Neuroprotection by safinamide in the 6-hydroxydopamine model of Parkinson's disease

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Abstract

Aims: Current therapies in Parkinson's disease mainly treat symptoms rather than provide effective neuroprotection. We examined the effects of safinamide (monoamine oxidase B and sodium channel blocker) on microglial activation and the degeneration of dopaminergic neurons in a rat model of PD *in vivo*, and on microglia *in vitro*. **Methods:** Rats received unilateral stereotaxic injection of 6-hydroxydopamine into the medial forebrain bundle on day 0: the contralateral side served as control. Safinamide or vehicle were delivered from days 0 or 1, for 7 days, via sub-cutaneous mini-pumps. **Results:** In vehicle-treated rats 6-hydroxydopamine caused a significant increase in the number of activated MHC-II$^+$ microglia compared with the contralateral side, and only 50% of the dopaminergic neurons survived in the ipsilateral SNc. In contrast, rats treated daily with safinamide 50 and 150 mg/ml (on day 0 or 1) exhibited a significantly reduced number of activated microglia (55% reduction at 150 mg/ml) and a significant protection of dopaminergic neurons (80% of neurons survived) ($p<0.001$) compared with vehicle-treated controls. Rasagiline, a monoamine oxidase B inhibitor, and lamotrigine, a sodium channel blocking drug, also protected dopaminergic neurons, indicating that safinamide may act by either or both mechanisms. Safinamide also reduced the activation of microglial cells in response to lipopolysaccharide exposure *in vitro*. **Conclusion:** Safinamide therapy suppresses microglial activation and protects dopaminergic neurons from degeneration in the 6-hydroxydopamine model of PD, suggesting that the drug not only treats symptoms, but also provide neuroprotection.

Introduction

Degeneration of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNc) is one of the pathological hallmarks of Parkinson’s disease (PD). Multiple factors including: oxidative stress [1], excitotoxicity [2], mitochondrial dysfunction [3] and inflammation [4] may be involved in the initiation and progression of pathology in PD, so drugs with multiple modes of action may be of particular value for neuroprotective therapy.Safinamide is approved in the EU for use in combination with dopamine agonists as a symptomatic therapy in PD. Safinamide's pharmacological activities include selective blockade of state-dependent voltage-gated sodium channels [5], inhibition of induced glutamate release *in vitro* and reversible inhibition of monoamine oxidase-B (MAO B) [6]. This pharmacological profile has suggested possible symptomatic benefits in PD, by increasing DA availability through inhibition of its metabolism by brain MAO-B [6], and neuroprotective benefits through reduction of sodium influx and excitotoxic glutamate release [6]. More recently there has been an appreciation of the role of blockers of voltage gated sodium channels on the activation of microglia [7][8], combined with the demonstration that safinamide can markedly reduce microglial activation and neuroinflammation in animal models of multiple sclerosis [9]. These observations suggest an additional potential role for safinamide in the therapy of PD, and other neurodegenerative diseases with significant microglial involvement. In this report we investigate the effects of safinamide in a widely used animal model of PD in which both neuroinflammation and neurodegeneration are induced in the SN by focal delivery of the neurotoxin 6-hydroxydopamine (6-OHDA). *In vitro* studies with purified microglia were used to establish a direct effect of safinamide on these cells. The results suggest that both the sodium channel and MAO-B activities of safinamide may contribute to a unique profile of efficacy, and that safinamide therapy protects against neurodegeneration, in addition to any symptomatic effects.

Methods

6-OHDA lesion induction

Male Sprague–Dawley rats (Harlan Ltd, UK), 200-230 g on the day of surgery, were housed five rats per cage and kept on a 12-h light/dark cycle, with food and water *ad libitum*. On arrival animals were allowed to acclimatize to the new environment for seven days before any experiments were conducted. Lesions were induced by stereotactic injection of 6-OHDA–hydrogen bromide free base (12 μg in final injection volume of 4 μl in 0.1% ascorbic acid/saline solution) into the left medial forebrain bundle (mfb), under isoflurane-anesthesia as previously described [10] using the following coordinates for the location of the mfb: 2.2 mm posterior, 1.5 mm lateral from bregma, and 7.9 mm ventral to the dura, according to the brain atlas of Paxinos and Watson (1986). The contralateral side served as an uninjected control. All experiments were performed in accordance with the UK Home Office Animals (Scientific Procedures) Act (1986) and approved by the local ethics committee (University College London).

Drug treatment regimens

Two treatment regimens were employed. For the first study, animals received a stereotactic injection of 6-OHDA into the left mfb as described above. The animals were divided randomly into three groups (n=6-8 per group) and, to ensure consistent drug levels, subcutaneous osmotic mini-pumps were implanted (delivery rate: 10 μl/hr, 7 days duration) (ALZET, Cupertino, CA), containing safinamide 50 or 150 mg/ml, or distilled water as vehicle (calculated delivery of 12 mg/day or 36 mg/day). Drug/vehicle release from the pumps is reported by the manufacturer to start 24 hours after implantation. The doses were chosen based on previous assays of the blood serum concentration (1.4-13.5 μM) of the drug following administration of safinamide by minipump in rats for 7 consecutive days in which it was observed that the drug reduced neuroinflammation and provided neuroprotection in another model when plasma levels were maintained above 10 μM [9], consistent with a sodium channel blocking mechanism (IC50 2-10 μM). These doses are clinically efficacious and tolerated in patients [6] . Safinamide/vehicle treatment (subcutaneous osmotic mini-pumps as before) was administered for seven consecutive days starting on the same day (day 0) as 6-OHDA lesion induction, and on day eight after 6-OHDA lesion induction the animals were killed under terminal anaesthesia by perfusion fixation. A seven-day dosing regimen after 6-OHDA lesioning was chosen because this duration covers the period over which the majority of DA neurons degenerate, and a high number of activated microglial cells are observed [10]. To validate the findings the first study (see above) was repeated.

For the second study, animals received a stereotactic injection of 6-OHDA into the left mfb as described above, and they were then divided randomly into two groups (n=6 per group). On the following day (day 1) osmotic mini-pumps (seven day duration, Alzet, Cupertino, CA) containing safinamide 150 mg/ml and/or vehicle were implanted sub-cutaneously and treatment was continued for seven consecutive days prior to sacrifice on day nine after 6-OHDA lesion induction. This study was designed to start safinamide treatment after a delay of 48 hours, to rule out possible drug interaction with 6-OHDA (which has a short half-life due to rapid oxidation). The dose of safinamide was chosen based on our finding from the first experiment (see results).

Safinamide combines activity against both MAO B and sodium channels, and so to determine whether the effects of safinamide in the preceding studies could be ascribed to one or the other mechanism a third experiment was performed to compare the results obtained above with those obtained from dosing with the MAO B inhibitor rasagiline, and, in a separate group of animals, with the sodium channel blocking agent lamotrigine. Male SD rats (200-230 g, n=18) were injected on day zero with 6-OHDA into the medial forebrain bundle as described above. The animals were divided randomly into three equal groups and, on day two, therapy was initiated with rasagiline (1 mg/kg one loading dose p.o., followed by 0.5 mg/kg, p.o. once daily for seven consecutive days (see [11]). To select a therapeutically relevant dose for rasagiline reference was made to human therapeutic and animal model data. Because of the irreversible mode of inhibition of rasagiline on monoamine oxidases, a low daily dose is used to accumulate significant inhibition of MAO-B without troublesome effects on MAO-A. Thus the recommended dose in PD therapy is 1 mg per day (approximately 0.015 mg/kg). In several reports on studies in rat models rasagiline has been reported affect biochemical and cellular processes in the CNS at doses ranging from 0.05 mg/kg/day [12] through 0.5 mg/kg/day [13], to 1 and 3 mg/kg/day [14]. Lamotrigine (10 mg/kg p.o. once daily for seven days) or vehicle (0.5 % methylcellulose p.o. once daily for seven days). All animals were killed by perfusion fixation (see below) while under deep terminal anaesthesia 9 days after the 6-OHDA lesion induction. The doses selected for lamotrigine and rasagiline were based on previous reports showing neuroprotective properties in rodent models of PD and clinically relevant and safe circulating concentrations in patients [15,16]. The adoption of different routes of drug administration was necessary due to lack of comparable data linking dose and circulating concentration for the different agents.

Tissue fixation and collection

At the end of each individual experiment animals were perfused transcardially with cold, phosphate-buffered saline (PBS 0.1M) followed by perfusion fixation with cold (4ºC) paraformaldehyde (4% in 0.1M PBS, pH 7.4). Brains were transferred to 4% paraformaldehyde solution (4% in 0.1M PBS, pH 7.4) and kept for 24-48 hours prior to cryoprotection in sucrose solution (30% in 0.1M PBS, containing sodium azide (0.1%) as preservative) until the block sank (48-72 hours). Coronal sections (20 µm thick) were cut throughout the entire SN using a cryostat and collected as free floating specimens.

