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Nicotine modulates neurogenesis in the central canal during EAE

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Abstract

Nicotine has been shown to attenuate experimental autoimmune encephalomyelitis (EAE) through inhibiting inflammation in microglial populations during the disease course. In this study, we investigated whether nicotine modified the regenerative process in EAE by examining nestin-expressing neural stem cells (NSCs) in the spinal cord, which is the primary area of demyelination and inflammation in EAE. Our results show that the endogenous neurogenic responses in the spinal cord after EAE are limited and delayed: while nestin expression is increased, the proliferation of ependymal cells is inhibited compared to healthy animals. Nicotine application significantly reduced nestin expression and partially allowed for the proliferation of ependymal cells. We found that reduction of ependymal cell proliferation correlated with inflammation in the same area, which was relieved by administration of nicotine. Further, increased numbers of oligodendrocytes (OLs) were observed after nicotine treatment. These findings give a new insight into the mechanism of how nicotine functions to attenuate EAE.

Keywords

Nicotine; EAE; Neural stem cells; central canal; oligodendrocytes

Introduction

Multiple sclerosis (MS) is an autoimmune demyelinating disease characterized by inflammation, demyelination and neurodegeneration within the central nervous system (CNS). Experimental autoimmune encephalomyelitis (EAE) is the most commonly used animal model for MS (Gao and Tsirka, 2011). Previous studies from our lab demonstrated that nicotine, an agonist of nicotinic acetylcholine receptors (nAChRs), attenuated clinical

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symptoms of EAE mice (Gao et al., 2014). Nicotine-treated EAE mice presented reduced EAE clinical scores, decreased inflammation, and significantly less demyelination in the WM of spinal cord. The mechanism of nicotine's effects involved inhibition of microglia activation and differentiation (Gao et al., 2014). However, results from recent studies suggested that protective roles of nicotine on MS/EAE might not only rely on suppressing microglia activity or inflammation. Expression of nAChR subunits was detected in both cultured undifferentiated neural stem/progenitor cells (NSCs) (Takarada et al., 2012) and differentiated oligodendrocyte progenitor cells (OPCs) (Rogers et al., 2001). Nicotine application not only suppressed proliferation of NSCs, but also promoted neuronal differentiation (Takarada et al., 2012). As NSCs and OPCs directly or indirectly give rise to myelinating oligodendrocytes under normal and pathological conditions (Grade et al., 2013), protective effects of nicotine on EAE might involve regulation of neuronal regeneration or remyelination (Li et al., 2002) during the disease. Using microarray experiments after chronic administration of nicotine increased production of inositol phosphates and increased expression of genes involved in remyelination and axonal growth were observed (Li et al., 2002).

Behaviors of NSCs in the subventricular zone (SVZ) during EAE are described in multiple studies (Picard-Riera et al., 2002, Pluchino et al., 2008, Rasmussen et al., 2011). Specifically the loss of SVZ architecture has been reported during the course of EAE (Rasmussen et al., 2011) coinciding with cell proliferation and oligodendrogenesis (Picard-Riera et al., 2002, Rasmussen et al., 2011) in the acute phase of symptoms. Moreover the accumulation of non-migratory neuroblasts has been reported (Pluchino et al., 2008). However, the plasticity of endogenous NSCs in the spinal cord, the major affected region in MS patients/EAE animals, is poorly characterized. It is accepted that ependymal cells around the central canal (CC) exhibit characteristics similar to those of NSCs in the SVZ (Hamilton et al., 2009). NSC markers at different stages were found expressed in the ependymal layer. Cultured ependymal cells could form neurospheres, which differentiated into different populations of neural cells upon stimulation (Hamilton et al., 2009). However, the organization of the ependymal layer is different from SVZ (Hamilton et al., 2009, Hugnot and Franzen, 2011). First, there is no clear sub-ependymal zone, and Nestin+GFAP+ cells, the "active" NSCs, are mostly located in the dorsal pole of ependymal layer. Further, proliferation of cells in the ependymal layer is low and concentrated in the dorsal side (Hamilton et al., 2009). These unique properties of ependymal cells might contribute to unique functions in MS/EAE.

