Neuroscience 297 (2015) 11–21

NICOTINE MODULATES NEUROGENESIS IN THE CENTRAL CANAL DURING EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

Z. GAO, [a,b†‡] **J. C. NISSEN,** [b‡] **L. LEGAKIS** [b¶] **AND S. E. TSIRKA** [a,b*]

[a] *Program in Neuroscience, Stony Brook University, Stony Brook, NY, United States*

[b] *Department of Pharmacological Sciences, Stony Brook University, Stony Brook, NY, United States*

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 the 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.

Key words: 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 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 white matter (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 (OLs) 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 the production of inositol phosphates and increased expression of genes involved in remyelination and axonal growth was 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

*Correspondence to: S. E. Tsirka, Department of Pharmacological Sciences, Stony Brook University, Stony Brook, NY, United States.
E-mail address: Styliani-anna.tsirka@stonybrook.edu (S. E. Tsirka).
† Current address: Division of Hematology, Cincinnati Children's Hospital, 3333 Burnet Avenue, Cincinnati, OH 45229-3039, United States.
‡ Equal contribution.
¶ Current address: Department Pharmacology, Virginia Commonwealth University, Richmond, VA, United States.

Abbreviations: CC, central canal; EAE, experimental autoimmune encephalomyelitis; GM, gray matter; MBP, myelin basic protein; MS, multiple sclerosis; nAChRs, nicotinic acetylcholine receptors; NSCs, neural stem cells; OLs, oligodendrocytes; OPCs, oligodendrocyte progenitor cells; PBS, phosphate buffered saline; SVZ, subventricular zone; TNF-α, tumor necrosis factor-alpha; WM, white matter.

http://dx.doi.org/10.1016/j.neuroscience.2015.03.031
0306-4522/

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) + OLs. 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.

EXPERIMENTAL PROCEDURES

Animals

C57BL/6 (wild-type) mice (Jackson Laboratory) were bred in-house under pathogen-free conditions on a 12-h 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 0–5 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

Two hundred millligram per milliliter nicotine ditartrate in saline was loaded into mini-osmotic pumps (28-day, infusion rate of 0.25 μl/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 83.8 ng/ml (14-day infusion) and 85.7 ng/ml (28-day infusion), which is comparable to that found in heavy smokers (80–100 ng/ml) (Paulson et al., 2010).

Immunofluorescence

Mice were transcardially perfused using 4% paraformaldehyde (PFA)/phosphate buffered saline (PBS) (pH 7.4). Spinal cords were isolated, post-fixed, and dehydrated in 30% sucrose at 4 °C. After meninges removal, coronal sections (25 μm) were prepared with a cryostat (Leica, Buffalo Grove, IL, USA).

Spinal cord sections were incubated in warm (80 °C) sodium citrate solution (pH 6.0) for 20 min for antigen retrieval. Samples were blocked in 3% BSA in PBS-T (0.3% TritionX-100/PBS) and then incubated with primary antibodies (rabbit anti-Iba1:500, Wako, Richmond, VA, USA; mouse anti-nestin 1:200, Developmental Studies Hybridoma bank (DSHB, University of Iowa, Iowa City, Iowa, USA); anti-GFAP 1:500, BD Biosciences, San Jose, CA, USA; anti-Dcx, 1:200, DSHB; anti-Olig2 1:200, DSHB; anti-CD45, 1:1000, BD systems, anti-MBP, 1:200, AbD Serotec, Raleigh, NC, USA, anti-Ki67 1:500, Abcam, Cambridge, MA, USA; anti-CC1, 1:250, Abcam; anti-NG2, 1:250, Millipore, Billerica, MA, USA) overnight at 4 °C. Incubation with fluorescence-conjugated secondary antibodies at 1:1000 was performed at room temperature for 1 h, followed by washing with PBS and mounting using Fluoromount-G with DAPI (Southern Biotech, Birmingham, AL, USA). 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 ± SEM). Two-tailed *t*-test was used for comparisons between two groups. A one-way analysis of variance (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 in blood vessels in both white and gray matter (GM) (Figs. 1C and 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