ORIGINAL ARTICLE

Presymptomatic MPTP Mice Show Neurotrophic S100B/mRAGE Striatal Levels

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SUMMARY

Aims: Astrocytic S100B and receptor for advanced glycation endproducts (RAGE) have been implicated in Parkinson's disease (PD) pathogenesis through yet unclear mechanisms. This study attempted to characterize S100B/mRAGE (signaling isoform) axis in a dying-back dopaminergic (DAergic) axonopathy setting, which mimics an early event of PD pathology. **Methods:** C57BL/6 mice were submitted to a chronic MPTP paradigm (20 mg/kg i.p., 2 i.d-12 h apart, 5 days/week for 2 weeks) and euthanized 7 days posttreatment to assess mRAGE cellular distribution and S100B/mRAGE density in striatum, after probing their locomotor activity (pole test and rotarod). Dopaminergic status, oxidative stress, and gliosis were also measured (HPLC-ED, WB, IHC). **Results:** This MPTP regimen triggered increased oxidative stress (augmented HNE levels), gliosis (GS/Iba1-reactive morphology), loss of DAergic fibers (decreased tyrosine hydroxylase levels), and severe hypodopaminergia. Biochemical deficits were not translated into motor abnormalities, mimicking a presymptomatic PD period. Remarkably, striatal neurotrophic S100B/mRAGE levels and major neuronal mRAGE localization coexist with compensatory responses (3-fold increase in DA turnover), which are important to maintain normal motor function. **Conclusion:** Our findings rule out the involvement of S100B/mRAGE axis in striatal reactive gliosis, DAergic axonopathy and warrant further exploration of its neurotrophic effects in a presymptomatic compensatory PD stage, which is a fundamental period for successful implementation of therapeutic strategies.

Introduction

RAGE (Receptor for advanced glycation endproducts) is a multiligand receptor of the immunoglobulin superfamily long implicated in the sustainment of glial activation, oxidative stress, and neurotoxicity [1–4]. RAGE is expressed in both full-length membrane-bound (mRAGE) and soluble (sRAGE) forms. Whereas mRAGE is the RAGE signaling isoform, sRAGE lacks the transmembrane domain and is a competitive inhibitor of mRAGE acting as a decoy for ligands [5]. Therefore, it is vital to dissect different RAGE isoforms as it is likely that subtle shifts in ratio of RAGE variants can significantly impact intracellular signaling.

Brain deleterious events have been linked to RAGE ligands (e.g., S100 proteins, amyloid peptide, HMGB-1) that get complex three-dimensional structures in oxidative settings [6]. RAGE activation following accumulation of such ligands perpetuates neurodegeneration by triggering its own upregulation via a positive feedback loop [7]. Nonetheless, a range of adult cell types shows low levels of RAGE expression underlying RAGE physiological function. Consistently, physiological levels of RAGE ligands may modulate brain plasticity and damage repair [8]. RAGE pleiotropic effects seem to be strongly dependent on the cell type and the context [9]. For example, brain physiological levels of S100B tonically activate trophic RAGE signaling in neurons [10]. In contrast, S100B overproduction causes excessive neuronal RAGE stimulation that culminates in overproduction of ROS and neurotoxicity in injured brain tissue [11]. Accordingly, S100B is envisaged as a double-edged sword because it could be a neurotrophin [12] or a damage-associated molecular protein [13], depending on its levels.

Increased expression of both S100B [14] and RAGE [15] were reported in patients with Parkinson's disease (PD), suggesting that S100B/RAGE axis can be involved in PD neurodegenerative process. Moreover, RAGE was proposed to play a role in microglia-mediated dopaminergic degeneration in ventral midbrain of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

(MPTP)-treated mice [16], a gold standard PD animal model [17]. Therefore, we hypothesized that continuous accumulation of S100B and subsequent RAGE upregulation would foster glial reactivity and DAergic axonopathy in striata. Hence, we attempted to characterize the neurochemical, immunohistochemical, and functional correlate of striatal S100B/mRAGE axis in a chronic MPTP mouse model of PD.

Materials and Methods

Animals and MPTP Protocol

Male adult C57BL/6J mice (3 months old; 24–28 g; Charles River Laboratories, Barcelona, Spain) were housed four per cage, under controlled environmental conditions [12-h light/dark schedule at room temperature (RT) of $23 \pm 1°C$, with food and water supplied *ad libitum*]. All experiments were approved by the Institutional Animal Care and Use Committee from Faculty of Medicine, Coimbra University, and were performed following the European Community directive (2010/63/EU). The animal procedures were performed in strict accordance with the "Guide for the Care and Use of Laboratory Animals" (Institute of Laboratory Animal Resources, National Academy Press 1996).

