Acute Electrical Stimulation of Nucleus Ambiguus Enhances Immune Function in Rats

Ying-Wu Mei, Zhan-Qing Yang, Wei Wang, De-Guang Song, Xu-Ming Deng, Ju-Xiong Liu

ABSTRACT: Background: Up to now, many “immunoactive” brain areas have been identified, such as hypothalamic nuclei, brain reward system; but the nucleus ambiguous (Amb), a nucleus nervi vagis of medulla oblongata, was less well studied in neuroimmunomodulation. Methods: In order to obtain more profound comprehension and more knowledge on Amb, we studied the effect of acute electrical stimulation of Amb on thymus and spleen activity in rat. A stimulator was applied to stimulate the Amb of the anaesthetized rats using the parameter at 100μA×5ms ×100 Hz every 1s for 1 min. The levels of TGF-β and thymosin-β4 mRNA in thymus, the release of IL-2 and IL-6 at splenocyte in vitro and splenic lymphocyte proliferation were measured at hour 0, 0.5, 1, 2, 3 following the electrical stimulation. Results: The results showed that concanavalin A (Con A)-induced splenic lymphocyte proliferation and the release of IL-2 and IL-6 were all significantly enhanced at 0.5, 1, and 2 h following effective Amb stimulation as compared to in the control group. However, as compared to in the control group, the levels of TGF-β and thymosin-β4 mRNA in the thymus were both remarkably reduced at 0.5, 1, and 2 h following effective Amb stimulation. Conclusions: These findings reveal that the Amb participates in the modulation of animal immune functions.

RÉSUMÉ: La stimulation électrique aiguë du noyau ambigu stimule la fonction immunitaire chez le rat. Contexte : Plusieurs zones immunoactives du cerveau ont été identifiées jusqu’à maintenant, dont les noyaux hypothalamiques (corps de Luys, noyau sous-thalamique), le système de récompense du cerveau. Cependant, il existe peu d’études sur le rôle du noyau ambigu (Amb), le noyau du nerf vague situé dans le bulbe rachidien, dans la neuro-immunomodulation. Méthodes : Nous avons étudié l’effet de la stimulation électrique aiguë de l’Amb sur l’activité du thymus et de la rate chez le rat afin de mieux connaître et de comprendre le rôle de l’Amb. Un stimulateur a été installé chez des rats anesthésiés pour stimuler l’Amb. Le paramètre utilisé était de 100μA x 5 ms x 100 Hz toutes les 1 s pendant 1 min. Les niveaux de TGF-β et d’ARNm de la thymosine β4 dans le thymus, la libération d’IL-2 et d’IL-6 par des splénocytes in vitro et la prolifération lymphocytaire splénique ont été mesurés aux temps 0,5 h, 1 h, 2 h et 3 h après la stimulation électrique. Résultats : La prolifération lymphocytaire splénique induite par la concanavaline A (Con A) et la libération d’IL-2 et d’IL-6 étaient significativement augmentées aux temps 0,5 h, 1 h et 2 h après une stimulation efficace de l’Amb par rapport au groupe témoins. Cependant, les niveaux de TGF-β et d’ARNm de la thymosine β dans le thymus étaient diminués de façon importante par rapport au groupe témoin aux temps 0,5 h, 1 h et 2 h. Conclusion : Selon ces observations, l’Amb participe à la modulation des fonctions immunitaires chez l’animal.

organ. Therefore, we speculated that the Amb may have immunoregulatory functions. The present study aimed to investigate this aspect.

The present study was designed to compare the changes in the levels of TGF-β and thymosin-β4 mRNA in the thymus, the release of IL-2 and IL-6 in the spleen, and splenic lymphocyte proliferation following acute electrical stimulation of the Amb of anesthetized rats. TGF-β, thymosin-β4, IL-2, IL-6, and splenic lymphocyte proliferation were determined concomitantly in the thymus and spleen of the same individuals.

