Research Report

Minocycline treatment reduces white matter damage after excitotoxic striatal injury

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ABSTRACT

We investigated the protective effects of minocycline following white matter damage (WMD) in the rat striatum. Excitotoxic lesions were induced by N-Methyl-D-Aspartate (NMDA) microinjections and caused striatal damage, concomitant with microglial/macrophage activation. The excitotoxic lesion both damaged oligodendrocytes (Tau-1+ cells) and caused a decrease in tissue reactivity for myelin basic protein (MBP) after post-lesional day 3 (PLD). Treatment with the semi-synthetic tetracycline antibiotic minocycline, however, led to oligodendrocyte preservation and decreased myelin impairment. Taken together, these results suggest that white matter damage (WMD) is an important component of the physiopathology of acute striatal damage and that microglial/macrophage activation contributes to this pathological phenomenon.

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1. Introduction

Damage to the white matter (WM) is an important factor contributing to the severity of functional deficits caused by acute injuries of the brain and the spinal cord, such as trauma and stroke (Guimarães et al., 2009; Coleman and Perry, 2002; Medana and Esiri, 2003). In humans, lesions restricted to the spinal cord's gray matter usually limit the reach of motor and sensory disturbances to the areas primarily affected, without compromising the functions controlled by neighboring segments. By contrast, white matter damage (WMD) may have a more devastating effect, sometimes rendering a person tetraplegic and incontinent (Sadowsky et al., 2002). WMD can also be elicited indirectly, for instance due to ionic imbalance and inflammatory events elicited by the injurious agent (Stys, 2004).

The inflammatory response elicited by injury is determinant to the secondary neuronal degeneration that affects neighboring areas in the central nervous system (CNS) (Popovich et al., 1999; Popovich et al., 2002; Gomes-Leal et al., 2005). Inhibition of hematogenous macrophage recruitment with silica dust (Bligh, 1994) or liposome encapsulated clodronate (Popovich et al., 1999), for instance, improves considerably the prognosis of acute spinal cord injury (SCI).
in rodents. Also, blockage of microglial activation using the semi-synthetic tetracycline antibiotic minocycline provides neuroprotection in a number of experimental models of acute and chronic neural disorders, including SCI (Lee et al., 2003; Stirling et al., 2004), stroke (Yrjanheikki et al., 1999; Hayakawa et al., 2008), excitotoxic injury (EI) (Tikka et al., 2002), amyotrophic lateral sclerosis (Kriz et al., 2002; Van Den Bosch et al., 2002), and Parkinson’s, Huntington’s and Alzheimer’s diseases (Yong et al., 2004). Recently, it has been shown that minocycline treatment is beneficial for humans during the acute phase of stroke (Lampl et al., 2007).

So far, however, few studies have directly investigated the possible causal relationship between inflammation and WMD (Popovich et al., 2002; Gomes-Leal et al., 2005; Hewlett and Corbett, 2006). In previous works, our laboratory described the related patterns of WMD and inflammatory response in different brain areas using both ischemic and excitotoxic models of acute neural disorders (Gomes-Leal et al., 2004; Gomes-Leal et al., 2005; Dos Santos et al., 2007; Lima et al., 2008; Souza-Rodrigues et al., 2008). For instance, we showed that macrophages seem to contribute to axonal damage in the dorsal horn of the rat spinal cord injected with N-Methyl-D-Aspartate (NMDA) (Gomes-Leal et al., 2005).

After excitotoxic injury (EI) and ischemia in the striatum, a region of the basal ganglia linked to a number of neurodegenerative diseases (Mahapatra et al., 2004), all components of the WM, including axons and associated oligodendrocytes, are heavily damaged (Irving et al., 1996, 1997, 2001; Lima et al., 2008; Souza-Rodrigues et al., 2008). The acute inflammatory response to focal excitotoxic lesions has already been described in this region (Bolton and Perry, 1998; Lima et al., 2008) and includes intense microglial activation in the first 7 days following the primary damage (Lima et al., 2008). We have recently shown that acute striatal excitotoxic injury causes conspicuous WMD concomitant with intense microglial activation in the first 7 days following the primary insult (Lima et al., 2008). Yet, to our knowledge, there has been no investigation addressing directly whether microglial activation contributes to WMD after an acute striatal EI.

