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Research Paper

Changes of amino acid concentrations in the rat medial vestibular nucleus following unilateral labyrinthectomy

YU Hai-Ling[1], AN Ying[2], JIANG Hai-Ying[2], JIN Qing-Hua[2], JIN Yuan-Zhe[2,*]

[1]Department of Pharmacology; [2]Department of Physiology, Yanbian University College of Medicine, Key Laboratory of Organism Functional Factors of the Changbai Mountain, Ministry of Education, Yanji 133000, China

Abstract: To understand the neurochemical mechanisms underlying the vestibular compensation, we determined the levels of amino acids such as aspartate, glutamate, glutamine, glycine, taurine, alanine in the medial vestibular nucleus (MVN) following unilateral labyrinthectomy (UL), by using *in vivo* brain microdialysis and high-performance liquid chromatography technique. Rats were pre-treated by infusing 2% lidocaine 1.2 mL or 10 mg arsanilic acid into the tympanic cavity to obstruct uni-periphery vestibular organ, and then the levels of amino acids were determined in MVN of normal control and ipsilateral or contralateral lesional (ipsi-/contra-lesional) rats. In the control experiment, the levels of aspartate, glutamate, glutamine, glycine, taurine, and alanine were (6.15±0.59), (18.13±1.21), (33.73±1.67), (9.26±0.65), (9.56±0.77) and (10.07±0.83) pmol/8 μL sample, respectively. The concentrations of aspartate and glutamate decreased, while the concentration of taurine increased in the ipsi-lesional MVN of rats 10 min after infusing 2% lidocaine into middle ear to obstruct uni-periphery vestibular organ. Whereas the concentration of glutamate increased, the concentrations of glycine and alanine decreased in the contra-lesional MVN, accompanied by imbalances of glutamate, glycine and alanine in the bilateral nuclei. In contrast, the levels of glutamate and alanine decreased, the level of glutamine increased in the ipsi-lesional MVN, and the level of glutamate decreased in the contra-lesional MVN of rats 2 weeks after infusing 10 mg arsanilic acid into the tympanic cavity to obstruct uni-periphery vestibular organ. Furthermore, the level of glutamine in the ipsi-lesional MVN was obviously higher than that in the contra-lesional MVN. These results demonstrate that an imbalance of different amino acids appeared in bilateral MVN after UL, and this imbalance decreased after the development of vestibular compensation. Whereas the imbalance of glutamine release in bilateral nuclei appeared after vestibular compensation.

Key words: amino acids; medial vestibular nucleus; unilateral labyrinthectomy; vestibular compensation

单侧迷路破坏后大鼠前庭神经内侧核区氨基酸含量的变化

于海玲[1]，安 英[2]，姜海英[2]，金清华[2]，金元哲[2,*]

延边大学医学院 [1]药理学教研室；[2]生理学教研室，长白山生物功能因子省部共建教育部重点实验室，延吉 133000

摘 要：本实验用脑部微量透析法和高效液相色谱法观察单侧迷路破坏(unilateral labyrinthectomy，经利多卡因或对氨基苯胂酸盐预处理以阻断单侧外周前庭器官)后大鼠同侧及对侧前庭神经内侧核(medial vestibular nucleus, MVN)区部分氨基酸(天冬氨酸、谷氨酸、谷氨酰胺、甘氨酸、牛磺酸和丙氨酸)含量的变化，以了解前庭代偿的部分神经化学机制。实验观察到，对照组大鼠 MVN 区天冬氨酸、谷氨酸、谷氨酰胺、甘氨酸、牛磺酸和丙氨酸浓度分别为(6.15±0.59)，(18.13±1.21)，(33.73±1.67)，(9.26±0.65)，(9.56±0.77)和(10.07±0.83) pmol/8 μL 透析样本。左侧中耳内灌注 2% 利多卡因后 10 min，同侧 MVN 区天冬氨酸、谷氨酸含量立即减少(P<0.05)，牛磺酸含量增加(P<0.05)；对侧 MVN 区谷氨酸含量立即增加(P<0.05)，甘氨酸和丙氨酸含量减少；双侧核团间谷氨酸、甘氨酸和丙氨酸含量失衡。而用对氨基苯胂酸盐永久阻断单侧前庭器官 2 周后，同侧 MVN 区谷氨酸和丙氨酸含量减少，谷氨酰胺含量增高；对侧 MVN 区谷氨酸含量也减少；同侧 MVN 区谷氨酰胺含量明显高于对侧 MVN 区。结果提示，单侧迷路破坏后双侧 MVN 区氨基酸含量立即失去平衡，随着前庭代偿的进展，此差异减少，但是在前庭代偿后却出现双侧前庭核区谷氨酰氨的含量失衡，说明在前庭代偿过程中氨基酸含量变化起着重要作用。