Herein we applied a new chronic MPTP paradigm (Figure 1). We considered this regimen as RAGE ligands accumulation occurs preferentially in chronically stressed environments, which more closely mimic the progressive nature of PD [7]. Briefly, animals were injected intraperitoneally (i.p.) with 20 mg/kg of MPTP hydrochloride in saline solution (Sigma-Aldrich, St. Louis, MO, USA; MPTP, n = 8) or with saline solution (0.9% NaCl; SAL, n = 8), twice daily 12 h apart for 10 days, and a pause time of 2 consecutive days at the end of the first 5 days of administration. Technical recommendations of MPTP safety handling were strictly enforced [18]. All animals survived this dosing regimen and none showed weight reductions. Sacrifice of both saline (n = 4)- and MPTP-treated mice (n = 4) were performed 7 days after last administration by decapitation, following motor function assessment. Brains were rapidly dissected and immediately frozen in liquid nitrogen and stored at −80°C until protein levels assessment and dopamine (DA) and metabolites quantification. Remaining animals (SAL; n = 4 and MPTP; n = 4) were deeply anesthetized with pentobarbital and transcardially perfused with 0.1 M PBS followed by 4% paraformaldehyde (PFA) in 0.1 M PBS. The brains were removed and postfixed for 24 h in 4% PFA and then dehydrated in 30% sucrose in 0.1 M PBS for 24 h for subsequent immunohistochemistry analysis. All chemicals (ultrapure and

Figure 1 Experimental design including MPTP administration protocol, locomotor behavior assessment, and euthanasia.

pro analysis quality) were purchased from Sigma-Aldrich and Merck AG (Darmstadt, Germany).

Locomotor Assessment: Rotarod and Pole Tests

Animals [SAL (n = 8) and MPTP (n = 8)] were submitted to rotarod and pole tests at day 7 posttreatment. These tests have been extensive and successfully used to probe motor performance and coordination following DA system lesions in MPTP-treated mice [19,20]. All tests were carried out between 9:00 and 17:00 h in a sound-attenuated room under low-intensity light (12 lx) and were scored by the same person in an observation room where the mice had been habituated for at least 1 h before the beginning of the tests.

Rotarod Test

Motor coordination and balance were evaluated using the rotarod test. The rotarod apparatus (Letica Scientific Instruments, LE 8200 model) consisted of a rotating spindle (3 cm of diameter distancing 15 cm of base) with 5 individual compartments distancing 3 cm so that 5 animals could be simultaneously tested. Animals were initially trained to maintain themselves on the rotating rod at 15 rotations per minute (RPM) for 120 seconds (habituation phase), prior to the trial. This assured that all the animals reached an analogous baseline. An increasing speed protocol was implemented herein, as the sensitivity to motor disability has been shown to improve when animals are forced to move at increasingly faster speeds [21]. Briefly, animals were placed on the rotating rod and tested using an increasing rotating speed (from 10 to 22 rpm) for 240 sec. The time that mice remained on the rotarod was recorded over 3 trials with an intertrial interval of approximately 5 min. Data are presented as the mean time on the rotating bar over 3 test trials.

Pole Test

Pole test was performed to determine the degree of bradykinesia and slight modifications to a previous protocol were implemented [20]. A metal pole (50 cm high and 8 mm diameter), doubly wrapped with cloth tape to prevent slipping and whose base was positioned in the home cages, was used for this test. Mice were placed head upward on the top of the pole. Thereafter, time taken to orientate the body completely downwards (Tturn) and to land on all four paws on the floor was recorded (TLA) with a cutoff of 60 seconds. The test consisted of 3 trials (20 min interval between each) and trial average was used for statistical analysis.

Quantification of Dopamine and Metabolites by HPLC-ED

Right striata were sonicated in ice-cold 0.2 M perchloric acid and centrifuged ($15.500 \times g$, 7 min, 4°C), and the supernatants were used to determine the content of dopamine (DA) and metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) by high-performance liquid chromatography (HPLC-ED) with an electrochemical detector, as previously described [22,23]. The pellet was resuspended in 1 M NaOH and stored at −80°C for
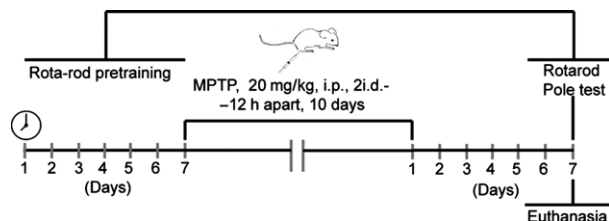
total protein quantification by the bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific, MA, Waltham, USA). Concentration of DA and its metabolites were determined by comparison with peak areas of standards and expressed in nanogram per mg of protein.