Immunohistochemical assessment of dopaminergic neuronal degeneration and microglial activation

Dopaminergic neurons were identified by the immunohistochemical detection of tyrosine hydroxylase (TH), as described previously [10]. Briefly, endogenous peroxidase activity in the nigral free-floating sections was blocked by incubation in H_2O_2 (1%) for 30 min. Nonspecific binding was blocked by incubation in normal goat serum (20%, diluted in PBS containing 0.1% Triton X-100) for 1 hr (Sigma, UK). Sections were subsequently incubated in rabbit polyclonal anti-TH antibody (Pel Freez, USA) diluted 1:1000 in normal goat serum (2% in 0.1% Triton X-100) overnight at room temperature. After washing with PBS with 0.1% Triton X-100, sections were incubated with biotinylated secondary antibody (anti-rabbit IgG; Sigma UK) diluted 1:200 in PBS, 0.1% Triton, for 1.5 h. After subsequent washes in PBS with 0.1% Triton, sections were incubated for 45 min in avidin biotin complex diluted 1:200 in PBS (Vectastain Elite ABC kit; Vector Laboratories, UK). Bound antibody was visualized utilizing 3, 3′-diaminobenzidine and hydrogen peroxide substrate. Activated microglia were distinguished by the expression of major histocompatibility complex II (MHC-II, OX-6 labeling), using a similar protocol to that described for TH, with the following amendments. Non-specific binding was blocked with normal horse serum (5% in PBS), and the monoclonal antibody directed against OX-6 (Abcam, UK) was made up in normal horse serum (1%) (Sigma) in a dilution of 1:750. The biotinylated secondary antibody was rat-adsorbed mouse monoclonal (Sigma).

Image capture and cell counts

Cell counts were conducted manually using photomicrographs prepared using a light microscope connected to a Nikon D300 camera at 10x (objective) magnification. To ensure non-biased and complete sampling of the entire SNc, nucleated TH-immunopositive neurons without a shrunken cell body were manually counted in both ipsilateral and contralateral hemispheres of the brain in each of the stereotactic regions of the SNc (−4.80, −5.30, −5.60, −5.80 and −6.30 mm from bregma) [17]. The stereotactic region in each section was determined by examination of the shape and distribution of TH immunoreactive neurons at low magnification with reference to a rat brain atlas. For consistency, and to minimize bias, one blinded operator performed all the analysis and was tested for the consistency of counting at each stereotactic level of the SNc. For each animal, 8-10 sections were counted at each of approximately five stereotactic levels, and the average number of neurons per section was determined. These averages were pooled to give a mean cell count per section per group, for the ipsilateral and the contralateral side. The same approach was utilized to count microglial cells in each group.

Statistical analysis of the data

The data are presented as mean ± SEM. Statistical significance was calculated by two-way analysis of variance (ANOVA) and post-tested with post-*hoc* parametric Newman-Keuls Multiple Comparison test using GraphPad Prism v5.00 (GraphPad software, USA) software. Differences within and between groups were considered statistically significant at p <0.05.

In vitro **studies**

Animals and materialsSprague Dawley rat pups (5-6 day old) were bred and reared in house from stock animals (Charles River UK, Kent, UK), and were sacrificed in accordance with schedule 1 of the Animals Scientific Procedures Act (1986) UK. Primary cultures of rat microglia and primary cultures of mixed glia-cerebellar granule neurons were prepared and seeded as previously described [18].The cells were used after 1 day *in vitro* (DIV; microglia) or at least 7 DIV (glial-neuronal cultures). Cell culture materials were from Invitrogen (Paisley, UK), FITC-conjugated isolectin B4 (ILB4) a marker of microglia [19], was from Sigma and anti-arginase-1, a marker for the M2 microglial phenotype characterized as a protective, regenerative phenotype (16;17) was from Santa Cruz Biotech (USA).

Cell treatment

Microglia were treated with safinamide (0.2-200 μM) or vehicle solvent (dd sterile H2O) ± lipopolysaccharide (LPS, final concentration 2 μg/ml) for 24 h. We used LPS as an activator of microglia as *in vivo* injection of LPS into the SNc or the striatum has been shown to produce a highly appropriate animal model for Parkinson's disease [20–22]. For immunofluorescence analysis of arginase-1 and ILB4 expression, treated microglia were processed using standard immunolocalisation protocols, the nuclei counterstained with DAPI, and images captured with a Zeiss Axioskop fluorescence microscope and x40 objective, with images captured under the same exposure conditions for all fields using Axiovision software.

Neuronal apoptosis was evaluated with 2,5'-Bi-1H-benzimidazole, 2'-(4-ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-/ 23491-52-3 (Hoechst 33342) [23], cell death by uptake of propidium iodide (PI). The percentage of cell death, determined by the number of PI-positive cells per field of view, was expressed as a percentage of the total number of Hoechst-staining cells (all cells per field of view) [23].

Results

Safinamide treatment protects TH+ve DA neurons in the SNc following 6-OHDA lesion induction

Unilateral injection of 6-OHDA caused approximately 50% loss of DA neurons in the SNc, compared with the number of neurons on the control side ($p<0.001$) in vehicle-treated animals. Significantly fewer neurons were lost in animals treated with either low or high doses of safinamide delivered by minipumps implanted on the day of 6-OHDA injection ($p<0.005$ and $p<0.001$ respectively; Fig1A, B), with greater protection (approx. 82% of neurons survived) achieved by the higher dose. Surviving neurons did not show morphological changes suggestive of a pre-degenerative state. Saline-injected controls showed no significant loss of DA neurons (data not shown).

Safinamide treatment (150 mg/ml) reduces the number of activated microglial cells in the SNc in 6-OHDA lesioned animals

The number of microglial cells expressing MHC class II (OX6$^+$; a marker of activation) was significantly increased in the lesioned SNc compared with the contralateral side ($p=<0.001$; Fig2A). The labelled cells were present in the medial and lateral portions of the SNc and they showed hypertrophied cell bodies and shortened processes. Treatment with safinamide at low dose (50 mg/ml for seven consecutive days starting on the same day as 6-OHDA lesion) had no significant effect on the number of activated microglia, but treatment with safinamide at high dose (150 mg/ml) caused a significant reduction (by approx. 45%) in the number of activated cells compared with vehicle-treated control animals (Fig 2B). In animals treated with safinamide at either low or high dose following 6-OHDA lesion induction, the microglial cells exhibited smaller cell bodies and more ramified processes than in vehicle-treated controls (Fig 2B inset).

Delayed administration of safinamide protects TH+ve dopaminergic neurons and reduces the number of activated microglia in the SNc following 6-OHDA lesion induction

To avoid the possibility that the neuronal and microglial protection was an artefact resulting from an unexpected interaction between safinamide and 6-OHDA, the implantation of the minipumps was delayed by 24 hours after 6-OHDA injection, thereby allowing an effective drug-free period of 48 hours in which the lesion could develop (see methods). In control animals, injection of 6-OHDA resulted in the degeneration of approximately 50% of the DA neurons ($p<0.001$), as described above, but even the delayed administration of safinamide at high dose (150 mg/ml; low dose was not employed) still provided significant neuronal and microglial protection. Thus safinamide-treated animals possessed significantly more surviving DA neurons than controls lacking safinamide (50% increase; $p<0.01$) (Fig 3A), and significantly fewer activated (OX6$^+$) microglial cells ($p=<0.01$) (Fig 3B). As before, the microglial cells in safinamide-treated animals also had a less activated morphological phenotype.

Effects of reference molecules rasagiline and lamotrigine on the survival of DA neurons and the activation of microglial cells

Rasagiline and lamotrigine were employed as relatively specific inhibitors of MAO B and sodium channels respectively, for comparison with safinamide. As before, the injection of 6-OHDA caused the degeneration of approximately 50% of DA neurons on the ipsilateral side ($p<0.001$; Fig 4A), but daily administration of rasagiline starting one day after 6-OHDA injection provided significant protection resulting in a 50% increase in the number of surviving neurons ($p<0.01$).

Interestingly, rasagiline also provided a significant reduction in the number of activated microglial cells ($p<0.001$) (Fig 4B). Similarly, lamotrigine therapy provided significant neuronal protection, with an increase of 37% in the number of surviving neurons ($p<0.01$) (Fig4A). Lamotrigine treatment also resulted in significantly fewer $OX6^+$ microglial cells within the lesion ($p<0.001$) (Fig 4B), and the microglial cells exhibited a less activated morphological phenotype (Fig 4C).

Neuroprotective effect of safinamide *in vitro*

Incubation of mixed neuronal-glial cultures with safinamide (0.2 - 20.0 μM) had no detrimental effect on cell survival; thus there was no statistically significant difference between cells cultured with or without safinamide. In fact the highest dose of safinamide used appeared to enhance cell survival above controls (Fig 5).

Incubation of the neuronal-glial cultures with 2 μg/ml LPS induced significant cell death after 24 h, but this was attenuated to control levels by 2-20.0 μM safinamide. Incubation of primary rat cultured microglia with 2 μg/ml LPS for 24 h reduced the expression of arginase 1 observed in control, non-stimulated microglia (Fig 6). However incubation of primary cultured microglia with 2 μg/ml LPS in the presence of 2μM safinamide promoted enhanced arginase expression as revealed by immunofluorescence. The co-labelling of the cultures with ILB4 was used to confirm that the cells were microglia.