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*Corresponding author. Tel: +86-433-2660586; E-mail: y-z-jin@ybu.edu.cn

关键词：氨基酸；前庭内侧核；单侧迷路破坏；前庭代偿
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Unilateral labyrinthectomy (UL) produces vestibular symptoms, including autonomic symptoms, oculomotor and postural asymmetry. Some of these symptoms gradually disappear over time[1], known as vestibular compensation, which might be considered as a functional reassembly of the central vestibular system and used as an experimental model of lesion-induced vestibular plasticity in the central nervous system (CNS)[2,3].

The medial vestibular nucleus (MVN), one of the most important nuclei in the vestibular nucleus complex in the brainstem[4], is the main nucleus for transmission of information from peripheral vestibular input to the central pathways and is mainly involved in monitoring the compensatory responses[5,6]. UL produces asymmetrical spatiotemporal changes in the expressions of several inducible or constitutive transcriptional factors in the vestibular nuclei (VN)[7,8]. Other phosphorylated proteins can also be detected in the MVN following UL[5,9]. Therefore, it is suggested that MVN mainly plays an important role in the process of vestibular compensation.

Numerous previous studies suggest that amino acids are the main neurotransmitters or neuromodulators in the CNS, including glutamate (Glu), aspartate (Asp), glycine (Gly), alanine (Ala), taurine (Tau), and γ-amino butyric acid (GABA)[10-12]. However, the detailed role of amino acids of VN in vestibular compensation is still not clear. Therefore, we investigated how the actual release of amino acids in MVN changes during vestibular compensation in order to determine the involvement of amino acids in the vestibular plasticity inducing the compensation following UL, by using an *in vivo* microdialysis technique and high-performance liquid chromatography (HPLC) technique.

1 MATERIALS AND METHODS

1.1 *Animals*

One hundred and eleven male Wistar rats (Crj: Wistar; yanji, China), 7-week old and weighing 200-300 g, were randomly divided into the lidocaine-UL group and the arsanilic acid-UL group [including ipsilateral and contralateral lesional (ipsi- and contra-lesional) groups, n=15 and n =7 rats, respectively], and the normal control group (n=67 rats).

1.2 *Implantation of microdialysis probes*

The rats were anesthetized with chloral hydrate (300 mg/kg, i.p.) and placed in a stereotaxic frame (Chengmo, Japan) with the incisor bar set at -3.3 mm. The skull from 1/2 posterior to the lambdoid suture of the parietal bone to the great occipital foramen was removed by micro electro-rotor (Saeyang, Korea), and cerebellum was partly absorbed by electric suction apparatus (DEX-23D, China) to expose vestibular area in the brainstem under an operating microscope (AE 993330901, China), and a microdialysis probe (the tip of the probe covered with a 1.5-mm length of hollow fibers, 200-μm outsider diameter, cellulose acetate membrane, 48 kDa molecular cut-off; Terumo, Japan) was stereotaxically implanted into the left MVN (2.9-3.0 mm anterior to the area postrema, 0.7-0.8 mm lateral to the midline, and 1.5-1.6 mm ventral to the surface) according to the atlas of Paxinos and Watson[13].

