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Minocycline Causes Widespread Cell Death and Increases Microglial Labeling in the Neonatal Mouse Brain

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Abstract

Minocycline, an antibiotic of the tetracycline family, inhibits microglia in many paradigms, and is among the most commonly used tools for examining the role of microglia in physiological processes. Microglia may play an active role in triggering developmental neuronal cell death, although findings have been contradictory. To determine whether microglia influence developmental cell death, we treated perinatal mice with minocycline (45 mg/kg) and quantified effects on dying cells and microglial labeling using immunohistochemistry for activated caspase-3 (AC3) and ionized calcium-binding adapter molecule 1 (Iba1), respectively. Contrary to our expectations, minocycline treatment from embryonic day 18 to postnatal day (P)1 caused a >10-fold increase in cell death 8 h after the last injection in all brain regions examined, including the primary sensory cortex (S1), septum, hippocampus and hypothalamus. Iba1 labeling was also increased in most regions. Similar effects, although of smaller magnitude, were seen when treatment was delayed to P3-P5. Minocycline treatment from P3-P5 also decreased overall cell number in the septum at weaning, suggesting lasting effects of the neonatal exposure. When administered at lower doses (4.5 or 22.5 mg/kg), or at the same dose one week later (P10-P12), minocycline no longer increased microglial markers or cell death. Taken together, the most commonly used microglial “inhibitor” increases cell death and Iba1 labeling in the neonatal mouse brain. Minocycline is used clinically in infant and pediatric populations; caution is warrented when using minocycline in developing animals, or extrapolating the effects of this drug across ages.

Keywords

cell death; minocycline; microglia; cortex; hippocampus; septum; hypothalamus

INTRODUCTION

Microglia are the primary immune cells of the brain, and have long been linked to neuronal cell death. As the brain’s resident macrophages, microglia were originally thought simply to phagocytose dead cells (Perry et al., 1985; Ashwell 1990; Ferrer et al., 1990). More recently, however, they have been implicated in actively determining whether neurons live or die,

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although most of the evidence pertains to injury or disease models (Venero et al., 2011; Neher et al., 2012; Loane and Kumar, 2016).

Much less is known about the role of microglia during naturally-occurring, developmental cell death, and the available evidence is contradictory. About 50% of the neurons initially produced degenerate during a period of cell death that occurs primarily during the first 10 postnatal days in mice (Buss et al., 2006; Ahern et al., 2013; Mosley et al., 2016). Several studies suggest that microglia actively contribute to developmental neuronal cell death (reviewed in Marín-Teva et al., 2011). For example, cell death is suppressed in cultured retina, spinal cord and cerebellum when microglia are reduced or eliminated (Frade and Barde, 1998; Sedel et al., 2004; Marín-Teva et al., 2004). In addition, developmental apoptosis *in vivo* is decreased in the hippocampus in mice deficient in an integrin expressed by microglia and required for their interaction with neurons (Wakselman et al., 2008). On the other hand, more recent studies suggest that microglia support the survival of neurons in the somatosensory cortex of neonatal mice (Ueno et al., 2013; Arnoux et al., 2014). It is not clear whether the discrepant outcomes reflect differences in the role of microglia in different neural regions (somatosensory cortex versus other neural regions), or are due to variations in experimental design (i.e., *in vitro* versus *in vivo*; rat versus mouse; means of eliminating or inhibiting microglia).

One of the most commonly used tools for studying the roles of microglia has been minocycline, a broad-spectrum antibiotic, which also has anti-inflammatory properties (see Möller et al., 2016 for review). Minocycline reduces markers of microglial activation *in vivo* and *in vitro*, and alleviates neurodegeneration following hypoxia-ischemia, excitotoxicity, brain injury, and in several mouse models of neurodegenerative disease (Yrjänheikki et al., 1999; Chen et al., 2000; Sanchez Mejia et al., 2001; He et al., 2001; Tikka et al., 2001; Du et al., 2001; Wu et al., 2002; Kriz et al., 2002; Van Den Bosch et al., 2002; Popovic et al., 2002; Arvin et al., 2002; Peng et al., 2006; Tang et al., 2010). These findings suggest that in the absence of microglial interference, more neurons would be viable after injury or disease. In other cases, however, deleterious effects of minocycline have been reported (Diguet et al., 2004; Chen and Trapp, 2016).

To test the hypothesis that microglia contribute to naturally occurring cell death, we attempted to inhibit microglia with minocycline during the peak period of neuronal cell death in neonatal mice. The effects of minocycline were unexpected, which led to further analyses as described below.

METHODS

Animals

C57BL/6 mice were housed in a breeding colony at Georgia State University. Animals were maintained on a 12:12-hour light:dark cycle (lights on at 0600 h) at 22°C with food and water available *ad libitum*. All procedures were approved by the Georgia State University Institutional Animal Care and Use Committee and are in accordance with national guidelines.

Experiment 1A: Effects of minocycline on cell death and microglia in perinatal mice

A dose of 45 mg/kg minocycline hydrochloride (Sigma, St. Louis, MO) was chosen for the first experiment based on previous studies (Arvin et al., 2002; Tsuji et al., 2004; Mishra and Basu, 2008; Ueno et al., 2013; Arnoux et al., 2014; Peng et al., 2014). In keeping with prior work, and the reported half-life of the drug (Agwuh and MacGowan, 2006), minocycline or the vehicle alone (0.9% saline) was injected subcutaneously twice a day at 0630 h and 1730 h. A total of five injections were given between the morning of embryonic day 18 (E18) and the morning of postnatal day 1 (P1), or between P3 and P5, and in both cases pups were sacrificed 8 h after the last injection. These ages were chosen because the highest rates of cell death are seen during the first 5 days of postnatal life in the mouse forebrain (Ahern et al., 2013; Mosley et al., 2016).

For embryonic treatments, timed pregnancies were generated by pairing breeders overnight and removing the male at 0900 h the next morning (designated E0). Injections were given to the dam (based on dam body weight) until the birth of the pups on E19 (= postnatal day 0), at which point injections were given directly to the pups (based on pup body weight). Minocycline is lipophilic and readily passes from mother to fetus; it is present in amniotic fluid and umbilical cord blood after treatment of pregnant females, as well as in milk during lactation (Saivin and Houin, 1988), although at concentrations that may be lower than that in maternal plasma.

For the P5 group, breeders were checked twice daily for new litters, and the day of birth designated as P0. Pups were injected from P3 to the morning of P5.

In this and all subsequent experiments, equal numbers of male and female offspring were included in all groups and body weights were recorded at sacrifice. Number of animals per group is indicated in the figure legends.

Experiment 1B: Long-term effects of perinatal minocycline treatment

An additional cohort of pups was injected with 45 mg/kg minocycline or vehicle between P3-P5, as described above, and then were undisturbed until weaning. Brains were collected on P21, and stereological cell counts were performed as described below to examine the long-term effects of neonatal minocycline treatment on cell number.

Experiment 2: Effects of a range of doses of minocycline on cell death and Iba1 labeling

The injection protocol followed that described above, except that pups received two daily subcutaneous injections of 0, 4.5 mg/kg, 22.5 mg/kg, or 45 mg/kg minocycline from P3-P5. A total of five injections were given and pups were killed 8 h after the last injection on P5, as above.

