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Research report

Cannabinoid receptor 1 contributes to sprouted innervation in endometrial ectopic growth through mitogen-activated protein kinase activation

Hongxiu Han [a,1], Xizi Liang [a,1], Juan Wang [b], Qianqian Zhao [a], Mei Yang [b], Weifang Rong [b], Guohua Zhang [b,*]

[a] Department of Pathology, Shanghai Ninth People's Hospital, Shanghai Jiaotong University School of Medicine, 659 Zhizhaoju Road, Shanghai 200011, China
[b] Department of Physiology, Shanghai Jiaotong University School of Medicine, 280 South Chongqing Road, Shanghai 200025, China

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ABSTRACT

The endocannabinoid system regulates neurite outgrowth and neurogenesis during development of the central nervous system. Cannabinoid receptor 1 (CB1R) is expressed in neurons, including the somata and fibers, that innervate the endometrial ectopic cyst in rats. Here, we investigated the contribution of CB1R and its downstream signaling to the innervation of endometrial ectopic growth. We found that intrathecal injection of a CB1R agonist enhanced both the density of protein gene product (PGP) 9.5-immunoreactive sprouted nerve fibers and the protein level of PGP 9.5 of the ectopic cyst, and the CB1R antagonist induced opposite effects. The CB1R agonist increased the expression of phosphorylated extracellular signal-regulated kinase (pERK) and c-Jun *N*-terminal kinase (pJNK), but not pp38, in dorsal root ganglion (DRG), whereas the CB1R antagonist only decreased the expression of pERK. In cultured DRG neurons, CB1R agonists dose-dependently increased neurite elongation. The mitogen-activated protein kinase (MAPK)/ERK kinase (MEK) and JNK inhibitors, but not the p38 inhibitor, attenuated CB1R agonist-induced neurite elongation. The inhibitions of CB1R and its downstream ERK and JNK signaling pathways may alleviate the sprouted innervation that has been involved in ENDO-associated pain. This finding may provide a new therapeutic target for patients with endometriosis.

1. Introduction

Endometriosis (ENDO) is defined by the presence of endometrial growths outside the uterus in women of childbearing age (Giudice, 2010; Stratton and Berkley, 2011). The major symptoms, including subfertility, severe dysmenorrheal (pain with menstruation), dyspareunia (vaginal hyperalgesia), dyschezia (pain with defecation), and chronic pelvic pain, often co-occur with other painful disorders. New treatments are badly needed because the pain is difficult to abate without treatment either from hormones that often produce severe side effects or from surgery that usually does not help (Kennedy et al., 2005; Giudice, 2010; Stratton and Berkley, 2011).

ENDO is induced in rats by autotransplanting small pieces of the uterine horn that grow into vascularized cysts on abdominal arteries (Vernon and Wilson, 1985). Rats with ENDO have similar symptoms to women with endometriosis (Sharpe-Timms, 2002; Cason et al., 2003; Nagabukuro and Berkley, 2007). The nerve supply is recruited in ectopic growths of both rats and women with ENDO (Berkley et al., 2004, 2005; Zhang et al., 2008). The axons from the sensory and sympathetic neurons located in the thoracic dorsal root ganglia (DRG) and celiac ganglion (CG) sprout branches into the ectopic cyst (Dmitrieva et al., 2010). Accumulated evidence does support the opinion that this innervation is involved in endometriosis-associated pain (McAllister et al., 2009; Stratton and Berkley, 2011; McAllister et al., 2012).