1.3 *Hemilabyrinthectomy*

Twenty-two rats were anesthetized with chloral hydrate (300 mg/kg, i.p.) and were right arm reclining, which were injected 0.1 mL arsanilic acid solution [100 mg/mL by adding arsanilic acid (Sigma) to 0.3 mol/L sodium carbonate solution] to rate the tympanic membrane through external ear into left middle ear. An absorbent cotton (diameter: 2 mm) was placed to prevent the medicament effuse. In this way, the membranous labyrinth was destroyed chemically. After recovering from anesthesia, as the labyrinthectomy was completed, the animals showed severe symptoms of unilateral vestibular loss (e.g., spontaneous nystagmus, body rolling and head yaw). The animal models prepared were used in the experiment after 2 weeks.

Twenty-two rats were anesthetized with chloral hydrate (300 mg/kg, i.p.) and were dorsal position. The skin of neck medisectted, left-lateral muscle intergroove of sternohyoid was stripped to expose the left tympanic bulla. An eyehole was drilled by micro electro-rotor and a polyethylene catheter (caliber 0.5 mm) was inserted into the left middle ear, another terminal was connected with a syringe that was filled with 2% lidocaine hydrochloric solution (0402131, Tianjin, China), the guide catheter was fitted by dental cement. Then, 1.2 mL (divided into 5 times in 10 min, 0.4 mL for the first time) 2% lidocaine was evenly pushed into the middle ear in the experimental course. The imbalance of bilateral eyeball position appeared immediately after lidocaine was injected.

1.4 *In vivo microdialysis study*

The MVN of each rat was perfused with modified Ringer's solution (147 mmol/L NaCl, 4 mmol/L KCl, 2.3 mmol/L

$CaCl_2$; pH 6.5) through the implanted microdialysis probe (exposed membrane 1.0 mm) at a constant rate of 1.5 μL/min using a microsyringe pump (ESP-64, Eicom, Japan). The dialysate was collected in an Eppendorf tube using a fraction collector (EFC-82, Eicom, Japan) every 10 min. The microdialysis schedule is shown in Fig.1. As shown in our previous experiments, the concentration of amino acids became stable after 90 min[14]. After a 90-min stabilization period, samples of dialysate were collected. All samples of collected dialysates were kept at -80 °C for later analysis.

1.5 *Amino acid analysis*

Amino acid levels were measured by using HPLC with an electrochemical detector (HPLC-ECD) system according to the precolumn derivatization method described by Jin *et al*[15]. In advance, 2 μmol/L standard solution [by adding *L*-Asp13.31 mg, *L*-Glu 14.71 mg, *L*-glutamine (Gln) 14.62 mg, Gly 7.51 mg, Tau 12.51 mg, *L*-Ala 8.91 mg to 50 mL of 0.1 mol/L HCl, diluted in artificial cerebral spinal fluid (ACSF) 1 000 times] and 40 mmol/L *o*-phthalaldehyde (OPA) solution [by adding 13.5 mg of OPA and 10 μL 2-mercaptoethanol to 2.5 mL of 0.1 mol/L K_2CO_3 buffer (pH 9.5) with 10% ethanol] were prepared. The solution was then stored at 0-4 ºC and diluted in 0.1 mol/L K_2CO_3 buffer to yield a 4 mmol/L OPA solution just before detection. The dialysate (12 μL) was mixed with 3 μL of 4 mmol/L OPA solution and allowed to react for 2.5 min at 25 ºC. After completing the reaction, 10 μL of the reaction mixture was manually injected into an HPLC system with an Eicompak MA-5 ODS column (4.6-mm inner diameter ×150 mm; Eicom, Japan) for assaying the amino acids. Detection was accomplished with an ECD (Eicom) with +700 mV Ag/AgCl electrodes. The mobile phase (0.1 mol/L $NaH_2PO_4 \cdot 2H_2O$, 0.1 mol/L $Na_2HPO_4 \cdot 12H_2O$, 30% MeOH, 0.5 mmol/L EDTA·2Na, adjusted to pH 6.0) was delivered at 1.0 mL/min through an HPLC pump and the potential of ECD was set at +700 mV.