Experiment 3: Effects of minocycline after the cell death period

Cell death throughout the mouse forebrain is elevated during the first postnatal week, and falls to very low levels in most brain regions by P9-P11 (Ahern et al., 2013). To examine effects of minocycline after the cell death period, we injected mice with 45 mg/kg

minocycline or vehicle alone every 12 hours from P10-P12. Animals received a total of five injections and were sacrificed 8 h after the last injection.

Brain Collection and Immunohistochemistry

Pups were rapidly decapitated (unless they were > 10 days old, in which case they were anesthetized via exposure to CO_2 before decapitation), and brains were removed and immersion-fixed in 5% acrolein (Alfa Aesar, Ward Hill, MA) in 0.1M phosphate buffer (PB) overnight. They were then transferred to a 30% sucrose solution in 0.1M PB at 4 °C until sectioning at 40 μm into four coronal series. Sections were stored in cryoprotectant (30% sucrose, 1% polyvinylpyrrolidone, 30% ethylene glycol in 0.1M PB at −20 °C) until use.

For detection of dying cells and microglia, alternate sections were immunocytochemically labeled for activated caspase 3 (AC3) or ionized calcium-binding adapter molecule 1 (Iba1), respectively. AC3 is a hallmark of apoptosis, and almost all AC3+ cells in the neonatal rodent brain also exhibit other markers of cell death (Srinivasan et al., 1998; Zuloaga et al., 2011). Iba1 is constitutively expressed by microglia and is upregulated following microglial activation (Ito et al., 1998; Imai and Kohsaka, 2002).

Sections were rinsed thoroughly in 1X Tris-buffered saline (TBS; pH 7.6), immersed in 0.05M sodium citrate in TBS for 30 minutes, and rinsed again. Sections were then immersed in 0.1M glycine in 1X TBS for 30 minutes, rinsed, and incubated in a concentrated blocking solution (1X TBS, 20% normal goat serum, 1% hydrogen peroxide, 0.3% Triton-X) before incubating overnight in a primary antibody solution (1X TBS, 2% normal goat serum, 0.02% Triton-X) to detect AC3 (anti-cleaved caspase-3, Cell Signaling, Beverly, MA; 1:20,000) or Iba1 (anti-Iba1, Wako, Chuo-Ku, Osaka, Japan; 1:20,000). Sections were washed in dilute blocking solution (1X TBS, 1% normal goat serum, 0.02% Triton-X) and incubated for 1 h in secondary antibody (biotinylated goat anti-rabbit, Vector Laboratories, Burlingame, CA; 1:500 in 1X TBS, 2% normal goat serum, 0.3% Triton-X). They were then rinsed, incubated for 1 h in an ABC solution (Vectastain Elite ABC kit, Vector Laboratories; 1:500, 1X TBS), and incubated in diaminobenzidine-nickel (DAB) solution (Vector Laboratories) for 20 min or until the reaction was complete.

Brain Regions Analyzed

We analyzed AC3 and Iba1 labeling in the primary somatosensory cortex (S1), hippocampus, septum, and hypothalamus. All regions were examined bilaterally and analyses were performed blind to treatment group. S1 was analyzed in three matching sections of each animal: the rostral-most section in which the dentate gyrus was clearly defined (Figure 41 in Paxinos and Franklin, 2001), and the next two alternate sections. For the hippocampus we included all sub-regions of the dorsal hippocampus from the rostral-most appearance of the dentate gyrus to the point where the hippocampus began to tip ventrally (Figure 41–Figure 43 in Paxinos and Franklin, 2001). The septum was analyzed from the point where the anterior commissure appeared in cross section just below the ventral-most tip of the lateral ventricles to the midline crossing of the anterior commissure (Figure 27–29), and the hypothalamus was analyzed ventral to the midline crossing of the

anterior commissure and comprised primarily the preoptic area (Figure 30–31 in Paxinos and Franklin, 2001).

Quantification of AC3 and Iba1 Labeling

We used several methods of quantification. AC3 immunostaining of minocycline-treated animals on P1 in Experiment 1A was so dense that individual cells could not be discerned; instead, gray level thresholding was used. Photomicrographs were taken of each region and Image J (National Institutes of Health, Bethesda, MD, USA) was used to draw a contour around the areas of interest. We then quantified the area covered by immunostaining above threshold within the region of interest, with threshold based on the default algorithm in ImageJ. For P5 animals in Experiment 1A, and all groups in Experiment 2, AC3 labeled cells were sufficiently sparse to count cells manually from photomicrographs. Areas of interest were traced in ImageJ and AC3+ cell counts were expressed as a density (# of AC3+ cells per unit area).

Iba1 immunostaining in Experiment 1A was quantified using the gray level thresholding method described above. The number of Iba1+ cells was counted in Experiment 2 using the particle counter function in Image J. Specifically, gray-level thresholding was performed and the number of “particles” within a window of 20–500 pixels was counted. This range was determined by measuring the number of pixels occupied by very small and very large Iba1+ cells and the method was validated by comparing automated particle counts to manual counts of cells in a subset of animals. The cross-sectional area of each region analyzed was measured using Image J and used to calculate the density of Iba1-positive cells.

Stereological Cell Counts and Microglial Morphology

We also performed unbiased stereology using the optical fractionator probe in StereoInvestigator (MBF Biosciences, Williston, VT, USA). For Iba1+ cell counts (Experiment 1A), contours were drawn around the region of interest using the 2.5x microscope objective and labeled cells were counted using the 20x objective. The counting frame was 200 μm x 200 μm and guard zones were set at 1.5 μm. The coefficient of error (CE) was < 0.10 for each animal. In addition to total Iba1+ cell counts, each labeled cell was classified based on morphology as amoeboid, stout, thick, or ramified, as in previous studies (Gómez-González and Escobar, 2010; Schwarz et al., 2012). Amoeboid, stout and thick microglia are thought to reflect more activated states than fully ramified microglia.

For analysis of long-term effects of minocycline (Experiment 1B), brains of P21 mice were frozen sectioned into four series and one series was stained with thionin. All cells with a neuronal morphology (large and multipolar) were counted by stereology, as above, except that counting was performed using the 100x objective and a counting frame of 30 μm x 30 μm.

Statistics

We first used 2-way ANOVAs (sex-by-treatment) to compare groups. Because no significant sex differences were found for any measure, data from males and females are combined in the analyses below. In Experiments 1 and 3, independent t-tests were used to compare

vehicle- and minocycline-treated groups on most measures. Stereological Iba1+ morphology counts (Experiment 1A) were analyzed using a mixed design 2-way ANOVA with group (vehicle versus minocycline) as the between-subjects factor and morphology (amoeboid, stout, thick or ramified microglia) as the within-subjects factor. In Experiment 2, one-way ANOVAs were used to compare effects of the different doses of minocycline. Post-hoc comparisons were performed using Fisher's Least Significant Difference. Significance was set at $P < 0.05$.