Western Blot Analysis

Left striata were used for total protein extracts as previously described [23] and total protein concentration was determined by the bicinchoninic acid (BCA) protein assay (Pierce Biotechnology, Rockford, IL, USA). Equal amounts of protein were separated using 8–15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to a polyvinylidene difluoride membrane (Merck Millipore, Madrid, Spain) and blocked by 5% defatted milk for 2 h (Silva et al. 2014). Membranes were incubated overnight at 4°C with the following primary antibodies: mouse anti-TH (1:1000; MAB318, Merck Millipore), rabbit anti-HNE (1:500; 393207, Calbiochem, Merck Millipore), mouse anti-GFAP (1:1000; IF03L, Calbiochem, Merck Millipore), rabbit anti-S100β (1:500; JBC1771181, Merck Millipore), and rabbit anti-RAGE raised against the C-terminal domain recognizing specifically the full-length membrane-bound isoform (1:500; Ab3611, Abcam, Cambridge, UK). Membranes were then incubated with alkaline phosphatase-conjugated IgG secondary antibodies (mouse or rabbit 1:10,000; GE Healthcare, Little Chalfont, UK). Finally, membranes were visualized on Typhoon FLA 9000 (GE Healthcare) and analyzed using Image Quant 5.0 software (Molecular Dynamics, Inc., Sunnyvale. CA, USA). Results were normalized against internal controls β-actin (1:1000; A5316, Sigma-Aldrich) or GAPDH (1:1000; MAB374, Merck Millipore) and then expressed as percentage of control.

Immunohistochemistry

Striatum anatomical limits were identified using a mouse brain atlas (AP, +1.32 to +0.5 mm). Striatal coronal sections of 40 μm thickness were collected from cryostat (Leica CM3050S, Nussloch, Germany) in 0.1 M PBS and used for free-floating immunohistochemistry. Briefly, slices were washed twice with 0.1 M PBS, blocked with 0.25% Triton X-100 and 5% normal fetal bovine serum (FBS) in 0.1 M PBS for 1 h at room temperature, and then incubated for 24 h at 4°C with the following primary antibodies: rabbit anti-RAGE (1:200; Ab3160, Abcam), mouse anti-GFAP (1:1000; IF03L, Calbiochem), mouse antiglutamine synthetase (GS; 1:500; MAB302, Millipore), goat antiionized calcium-binding adapter molecule 1 (Iba1; 1:200, Ab5076, Abcam), mouse anti-NeuN (1:500; MAB377, Millipore), or mouse anti-TH (1:500; MAB318, Millipore).

Sections were then rinsed with 0.1 M PBS (3 × 10 min) and incubated with 4′,6-diamidino-2-phenylindole (DAPI; 1:5000; D1306, Invitrogen, Carlsbad, CA, USA) for nuclear staining and the secondary fluorescent antibodies for 2 h at room. Finally, sections were rinsed with 0.1 M PBS (3 × 10 min) and mounted with glycergel (Dako mounting medium). Samples were imaged using a confocal laser scanning microscope (LSM 710 Meta, Carl Zeiss, Gottingen, Germany).

Statistical Analysis

Differences in locomotor and biochemical data were compared using unpaired Student's *t*-test. The accepted level of significance for the tests was $P < 0.05$. Data are expressed as means $\pm$ SEM, and analyses were performed using GraphPad Prism 5.0 software for Windows.

Results

Mice Chronically Exposed to MPTP Maintain Normal Motor Function Regardless Severe Disruption of Dopaminergic Nerve Terminals

MPTP-treated animals displayed motor performances similar to control animals in both tests ($P > 0.05$, Figure 2A,B). Nonetheless, MPTP imposed a severe disturbance of striatal dopaminergic system as demonstrated by a significant reduction in TH (~68% of control, $P < 0.01$), DA, and its metabolites levels (~11%, ~21%, and ~40% of control, respectively; $P < 0.001$) and a significant increase in DA turnover (DOPAC+HVA/DA ratio, ~300% of control; $P < 0.05$) 7 days after the toxic insult (Figure 2C–G).