Discussion

In PD, the underlying neurodegenerative processes continue even in treated patients, leading to increasingly drastic losses of vulnerable cells and synapses, and disability that cannot be adequately controlled with current drugs. The need for treatments that will reduce the underlying cell loss is widely recognized. Some compounds have been shown to be effective in toxin-induced models of DA neuron degeneration, and although none has yet been accepted to provide significant neuroprotection in PD patients, a “delayed start” clinical study has suggested that rasagiline treatment may have had an effect on disease progression [24]. Thus there is a need for drugs with additional neuroprotective activities. The results described above show that microglial activation and DA neuron loss can both be reduced by administration of activity-dependent sodium channel blockers (lamotrigine and safinamide), and MAO B inhibitors (rasagiline and safinamide). A single agent combining both of these activities might therefore have potential for long-term benefits in PD and other neurodegenerative diseases with involvement of microglial activation.

DA neurons are particularly vulnerable to mitochondrial damage because of their high energy demand [25]. This is indeed evident in many PD patients, where there is a well-documented deficiency in mitochondrial complex I in the SNc [26]. In the 6-OHDA model of PD one of the mechanisms that leads to DA neuronal death is inhibition of mitochondrial complexes and Glinka and Youdim [27] have shown in isolated brain mitochondria that complexes I and IV are completely inhibited by 6-OHDA. Such inhibition decreases ATP production resulting in inadequate functioning of the Na+/K+ ATPase (the "sodium pump") which normally consumes the majority of ATP production within neurons [28]. This inadequacy results in excessive accumulation of intraneuronal sodium ions which can cause reverse-mode operation of the sodium-calcium exchanger, thereby importing harmful concentrations of calcium, culminating in neurodegeneration. Blockade of sodium channels by safinamide would be expected to promote neuronal survival, as observed, by reducing sodium influx and so reducing the likelihood of calcium importation. Several sodium channel blocking agents are known to provide neuroprotection in models of multiple sclerosis, including flecainide [29], phenytoin [30],[31], lamotrigine [32]and safinamide [9]. The protection mediated by safinamide is achieved even with delayed administration of safinamide, which indicates that the neuroprotective effect is not due to an interference with 6-OHDA itself, but rather that it is the result of the effect of safinamide of neurons and glia. Such a mechanism is supported by the observation that another, more specific, sodium channel blocking agent, lamotrigine, had similar neuroprotective effects. Similarly, Lagrue et al (2007) [33] have shown that lamotrigine protects DA neurons in the MPTP model of PD, and lamotrigine also provides axonal protection in an animal model of MS [32]. Furthermore, lamotrigine at high doses can reduce cortical infarct volume, and protect against global cerebral ischaemia [34].

We compared the effect of safinamide in the 6-OHDA model with rasagiline, a MAO-B inhibitor commonly used in PD therapy. Rasagiline administered at 0.5 mg/kg daily p.o. showed similar effects on the loss of TH neurons and microglial activation to those of high dose safinamide. It is thus possible that MAO-B inhibition is part of the neuroprotective mechanism of safinamide. However since a MAO-B-independent neuroprotective mechanism has been postulated for rasagiline [35], further investigation is necessary before the role of MAO-B inhibition in the microglial and neuroprotective effects reported here for safinamide are fully understood.

The findings show that treatment with high dose safinamide not only protected DA neurons, but also significantly reduced inflammation as assessed by a measure of microglial activation, namely the expression of MHC class II. In this study we did not assess whether the microglial activation preceded the neuronal degeneration or whether it occurred in response to it, but we have previously reported that following 6-OHDA lesion induction microglial activation occurred within 3 days whereas neuronal loss was detected from day 7 following lesioning [10]. This finding therefore indicates that the anti-inflammatory effect of safinamide may be a direct effect on microglia. Low dose safinamide provided partial protection of TH neurons, and had a partial effect on microglia with changes in size and morphology of individual MHC class II positive cells, without reducing their total number.

Microglial activation can initiate and perpetuate neuronal loss by a release of cytotoxic substances, such as pro-inflammatory cytokines and oxygen-derived reactive species that are able to induce neuronal cell death *in vitro* [36] and *in vivo* [37]. Indeed, LPS-induced microglial activation has been associated with the destruction of DA neurons in the SNc [20,21,37] and microglial suppression can retard dopaminergic cell death in the SNc in the 6-OHDA model [38]. The suppression of microglial activation by safinamide may therefore be clinically important because microglia are implicated in causing the neuronal loss in idiopathic PD [4], as they are in the loss following MPTP intoxication of humans [39]. Cultured microglia express the Nav1.5 and 1.6 subtypes [40] and Nav1.5 sodium channels are present on macrophages [40] and furthermore a robust increase in the expression of Nav1.6 has been found in activated microglia and macrophages [30] associated with phagocytic and migratory activity and cytokine release [41]. Blockade of these channels may contribute to the neuroprotective effects of sodium channel blockade, (reviewed in [42]). In addition safinamide enhanced the development of a phagocytic phenotype in microglia *in vitro*, with enhanced arginase-1 expression, features of a neuroprotective microglial phenotype sometimes referred to as M2. These findings of an altered microglial phenotype and reduced neurotoxicity concur with the *in vivo* data presented here. Furthermore whilst there was a reduction in the number of microglia *in vivo* following safinamide treatment, this was only of those microglia positive for OX-6 and does not take into account movement of protective alternatively activated microglia which may be negative for OX-6 antigen. It is possible that the reduction in the number of degenerating neurons in safinamide-treated animals may have contributed to the reduction in microglial activation.

Neuroprotection is one of the unmet needs in PD and current therapies do not address the progressive neurodegenerative process. Our data show that in addition to modulating the symptoms in patients[43], safinamide also protects DA neurons and reduces inflammation in the 6-OHDA model of PD. We note that safinamide is now approved for use in PD therapy in Europe and that it is well tolerated in these patients at a similar plasma concentration to that shown to be neuroprotective in the current study.

Figure legends

Figure 1: **A:** Treatment with safinamide (50 and 150 mg/ml) for 7 days protects TH+ve DA neurons in SNc following 6-OHDA lesion induction in comparison with vehicle treated controls. Results are expressed as the mean number of TH+ve DA neurons ±SEM in the non-lesioned side of the brain (grey bars) compared with the lesioned side of the brain (black bars). Level of significance was determined by repeated measure two-way ANOVA test followed by Newman-Keuls Multiple Comparison post-hoc post-test. $^{**}p < 0.01$, $^{***}p < 0.001$, n = 6 animals/per group. **B**: Upper right: TH+ve DA neurons in the non-lesion side of the SN in a vehicle treated animal. DA neurons show normal morphology with intact cell bodies and axons and dendrites (x100). Inset: Healthy multipolar TH+ve DA neuron with an intact cell body and processes (x200). Upper left: Degenerating TH+ve DA neurons in the SN on the side of the 6-OHDA lesion induction (x100). Inset: DA neurons are morphologically compromised with round and smaller cell bodies and reduced length and number of processes (x200). Lower right: TH+ve DA neurons in the non lesion side of SN in safinamide treated animals. DA neurons show normal morphology with intact cell bodies and process (x100). Lower left: TH+ve DA neurons in the SN on the side of the 6-OHDA lesion in safinamide treated animals (x100). Inset: DA neurons show healthy morphology with intact cell bodies and processes (x200).

Figure 2: **A**: Treatment with safinamide (150 mg/ml) for 7 days reduced the number of activated OX6+ve microglial cells in the in SNc following 6-OHDA lesion induction in comparison with vehicle and low dose safinamide (50mg/ml) treated animals. Results are expressed as the mean number of OX6+ve activated microglial cells ±SEM in the non-lesioned side of the brain (grey bars) compared with the lesioned side of the brain (black bars). Level of significance was determined by repeated measure two-way ANOVA test followed by Newman-Keuls *post hoc* Multiple Comparison test . $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, n = 6 animals/per group. **B:** Upper right: Very few OX6+ve activated microglial cells in the non-lesion side of the SN in vehicle treated animals (x100).

Upper left: Increased number of OX6+ve activated microglial cells in the medial and lateral tiers of the SNc on the side of the 6-OHDA lesion induction (x100). Inset: Activated microglial cells show a hypertrophied cell body and shorter processes (x200). Lower left image: Following safinamide treatment (150mg/ml) the number of OX+ve microglial cells is reduced in the SN on the side of the 6-OHDA lesion (x100). Inset: Morphologically OX6+ve cells show smaller cell bodies and more ramified processes in the SNc (x200).

Figure 3: **A:** Delayed administration of safinamide (150 mg/ml) protects TH+ve DA neurons in SNc following 6-OHDA lesion induction in comparison with vehicle treated animals. Results are expressed as the mean number of TH+ve DA neurons ±SEM in the non-lesioned side of the brain (grey bars) compared with the lesioned side of the brain (black bars). Level of significance was determined by repeated measure two-way ANOVA test followed by Newman-Keuls *post hoc* Multiple Comparison test .**$p < 0.01$, ***$p < 0.001$, $n = 6$ animals/per group. **B:** Delayed administration of safinamide (150 mg/ml) reduced the number of activated OX6+ve microglial cells in the in SNc following 6-OHDA lesion induction in comparison with vehicle and low dose safinamide (50 mg/ml) treated animals. Results are expressed as the mean number of OX6+ve activated microglial cells ±SEM in the non-lesioned side of the brain (grey bars) compared with the lesioned side of the brain (black bars). Level of significance was determined by repeated measure two-way ANOVA test followed by Newman-Keuls *post hoc* Multiple Comparison test, **$p < 0.01$, ***$p < 0.001$, $n = 6$ animals/per group.

