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# GABAergic neurons in cerebellar interposed nucleus modulate cellular and humoral immunity via hypothalamic and sympathetic pathways

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ABSTRACT

Our previous work has shown that cerebellar interposed nucleus (IN) modulates immune function. Herein, we reveal mechanism underlying the immunomodulation. Treatment of bilateral cerebellar IN of rats with 3-mercaptopropionic acid (3-MP), a glutamic acid decarboxylase antagonist that reduces γ-aminobutyric acid (GABA) synthesis, enhanced cellular and humoral immune responses to bovine serum albumin, whereas injection of vigabatrin, a GABA-transaminase inhibitor that inhibits GABA degradation, in bilateral cerebellar IN attenuated the immune responses. The 3-MP or vigabatrin administrations in the cerebellar IN decreased or increased hypothalamic GABA content and lymphoid tissues' norepinephrine content, respectively, but did not alter adrenocortical or thyroid hormone levels in serum. In addition, a direct GABAergic projection from cerebellar IN to hypothalamus was found. These findings suggest that GABAergic neurons in cerebellar IN regulate immune system via hypothalamic and sympathetic pathways.



## 1. Introduction

The cerebellum, the largest subcortical center for motor control, has been found in recent decade to be involved in regulation of non-somatic functions, such as visceral activities (Xu and Frazier, 2002; Zhu et al., 2006), feeding behavior (Zhu and Wang, 2008), and cognition and working memory (Alexander et al., 2012; Thürling et al., 2012). Immune function, which can be considered as a non-somatic activity, has been largely reported on its regulation by the central nervous system, especially by the hypothalamus (Wrona, 2006; Wood et al., 2013). A few literatures have presented that the cerebellum is also associated to immune modulation. For example, a suppressed T cell and macrophage function is observed in the "reeler" mutant, a murine strain with elevated norepinephrine (NE) concentration in the cerebellum (Green-Johnson et al., 1995). Lesion of the vestibulocerebellum of rats with kainic acid (KA) induces immunosuppressive effect (Ghoshal et al., 1998). However, the existing knowledge concerning immunomodulation by the cerebellum is poorly understood. In recent years, we have focused on the cerebellar nuclei, fastigial nucleus (FN) and interposed nucleus (IN) that are the sites for final output of cerebellar information, to reveal immune regulation by the cerebellum. We found that lesions of FN with KA enhance immune function, including cellular and humoral immunity (Peng et al., 2005; Ni et al., 2010), whereas lesions of IN inhibit the immune function (Peng et al., 2006). The positive and negative immunoregulations by the two different cerebellar nuclei strongly shows that the cerebellum involves an elaborate and critical modulation of immune homeostasis.

Nevertheless, dissecting the pathways or mechanisms underlying cerebellar immunomodulation is daunting, because there is no direct structural connection from the cerebellum to immune cells. A direct bidirectional connection between the cerebellum and the hypothalamus, termed cerebellar–hypothalamic projections and hypothalamic–cerebellar projections, has been presented (Dietrichs and Haines, 1984; Dietrichs et al., 1994; Cavdar et al., 2001a,b). The cerebellar-hypothalamic circuits play an important role in conveying and integrating the information on motor and visceral modulation (Dietrichs et al., 1994; Zhu et al., 2006; Zhu and Wang, 2008). The direct cerebellar–hypothalamic projections arise from all the three cerebellar nuclei, FN, IN and dentate nucleus (DN), and terminate to extensive regions of the hypothalamus, such as lateral, posterior and dorsal hypothalamic areas (LHA, PHA and DHA, respectively), as well as the dorsomedial and paraventricular nuclei (Cavdar et al., 2001a,b; Onat and Cavdar, 2003). The cerebellar–hypothalamic projections transmit non-somatic regulatory information from the cerebellum, such as respiratory, cardiovascular, gastrointestinal, and behavioral regulatory information (Gaytán et al., 2002; Wen et al., 2004; Zhu and Wang, 2008). On the basis of these reports, we