In the present study we try to overcome this deficiency by first establishing the patterns of macrophage/microglial activation, oligodendrocyte degeneration and myelin damage at 1, 3 and 7 days following acute microinjections of NMDA into the rat striatum. We then blocked microglial activation with minocycline, in search of a possible direct link between microglia recruitment and WMD. Our results show that minocycline treatment significantly reduces WMD in the striatum following the acute phase of an EI.

2. Results

2.1. Lesion area

We measured the areal limits of excitotoxic lesions induced by the NMDA microinjections in brain sections stained with hematoxylin–eosin (Fig. 1). Excitotoxic damage was characterized by tissue rarefaction caused by the loss of cell bodies and necrosis and evidenced by a distinctive pallor (Fig. 1). The area compromised by tissue loss became increasingly larger in later survival times.

2.2. Microglial activation

The NMDA microinjections induced a conspicuous inflammatory response in the striatum, characterized by intense microglial reaction as revealed by the anti-ED1 immunohistochemistry (Figs. 2B–E). ED1+ microglial cells were present in both the gray and WM and their number progressively increased from PLDs 1 to 7 (Figs. 2B–E). In control animals, this inflammatory reaction was negligible, with few ED1+ cells in the striatum of vehicle-treated animals (8±0.5 cells/mm²) (Fig. 2A). A considerable number of ED1+ cells were already present in the damaged striatum in PLD 1 (181±11 cells/mm²) (Fig. 2E), and their number only increased afterwards, reaching a density of 239±15 cells/mm² in PLD 3 and 192±12 cells/mm² in PLD 7 (Fig. 2E). The temporal increase in ED1+ cell density for PLDs 1, 2 and 3 was statistically significant compared to vehicle (p<0.05). Comparisons between PLDs 3 and 2 were statistically significant (p<0.05), but not between PLDs 7 and 3 (p>0.05).

2.3. Damage to oligodendrocytes

It has been shown elsewhere that, among glial cells, only damaged oligodendrocytes express the dephosphorylated form of the Tau protein (Tau-1) following acute neural disorders, including stroke and EI (Irving et al., 1997; Valeriani et al., 2000). After the NMDA injections, as expected, we found Tau-1+ cells displaying the conventional oligodendrocyte morphology in every PLD we analyzed (Fig. 3). Tau-1+ cells were present in the striatum of NMDA-treated, but not vehicle-treated animals, mainly in PLDs 3 (305±21 cells/mm²) and 7 (148±8 cells/mm²) (Fig. 3). A few Tau-1+ cells were observed in PLD 1 (20±3 cells/mm²) (Figs. 3B, E). Quantitative analysis revealed a considerable increase in the number of Tau-1+ cells from PLDs 1 to 7 (Fig. 3E), when compared to vehicle-treated animals (p<0.05). We observed the maximum number of Tau-1+ cells 3 days after the NMDA injection (Figs. 3C, E).

We performed immunocytochemistry against CNPase, an enzyme highly expressed in the cytoplasm of normal oligodendrocytes (Sprinkle, 1989), as an additional morphological marker of these glial cells (Fig. 4). Oligodendrocytes displaying cytoplasmic alterations with nucleus preservation (right arrow) or advanced stage of pathology with pyknotic nucleus were observed at PLD 3 and PLD 7 after NMDA injection (Fig. 4A). Caspase 3 positive cells with morphology resembling that of oligodendrocytes were present at the same survival times (Fig. 4B).