1.6 *Histology*

At the end of the experiment, the animals were sacrificed with an overdose injection of chloral hydrate. The brains were removed and fixed in 10% neutral phosphate-buffered formalin. After 3 d, the brains were placed in a 30% sucrose solution and cut into 50-μm sections in order to ascertain the location of the tip of dialysis probe using a cryomicrotome (Wheel microtome, KD-1508, China). Sections were stained with neutral red and then examined using light microscopy. Only the data obtained from animals in which the microdialysis probe was positioned correctly in the appropriate dialysis site were processed and included in the results, and other samples were discarded.

1.7 *Statistical analysis*

The area of each peak of the HPLC chromatograms representing the sample content of amino acids was automatically integrated and compared with that of external standards. All data were expressed as means±SEM, and data analysis was performed by a one-way analysis of variance (ANOVA) for repeated measures, with treatment as main factors, followed by the least-significant difference test of multiple comparison [Fisher's least significant differences test (LSD) protected *t*-test]. A probability level of $P<0.05$ was considered statistically significant. All statistical procedures employed the Stat View version 11.5 software for SPSS (SPSS Inc., Chicago, USA).

2 RESULTS

Animals appeared immediately ocular asymmetries (descending in the ipsi-lesional eyeball and ascending in the contra-lesional eyeball) after infusing lidocaine into the left middle ear. In contrast, the vestibular symptoms, including oculomotor and postural asymmetry, were observed from 6 h to 168 h after infusing arsanilic acid into the tympanic cavity. Some of these symptoms gradually disappeared over time, but others continued. Frequency of

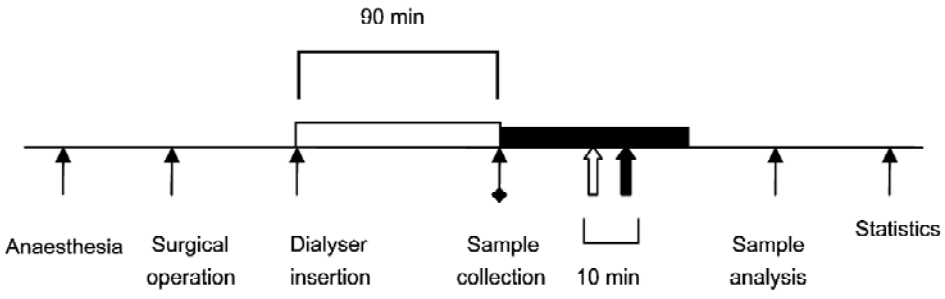

Fig. 1. The experimental protocols.

nystagmus gradually enhanced from its appearance, the high incidence of nystagmus appeared at 24 h after UL, then reduced and disappeared at 120 h. The head yaw gradually augmented, the peak time appeared at 120 h, and did not restore until 168 h. The body rolling came forth two peak times at 24 h and 96 h, respectively. The apex was 24 h, then reduced and disappeared over 168 h after UL (Fig.2).

The basal levels of Asp, Glu, Gln, Gly, Tau, and Ala in dialysis samples of the MVN were (6.15±0.59), (18.13± 1.21), (33.73±1.67), (9.26±0.65), (9.56±0.77) and (10.07± 0.83) pmol/8 μL, respectively (n=67). The concentrations of Asp and Glu decreased, and the concentration of Tau increased in the ipsi-lesional MVN of rats 10 min after infusing lidocaine into middle ear to obstruct unilateral vestibular end-organ (P <0.05). In contrast, the concentration of Glu increased, and the concentrations of Gly and Ala decreased in the contra-lesional MVN, accompanied by imbalances of Glu, Gly and Ala in the bilateral nuclei of rats (P<0.05) (Fig.3 and 4).