In this study, we investigated whether nicotine regulated the activity of ependymal cells in mouse spinal cord during EAE. Using nestin as a marker to examine the response of NSCs to EAE, results showed that there was an impaired activation of nestin+ ependymal cells during the disease. While nestin expression overall increased, proliferation of the nestin+ cells was suppressed. On the other hand, nicotine application significantly altered the EAE responses of nestin-expressing cells by disinhibiting their proliferative ability. Further, we show that nicotine application during EAE promotes the generation of mature myelin basic protein (MBP)+ oligodendrocytes. Overall, our data indicate that ependymal cell activity in the spinal cord can be suppressed by an inflammatory microenvironment, which is reversed by the anti-inflammatory mediator nicotine.

Material and methods

Animals

C57BL/6 (wild-type) mice (Jackson Laboratory) were bred in-house under pathogen-free conditions on a 12-hour light/dark cycle. Protocols were approved by the Stony Brook University Institutional Animal Care and Use Committee (IACUC) and the Division of Laboratory Animal Research.

EAE induction

EAE was actively induced by subcutaneous injection of MOG35-55 peptide (MEVGWYRSPFSRVVHLYRNGK, Yale University Peptide Synthesis Facility) in 8 week-old female mice as previously described (Lu et al., 2002, Bhasin et al., 2007, Wu et al., 2012, Nissen et al., 2013, Gao et al., 2014). EAE clinical symptoms were scored on a scale of 0 to 5 with gradations of 0.5 for intermediate symptoms. 0, no detectable symptoms; 1, loss of tail tone; 2, hindlimb weakness or abnormal gait; 3, complete paralysis of the hindlimbs; 4, complete hindlimb paralysis with forelimb weakness or paralysis; 5, moribund or dead.

Nicotine delivery

200mg/ml nicotine ditartrate in saline was loaded into mini-osmotic pumps (28-day, infusion rate of 0.25 μ L/h, Alzet)(Shi et al., 2009, Gao et al., 2014) and then implanted subcutaneously in the back of the mice. The serum cotinine levels were measured at 83.8ng/ml (14-day infusion) and 85.7ng/ml (28-day infusion), which is comparable to that found in heavy smokers (80–100ng/ml)(Paulson et al., 2010).

Immunofluorescence

Mice were transcardially perfused using 4% paraformaldehyde (PFA)/PBS (pH 7.4). Spinal cords were isolated, post-fixed, and dehydrated in 30% sucrose at 4°. After meninges removal, coronal sections (25 μ m) were prepared with a cryostat (Leica).

Spinal cord sections were incubated in warm (80°) sodium citrate solution (pH 6.0) for 20min for antigen retrieval. Samples were blocked in 3% BSA in PBS-T (0.3% TritonX-100/PBS) and then incubated with primary antibodies (rabbit anti-Iba1:500, Wako; mouse anti-nestin 1:200, Developmental Studies Hybridoma bank (DSHB); anti-GFAP 1:500, BD Biosciences; anti-Dcx, 1:200, DSHB; anti-Olig2 1:200, DSHB; anti-CD45, 1:1000, BD systems, anti-MBP, 1:200, AbDSerotec, anti-Ki67 1:500, Abcam; anti-CC1, 1:250, Abcam; anti-NG2, 1:250, Millipore) overnight at 4°C. Incubation with fluorescence-conjugated secondary antibodies at 1:1000 was performed at room temperature for 1hour, followed by washing with PBS and mounting using Fluoromount-G with DAPI (Southern Biotech). 4–8 spinal cord sections in the lumbar region were imaged per biological replicate. ImageJ (NIH) was used to quantify the intensity of fluorescent signals.

