

The hypothalamus mediates the effect of cerebellar fastigial nuclear glutamatergic neurons on humoral immunity

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Abstract

OBJECTIVES: We explored effect of glutamatergic neurons in the fastigial nucleus (FN), one of three cerebellar nuclei, on humoral immunity and revealed that this effect was mediated by the hypothalamus via FN-hypothalamic glutamatergic transmission.

METHODS: Rats were immunized with bovine serum albumin (BSA). On the third day after the immunization, 6-diazo-5-oxo-L-norleucine (DON), an inhibitor of glutaminase for glutamate synthesis, was microinjected in bilateral FN and D,L-threo-β-hydroxyaspartic acid (THA), an inhibitor of glutamate transporters on plasma membrane, was microinjected in both sides of lateral hypothalamic area (LHA). Glutamate content in the hypothalamus was examined by high-performance liquid chromatography (HPLC). Flow cytometry and enzyme-linked immunosorbent assay (ELISA) were used to measure B lymphocyte percentage in mononuclear cells of peripheral blood and levels of anti-BSA IgM and IgG antibodies in the serum, respectively.

RESULTS: DON injection in bilateral FN reduced B lymphocyte percentage and anti-BSA IgM and IgG levels, and simultaneously decreased glutamate content in the hypothalamus. Combined treatment with DON in the FN and with THA in the LHA elevated B cell number and anti-BSA IgM and IgG levels and increased hypothalamic glutamate content compared with DON treatment alone. However, combined treatment with DON in the FN and with THA in the ventrolateral thalamic nuclei (VL) did not significantly alter DON-dependent changes in B cell number and antibody levels, although the co-treatment altered DON-dependent glutamate content in the thalamus.

CONCLUSION: Cerebellar FN glutamatergic neurons participate in modulation of humoral immunity and this effect is mediated by the hypothalamus via FN-hypothalamic glutamatergic transmission.

INTRODUCTION

Modulation of the immune system by the central nervous system has been well recognized (Madden & Felten, 1995; Jiang *et al.* 1998; Dantzer, 2004; Wrona, 2006). However, the cerebellum, a largest subcortical center for motor control, has been not well clarified on its role in immunomodulation, although early several reports suggest a relationship between the cerebellum and immune system (Green-Johnson *et al.* 1995; Ghoshal *et al.* 1998). Recently, we have shown that the cerebellar fastigial nucleus (FN) and the interposed nucleus (IN), two of three cerebellar nuclei, regulate differentiation and function of T cells and cytotoxicity of natural killer (NK) cells (Peng *et al.* 2005, 2006; Ni *et al.* 2010). But more evidence for the effect of cerebellum on the immune system including humoral immunity needs to be provided to better understand cerebellar role in immunomodulation.

On the other hand, since there are no direct structural connections between the cerebellum and the immune system, pathway and mechanism mediating cerebellar immunoregulatory information predominantly require to be explored. Over several decades, the hypothalamus has been well investigated on its role in immunomodulation and considered as one of the most important immunoregulatory centers (Stein *et al.* 1981; Yang *et al.* 1997; Hori *et al.* 1998; Hirokawa *et al.* 2001; Baciu *et al.* 2003; Wrona, 2006). Importantly, between the cerebellum and the hypothalamus, direct bidirectional connections have been found. The connections include hypothalamocerebellar projections and cerebellohypothalamic projections, which constitute the cerebellar-hypothalamic circuits (Dietrichs *et al.* 1994; Haines *et al.* 1997; Cavdar *et al.* 2001a, b). Among those, the direct cerebellohypothalamic projections, arising from all three cerebellar nuclei and projecting to hypothalamic extensive areas and nuclei, have been reported to be neuroanatomical substrates underlying cerebellar modulation of nonsomatic activities, such as visceral activities (Zhu *et al.* 2006). Thus, the central status of the hypothalamus in immunomodulation and the important findings of direct cerebellohypothalamic projections provide a clue for our hypothesis that the cerebellum conveys its immunoregulatory information to the hypothalamus via the direct cerebellohypothalamic projections.

