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RESEARCH ARTICLE

Stress injuries and autophagy in mouse hippocampus after chronic cold exposure

Ting-ting Qu[1, 2, #], Jie-xin Deng[1, #], Rui-ling Li[1], Zhan-jun Cui[1], Xiao-qing Wang[1], Lai Wang[1, *], Jin-bo Deng[1, *]
1 Institute of Neurobiology, College of Life Science, Henan University, Kaifeng, Henan Province, China
2 Nursing College, Henan Vocational College of Applied Technology, Zhengzhou, Henan Province, China

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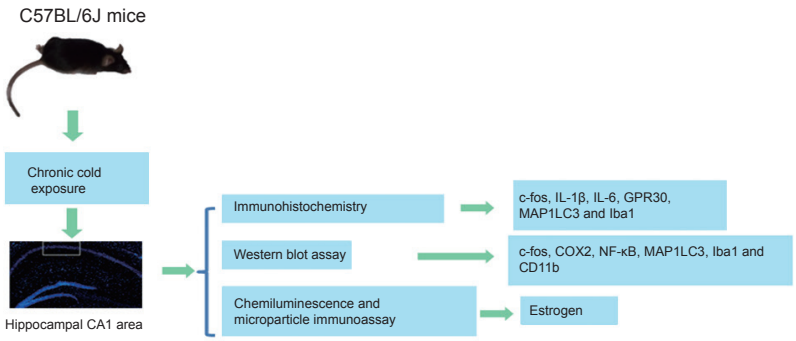
Graphical Abstract

Changes of oxidative stress, autophagy and estrogen level in mouse hippocampus after chronic cold exposure



Correspondence to:
Lai Wang, M.D. or Jin-bo Deng, M.D., wanglai@henu.edu.cn or jinbo_deng@henu.edu.cn.

#These authors contributed equally to this study.

orcid:
0000-0002-3323-7210 (Lai Wang)
0000-0002-3166-2686 (Jin-bo Deng)

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Abstract

Cold exposure is an external stress factor that causes skin frostbite as well as a variety of diseases. Estrogen might participate in neuroprotection after cold exposure, but its precise mechanism remains unclear. In this study, mice were exposed to 10°C for 7 days and 0–4°C for 30 days to induce a model of chronic cold exposure. Results showed that oxidative stress-related c-fos and cyclooxygenase 2 expressions, MAP1LC3-labeled autophagic cells, Iba1-labeled activated microglia, and interleukin-1β-positive pyramidal cells were increased in the hippocampal CA1 area. Chronic cold exposure markedly elevated the levels of estrogen in the blood and the estrogen receptor, G protein-coupled receptor 30. These results indicate that neuroimmunoreactivity is involved in chronic cold exposure-induced pathological alterations, including oxidative stress, neuronal autophagy, and neuroimmunoreactivity. Moreover, estrogen exerts a neuroprotective effect on cold exposure.

Key Words: *nerve regeneration; chronic cold exposure; oxidative stress; autophagy; microglial cells; neuroimmunoreactivity; hippocampal CA1 area; estrogen; neural regeneration*

Introduction

Stress response refers to an organism's reaction to external or internal environmental alterations. Physiological functions are optimal when performed under constant body temperature (Varela et al., 2015). Cold exposure is an external stress factor that causes skin frostbite and damages various physiological processes including movement, and the cardiovascular, immune, and nervous systems, resulting in various diseases (Mohr et al., 2009; Brazaitis et al., 2015). Therefore, it is of great significance to understand the risks of cold exposure to health.

The mechanisms of an individual's response to cold stress are very complex because many cells, organs, and systems are involved. At the system and organ level, cold stress activates the hypothalamic-pituitary-adrenal axis and excites sympathetic nerves. In addition, vasoconstriction reduces heat radiation. At the cellular level, a cold stimulus can induce oxidative stress, resulting in cell injury and even apoptosis (Venditti et al., 2007; Mihailidou et al., 2009; Ouellet et al., 2011).

Terrien et al. (2011) and Sugama et al. (2011) reported that acute cold induced microglial activation as early as 30

minutes after exposure. In addition, cold exposure increased interleukin (IL)-1β immunoreactivity in the hippocampus and hypothalamus. Our previous study reported that estrogen might be involved in neuroprotection after chronic cold exposure (Cui et al., 2014).