Statistical Analysis

Results are presented as an average with error bars indicating the standard error of the mean (Mean $\pm$ SEM). Two-tailed t-test was used for comparisons between two groups. One-way

ANOVA was performed for comparisons between multiple groups. Significance is indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Results

Temporal responses of nestin-expressing NSCs around the CC during EAE

As previously reported (Hamilton et al., 2009), nestin-expressing cells were mainly located dorsally in the ependymal layer (Fig. 1A) under physiological conditions. These cells were GFAP+ (Fig. 1B) and sent out processes both toward the dorsal column and lumen (Fig. 1A, B). Nestin+ signals were also observed in the lateral and ventral poles of the ependymal layer, but signals in these areas were weaker than those in the dorsal pole. Moreover, the nestin+ cells in lateral and ventral sides did not express GFAP (Fig. 1B). Nestin immunoreactivity was also detected on blood vessels in both white and grey matter (Figs. 1C, 6B), consistent with previous studies (Patschan et al., 2007).

Clinical deficits consistent with EAE were evident at Day 7 after EAE onset, reached the peak around Day 21 with subsequent recovery (Gao et al., 2014). To assess how NSCs responded to EAE, nestin expression was examined in spinal cord samples collected at different timepoints (Day 0, Day 14, Day 21, Day 28) after EAE induction (Fig. 2). The sections were selected from lumbar locations with evident demyelination during EAE. Our results show that there was no significant change of nestin expression until after Day 14. A dramatic increase of nestin+ signals was observed at the peak of the disease (Day 21). The increase of nestin signals in Day 21 samples was not only observed in the dorsal side of ependymal zone, but also in the ventral pole, indicated by the signal intensity map (Fig. 2A). Additionally there were numerous and longer nestin+ processes emanating from the ependymal layer. As recovery progressed (Day 28), nestin expression decreased (Fig. 2A). The increase in nestin expression was statistically significant on day 21 (Fig. 2B), but not at other time points.

EAE reduces ependymal cell proliferation around the CC

The proliferation of ependymal cells was evaluated by counting Ki67+ cells in the ependymal layer, which labels cells in all active phases of the cell cycle- G1, S, G2, and M phases but not during the resting phase G0 (Scholzen and Gerdes, 2000, Eisch and Mandyam, 2006, Jonat and Arnold, 2011). Proliferation around the CC was low under normal conditions (Fig. 3A). No signal or only one or two Ki67+ cells were observed on more than 40% of the sections (Table 1). However, the numbers of proliferating cells during EAE were even lower, since no Ki67+ cells were detectable in more than 70% of sections quantified, suggesting that EAE impaired ependymal proliferative activity (Fig. 3B, Table 1), in agreement with the literature (Lacroix et al., 2014).

Inflammation is correlated with changes in nestin expression during EAE

Compared to other hallmarks of EAE occurring in the WM (demyelination, inflammation), the observed NSC responses were significant but impaired only at the peak of the disease. This limited reaction may be due to the fact that the stem cell niche is not in proximity to the

demyelinated areas. This possibility led us to investigate what component of the spinal cord microenvironment may regulate the ependymal cell response during EAE.

Previous studies indicated that inflammation is a key regulator of NSC activity under pathological conditions (Gao et al., 2009, Yang et al., 2009, Gao and Tsirka, 2011). To examine the interactions between ependymal cells and inflammatory cells, we used antibodies for Iba1 and CD45 to label the microglia/macrophages and infiltrating leukocytes, respectively, around the CC. As shown in Fig. 4, resting microglia were in close contact with the ependymal layer before the onset of EAE (Day 0). As the disease progressed to Day 14, microglia around the CC showed partially-activated morphology with larger cell bodies and retracted processes, although the number of microglia did not change significantly. At the peak of disease (Day 21) massive microglial activation was observed in the ependymal zone. After the peak (Day 28), microglial activation decreased, indicated by their relative resting morphology. Microglial/macrophage cell numbers were counted in the ependymal zone and adjacent grey matter (quantification in Fig. 8B). The number of activated microglia/macrophages clustered around the CC at the peak of EAE was three times higher than that of microglia in healthy samples. On the other hand, reduced numbers of CD45+ leukocytes (indicated by arrows) were observed in the ependymal zone during the whole EAE course, even at the peak of the disease (Fig. 4). The number of CD45+ cells in the ependymal zone and adjacent grey matter was only one third of that of microglia in the same area (Fig. 8C). These results suggested that changes of local inflammation around the CC may correlate with impaired NSCs response during EAE.

