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Gonadectomy affects brain derived neurotrophic factor in rats after chronic constriction nerve injury

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KEY WORDS gonadectomy; estrogens; brain-derived neurotrophic factor; gene expression regulation; pain; chronic constriction injury

ABSTRACT

AIM: To assess the effect of gonadectomy on brain derived neurotrophic factor (BDNF) expression in neuropathic pain. **METHODS:** Using chronic constriction injury (CCI) model, we detected BDNF mRNA in dorsal root ganglion and protein content in spinal cord by reverse transcription polymerase chain reaction and enzyme-linked immunosorbent assay respectively. The time point we chose was post CCI operation d 0, 3, 7, 14, and 21. **RESULTS:** After CCI surgery, BDNF mRNA in ipsilateral DRGs was upregulated and reached its maximum on post operation d 7. BDNF protein level in ipsilateral spinal cord was also increased and reached its maximum on post operation d 14. The magnitude of this increase in gonadectomy (GDX) rats was significantly smaller than the GDX-sham rats at each time point. **CONCLUSION:** Gonadectomy reduced the BDNF increment after CCI surgery. Estrogen may affect nociceptive processing by its effect on BDNF.

INTRODUCTION

The development of animal models of neuropathic pain has been of value in characterizing the neural mechanisms involved in neuropathic pain syndromes and associated processes following peripheral nerve injury. The chronic constriction injury (CCI) model^[1], produced by loose ligation of the sciatic nerve with four chromic gut sutures, has been known as one of the most reliable models of neuropathic pain. With CCI of the rat sciatic nerve, animals show symptoms similar to the clinical features of human causalgia and reflex sympathetic dystrophy. Behavioral signs include hypersensitivity to

high and low temperatures^[1,2] as well as to noxious and non-noxious mechanical^[2] stimulation.

Estrogen appears to have a role in processing noxious stimuli. For instance, studies on human subjects have demonstrated that subjective experience of pain varied during the menstrual cycle^[3,4] and a meta-analysis of the majority of studies on experiment-induced pain indicates that there are gender differences in pain sensitivity, with female displaying greater sensitivity^[5,6]. Although cultural as well as social differences may be plausible explanations to this phenomenon, experimental animal studies indicate a biological component. Studies in mice indicate that males and females use functionally distinct pain pathway, and estrogen play a major role in regulating the pathway^[7]. Estrogen modulates numerous molecular mechanisms involved in pain. It regulates neurotransmitter and neuromodulator and

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exerts its action via genomic or nongenomic mechanisms^[8-10].

A common gene product regulated by estrogen is brain derived neurotrophic factor (BDNF)^[10]. It is a type of neurotrophin that has been studied in terms of the roles in neuronal survival and development^[11,12]. Recently, much attention has been focused on its role as a new neuromodulator in the spinal dorsal horn^[13], especially in inflammatory pain state. Zhou and Rush^[14] demonstrated that BDNF was synthesized in the dorsal root ganglion (DRG) and transported to terminals in the periphery and spinal cord. Therefore, it is possible that peripheral inflammation may induce an increase in BDNF synthesis in DRGs and consequently enhance that level of BDNF in the spinal cord.

The aim of the present study was to investigate the effect of gonadectomy on BDNF mRNA in DRGs and BDNF protein content in spinal cord in CCI rats.

MATERIALS AND METHODS

Animals Experiments were performed on female ($n=60$) Sprague-Dawley rats (Clean grade, Shanghai Experimental Animal Center, Chinese Academy of Sciences) 180-200 g between the age of 8 and 10 weeks. They were maintained under a 12 h light/12 h dark cycle with lights on at 6:00 AM, with food and water available *ad libitum* and were housed for a minimum of one-week prior to use. The rats were randomly assigned to sham surgery (GDX-sham) or gonadectomy (GDX) group ($n=30$ rats/condition). All the rats received CCI surgery. Particular efforts were made to minimize animal suffering and to reduce the number of animals used. All animal experiments were performed in accordance with the national legislation, and with the National Institutes of Health Guide regarding the care and use of animals for experimental procedures^[15].