Decreased levels of Glu and Ala (P<0.01, P<0.05) but increased level of Gln (P<0.05) were measured in the ipsi-

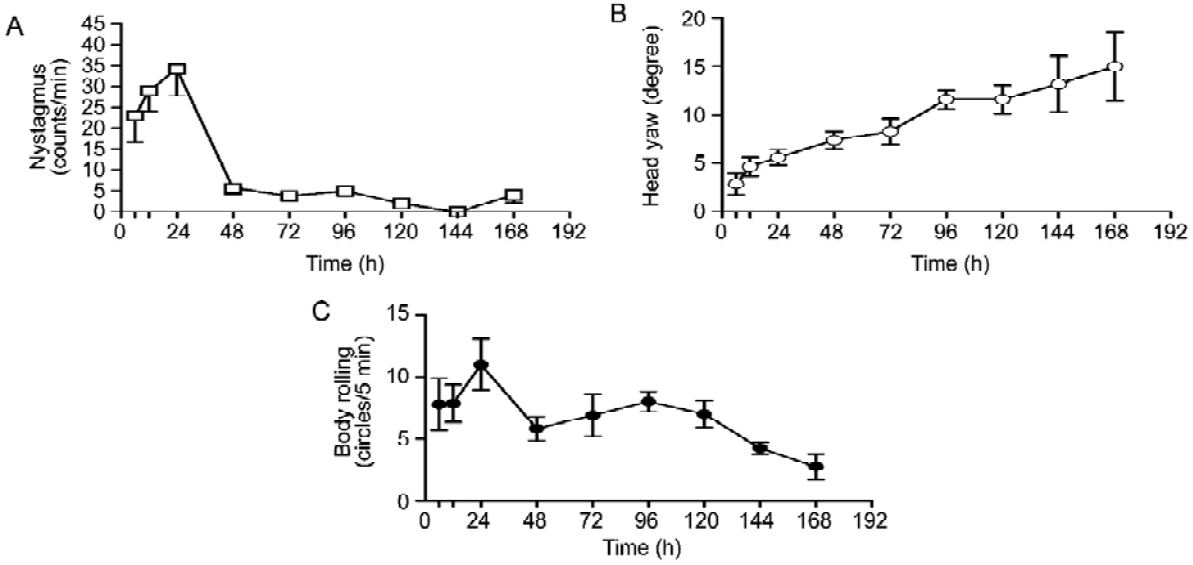

Fig.2. Changes after obstruct of unilateral vestibular end-organ by infusing arsanilic acid into the tympanic cavity in rats (n=10). Frequency of spontaneous nystagmus gradually enhanced from its appearance. The high incidence of nystagmus appeared at 24 h after UL, then reduced and disappeared at 120 h. The head yaw gradually augmented, the peak time appeared at 120 h, and did not restore until 168 h. The body rolling came forth two peak times at 24 h and 96 h, respectively. The apex was 24 h, then reduced and disappeared over 168 h after UL.

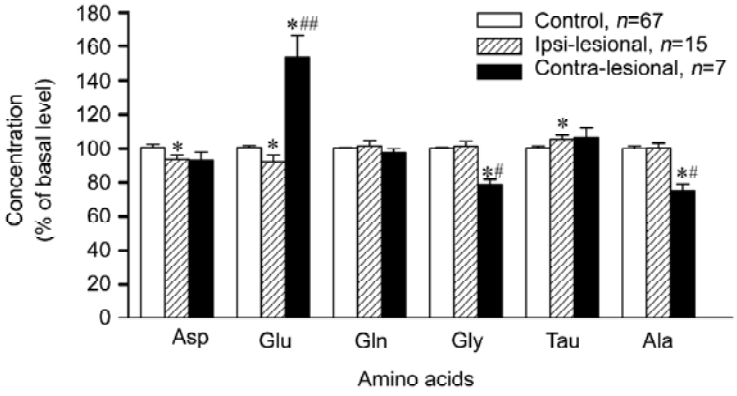

Fig. 3. Comparison of amino acid levels in the dialysate of bilateral MVN in the immediate post-UL rats that were pretreated by infusing lidocaine into the left middle ear. All values are presented as a percentage of the average of basal samples. means±SEM. $^{*}P$<0.05 compared with control; $^{\#}P$<0.05, $^{\#\#}P$<0.01 compared with ipsi-lesional MVN.

