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Modulation of immune function by glutamatergic neurons in the cerebellar interposed nucleus via hypothalamic and sympathetic pathways

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ABSTRACT

Our recent work has shown that the cerebellar interposed nucleus (IN) contains glutamatergic neurons that send axons directly to the hypothalamus. In the present study, we aimed to demonstrate modulation of cellular and humoral immunity by glutamatergic neurons in the cerebellar IN by means of gene interventions of glutaminase (GLS), an enzyme for glutamate synthesis, and to reveal pathways transmitting the immunomodulation. Injection of GLS-shRNA lentiviral vector into bilateral cerebellar IN downregulated GLS expression in the IN. The silencing of GLS gene in the cerebellar IN decreased interleukin (IL)-2 and interferon (IFN)-γ production, B-cell number, and IgM antibody level in response to antigen bovine serum albumin (BSA). On the contrary, injection of GLS lentiviral vector into bilateral cerebellar IN upregulated GLS expression in the IN. The GLS gene overexpression in the IN caused opposite immune effects to the GLS gene knockdown. Simultaneously, the GLS gene silencing in the cerebellar IN reduced and the GLS overexpression elevated glutamate content in the hypothalamus, but they both did not affect glycine and GABA contents in the hypothalamus. In addition, the immune changes caused by the GLS gene interventions in the IN were accompanied by alteration in norepinephrine content in the spleen and mesenteric lymph nodes but not by changes in adrenocortical and thyroid hormone levels in serum. These findings indicate that glutamatergic neurons in the cerebellar IN regulate cellular and humoral immune responses and suggest that such immunoregulation may be conveyed by cerebellar IN-hypothalamic glutamatergic projections and sympathetic nerves that innervate lymphoid tissues.

1. Introduction

The immune system, as an important defense system, is widely regulated by nervous and endocrine systems (Bellavance and Rivest, 2012; Forsythe, 2012; Rivest, 2010; Webster et al., 2002). The cerebellum, the largest subcortical center for motor control, has been reported to regulate non-somatic activities, including cardiovascular, respiratory and gastrointestinal activities (Holmes et al., 2002; Ladabaum et al., 2001; Rosenberger et al., 2013; Tsubota et al., 2012; Xu and Frazier, 2002; Zhuang et al., 2008). A few reports propose a functional relationship between the cerebellum and immune system. For example, in "reeler" mice, a neurological mutant strain with an abnormally high concentration of cerebellar norepinephrine (NE), function of T lymphocytes and macrophages is suppressed (Green-Johnson et al., 1995); lesion of the vestibulocerebellum of rats causes an immunosuppressive effect (Ghoshal et al., 1998). In recent years, we have focused on the two cerebellar nuclei, fastigial nucleus (FN) and interposed nucleus (IN), to show immunomodulation by the cerebellum. We found that lesions of the cerebellar FN with kainic acid caused an enhancement of T, B and natural killer cells (Peng et al., 2005), while lesions of the cerebellar IN led to a suppression of these cells (Peng et al., 2006). Since the cerebellar nuclei are final output sites by which cerebellar regulatory information is exported, immune changes caused by damage to cerebellar nuclei represent a regulation of immune system by the cerebellum.

Although the cerebellar IN has been shown to have immune regulatory effect, what types of neurons in the IN contribute to the immunomodulation remains to be clarified. Recently, we have reported that microinjection of 6-diazo-5-oxo-L-norleucine (DON), an inhibitor of glutaminase (GLS) that is an enzyme for glutamate synthesis, in bilateral cerebellar IN results in suppression of T and B cells (Lu et al., 2012). These findings suggest that glutamatergic neurons in the cerebellar IN participate in regulation of immune

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function. However, this immune regulation by glutamatergic neurons in the cerebellar IN mainly represents a role in resting immune state. In most state, immune system depends on adaptive immune responses, which are executed by T and B lymphocytes, to eliminate foreign antigens and pathogens. To further demonstrate the regulation of adaptive immune responses by glutamatergic neurons in the cerebellar IN, in this study we assessed cellular and humoral immunity in response to antigen bovine serum albumin (BSA) after interventions of GLS gene in the cerebellar IN.

As the cerebellum has no direct structural connection with immune cells, the immunomodulation by the cerebellum needs to be further explained. We hypothesized that the hypothalamus is a relay station that connects the cerebellum and immune system. This hypothesis was presented on the basis of the findings that the hypothalamus is a crucial center of adjusting immune system (Cross et al., 1984; Rivest, 2010; Stein et al., 1981; Wrona and Trojniar, 2003, 2005; Wrona et al., 1994; Yang et al., 2011) and that a direct cerebellar–hypothalamic projection exists from the cerebellum to the hypothalamus (Cavdar et al., 2001; Dietrichs and Haines, 1984; Haines et al., 1985, 1990). Recently, by using anterograde and retrograde tracing of nerve tracts combined with glutamate fluorescent immunohistochemistry, we have not only demonstrated the direct neuronal projections from the cerebellar IN to the hypothalamus, but also shown that these projections use glutamate as a neurotransmitter (Lu et al., 2012). Impairment of the cerebellar IN-hypothalamic glutamatergic transmission by injection of DON in bilateral cerebellar IN attenuates immune function (Lu et al., 2012), similar to that of lesions of whole cerebellar IN (Peng et al., 2006). This suggests that cerebellar IN glutamatergic projections to the hypothalamus may mediate cerebellar IN immunomodulation. However, it still requires clarification whether the cerebellar IN-hypothalamic glutamatergic pathway is also involved in transmitting the modulation of specific immune responses to antigen challenge.