Recently, we have found that direct cerebellohypothalamic GABAergic and glutamatergic projections, originating from the cerebellar IN and mainly terminating in the lateral hypothalamic area (LHA), are involved in mediation of cerebellar immunomodulation (Wang *et al.* 2011; Lu *et al.* 2012). However, whether the hypothalamus also mediates cerebellar FN immunomodulation through cerebellar-hypothalamic

projections is not clear. In the cerebellar FN, there are glutamatergic and GABAergic neurons (Chen & Hillman, 1993; Sultan *et al.* 2002; Uusisaari *et al.* 2007; Uusisaari & Knöpfel, 2008; Chung *et al.* 2009). Electrophysiological studies have revealed that stimulation of the FN evokes a monosynaptic inhibitory or excitatory response of LHA neurons (Min *et al.* 1989; Katafuchi & Koizumi 1990; Zhang *et al.* 2003). These findings suggest that direct FN-LHA GABAergic and glutamatergic projections exist between the cerebellum and hypothalamus. In the present study, we focused on the FN-LHA glutamatergic projections that transmit FN immunoregulatory information to the hypothalamus. A humoral immune response to bovine serum albumin (BSA) and glutamate content in the hypothalamus were simultaneously measured after bilateral cerebellar FN was microinjected with 6-diazo-5-oxo-L-norleucine (DON), an inhibitor of glutaminase for glutamate synthesis (Conti & Minelli, 1994), or after combined treatment with DON in the FN and with D,L-threo- β -hydroxyaspartic acid (THA), an inhibitor of glutamate transporters on plasma membrane (Shimamoto *et al.* 1998), in the LHA.

MATERIALS AND METHODS

Animals

Adult Sprague-Dawley rats weighing 220–240 g of either sex (Center of Experimental Animals, Nantong University, China) were used in this study. The animals were housed one per cage on 12 h light-dark cycles and had free access to food and water. The research described in this report was conducted in compliance with the policy guidelines of the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996. In the present study, 49 rats were used to analyze simultaneously B lymphocyte percentage in peripheral mononuclear cells, anti-BSA IgM antibody, and glutamate content in the hypothalamus and thalamus. Additional 42 rats were used to analyze anti-BSA IgG antibody. The rats in all the experiments were randomly divided into seven different groups: intact group (without any treatment); FN saline group (saline was microinjected into bilateral FN); FN DON group (DON was microinjected into bilateral FN); LHA THA group (THA was microinjected into bilateral LHA); FN DON + LHA THA group (DON and THA were microinjected into bilateral FN and LHA, respectively); VL THA group (THA was microinjected into bilateral VL); FN DON + VL THA group (DON and THA were microinjected into bilateral FN and VL, respectively).

Immunization with BSA

Rats were immunized with BSA (Sigma). Briefly, 0.25 ml of 4 mg/ml BSA was emulsified with an equal volume of complete Freund's adjuvant (CFA, Sigma) and injected intraperitoneally per rat. On the third day

following BSA immunization, DON and THA were injected into the corresponding brain nuclei. 3 days later, blood was collected for measurement of B lymphocyte percentage in mononuclear cells of peripheral blood and anti-BSA IgM antibody. For examination of anti-BSA IgG antibody, on day 10 after BSA immunization, rats were immunized again with intraperitoneal injection of 0.25 ml of 4 mg/ml BSA emulsified with an equal volume of the incomplete Freund's adjuvant (IFA, Sigma). On day 13 DON and THA were strengthened and on day 16 the blood was taken for IgG measurement.

Orientation and microinjection of brain nuclei

On the third day following BSA immunization, the rats were anesthetized with pentobarbital (55 mg/kg, i.p.) and then fixed onto a stereotaxic apparatus (David Kopf 902-A, USA). The scalp was incised and a small hole was drilled in the cranium dorsal of the target site. The coordinates for the placement parameters of the FN, LHA and VL were obtained according to the rat brain atlas in stereotaxic coordinates (Paxinos & Watson, 1998) as follows: AP 11.5 mm, LR 1.1 mm, H 6.3 mm; AP 4.2 mm, LR 1.2 mm, H 8.1 mm; AP 2.8 mm, LR 1.5 mm, H 6.5 mm, respectively. The incisor bar positioned 3.3 mm below the center of the aural bars. A volume of 0.3 µl of 50 mM DON (Sigma) was delivered into each side of the FN through a microsyringe. Similarly, a volume of 0.4 µl of 10 mM THA (Sigma) was microinjected into each side of the LHA or VL. All the injections lasted for 4 min and the needle remained in the target locations for 5 min to prevent backflow. The rats were bred for 3 days conventionally.