lesional MVN, and decreased level of Glu (P<0.01) was observed in the contra-lesional MVN of rats 2 weeks after infusing 10 mg arsanilic acid into the tympanic cavity to obstruct unilateral vestibular end-organ. In contrast, the level of Gln in the ipsi-lesional MVN increased more significantly than that in the contra-lesional MVN (P<0.05), and Gln lost its balance between ipsi- and contra-lesional MVN 2 weeks after UL (Fig.5 and 6).

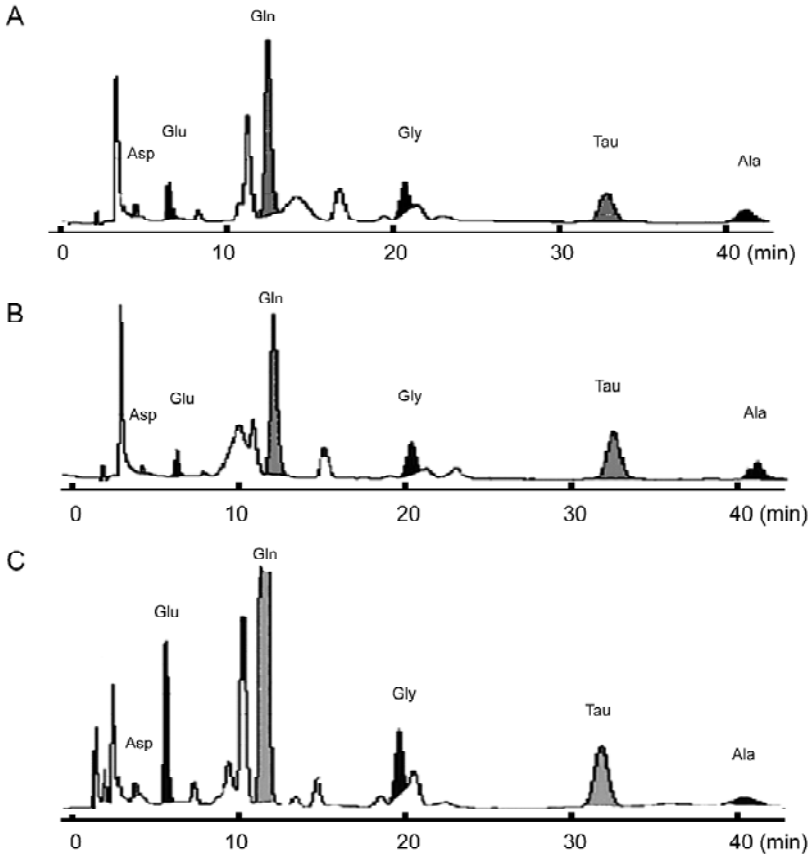

Fig.4. Typical chromatogram of amino acid levels in the dialysate of bilateral MVN in the immediate post-UL rats that were pretreated by infusing lidocaine into the left middle ear. *A*: Basal levels. *B*: Amino acid levels in the ipsi-lesional MVN after 1.2 mL lidocaine perfusion. *C*: Amino acid levels in the contra-lesional MVN after 1.2 mL lidocaine perfusion. Asp, aspartate; Glu, glutamate; Gln, glutamine; Gly, glycine; Tau, taurine; Ala, alanine.

