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Cholinergic imbalance in lumbar spinal cord of a rat model of multiple sclerosis

Chunling Liu[1,2], Hui Liu[1], Hongjun Jin, Xuyi Yue, Zonghua Luo, Zhude Tu*

Department of Radiology, Washington University School of Medicine, St. Louis, MO 63110, USA

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ABSTRACT

Cholinergic dysfunction in the central nervous system is an important characteristic of multiple sclerosis and experimental autoimmune encephalomyelitis (EAE). By using a rat EAE model, upregulation of vesicular acetylcholine transporter (VAChT) level in the EAE rat lumbar spinal cord was detected by western blot and immunostaining, and was associated with lymphocyte filtration and glial activation. *Ex vivo* and *in vitro* autoradiography studies with [^{18}F]VAT, a VAChT-specific radioligand, also revealed increased tracer uptake in EAE rat lumbar spinal cord compared with shams. These studies on VAChT expression suggest central cholinergic imbalance during EAE progression.

List of abbreviations

ACh	acetylcholine
AD	Alzheimer's disease
BBB	blood-brain barrier
CFA	complete Freund's adjuvant
ChAT	choline-acetyltransferase
CNS	central nervous system
EAE	experimental autoimmune encephalomyelitis
H&E	Hematoxylin and eosin
HRP	horseradish peroxidase
IHC	immunohistochemistry
IF	immunofluorescence
LPS	lipopolysaccharide
MAP	maximum *a posteriori*
MBP	myelin basic protein
MS	multiple sclerosis
nAChR	nicotinic acetylcholine receptor
NHP	nonhuman primate
OCT	optimal cutting temperature
PBS	phosphate-buffered saline
PSL/mm^2	photo-stimulated luminescence signals per square millimeter
PVDF	polyvinylidene difluoride
SPECT	single photon emission computed tomography
VAChT	vesicular acetylcholine transporter

1. Introduction

Multiple sclerosis (MS) is the most common demyelinating disease of the central nervous system (CNS) and the leading cause of non-traumatic neurological disability among young adults (Karussis, 2014). The pathology of MS lesions is characterized by demyelination, inflammation and axonal/neuronal pathology (Mahad et al., 2015). Cholinergic dysfunction is commonly observed in the CNS of MS patients and animal models of experimental autoimmune encephalomyelitis (EAE) (Tata et al., 2014; Vogt et al., 2009). Lower motor neurons, which are cholinergic neurons and located in the ventral horn of the spinal cord, undergoes substantial loss ranging from 15% to 48% in MS cases compared to healthy controls (Vogt et al., 2009). In MS brain, activity and protein expression of choline-acetyltransferase (ChAT) is also decreased in hippocampus (Kooi et al., 2011). Meanwhile, evidence that cholinergic modulation of inflammatory response contributes to MS progression is accumulating. The ChAT protein level in the cerebrospinal fluid of MS patients is more than two-fold higher compared to controls (Vijayaraghavan et al., 2013). Moreover, activation of α7 nicotinic acetylcholine receptors (nAChRs) by nicotine suppresses production of pro-inflammatory cytokine, infiltration of proinflammatory monocytes and neutrophils into the CNS, and alleviates the severity of EAE (Jiang et al., 2016; Nizri et al., 2009; Reale

* Corresponding author.
E-mail address: tuz@mir.wustl.edu (Z. Tu).
[1] Co-first authors.
[2] Present address: Department of Neurology, The Second Affiliated Hospital of Zhengzhou University, 2# Jingba Road, Zhengzhou 450014, China.

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et al., 2015). While cholinergic imbalance in the course of MS is complex and reflects the heterogeneity of MS pathophysiology, it provides important information for evaluation of disease severity of MS and potentially could be a therapeutic target for MS.