RESULTS

Experiment 1A: Effects of minocycline on cell death and microglia in perinatal mice

Cell Death—Treatment of perinatal mice with 45 mg/kg minocycline from E18 – P1 led to a massive increase in AC3+ cells on P1 that was evident in stained sections with the naked eye (Figure 1A–D). While AC3 labeling was increased throughout the brain, the sensory cortex and hippocampus appeared most severely affected (Figure 1B). An effect in the same direction, but of smaller magnitude, was evident when pups were treated with minocycline from P3-P5 and examined 8 h after the last injection (Figure 1E–J).

Quantification confirmed our qualitative assessments: AC3 labeling in minocycline-treated pups was increased more than 10-fold in all areas examined on P1 (S1, $P < 0.002$; septum, $P < 0.02$; hippocampus, $P < 0.005$; and hypothalamus, $P < 0.005$; Figure 2A,C,E,G). The number of AC3+ cells per unit area was also increased in all areas on P5 (S1, $P < 0.03$; septum, $P < 0.0001$; hippocampus, $P < 0.05$; and hypothalamus, $P < 0.002$; Figure 2B,D,F,H).

Microglia—Treatment with 45 mg/kg minocycline increased labeling for Iba1 on P1 (Figure 3A–D) and P5 (Figure 3E–J). Visual inspection suggested darker label per cell as well as more Iba1+ cells in minocycline-treated animals (Figure 3). A larger number and more uniform distribution of Iba1+ cells at P5 than in P1 was also evident from visual inspection.

Gray level thresholding confirmed increased Iba1 labeling in minocycline-treated pups in S1 ($P < 0.002$), the septum ($P < 0.0005$) and hippocampus ($P < 0.05$) on P1, and in S1 ($P < 0.005$) and the septum ($P < 0.04$) on P5 (Figure 4). Although effects in the same direction were seen for the hippocampus on P5, and the hypothalamus on P1 and P5, these comparisons did not reach significance (Figure 4).

We also used stereology to determine whether the increased Iba1 labeling seen in minocycline-treated pups was due to changes in the number and/or morphology of microglia. We counted the number of microglia on P5 in each of four different morphological classifications: amoeboid, stout, thick, and fully ramified (Figure 5). The septum was chosen for this analysis because this area showed a significant effect of minocycline on Iba1 labeling on both P1 and P5, and also contained a large enough number of microglia to meaningfully divide counts into four sub-groups. In agreement with previous findings in the postnatal rodent brain (Dalmau et al., 1998), the majority of microglia in both the saline and minocycline-treated neonates were stout or thick, with only small numbers of

amoeboid or fully ramified microglia (Figure 5). We found a main effect of treatment, with 43% more microglia overall in minocycline- than in vehicle-treated animals ($F_{1,19} = 6.95$; $P < 0.02$; Figure 5). We also found a treatment-by-morphological category interaction ($F_{3,57} = 5.90$; $P < 0.002$) due to the fact that minocycline-treated animals had more amoeboid, stout and thick microglia than did vehicle controls, whereas the reverse trend was seen for ramified microglia. Posthoc comparisons show that the difference between groups was significant only for stout and thick microglia ($P \leq 0.005$ in each case; Figure 5). Taken together, the effects of minocycline on Iba1 labeling are likely due to the combined effects of greater labeling per cell and an increase in the number of stout or thick microglia in minocycline-treated animals.

Experiment 1B: Long-term effects of postnatal minocycline treatment

We next asked whether the increased cell death seen after neonatal minocycline treatment leads to long-term changes in cell number. Mice were treated with saline or 45 mg/kg minocycline from P3-P5, as previously, and brains were collected on P21. Brain sections were thionin stained and stereological cell counts were made of the number of cells with a neuron-like morphology in the septum, which was the brain region exhibiting the most statistically robust effect of minocycline on cell death at P5 (Experiment 1A). We found a significant 15% reduction in cell number in the septum of minocycline-treated mice ($313 \times 10^4 \pm 12.7 \times 10^4$) compared to saline controls ($369 \times 10^4 \pm 22.2 \times 10^4$; $P < 0.05$).

Body Weight—There were no differences in body weight between minocycline and vehicle-treated pups in Experiment 1A or 1B (P1: $P = 0.751$; P5: $P = 0.296$; P21: $P = 0.970$).

Experiment 2: Effects of a range of doses of minocycline on cell death and Iba1 labeling

We next considered the possibility that 45 mg/kg minocycline might be too high a dose for neonates and determined whether lower doses might spare neurons and/or inhibit microglia during the cell death period. Mice were administered 0, 4.5, 22.5, or 45 mg/kg minocycline every 12 hours from P3-P5. Pups were killed 8 h after the last injection and effects on cell death and microglia were examined in the S1 and septum; two regions showing effects of minocycline on both AC3+ and Iba1+ cell number on P5 in Experiment 1.

ANOVA revealed an effect of treatment on AC3+ cell number in the S1 ($F_{3,57} = 4.93$, $P < 0.0001$). Minocycline-treated pups had higher AC3+ cell counts than vehicle controls, but this was significant only for the 45 mg/kg dose of minocycline ($P < 0.0001$; Figure 6A). In the septum, the overall ANOVA did not reach significance ($F_{3,51} = 2.038$, $P = 0.090$; Figure 6B). If a post-hoc test is nonetheless performed, pups receiving the 45 mg/kg dose had higher AC3+ counts than did vehicle controls ($P < 0.02$), confirming the observations of Experiment 1A.

ANOVA also identified significant effects of minocycline on Iba1+ cell counts in the S1 ($F_{3,41} = 6.14$, $P < 0.002$) and septum ($F_{3,40} = 3.60$, $P < 0.03$). Only the 45 mg/kg group was significantly different from vehicle controls in post hoc tests ($P < 0.001$ and $P < .003$ for the S1 and septum, respectively; Figure 6C,D), although there was also a strong trend for increased Iba1+ labeling in the S1 of mice receiving 22.5 mg/kg minocycline ($P = 0.059$).

Experiment 3: Effects of minocycline after the cell death period

To evaluate the effects of minocycline at later ages, we administered the effective dose in the experiments above (45 mg/kg) from P10-P12, and examined the S1 and septum on P12. We found no effect of minocycline on Iba1+ cell counts in either brain region (Figure 7A,B). We also examined AC3+ labeling in these animals. Cell death is very low by P9 in most forebrain regions (Ahern et al., 2013) and, in confirmation, there were virtually no AC3+ positive cells in the S1 or septum of control animals at P12 (not shown). We also found no cell death in minocycline treated animals, indicating that the 45 mg/kg minocycline dose does not alter cell death or microglia at this age.

DISCUSSION

Treatment with minocycline increased AC3 labeling in the neonatal mouse brain. The effects were age-dependent, with 45 mg/kg minocycline causing massive effects when administered from E18-P1, moderately large effects from P3-P5, and no effect from P10-P12. Effects were also dose-dependent, as minocycline treatment from P3-P5 did not significantly alter cell death at the lower doses tested.