A previous study in which cannabinoid receptor 1 (CB1R) was found in the rodent uterus and blastocyst indicated that CB1R contributes to reproductive function and dysfunction (Paria et al., 2002). Cannabinoids have long been used to alleviate dysmenorrheal in women (Russo, 2002). Intriguingly, CB1R is found in both axons of endometriosis' abnormal growths and neurons that innervate the ectopic cyst (Dmitrieva et al., 2010). This finding suggests that the cannabinoid system is associated with the development of sprouted innervations (Dmitrieva et al., 2010). In support of this hypothesis, CB1R stimulation is involved in neurite outgrowth in various neurons including mouse Neuro2A cells, pyramidal cells

* Corresponding author at: Department of Physiology, Shanghai Jiaotong University School of Medicine, 280 South Chongqing Road, Shanghai 200025, China.
E-mail address: ghzhang2009@shsmu.edu.cn (G. Zhang).
[1] These authors equally contributed to the work.

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and retinal ganglion cells (He et al., 2005; Jordan et al., 2005; Mulder et al., 2008; Argaw et al., 2011). CB1R signaling plays an important role either during central nervous system development or in the adult brain (Chevaleyre et al., 2006; Harkany et al., 2007, 2008). The cellular signaling pathways, such as mitogen-activated protein kinases (MAPKs), Akt and Src are indicated to be associated with CB1R-induced neurite growth (Bromberg et al., 2008).

Here, in the rat model of ENDO, we tested the hypothesis that peripheral CB1R is involved in sprouted innervation of ectopic growths via the downstream signaling MAPKs, including extracellular signal-regulated protein kinase (ERK), p38 and c-Jun *N*-terminal kinase (JNK). An in vivo pharmacological study in vivo was performed to investigate whether CB1R is involved in the sprouted axons of ectopic growth by immunofluorescence (IF) and western blot (WB). Furthermore, WB was used to examine the effects of CB1R on the activation of the MAPK signaling pathways of DRG. Finally, we investigated if MAPK signaling contributes to the neurite outgrowth of cultured neuron from DRG induced by CB1R stimulation.

2. Results

2.1. The contribution of CB1R to nerve growth within an ectopic cyst

First, the expression of PGP 9.5 in the ectopic cyst was examined by immunofluorescence. IF staining revealed that PGP 9.5-immunoreactive nerve fibers were mainly distributed in the hilus of the ectopic cyst. The nerve fibers entered the cyst at the hilus and through the cyst wall (Fig. 1).

Intrathecal injection of a CB1R agonist, ACPA (0.1 nmol, once a day for a week), significantly promoted the density of nerve fibers, whereas intrathecal injection of a CB1R antagonist, AM 251 (0.1 nmol, once a day for a week), attenuated the density of nerve fibers (ACPA: 516 ± 103, control: 360 ± 84, AM251: 193 ± 72, PGP 9.5^{+}-neurites/mm^2; $P = 0.002$, ACPA vs. control; $P = 0.002$, AM251 vs. control). AM251 reversed the ACPA-induced increase in the density of nerve fibers in ectopic cysts (ACPA + AM251: 347 ± 96 PGP 9.5^{+}-neurites/mm^2, Fig. 2).

Fig. 3 shows the WB results that i.t. injection of ACPA dose-dependently increased the protein level of PGP 9.5 in the ectopic cyst ($P = 0.033$, ACPA 0.1 nmol vs. control). However, AM251 apparently decreased the protein level of PGP 9.5 ($P < 0.001$, AM251 0.1 nmol vs. control; $P = 0.035$, AM251 0.03 nmol vs. control). AM251 reversed the ACPA-induced increase in the protein level of PGP 9.5.

2.2. The effects of CB1R on the expression of pMAPKs

Upon activation, CB1Rs raise second-messenger cascades linking numerous downstream kinase interactions. The roles of MAPK signaling cascades, including ERK, p38 and JNK, were tested here by western blot analysis. First, CB1R stimulation by ACPA 0.1 nmol (i.t. injection) significantly induced an increase in the protein level of pERK in DRG ($P = 0.032$, ACPA vs. control), whereas CB1R antag-