Nicotine application suppressed nestin expression but increased proliferation in the ependymal layer during EAE

We have recently reported that nicotine attenuates the inflammatory reactions that take place during EAE and improves the score of EAE (Fig 5A, published in (Gao et al., 2014)). To test whether nicotine affects the NSC behavior around the CC, we first examined how nicotine regulated nestin expression during EAE. Nicotine was infused into EAE mice at Day 0 of EAE and the drug delivery lasted for 28 days (Gao et al., 2014). As described above (Fig. 2), increased nestin expression around the CC was observed on both Day 21 and Day 28 in vehicle-treated EAE samples. In contrast, there was no change of nestin expression during EAE in nicotine-treated samples compared with samples collected before EAE induction (please compare Fig. 5B and Fig. 1). This result suggested that nicotine application suppressed nestin expression during EAE. Further, Ki67 positive cells at the central canal in nicotine-treated animals are co-localized with nestin, indicating that proliferation is occurring in NSCs (Fig. 5C). Nicotine treatment alone did not have significant effects on nestin expression or the number of nestin+ cells. Nestin+ cells in nicotine-treated samples consist of the NSCs in the ependymal layer and the endothelial progenitor cells in the white matter (WM) and gray matter (GM) (Fig. 6A, B).

We also examined the proliferation of ependymal cells during nicotine application. We found that approximately 67.5% of nicotine-treated EAE sections presented less than three Ki67+ cells (Table 1). This result is distinct than the one observed in vehicle-treated EAE sections, where 100% of the sections contained less than three Ki67+ cells (Table 1).

Therefore proliferation was still evident in nicotine-treated EAE tissue. This result suggested that nicotine application disinhibited the proliferation of ependymal cells during EAE. However, the extent of proliferation in nicotine-treated samples was still lower than that in healthy spinal cords, indicating that nicotine application itself may possibly regulate NSC proliferation. Indeed, staining for Ki67 in sections after treatment with nicotine alone for 28 days revealed that nicotine decreased the proliferation of ependymal cells to levels comparable to those in nicotine-treated EAE samples: ~67.5% sections had less than three Ki67+ cells (Table 1). Nevertheless, taken together, nicotine by itself appears to impair the proliferation of ependymal cells in physiological settings, but allows for their proliferation during EAE (compare numbers in Table 1), most likely through the promotion of anti-inflammatory environment.

Nicotine enhances the presence of mature MBP+ oligodendrocytes during EAE

We evaluated the expression of differentiation markers at the different time points to assess whether nicotine may affect the differentiation of nestin+ cells. As shown in Fig. 7A, nicotine treatment resulted in decrease in the numbers of nestin+GFAP+ cells normally associated with EAE at D21, and had no significant effect on differentiation towards neuroblast (Dcx+) or oligodendrocyte lineage (Olig2+)(Fig. 7B).

Induction of acute EAE disease can promote the differentiation of mature oligodendrocytes (OLs) in the SVZ neurogenic niche (Rasmussen et al., 2011). We hypothesized as mature myelinating oligodendrocytes are the most relevant cells in EAE recovery, that nicotine may affect the numbers of mature oligodendrocytes. We examined spinal cord sections of a healthy control mouse as well as those at the peak of disease that received saline or nicotine treatment for MBP expression as a marker for mature OLs. We found a significant decrease in MBP in saline-infused EAE animals, while nicotine-infused animals had a significant increase in MBP expression even over control (Fig. 7C). To further determine nicotine's effects on oligodendrocyte lineage, we observed levels of NG2+ oligodendrocyte precursors and CC1+ mature oligodendrocytes at the central canal during EAE. We found that nicotine administration appreciably increased both NG2 and CC1 staining, indicative of its promotion of the oligodendrocyte lineage (Fig. 7D). These data indicate that nicotine may promote disease recovery, either by enhancing differentiation towards oligodendrocytes from OPCs (Barnabe-Heider et al., 2010), or preventing the loss of oligodendrocytes (Nizri et al., 2009).