Functions for AC3 unrelated to cell death have been reported (e.g., Li and Sheng, 2012). However, almost all cells that are AC3+ in the newborn rodent brain also exhibit other markers of cell death (Srinivasan et al., 1998; Zacharaki et al., 2010; Zuloaga et al., 2011), and over 95% of AC3+ cells throughout the neonatal forebrain are eliminated in mice with a deletion of the pro-death gene *Bax* (Ahern et al., 2013). This, combined with the observation that total cell number was reduced in minocycline-treated mice at weaning, supports the conclusion that minocycline increases cell death in the neonatal mouse brain.

Neonatal minocycline administration paradoxically *increased* Iba1 labeling in all brain areas examined, and also increased the number of stout or thick microglia. Although this was specific to the 45 mg/kg dose, at no dose did we see inhibition of microglia in newborn mice. Minocycline is a long-acting tetracycline that readily crosses the blood-brain-barrier (Yrjanheikki et al., 1999; Kielian et al., 2007). Although often considered a specific microglial inhibitor without direct actions on astroglia or neurons, other modes of action are possible, especially after systemic administration (Möller et al., 2016). If minocycline acted directly, and only, on microglia, our results would suggest that microglial activation increases cell death in the neonatal brain. On the other hand, the effects on microglial labeling seen here may have been secondary to effects of minocycline on cell death. Dying neurons are thought to provide “find me” signals that trigger microglial infiltration and activation (reviewed in Casano and Peri, 2015); if minocycline directly kills neurons in the neonatal brain, the effects on microglia seen here could have been indirect.

Regardless of the mechanism(s) of action, our current findings are in contrast to the microglial inhibition and neuroprotection reported in many previous rodent studies using similar doses of minocycline (30 – 90 mg/kg; studies referenced above as well as more recent findings by Reinebrant et al, 2012; Ma et al., 2012; Kobayashi et al., 2013; Azevedo et al., 2013; Yin et al., 2013; Peng et al., 2014). The present observations are in accord, however, with those of two recent studies reporting increased cell death specifically in layer

V of the mouse sensorimotor or somatosensory cortex following neonatal minocycline treatment (Ueno et al., 2013; Arnoux et al., 2014). We find that the effects are not limited to S1, with increased cell death also seen in the septum, hippocampus, hypothalamus and, based on visual inspection, likely in other regions that we did not quantify. In addition, although Ueno et al. (2013) and Arnoux et al. (2014) both observed increased cell death in neonatal sensory cortex after minocycline treatment, they found opposite effects on microglia: Arnoux et al. (2014) report microglial activation and Ueno et al. (2013) report microglial inhibition in response to minocycline.

Age-dependent effects of minocycline may be related to developmental changes in microglia and/or neurons

Microglial progenitors proliferate rapidly during late prenatal and early postnatal life, with 10- to 20-fold increases in the number of microglia observed during the first two weeks of life, depending on brain region (Alliot et al., 1999; Dalmau et al., 2003; Sharaf et al., 2013). Microglial morphology also changes developmentally, with more amoeboid and stout microglia (usually suggestive of an activated or mobile state) perinatally, and more ramified microglia with long, thin processes seen at later ages (Dalmau et al., 2003; Schwarz et al., 2012). Following hypoxia-ischemia, there is a greater microglial response in newborn mice than in juveniles (Ferrazzano et al., 2013), and a growing body of literature reveals differences in gene expression between neonatal and adolescent or adult microglia (Parakalan et al., 2012; Crain et al., 2013; Butovsky et al., 2014). Developmental changes in microglia may therefore explain the different responses to minocycline seen in mice of different ages in the current study.

In addition, the large majority of previous studies reporting inhibition of microglia by minocycline examined adults or developing rodents following injury or disease. In the one exception we are aware of, introcerebroventricular administration of minocycline reduced Iba1 protein in the neonatal rat brain, as determine by Western blot (Lenz et al., 2013). Microglia become activated and increase their production of a wide array of chemokines and cytokines following neural injury or perturbation (Lucin and Wyss-Coray, 2009), so differences in the transcriptional state of activated versus non-activated microglia could explain the different effects of minocycline under normal and injury-induced conditions.

Our results may also reflect a developmental switch in neurons. Developmental cell death peaks between P1 - P5, and drops to very low levels by P9 in most regions of the mouse forebrain (Ahern et al., 2013; Mosley et al., 2016). The varying effects of minocycline on cell death seen at different ages here may reflect greater vulnerability of neurons during the cell death period. One of the most striking effects of minocycline was seen in the oriens layer of the hippocampus on P1 (although we did not separately analyze the different hippocampal cell layers, this layer appeared most affected based on visual inspection; see Figure 1). Interestingly, the hippocampal oriens has an exceptionally high rate of naturally-occurring cell death that peaks on P1 in the mouse (Mosley et al., 2016), suggesting that minocycline did not induce cell death *de novo*, but exaggerated an ongoing process. This is in accord with previous suggestions that the period of neuronal cell death identifies a window during which exposure to drugs, injuries, infections, or variations in environmental

factors may have greatest impact (McDonald et al., 1988; Yakovlev et al., 2001; Olney, 2002).

As noted above, most previous studies reporting neuroprotection by minocycline examined induced or pathological neurodegeneration (Yrjänheikki et al., 1999; Chen et al., 2000; Sanchez Mejia et al., 2001; He et al., 2001; Tikka et al., 2001; Du et al., 2001; Wu et al., 2002; Kriz et al., 2002; Van Den Bosch et al., 2002; Popovic et al., 2002; Arvin et al., 2002; Peng et al., 2006; Tang et al., 2010; Reinebrant et al., 2012; Lopez-Rodriguez et al., 2013; Yin et al., 2013). Different molecular mechanisms may underlie neuronal cell death depending on whether it is naturally-occurring or due to injury, disease, or excitotoxicity (e.g., Gibson et al., 2001; Cregan et al., 2002). Thus, while minocycline often protects against pathological cell death, our results suggest it may increase naturally occurring cell death in the perinatal brain.

Regional differences in effects of minocycline

Although effects of minocycline appeared widespread, we also observed evidence of regional variation, with Iba1 labeling in the hypothalamus less affected than the other regions examined here. Microglia from different brain regions have distinct immunoregulatory gene expression profiles at rest and under stimulated conditions (Ren et al., 1999; de Haas et al., 2008; Lai et al., 2011). Thus, it is possible that regional diversity in microglia contributed to the region-specific responses to minocycline seen here. The cellular density of microglia can also vary as much as 5-fold across different regions of the mouse brain (Lawson et al., 1990), and the response to an immune challenge correlates with microglial density (Olah et al., 2011). Differences in microglial density are unlikely to explain the current results, however, as we did not observe large differences among the regions quantified here (see Figure 4).

Species differences?

Some of the apparent contradictions in the literature regarding whether minocycline is neurotoxic or neuroprotective, or activates or inhibits microglia, could be due to species differences. When administered in doses shown to provide neuroprotection after hypoxia-ischemia in postnatal rats, minocycline increased hypoxic/ischemic injury in the P8 mouse brain (Tsuji et al., 2004). In a more recent study, however, a similar dose of minocycline suppressed the microglial response to hypoxia/ischemia injury in P9 mice (Cikla et al., 2016). Minocycline is also neuroprotective in the adult mouse in models of traumatic brain injury, Parkinson disease, Huntington disease, amyotrophic lateral sclerosis, and other conditions (Chen et al., 2000; Du et al., 2001; Sanchez Mejia et al., 2001; Kriz et al., 2002). Taken together, we conclude that the varying effects of minocycline are more likely to be due to developmental changes and/or molecular differences between naturally occurring and induced neuronal cell death than to species differences.

