**Research Article**

# Minocycline Ameliorates Neuronal Loss after Pilocarpine-Induced *Status epilepticus*

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## Abstract

Minocycline is an antibiotic agent that has been shown to have neuroprotective properties in animal models of ischemia, Huntington's disease and Parkinson's disease. However, data are lacking regarding the neuroprotective effects of minocycline treatment following pilocarpine-induced *status epilepticus*. Rats were treated with 25 mg/kg minocycline 2 hours after the onset of pilocarpine-induced SE. The hippocampus was examined for neuronal loss and microglia proliferation. The rats were monitored for the development of spontaneous recurrent seizures.

Minocycline treatment was found to reduce seizure-induced neuronal loss in the hippocampus. Although minocycline treatment did not significantly inhibit microglia activation, a modest reduction in microglial cell number was related to greater neuroprotection. Finally, a single-dose of minocycline did not prevent the occurrence of spontaneous behavioral seizures. Taken together, the findings suggest that Minocycline treatment is neuroprotective after SE.

## ABBREVIATIONS

**ANOVA:** Analysis of Variance; Min: Minutes; Mino: Minocycline; NeuN: Neuronal Nuclei; PBS: Phosphate Buffered Saline; SAL: Saline; SE: Status Epilepticus; SEM: Standard Error of the Mean; SRS: Spontaneous Recurrent Seizures

## INTRODUCTION

Minocycline is a tetracycline derived antibiotic that recently was shown to have neuroprotective properties in different neuropathological experimental models, including cerebral ischemia [1], Parkinson's disease [2] and Huntington's disease [3]. In addition, minocycline has been shown to be neuroprotective in the hippocampus when administered as a prophylactic agent prior to seizures induced by intrahippocampal injections of kainic acid [4].

The neuroprotective action of minocycline appears to result from inhibition of caspase- dependent, and -independent programmed cell death [4-6], and through microglia inhibition [7,8]. Moreover, minocycline inhibits neuroinflammation, suppresses free-radical production and inhibits metalloproteinases (for review see Yong et al. [9]). Thus,

minocycline might be a useful neuroprotective agent in diseases that exhibit neurodegeneration and neuroinflammation.

Neuronal loss in the hippocampus and piriform cortex has been described after seizures in humans [10-12]. This neuronal loss can be replicated in animal models of epilepsy, including the pilocarpine model of temporal lobe epilepsy [13-17]. Similar sclerosis is seen in numerous limbic structures and might be involved in seizure generation, seizure facilitation and the progression of temporal lobe epilepsy [18-20]. Thus, neuroprotective strategies might inhibit the development of epilepsy.

This study tested the hypothesis that minocycline treatment would ameliorate neuronal loss in the hippocampus following pilocarpine-induced SE. In addition because minocycline has been shown to inhibit microglial cells in vitro, this study analyzed microglial cell number after seizures to see if minocycline treatment would influence seizure-induced microglial cell activation. Lastly, spontaneous recurrent behavioral seizures were analyzed to determine if minocycline treatment would inhibit the epileptogenic progression. Our results show that a single dose of minocycline given shortly after Status Epilepticus

(SE) is neuroprotective in the hippocampus, but does not inhibit microglia proliferation and does not prevent the development of spontaneous seizures.

## MATERIALS AND METHODS

Male Sprague-Dawley rats (230-235 g) were used in this study. The animals were maintained in a 12 hour light-dark cycle (06:00-18:00) with food and water ad libitum. The experimental procedure was approved by the Internal Animal Care and Use Committee of the Texas A&M University Health Science Center (Permit number: 2008-002-R).

Systemic injection of pilocarpine was used to induce SE as previously described [21]. All animals were treated with 10 mg/kg diazepam i.p. 90 min after the onset of SE and were randomly assigned to receive either minocycline or saline. Rats that did not develop SE were excluded from the study. At 20 min after administration of diazepam, non-treated seizure animals received saline injection (SE group) and treated animals received a single minocycline injection of 25 mg/kg i.p. (SE+Mino). The minocycline dose of 25 mg/kg was chosen because this dose was shown to be safe and effective [22-24]. Alternatively, doses exceeding 25 mg/kg were showed to potentiate weight loss [25,26], cause skin irritation and liver toxicity [23,27].