2.4. Diminished reactivity to myelin basic protein (MBP) in the striatum

We used a monoclonal antibody raised against MBP to investigate the pattern of myelin impairment following the NMDA microinjections in the rat striatum. As expected (Irving et al., 2001), there was a progressive loss of MBP reactivity in WM tracts of NMDA-treated (Figs. 4B–D), but not in vehicle-treated animals (Fig. 4A). This decrease in MBP reactivity was more conspicuous in PLD 7 (Fig. 4D).
2.5. Minocycline protection

Since microinjections of NMDA induced intense microglial activation concomitant with damage to the WM in the rat striatum, we investigated whether there is a causal relationship between these two events by administering either minocycline or sterile saline into rats during the 2 days following the NMDA injection. Animals were perfused at PLD 3, in which, as we have shown above, both microglial activation and oligodendrocyte damage reach a peak.

The minocycline treatment significantly reduced the number of ED1+ cells in the striatum 3 days after the NMDA injection (Figs. 5A–C). Quantitative analysis revealed a 52% decrease in the number of ED1+ cells in animals treated with minocycline (110±5 cells/mm²) compared to saline-treated animals (230±10 cells/mm²) (Fig. 5C). In minocycline-treated rats (Fig. 6B), there was also a decline in the number of Tau-1+ cells and concomitant myelin preservation (see Fig. 6). Additional quantification revealed that minocycline treatment decreased in about 50% of the number of pathological oligodendrocytes (Tau-1+ cells) in the striatum (158±14 cells/mm²), as compared to saline-treated animals (305±28 cells/mm²) (Fig. 7). These results suggest that minocycline protects the striatal WM tracts following an EI.

3. Discussion

In the present study, we used spatially precise excitotoxic lesions to study the contribution of subsequent inflammatory response to oligodendrocyte damage and myelin impairment in the rat striatum. More specifically, we investigated whether microglial activation contributes directly to WMD following striatal injury. We used the anti-inflammatory drug minocycline to inhibit inflammatory response and microglial activation. One advantage of using the non-traumatic method of making targeted microinjections of NMDA with a thin-glass capillary is to minimize the possibility of mechanical trauma and thus guarantee that the inflammatory response is mostly due to the excitotoxic lesion. This methodology has been successfully used in previous studies in our laboratory in the spinal cord (Gomes-Leal et al., 2004; Gomes-Leal et al., 2005), brain stem (Dos Santos et al., 2007), and striatum (Lima et al., 2008).

Here we show that after acute striatal excitotoxic injury, oligodendrocytes become immunoreactive for Tau-1, the dephosphorylated form of Tau protein, with a peak at PLD 3, and a decrease at PLD 7. This is in agreement with previous studies showing that Tau-1 immunoreactivity is expressed in oligodendrocytes in early survival times after stroke and other...

Although the expression of Tau-1 in oligodendrocytes has been considered a morphological marker of damaged oligodendrocytes (Irving et al., 1996, 1997; Valeriani et al., 2000; Irving et al., 2001), the exact meaning of this phenomenon is unknown. Tau-1 is preferentially expressed in oligodendrocytes after stroke and other acute neural disorders. Double labeling studies have shown that oligodendrocytes, but not astrocytes and microglia, are immunoreactive for Tau-1 following stroke (Valeriani et al., 2000). Tau-1 immunoreactivity may underly pathological changes on the cytoskeleton of these glial cells (Irving et al., 1996, 1997; Valeriani et al., 2000; Irving et al., 2001). Pretreatment with the spin trap agent