Vesicular acetylcholine transporter (VAChT) which loads acetylcholine (ACh) into vesicles, is a reliable biomarker for cholinergic system and has been investigated extensively in cognitive dysfunction (Efange, 2000). A single photon emission computed tomography (SPECT) study using a specific VAChT radioligand [^{123}I]IBVM demonstrated early cholinergic degeneration occurs in the early stage of Alzheimer's disease (AD) (Mazere et al., 2008). As the disease progresses in AD patients, VAChT expression level change in a similar fashion and magnitude to other cholinergic maker proteins, in particular ChAT (Kuhl et al., 1999; Kuhl et al., 1996). Recent studies revealed that VAChT may participate in the pathogenesis of inflammatory response. Rodents with acute lung inflammation showed reduced VAChT expression (Lips et al., 2007). On the other hand, transgenic mice with reduced VAChT levels (VAChT KD^{hom} mice) show airway inflammation with enhanced inflammatory markers, hyper-responsiveness and increased collagen and elastic fiber deposition, indicating cholinergic deficiency impair lung function and produce local inflammation (Pinheiro et al., 2015). VAChT KD^{hom} mice also developed severe kidney disease with a prominent inflammatory response and high expression of pro-inflammatory cytokines (Silva et al., 2016). Moreover, the decreased ability to release ACh in VAChT knock down mice exacerbates systemic and cerebral inflammation and are more susceptible to inflammation and sickness behavior induced by lipopolysaccharide (LPS) (Leite et al., 2016). Very recently, cholinergic PET imaging using [^{11}C]donepezil (an acetylcholinesterase (AChE) radioligand) and [^{18}F]FEOBV (an VAChT radioligand) shows distinct accumulation of both tracers in a murine abscess model (Jorgensen et al., 2017).

Accordingly, we performed a pilot study to investigate the change of VAChT protein expression in rat lumbar spinal cord in response to demyelination and neuroinflammation induced by EAE, using western blot and immunostaining. In addition, we further performed autoradiography studies to assess the change of VAChT expression in EAE rat spinal cord. The promising VAChT radiotracer,(-)-(1-(8-(2-[^{18}F]fluoroethoxy)-3-hydroxy-1,2,3,4-tetrahydronaphthalen-2-yl)-piperidin-4-yl)(4-fluorophenyl)-methanone ([^{18}F]VAT, Fig. 5), possessing high potency (K_i = 0.59 nM), high selectivity (> 10,000-fold selectivity for VAChT *versus* sigma receptors), and favorable *in vivo* binding profile (Jin et al., 2015; Tu et al., 2015), was utilized for autoradiography studies.

2. Methods

2.1. EAE rat model

All animal experiments were conducted in compliance with the Guidelines for the Care and Use of Research Animals under protocols approved by Washington University's Institutional Animal Care and Use Committee. The EAE rat model was established as previous reported (Liu et al., 2016). In brief, female Lewis rats (Charles River Laboratories, Inc., Wilmington, MA), weighing 100–125 g, were used for EAE induction. Before each immunization, a myelin basic protein (MBP) emulsion was freshly prepared using a MBP fragment (MBP68–86, AnaSpec Inc., San Jose, CA) and complete Freund's adjuvant (CFA), containing 1 mg/mL heat-inactivated Mycobacterium tuberculosis H37Ra (Difco Laboratories, Detroit, MI). Immunization of rats was performed under anesthesia (2–3% isoflurane in O_2), by injecting 200 μL of emulsion, divided equally between the hind footpads. Control rats were injected with an identical volume of a PBS/CFA emulsion.