The non-seizure animals were injected with saline instead of pilocarpine 30 min after methyl-scopolamine. These animals also received diazepam 90 min after the saline injections (Sal group) and minocycline injection 20 min after diazepam (Mino group). Thus, we had four experimental groups for these experiments, a Sal group, a Mino group, a SE group, and a SE+Mino group. Animals were weighed prior to treatment and once daily at the same time each day until sacrifice.

For immunohistochemical analysis, animals were deeply anesthetized five days after SE and perfused through the heart with 0.9% NaCl for 1 min, followed by 200 ml of 4% paraformaldehyde in PB (0.1 M, pH 7.4). The brains were removed, postfixed and cryoprotected in sucrose solution. Cryostat sections were cut at 40 μm and stored in PBS. Brain sections were processed for Neuronal Nuclei (NeuN) marker and ionized calcium binding adaptor molecule 1(Iba1), a marker of microglia. Free-floating sections were incubated in 1% H2O2 for 1,5 h, rinsed in PBS and then incubated for 1 hr in 3% normal horse serum (Vector Labs), in PBS. Sections were next incubated in primary antibody (mouse anti-NeuN, 1:1000, Chemicon; goat anti-Iba1, 1:500, Abcam), in PBS with 3% normal horse serum, for 24 h. For Iba1 staining, sections were then washed in PBS and incubated with secondary antibody (donkey anti-goat Alexa 555, 1:200, Millipore) for 90 min. For NeuN staining the tissue was incubated with secondary antibody (donkey anti-mouse, 1:200, Vector Labs), rinsed in PBS baths, incubated in avidin-biotin conjugated solution (ABC kit, Vector Labs), rinsed and developed with diaminobenzidine (DAB, Sigma). Brain sections were then mounted onto glass slides and cover slips were applied using permount.

Stereological analysis of NeuN-labeled cells was used to determine the extent of neuronal loss in the hippocampus using the optical fractionator method [28]. Analysis was performed using a microscope (Nikon Eclipse MU) with a motorized stage connected to a computer running the Stereo Investigator

software (MBF Bioscience). Briefly, hippocampal subregions (hilus, CA1 and CA3) were examined in 4 to 7 sections per rat. A counting frame of 40 x 40 μm was distributed in a randomly positioned lattice that varied in size according with the subregion (hilus: 100 x 100 μm; CA1 and CA3: 250 x 250 μm). NeuN positive cells were counted using as criteria staining in defined round cell nucleus. Results are presented as mean density of NeuN positive cells /mm3 ± SEM.

For microglia quantification, images of Iba1+ stained cells were captured using a laser scanning confocal microscope (Olympus Fluoview 1000). For each subregion, a 20x objective lens and 3x digital zoom were used to capture two random fields per section in a total of three sections per rat separated by at least 960 μm each. Ten frames in the Z dimension were acquired in each counting field. The three-dimensional confocal images were processed and analyzed with an Olympus Fluoview microscope and software (FV10-ASW; Version 1.7a). Hippocampal subregions (hilus, CA1 and CA3) were traced using the Free Area tool and Iba1+ cells were quantified using the Point tool. The Measurement command calculated the area delimitated in each counting field for each subregion and the total number of cells marked. The measurements of two counting fields captured per region in each section were summed. Total values for each category over each section were averaged across subjects in a group and compared between groups. The investigator was blind to the animal condition at the time of quantification. Results are presented as number of Iba1 positive cells / mm$^2$ ± SEM.

For the Spontaneous Recurrent Seizure investigation (SRS), two additional groups of rats (SE, n=6 and SE+Mino, n=6) were treated as previously described but were kept alive for 30 days after SE. Beginning one week after SE, the behavior of these rats was video-recorded using a digital camera for approximately 5h per day until 30 days. Class 3 motor seizure or greater in Racine's scale of limbic seizures [29] were considered for SRS frequency analyses.

Comparisons between Sal and Mino groups were made with t-test. Comparisons of histological parameters were made with nonparametric analysis of variance (ANOVA), followed by post-hoc Bonferroni test, where appropriate. Spearman rank order correlation test was used to investigate the correlation of neuronal loss and microglia proliferation. Spontaneous Recurrent Seizure (SRS) activity, including the incidence and number of seizures was analyzed using the Student - t test. Results for all analyses were considered significant at a level of 0.05.

