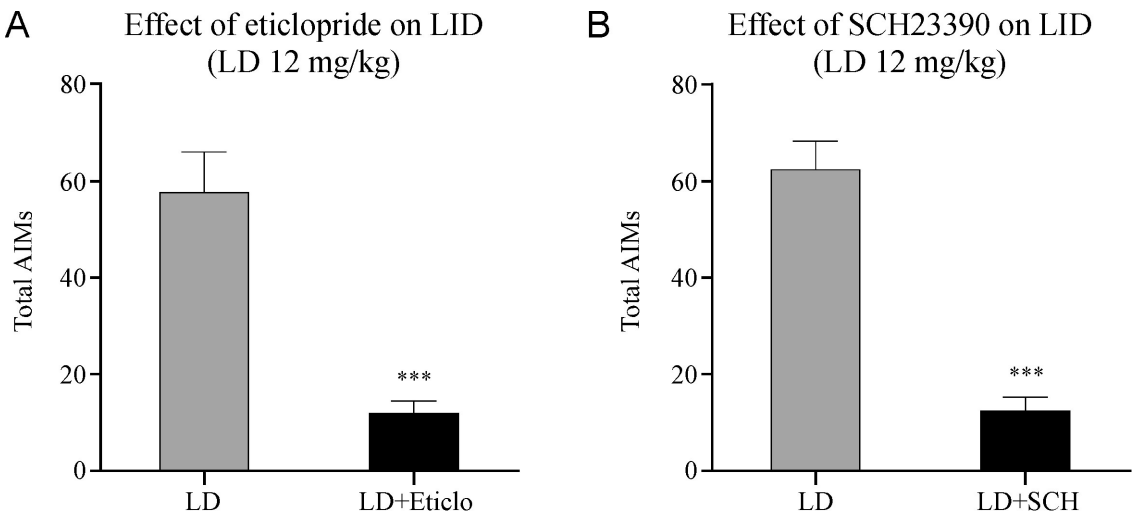
***
LD
LD + SCH
LD
LD + SCH
Control
Grafted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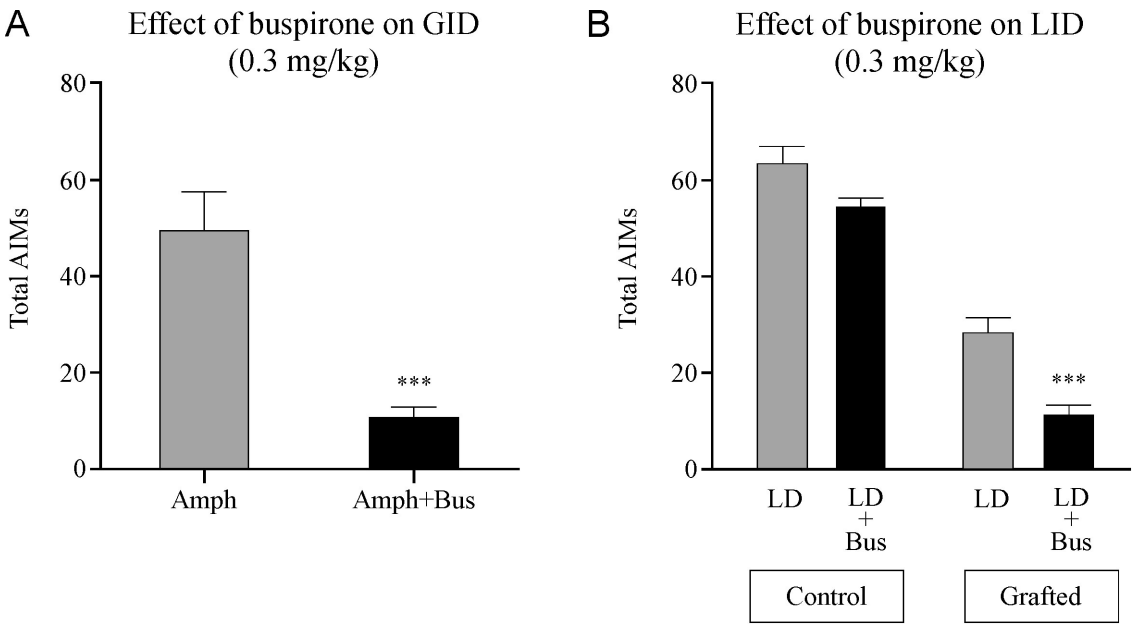
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***
Amph
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Effect of buspirone on LID