Materials TriPure Isolation Reagent was purchased from Roche; Primers of BDNF and GAPDH were synthesized by Sangon Bioengineering Co Ltd (Shanghai, China); Reverse Transcription System, MgCl₂, *Taq* DNA polymerase, dNTP, *Taq* DNA polymerase magnesium-free buffer, agarose, 100-bp DNA ladder, and E-Max ELISA kit were obtained from Promega; micro-BCA protein assay was from Pierce.

Ovariectomy On the first day of the research, ovariectomies were performed under urethane anesthesia (1 g/kg, ip). The ovaries were removed through bilateral incisions in the dorsal flank. For sham-surgery, the ovaries were exposed but not removed.

CCI surgery CCI surgery was performed 3 weeks after the ovariectomy, a time lag sufficient to deplete circulating estrogen^[16]. The surgery to produce CCI was first described by Bennett and Xie^[1]. Under urethane (1 g/kg, ip) anesthesia, the right sciatic nerve was exposed and proximal to the trifurcation, approximately 7 mm of the common sciatic nerve was freed from adhering tissue. Four 4-0 chromic gut sutures were tied around the nerve at intervals of approximately 1 mm, and ligatures were tied loosely enough so that, on visual inspection, blood flow was not obstructed. The left sciatic, used as control, was exposed but not ligated. The surgical incision was sutured and postsurgical recuperation was monitored daily. Special attention was paid to prevent infection, thus minimizing the influence of inflammation.

Tissue harvest Animals were allowed to survive for 0, 3, 7, 14, and 21 d after CCI surgery. After each survival time, the animals were deeply anesthetized with urethane (1.25 g/kg, ip) and then decapitated ($n=6$ per group). The lumbar spinal cord was exposed by laminectomy, about 8 mm piece of the lumbar spinal cord containing the L4-L5 segments was removed, and tissue was cut along the midline into ipsilateral and contralateral side. At the same time, fresh L4 and L5 DRGs were dissected bilaterally. Identical landmarks were used for tissue removal in each experimental group. Both spinal cord and DRGs were quickly removed to liquid nitrogen.

RT-PCR analysis of BDNF mRNA BDNF mRNA expression in DRGs was analysed with reverse transcription polymerase chain reaction (RT-PCR) method. Total RNA was extracted with TriPure Isolation Reagent and quantified by absorption at 260 nm. Reverse-transcription (RT) was implemented using Reverse Transcription System according to the protocol. We used glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as normalization control. The sequences of the primers were: BDNF forward CGTGATCGAGGAGCTGT-TGG, BDNF reverse CTGCTTCAGTTGGC-CTTTTCG; GAPDH forward CACGGCAAGTTCAATG-GCACA, GAPDH reverse GAATTGTGAGGGAGA-GTGCTC. A total volume of 100 μ L reaction contained 2 μ L of RT product, MgCl₂ 1.5 mmol/L, 2.5 U *Taq* DNA polymerase, dNTP 100 μ mol/L, primer for BDNF 0.1 μ mol/L and primer for GAPDH 0.08 μ mol/L, and 1 \times *Taq* DNA polymerase magnesium-free buffer. Then the reaction mixture was overlaid with two drops of mineral oil and incubated in thermocycler (MiniCycler PTC 150, MJ

Research Inc, USA) programmed to predenature at 94 °C for 2 min, denature at 94 °C for 1 min, anneal at 56 °C for 1 min and extend at 72 °C for 2 min for a total of 25 cycles. The last cycle was followed by a final incubation at 72 °C for 5 min and cooled to 4 °C. Pilot experiment had proved that this cycle number allowed the product detection within the linear phase of amplification. All experiments included negative controls, where template RNA or reverse transcriptase was omitted. The PCR products were electrophoresed on a 1.5 % agarose gel stained with ethidium bromide. They yielded a single band corresponding to a 342 (BDNF) and 970 (GAPDH) bp cDNA fragment, respectively. The gel was captured as a digital image and analysed using Scion Image software (Maryland, USA). Values in each sample were normalized with GAPDH control. The results were expressed as a ratio of the signal of BDNF PCR product to the signal of GAPDH PCR product.