## RESULTS AND DISCUSSION

NeuN immunohistochemistry was used to assess neuronal loss after SE. As can be seen in Figure 1, rats present a robust cell loss in the hippocampus five days after SE, compared to control rats. Most neuronal loss occurred in the hilus and pyramidal cell layer of CA1 (Figure 1). Minocycline treatment partially ameliorated this neuronal loss (Figure 1). Extensive neuronal loss was also observed in the piriform cortex of SE rats (**Figure 1**). Minocycline also partially protected piriform cortex neurons after seizures (Figure 1E-H).

In order to quantify neuronal loss in the hippocampus, NeuN-labeled cells were counted using stereological analyses through
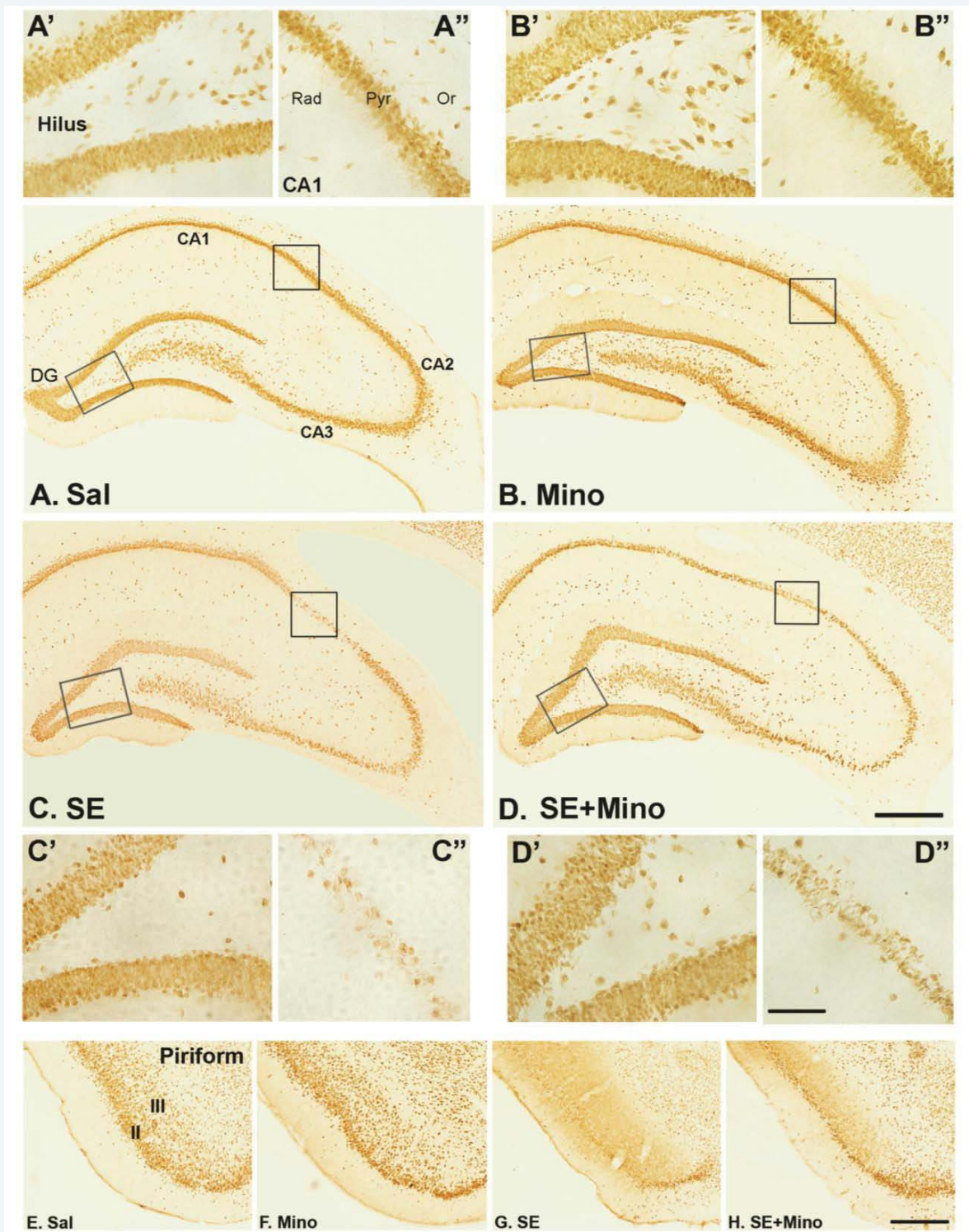
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**Figure 1** Neuronal nuclei (NeuN) immunostaining in hippocampus and piriform cortex. Images of the hippocampus of all treatment groups are shown in A-D. Inset areas depicting details from hilus (') and CA1 (") are presented in higher magnification with corresponding group labels. Control rats that received saline (A) or minocycline (B) injections present normal NeuN distribution with dense granular and pyramidal cell layers and relatively dispersed neurons in the hilus of the dentate gyrus, and CA1 oriens and radiatum. Note the neuronal loss in SE rats (C) compared to control rats (A), where relatively few neurons can be observed in the hilus (C') and CA1 regions (C"). Minocycline treatment partially ameliorated neuronal loss in the hippocampus after seizures (D). Note that relative to SE rats (C), more cells are present in the minocycline treated SE rats in the hilus (D') and in CA1 (D"). Lower panels present low magnification images of NeuN staining in the piriform cortex of all treatment groups. Control groups (E and F) present a typical dense neuronal population in piriform cortex layers II and III. As in the case of the hippocampus, after SE, cell layers of the piriform cortex show robust neuronal loss (G). This neuronal loss in the piriform cortex after SE was partially ameliorated by minocycline treatment (H). DG, dentate gyrus; Rad, radiatum; Pyr, pyramidal cell layer; Or, oriens. Scale bars in D, H: 1mm; in D": 200 μm.