MATERIALS AND METHODS

2.1. Animals

The experiment was conducted on 42 female Wistar rats (Center of Experimental Animals, Jilin University, China), weighing 240–260 g were used in this study. Before the experiment, the animals were housed in individual cages under conditions of constant temperature (20°C) and a 12 hour (h)-light/12 h-dark illumination cycle; they had access to food and water ad libitum. Housing and experimental procedures were in accordance with the policy guidelines of the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23), as revised in 1996.

2.2. Surgery and electrical stimulation

The rats were randomly divided into two groups: the electrical stimulation group (36 rats) and the sham control group (six rats); and the rats of electrical stimulation group were randomly divided into four groups again (L0.5h=10, L1h=9, L2h=10, L3h=7). Except for the electrical stimulation, the sham control group received the same treatment as the electrical stimulation group; In addition, non-operated rats were acted as normal control. Following the administration of Nembutal (30 mg/kg, i.p.), the rats were placed in a stereotaxic apparatus (David Kopf 902-A, USA). A stimulating electrode (monopolar stainless steel electrodes, 0.2 mm in diameter and insulated along the entire length except for the square-cut tip) was aimed at the Amb. The Paxinos and Watson10 stereotaxic coordinates were as follows: 12.10–12.84 mm posterior to the bregma, 1.98–2.20 mm lateral to the midline, and 9.22–9.68 mm below the skull surface. Using a Nihon Kohden SEN-1703 stimulator, the rats were stimulated at 100 μA × 5 ms × 100 Hz every 1 second (s) for 1 minute.

Immunological features were assessed 0.5, 1, 2, and 3 h after the experiment in both the electrical stimulation and control groups.

Figure 1: Localization of the tips of the stimulation electrodes, A~D is 30um thick section and strained with hematoxylin and eosin. E schematically shows the position of the electrode tip.
2.3 A 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay for the measurement of Con A-induced proliferation of splenic lymphocytes

A quantitative MTT colorimetric assay was performed as described by Mosmann. Briefly, the rats of the electrical stimulation and control groups were killed with an overdose of pentobarbital (100 mg/kg, i.p.) following electrical stimulation at 0.5, 1, 2 and 3 h, and their spleens were harvested by celiotomy. Splenic lymphocytes were obtained by gently squeezing the spleen, and they were then washed three times with RPMI 1640 culture medium (Gibco). The erythrocytes were lysed using sterilized distilled water. The lymphocytes were then resuspended in complete culture medium that comprised RPMI 1640 medium, 10% heat-inactivated fetal calf serum, 2.5 × 10–2 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Sigma), 1 × 10–3 M sodium pyruvate, 5 × 10–5 M b-mercaptoethanol, and antibiotics (100 U/mL penicillin, 100 U/mL streptomycin) at a concentration of 10 × 10^6 cells/mL. Cell suspension (50 µL) and 50 µL Con A (10 µg/mL) were added in triplicate to 96-well flat-bottom sterile plates. The plates were incubated in an incubator (ESPEC BNA-311, Japan) at 37°C in 5% carbon dioxide (CO₂) for 44 h. After incubation, 20 µL of 5 mg/mL MTT solution was added to each well, and the cells were incubated for an additional 4 h. Thereafter, 100 µL dimethyl sulfoxide (DMSO) was added to each well, and the optical density (OD) was read on a Universal Microplate Reader (Elx 800, Bio-TEK Instruments, Inc., USA) by using a test wavelength of 490 nm after 10 min. Proliferation is expressed as the stimulation index (percentage of the unstimulated control), which is the OD of the treated wells/the OD of the unstimulated control wells (cells that received no mitogen).