Immunoregulatory information from the central nervous system requires to be finally transmitted to immune cells, and thus this regulation can be achieved. Therefore, in addition to the central pathway, the peripheral pathways conveying the immunoregulatory information that has arrived in the hypothalamus via the direct cerebellar IN-hypothalamic glutamatergic projections need to be revealed to better understand the cerebellar immunoregulation. In general, the hypothalamus exerts its immune regulation by sympathetic nerves that directly innervate immune cells and by adrenocortical and thyroid hormones that directly act on immunocytes (Armstrong and Klein, 2001; Haddad et al., 2002; Klecha et al., 2006; Okamoto et al., 1996; Rivest, 2010). Accordingly, we presumed that sympathetic nerves and/or adrenocortical/thyroid hormones are potent candidates for transmission of the immunoregulatory information from the cerebellar IN-hypothalamic glutamatergic projections to immune cells via a relay of the hypothalamus. This presumption remains to be elucidated.

2. Materials and methods

2.1. Animals

Total 100 Sprague–Dawley rats (Center of Experimental Animals, Nantong University, China) weighing 200–220 g were used in this study. The animals were housed one per cage after surgery under conditions of constant temperature (22 °C), light from 6:00 A.M. to 6:00 P.M., and access to food and water ad libitum. Among these rats, 10 were used to identify the injection location of GLS gene interventions; 48 were employed in GLS gene silencing experiments, which were randomly divided into the three groups, intact, scrambled-shRNA (Scr-shRNA) and GLS-shRNA, with 16 for each group; and 42 were for the GLS gene overexpression experiments, which were also randomly divided into the three groups, intact, lentiviral vector and lentiviral vector expressing GLS, with 14 for each group. In the experiments, results from the rats that had not accurately been localized in bilateral cerebellar IN with the injections of GLS gene interventions were not included in statistical analysis.

2.2. Construction of lentiviral vectors expressing GLS-shRNA or GLS

The shRNA sequence targeting rat GLS (Gene Bank Accession NM_012569) was 5′-ACG AGA AAG TGG AGA CCG A-3′. Lentiviral vector expressing Scr-shRNA (5′-TTC TCC GAA CGT GTC ACG T-3′) or just lentiviral vector was used as a negative control for GLS-shRNA (GLS gene silencing) or GLS (GLS gene overexpression), respectively. The GLS-shRNA and GLS lentiviral vectors were generated by Genechem Co. Ltd. (Shanghai, China). The recombinant lentiviruses expressing GLS-shRNA or Scr-shRNA were prepared to a titer of 8×10^8 transducing units/ml, and the lentiviral vector expressing GLS or just lentiviral vector was 2×10^8 transducing units/ml.

2.3. Injections of GLS-shRNA lentiviral vector or GLS lentiviral vector in bilateral cerebellar IN

Following the administration of pentobarbital (55 mg/kg, i.p.), rats were placed in a stereotaxic apparatus (David Kopf 902-A, USA). A volume of 0.3 μl GLS-shRNA lentiviral vector or GLS lentiviral vector was bilaterally delivered into the cerebellar IN of rats using 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, based on the atlas of Paxinos and Waston (1998). The injection was made over 3 min and the needle was then left in the place for additional 5 min. The same volume of Scr-shRNA lentiviral vector or just lentiviral vector was injected into bilateral cerebellar IN as a negative control for GLS-shRNA lentiviral vector or GLS lentiviral vector, respectively. All immune parameters and other observations listed below were measured on the 7th day following the lentiviral vector injections.

To identify the injection locations of GLS gene interventions, we performed histological observation on cerebellar sections. Briefly, the rats that had been injected with GLS gene interventions in bilateral cerebellar IN were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 h under deep anesthesia. The cerebellum was removed and post-fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 4 h, and then dehydrated at 20% and 30% sucrose solution successively. Subsequently, the cerebellum was cut into 30 μm-thick coronal plane slices with a freezing microtome (Leica CM 1900, Germany). Both sides of cerebellar nuclei were observed under a fluorescence microscope (Leica DML, Germany) by green fluorescent protein carried by the lentiviral vectors. After each experiment, each rat was checked on both sides of the cerebellar IN about the injection places of GLS gene interventions by means of the injection needle passages. If the injections of the lentiviral vectors were not accurately placed in either side of the cerebellar IN, all the observed data were excluded in statistical analysis.

2.4. Immunization with bovine serum albumin (BSA)

On the first day after injections of GLS-shRNA or GLS lentiviral vectors in bilateral cerebellar IN, all the animals were immunized with BSA. Briefly, BSA at 4 mg/ml in saline was emulsified in an equal volume of Freund's complete adjuvant. A volume of 0.5 ml emulsion containing 1 mg BSA was intraperitoneally injected into each rat. On the sixth day following the immunization, the