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the optical fractionator method. There was no difference between groups in the volume of any hippocampal region analyzed (data not shown). In addition, there was no significant difference in the density of neurons between control rats (Sal and Mino rats not given seizures) in any of the hippocampal regions analyzed (Sal - Hilus: $18723\pm739$ neurons/mm$^3$; Mino - Hilus: $23136\pm2590$ neurons/mm$^3$; Sal - CA1: $22703\pm1732$ neurons/mm$^3$; Mino - CA1: $28214\pm2249$ neurons/mm$^3$; Sal - CA3: $26941\pm3201$ neurons/ mm$^3$; Mino - CA3: $35547\pm1706$ neurons/mm$^3$; p>0.05 for all). Thus, Sal and Mino rats were grouped together for subsequent comparisons. This combined group is referred to as a single Control group. Graphs showing the neuronal density of each hippocampal subfield are illustrated in Figure 2.

Consistent with the qualitative analysis, stereological quantification showed a significant neuronal loss in the SE group compared with Controls in all hippocampal regions (p<0.05). In the hilus, SE+Mino rats showed significantly less neuronal loss than SE rats (p=0.029). In CA1 and CA3, SE+Mino rats also presented some neuronal loss but it was not significant different from Control or SE rats (p>0.05).

In order to examine the effects of minocycline treatment on the proliferation of microglial cells after seizures, Iba-1 labeled microglial cells were examined qualitatively and quantitatively. As can be seen in Figure 3, control rats, including those given minocycline, present microglia cells with defined round cell bodies, long processes and each microglial cell appears to occupy specific domains with minimal overlap. Five days after SE, microglia cells appear hypertrophied, with enlarged cell bodies and smaller processes (Figure 3). In addition, there appeared to be many more microglia and they demonstrate substantial overlap. The proliferation and activation of Iba-1 labeled microglial cells in SE+ Mino rats was less robust than SE rats, but still detectable relative to control rats (Figure 3). This observation was consistent in all hippocampal regions, including the hilus of the dentate gyrus, CA1 and CA3 areas (Figure 3).