Conclusion

The increased Iba1 labeling after minocycline treatment seen here prevented us from answering our original question of whether inhibition of microglia affects naturally occurring neuronal cell death. Our results instead point to a developmental switch in

responsiveness to minocycline, which may represent age-related changes in microglia, neurons, or both. Minocycline is administered to infants and young children to treat bacterial infections (Okada et al., 2012; Keneko et al., 2012), and has been used in preliminary clinical trials for pediatric disorders (e.g., Chacon et al., 2011; Grieco et al., 2014; Hagelman and Polussa, 2015). Although generally considered safe, some adverse effects of minocycline in pediatric patients have been reported (e.g., Chamberlain et al., 2006; El-Halak et al., 2008). Our results suggest caution when extrapolating the effects of minocycline from one developmental age to another.

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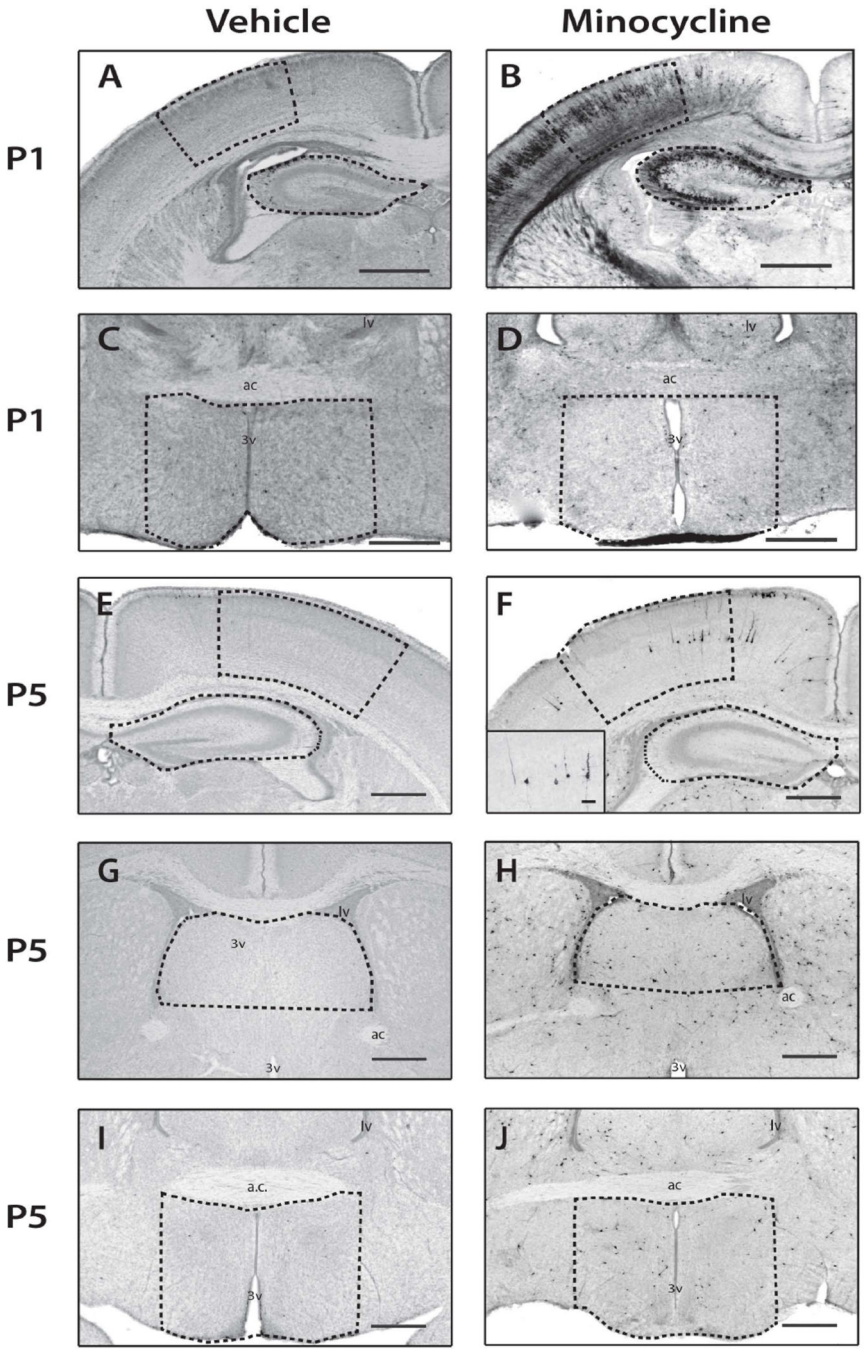

Figure 1.

Minocyline treatment of perinatal mice increased AC3 labeling across many brain regions. Photomicrographs of sections through the forebrain reveal many more AC3 labeled cells in minocycline-treated mice (right) than in vehicle-treated controls (left) on both P1 (A–D) and P5 (E–J). Dotted lines depict the regions quantified: S1 and hippocampus (A, B, E, F); hypothalamus (C, D, I, J); septum (G, H). Inset in F is a higher magnification view of labeled cells in the boxed area. Scale bars = 500 μm for A-J and 50 μm for F inset. Abbreviations: 3v, third ventricle; ac, anterior commissure; lv, lateral ventricle.

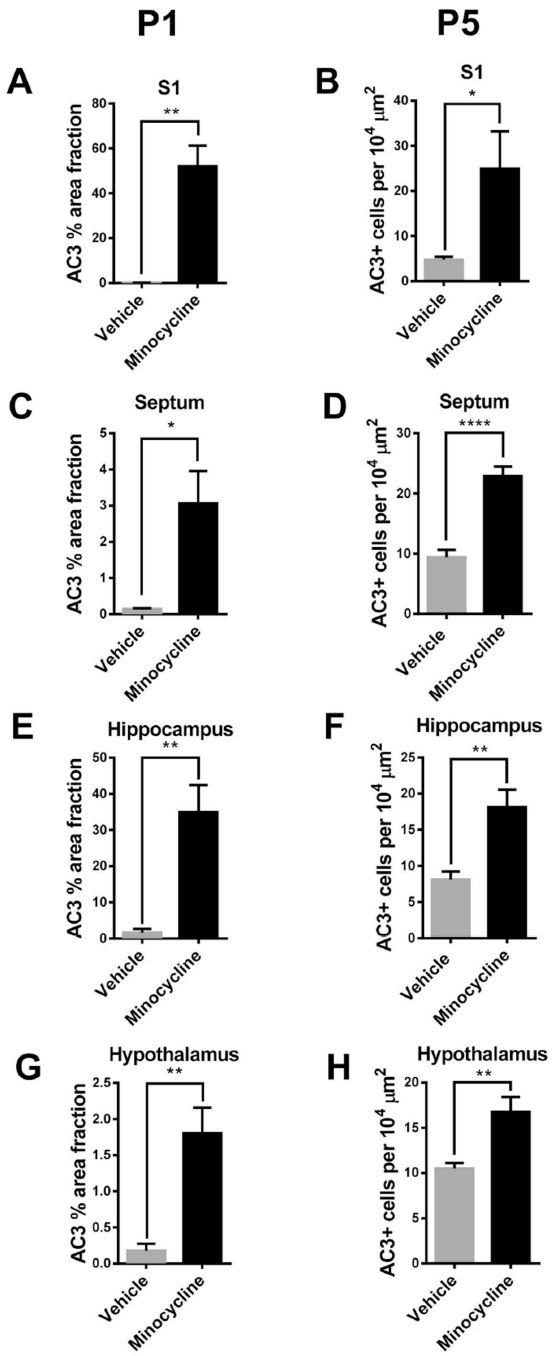

Figure 2.