Protein assessment BDNF protein was assessed using the E-Max ELISA kit according to manufacturer's recommendations. For protein extraction, dissected spinal cord tissue was homogenized in lysis buffer (18 mL/g tissue) containing NaCl 137 mmol/L, Tris-HCl 20 mmol/L (pH 8.0), 1 % NP40, 10 % glycerol, PMSF 1 mmol/L, leupeptin 1 g/L, sodium vanadate 0.5 mmol/L, and AEBSF 100 g/L. Homogenized samples were diluted in two volumes of DPBS buffer (0.2 g KCl, 8.0 g NaCl, 0.2 g KH₂PO₄, 1.15 g Na₂HPO₄, 654 mL 1 mol/L MgCl₂, 905 mL 1 mol/L CaCl₂) and acid-treated by the addition of 1 µL of 1 mol/L HCl per 50 µL of sample for 15 min at room temperature, and then neutralized to a pH of 7.6 using 1 mol/L NaOH. After centrifugation at 1500×g for 15 min at room temperature, supernatant was collected and diluted 1:2 in block and sample buffer. Twenty microliter of each sample was removed to determine the total protein concentration using the micro-BCA protein assay. For the ELISA, 96-well, flat-bottomed plates (Corning Costar, USA) were incubated overnight at 4 °C with carbonate coating buffer containing anti-BDNF monoclonal antibody. Plates were blocked for 1 h with block and sample buffer, followed by incubation with samples and BDNF standards for 2 h at room temperature with shaking. A standard curve was established using serial dilutions of known amounts of BDNF ranging from 0 to 500 ng/L, diluted in block and sample buffer. Plates were washed five times with TBST (Tris-HCl 20 mmol/L, NaCl 150 mmol/L, 0.05 % Tween-20), followed by 2 h incubation (room tempera-

ture) with anti-human BDNF polyclonal antibody, five washes with TBST, and 1 h incubation (room temperature) with horseradish peroxidase. Enzyme solution (1:1 TMB and peroxidase substrate) was prepared at 1 h in advance and subsequently incubated on the plate for 10 min (room temperature). After samples turned blue, the reaction was stopped with phosphoric acid and absorbance was read at 450 nm using a plate reader. The BDNF protein content was normalized to the total protein concentration and the results were expressed as pg/g (total protein).

Statistical analysis Data were presented as mean±SD. Statistical analysis was performed by Student's *t*-test. Statistical significance was assessed at *P*<0.05.

RESULTS

Effects of gonadectomy on BDNF mRNA expression in DRGs after CCI surgery In all amplifications, single bands of 342 and 970 bp corresponding to the expected size fragments of BDNF and GAPDH were obtained (Fig 1). No band was observed in the negative controls. L5 DRG showed similar changes in BDNF mRNA expression with L4 DRG.

In ipsilateral DRGs, the BDNF mRNA expression of GDX rats was significantly lower than that of the GDX-sham ones (*P*<0.05, Fig 2A) at each time point. At the same time, gonadectomy significantly reduced the increment of BDNF mRNA in contralateral side (*P*<0.05, Fig 2B).

Compared to PO d 0, the BDNF mRNA expression in ipsilateral DRGs of both GDX and GDX-sham rats was significantly higher on PO d 3, 7, 14, and 21 (*P*<0.05, Fig 2A). However, the data revealed its level in the contralateral side did not differ from PO d 0 (*P*>0.05, Fig 2B).

From post CCI operation (PO) d 3, the BDNF mRNA in the ipsilateral DRGs increased significantly compared to the contralateral side in GDX rats (*P*<0.05, Fig 3A). And the changing process occurred quite similarly in the GDX-sham rats (*P*<0.05, Fig 3B).