The current study investigated changes in oxidative stress, neuronal autophagy, neuroimmunoreactivity, and estrogen levels after chronic cold exposure to elucidate the involved pathological alterations, cell stress responses, and estrogen-mediated neuroprotection mechanisms.

Materials and Methods

Establishment of a chronic cold exposure model

All experiments were carried out in accordance with the Institutional Guidelines of Henan University for Animal Welfare (MEWEAHUM2014-000). Adult male C57BL/6J mice (25–30 g) at postnatal day 40–50 were fed in standard laboratory animal housing with a 12-hour light/dark cycle at 20–25°C. The mice were randomly divided into a control group and a cold exposure group, with 20 mice in each group. To decrease the mortality from sudden exposure to 0–4°C, pre-exposure at 10°C was carried out for the cold exposure group. After pre-exposure for 7 days, the treatment mice were exposed to 0–4°C for 30 days. During cold exposure, each experimental mouse was housed in a separate cage to avoid mutual contact for warmth. During cold exposure, behavior and mortality were recorded. The control mice were housed under standard conditions at 20–25°C.

Sample collection

Both control and treatment mice were intraperitoneally anesthetized with sodium pentobarbital (20 mg/kg) after intervention. Mice were perfused transcardially with 4% paraformaldehyde. After brains were removed, immersion fixation was carried out at 4°C for 1–2 days. The hippocampus is an important organ for learning and memory, and the CA1 area of the hippocampus is very sensitive to stress, such as ischemia and hypoxia (Schmidt-Kastner, 2015). Therefore, the CA1 area (Schmidt-Kastner, 2015) was selected as a target for the measurement.

Immunofluorescence assay

Hippocampal coronal sections were cut and rinsed with 0.01 M phosphate buffer. Nonspecific antigens were blocked with 10% normal goat serum (with 0.3% Triton X-100, 1% bovine serum albumin in 0.1 M phosphate buffer) for 30 minutes. The slices were then incubated with primary antibodies at 4°C overnight. After rinsing three times, the slices were incubated with secondary antibodies for 3 hours at room temperature. The following primary antibodies were used: rabbit anti-IL-1β polyclonal antibody (1:500; Abcam, Cambridge, UK), rabbit anti-c-fos polyclonal antibody (1:500; Abcam, Cambridge, UK), rabbit anti-cyclooxygenase 2 (COX2) polyclonal antibody (1:500; Abcam), rabbit anti-MAP1LC3 polyclonal antibody (1:500; Abcam), rabbit anti-Iba1 polyclonal antibody (1:400; Abcam), rabbit anti-IL-6 polyclonal antibody (1:200; Abcam), and rabbit anti-G protein-coupled receptor 30 (GPR30) polyclonal antibody (1:300; Abcam). Secondary antibodies used were donkey anti-rabbit IgG Alexa Fluor 568 (1:600; Invitrogen, Carlsbad, CA, USA), and donkey anti-goat IgG Alexa Fluor 488 (1:500; Invitrogen). Sections were coverslipped with medium (65% glycerol in 0.01 M phosphate buffer + 1:10,000 DAPI for counterstaining). Then, cells were imaged with an epifluorescence microscope (BX61; Olympus, Tokyo, Japan) under rhodamine or fluorescein isothiocyanate excitation. High-quality sections were photographed with an Olympus laser confocal microscope (FV1000; Olympus). The numbers of c-fos-, COX2-, MAP1LC3-, Iba1-, IL-1β-, IL-6- and GPR30-immunoreactive cells in the unit area were calculated. The number of immunoreactive cells was equal to the cell numbers/measured area (cells/mm^2). For cell measurement, 20 mice in each group and 5 sections per mouse were used.