Quantification of AC3 labeling revealed that perinatal minocycline treatment significantly increased cell death on P1 and P5 in the S1 (A, B), septum (C, D), hippocampus (E, F) and hypothalamus (G, H). Number of animals per group = 5 P1 vehicle, 4 P1 minocycline, 12 P5 vehicle, 11 P5 minocycline. Asterisks: $*P < 0.05$, $**P < 0.005$.

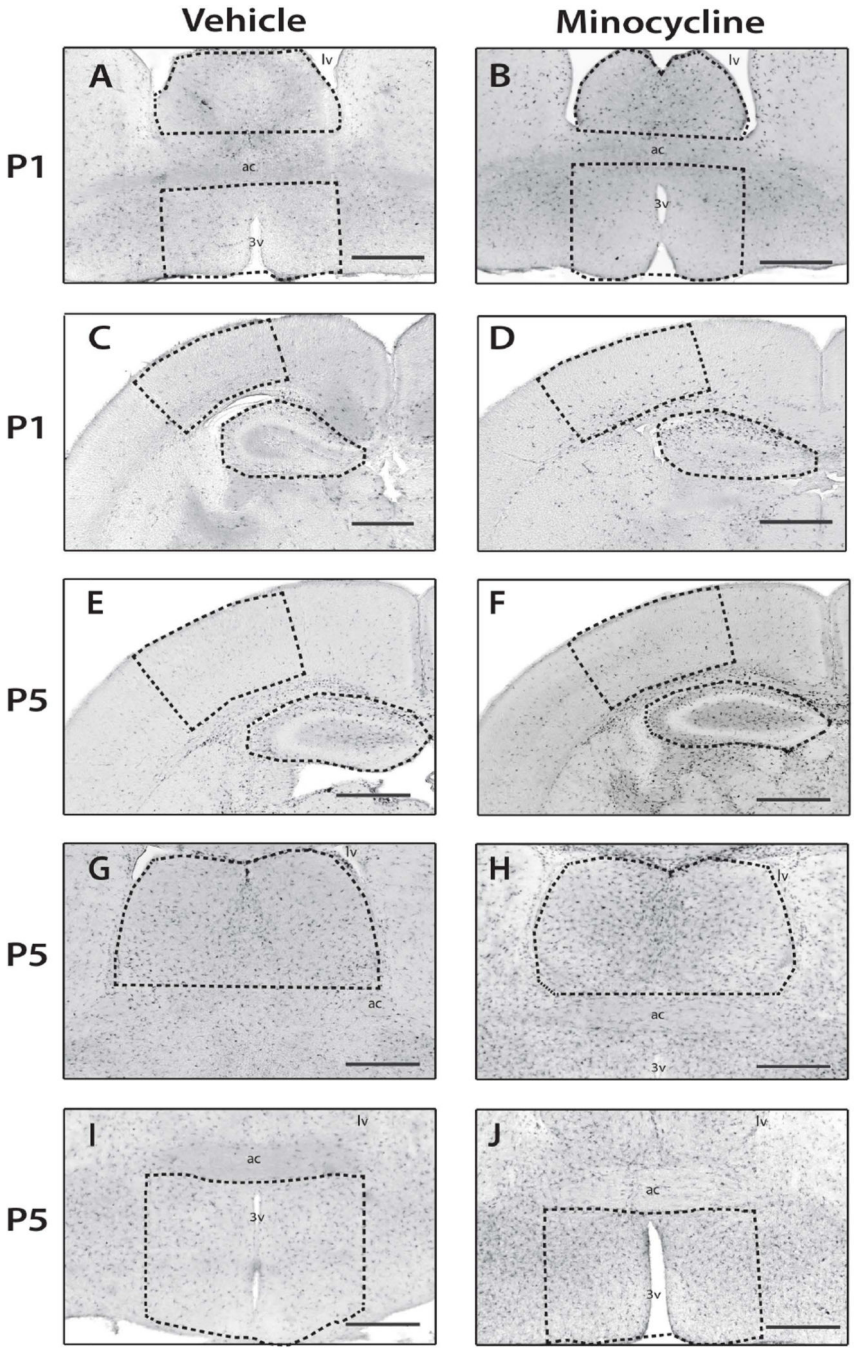

Figure 3.

Perinatal minocycline treatment increased Iba1 labeling. Photomicrographs of sections through the forebrain reveal more Iba1 labeling in minocycline-treated mice (right) than in vehicle-treated controls (left) on both P1 (A–D) and P5 (E–J). Dotted lines depict the regions quantified: S1 and hippocampus (C, D, E, F); hypothalamus (A, B, I, J); septum (A, B, G, H). Scale bars = 500 μm. Abbreviations: 3v, third ventricle; ac, anterior commissure; lv, lateral ventricle.

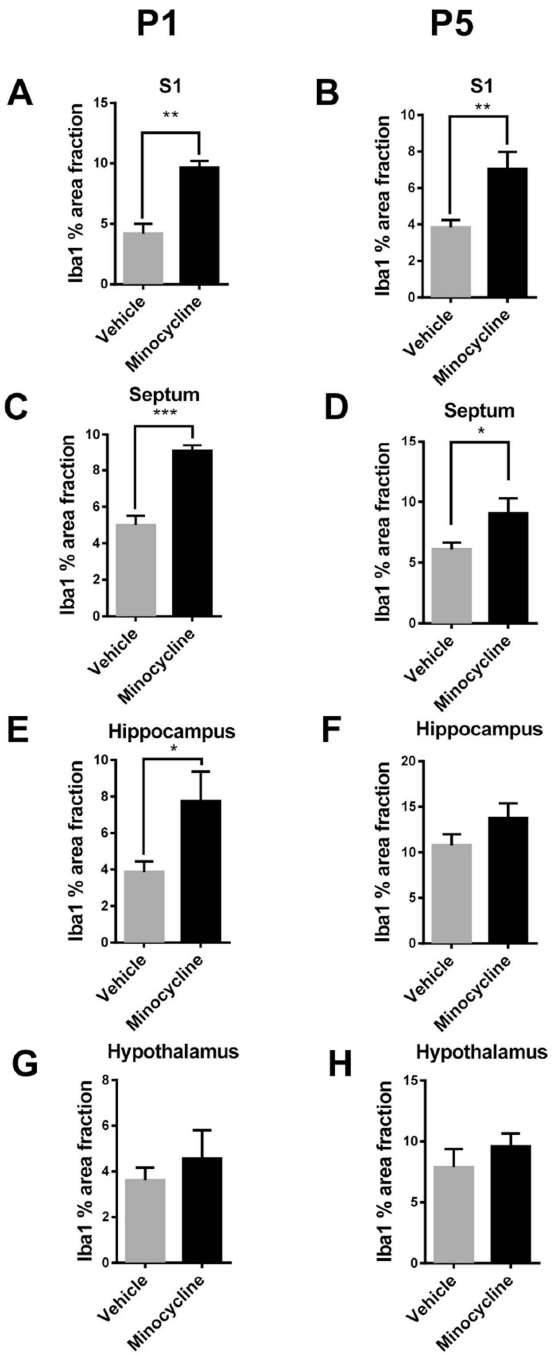

Figure 4.