Effects of gonadectomy on BDNF protein content in spinal cord following CCI surgery There was significant difference between the two groups both in ipsilateral side (*P*<0.05, Fig 4A) and contralateral side (*P*<0.05, Fig 4B) throughout the research. The BDNF protein level of GDX rats was significantly lower than the GDX-sham ones (*P*<0.05, Fig 4A, 4B).

BDNF protein level in contralateral side of each

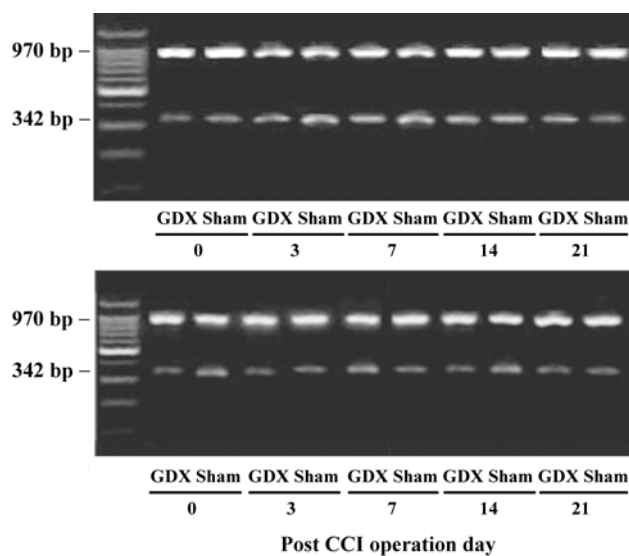


Fig 1. GDX inhibited BDNF mRNA increase in ipsilateral (A) and contralateral (B) DRGs after CCI surgery. Total RNA of DRGs was extracted and BDNF mRNA was co-amplified with GAPDH by RT-PCR. Amplified DNA was visualized through 1.5 % agarose gels stained with ethidium bromide. The photograph presents BDNF mRNA (342 bp) and GAPDH (970 bp) on PO d 0, 3, 7, 14, and 21. The marker shows a 100-bp DNA ladder indicating the size of RT-PCR products. BDNF: brain derived neurotrophic factor; DRG: dorsal root ganglion; GDX: gonadectomy; PO: post CCI operation; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

group had no significant changes during the time course ($P>0.05$, Fig 4B).

On PO d 3, 7, 14, and 21, BDNF protein level in ipsilateral spinal cord of the two groups was significantly higher from PO d 0 ($P<0.05$, Fig 4A).

On PO d 0, we measured the baseline of BDNF protein level from each group. There was no difference between the ipsilateral and the contralateral spinal ($P>0.05$). From PO d 3, BDNF protein level in ipsilateral side of both groups increased significantly compared with the contralateral side and decreased on PO day 21 (Fig 5A, 5B).

DISCUSSION

Our results demonstrate that gonadectomy affects BDNF mRNA expression in DRGs and protein content in spinal cord. In DRGs, BDNF mRNA expression increased significantly in the ipsilateral side from PO d 3, reached the maximum on PO d 7 and maintained for at least 3 weeks. Meanwhile, BDNF protein in ipsilateral spinal cord had a smaller but significant increase on PO d 3, and then reached the maximum on PO d 14. The