Quantification of Iba-1 labeled microglia confirmed a significant increase in the number of Iba-1-labeled microglial cells after seizures. Sal and Mino groups were again combined for this analysis ("Control") because no difference was observed between the two groups (Sal - Hilus: $440\pm57$ microglia/mm$^2$; Mino - Hilus: $444\pm14$ microglia/mm$^2$; Sal - CA1: $354\pm41$microglia/mm$^2$; Mino - CA1: $340\pm17$ microglia/mm$^2$; Sal - CA3: $330\pm20$ microglia/ mm$^2$; Mino - CA3: $329\pm13$ microglia/mm$^2$; p>0.05 for all). As can be seen in Figure 3F, Control rats has significantly less microglia cells in all hippocampal regions analyzed. In SE rats, the number of microglia cells increased significantly in all hippocampal regions (p<0.000 for all regions examined). In SE+Mino rats, a trend toward a decrease in the number of Iba-1 cells was observed in CA3 (p=0.088) but the decrease did not reached statistical significance in other hippocampal regions analyzed (hilus, p=0.701; CA1, p=0.299). Correlation analysis showed that when the amount of neuronal loss was correlated with the number of the microglia cells, a negative correlation coefficient was apparent between the two variables in all hippocampal regions analyzed (hilus: R= -0.520, p<0.05; CA3: R= -0.571, p<0.01; CA1: R= -0.659, p<0.01), meaning that more neuronal loss was correlated with more microglial activation.
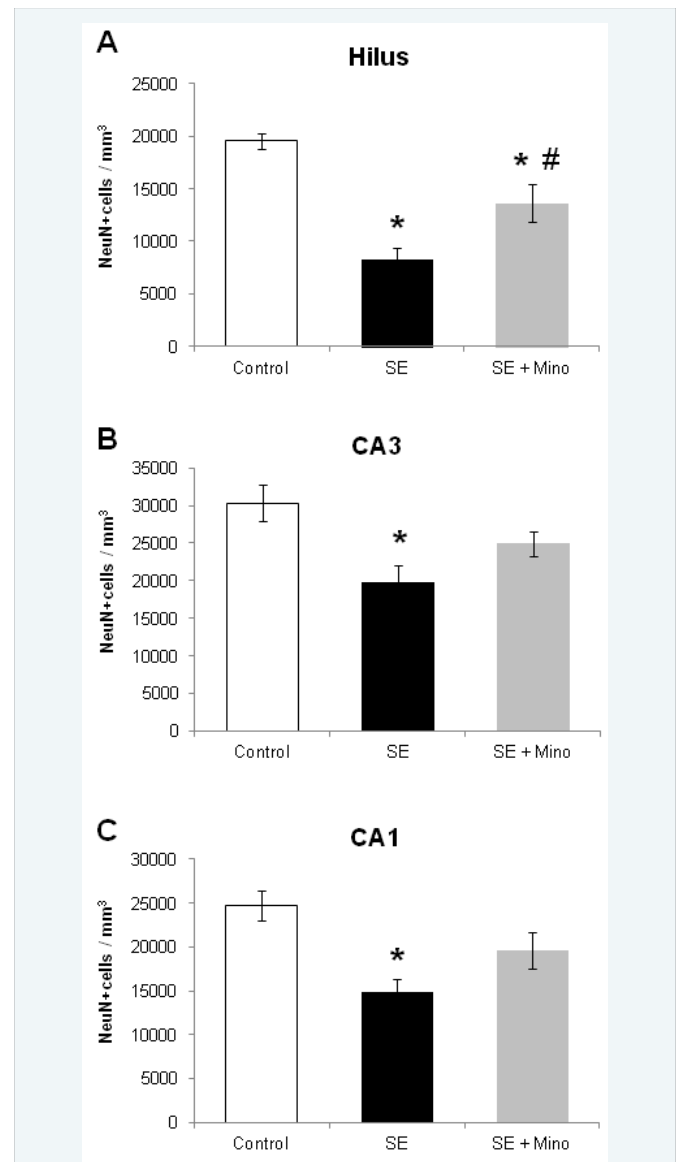


**Figure 2** Stereological counting of NeuN-positive cells in the hippocampus. Stereological quantification confirmed a significant neuronal loss in all regions of the hippocampus of SE animals compared to control rats (A-C). The neuronal loss in the hippocampus was partially ameliorated by minocycline treatment, although the difference in the number of neurons in the between SE and SE+Mino groups only reached significance in the hilus (A). Values: mean number of NeuN positive cells/mm$^3$ ± SEM. *p<0.05 to Control; #p<0.05 to SE group.