Western blot assay

To verify the results of the immunofluorescence assay, the expressions of c-fos, COX2, MAP1LC3, Iba1, nuclear factor-kappa B (NF-κB)-p65 and CD11b in hippocampus tissues were investigated using western blot assay. The proteins of hippocampal tissue were extracted with a cytoplasmic protein extraction kit (Beyotime Institute of Biotechnology, Haimen, China). The bicinchoninic acid assay was used to measure total protein concentrations according to a standard curve. The samples were subjected to electrophoresis and transferred to membranes. Primary antibodies, rabbit anti-c-fos polyclonal antibody (1:1,000; Abcam), rabbit anti-COX2 polyclonal antibody (1:2,000; Abcam), rabbit anti-MAP1LC3 monoclonal antibody (1:2,000; Abcam), rabbit anti-Iba1 (1:2,000; Abcam), rabbit anti-NF-κB-p65 polyclonal antibody (1:500; Santa Cruz Biotechnology, Dallas, TX, USA) or mouse anti-CD11b (1:1,000; Abcam), were incubated at 4°C overnight. After washing with Tris Buffered Saline with Tween 20 three times (15 minutes each), corresponding horseradish peroxidase-labeled goat anti-rabbit IgG (1:2,000; Beyotime Institute of Biotechnology) and horseradish peroxidase-labeled rabbit anti-mouse IgG (1:1,000; Zhongshan Golden Bridge Biotechnology, Beijing, China), were incubated at room temperature for 2 hours. Finally, the membranes were incubated in enhanced chemiluminescence reagent for 3 minutes, and X-ray films were exposed and developed. β-Actin (Beyotime Institute of Biotechnology) was used as an internal reference. The grayscale ratio of target bands with an internal reference indicated the relative gray value of target bands. Objective cells in the CA1 area of the hippocampus were measured with ImageJ ProPlus 6.0 software (Media Cybernetics, Rockville, MD, USA). Protein expression levels were equal to the optical density value of the target band/β-actin.

Blood estrogen test

After anesthetization with sodium pentobarbital (20 mg/kg, intraperitoneally), the eyeballs of mice were removed, and 2 mL of blood was collected from the orbit. Blood was self-clotted at room temperature for 1 hour, and centrifuged at 1,500 × *g* for 15 minutes. Afterwards, 400–500

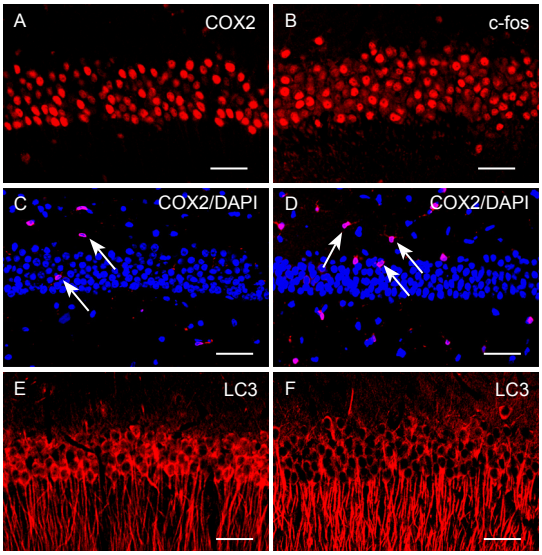


Figure 1 Cold-induced cellular oxidative stress, inflammatory injury, and autophagy in the hippocampal CA1 area (immunofluorescence assay).
(A, B) c-fos-immunoreactive cells (red) in the control group (A) and cold exposure group (B); (C, D) COX2-immunoreactive cells (red) (arrows) in the control group (C) and cold exposure group (D). The pyramidal layer is visualized by DAPI counterstaining (blue); (E, F) MAP1LC3-immunoreactive cells (red) in the control group (E) and cold exposure group (F). After cold exposure, the numbers of c-fos-immunoreactive cells, COX2-immunoreactive cells, and MAP1LC3-immunoreactive cells are increased. Scale bars: 50 μm. COX2: Cyclooxygenase 2; DAPI: 4′,6-diamidino-2-phenylindole.

μL of serum was taken. Finally, chemiluminescence and microparticle immunoassays (Takeda et al., 2013) were used to measure the serum estrogen level using a specific kit and automated immunoassay analyzer (ARCHITECT, Abbott Park, IL, USA).

Statistical analysis

The data were expressed as the mean ± SD. SPSS v11.5 statistical software (SPSS, Chicago, IL, USA) was used to compare measurements between control and cold exposure groups, and an independent-sample *t*-test analysis was carried out. A value of $P < 0.05$ was accepted as statistically significant.