Striata of Chronically MPTP-Treated Mice Show an Oxidant and Gliotic Profile

Chronic MPTP treatment triggered an accumulation of phospholipid peroxidation 4-hydroxy-2-nonenal (HNE) adducts (≈75 and 200 kDa) in striata when compared with control animals (122 and 125% of control, respectively; $P < 0.05$), 7 days postdosing (Figure 3A). This oxidant setting paralleled a robust gliotic profile. In fact, a significant increase in glial fibrillary acidic protein (GFAP) levels (175%, $P < 0.05$; Figure 3B) and profound astrocytic morphological changes comprising enlarged cell bodies and increased ramified processes were observed upon MPTP, as highlighted by GFAP and glutamine synthetase (GS) immunostaining (Figure 3C). Reactive profile of microglia cells, classically described to respond prior to astrogliosis in the acute phase of MPTP insult [24], is less robust than the astrocytic one. Nevertheless, actincross-linking ionized calcium-binding adaptor molecule 1 (Iba1) immunostaining showed a reduced complexity of microglia shape with shortened and thicker processes branching off from soma in striata of MPTP-treated mice when compared to control mice (Figure 3D).

Mice Chronically Exposed to MPTP Display Striatal Physiological S100B Levels and Basal Neuronal mRAGE Expression

Impact of chronic MPTP on S100B/mRAGE protein levels and mRAGE cellular localization was assessed by Western blot and double-labeling immunohistochemistry. Both S100B and mRAGE remained at physiological levels (107 and 101% of control, respectively; $P > 0.05$; Figure 4A,B) regardless the oxidant and gliotic profile MPTP driven. mRAGE was predominantly found in striatal neurons from saline- and MPTP-treated mice and showed a punctuated distribution mostly localized in nuclei and perikarya of nearly all neuron-specific protein (NeuN)-positive cells
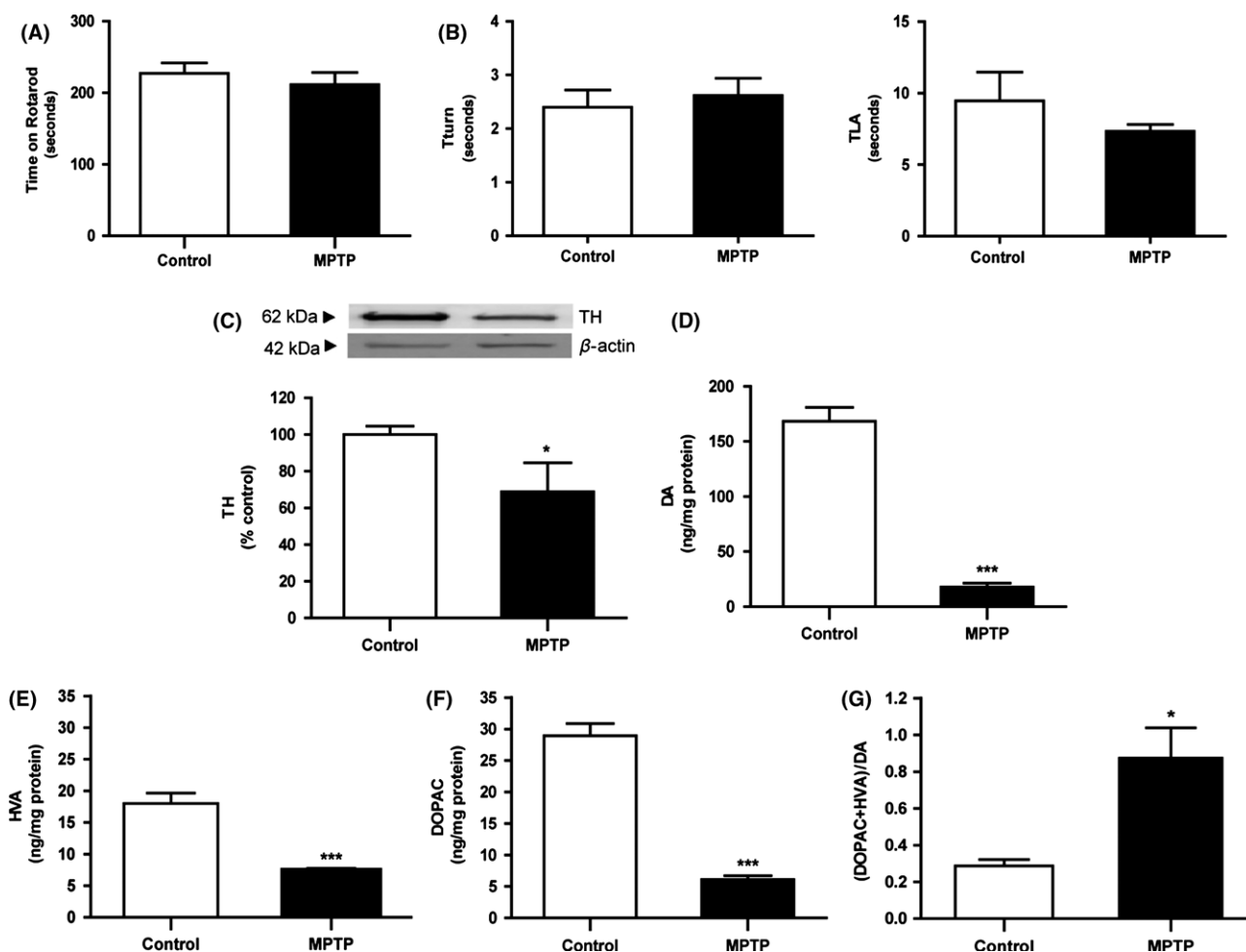
Figure 2 Effect of MPTP chronic treatment on motor skills and striatal dopamine status 7 days post-MPTP. MPTP-treated mice showed normal motor function, exhibiting similar performances of control animals in (**A**) time spent on rotarod and (**B**) time until they turned completely downward (Tturn) and landed floor (TLA) in pole test. Normal motor function occurred in the presence of striatal dopaminergic disruption MPTP driven, as shown by reduced (**C**) TH, (**D**) DA, (**E**) DOPAC, and (**F**) HVA tissue levels and a significant increase in (**G**) (DOPAC+HVA)/DA, which reflects dopamine turnover. TH protein levels were assessed by Western blot, normalized with β-actin levels, and expressed as % of control. DA and metabolites were assessed by HPLC-ED and expressed as ng/mg protein. Results *$P < 0.05$, ***$P < 0.001$ versus SAL-treated animals, using an unpaired Student's *t*-test.

