THE JOURNAL OF
SEXUAL MEDICINE

ORIGINAL RESEARCH

BASIC SCIENCE

# M1 Macrophages Are Predominantly Recruited to the Major Pelvic Ganglion of the Rat Following Cavernous Nerve Injury

Hotaka Matsui, MD,[1,2,3,*] Nikolai A. Sopko, MD, PhD,[1,*] Johanna L. Hannan, PhD,[1,4] Allison A. Reinhardt, BS,[5] Max Kates, MD,[1] Takahiro Yoshida, MD, PhD,[1] Xiaopu Liu, MA,[1] Fabio Castiglione, MD, PhD,[6] Petter Hedlund, PhD,[7] Emmanuel Weyne, MD, PhD,[6] Maarten Albersen, MD, PhD,[6] and Trinity J. Bivalacqua, MD, PhD[1]

## ABSTRACT

**Introduction:** Neurogenic erectile dysfunction is a common sequela of radical prostatectomy. The etiology involves injury to the autonomic cavernous nerves, which arise from the major pelvic ganglion (MPG), and subsequent neuroinflammation, which leads to recruitment of macrophages to the injury site. Currently, two macrophage phenotypes are known: neurotoxic M1 macrophages and neuroprotective M2 macrophages.

**Aim:** To examine whether bilateral cavernous nerve injury (BCNI) in a rat model of erectile dysfunction would increase recruitment of neurotoxic M1 macrophages to the MPG.

**Methods:** Male Sprague-Dawley rats underwent BCNI and the MPG was harvested at various time points after injury. The corpora cavernosa was used to evaluate tissue myographic responses to electrical field stimulation ex vivo. Quantitative real-time polymerase chain reaction was used to examine the gene expression of global macrophage markers, M1 macrophage markers, M2 macrophage markers, and cytokines and chemokines in the MPG. Mathematical calculation of the M1/M2 index was used to quantify macrophage changes temporally. Western blot of MPG tissues was used to evaluate the protein amount of M1 and M2 macrophage markers quantitatively. Immunohistochemistry staining of MPGs for CD68, CD86, and CD206 was used to characterize M1 and M2 macrophage infiltration.

**Main Outcome Measures:** Corpora cavernosa responsiveness ex vivo; gene (quantitative real-time polymerase chain reaction) and protein (western blot) expressions of M1 and M2 markers, cytokines, and chemokines; and immunohistochemical localization of M1 and M2 macrophages.

**Results:** BCNI impaired the corporal parasympathetic-mediated relaxation response to electrical field stimulation and enhanced the contraction response to electrical field stimulation. Gene expression of proinflammatory (*Il1b, Il16, Tnfa, Tgfb, Ccl2, Ccr2*) and anti-inflammatory (*Il10*) cytokines was upregulated in the MPG 48 hours after injury. M1 markers (CD86, inducible nitric oxide synthase, interleukin-1$\beta$) and M2 markers (CD206, arginase-1, interleukin-10) were increased after BCNI in the MPG, with the M1/M2 index above 1.0 indicating that more M1 than M2 macrophages were recruited to the MPG. Protein expression of the M1 macrophage marker (inducible nitric oxide synthase) was increased in MPGs after BCNI. However, the protein amount of M2 macrophage markers (arginase-1) remained unchanged. Immunohistochemical characterization demonstrated predominant increases in M1 ($CD68^{+}CD86^{+}$) macrophages in the MPG after BCNI.

**Conclusion:** These results suggest that an increase in M1 macrophage infiltration of the MPG after BCNI is associated with impaired neurogenically mediated erectile tissue physiology ex vivo and thus has significant implications for cavernous nerve axonal repair. Future studies are needed to demonstrate that inhibition of M1 macrophage recruitment prevents erectile dysfunction after CNI. **Matsui H, Sopko NA, Hannan JL, et al.**

Received September 4, 2016. Accepted December 16, 2016.

[1]The James Buchanan Brady Urological Institute and Department of Urology, The Johns Hopkins School of Medicine, Baltimore, MD, USA;

[2]Department of Urology, The University of Tokyo, Tokyo, Japan;

[3]Department of Urology, Doai Memorial Hospital, Tokyo, Japan;

[4]Department of Physiology, Brody School of Medicine, East Carolina University, Greenville, NC, USA;

[5]Department of Chemistry, Ripon College, Ripon, WI, USA;

[6]Laboratory for Experimental Urology, Department of Development and Regeneration, University of Leuven, Leuven, Belgium;

[7]Department of Clinical and Experimental Pharmacology, Lund University, Lund, Sweden

*Equivalent contribution.



http://dx.doi.org/10.1016/j.jsxm.2016.12.012

**M1 Macrophages Are Predominantly Recruited to the Major Pelvic Ganglion of the Rat Following Cavernous Nerve Injury. J Sex Med 2017;14:187−195.**



**Key Words:** Peripheral Nerve Injury; Neuroinflammation; Macrophage Markers; Neuroprotective; Major Pelvic Ganglion; Erectile Dysfunction