Results

Alterations of oxidative stress and autophagy after chronic cold exposure

To determine the effect of oxidative stress and inflammatory damage to neurons in the CA1 area after chronic cold exposure, an immunofluorescence assay was used to measure the relative levels of proteins including c-fos, COX2, and NF-κB. We found that c-fos-immunoreactive cells were mainly pyramidal cells and granule cells in the pyramidal and granular layers, but that COX2-immunoreactive cells were polygonal interneurons distributed in the polymorphic, pyramidal, and molecular layers. After cold exposure, numbers of c-fos-immunoreactive cells and COX2-immunoreactive cells in the CA1 region were increased significantly (**Figure 1A–D**). To confirm our observation, these immunoreactive cells were measured (**Figure 2A**, **B**). There was a statistically significant difference between the cold exposure and control groups ($n = 20$, $P < 0.01$). Western blot assay supported the conclusions from the immunofluorescence assay ($n = 10$, $P < 0.01$) (**Figure 2C**, **D**), suggesting that chronic cold exposure increased oxidative stress and inflammatory injury in hippocampal neurons.

We also used MAP1LC3 to label autophagic neurons with immunocytochemistry. MAP1LC3 was mainly expressed in the somas and dendrites of pyramidal cells. Compared with the control group, numbers of MAP1LC3-immunoreactive cells were significantly increased after cold exposure (**Figure 1E**, **F**). There was a significant difference between the cold exposure and control groups ($n = 20$, $P < 0.01$) (**Figure 3A**). Western blot assay showed that MAP1LC3 was over-expressed in the cold exposure group, supporting the immunofluorescence assay results ($n = 10$, $P < 0.01$) (**Figure 3B**, **C**).

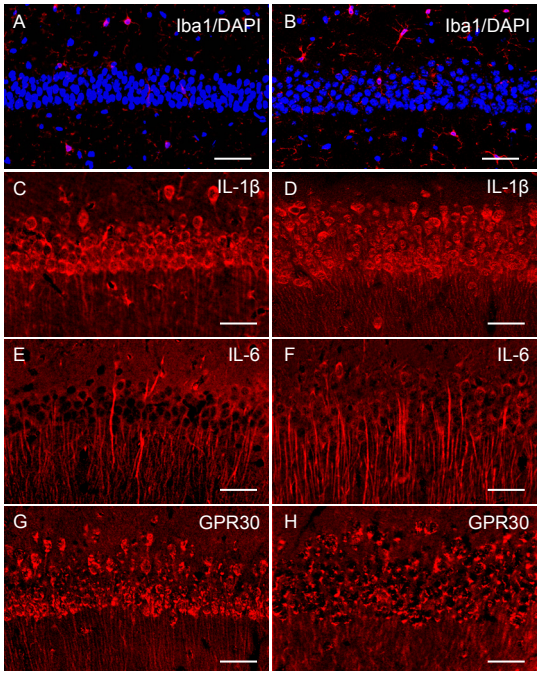


Figure 4 Cold exposure increases neuroimmunoreactivity and estrogen receptor GPR30 expression (immunofluorescence assay).
(A, B) Iba1-labeled microglial cells (red) in the control group (A) and cold exposure group (B). The pyramidal layer is visualized by DAPI counter-staining (blue). The number of microglial cells (red) was significantly increased after chronic cold exposure. (C, D) IL-1β-immunoreactive cells (red) in the control group (C) and cold exposure group (D). The number of IL-6-immunoreactive cells (red) was significantly increased after chronic cold exposure. (E, F) IL-6-immunoreactive cells (red) in the control group (E) and cold exposure group (F). Cold exposure induced an increase of IL-6-immunoreactive cells. (G, H) GPR30-immunoreactive cells (red) in the control (G) and cold exposure (H) groups. GPR30 expression was increased after cold exposure. Scale bars: 50 μm. DAPI: 4′,6-Diamidino-2-phenylindole; IL: interleukin; GPR30: G protein-coupled receptor 30.