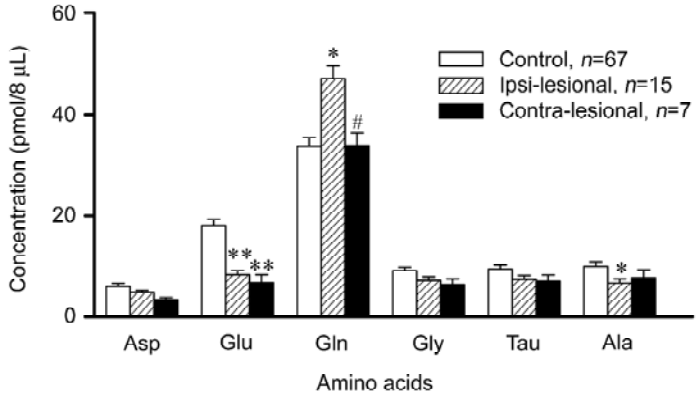

Fig.5. Comparison of amino acid content in the dialysate of bilateral MVN in the 2 weeks post-UL rats that were pretreated by infusing arsanilic acid into the tympanic cavity. All values are presented as the average of sample concentration (pmol/8 μL). means±SEM. $^{*}P$<0.05, $^{**}P$<0.01 compared with control; $^{\#}P$<0.05 compared with ipsi-lesional MVN.

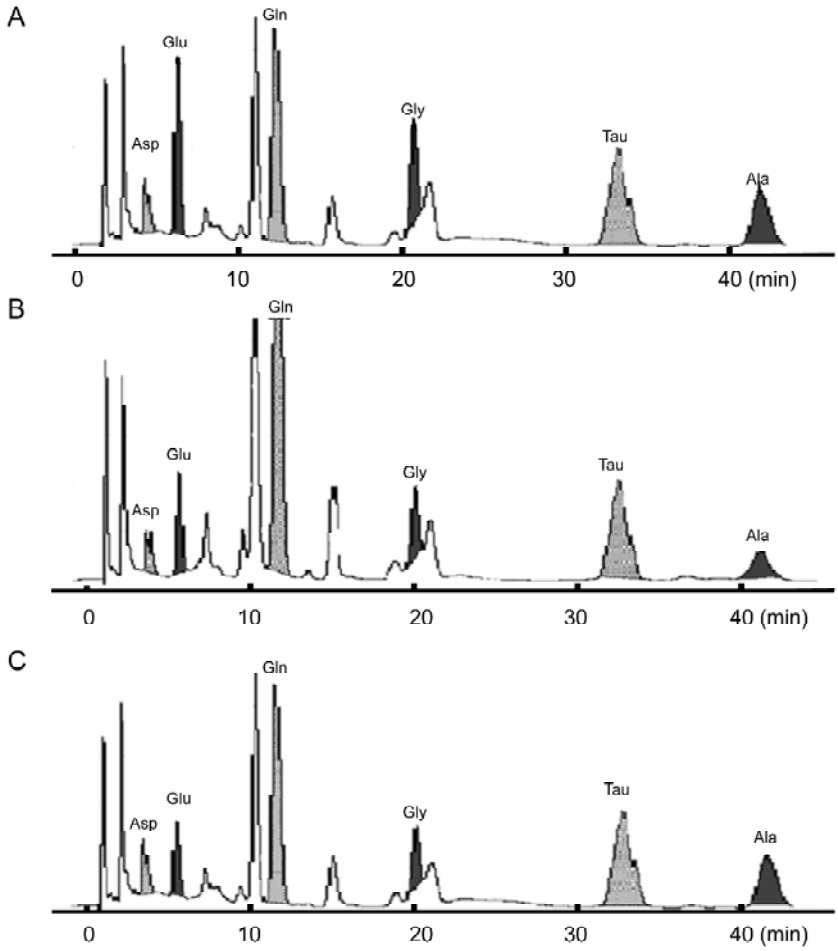

Fig.6. Typical chromatogram of amino acid content in the dialysate of bilateral MVN in the 2 weeks post-UL rats that were pretreated by infusing arsanilic acid into the tympanic cavity. *A*: Basal levels. *B*: Amino acid levels in the ipsi-lesional MVN. C: Amino acid levels in the contra-lesional MVN. Asp, aspartate; Glu, glutamate; Gln, glutamine; Gly, glycine; Tau, taurine; Ala, alanine.