Figure 4: **A:** Treatment with rasagiline (1 mg/kg one loading dose p.o., followed by 0.5 mg/kg, p.o. once daily for seven consecutive days), lamotrigine (10 mg/kg p.o. once daily for seven days) and safinamide (150 mg/ml) protected TH+ve DA neurons following 6-OHDA lesion induction compared with vehicle treated animals. Results are expressed as the mean number of TH+ve DA neurons ±SEM in the non-lesioned side of the brain (grey bars) compared with the lesioned side of the brain (black bars). **B:** Treatment with rasagiline (1 mg/kg one loading dose p.o., followed by 0.5 mg/kg, p.o. once daily for seven consecutive days), lamotrigine (10 mg/kg p.o. once daily for seven days) and safinamide (150 mg/ml) reduced the number of activated OX6+ve microglial cells in the in SNc following 6-OHDA lesion induction in comparison with vehicle treated animals. Results are expressed as the mean number of cells ±SEM in the non-lesioned side of the brain (grey bars) compared with the lesioned side of the brain (black bars). Level of significance was determined by

repeated measure two-way ANOVA test followed by Newman-Keuls *post hoc* Multiple Comparison test. *p < 0.05, **p < 0.01, ***p < 0.001, n = 6 animals/per group. **C:** Lamotrigine treatment reduces the number of activated microglial cells and the remaining OX6+ve microglia show a less activated phenotype with smaller cell bodies and ramified processes compared with the vehicle treated animals where OX6+ve microglia are morphologically activated with hypertrophied cell bodies and shorter processes (x200).

Figure 5: Mixed cerebellar glial –neuronal cultures (7 DIV) were treated as follows, ctrl; basal untreated cells; solv ctrl, 10µl dd H_2O, safinamide (0.2-20µM) ± lipopolysaccharide (LPS, 2µg/ml) for 24 h. The total number of live cells was determined following fluorescence imaging of Hoechst 33342 (total cell number) and propidium iodide (for cell death) staining. The percentage of live cells per field of view per coverslip was determined and data analysed by ANOVA and paired t-test to controls or as indicated. Data from one representative experiment of three and represent the mean ± SEM of three coverslips per condition with 20 fields of view per coverslip and levels of significance are p<0.01, ** or p<0.05 *.

Figure 6: Safinamide induces an M2 type phenotype as evidenced by enhanced staining of arginase I, a marker of this microglial phenotype. 1-DIV primary rat microglia were untreated (Control) or treated with 2 µg/ml lipopolysaccharide (LPS) ± 2µM safinamide (LPS + Saf 2 µM) for 24 h. Fixed and permeabilised cells were immunolabelled for arginase-I (red) and the microglial marker ILB4 (green), with nuclei labelled with DAPI (blue) and images captured by fluorescence microscopy using set manual exposures for arginase-I and ILB4 and automatic exposure for DAPI. Left hand column, representative low power fields, middle column, Arginase and DAPI, right hand column, arginase, DAPI and ILB4 overlay. Scale bar = 50 µm

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Author contributions

MS performed the *in vivo* experiments, assisted with experimental design and the immunohistochemical analysis, and drafted the manuscript.

GM assisted with the histological assessments.

JMP performed the cell culture studies and assisted with the drafting of the manuscript.

TP assisted with the cell culture studies.

AR assisted with the design of the experiments, assessment of the data and review of the manuscript.

KJS had the overall control of the design and execution of the experimental studies, assessment of the data and the final production of the manuscript.

Conflict of interest

This study was partly funded by MerckSerono, Dr A Roach was employed by MerckSerono at the time.

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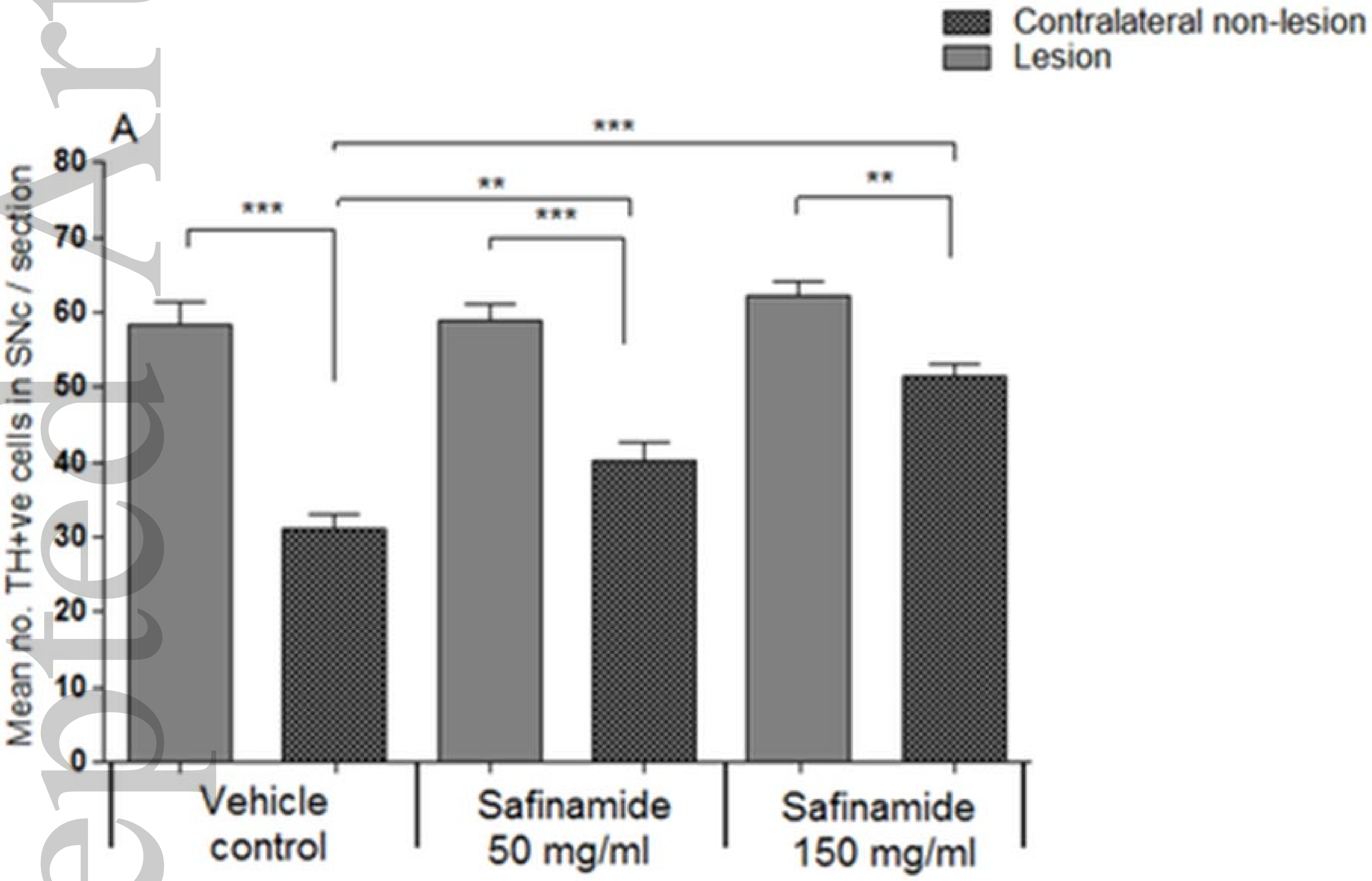