Nicotine suppresses inflammation around the CC during EAE

To assess whether nicotine's effects on ependymal cells were correlated with the inflammatory environment around the CC, activities of Iba1+ microglia and CD45+ leukocytes were evaluated. We found that, compared with control samples, there was attenuated microglial/macrophage activation and rare leukocyte infiltration around the CC in nicotine-treated samples (Fig. 8A), correlating with the decreased levels of nestin immunoreactivity in the same area (Fig. 5). Quantification of the immunopositive cells showed that the numbers of microglia/macrophages around the CC and adjacent GM were higher than those of infiltrating leukocytes in both vehicle and nicotine-treated samples (Fig. 8B, C). Nicotine application significantly decreased the number of microglia, but only had a

modest effect on infiltrating leukocytes (Fig. 8B, C). Further, in saline-infused animals, microglia are primarily of the M1, inflammatory subtype, as indicated by iNOS staining, while those treated with nicotine are primarily of the anti-inflammatory, M2 subtype as noted by arginase-1 staining, which is consistent with previous reports (Gao et al., 2014) (Fig. 8D).

Discussion

In this study, we examined the modulation of temporal responses of NSCs in mouse spinal cord during EAE after administration of nicotine. We found that nestin+ ependymal cells around the CC had a significant response at the peak of the disease, in agreement with previous reports (Guo et al., 2011). The cells exhibited increased expression of nestin and longer processes but decreased proliferation, suggesting that NSC responses in EAE may be delayed relative to behavioral symptoms and inflammatory events (Pluchino et al., 2008, Rasmussen et al., 2011). We also observed an increase of GFAP+ cells around the CC during symptom peak compared to pre-disease levels, in agreement with data from spinal cord injury protocols (Meletis et al., 2008). The ependymal cell activity change was associated with local elevated microglial/macrophage activation. Although infiltrating leukocytes were also present, their numbers were much lower than those of microglia/macrophages, suggesting that microglia were the predominant immune cells regulating NSCs responses during EAE. We also show that nicotine promotes increase of mature MBP + OLs around the CC.

Microglia/macrophages potentially regulate NSCs functions in two ways: We observed close contacts between microglia/macrophages and cells of the ependymal layer. As we have shown that microglia shaped adult hippocampal neurogenesis through phagocytosis (Sierra et al., 2010), microglia/macrophages could affect NSC numbers in EAE via direct interaction. Another mechanism of their effect on NSC could be through regulating the inflammatory environment: two extremes of activated microglia/macrophages have been identified: M1 pro-inflammatory microglia and M2 anti-inflammatory microglia (Colton and Wilcock, 2010) along with a continuum of states between M1 and M2. M1 and M2 microglia have opposite effects on NSCs (Bauer et al., 1994, Butovsky et al., 2006b, Mikita et al., 2011). Media from cultured M1 microglia significantly reduced NSC differentiation towards oligodendrocytes. TNF- α inhibition significantly down-regulated this effect, suggesting that TNF- α primarily mediated the detrimental effects of M1 microglia on NSCs (Butovsky et al., 2006b). In contrast, injection of M2 microglia resulted in increased oligogenesis in the spinal cord during EAE (Butovsky et al., 2006a). Studies by our lab (Gao et al., 2014) and others (Murphy et al., 2010, Mikita et al., 2011) demonstrated that M1 microglia were dominant during EAE. M1 microglia might be responsible for the impaired reaction of ependymal cells in EAE. As nicotine can reduce M1 numbers (Gao et al., 2014), it is possible that this is the mechanism by which it promotes mature OLs increase.