3 DISCUSSION

Previously, a pharmacological study using HPLC detected Glu in the VN[16,17]. Histochemical and electrophysiological studies also showed that Glu, a primary afferent neurotransmitter from the vestibular nerve to the MVN, exists in the vestibular nerve terminal[18,19]. There is direct experimental evidence regarding the actual Glu release from MVN during the vestibular compensation process over 48 h following UL[16]. Inoue *et al.* reported that the imbalance of Glu release in bilateral nuclei induced spontaneous nystagmus associated with the rapid development of vestibular compensation[16]. However, the research on other amino acids in MVN participating in vestibular compensation is not yet reported.

Microdialysis is a useful method to assess and manipulate pharmacologically extracellular amino acid levels in the rat brain[20-23]. The microdialysis technique was used together with HPLC and ECD to separate and quantify different amino acids such as Asp, Glu, Gln, Gly, Tau and Ala. Thus, we used the microdialysis technique in the present study to investigate the release profile of amino acids. UL leads to the alteration in the release of amino acids in the ipsi- and contra-lesional VN and an imbalance of different excitatory and inhibitory amino acids results in neurochemical changes. These imbalances of different amino acids and neurotransmitters are behaviourally expressed in the form of UL and its symptoms.

Our previous study demonstrated that amino acid content markedly changed in 90 min after microdialysis probe inserted, but tended to stabilization after 90 min[14]. Therefore, all the samples were collected 90 min after microdialysis probe inserted into the MVN in this experiment. In addition, the symptoms of animals after UL were similar with reports from other researchers[24]. It proves that the models applied are credible in our experiments.

In the experiment, the concentrations of Asp and Glu

decreased, and the concentration of Tau increased in the ipsi-lesional rat VN neurons 10 min after infusing lidocaine into middle ear to obstruct unilateral vestibular end-organ, due to deficits in peripheral afferent input. Therefore, in the MVN ipsilateral to the lesion site, the decrease in the levels of the excitatory amino acids, Asp and Glu, is assumed to be due to a UL-induced loss of input from the vestibular nerve, while the increased level of the inhibitory amino acid, Tau, could more debase the excitability of MVN ipsi-lesional to the lesion. On the other hand, in the MVN contralateral to the lesion, the concentration of Glu increased after UL, and the concentrations of Gly and Ala decreased. These changes could contribute to enhance the excitability of MVN contralateral to the lesion. This shows that the excitability of ipsi-lesional MVN immediately reduced due to deficits in peripheral afferent input from the vestibular nerve of rats, but the excitability of contra-lesional MVN immediately enhanced for the vestibular functional compensation, which suggests that internuclear neuronal compensation could occur mostly in the early phase of UL.

In contrast, the levels of Glu and Ala decreased, but the level of Gln increased in the ipsi-lesional MVN of rats which were pretreated by infusing 10 mg arsanilic acid into the tympanic cavity to obstruct unilateral periphery vestibular organ, and the level of Glu decreased in the contra-lesional MVN 2 weeks after UL. These results suggest that the level of excitatory amino acid, Glu, reduced due to the loss of input from the vestibular nerve caused by UL in the ipsi-lesional MVN, and did not recover to the basal level after vestibular compensation (2 weeks after UL). The level of Glu reduced in the contra-lesional MVN was presumably due to the depression of neuronal activity in the contra-lesional MVN through inhibitory input from the cerebellum or other sources[25] to accommodate with the ipsi-lesional MVN. In addition, the level of Gln in the ipsi-lesional MVN increased more significantly than that in the contra-lesional MVN, and the Gln lost its balance in ipsi-and contra-lesional MVN 2 weeks after UL, suggesting that a change in the release of Gln, a precursor of Glu synthesis[26], in the bilateral nuclei may exert an important influence on the plasticity in the central vestibular system and participate in vestibular compensation. The role of Ala in the MVN is still largely unknown. It could be functionally related to the increased Glu level during vestibular compensation, since Ala may be a precursor of Glu and could produce an effect on the concentration of Glu[27,28].

These results suggest that the alteration in function of glutamatergic system in the ipsi- and contra-lesional MVN, a neurochemical change after UL, plays an important role in the formation and development of vestibular compensation.

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