Analysis of behavioral seizures revealed that a single dose of minocycline given two hours after pilocarpine-induced seizures did not affect the development or severity of SRS. The behavioral analysis of spontaneous seizures in rats (stage 3 or higher) showed that both SE and SE+Mino, presented similar frequency of spontaneous seizures (p>0.6; Figure 4), similar total number of spontaneous seizures (p>0.6, Figure 4) and similar seizure severity (Figure 4).

Overall, in the SE group, 5 of the 6 rats, and 4 out of the 5 rats in the SE+Mino group developed spontaneous seizures (Figure 4).
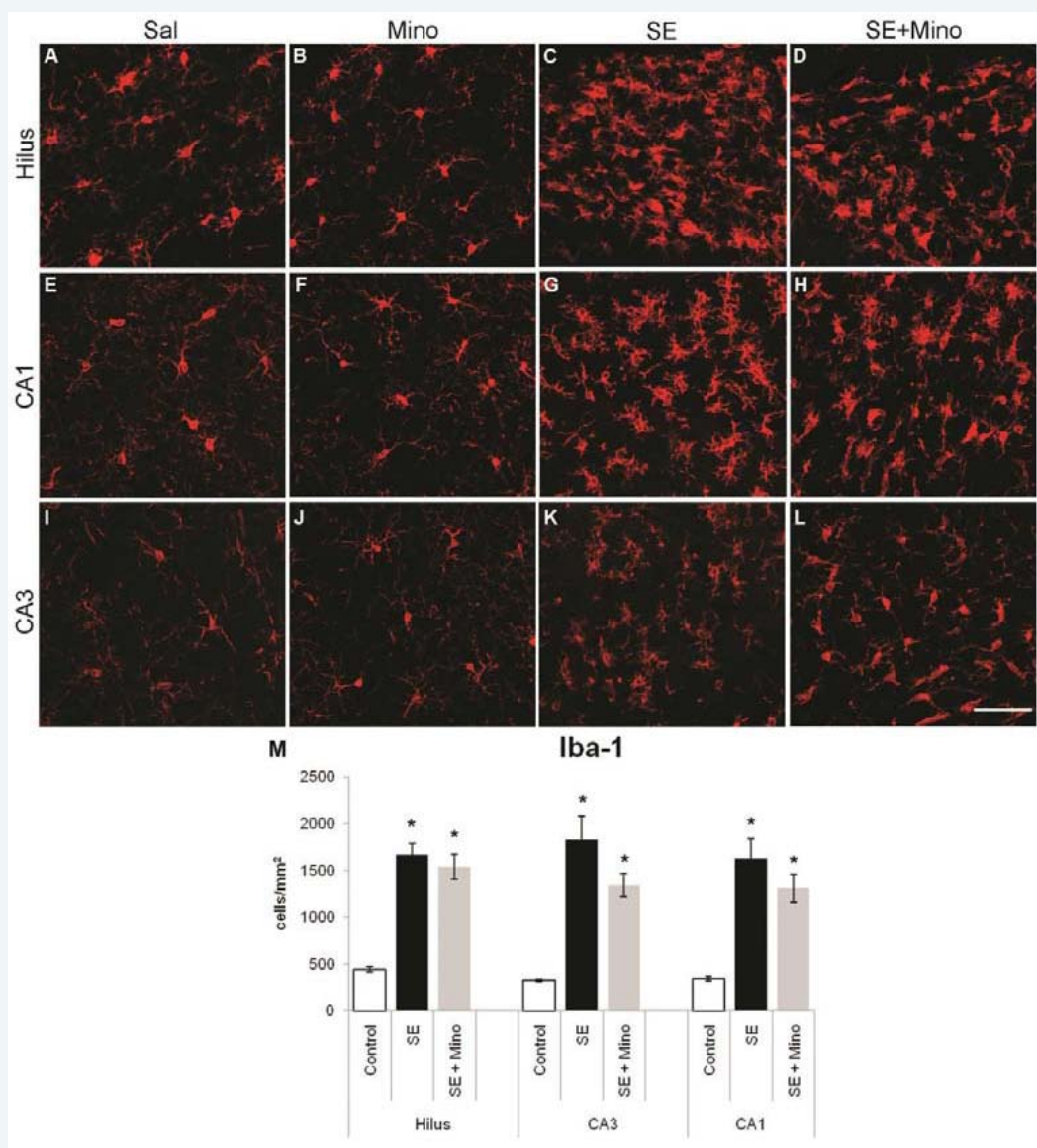
**Figure 3** Representative confocal micrographs and stereological quantification of Iba-1 microglia immunostaining in the hippocampus. Images of the hilus (A-D), CA1 (E-H) and CA3 (I-L) are shown. In normal conditions microglia cells present a well delineated round cell body, with many long and thin processes, and minimal overlap (A, E, I). Minocycline injection does not change this pattern of microglial expression (B, F, J). Note, that after SE (C, G, K), microglia cells present an activated morphology with bigger and longer cell bodies, shorter processes and increased numbers relative to control groups. Interestingly, a relatively sparse distribution and reduced numbers of microglia cells are visible after minocycline treatment following seizures (D, H, L). Scale bar = 50 μm. In M, stereological quantification of microglial cells confirm the robust increase in the number of microglia cells after seizures in the hilus, CA3 and CA1 regions of the hippocampus. Although Minocycline treatment decreased the number of microglia cells compared with SE group, the difference did not reach significance. Values: mean number of Iba-1positive cells/mm$^2$ ± SEM. *p<0.05 to Control.