Fig. 2 – Microglia/macrophage activation following acute striatal excitotoxic damage, as revealed by anti-ED1 immunocytochemistry. Vehicle (A) and NMDA-treated animals at PLD 1 (B), PLD 3 (C) and PLD 7 (D). The maximum peak of macrophage recruitment occurred between PLDs 3 and 7 (C–D), as confirmed by quantitative analysis (E) (*p < 0.05, compared to vehicle). Comparisons between PLDs 3 and 1 were statistically significant (*p < 0.05), but not between PLDs 7 and 3 (*p > 0.05). Arrows point to ED1 positive cells. Scale bar: 50 µm.
alpha-phenyl-tert-butyl-nitrone (PBN) (100 mg/kg) reduced the number of Tau-1 positive oligodendrocytes by 55% in the subcortical white matter of the ischemic hemisphere compared with untreated animals at 40 min after middle cerebral artery occlusion (MCAO). This result suggests that free-radical mediated mechanisms are involved in the biochemical cascade leading to expression of Tau-1 in oligodendrocytes during ischemia. In addition, it has been proposed that Tau-1 immunoreactivity in these glial cells might represent an attempt to preserve cytoskeleton stability affected by the pathological insult (Irving et al., 2001). This hypothesis is supported by the finding that Tau-1 binds to and stabilizes microtubules in vitro (Weingarten et al., 1975). It is likely that expression of Tau-1 in oligodendrocytes represent an early attempt of the cells to preserve cytoskeleton integrity affected by the pathological insult.

Fig. 3 – Oligodendrocyte damage following an acute excitotoxic striatal injury, as revealed by anti-Tau-1 immunocytochemistry. Vehicle (A) and NMDA-treated animals at PLD 1 (B), PLD 3 (C) and PLD 7 (D). The maximum peak in the number of Tau-1+ cells occurred at PLD 3 (C), as confirmed by quantitative analysis (E) (*p < 0.05). Arrows point to damaged oligodendrocytes (Tau-1+ cells). Scale bar: 50 µm.
The possibility that oligodendrocytes are just overwhelmed by ischemia and fail to control effectively the action of free radicals can be a key factor to their degeneration in altered conditions (Dewar et al., 1999; Stys, 2004).

We found CNPase positive oligodendrocytes displaying conspicuous pathological alterations in both cytoplasm and nucleus as well as caspase-3 positive cells with morphology resembling that of oligodendrocytes in later survival times after NMDA injections, which is in agreement with these previous reports (Shuman et al., 1997; Emery et al., 1998; Beattie et al., 2000; Stirling et al., 2004; Goldenberg-Cohen et al., 2005; Yune et al., 2007). Nevertheless, further studies using triple and double immunofluorescence for apoptosis, Tau-1 and other oligodendroglial markers, in different survival times, are necessary to establish the final fate of Tau-1+ oligodendrocytes after acute neural disorders.

We found a pronounced loss of MBP reactivity especially in later PLDs after EI, corroborating previous studies suggesting that lesion of the WM can involve excitotoxic/inflammatory mechanisms occurring after neural disorders and directly related to the breakdown of the myelin sheath and induced by the increased flux of calcium ions (Gomes-Leal et al., 2004, 2005; Lima et al., 2008; Souza-Rodrigues et al., 2008).

Even though there are several evidences in the literature that show that treatment with the anti-inflammatory drug minocycline reduces microglial activation with concomitant neuroprotection (Tikka et al., 2001; Stirling et al., 2004; Hayakawa et al., 2008), only few studies have investigated the effect of minocycline on WM pathology (Stirling et al., 2004; Hewlett and Corbett, 2006). One of the neuroprotective effects mediated by minocycline is associated with the inhibition of inflammatory enzymes such as metalloproteinases, cyclooxygenase-2 (COOX-2), inducible nitric oxide synthase (iNOS), tumor necrosis factor alpha (TNF-α) and interleukin 1-beta (IL-1β). This action is also commonly seen after the blockade of microglial activation (Stirling et al., 2004; Suk et al., 2004). For instance, since activated microglia is the major source of iNOS and other species of reactive oxygen (ROs) (Kreutzberg, 1996), the minocycline-induced inactivation/reduction of microglial responses in the CNS reduces the expression of inflammatory enzymes, preventing cell death (Stirling et al., 2004). In addition, minocycline can increase the expression of anti-inflammatory factors such as interleukin-10 (IL-10), reducing the area of lesion and promoting the functional recovery of rats submitted to spinal cord lesion (Lee et al., 2003; Wells et al., 2003). Minocycline can also prevent apoptosis of both oligodendrocytes and neurons by inhibiting the formation of mitochondrial transitory permeability pore and blocking the release of cytochrome c to the cytoplasm (Zhu et al., 2002). Our present results show that minocycline effectively safeguards oligodendrocytes from these negative outcomes of the EI, as the number of ‘Tau-1+’ cells considerably decreased after PLD 3. Although there is no definitive evidence showing that this is due either to cell loss or oligodendrocyte survival and reversion to their normal morphology, our results suggest that the later hypothesis is true, since the number of pathological oligodendrocytes is strongly diminished after minocycline administration in PLD 3, which is the critical period for cell degeneration.