Quantification demonstrated significantly greater Iba1 immunoreactivity in minocycline-treated animals than in vehicle controls in the S1 (A), septum (C), and hippocampus (E) on P1 and in the S1 (B) and septum (D) on P5. Effects of minocycline were not significant in the hypothalamus (G, H). Number of animals per group = 5 P1 vehicle, 4 P1 minocycline, 12 P5 vehicle, 11 P5 minocycline. Asterisks: $*P < 0.05$, $**P < 0.005$, $***\ P < 0.0005$.

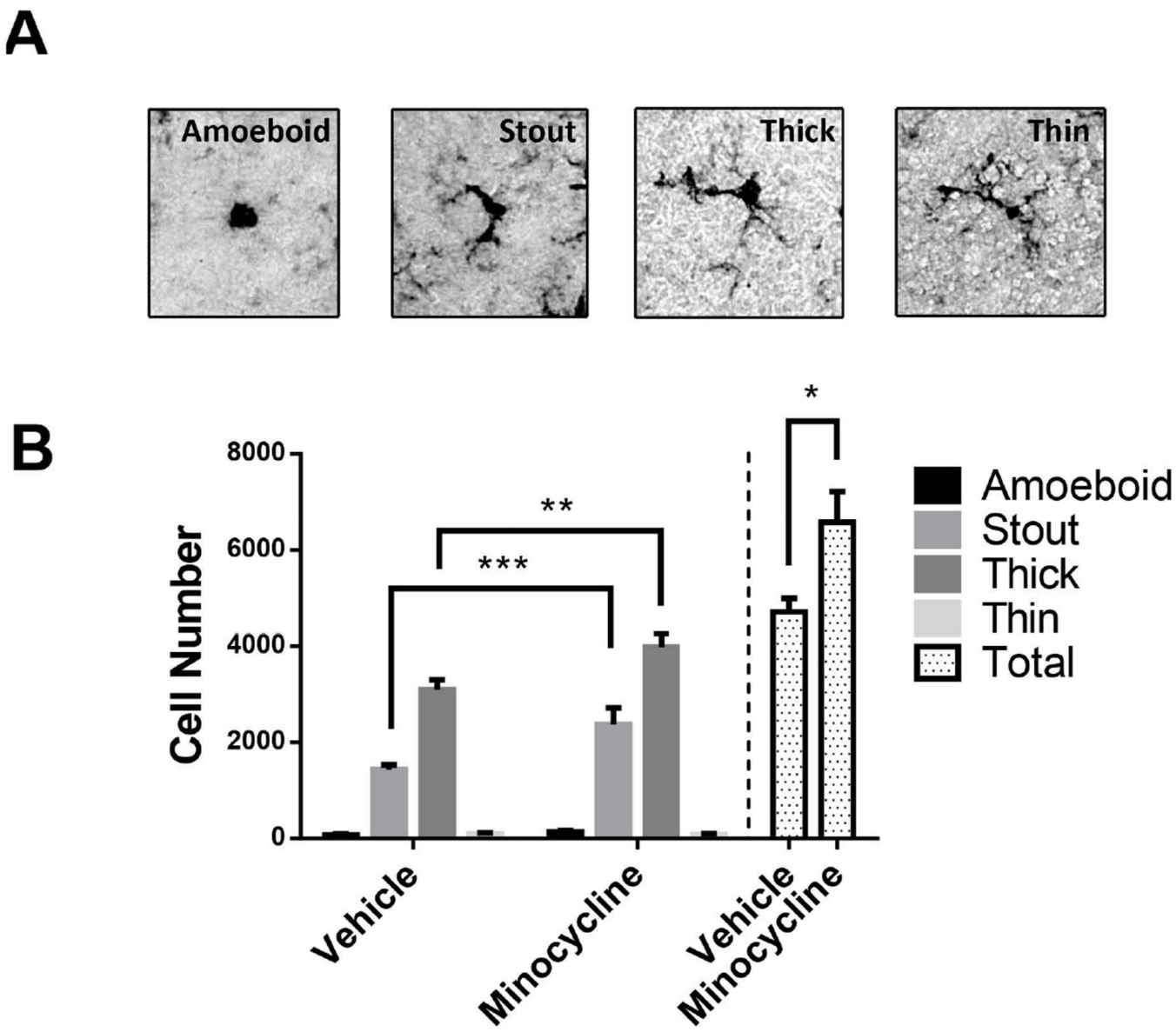

Figure 5.

Minocycline treatment increased the number of stout and thick microglia. (A) Photomicrographs depicting four morphological categories of micoglia. (B) Stereological cell counts of microglia in the septum of vehicle- and minocycline-treated pups on P5. Minocycline treatment increased the number of microglia with a stout or thick morphology, as well as total microglial cell number. Number of animals per group = 5 for vehicle and 6 for minocycline. Asterisks: $*P < 0.05$, $**P = 0.005$, $***P < 0.0005$.