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LD
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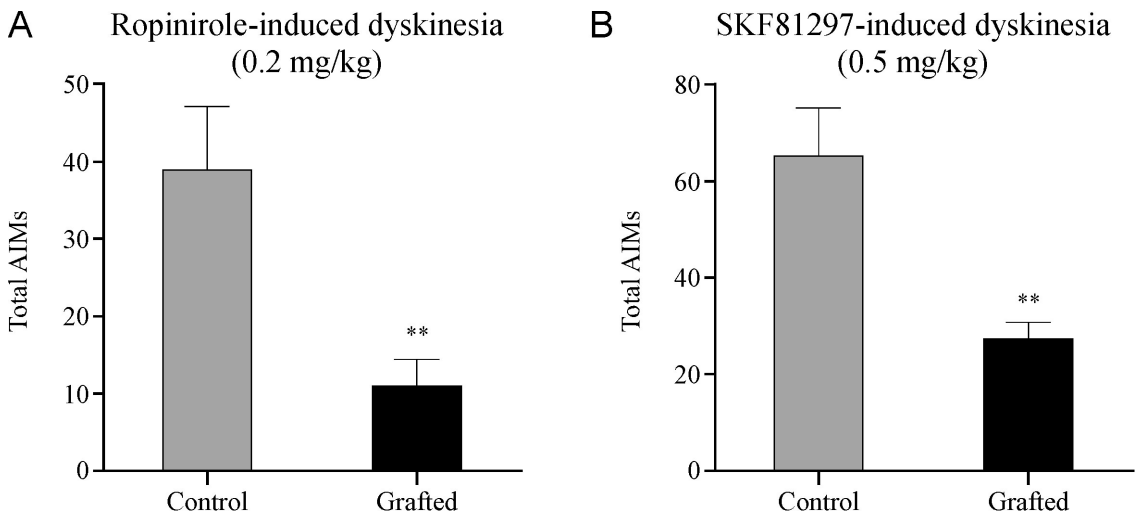
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Control
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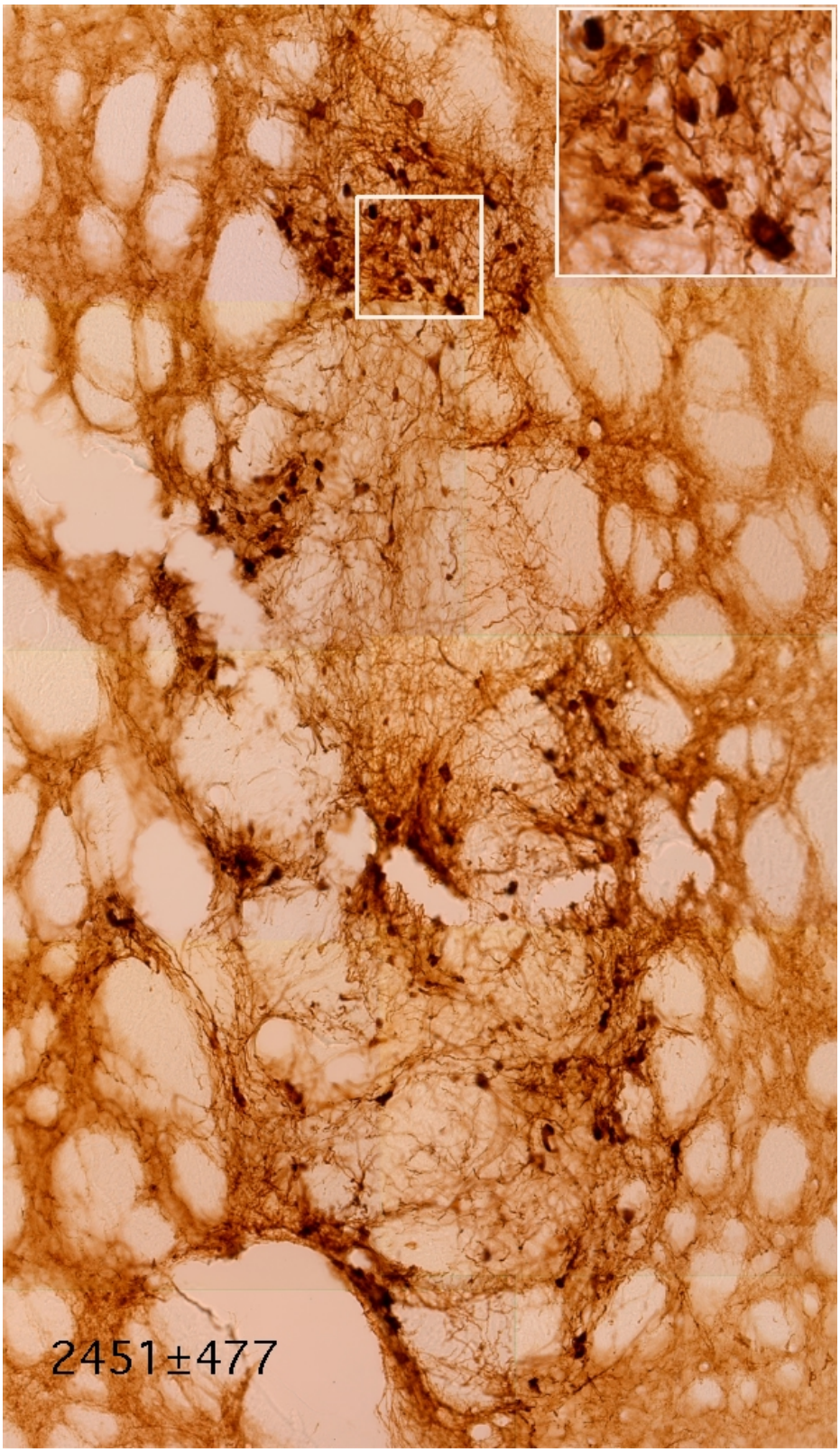
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