2.4 Measurement of IL-2 and IL-6 production in splenic lymphocytes in vitro

To assess cytokine production, the splenocytes (10 × 10^6 cells/mL) were incubated for 48 h (37°C, 5% CO₂) in 48-well tissue culture plates (0.5 mL/well) in the presence of Con A at a concentration of 5 µg/mL. After incubation, the supernatants were collected, centrifuged, and stored at –20°C until analysis. Rat enzyme-linked immunosorbent assay (ELISA) mini kits (Boster, WuHan, China) were used for this assay.
2.5 Real-time polymerase chain reaction (PCR) analysis of thymosin-β4 and TGF-β mRNA in the thymus

A realtime PCR assay was performed as described. Rats in the electrical stimulation and control groups were sacrificed at 0.5, 1, 2, and 3 h after receiving the electrical stimulation. Each thymus was rapidly removed and frozen in liquid nitrogen. Total RNA was extracted from the dissected tissue of 30 mg using TRIzol reagent (Gibco BRL, Grand Island, NY) according to the manufacturer’s instructions. The integrity and quality of the isolated RNA was examined by quantitating the A260/280 ratio (BioPhotometer, Eppendorf, Hamburg, Germany) and resolving the RNA on a 1% agarose gel. The cDNA was prepared from 2.0 µg RNA (OD=1.9~2.0) in the presence of 2.5 µM oligo (dT) primer and 200 U Moloney murine leukemia virus reverse transcriptase (M-MLV; Promega, Madison, WI) in a total volume of 20 µL. The reaction mixture was incubated for 1 h at 42°C and stopped by heating at 90°C for 5 min. The real-time PCR primers corresponding to rat TGF-β, thymosin-β4, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are listed in the Table. Specific controls were constructed for TGF-β, thymosin-β4 and GAPDH by cloning products of PCR reactions. The PCR products were cloned into a pMD18-T vector (TaKaRa). Twenty-five milliliter LB-broth cultures containing single colonies were grown up overnight, shaking at 200 rpm at 37°C and plasmids were subsequently purified using the MiniBEST Plasmid
Purification Kit Ver. 2.0 (TaKaRa). Plasmid DNAs were solubilised in 150 µl TE buffer. DNA sequencing confirmed the sequences of the cloned products. Purified plasmid clones were quantified using a BioPhotometer. (Eppendorf, Hamburg, Germany)

Real-time PCR runs were performed in 96-well optical plates in triplicate (each containing 1×SYBR real-time PCR master mix (ToYoBo), 0.2 µl of each primer (10 mM) and 2 µl template DNA (either cDNA diluted 1:2 and plasmid DNA dilutions ranging from 1: 2 to 1:128 in a final volume of 25 µl). After pre-incubation at 95°C for 60 s, PCR was performed as follows: 40 cycles of denaturation at 95°C for 15 s, annealing at 54°C (TGF-β, thymosin-β4, and GAPDH), and elongation at 72°C for 45s; dissociation data was automatically captured at the end of the PCR run. Detection was performed using an ABI PRISM 7000 Sequence Detector.

Three standard curves were drawn by plotting the threshold cycle (CT) against the log of the concentration of the plasmids (Figure 4). The CT was defined as the cycle at which a statistically significant increase in the magnitude of the signal generated by the PCR reaction was first detected. The CT was calculated under default settings for the real-time sequence detection software (Applied Biosystems). The equation drawn from the graph was used to calculate the precise concentration of specific cDNA present per microgram of total oligo-dT primed cDNA, tested in the same reaction plate as the standard.

In order to eliminate the error brought by manipulation process, final values were calculated with the specific RNA levels relative to GAPDH RNA.

### 2.6 Histological verification

At 0.5, 1, 2, 3 hours following the electrical stimulation, the animals were sacrificed, and the localization of the stimulating electrodes was determined. The electrode’s tips were marked according to the following procedure. A direct anodal current of 100 µA was passed for 20 s through the electrode of the animals that were previously anesthetized with Nembutal. Subsequently, the brains were removed from the skulls and fixed in 10% formalin solution. After fixing, the brains were frozen in liquid nitrogen, and sections of 30-nm thickness were cut and stained with hematoxylin and eosin to determine the electrode placements.