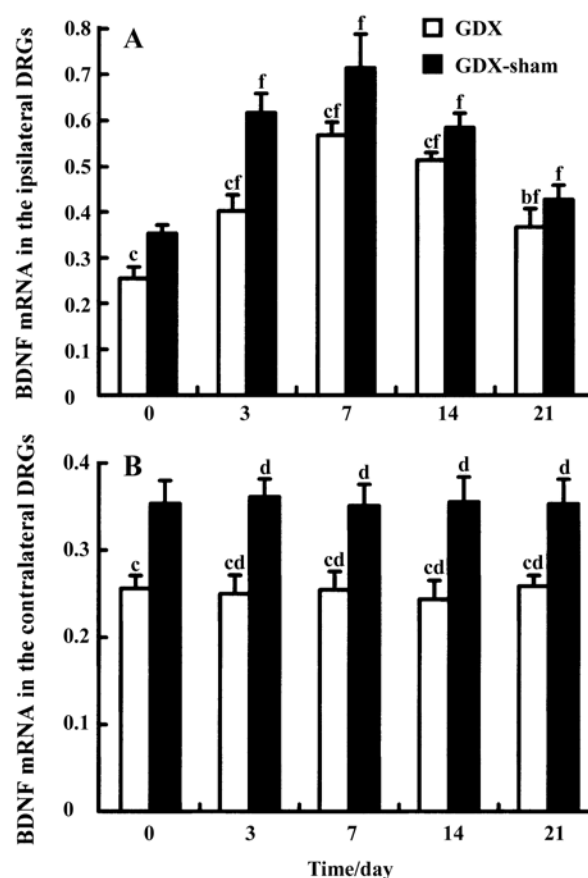


Fig 2. BDNF mRNA expression in ipsilateral (A) and contralateral (B) DRGs from the GDX and GDX-sham group on PO d 0, 3, 7, 14, and 21, expressed as a ratio of the density of BDNF to GAPDH. $n=6$. Mean \pm SD. BDNF: brain derived neurotrophic factor; DRG: dorsal root ganglion; GDX: gonadectomy; PO: post CCI operation. ^b $P<0.05$, ^c $P<0.01$ vs GDX-sham. ^d $P>0.05$, ^f $P<0.01$ vs the matched group on PO d 0.

induction in GDX group was smaller in quantity as compared to the GDX-sham ones. Using CCI model, we were unable to find significant differences between the two groups in the changing tendency of both the BDNF mRNA and protein. However, the extent of BDNF expression differed as a function of different estrogen levels. Rats with low estrogen level of GDX group got a lower expression.

CCI model has been widely used in studying many aspects of pain-related behaviors^[1,2,17,18]. Tying loosely constrictive ligatures around the rat sciatic nerve easily simulates a nerve injury. The inflammatory and the associated immune responses elicited by unilateral ligation of the sciatic nerve are crucial for the development of neuropathic pain^[19,20]. And also the phenotypic changes of DRG neurons have been extensively studied as an explanation for this pain state. These pheno-