Examination of B lymphocyte percentage in mononuclear cells of peripheral blood by immunofluorescence staining and flow cytometry

On day 3 after DON and THA microinjections, blood samples were collected into sterile heparinized tubes by endocardiac puncture. The mononuclear cells of peripheral blood were isolated by density gradient centrifugation on a Ficoll-Hypaque gradient. The interface mononuclear cells were collected and washed twice with 0.01 M phosphate-buffered saline (PBS) and then suspended in PBS at a concentration of 1×10^7 cells/ml. The mononuclear cells were incubated with the mouse anti-rat CD45RA-FITC antibody (0.5 µg in 100 µl PBS, clone OX-33, Invitrogen) at 25 °C in darkness for 15–20 min. After washing, the cells were resuspended in 500 µl of 0.01 M PBS per sample until analysis by flow cytometry. Analyses were performed with a FACSCalibur flow cytometer (BD Biosciences) by acquiring a minimal of 5,000 events from each sample. FACS data analysis was performed using CellQuest software (BD Biosciences).

Measurement of anti-BSA antibodies by enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) was employed to assess production of anti-BSA antibodies. Blood samples were collected from the right atrium and the sera were used to measure anti-BSA IgM and IgG antibodies. A 96-well microtiter plate (Costar, Corning Incorporated, USA) was coated with 125 µl of BSA (2 mg/ml) in 0.01 M PBS per well for 16 h at 4 °C. After washed three times with PBS-T, the plate was blocked by adding 125 µl of PBS-T with 10% nonfat milk per well for 1 h to avoid non-specific binding. The plate was washed and 125 µl of diluted serum sample (1:500) was added to each well. After incubation for 2 h at 37 °C, the plate was washed and 100 µl of freshly diluted (1:3,000) horseradish peroxidase-conjugated goat anti-rat Ig isotype-specific antibodies (Serotec, Oxford, UK) were added to each well, and incubated during 2 h at 37 °C. Then the plate was washed three times with PBS-T. 100 µl of 3,3',5,5'-tetramethylbenzidine (TMB) was added to each well and the plate was held at 37 °C for 10 min. The reaction was stopped with 1 N H₂SO₄ and absorbance was determined with a Universal Microplate Reader at 450 nm.

Determination of glutamate content in the hypothalamus or thalamus by high-performance liquid chromatography assay

When the peripheral blood was harvested for measurement of B cell number and anti-BSA IgM antibody level, the hypothalamus and thalamus in the rats were simultaneously dissected on ice. The tissues were respectively homogenized in 0.4 M perchloric acid with 0.1 mM EDTA (10 µl per 1 mg tissue) and centrifuged for 20 min (15,000 rpm) at 4 °C. The supernatants were stored at -80 °C until analysis. High-performance liquid chromatography (HPLC) supplemented with a fluorescent detection (Waters, USA) was used to determine glutamate content. Briefly, after derivatization with 6-aminoquinolyl-N-hydroxy-succinimidyl carbamate, 10 µl of each extract from the hypothalamus or thalamus was injected into HPLC equipment with an amino acid analysis column (AccQ TagTM, 3.9×150 mm, 4 µm beads, Waters), and mobile phase was pumped at a flow rate of 1 ml/min. A gradient elution was used to separate the mixture of amino acid. Mobile phase A consisted of sodium acetate buffer. Mobile phase B was acetonitrile. The amino acid was detected fluorometrically at an excitation wavelength of 250 nm and an emission wavelength of 395 nm.

Statistical analysis

All results were expressed as mean ± standard deviation. Statistical analysis was carried out with Statistics Package for Social Science (SPSS, 16.0). The data were submitted to the one-way analysis of variance (ANOVA) to compare the differences of all groups relative to each other. Following ANOVA, the Student-

Newman-Keul's test was used for pairwise comparisons of data. Differences were considered statistically significant at $p<0.05$.