### 2.7 Statistical analysis

The data were expressed as mean standard deviation. Statistical analysis was carried out with SPSS 11.5 software. The data were analyzed either by the Student’s t-test or two-way analysis of variance. The Student-Newman-Keul’s test was also used to compare the data between the groups. Differences were considered statistically significant at P < 0.05.

### RESULTS

#### 3.1 Observations of the electrode placements

After the experiments were complete, correct placement of the tip of the electrode was verified microscopically in the brain sections stained with hematoxylin and eosin. Figure 1 schematically shows the position of the electrode tip for a stimulatedAmb.

#### 3.2 Results of the measurement of Con A-induced proliferation of splenic lymphocytes

After the Amb was electrically stimulated, the lymphocytes were prepared from the spleen, and their proliferative response to a T-cell mitogen and Con A was examined. As shown in Figure 2, the MTT assay was performed for the electrical stimulation and control groups at 0.5, 1, 2, and 3 h after the Amb stimulation of the electrical control group; the MTT assay was performed to evaluate lymphocyte proliferation. In the rats of the electrical stimulation group, the stimulation index (SI) values were all significantly increased at 0.5, 1, and 2 h after the electrical stimulation of the Amb as compared to in the corresponding control rats that received no stimulation. Subsequently, the SI values began to decrease gradually. These results indicate that the acute electrical stimulation of the Amb enhanced the effect of post-stimulation time on the proliferation of splenic T lymphocytes.

#### 3.3 Alteration in the production of the cytokines IL-2 and IL-6 in vitro

Splenocytes from both groups were stimulated with Con A, and the production of IL-2 and IL-6 were determined by rat ELISA mini kits. As shown in Figure 3, as compared to the control group, the production of IL-2 and IL-6 in the electrical stimulation group gradually increased. IL-2 production peaked 1 h after stimulation, and IL-6 production peaked at 2 h.

<table>
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<th>Gene</th>
<th>Sense (5’-3’)</th>
<th>Anti sense (5’-3’)</th>
<th>Size (b p)</th>
<th>Gene bank accession no.</th>
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<td>GAPDH</td>
<td>CTGATGCCTCCATGTTTGTG</td>
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<td>thymosin-β4</td>
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<td>AAGGCAATGCTCGGAAT</td>
<td>135</td>
<td>BC058137</td>
</tr>
</tbody>
</table>

Table: Primers for the real time RT-PCR analysis
Subsequently, the values began decreasing. These results demonstrated that acute electrical stimulation of the Amb may increase the amounts of IL-2 and IL-6 released in splenic lymphocytes in vitro.

### 3.4 Alterations in the levels of thymosin-β4 and TGF-β mRNA in the thymus

Real time reverse transcriptase (RT)-PCR analysis demonstrated that the expression of thymosin-β4 and TGF-β mRNA decreased after electrical stimulation of the Amb, as shown in Figure 5. The highest expressions were observed in the control group. Irrespective of thymosin-β4 or TGF-β mRNA, their expression rapidly decreased and persisted until at least 2 h after electrical stimulus. Subsequently, at 3 h after the electrical stimulus, the expressions began to recover.

**DISCUSSION**

Studies utilizing CNS lesions or stimulation have suggested that many specific regions of the brain, particularly regions of the hypothalamus and limbic system, may modulate immune activity. However, there are limited reports regarding the effect of Amb-involved immunoregulation thus far. This study evaluates the effects of acute electrical stimulation of the Amb on the expression of TGF-β and thymosin-β4 mRNA in the thymus, the release of IL-2 and IL-6 in the spleen, and splenic lymphocyte proliferation in anesthetized rats.