Body weight and neurological symptoms were checked daily on the animal post-injection of MBP. Neurological impairment was graded as follows: 0, no symptoms; 1, flaccid tail; 2, hindlimb weakness; 3, paraparesis; 3.5, unilateral hindlimb paralysis; 4, bilateral hindlimbs paralysis; and 5, bilateral hindlimbs paralysis and incontinence. Rats with score ≥ 4.0 in EAE group were euthanized and processed for western blot (n = 4), immnohistological studies (n = 4), as well as *in vitro* autoradiography (n = 2). Lumbar spinal cords were rapidly dissected on ice. Isolated tissues were then dissected and cut in half. One half of the lumbar spinal cord was fixed in 10% formalin and processed for immunohistological studies. The other half was snap frozen and used for Western blot and *in vitro* autoradiographic studies. Additional groups of EAE and sham rats were used for *ex vivo* autoradiography (n = 4). The total number of rats that used in this study was 28, n = 14 for EAE and sham groups.

2.2. Western blot quantification of VAChT protein expression

Rats were euthanized and lumbar spinal cords were frozen for western blot. Frozen tissues were homogenized in cold RIPA buffer containing 25 mM Tris-HCl, pH 7.6, 150 nM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS with protease inhibitor cocktails, and centrifuged at 12,000*g* for 20 min at 4 °C to remove the insoluble material. The pellets were discarded and supernatant was further mixed with the loading buffer containing 65.8 mM Tris-HCl, pH 6.8, 2.1% w/v SDS, 26.3% v/v glycerol, 0.01% w/v bromophenol blue and boiled at 95 °C for 5–7 min. The prepared samples were electrophoresed on 12% SDS-PAGE, before transfer to polyvinylidene difluoride (PVDF) membrane and blocked with 5% nonfat dry milk for 1 h at room temperature. After washing, the membrane was incubated with a mouse anti-rat VAChT antibody (1:500, Abcam, Cambridge, MA) for 16 h at 4 °C followed by incubation with horseradish peroxidase (HRP) linked secondary antibody (1:10,000, R&D systems, Minneapolis, MN) at room temperature for 1 h. After the incubation, blots were washed and developed using Immobilon Western chemiluminescent HRP substrate (Thermo scientific, Pittsburgh, PA) following the recommended procedure. To normalize the protein bands to gel loading control, β actin was probed as an internal control. A digital gel image J 1.49 V (NIH, Bethesda, MD) analysis software was used for semi-quantification of VAChT immunoreactivity.

2.3. Histological analysis

Hematoxylin and eosin (H&E) staining was performed using a routine procedure to visually check lesion morphology surrounding spinal cord lesions. To determine the VAChT expression change in EAE rat spinal cord, immunohistochemistry was carried out. Sections were deparaffinized in xylene and rehydrated through a graded alcohol series to water, followed by antigen retrieval. Endogenous peroxidase activity was quenched with a solution of 3% H_2O_2 in PBS for 15 min. Sections were incubated in blocking buffer for 15 min before incubating with the mouse anti-rat VAChT antibody (1:100, Abcam, Cambridge, MA) overnight at 4 °C. Primary antibody binding was detected using an anti–mouse HRP-DAB staining kit (R&D Systems, Minneapolis, MN) following the manufacturer's instructions.

To further identify the cellular location of VAChT expression, immunofluorescence was performed on deparaffinized and hydrated slides. The primary antibodies were added as a mixture and incubated with the sections at 4 °C overnight. The secondary fluorescently labeled antibodies were then added and incubated in the dark for 60 min. The primary antibodies and secondary antibodies used in this study were: 1) the mouse anti-rat VAChT antibody (1:100) with a fluorescein-conjugated goat anti-mouse IgG antibody (1:100, Jackson ImmunoResearch Lab, West Grove, PA); 2) a rabbit anti-rat CD19 antibody (1:100, Abnova, Taipei, Taiwan) or a rabbit anti-rat CD3 antibody (1:100, Abcam, Cambridge, MA) with a Dylight 594 Horse anti-rabbit antibody (1:100, Vector Laboratories, Burlingame, CA); 3) a goat anti-rat VAChT antibody (1:500, Millipore, Billerica, MA) with a Dylight 488 horse anti-goat antibody (1:100, Vector Laboratories, Burlingame, CA); 4) a mouse anti-rat GFAP antibody (1:400, Millipore,