RESULTS

DON injection in bilateral FN reduces B lymphocyte percentage and this effect is mediated by the LHA

Rats were immunized with BSA and 3 days later, DON was microinjected into bilateral FN and simultaneously, THA was microinjected into bilateral LHA. On day 3 after the injections, B lymphocyte percentage in peripheral mononuclear cells was measured by CD45RA labeling flow cytometry. DON treatment in the FN alone reduced B lymphocyte percentage but THA treatment in the LHA alone elevated B cell number relative to intact or saline-treated rats (Figure 1). Combined

treatment with DON in the FN and with THA in the LHA increased B cell percentage compared with DON injection alone but decreased B cell number relative to THA injection alone (Figure 1). Since THA treatment in the LHA alone also produced a change of B cell number, whether the DON-dependent change in B cell number was indeed mediated by the LHA was further statistically analyzed. The difference in B cell number between the THA treatment alone and the co-treatment with DON and THA was statistically smaller than that between intact and the DON treatment alone (Table 1), indicating that the decreased B cell effect by DON injection in the FN was weakened by THA treatment in the LHA. This suggests that DON-dependent change in B cell number is mediated by FN-LHA glutamatergic transmission.

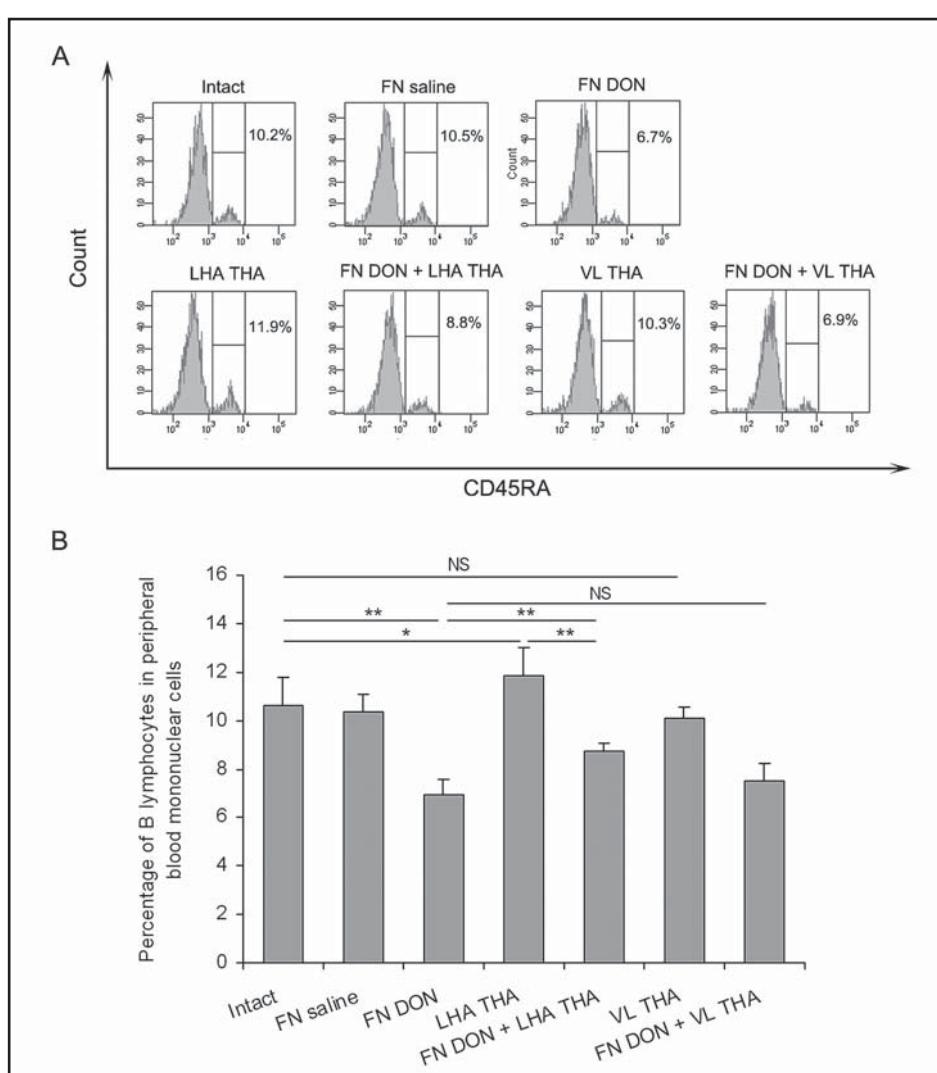


Fig. 1. Effect of DON injection in the FN on B lymphocyte percentage is mediated by the LHA. On the third day after treatment with DON in bilateral FN and with THA in bilateral LHA, blood samples were taken and B lymphocyte percentage in peripheral mononuclear cells was measured by flow cytometry with anti-CD45RA antibody labeling. The fluorescent images in (A) illustrate the percentage of CD45RA-positive lymphocytes (B lymphocytes) in mononuclear cells. The data in (B) are the mean and standard deviation of five independent experiments. * $p<0.05$, ** $p<0.01$, and NS no significance between the compared groups.

To further demonstrate that the DON-dependent effect is mediated specifically by LHA, we observed role of the VL, which is a major site receiving FN projections in the thalamus (Onat & Cavdar, 2003; Strick *et al.* 2009), in DON-dependent change. The THA injection in the VL alone did not significantly alter B cell percentage, and the co-injection with DON in the FN and with THA in the VL did not alter the DON-dependent decrease in B cell percentage either (Figure 1), showing that the FN DON-dependent effect was not mediated by the VL.