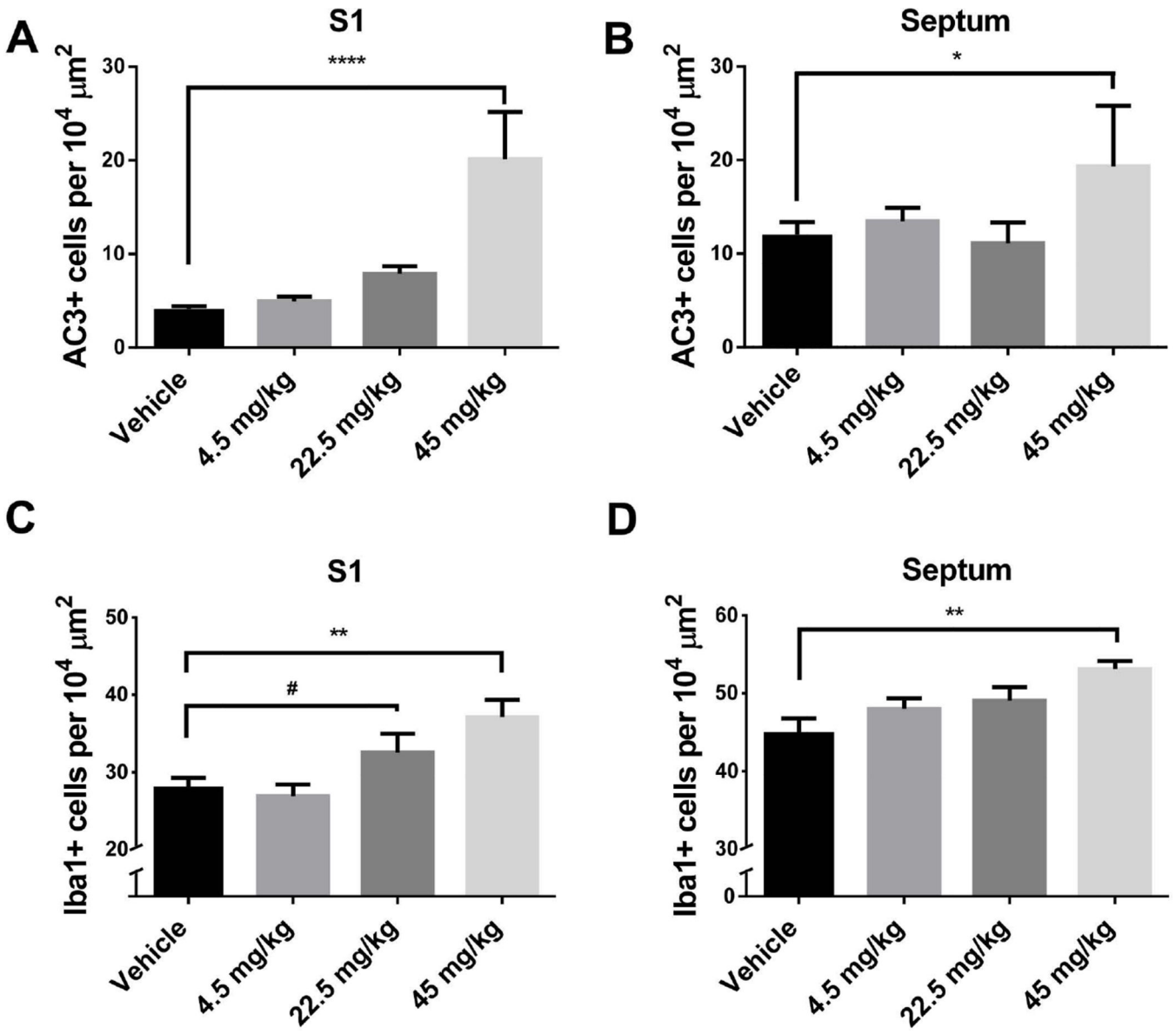

Figure 6.

Effects of neonatal minocycline treatment on cell death and microglial labeling were dose-dependent. The density of AC3+ cells (A, B) and Iba1+ cells (C, D) in the S1 and septum in mice treated with 0 (vehicle), 4.5 mg/kg, 22.5 mg/kg or 45 mg/kg minocycline from P3-P5. Significant effects were seen only for the 45 mg/kg dose. N=20 vehicle, N=14 4.5 mg/kg, N=13 22.5 mg/kg, and N=9 45 mg/kg. Asterisks: $*P < 0.05$, $**P < 0.005$, $****P < 0.0001$, $\#P = 0.059$.

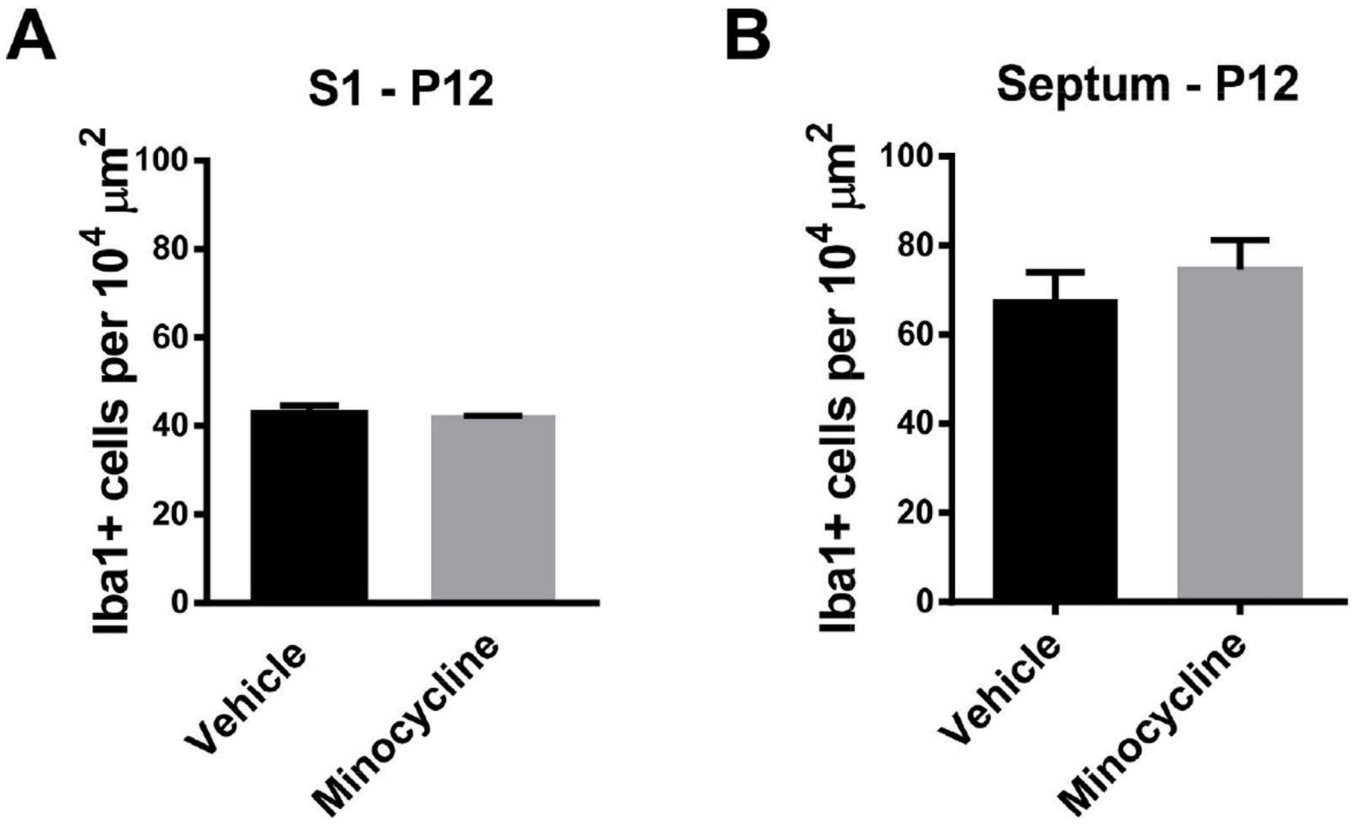

Figure 7.
Minocycline had no effect on Iba1 cell number in the S1 (A) or septum (B) when administered from P10-P12. N = 5 vehicle and 7 minocycline.