Neuroimmunoreactivity after chronic cold exposure

Neuroimmunoreactivity may be involved in cold stress, as previously reported (Sugama et al., 2011). Immunofluorescence staining was used to identify microglial cells ($Iba1^+$), stress cytokines (IL-6, IL-1β), and CD11b, which reflected the neuroimmunoreactivity present. The expressions of

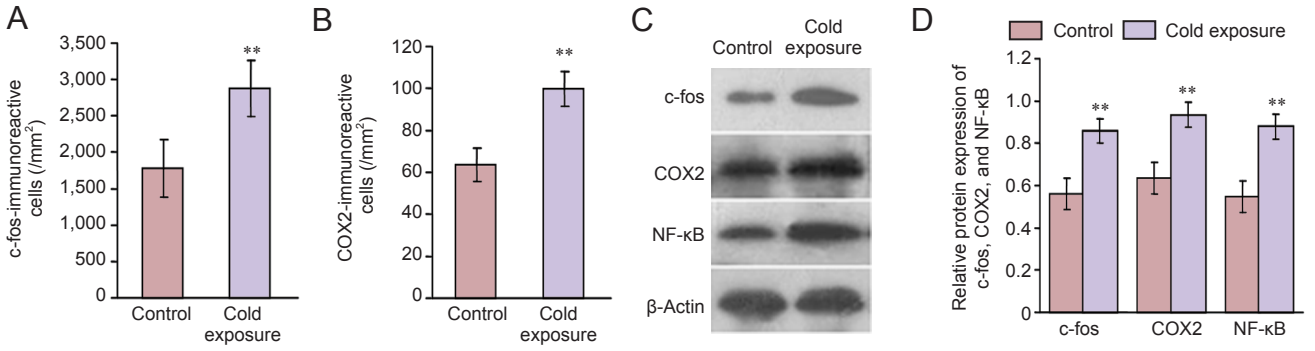
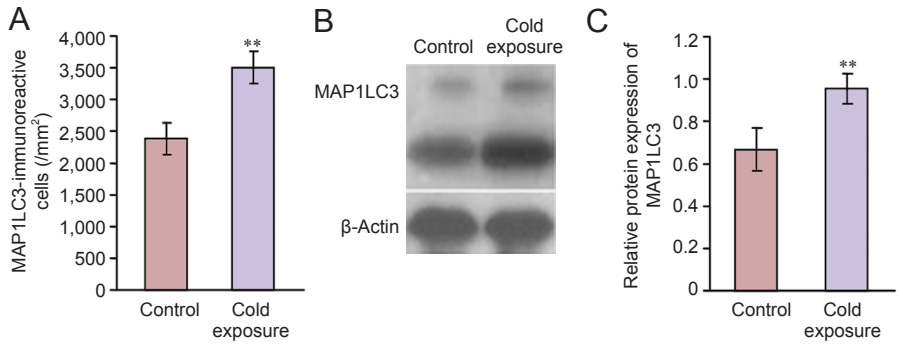


Figure 2 Expression of oxidative stress-related proteins after chronic cold exposure.
(A, B) The numbers of c-fos- (A) and COX2-immunoreactive cells (B) in the CA1 area after chronic cold exposure (immunofluorescence assay). (C) Western blots of c-fos, COX2, and NF-κB expression in the mouse hippocampus. (D) Semi-quantitative analysis of relative proteins by western blot assay. Relative protein expression is represented as the optical density ratio of targeted protein to β-actin. Data are expressed as the mean ± SD. $^{**}P < 0.01$, *vs.* control group (independent-sample *t*-test). NF-κB: Nuclear factor kappa B; COX2: cyclooxygenase 2.



(A) Quantitation of MAP1LC3-immunoreactive cells in the hippocampal CA1 area after chronic cold exposure (immunofluorescence assay). (B) Western blots of MAP1LC3 expression in the mouse hippocampus. (C) Semi-quantitative analyses of MAP1LC3 (western blot assay). Data are expressed as the mean ± SD. $^{**}P < 0.01$, *vs.* control group (independent-sample *t*-test). Relative protein expression is represented as the optical density ratio to β-actin.

Figure 3 Expression of autophagy-related proteins after chronic cold exposure.