Nicotine is protective in the course of EAE (Shi et al., 2009, Gao et al., 2014). Mechanisms of nicotine's effects involve inhibition of activation and differentiation of inflammatory cells including T cells (Butovsky et al., 2006b) and microglia/macrophages (Gao et al., 2014). In this study, we demonstrate that nicotine, directly or indirectly, regulated ependymal cell

activities during EAE. Nicotine application significantly inhibited nestin expression and partially allowed for proliferation of ependymal cells. As changes of ependymal cells activity after nicotine treatment correlated with inflammation in the same area, it is possible that nicotine's effects on NSC are not direct, as the expansion of nestin+ cells could mainly be a result of reduced overall inflammation. This concept is supported by our results that indicate nicotine's effects on nestin levels in physiological conditions is negligible, while it suppressed nestin levels during active inflammation in EAE. We have previously shown that inhibition of microglial/macrophage activation in the spinal cord by nicotine was observed starting at Day 14 of EAE (Gao et al., 2014), which is an earlier event compared with the change of ependymal cells. Therefore, "suppressed" responses of ependymal cells by nicotine are dependent on nicotine's downregulation on inflammation: ependymal cells in nicotine-treated mice did not need to react since the inflammation in the microenvironment was not intense enough to trigger a response.

However, we could not rule out the possibility of direct NSC regulation by nicotine, since decreased proliferation in the ependymal layer was observed after the 28-day nicotine. Our result agreed with previous studies showing that nicotine significantly decreased proliferation of NSCs both *in vivo* in the hippocampus and *in vitro* (Abrous et al., 2002, Takarada et al., 2012). This does not mean that nicotine alone impaired NSC functions. Takarada et al. demonstrated that nicotine application both decreased proliferation of neurospheres, and promoted differentiation into a neuronal lineage (Takarada et al., 2012). Further, recent reports indicate, using brain neurospheres which were exposed to nicotine in culture, that nicotine inhibited proliferation of mouse neural stem cells (Lee et al., 2014). However, our results suggest that NSC differentiation towards neuroblasts was not affected, while astrocytic fate was suppressed resulting in cell numbers reminiscent of a normal, non-EAE spinal cord. Ependymal cells undergo a variety of changes as a result of disease or damage. For example, following spinal cord injury proliferating ependymal cells differentiate into astrocytes and oligodendrocytes (Meletis et al., 2008, Barnabe-Heider et al., 2010). However, when undergoing EAE, they primarily become myelinating oligodendrocytes (Guo et al., 2011). We hypothesize that nicotine can promote this switch both through direct effects and through reduced inflammation.

Overall our study indicates that the endogenous neurogenic responses after EAE are limited, delayed and primarily regulated by the ensuing inflammatory cascades.

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Highlights

- Proliferation of ependymal cells is reduced in the central canal following EAE
- Ependymal cell proliferation is reduced in areas of inflammation
- Nicotine treatment in EAE reduced nestin expression and promoted ependymal cell proliferation
- Nicotine treatment resulted in increased numbers of mature oligodendrocytes

Figure 1. Characterization of nestin⁺ cells in the normal spinal cord

Frozen sections of spinal cords were isolated from 8-week-old mice and stained with nestin antibodies (green). Representative images were taken in area around the CC (**A**) and in white matter/ gray matter (**C**). GFAP expression (red) in nestin-expressing cells in ependymal layer was shown in (**B**). DAPI: blue; Bar = 50 μm

Figure 2. Temporal expression of nestin around the CC during EAE

(A) Nestin expression (green) around the CC was examined at different time points (Day 0, Day 14, Day 21 and Day 28) during EAE. Signal intensity was measured over a rectangular area covering the CC from dorsal to ventral as indicated by arrows. D: dorsal V: ventral. (B) Quantification of nestin signal intensity. * indicates significance between nicotine- and vehicle-treated samples. n=3; DAPI: blue; Bar = 50 μ m

Figure 3. Change of proliferation of ependymal cells during EAE
Ki67+ cells in the ependymal layer at Day 0 and Day 21 of EAE were examined.
Representative confocal images were shown in (A) and (B); n=3. DAPI: blue; Bar = 50 μ m.