(Figure 4C). mRAGE immunofluorescence was further detected in somas of both resting and reactive astrocytes (Figure 4D). mRAGE immunofluorescence does not seem to be localized in microglia cells (Figure 4E) nor in DAergic axons (Figure 4F) in basal conditions, a distribution pattern that remained unchanged after MPTP treatment.

Conclusion

Nearly all of the available treatments for PD progression are symptomatic in nature and do not appear to slow or reverse its natural course. Novel therapeutic strategies for PD are therefore critical. This prompted us to characterize striatal S100B/mRAGE axis in a chronic MPTP mouse model of PD. Chronic MPTP protocols provide a sustained oxidative setting that favor accumulation of RAGE ligands in striatum, a brain area comprising early events in PD-related pathology [25]. Our results clearly demonstrate that

this MPTP model triggered a severe DAergic disruption as shown by depletion of striatal DA and its metabolites DOPAC and HVA. These findings are aligned with DAergic toxicity reported for other chronic and acute MPTP mice models [26]. DA depletion surmounts the range of decrease in TH suggesting that DAergic dysfunction is more prominent than DAergic terminal degeneration in this MPTP regimen.

Most of these biochemical events occur over several years prior to diagnosis of typical PD motor symptoms [27]. Multifactorial mechanisms occurring at corticobasal ganglia-thalamo loop are suggested to display key compensatory roles for motor function until striatal DAergic deficits reach a critical threshold [28]. MPTP experimental models revolve around a controversial correlation between motor deficits and striatal DA levels. In fact, motor disabilities are transient and identified shortly after treatment, sometimes when mice are still intoxicated by MPTP [29,30]. Not surprisingly, our chronic MPTP model displayed normal motor