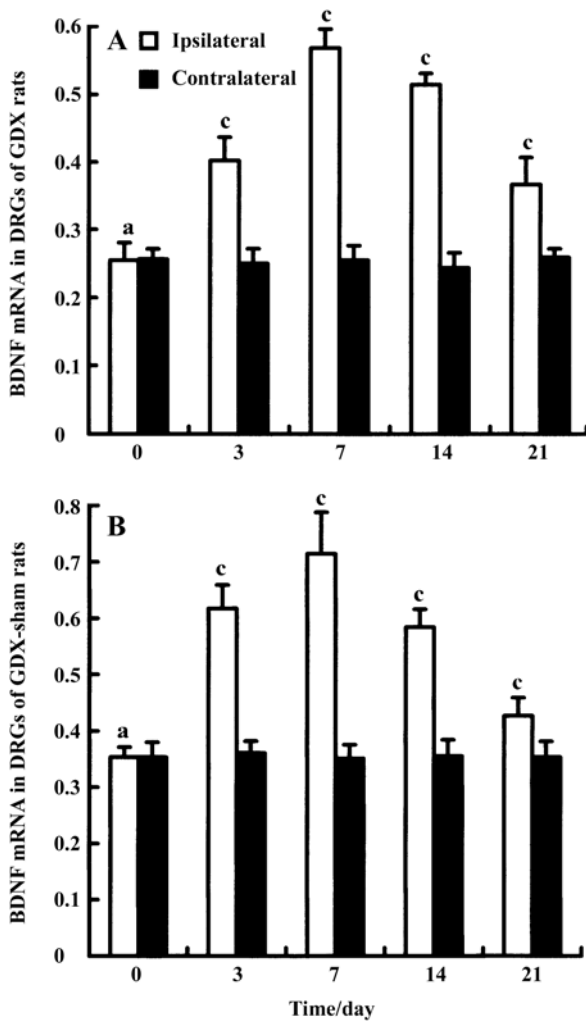


Fig 3. BDNF mRNA expression in ipsilateral and contralateral DRGs from the GDX (A) and GDX-sham (B) group on PO d 0, 3, 7, 14, and 21, expressed as a ratio of the density of BDNF to GAPDH. *n*=3. Mean±SD. BDNF: brain derived neurotrophic factor; DRG: dorsal root ganglion; GDX: gonadectomy; PO: post CCI operation. ^a*P*>0.05, ^c*P*<0.01 vs contralateral.

typic changes may contribute to development and maintenance of spontaneous pain and may have roles in central sensitization in the spinal cord.

BDNF is an endogenous modulator of nociceptive responses and is necessary for central sensitization in the spinal cord^[13], where postsynaptic cells express its receptor (*trkB*)^[21]. The increased activity of postsynaptic neurons leads to the release of BDNF and translocation of *trkB* receptors to the cell surface. This will modulate synaptic transmission and regulate neuronal activity in the areas responding to peripheral stimulation of the injured peripheral nerve. BDNF also has a role as a central modulator of tactile stimulus-induced

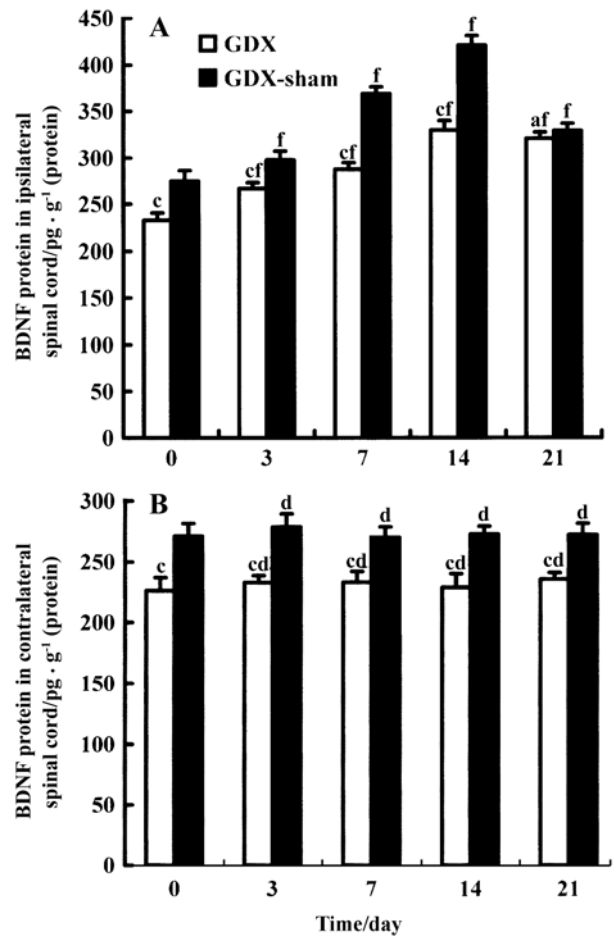


Fig 4. BDNF protein content in ipsilateral (A) and contralateral (B) side of spinal cord from GDX and GDX-sham group on PO d 0, 3, 7, 14, and 21. It was normalized to the total protein concentration and expressed as pg/g (total protein). *n*=3. Mean±SD. BDNF: brain derived neurotrophic factor; DRG: dorsal root ganglion; GDX: gonadectomy; PO: post CCI operation. ^a*P*>0.05, ^c*P*<0.01 vs GDX-sham. ^d*P*>0.05, ^f*P*<0.01 vs the same group on PO d 0.

inflammatory pain hypersensitivity^[22]. Additionally, substantial evidence suggests that BDNF is involved in the neurotransmission of nociceptive functions. In an *in vitro* spinal cord preparation, BDNF can potentiate the nociceptive spinal reflex responses. *N*-methyl-*D*-aspartate (NMDA) receptor-mediated responses, known as a major contributor to central sensitization, can be enhanced by exogenous BDNF^[23].