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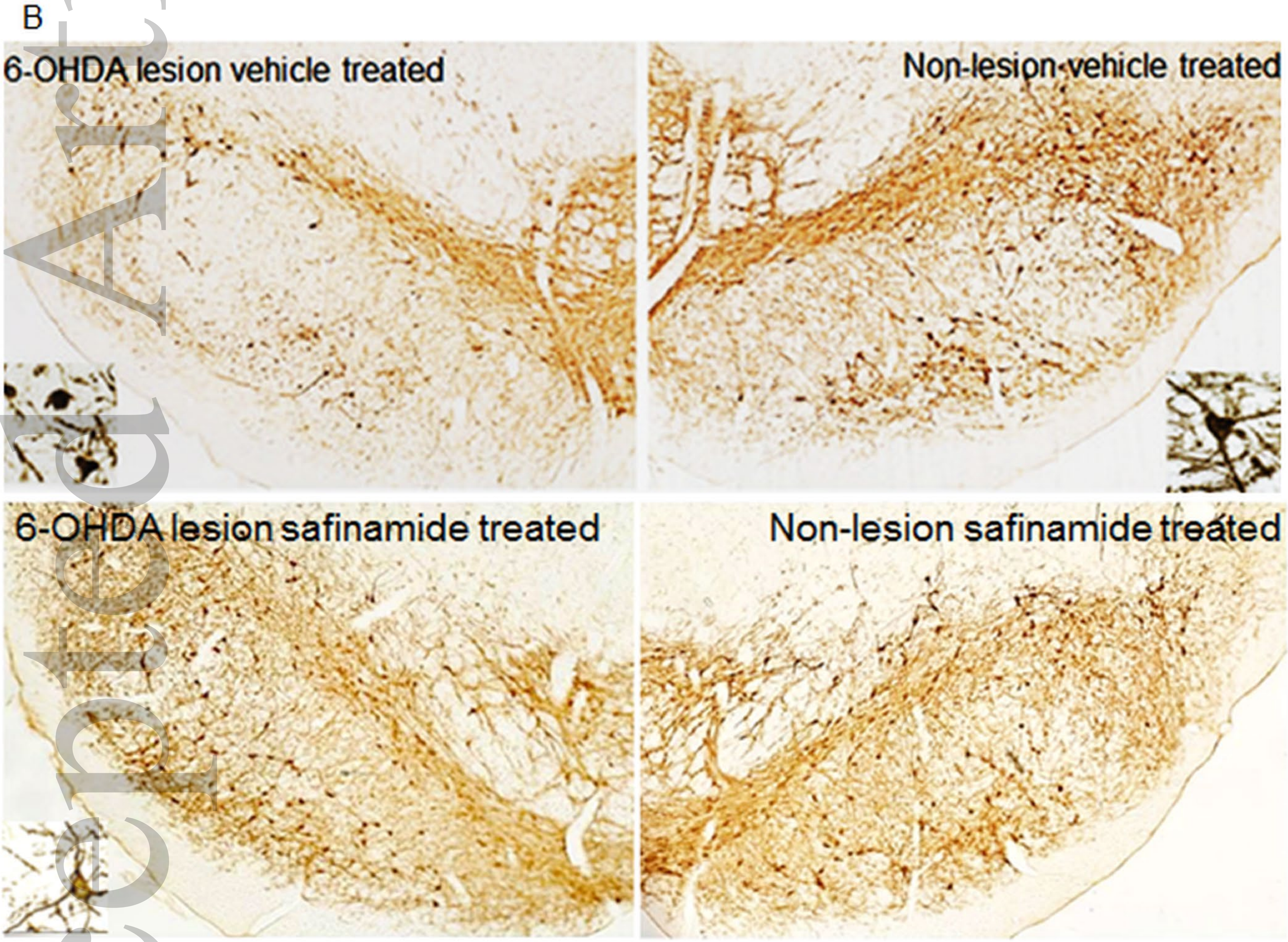
B
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Non-lesion vehicle treated
6-OHDA lesion safinamide treated
Non-lesion safinamide treated

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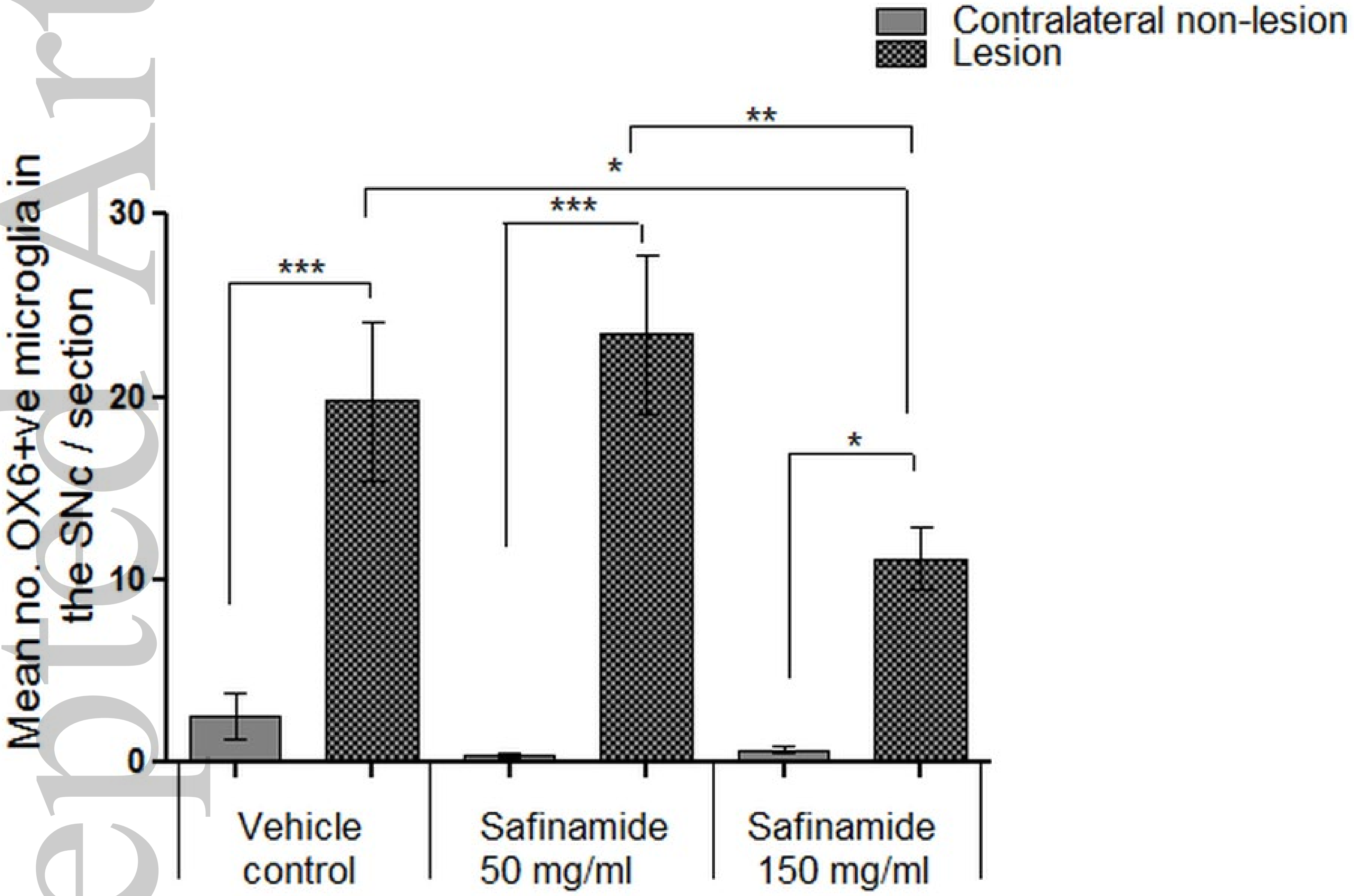

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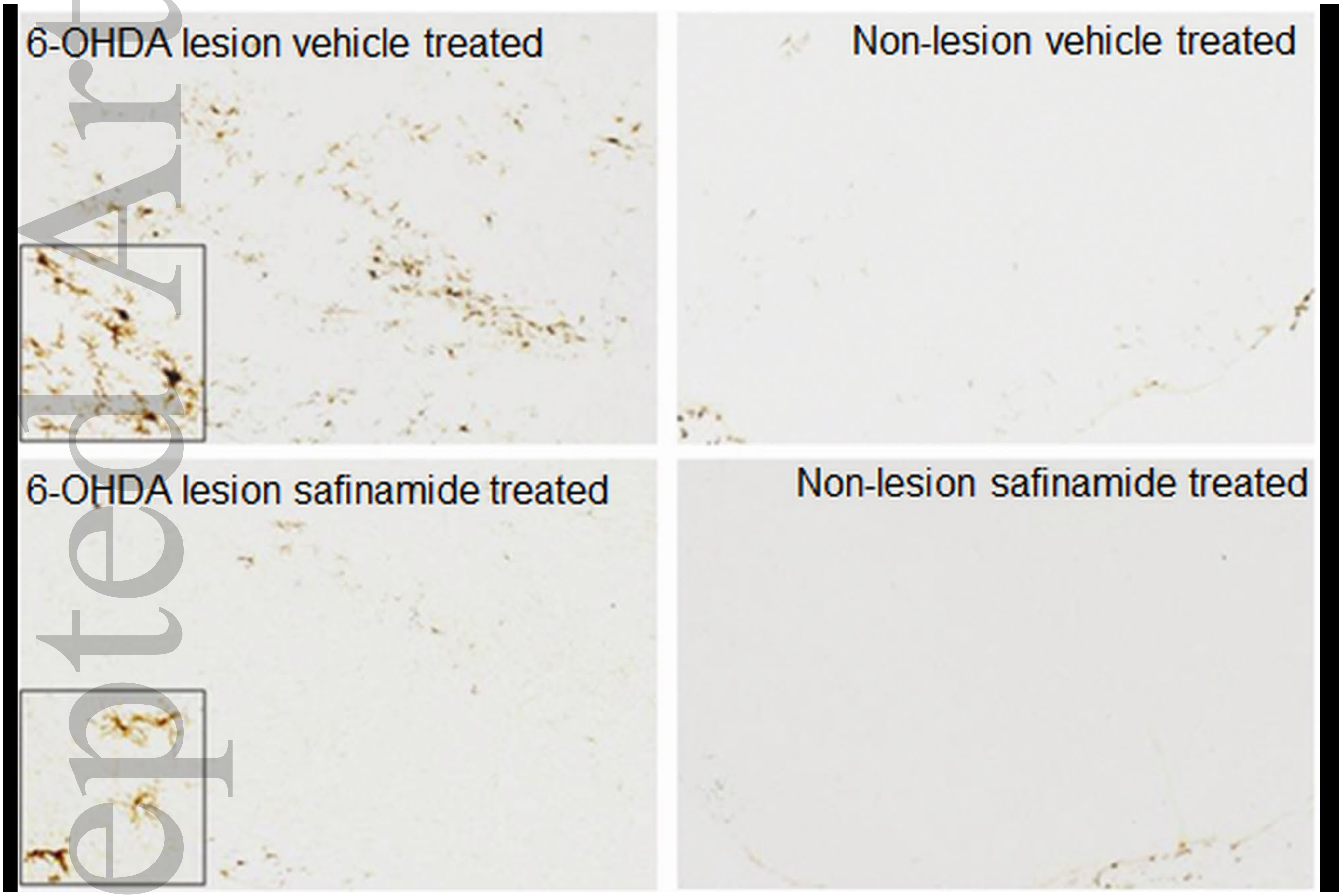
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Non-lesion safinamide treated