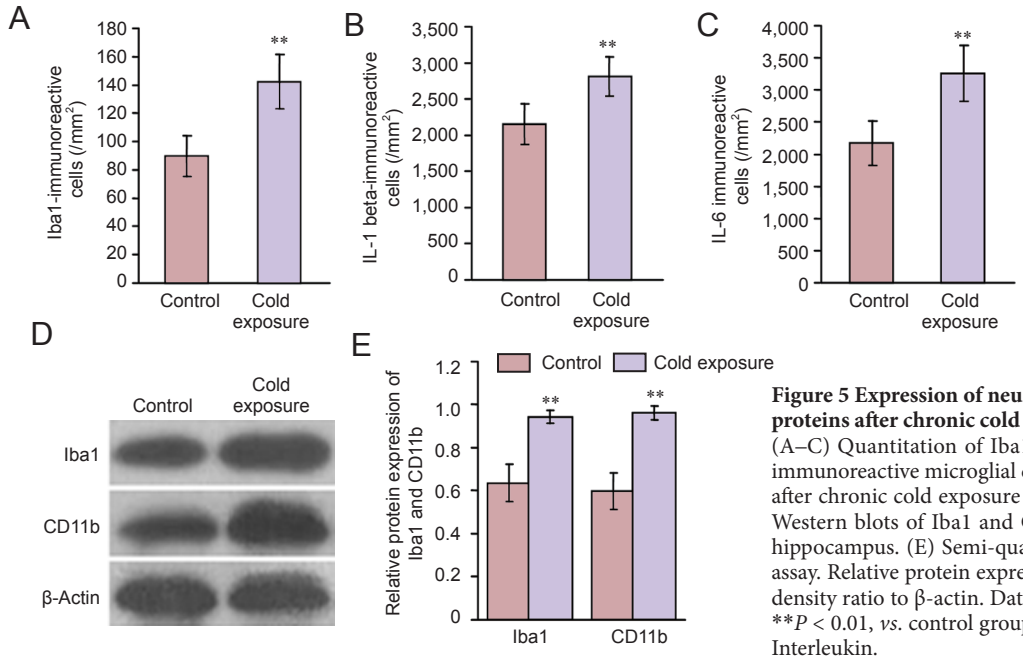


Figure 5 Expression of neuroimmunoreactivity-related proteins after chronic cold exposure.
(A–C) Quantitation of Iba1- (A), IL-1β- (B), and IL-6- (C) immunoreactive microglial cells in the hippocampal CA1 area after chronic cold exposure (immunofluorescence assay). (D) Western blots of Iba1 and CD11b expressions in the mouse hippocampus. (E) Semi-quantitative analyses of western blot assay. Relative protein expression is represented as the optical density ratio to β-actin. Data are expressed as the mean ± SD. $^{**}P < 0.01$, *vs.* control group (independent-sample *t*-test). IL: Interleukin.

Iba1 and CD11b were also detected by western blot assay. Iba1-positive microglial cells were evenly distributed in the hippocampus with a star-like shape, and IL-1β and IL-6-immunoreactive cells were mainly located in the pyramidal and granule layers. The numbers of microglial cells and IL-1β-immunoreactive pyramidal cells in the CA1 region was increased significantly after cold exposure (**Figure 4A–F**). Statistical analyses confirmed these observations ($P < 0.01$) (**Figure 5A–C**). Western blot assay results were consistent with the immunofluorescence assay ($P < 0.01$) (**Figure**

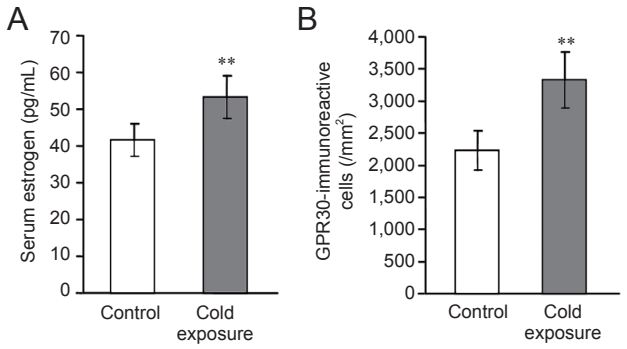


Figure 6 Estrogen levels and GPR30 expression after chronic cold exposure (immunofluorescence assay).
(A) Estrogen levels in the blood after cold exposure detected by immunoassay. (B) Alterations of GPR30-immunoreactive cells in the hippocampal CA1 area after chronic cold exposure detected by immunofluorescence assay. Data are expressed as the mean ± SD. $^{**}P < 0.01$, *vs.* control group (independent-sample *t*-test); GPR30: G protein-coupled receptor 30.