Considering that WMD is a key parameter in defining the prognosis of human brain diseases (Coleman and Perry, 2002; Coleman et al., 2006; Coleman et al., 2005; Yang et al., 2007), the CNPase positive cells with morphology resembling that of oligodendrocytes in later survival times may be related to the loss of oligodendrocytes. There are recent reports showing the co-labeling of Tau-1+ oligodendrocytes with other pathological markers after SCI (Cheng et al., 2008; Li et al., 2008). In addition, several studies have shown that a great number of oligodendrocytes undergo apoptosis even in later survival times after acute neural disorders, including stroke and spinal cord injury (Shuman et al., 1997; Emery et al., 1998; Beattie et al., 2000; Stirling et al., 2004; Goldenberg-Cohen et al., 2005; Yune et al., 2007). The exact mechanism by which oligodendrocytes are damaged after acute neural disorders is not completely established. Nevertheless, it has been shown that these glial cells express glutamate receptors and are thus susceptible to excitotoxicity mediated by this amino acid (Matute, 2006; Matute et al., 2007; Bakiri et al., 2008). Oligodendrocytes are also highly vulnerable to oxidative stress caused by excitotoxicity-mediated ischemia (Dewar and Dawson, 1997; Irving et al., 1997), mostly due to high metabolic activity, low intracellular concentrations of glutathione, high concentration of lipids, and high iron content (Dewar et al., 1999; Stys, 2004). The possibility that oligodendrocytes are damaged after acute neural disorders is not completely established. Nevertheless, it has been shown that these glial cells express glutamate receptors and are thus susceptible to excitotoxicity mediated by this amino acid (Matute, 2006; Matute et al., 2007; Bakiri et al., 2008). Oligodendrocytes are also highly vulnerable to oxidative stress caused by excitotoxicity-mediated ischemia (Dewar and Dawson, 1997; Irving et al., 1997), mostly due to high metabolic activity, low intracellular concentrations of glutathione, high concentration of lipids, and high iron content (Dewar et al., 1999; Stys, 2004). The possibility that oligodendrocytes are damaged after acute neural disorders is not completely established. Nevertheless, it has been shown that these glial cells express glutamate receptors and are thus susceptible to excitotoxicity mediated by this amino acid (Matute, 2006; Matute et al., 2007; Bakiri et al., 2008). Oligodendrocytes are also highly vulnerable to oxidative stress caused by excitotoxicity-mediated ischemia (Dewar and Dawson, 1997; Irving et al., 1997), mostly due to high metabolic activity, low intracellular concentrations of glutathione, high concentration of lipids, and high iron content (Dewar et al., 1999; Stys, 2004).
Medana and Esiri, 2003), the role played by minocycline in protecting the WM has a very important clinical implication (Dewar et al., 1999; Coleman and Perry, 2002; Medana and Esiri, 2003). Minocycline-induced WM protection should also be investigated using different models of neural disorders and using different temporal windows for a solid confirmation of the present results. In addition, although minocycline treatment decreased the number of pathological oligodendrocytes in half, it is conceivable that other factors beyond microglial activation could also be involved.