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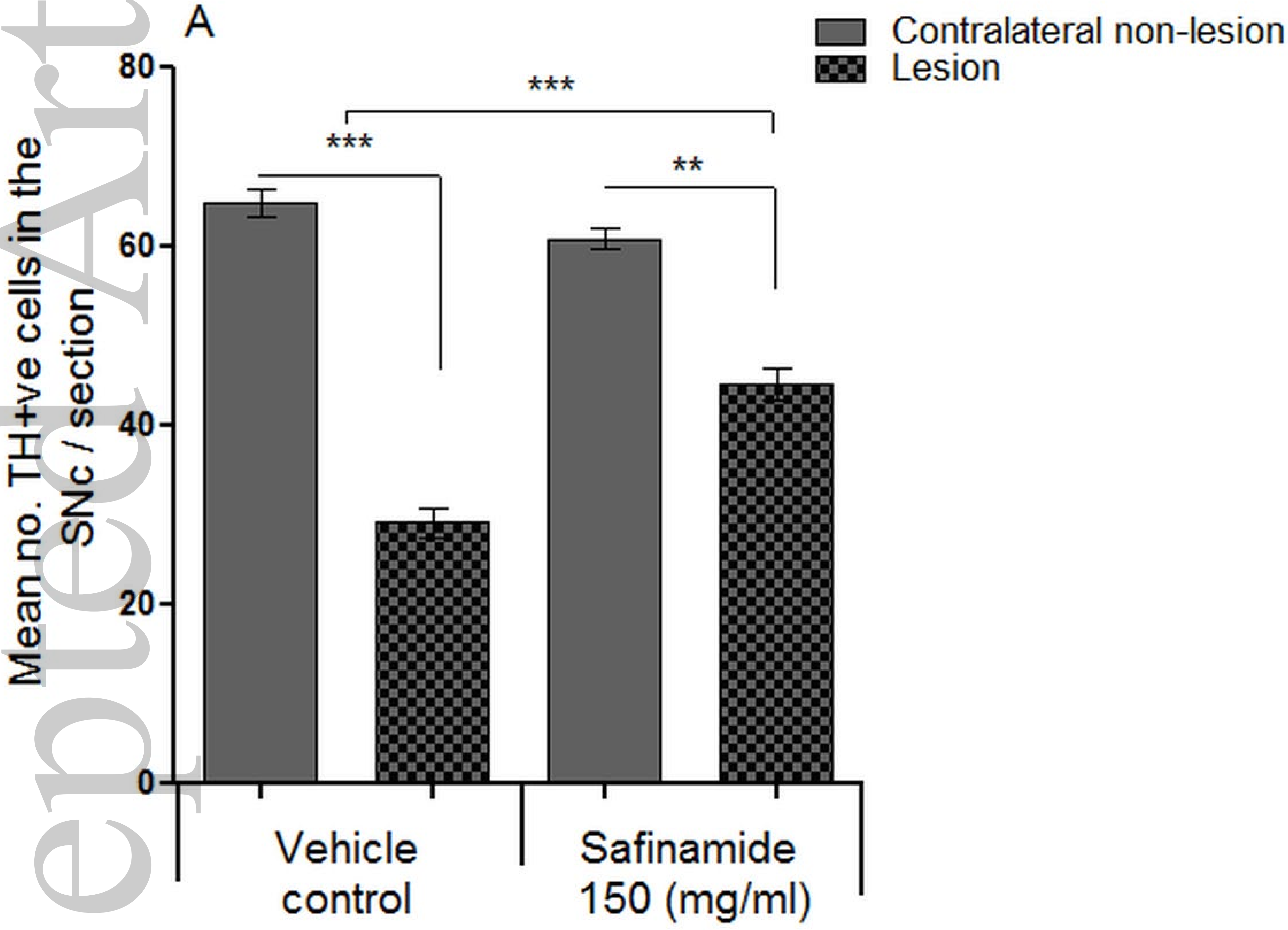

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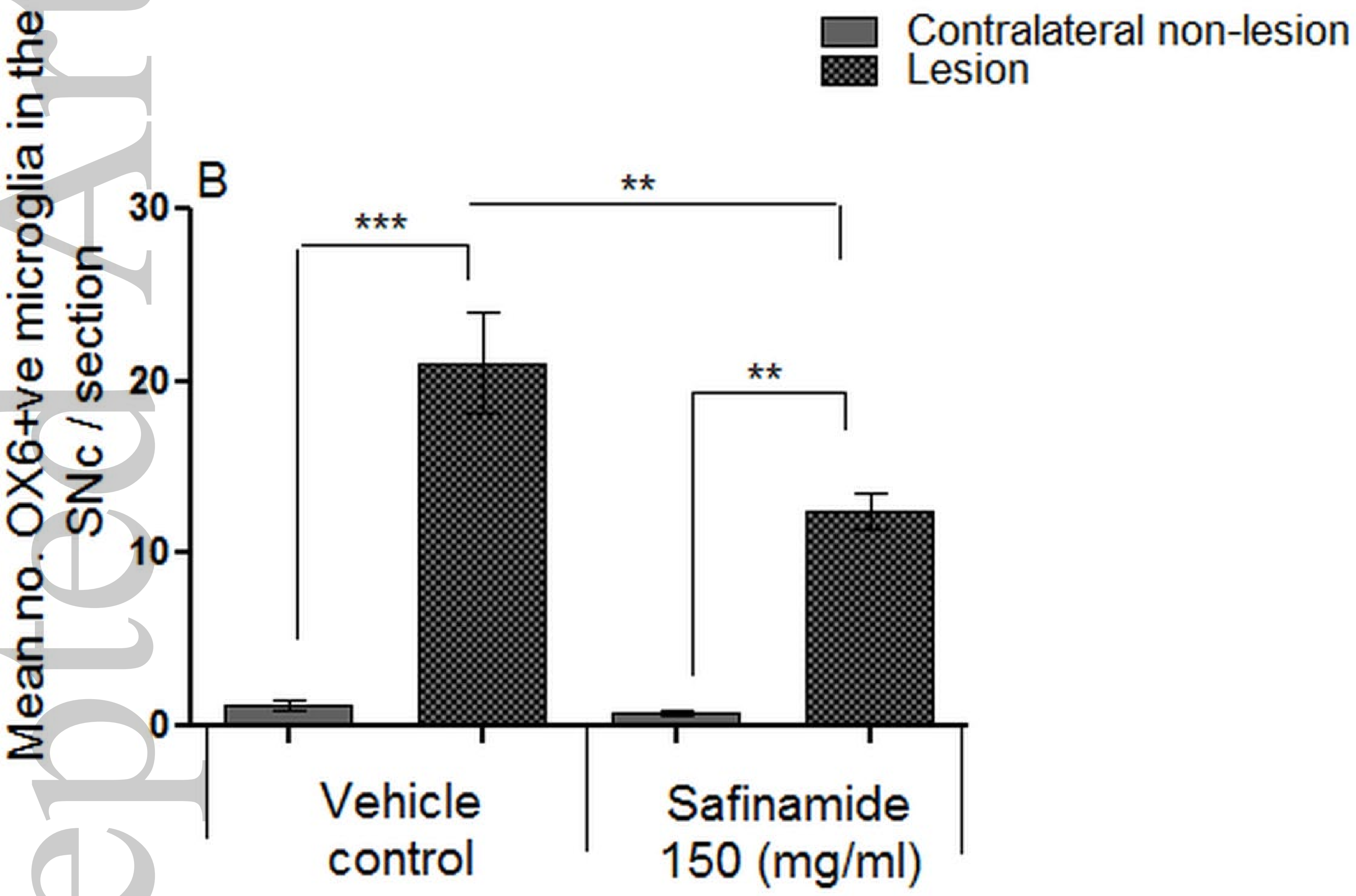