DON injection in bilateral FN lowers IgM and IgG levels and the effect is mediated by the LHA

Levels of anti-BSA IgM and IgG antibodies in the serum, which were measured by ELISA, were markedly lower in rats with DON-injected FN than those of control rats with intact or saline-treated FN (Figure 2). THA injection in bilateral LHA increased levels of anti-BSA IgM and IgG antibodies. Combined injection with DON in the FN and with THA in the LHA elevated IgM and IgG levels with respect to DON treatment alone but diminished the antibody levels relative to THA treatment alone (Figure 2). Further statistical analysis showed that the difference in IgM or IgG levels between the THA injection alone and the co-injection with DON and THA was statistically smaller than that between intact and the DON injection alone (Table 1), suggesting that the FN DON-dependent effect on IgM and IgG levels was mediated by the LHA.

Similarly, THA injection in the VL alone did not significantly alter levels of anti-BSA IgM or IgG antibodies in the serum, and the co-treatment with DON in the FN and with THA in the VL did not alter the DON-dependent reduction in IgM or IgG levels either

(Figure 2). This indicates that the VL is not involved in the FN DON-dependent effect on antibody response and on the other hand, it provides further evidence for the specific mediation of FN glutamatergic effect by the LHA.

DON injection in bilateral FN decreases glutamate content in the hypothalamus

Compared with intact or FN-saline rats, DON injection in bilateral FN reduced glutamate content in the hypothalamus (Figure 3), suggesting a glutamatergic transmission from the FN to the hypothalamus. As expected, THA injection in bilateral LHA increased glutamate content in the hypothalamus (Figure 3). Further, combined treatment with DON in the FN and with THA in the LHA elevated glutamate content in the

Tab. 1. Statistical analysis of the differences in B cell number and antibody levels between the different treatments.

Item	Difference between		
	Difference between intact and DON-injected FN	THA-injected LHA and DON-injected FN	DON plus THA-injected LHA
B lymphocyte percentage in peripheral mononuclear cells	3.68% ± 0.94%	3.12% ± 0.97%*	
Anti-BSA IgM antibody level in the serum	0.49 ± 0.08	0.37 ± 0.12*	
Anti-BSA IgG antibody level in the serum	0.88 ± 0.21	0.63 ± 0.26**	

The data are from figures 1 and 2. *p<0.05, **p<0.01, compared with the difference between intact and DON-injected FN.