4. Conclusion

We have investigated the patterns of inflammatory response, myelin impairment and oligodendrocyte damage after an
acute excitotoxic striatal injury. We have observed that NMDA microinjections induce progressive decrease of MBP immunoreactivity and increase in Tau-1 expression on oligodendrocyte in the striatum of adult rats. These phenomena may be related to NMDA-induced WM damage. Blockage of microglial activation with the tetracycline derivative minocycline

Fig. 6 - Progressive myelin impairment following striatal excitotoxic injury and myelin preservation by minocycline. Photomicrographs showing the differences in myelin staining in control (A), PLD 1 (B), PLD 3 (C) and PLD 7 (D) and minocycline-treated animals (E). Notice the progressive loss in myelin staining in later survival times. Also notice the relative preservation of myelin in a minocycline-treated animal (E). Arrows point to white matter tracts. Scale bar: 300 μm.
protected the WM, suggesting a role for these glial cells on the genesis of WM injury after an excitotoxic lesion. Taken together, our findings suggest that microglial/macrophage activation contributes to WM damage and that early administration of minocycline could improve prognosis after acute brain injury.

5. Experimental procedures

5.1. Experimental animals

Male adult Wistar rats were obtained from the Central Animal Facility in the Federal University of Pará. All animals were housed under standard conditions with food and water available ad libitum. All experimental procedures were carried out in accordance with the Principles of laboratory animal care (NIH Publication No. 86-23, revised 1985), under license of the Ethics Committee on Experimental Animals of the Federal University of Pará. All efforts were made to avoid animal suffering and distress.

5.2. Surgical procedures and the model of NMDA-induced excitotoxic injury

In our previous studies, we have characterized the present model of NMDA-induced excitotoxic injury in both brain (Dos Santos et al., 2007; Lima et al., 2008) and spinal cord (Gomes-Leal et al., 2004, 2005). In short, the animals were deeply anesthetized with an intraperitoneal injection of a mixture of ketamine chloride (90 mg/kg) and xylazine chloride (10 mg/kg) and positioned in a stereotaxic apparatus. A thermal blanket was used to maintain body temperature within the physiological range with the help of a rectal thermometer. After craniotomy, 80 nmol of NMDA (Sigma Company, USA) in 1 μl of sterile saline were injected into the rat striatum over a period of 2 min using a glass capillary micropipette (n = 6 by survival time). The pipette was left in place for 3 min before being slowly withdrawn. Control animals were injected with the same volume of sterile saline (n = 4 per survival time). We used the following stereotaxic coordinates for the injection (in millimeters relative to bregma): 4.0 mm lateral; 0.5 mm posterior; and 5.5 mm below the pial surface. To allow the posterior identification of the injection site, a small quantity of colanyl blue was added to the injected solutions. After surgery, animals were allowed to rest in their cages with water and food ad libitum until post-lesion days (PLDs) 1, 3 and 7.

5.3. Perfusion and histological analysis

After the specified survival times, animals were deeply anesthetized with an overdose of ketamine/xylazine and were perfused transcardially with 0.9% heparinized saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. The brains were removed from the skull, washed in 0.1 M PB for 5 min and cryoprotected by immersions in increasing concentrations of sucrose solution. Both coronal and parasagittal sections were obtained using a cryostat (Carl Zeiss Microm, Germany). Sections were directly collected onto gelatinized slides over microtomy and air dried for 24 h. After, slides were stored at −20 °C for posterior analysis.