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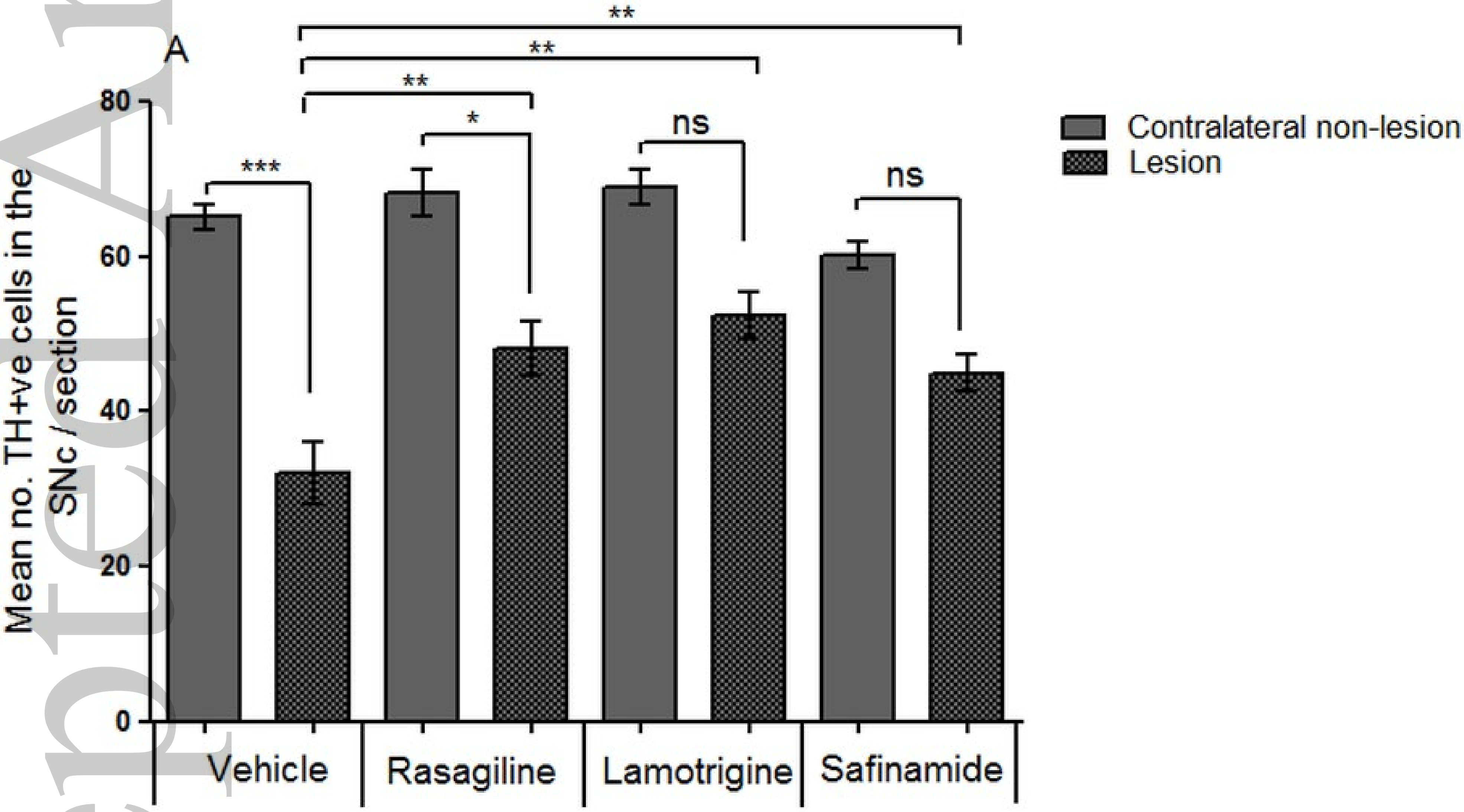

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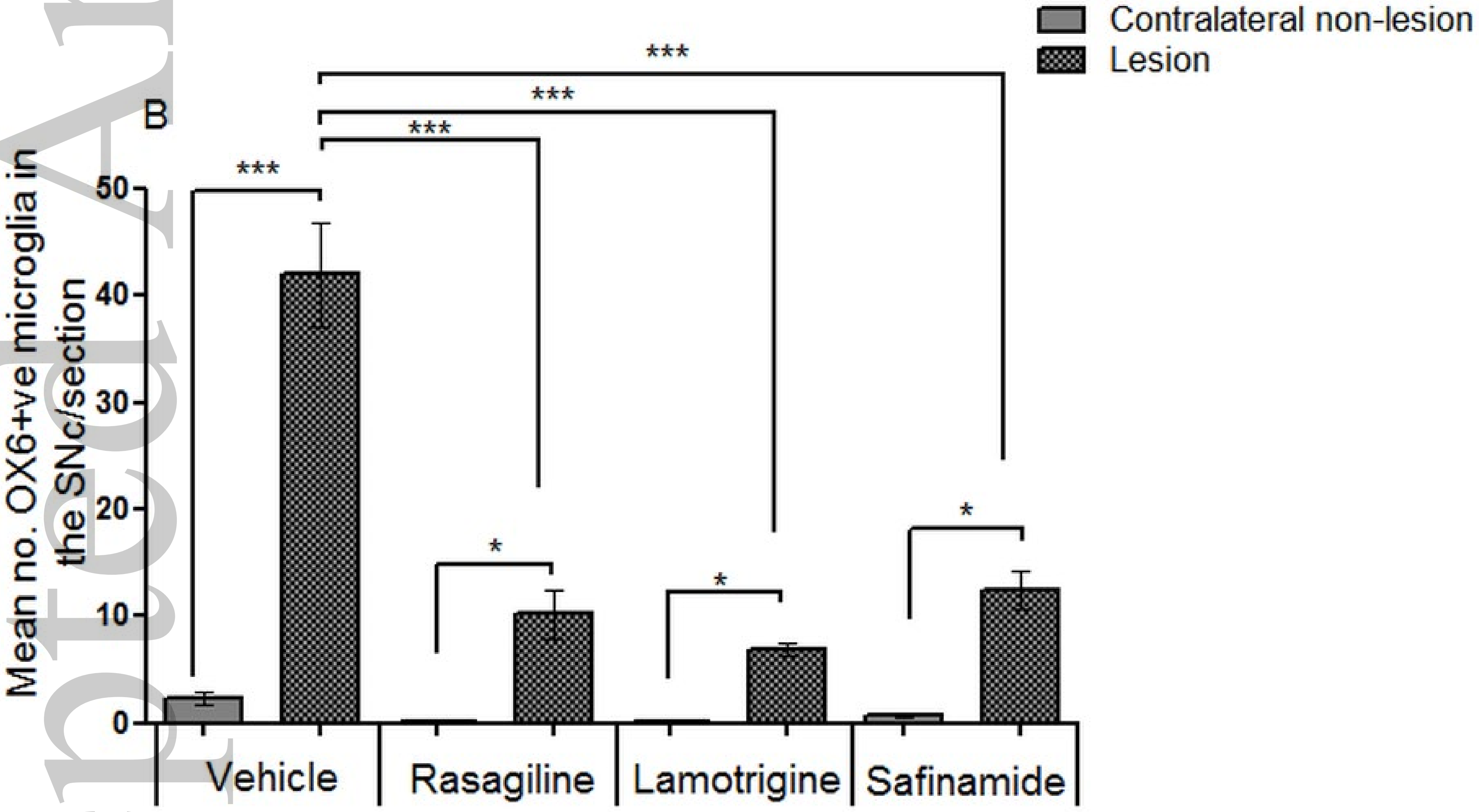