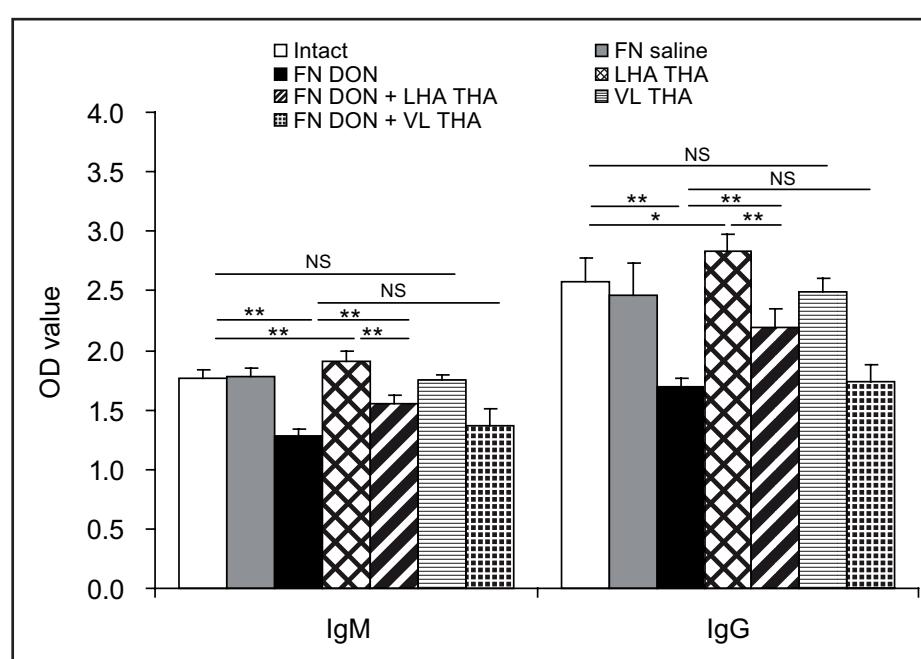


Fig. 2. Effect of DON injection in the FN on production of anti-BSA antibodies is mediated by the LHA. BSA-specific IgM and IgG antibodies in the serum were measured by ELISA. Each bar indicates the mean and standard deviation of six replications. *p<0.05, **p<0.01, and NS no significance between the compared groups.