The results from this study show that minocycline, administered two hours after SE, provides partial protection of neuronal loss in the hippocampus and piriform cortex. This effect is correlated with a modest decrease in microglial cell number. Despite the partial neuroprotective and anti-inflammatory effects, this dosage of minocycline did not inhibit the development of spontaneous behavioral seizures following SE.

The administration of minocycline as a treatment for neurodegenerative disorders has become the subject of investigation because of its neuroprotective effects in different pathological models [1-3]. In the epilepsy field, minocycline has been shown to be neuroprotective in the hippocampus when administered as a prophylactic agent prior to seizures induced by intrahippocampal injections of kainic acid [4]. In addition, injection of relatively high doses of minocycline following penicillin-induced seizures was effective in protecting hippocampal neurons [30]. Consistent with these studies, the data from the present study demonstrates for the first time, the neuroprotective effects of minocycline in the hippocampus and piriform cortex following pilocarpine-induced SE.
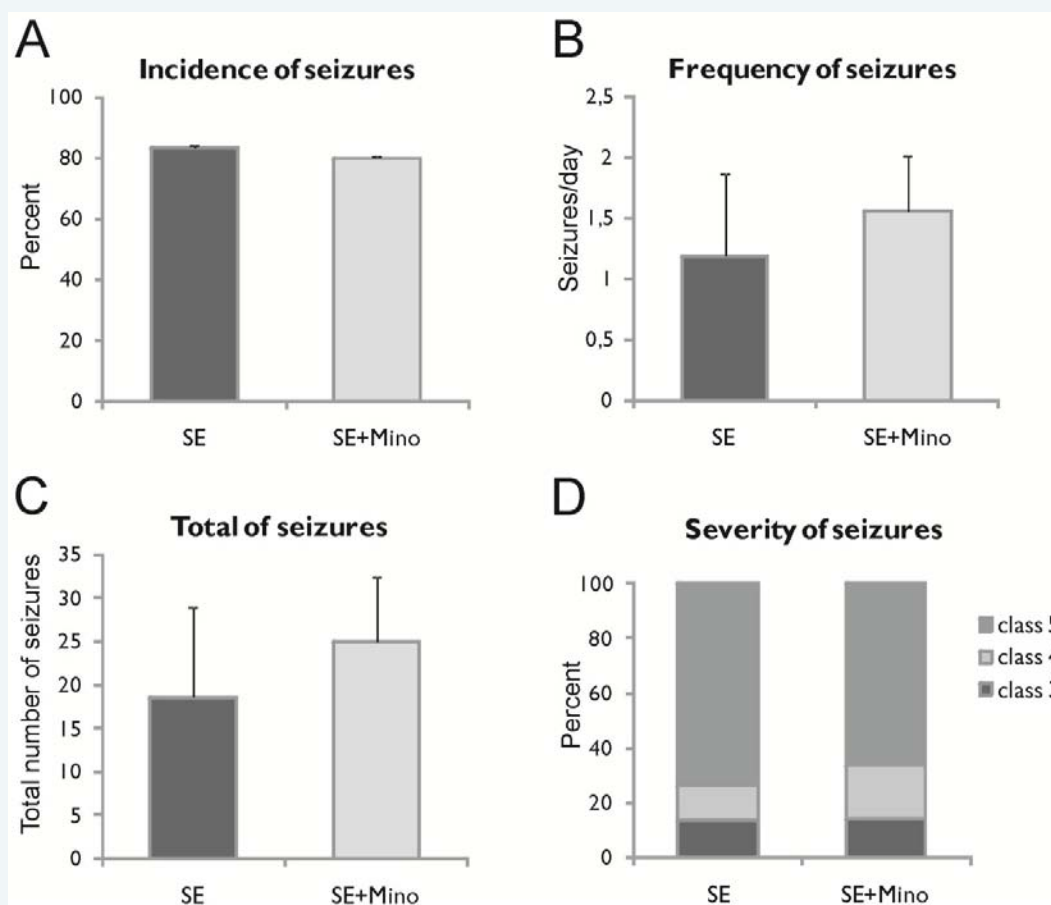
**Figure 4** Quantification of Spontaneous Recurrent Seizures (SRS) in saline and minocycline treated rats. The incidence (A), frequency (B), total number (C), and severity (D) of SRS are shown according to the Racine scale (1972). Class 3: forelimbs myoclonus; class 4: rearing; class 5: rearing followed by falling. There was no significant difference between groups in any category analyzed. Values (B and C): mean ± SEM.