In this study, we found that acute electrical stimulation of the Amb caused a dramatic decrease in the levels of thymosin-β4 and TGF-β mRNA in the thymus and an increase in Con A-induced lymphocyte proliferation and the production of IL-2 and IL-6. Thymosin-β4 was first isolated from calf thymus fraction 5; it is also found in various organs such as the spleen, thymus, liver that exhibit biological activity on the immune system cells. Thymosin-β4 has been reported to suppress the mixed lymphocyte proliferative response, induce the expression of terminal deoxynucleotidyl transferase in T cells, inhibit the migration of macrophages in vitro, and inhibit cytokine production by neutrophils after tumor necrosis factor (TNF)-α stimulation. TGF-β, which can be released by thymocyte and thymic epithelial cells, almost inhibits all lymphocyte proliferation, including mitogen- and allogenic antigen-induced T-cell proliferation and *Staphylococcus aureus* Cowan I (SAC)-induced and IL-2 independent B-cell proliferation. Further, TGF-β can inhibit the functions of cytotoxic T lymphocyte (CTL), natural killer, and lymphokine-activated killer cells and macrophage activity. Further, the immune enhancement activity of IL-2 and IL-6 is now well accepted. Thus, the synchronous decrease in the levels of thymosin-β4 and TGF-β mRNA along with the synchronous enhancement of Con A-induced lymphocyte proliferation and the production of IL-2 and IL-6 indicates that acute electrical stimulation of the Amb can lower the secretion of some immunosuppressive factors and enhance that of others. This may promote immune activity in rats.

The nucleus ambiguus that belongs to a nucleus of the vagus nerve of the medulla oblongata and is an important visceral and somatic motor nucleus that establishes extensive connections with the CNS and peripheral organs. The vagus nerve will be activated by the acute electrical stimulation of the Amb. However, the vagal innervation of the thymus has been debated, the splenic nerve comprises approximately 98% of sympathetic nerve fibers and there is no direct experimental evidence that supports modulation of thymus and spleen activitives by the vagus nerve. Therefore, we can speculate that there must be other factors underlying this phenomenon. The most convincing is that the hypothalamus plays an important part in this modulation because of its direct or indirect connections with the Amb. In addition, retrograde tract-tracing techniques combined with immuno-histochemistry were used to find that hypothalamic hcr1-1-containing neurons send collateral axonal projections to cardiovascular sites in the Amb in the rat. In fact, the effect of neuroimmunomodulation on every region of the hypothalamus has been studied in detail. Destruction of the hypothalamic arcuate nucleus with the neurotoxin monosodium glutamate results in depressed NKCC and proliferation of the large granular lymphocytes (LGL) and in the disappearance of its age-dependent pattern. Hypophysectomy or adrenalectomy could reverse the effects of the preoptic/anterior hypothalamic lesions, suggesting that the effects of these lesions on the immune system were mediated via the pituitary hormones or peptides or other neuroendocrine routes. Mechanical lesion or isolation of the rat hypothalamic paraventricular nucleus (PVN) can selectively reduce the circulating white blood cells and the primary immune response, while it enhances cell-mediated immune function. The authors believe that PVN enhanced cell-mediated immunity functions by altering both the peripheral sympathetic tone and thyroid hormone secretion, and they suggested that the PVN represented an integral part of the neuroendocrine circuit that modulates the immune function of an organism. The lateral and the ventral tegmental area are termed as the “brain reward system.” These brain structures are related to positive reinforcement (reward) and have beneficial effects on the immune response, including the antiviral and antitumor cytotoxic activitives of lymphocytes. Additionally, the hypothalamus could also control the release of humoral immunostimulatory agents such as the adrenocorticotropic hormone, endogenous opioids (particularly b-endorphin), growth hormone, and prolactin, as well as some proinflammatory cytokines. Growth hormone and prolactin are known to stimulate immune responses.

In summary, electrical stimulation of the Amb can decrease the secretion of some immunsuppressive factors in the thymus and enhance splenic lymphocyte activity. These findings strongly indicate that the Amb participates in the modulation of animal immune functions.

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