5.4. Histopathological analysis and immunohistochemistry

The lesion area was visualized in either coronal or parasagittal sections (20 and 50 μm thick) stained with cresyl violet or hematoxylin. The site of NMDA injection was recognized by the presence of colanyl blue in the tissue and through the pallor associated with loss of cell bodies.
In order to evaluate microglial activation, myelin impairment and oligodendrocyte damage, we resorted to standard immunohistochemical procedures. Activated microglia/macrophages were labeled using the antibody ED1 (1:500, Serotec, UK), an antibody that binds to an epitope on the lysosomal membrane of activated macrophages and microglia (Robinson et al., 1986). Oligodendrocytes were revealed through CNPase immunocytochemistry (1:100, Chemicon, USA), an enzyme highly expressed in the cytoplasm of oligodendrocytes and Schwann cells (Sprinkle, 1989). Based on previous reports showing that oligodendrocytes become Tau-1 positive after brain trauma and ischemia (Irving et al., 1996, 1997, 2001), we used the Tau-1 antibody (1:500, Chemicon, USA) to label dephosphorylated epitopes on pathological oligodendrocytes. Apoptotic cells were labeled using an antibody anti-active caspase 3 (1:250, Promega, USA) (Lalancette-Hebert et al., 2007). Myelin impairment was evaluated using an antibody against myelin basic protein (MBP), an important component of compact myelin (1:100, Serotec, UK) (Irving et al., 1997; Gomes-Leal et al., 2005).

5.5. Labeling protocol

The slide-mounted sections were removed from the freezer, kept in an oven at 37 °C for 30 min and rinsed in 0.1 M phosphate buffer saline (PBS) for 5 min. To improve the labeling intensity, sections were treated with 0.2 M boric acid (pH 9.0) previously heated to 65 °C for 25 min. This temperature was maintained constant over the treatment period. Sections were allowed to cool down for about 20 min and incubated under constant agitation in a 1% hydrogen peroxide solution in methanol for 20 min. Sections were then rinsed in 0.05% PBS/Tween (Sigma Company, USA) solution for 3 min for three times and incubated with 10% normal horse serum in PBS for 30 min. Without further rinsing, sections were then incubated with the primary antibody in PBS for 2 h, rinsed in PBS/Tween solution for 3 min (3 times), and incubated with biotinylated horse anti-mouse secondary antibody (Vector Laboratories, USA) diluted at 1:100 in PBS for 1 h. As a negative control, normal horse serum, rather the primary antibody, was used in some sections. Sections were rinsed again for 3 min (three times) and incubated in the avidin–biotin–peroxidase complex (ABC Kit, Vector Laboratories, USA) for 45 min. Sections were then rinsed four times (3 min each) and revealed with DAB (Gomes-Leal et al., 2004). After the DAB reaction, sections were rinsed three times (3 min each) in 0.1 M PB, dehydrated and coverslipped with Entellan (Merck, Germany). Some sections were counterstained with hematoxylin or cresyl violet (Nissl staining).

5.6. Minocycline treatment

Animals (n=5) received intraperitoneal injections of either minocycline (90 mg/kg) or sterile saline (n=4) 2 h after the NMDA injection, followed by supplementary doses (45 mg/kg) at 12 h intervals for two days. Animals were perfused after 3 days (24 h after the last dose of minocycline). This survival time was chosen to inhibit microglia activation, because the activation of this glial cell peaks at 3–7 days after NMDA injection. In addition, maximum of oligodendrocyte damage has been observed at 3 days in the present model of acute striatal injury.

5.7. Qualitative and quantitative analysis

All sections were initially surveyed by light microscopy, illustrative images from all experimental groups were acquired using a digital camera (Nikon Coolpix 950E) attached to a microcomputer (Nikon AFX-DX Optiphot-2).

We used coronal sections containing the damaged striatum to establish the areal density of activated macrophages/microglia (ED1+ cells/mm²) and pathological oligodendrocytes (Tau-1+ cells/mm²), using a 1 mm² graticule attached to the eyepiece (objective 40×). We counted 3 fields per section and 3 sections/animal (n=5–6 animals by group). The regions of interest had the highest cell density along a line passing through the lesion center (central field), and 2 additional fields were chosen at 100 µm intervals (100 µm medially and 100 laterally). These counts were performed for both minocycline and saline-treated groups.

Average values for all measurements were assessed with analysis of variance (ANOVA) with the Newman–Keuls post hoc test. The criterion for statistical significance was preset at an alpha level of 0.05. Average values are expressed as mean ± S.E.M.

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