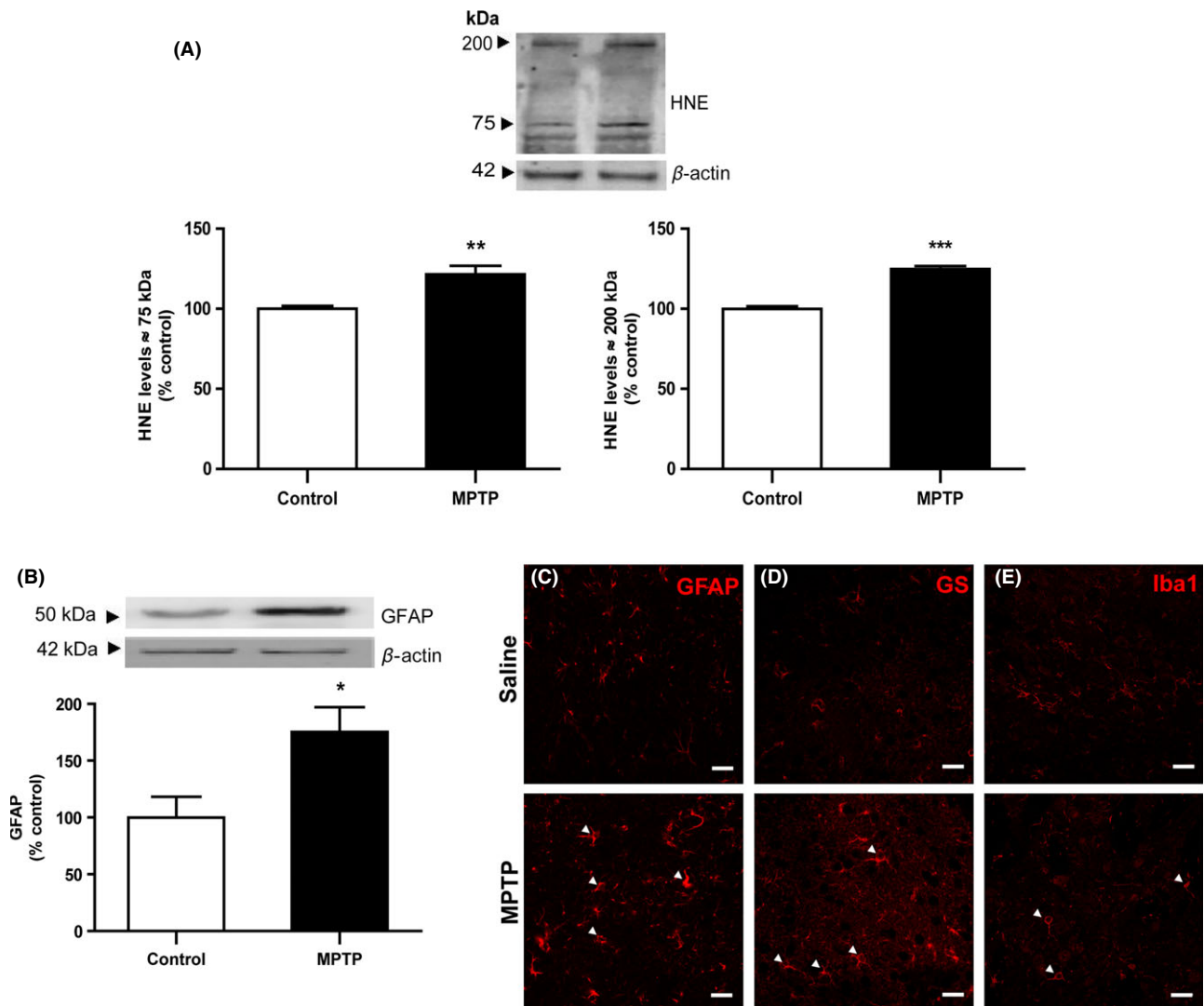
Figure 3 Effect of chronic MPTP treatment on striatal oxidative status and glial reactivity. MPTP triggered an oxidant setting confirmed by the accumulation of HNE protein adducts (75 and 200 kDa) in intoxicated animals (**A**). This oxidative status concurred with a gliotic profile, typified by increased GFAP levels (**B**) and enlarged astrocytic cell bodies with increased ramified processes (**C,D**—GFAP and GS immunofluorescence, respectively), and shortened and thicker microglia processes (**E**—Iba1 immunofluorescence), in MPTP-treated animals when compared to control animals (arrows in MPTP conditions). HNE protein levels were assessed by Western blot, normalized with β-actin levels, and expressed as % of control. Results are expressed as mean $\pm$ SEM of 4 animals per group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus SAL-treated animals, using an unpaired Student's *t*-test. Confocal images are representative of four animals per group. Scale bar: 20 μm.

function 7 days posttreatment, in spite of profound striatal hypodopaminergia. Increased DA turnover is one of the striatal compensatory mechanisms engaged in maintaining the minimum concentration of DA required for normal motor function in PD [19,31,32]. Consistently, we report a 3-fold increase in DA to metabolite ratio in MPTP-treated mice, suggesting that such striatal compensatory actions are recruited in this experimental setting and contribute to maintain motor skills. Overall, this MPTP model presents biochemical and locomotor similarities with presymptomatic PD stage, fundamental for successful implementation of strategies aimed at altering the progressive evolution of PD [27].