Figure 4. Inflammation around the CC during EAE

Anti-Iba1 (green) and anti-CD45 (red) were used to label microglia and infiltrating leukocytes respectively in samples collected at different time points (Day 0, Day 14, Day 21, Day 28) during EAE. CD45+ cells are indicated by arrows. DAPI: blue; Bar = 50 μ m

Figure 5. Nicotine application decreased nestin expression in EAE

Nicotine infusion started at Day 0 of EAE and lasted for 28 days. **(A)** Behavioral scores of EAE progression, as published in (Gao et al., 2014). **(B)** Representative images of nestin expression around CC were taken from vehicle and nicotine-treated samples collected on Day 21 and Day 28 of EAE. Nestin: green; DAPI: blue; Bar = 50 μm. **(C)** Proliferation of nestin-positive cells at the central canal. Anti-nestin (green) and anti-Ki67 (red) antibodies were used to label NSCs and proliferating cells respectively in samples collected at Day 21 during EAE in saline- or nicotine-infused animals. DAPI: blue; Bar = 50 μm

Figure 6. Nestin expression in control animals receiving nicotine for 28 days

Nestin expression around the CC (**A**) and in the adjacent spinal cord tissue (**B**) was examined in samples treated with nicotine alone for 28 days. Bar = 50 μ m, n=3–4.

Figure 7. Increased mature oligodendrocytes at the CC following nicotine treatment in EAE Spinal cord sections from healthy control animals as well as those from D21 following EAE induction with either saline or nicotine infusion were stained for differentiation markers. **(A)** Differentiation markers (Dcx for neuroblasts, GFAP for astrocytes, and Olig2 for oligodendrocyte lineage) were evaluated in nestin+ spinal cord sections around the CC from D0 and D21 control or nicotine-treated animals. ** $p < 0.01$. $n = 3-4$. **(B)** Lineage markers at the central canal. Anti-nestin (green) was used to label NSCs in samples collected at Day 21 during EAE in saline- or nicotine-infused animals. Sections were also stained with anti-GFAP, anti-Olig2, or anti-DCX (red). DAPI: blue; Bar = 50 μm . **(C)** Intensity of MBP staining was also quantified. MBP (red) and DAPI (blue) *** $p < 0.001$, ** $p < 0.01$, ns=not significant. $n = 3$. Bar = 50 μm . **(D)** Oligodendrocyte lineage markers at the central canal. Anti-NG2 (green) was used to label oligodendrocyte progenitors and anti-CC1 (green) to label cells of the oligodendrocyte lineage in samples collected at Day 21 during EAE in saline- or nicotine-infused animals. DAPI: blue; Bar = 50 μm

Figure 8. Nicotine application attenuated inflammation around the CC

Nicotine was infused into the animals at Day 0 and lasted till Day 28 of EAE.

Representative images from vehicle- and nicotine-treated samples are shown in (A) and in Figure 4. Anti-Iba1 and anti-CD45 were used to label microglia and infiltrating leukocytes respectively. Cell numbers of microglia (B) and leukocytes (C) in the ependymal zone and adjacent GM were quantified in the lumbar region of the spinal cord in sections with evident demyelination. * indicates significance between nicotine- and vehicle-treated samples.

** $p < 0.01$, * $p < 0.05$; n = 3; n.s.=not significant. (D) Microglial polarization at the CC. Anti-Iba1 (green) was used to label microglia, and iNOS (red) for M1 or Arg1 (red) for M2 in samples collected at Day 21 during EAE in saline- or nicotine-infused animals. DAPI: blue; Bar = 50 μ m

Table 1
Percentage of sections that carry the indicated number of Ki67+ cells/section

Quantification of Ki67+ cells in the ependymal layer in the lumbar spinal cord region at Day 0 (Control) and Day 21 (EAE) of EAE, as well as in nicotine-treated Day 21 sections (Nicotine EAE), and in sections of animals treated with nicotine alone (Nicotine Control). n=3–4. All the Ki67+ cells on each section were counted at a distance of 50µm on either side of the CC.

<u>Ki67+ cells/ section</u>	0	1	2	3	4	5	6	>7
Treatment								
Control	13	16	16	9	13	16	9	8
EAE	70	25	5	0	0	0	0	0
Nicotine Control	23	41	12	6	0	0	12	6
Nicotine EAE	35	23	18	5	5	14	0	0