One mechanism by which the neuroprotective actions of minocycline are known to occur is through inhibition of programmed cell death [1,5]. Minocycline inhibits activity of caspase-1, caspase-3 [3], caspase-independent cell death pathways [4] and mitochondrial cytochrome c release [6,31], all mechanisms involved in cell death cascades. Since apoptosis in the hippocampus and piriform cortex has been previously described after pilocarpine-induced seizures [32] it is plausible that this mechanism is related to the neuroprotective actions of minocycline after status epilepticus.

Despite the neuroprotective effects, a single dose of minocycline after pilocarpine- induced SE does not prevent the development, incidence, frequency, or severity of spontaneous behavioral seizures. These data are not entirely surprising considering the lack of anti- epileptogenic effects in other seizure models that used different neuroprotective compounds [33-37]. Interestingly, pre-treatment with minocycline was shown to attenuate second-hit seizure susceptibility induced by pentylenetetrazole [38,39]. It was also shown that minocycline treatment after kainic acid-induced status epilepticus in young animals attenuates microglia activation and decreases susceptibility to a second seizure induced later in life [40]. In addition, a recent clinical report showed a marked reduction in seizure frequency in response to minocycline therapy [41].

Although no reduction in spontaneous behavioral seizures was observed for a single-dose of minocycline after seizures, there is evidence supporting the idea that minocycline is not only neuroprotective, but may also be useful at reducing seizure susceptibility. Other dosing regimens might yield more beneficial anti-seizure effects in the pilocarpine model.

Minocycline has also been shown to inhibit microglial cells [1,8,40]. Microglial inhibition might provide secondary neuroprotection by inhibiting the production and release of detrimental neuroinflammatory proteins [42-44]. In the present study, microglia proliferation was negatively correlated with neuronal loss. However, our results did not show a significant inhibition of microglial cells following a single-dose of minocycline after SE, although a modest reduction of microglia cells was observed. It is possible that because of the severity of SE, higher doses, or longer treatment with minocycline may be necessary to have significant effects on the microglia cells of adult rats.

## CONCLUSION

Considering that seizure-induced cognitive deficits can be associated with cell loss throughout the limbic system [45,46], and cell loss may contribute to seizures, neuroprotective strategies might be beneficial as an augmentative treatment in

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epilepsy. Future studies should examine higher or longer dosage regimens and combination therapy to further determine the therapeutic potential of minocycline in epilepsy.

## ACKNOWLEDGEMENT

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