## INTRODUCTION

The mechanisms of axonal loss and denervation of target organs from peripheral nerve injury are well described.[1] Schwann cells are activated in response to peripheral nerve injury.[2] Inflammatory cytokines and chemokines are secreted by Schwann cells in the process of cleaning myelin or debris of dead neurons. Then, macrophages are recruited to induce local inflammation.[3] Two types of macrophages, M1 and M2, are currently known in the setting of nerve injury. M1 macrophages, also called classically activated macrophages, have been found to play a detrimental role in nerve regeneration.[4] M2 macrophages, also known as activated macrophages, have neuroprotective effects after nerve injury.[5]

Neurogenic erectile dysfunction (ED) is a common sequela after radical prostatectomy (RP) for the treatment of clinically localized prostate cancer.[6,7] The etiology of neurogenic ED after RP is damage to the neurovascular bundle or autonomic cavernous nerves (CNs) arising from the pelvic ganglia, which innervate the end organ penis.[7,8] Bilateral CN injury (BCNI) leads to axonal damage and decreased density of nitrergic nerves innervating the corpora cavernosa of the penis, with subsequent smooth muscle cell loss and fibrosis.[9,10] Most studies have investigated end organ changes of the penis after BCNI; however, the true etiologic mechanisms of BCNI-induced axonal loss are unknown.

In the major pelvic ganglion (MPG), different proinflammatory cytokines and chemokines are increased as soon as 24 to 48 hours after BCNI in rodents.[11,12] This increase in proinflammatory cytokines and chemokines can alter macrophage phenotypes toward neurotoxic M1 macrophages.[13] Based on these findings, we hypothesized that an increase in proinflammatory cytokines and chemokines after BCNI would enhance recruitment of neurotoxic M1 macrophages to the lesion and potentially contribute to the pathogenesis of CNI-induced ED. To test this hypothesis, we evaluated the effect of BCNI on the terminal organ (ie, penis) by ex vivo myography, temporal transcriptional changes of key cytokines and chemokines, and characterization of the macrophage phenotype involved in neuroinflammation in the MPG by quantitative real-time polymerase chain reaction (qPCR) and macrophage polarization in the MPG by immunohistochemistry and western blot.

## METHODS

### Animals

Male Sprague-Dawley rats (age = 8 weeks, weight = 300−350 g; Charles River, Wilmington, MA, USA) were used in this study. Rats were randomly separated into four groups (n = 10 per group): (i) sham; (ii) 48 hours after BCNI (BCNI-48h); (iii) 7 days after BCNI (BCNI-7d); and (iv) 14 days after BCNI (BCNI-14d). The MPGs and penises of the sham, BCNI-48h, BCNI-7d, and BCNI-14d groups were harvested after surgery as previously described.[14,15] All experiments were conducted in accordance with the The Johns Hopkins Medical Institutions Guidelines for Animal Care and Use and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

### BCNI Model

Under 3% isoflurane anesthesia, the prostate was exposed by midline laparotomy. The right CN was identified posterolateral to the prostate.[14] To induce BCNI, the right and left CNs (2−3 mm distal to the MPG) were crushed with Dumont #5 forceps. The forceps were closed completely three times for 15 seconds each.[15] The same surgeon performed all BCNI surgeries. In sham surgery, abdomens were closed after identifying the MPGs and CNs without crushing.

### Myographic Experiments

Contractile response to KCl, electrical field stimulation (EFS), phenylephrine, and parasympathetic-mediated relaxation of the penis were assessed with a muscle-strip myograph (820M, Danish Myograph Technology, Aarhus, Denmark). The penises were carefully removed (length = 10 mm) and placed in a physiologic Krebs solution (NaCl 130 mmol/L, KCl 4.7 mmol/L, $CaCl_2$ 1.56 mmol/L, $MgSO_4$ 1.18 mmol/L, $KH_2PO_4$ 1.18 mmol/L, $NaHCO_3$ 14.9 mmol/L, dextrose 5.6 mmol/L).[15−17] Under a dissecting microscope, the urethras including the corpus spongiosum, dorsal nerves, dorsal arteries, dorsal veins, and glans were carefully removed from the penises. Excised penises were cut in half along the urethral bed once the urethra and all supporting structures were removed with microscissors.[17] The penile sections were mounted in a muscle-strip myograph and bathed in Krebs solution 5 mL, maintained at 37°C, and bubbled with a mixture of 95% oxygen and 5% carbon dioxide. The penile sections were left untensioned for 30 minutes and stretched to an optimal resting tension of 4 mN for 60 minutes. The penile sections were exposed to KCl 120 mmol/L to evaluate the contractile responses. Once the contraction reached the plateau, the sections were washed with Krebs solution every 15 minutes. Contractile response to EFS was measured with frequencies of 4.0, 8.0, 16.0, and 32 Hz (for 10 seconds at 40 V, 2 mS; Grass S88 Stimulator, Grass Medical