*Abbreviations:* ACTH, adrenocorticotropic hormone; BSA, bovine serum albumin; Con A, concanavalin A; DHA, dorsal hypothalamic areas; DN, dentate nucleus; ELISA, enzyme-linked immunosorbent assay; FN, fastigial nucleus; FR, fluoro-ruby; GABA, γ-aminobutyric acid; HPLC, high performance liquid chromatography; IFN, interferon; IL, interleukin; IN, interposed nucleus; KA, kainic acid; LHA, lateral hypothalamic areas; 3-MP, 3-mercaptopropionic acid; NE, norepinephrine; OD, optical density; PB, phosphate buffer; PBMCs, peripheral blood mononuclear cells; PBS, phosphate-balanced solution; PHA, posterior hypothalamic areas; SCP, superior cerebellar peduncle; T3, 3, 5, 3′-triiodothyronine; T4, 3, 5, 3′, 5′-tetraiodothyronine; TMB, 3, 3′, 5, 5′-tetramethylbenzidine; TSH, thyroid stimulating hormone.

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hypothesized that the direct cerebellar–hypothalamic projections may be a central pathway transmitting cerebellar immunoregulation.

Although electrophysiological studies suggest that the direct cerebellar–hypothalamic projections may be glutamatergic and γ-aminobutyric acid (GABA)ergic (Pu et al., 1995; Wang et al., 1997; Zhang et al., 2005), exact neurotransmitters used by the projections are less clear. Recently, by using anterograde and retrograde tracings of nerve tracts combined with glutamate and GABA fluorescent immunohistochemistry, we have revealed that the cerebellar FN has direct glutamatergic and GABAergic projections to the LHA and that these projections transmit regulation of immune function by the cerebellar FN (Cao et al., 2012, 2013). However, regarding the cerebellar IN, it is unclear whether GABAergic neurons mediate the immunoregulation via their direct projections to the hypothalamus.

In addition, peripheral pathways from the hypothalamus to immune cells in conveying the cerebellar immunomodulation are not known. In general, the hypothalamus, one of the most important centers for immunomodulation, has two peripheral routes to immune cells, the sympathetic nerves and adrenocortical/thyroid hormones (Klecha et al., 2006; Karanikas et al., 2009; Sarkar et al., 2011). Accordingly, we hypothesized that the hypothalamus is an intermediary position that plays a connecting link between the cerebellum and immune cells by direct cerebellar GABAergic–hypothalamic projections and hypothalamic–sympathetic/hormone axes. Clarifying this hypothesis is significant for extending our understanding of immune modulation by the central nervous system.

## 2. Materials and methods

### 2.1. Animals

Sprague–Dawley rats (Center of Experimental Animals, Nantong University, China) weighing 200–220 g were housed in a temperature- and light-controlled room (22 °C; 12/12 h light/dark cycle) with food and water provided ad libitum. Housing and experimental procedures were carried out in accordance with the policy guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996.

### 2.2. Injection of 3-mercaptopropionic acid (3-MP) or vigabatrin in bilateral cerebellar IN

Rats were randomly divided into the four groups, intact, saline, 3-MP (a glutamic acid decarboxylase antagonist that reduces GABA synthesis) and vigabatrin (a GABA-transaminase inhibitor that inhibits GABA degradation). The rats were anesthetized with pentobarbital (Sigma; 40 mg/kg, i.p.) and placed in a stereotaxic apparatus (David Kopf 902-A, USA). 3-MP (Sigma; 60 μg in 0.3 μl saline for each side) or vigabatrin (Invitrogen; 12 μg in 0.3 μl saline for each side) was injected into bilateral IN according to the following stereotaxic coordinates: 11.2 mm posterior to the bregma, 2.2 mm left/right to the midline, and 6.3 mm ventral to the bregma (Paxinos and Waston, 1998). Intact and saline animals that were injected with saline in bilateral IN were used as controls.

### 2.3. Immunization with bovine serum albumin (BSA)

BSA (Sigma) was dissolved in 0.9% sodium chloride and emulsified with an equal volume of Freund's complete adjuvant (Sigma) under sterile conditions. For measurement of cellular immune response, each rat was immediately immunized with the BSA emulsion of 0.5 ml containing 1 mg BSA via intraperitoneal injection after the rat was treated with 3-MP or vigabatrin in bilateral cerebellar IN. For measurement of humoral immune response, the rats were immunized with the BSA emulsion 2 days before they were treated with 3-MP or vigabatrin in bilateral cerebellar IN. All the measurements described below were performed on day 3 after 3-MP or vigabatrin treatment in bilateral cerebellar IN.