5D, **E**), indicating an overexpression of Iba1 and CD11b after cold exposure. These results suggest that chronic cold exposure induces microglia and stress cytokine secretion.

Changes of estrogen and estrogen receptor levels after chronic cold exposure

Estrogen is an important steroid hormone and might be involved in responses to cold stress (Uchida et al., 2010). To understand this further, estrogen levels in the blood and estrogen receptor, GPR30 expression in the CA1 area were measured. In this study, chronic cold exposure significantly enhanced blood estrogen levels ($P < 0.01$; **Figure 6A**). Because GPR30 is an active target of estrogen (Lee et al., 2012), the expression of GPR30 in the CA1 area was also measured. GPR30 was mainly located on pyramidal cells and their projections toward the molecular layer. After cold exposure, numbers of GPR30-immunoreactive cells were significantly increased ($P < 0.01$) (**Figures 4G**, **H** and **6B**).

Discussion

Chronic cold exposure induced oxidative stress and neuronal autophagy

Oxidative stress is regarded as an early protective reaction to defend against injury caused by environmental changes. Subsequently, oxidative stress will develop into a cell inflammatory reaction *via* participation of the immune system (Loane et al., 2014; Lee et al., 2015; Lu and Black, 2016). During oxidative and inflammatory reactions, cytokines are secreted. c-fos is an early/immediate stress gene involved in oxidative stress, which induces cell growth and differentiation (Gil-Mohapel et al., 2013). NF-κB and COX2 are also regarded as oxidative stress and inflammatory cytokines (Alvira, 2014; Zhou et al., 2014; Li et al., 2015). Once stress and inflammatory cytokine levels reach optimal levels, apoptosis and cellular and tissue damage occurs (Town, 2010; Vianna et al., 2011; Cho et al., 2015).

In our study, the expressions of c-fos, NF-κB, and COX2 were investigated using immunocytochemistry and western blot assay. Chronic cold exposure increased the expression of these proteins, suggesting that oxidative stress and inflammatory injuries occur in the hippocampus. Reactive oxygen species are critical for oxidative stress (Cano et al., 2014), and reactive oxygen species might be induced by cold exposure (Wang et al., 2015). Furthermore, intensive lipid peroxidation induced by cold stimuli might result in an increase in reactive oxygen species in cells. In this study, cold exposure induced neuronal autophagy. Cell autophagy often occurs during nutritional deficiencies. During autophagy, cells can survive by digesting their own organelles to obtain necessary nutrition and energy (Parzych and Klionsky, 2014). Excessive autophagy can trigger the release of hydrolase from lysosomes causing cell death (Levine and Kroemer, 2008). In a previous study, cold exposure induced autophagy (Kumar, 2007). A link was also reported between cell oxidative stress and autophagy (Lv and Zhou, 2012). Oxidative stress and inflammatory reactions can probably induce the autophagy pathway under a cold stimulus (Kathiria et al., 2012; Lu and Xu, 2013). We believe that cold-induced autophagy probably has an important neuroprotective function and provides sufficient nutrition and energy for body and cell survival.

Cold induced-oxidative stress caused neuroimmunoreactivity

Neural immunity is very important for an organism to maintain microenvironmental stability in the brain, and microglia are key for neural immunity in the central nervous system. Microglia are derived from macrophages and are widely distributed in the central nervous system (Lawson et al., 1992). Microglia are rapidly activated upon infection by bacteria and viruses (Glass et al., 2010). As calcium-binding proteins and markers of microglia, Iba1 and CD11b have specific functions in neural immunity. Activated microglia express Iba1 and CD11b (Blanchard et al., 2014). In addition, the inflammatory cytokines, IL-1β and IL-6, are also secreted by activated microglia (Awada et al., 2014), suggesting a close relationship between oxidative stress/inflammatory reaction and neuroimmunoreactivity.