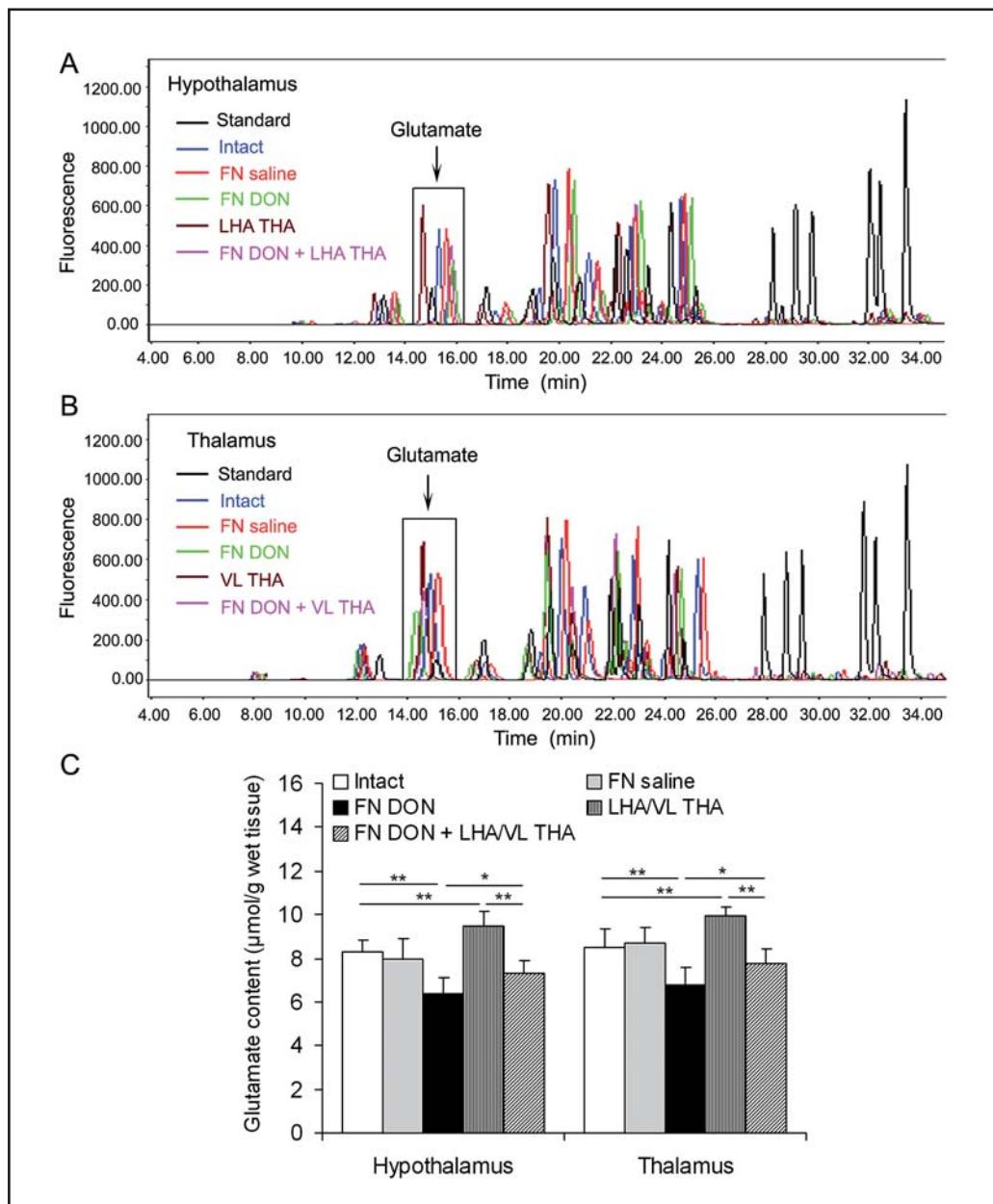


Fig. 3. Changes of glutamate content in the hypothalamus or thalamus following various treatments. HPLC assay was used to measure glutamate content in the hypothalamus or thalamus on the third day following the treatment with DON in the FN or the combined treatment with DON in the FN and with THA in the LHA/VL. Glutamate was eluted at the second peak according to standard amino acid sample. (**A**) and (**B**) display glutamate peaks in the hypothalamus and thalamus, respectively. (**C**) is the mean and standard deviation from seven replications as exhibited in (**A**) and (**B**). * $p<0.05$ and ** $p<0.01$.

hypothalamus with respect to DON treatment alone but reduced glutamate content relative to THA injection alone (Figure 3). This demonstrated the glutamatergic transmission from the FN to the LHA and confirmed that the DON injection in bilateral FN impaired the transmission.

As a positive control, glutamate level in the thalamus was also measured. DON injection in the FN also reduced glutamate level in the thalamus compared with intact or FN-saline rats (Figure 3). It suggests that there

is also a glutamatergic transmission from the FN to the thalamus. THA injection in the VL increased glutamate content in the thalamus. Thalamic glutamate content of rats with FN DON injection plus VL THA injection was obviously higher than that with DON treatment alone but lower than that with THA treatment alone (Figure 3). These data testify the glutamatergic transmission from the FN to the VL and demonstrate that the DON treatment in bilateral FN also weakened the transmission.

DISCUSSION

The cerebellum has been reported to be related to the immune system (Green-Johnson *et al.* 1995; Ghoshal *et al.* 1998). Since ultimate output of all cerebellar regulatory information is via the three cerebellar nuclei, FN, IN, and dentate nucleus, we have focused on these cerebellar nuclei to investigate their modulation of the immune system. Lesions of bilateral cerebellar FN with kainic acid enhance lymphocyte differentiation, maturity, and function (Peng *et al.* 2005; Ni *et al.* 2010). But lesions of bilateral cerebellar IN attenuate lymphocyte function (Peng *et al.* 2006). These results strongly show that the cerebellum participates in adjustment of the immune system. As there is no a direct connection in structure between the cerebellum and the immune system, the immunomodulating information from the cerebellum needs to be conveyed by some structures. In our recent studies, we have found that there are direct cerebellohypothalamic glutamatergic and GABAergic projections from the cerebellar IN to the LHA and that by these projections cerebellar immunomodulating information is transmitted to the hypothalamus (Wang *et al.* 2011; Lu *et al.* 2012). Therefore, the hypothesis that the hypothalamus also conveys FN glutamatergic immunomodulating information by cerebellohypothalamic projections was presented and explored in the present study.