### 2.4. Flow cytometric assay for T and B lymphocyte percentages

Blood was taken from the right ventricle and isolated by Ficoll–Hypaque density gradient centrifugation (specific gravity: 1.085) to obtain peripheral blood mononuclear cells (PBMCs). After washed twice with 0.01 M phosphate-balanced solution (PBS, pH 7.3), the PBMCs were incubated with anti-rat CD3-PE antibody (eBioscience; 0.25 μg in 100 μl PBS) and anti-rat CD45RA-FITC antibody (AbD Serotec; 1 μg in 100 μl PBS) for 30 min at room temperature protecting from light. These cells were then determined by a FACSCalibur flow cytometer (BD Biosciences, USA) equipped with CellQuest software.

### 2.5. Proliferative response assay for T lymphocytes

Carboxyfluorescein diacetate succinimidyl ester (CFSE, Invitrogen) and CD3 double labeling was employed to measure concanavalin A (Con A)-induced T lymphocyte proliferation. Single lymphocytes from the mesenteric lymph nodes were obtained by passing the tissue through a 200 μm nylon mesh screen, washing twice with RPMI 1640 medium (Gibco, Langley, OK, USA), and lysing erythrocytes with sterilized distilled water. The lymphocytes ($10^7$/ml) were incubated with CFSE (2 μM) for 15 min at 37 °C in the dark. The labeling reaction was terminated by adding equal volume of calf serum for 1 min. The cells ($10^6$/ml) were incubated with 5 μg/ml Con A (Sigma) at 37 °C in 5% $CO_2$ for 72 h. After centrifugated, the lymphocytes were incubated with anti-rat CD3-PE antibody (0.25 μg in 100 μl PBS) for 30 min at room temperature protecting from light. Fluorescence intensity of CFSE-labeled cells was decreased by one half with each cell division, and thus proliferation (or division) rate of the $CD3^+$ cells (T lymphocytes) was analyzed on a flow cytometer.

### 2.6. Real-time PCR for mRNA expression of T cell cytokines

The isolated lymphocytes from the mesenteric lymph nodes were obtained as described above. The cells were incubated with 5 μg/ml Con A at 37 °C in 5% $CO_2$ for 48 h. Total RNA was isolated from the cells using total RNA isolation kit (Invitrogen), and reversely transcribed using murine myelomonocytic lymphoma virus reverse transcriptase (Promega, Madison, WI, USA). Thermocycler conditions comprised an initial step at 95 °C for 5 min, followed by 40 cycles of 94 °C for 15 s, 60 °C for 20 s, and 72 °C for 20 s. The primers for the T cell cytokines, interleukin (IL)-2, interferon (IFN)-γ, IL-4 and IL-10, are summarized in Table 1. Accumulation of PCR products was detected in real time and the results were analyzed with the instrument's software (Rotor-Gene software, version 6.0) and presented as $2^{-\Delta\Delta Ct}$, as described by the manufacturer (User Bulletin).

### 2.7. Enzyme-linked immunosorbent assay (ELISA) for levels of T cell cytokines

The isolated lymphocytes ($5 \times 10^6$/ml) from the mesenteric lymph nodes were incubated with 5 μg/ml Con A at 37 °C in 5% $CO_2$ for 72 h. According to the instruction booklets (eBioscience), the culture supernatant

**Table 1**
Primer sequences used for real-time PCR.

| Gene | Sense | Antisense |
|---|---|---|
| IL-2 | 5′-CCATGATGCTCACGTTTAAATTTT-3′ | 5′-CATTTTCCAGGCACTGAAGATG-3′ |
| IFN-γ | 5′-GCCCTCTCTGGCTGTTACTG-3′ | 5′-TACCGTCCTTTTGCCAGTTC-3′ |
| IL-4 | 5′-ACCTTGCTGTCACCCTGTTCT-3′ | 5′-CTCTCTCAGAGGGCTGTCGTTA-3′ |
| IL-10 | 5′-TGGCAACCCAAGTAACCCT-3′ | 5′-CACCCACTTCCCAGTCAGC-3′ |
| β-Actin | 5′-CGTTGACATCCGTAAAGACC-3′ | 5′-TAGAGCCACCAATCCACAC-3′ |