Evidences arising from both patients with PD [15] and experimental PD models [18] suggest oxidative stress as a key player in PD pathology. This was observed in our model by strong HNE-immunoreactive bands in MPTP-treated animals, probably reflecting distinct protein adducts arising from lipoxidative damage, as previously reported in early stages of human parkinsonism [15]. Glial cells also contribute to PD progression with release of oxidative stress mediators involved in inflammatory processes [33–35]. Consistently, our immunohistochemical data clearly showed a sustained gliotic profile in MPTP-treated animals, lending additional credence to the protocol used herein. As environments laden with oxidative stress are prone to accumulation of RAGE
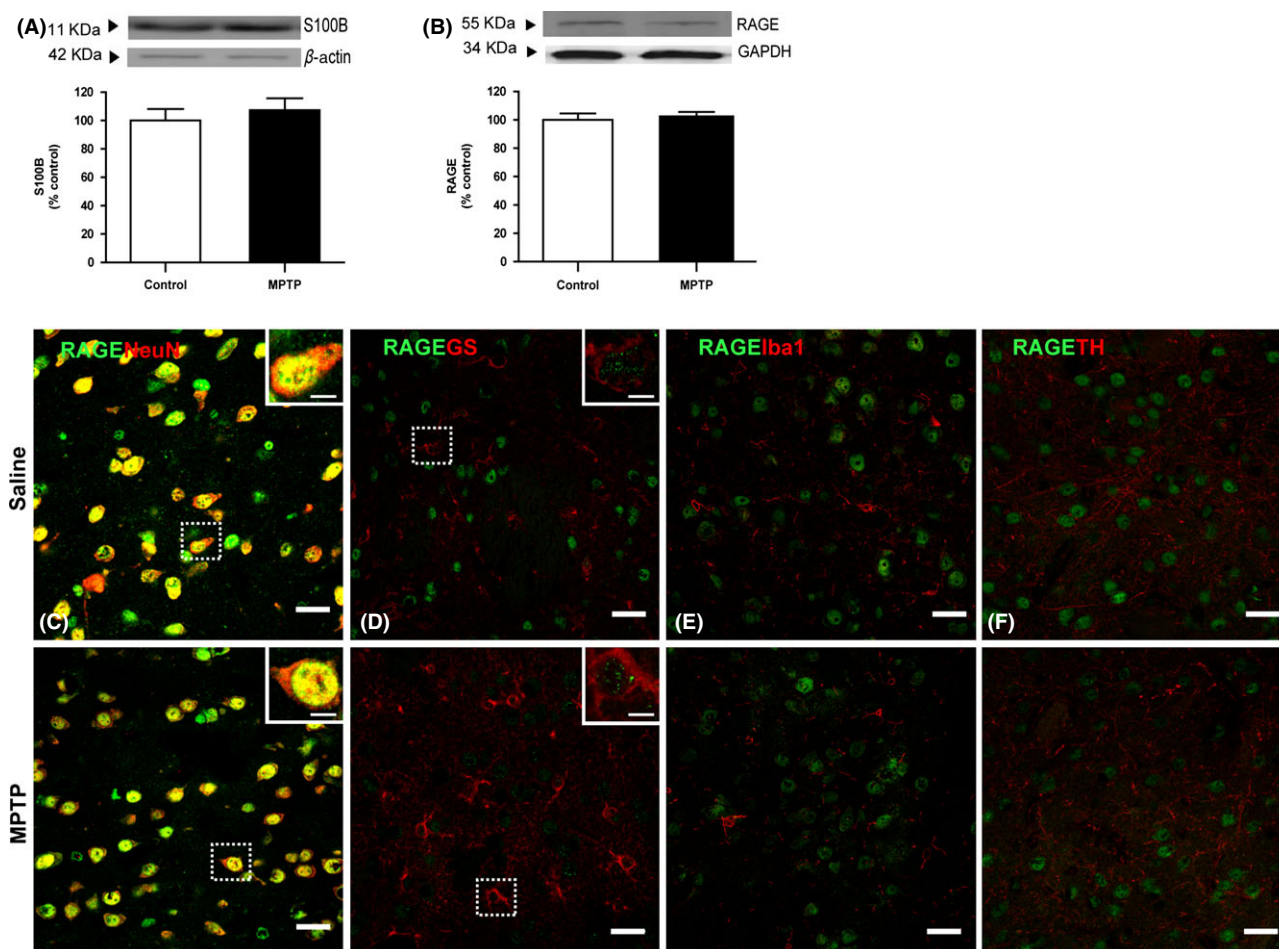
Figure 4 Effect of chronic MPTP treatment on S100B/RAGE axis. Physiological levels of both (**A**) S100B and (**B**) mRAGE proteins were observed in striatal total homogenates of MPTP-treated animals. Double immunofluorescent staining for RAGE (green) and neuronal marker NeuN, astrocytic GS, microglial Iba1, or TH-positive fibers (red) in striata of saline- and MPTP-treated mice was performed. mRAGE was found to be highly expressed in striatal neurons, showing a punctate distribution mostly localized in nuclei and perikarya of NeuN-positive cells (colabeling is in yellow) in both saline- and MPTP-treated animals (**C**). RAGE immunostaining was also found in soma of GS-positive cells in both saline- and MPTP-treated mice (**D**), regardless of the astrocytic reactive status. RAGE does not seem to be localized in Iba1-positive cells (**E**) nor in TH-positive fibers from both saline- and MPTP-injured striata (**F**). S100B and RAGE protein levels were assessed by Western blot, normalized with β-actin/GAPDH levels, and expressed as % of control. Results are expressed as mean $\pm$ SEM of 4 animals per group. Confocal images are representative of four animals per group. Scale bar 20 μm and 5 μm for zoomed images.

ligands [6], we investigated the likely accumulation of ligands/mRAGE in MPTP chronically stressed striata. Remarkably, we found normal density of astrocytic ligand S100B and mRAGE in spite of an oxidative and gliotic setting.