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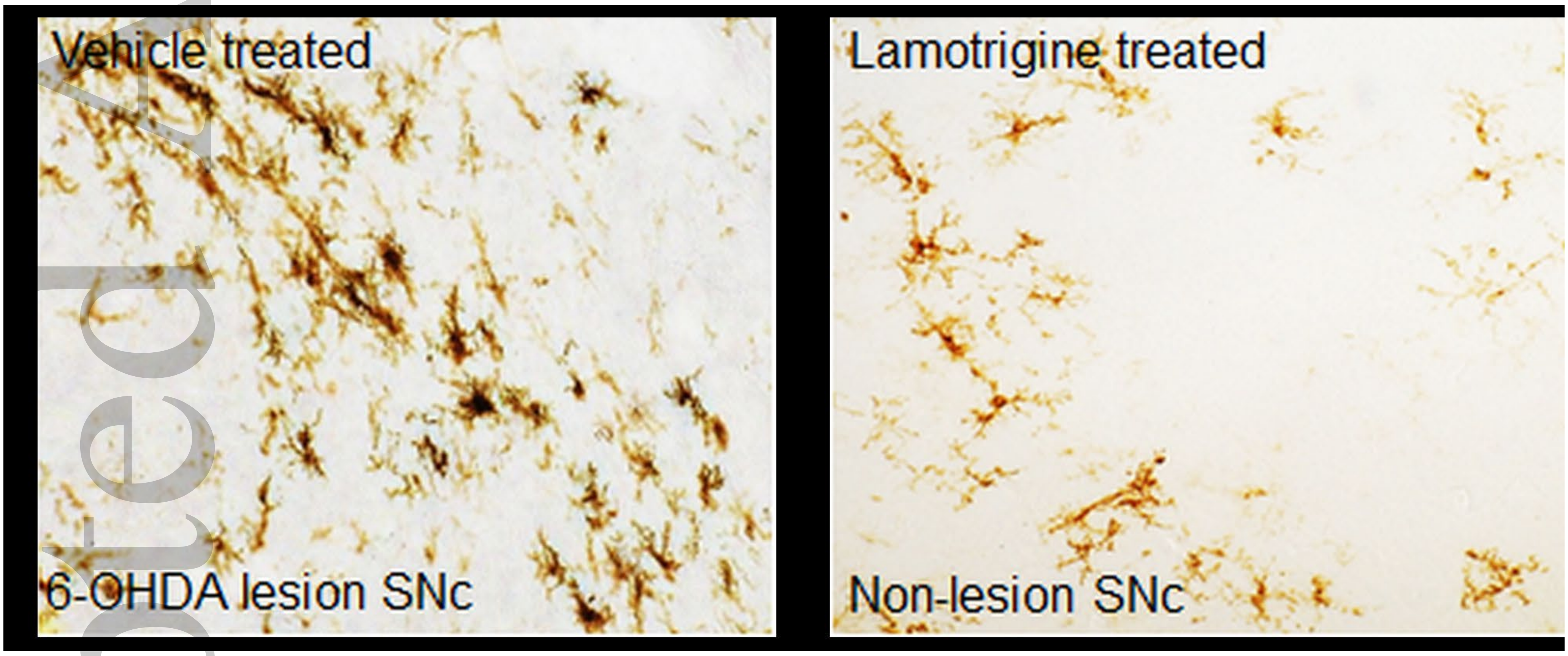
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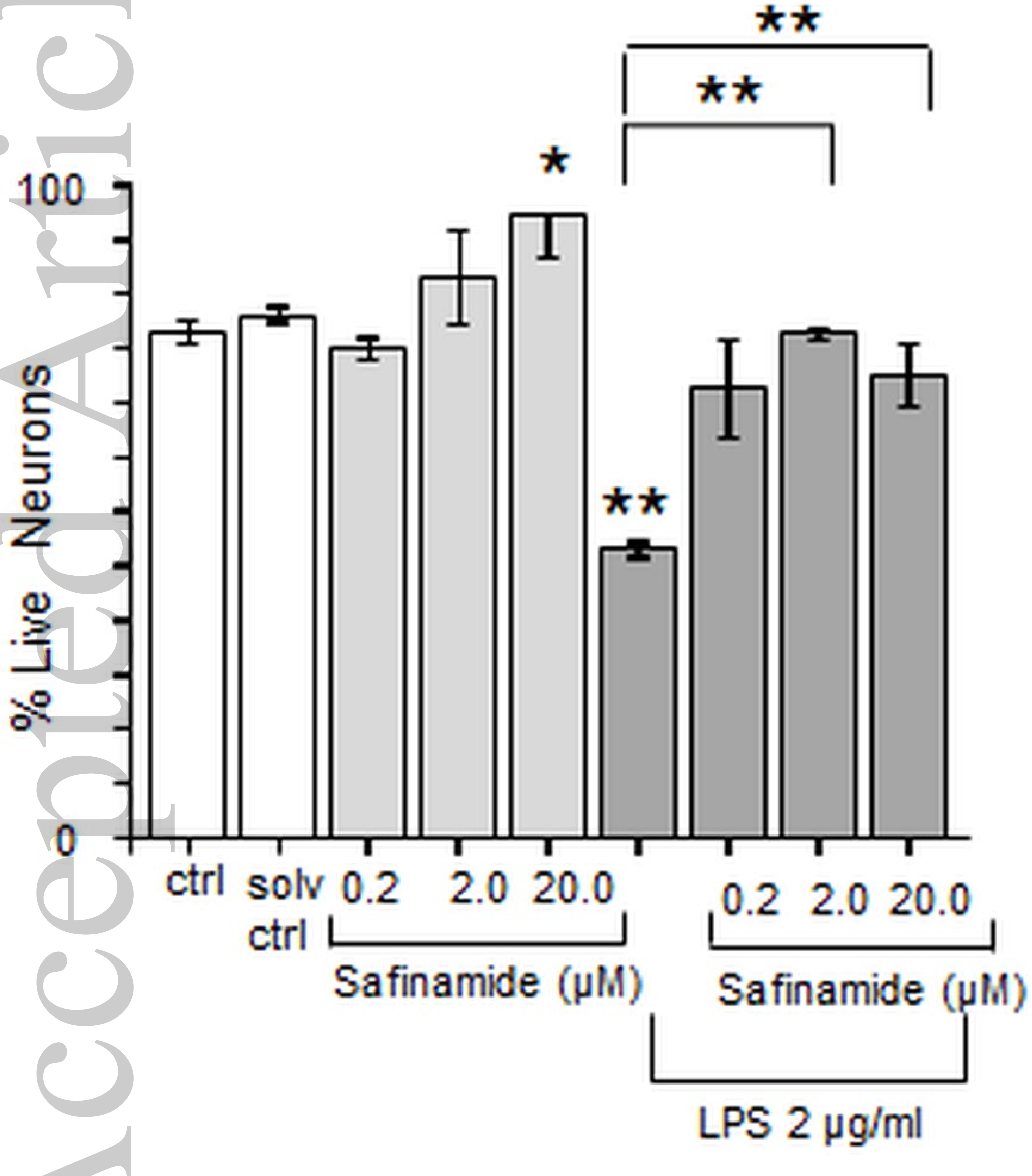

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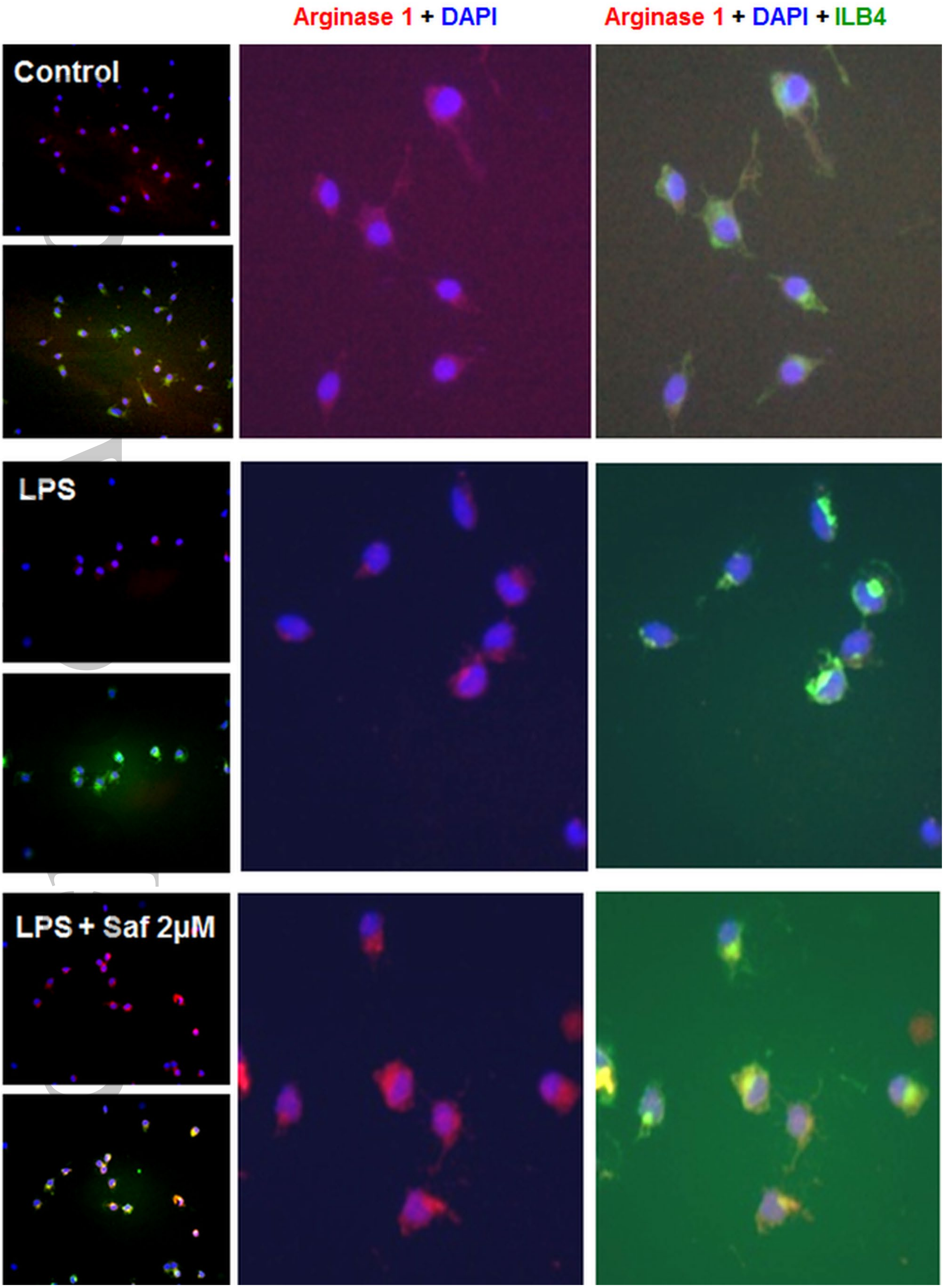
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