In the brain, glutamate is synthesized in the cytosol of glutamatergic axonal terminals by glutaminase. In general, glutaminase is synthesized in neuronal somas and then transported to axonal terminals, where the enzyme hydrolyzes glutamine into glutamate (Behar & Rothman, 2001). In response of glutamatergic neurons to stimulation, the neurons release glutamate out of the axonal terminals and after it acts on postsynaptic neurons, the glutamate is transported into glial cells or neurons by glutamate transporters on plasma membrane to cease its action (Kidd & Isaac, 2000; Behar & Rothman, 2001; Öngür *et al.* 2011). In the cerebellar FN, there are glutamatergic and GABAergic neurons (Chen & Hillman, 1993; Sultan *et al.* 2002; Uusisaari *et al.* 2007; Uusisaari & Knöpfel, 2008; Chung *et al.* 2009). Stimulation of the FN evokes a monosynaptic inhibitory or excitatory response of LHA neurons, suggesting a direct GABAergic or glutamatergic projection from the FN to the LHA (Min *et al.* 1989; Katafuchi & Koizumi 1990; Zhang *et al.* 2003). These reported results represent that neuronal somas in the cerebellar FN synthesize glutaminase and then the enzyme is transported to axonal terminals located in the LHA to synthesize glutamate. DON is an inhibitor of glutaminase (Conti & Minelli, 1994), and local injection of DON has a remote effect by its transportation in neurons (Kaneko *et al.* 1992). Thus, the DON injection in bilateral cerebellar FN in this study can inhibit activity of glutaminase both in the FN and in the LHA. As expected, the infusion of bilateral cerebellar FN

with DON decreased glutamate content in the hypothalamus, demonstrating a glutamatergic connection from the FN to the hypothalamus and suggesting that the DON injection in bilateral FN impaired FN-hypothalamic glutamatergic transmission. To identify this view, we injected DON in the FN and simultaneously infused THA, an inhibitor of glutamate transporters on plasma membrane (Hirata *et al.* 1997; Shimamoto *et al.* 1998), in bilateral LHA. The combined treatment weakened both effects of DON-dependent glutamate reduction and THA-dependent glutamate elevation in the hypothalamus. These results fully show a glutamatergic neuronal connection originating from the FN and terminating to the LHA.

DON injection in bilateral FN reduced B lymphocyte percentage in mononuclear cells of peripheral blood and anti-BSA IgM and IgG levels in the serum, indicating that glutamatergic neurons in the FN are involved in modulation of humoral immunity. Since the DON treatment diminished glutamate level in the hypothalamus, the impaired humoral immunity by DON is related to weakened FN-hypothalamic glutamatergic transmission. This point is supported by further data that the co-treatment with DON in the FN and with THA in the LHA remarkably altered the DON-dependent effect on humoral immunity.

The thalamus also receives a direct projection deriving from the cerebellar FN. This projection mainly terminates in the VL and regulates motor and nonmotor behavior (Onat & Cavdar, 2003; Strick *et al.* 2009). Our present results revealed that DON injection in bilateral FN reduced glutamate content in the thalamus and co-injection with DON in the FN and with THA in the VL significantly altered the DON-dependent reduction in thalamic glutamate level. This supports these reports on a glutamatergic projection from the cerebellar FN to the thalamus. However, the change in the DON-dependent reduction of thalamic glutamate level by the combined treatment did not significantly alter the DON-dependent attenuation of B cell number and antibody production. These findings demonstrate that the VL does not mediate the effect of glutamatergic neurons in the cerebellar FN on humoral immunity and therefore, at the thalamic profile we clarify that immunomodulation by cerebellar FN glutamatergic neurons is specifically conveyed by the hypothalamus.

Taken together, our current findings show that cerebellar FN glutamatergic neurons regulate humoral immunity and suggest that this effect is mediated by the hypothalamus via FN-hypothalamic glutamatergic transmission.

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