In our study, Iba1-positive microglia were significantly increased in the CA1 area after chronic cold exposure, and this was consistent with the expressions of Iba1 and CD11b detected by western blot assay. This indicates causality between cold-induced oxidative stress and immune responses in the central nervous system. Indeed, during oxidative stress, transcription factor and inflammatory cytokines, such as NF-κB (Wang et al., 2010), IL-1β, IL-6, COX2, TNF-α (Jensen et al., 2011; Luheshi et al., 2011) and inducible nitric oxide synthase (Loane and Byrnes, 2010), are released by neurons in the central nervous system. These stress cytokines activate microglia in the central nervous system causing neurotoxicity (Xie et al., 2014), consistent with the results of our study. These cytokines also further activate glial cells and produce more inflammatory mediators (Morris et al., 2015; Yang et al., 2015a). Finally, these inflammatory mediators induce anti-inflammatory cytokines and neurotrophic factors that are neuroprotective (Correale, 2014). Excessive amounts of cytokines cause neurotoxicity and inflammatory injuries in

the brain (Xie et al., 2014). For example, the over-expression of NF-κB IL-1β, and IL-6 promotes the production of oxygen free radicals, which cause inflammatory damage to endothelial cells in the blood-brain barrier (Gil-Núñez and Villanueva, 2001; Yang et al., 2015b).

Regulation of estrogen to autophagy and neural immunity

Our previous study showed that after chronic cold exposure, the mortality of male mice was significantly higher than that of females, suggesting that estrogen was involved in cold stress responses (Deng et al., 2011). Previous studies also confirmed that stress and inflammatory responses are stronger in males than in females (Ide et al., 2002), and stronger post-menopause than pre-menopause (Bombelli et al., 2005), suggesting a close correlation between estrogen and oxidative stress. Therefore, we hypothesize that estrogen protects against cold-induced neural injury. Estradiol is a steroid hormone with an important role in the reproductive, circulatory, and nervous systems (Brann et al., 2007). GPR30 is an estrogen receptor mainly distributed in the brain, such as the cerebral cortex and hypothalamus. GPR30 is required for the neurophysiological functions of estrogen. Previous studies confirmed that estrogen participates in neuronal activity of the hypothalamic-pituitary-adrenal axis after signaling through GPR30 (Lebesgue et al., 2009; Tang et al., 2014), to maintain body temperature (Handa et al., 2011; Shivers et al., 2015).

Because the activity of estrogen requires GPR30 expression, we detected estrogen levels in the blood and quantified GPR30 in the CA1 area. Our study showed that estrogen levels in the blood and GPR30 expression in the CA1 region were increased significantly after chronic cold exposure. Considering neural oxidative stress responses and the appearance of neuronal autophagy after cold exposure, estrogen probably mediates oxidative stress responses and neuronal autophagy in the central nervous system during cold exposure. The regulative mechanism of estrogen for neural immune responses and autophagy is not fully understood. We hypothesize that the GPR30 pathway is activated once estrogen signals through its receptor (Lee et al., 2012; Pupo et al., 2016). Estrogen mediates neural immunity and has neuroprotective functions *via* the GPR30 pathway by the production of estrogen-induced cytoprotective factors, such as heat shock proteins (Gragasin et al., 2003) and neurotrophic factors (Hou et al., 2010). Estrogen increases the expression of Bcl-2, a negative regulator of cell apoptosis including autophagy (Bei et al., 2012; Brann et al., 2012; Naderi et al., 2015). Moreover, estrogen improves cerebral blood circulation in cortical infarct areas by increasing nitric oxide release (McNeill et al., 1999).

In summary, chronic cold exposure induces neural oxidative stress, inflammatory damage, and autophagy. Immune responses are induced by an increase in the numbers of microglia and IL-1β- and IL-6-positive cells. Furthermore, blood estrogen levels and GPR30 expression were increased after cold exposure. Our study suggests that oxidative stress, autophagy, and immune responses mediated *via* estrogen have neuroprotective functions during cold exposure responses. Our data further contributes to our knowledge of cryomedicine and new clinical treatment strategies. Furthermore, these data will be useful in research of military survival and athletic performance in extreme cold environments.

Author contributions: *TTQ collected the main experimental data. JXD, RLL, ZJC and XQW provided technical assistances. LW and JBD wrote the paper and designed the assay. All authors approved the final version of the paper.*
Conflicts of interest: *None declared.*
Plagiarism check: *This paper was screened twice using CrossCheck to verify originality before publication.*
Peer review: *This paper was double-blinded and stringently reviewed by international expert reviewers.*

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