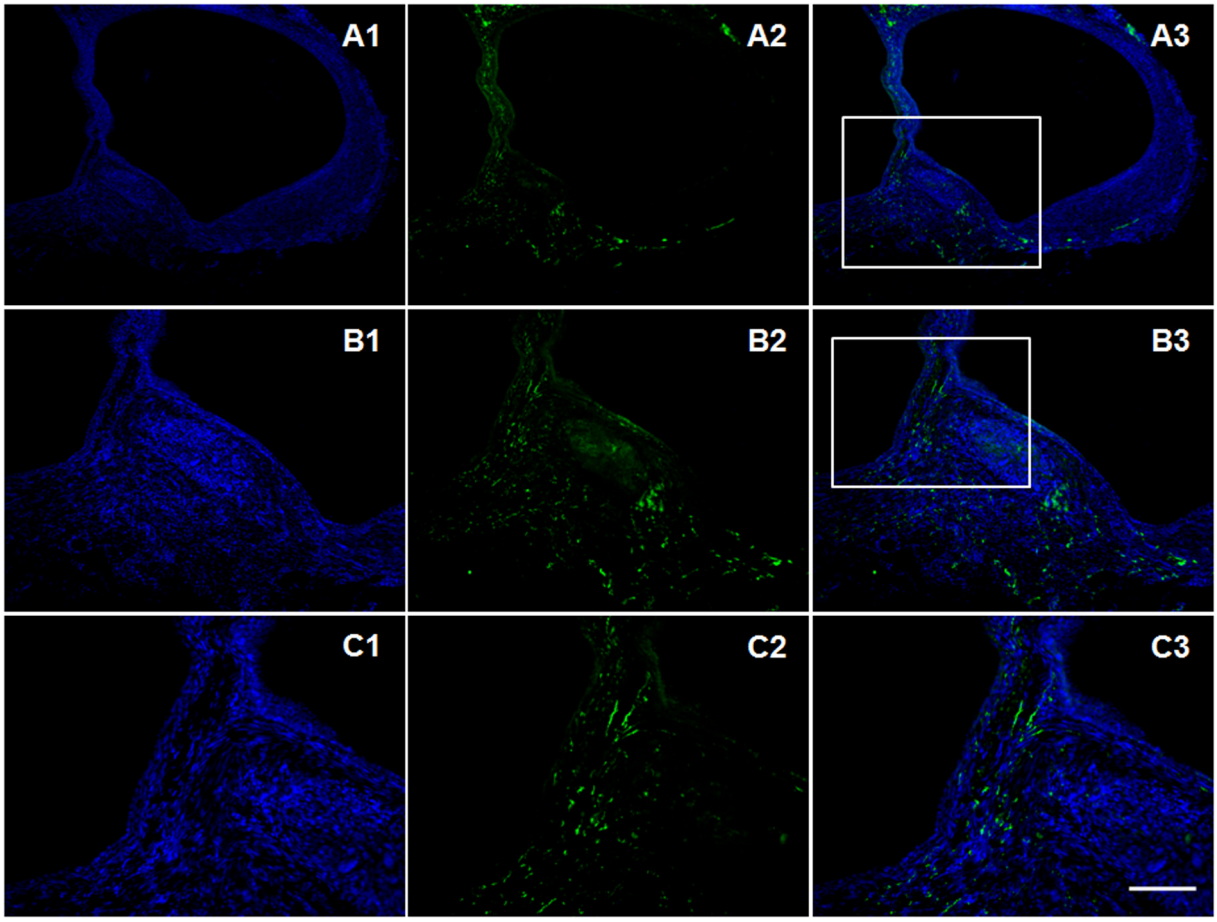

Fig. 1. The photomicrographs showing that PGP 9.5-immunoreactive nerve fibers were mainly distributed in the hilus of the ectopic cyst. A1–A3: Intact ectopic cyst; B1–B3: High-power view of hilus in the panel shown in A3; C1–C3: High-power view of area in the panel shown in B3. A1, B1, C1: DAPI (blue); A2, B2, C2: PGP 9.5 (green); A3, B3, C3: Merge. Calibration bar: 400 μm for A1–A3; 200 μm for B1–B3; 100 μm for C1–C3.