Identity of RAGE-bearing cells also dictates the outcome of RAGE biology [8]. Our results clearly showed a prominent neuronal mRAGE localization as RAGE is present virtually in all NeuN-positive cells in both saline- and MPTP-treated striata. This finding is consistent with RAGE being distributed in 98% of striatal spiny projection neurons and in all interneurons both in WT and in Huntington's disease (HD) animal model [36]. Importantly, a robust neuronal localization for RAGE was already described in nigral DAergic neurons following MPTP [16]. Nevertheless, RAGE immunofluorescence did not extend to striatal TH-positive axons, which was further confirmed by our findings. Regarding glial cells, evenly distribution of mRAGE in soma of astrocytes was

unchanged following astrogliosis in striata from MPTP mice. Therefore, even though RAGE is envisaged as a central player in glial reactivity [2–4], our observations rule out a chief role for S100B/RAGE axis in sustaining striatal reactive gliosis. Our data further exclude a direct involvement of RAGE in DAergic axonopathy under the present experimental setting.

Previous works have demonstrated an early upregulation of striatal S100B contents returning to basal levels at 7 days, in an acute MPTP model [35,37]. These findings are aligned with normal S100B reported here 7 days postchronic MPTP model. However, initial increase in S100B reported by former authors suggests earlier changes in ligands/RAGE levels are likely to occur in striatum in the acute phase of MPTP toxic insult. Notably, a long-lasting repression of S100B and RAGE gene expression after a transient increase was also reported in mesencephalon of MPTP-treated mice [16]. S100B and RAGE gene are both under complex

transcriptional regulation as negative regulatory elements within promoters are responsible of suppressing their expression, maintaining low levels of protein [11,38]. Altogether, these findings imply the existence of active compensatory processes tightly regulating S100B/RAGE axis toward homeostasis within striatonigral injury.

RAGE upregulation in chronically injured tissues led to a large number of studies focusing on RAGE-mediated neurotoxicity. For instance, a link between increased neuronal RAGE staining and neuronal death in preclinical and clinical HD settings is already established in striatum [36,39]. Nevertheless, RAGE's protective effects in homeostasis and repair after injury have been recently highlighted, substantiating the pleiotropism of this receptor [9,40]. In this chronic MPTP model, basal levels of S100B, endowed with neurotrophic activity [11,41], and normal neuronal RAGE density are more likely to be engaged in striatal neuroprotective mechanisms rather than in toxic events. For example, S100B/RAGE axis was recently linked to dendritic rearborization in cortical neurons following an excitotoxic glutamatergic insult [42]. Curiously, dendritic remodeling in striatal spiny projection neurons was correlated with mice locomotor spontaneous recovery 7 days postacute MPTP [29]. Therefore, RAGE prevalent neuronal expression in striatum after MPTP exposure warrants future studies to explore whether S100B/RAGE axis is playing a role in presymptomatic compensatory responses in PD neuropathology.

Nevertheless, increased levels of RAGE and ligands reported in patients with PD [14,15] necessarily imply failure of these overwhelmed repair-driven pathways in the course of human PD neuropathology. The next imperative question is as follows: Which RAGE isoform is we talking about when addressing RAGE axis? In fact, RAGE signaling is dependent upon the interplay between the membrane-bound isoform (mRAGE, which induces cellular signaling) and the soluble one (sRAGE, which is able to scavenge RAGE ligands thereby antagonizing mRAGE signaling) [5–7]. In fact, RAGE ligands may either accumulate in PD due to upregulated biosynthesis or impaired clearance systems (e.g., sRAGE). Unfortunately, most of previous studies used antibodies, which are incapable of distinguishing both RAGE isoforms, thus neglecting this RAGE complexity.

We newly characterize the biology of mRAGE isoform and its astrocytic S100B ligand in striatum of experimental PD. Our findings rule out the involvement of S100B/mRAGE axis in striatal gliosis and DAergic axonopathy, early events of PD neuropathology. Instead, neurotrophic S100B/mRAGE protein levels observed herein warrant further exploration of its putative neuroprotective role in a presymptomatic compensatory PD stage, a fundamental period for successful implementation of therapeutic strategies.

Acknowledgments

Conflict of Interest

The authors declare no conflict of interest.

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