When exposed to steroid hormones, many neural pathways are differently influenced. The presence of an estrogen response element in the BDNF gene provides a direct link between estrogen and BDNF^[24]. In addition, estrogen can regulate the expression of BDNF indirectly through a step involving other transcription

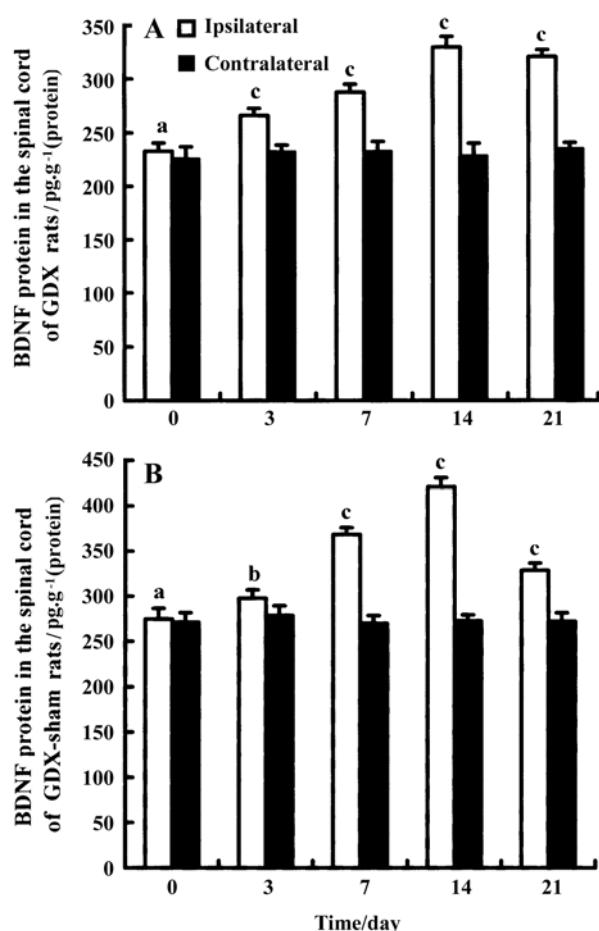


Fig 5. BDNF protein content in ipsilateral and contralateral side of spinal cord from GDX (A) and GDX-sham (B) group on PO d 0, 3, 7, 14, and 21. It was normalized to the total protein concentration and expressed as pg/g (total protein). $n=3$. Mean \pm SD. BDNF: brain derived neurotrophic factor; DRG: dorsal root ganglion; GDX: gonadectomy; PO: post CCI operation. ^a $P>0.05$, ^b $P<0.05$, ^c $P<0.01$ vs contralateral.

regulating factors^[25]. Besides its effects on BDNF, estrogen has been shown to influence the expression of neurotrophin receptors^[26].

Increases in the concentration of brain derived neurotrophic factor in the lumbar spinal dorsal horn are associated with pain behavior following chronic constriction injury in rats^[27]. BDNF may contribute to injury-induced plasticity in sensory information processing in the spinal dorsal horn, and ultimately perhaps to the development of persistent pain. Our results provide evidence that in neuropathic pain, estrogen can affect BDNF expression. It can be explained in two aspects. On one hand, estrogen may influence pain situation by its interaction with BDNF. BDNF serves as both neurotransmitter and neuromodulator in the nociceptive

process. On the other hand, injured nerve needs more neurotrophic factor to repair the injury. We are now trying to develop rat model to investigate how different estrogen level affects pain behavior and BDNF expression.

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