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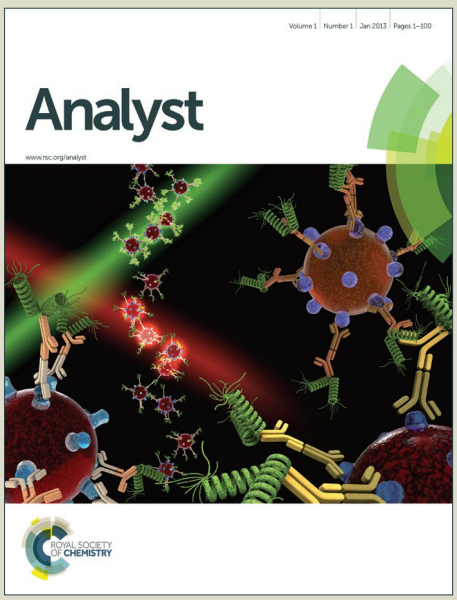

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Unilateral horizontal semicircular canal occlusion induces serotonin increase in medial vestibular nuclei: studied on microdialysis in vivo coupled with HPLC–ECD detection

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Abstract. Unilateral single semicircular canal occlusion (USSCO) is an effective treatment to some cases with intractable vertigo. All patients suffer behavioural imbalance caused by surgery, and then recover with a resumption of vestibular function. However, the compensation mechanism has not been fully evaluated. Findings suggest that serotonin (5-HT) is released from nerve terminals, and plays vital role in the plasticity of central nerve. In this study, we performed surgery of unilateral single semicircular canal occlusion (USSCO) on guinea pigs, and investigated the change of 5-HT with *in vivo* microdialysis of medial vestibular nucleus (MVN) coupled with high-performance liquid chromatography and electrochemical detector (HPLC-ECD). A total of 12 guinea pigs were divided randomly into two groups, namely the USSCO group and the control group. Animals in the USSCO group underwent surgery of lateral horizontal semicircular canal occlusion. And those in the control group experienced the same operation but just to expose the horizontal semicircular canal without occlusion. Vestibular disturbance symptoms were observed in cases of the USSCO group, e.g., head tilting, and forced circular movements and spontaneous nystagmus at postoperative days 1 and 3. The basal level of 5-HT was determined to be 316.78 ± 16.62 nM. It elevated to 448.85 ± 24.56 nM at one day following occlusion (P = 0.001). The increase was completely abolished with the vestibular dysfunction recovery. The results showed that unilateral horizontal semicircular canal occlusion could increase the 5-HT level in MVN. 5-HT may play significant role in the process of central vestibular compensation with residual vestibular function.

Introduction

Vertigo, one of the most common inner ear diseases, vastly affects 3 to 10% in the 18-to-39 age group and 35.4% for the older population.[2] Specifically, it may have a significant impact on quality of life and ability to work, and significantly increases the likelihood of falls. Ménière's disease, vestibular migraine, and benign paroxysmal positional vertigo (BPPV) are the common causes of vestibular peripheral vertigo, of which the annual incidence estimates range from 0.12 to 0.5%, 0.98%, and 0.06 to 0.6%, respectively.[1]

The conventional treatment of vertigo is comprehensive pharmacotherapy, mainly aiming to regulate the autonomic nervous function and thereby improve the inner ear microcirculation. However, when vertigo becomes recurrent, it may exacerbate the symptoms. In these cases of recurrence, a surgical approach may be considered to treat the intractable vertigo. At present, Unilateral Single Semicircular Canal Occlusion (USSCO) is gradually becoming a hot research topic. It is proved to be less destructive and preserves hearing. Especially, USSCO is safe and effective for treating intractable BPPV. However, all patients experience the syndrome of oculomotor and postural deficits, such as transient postoperative disequilibrium, nausea, and vomiting. Although being able to rapidly resolve within two weeks, [3,4] all of these imbalance symptoms are very painful in the process of recovery. In this context, investigation on the neurotransmitters that is involved in imbalance symptoms would be clinically beneficial and helpful to shorten the duration of acute vestibular disturbance symptoms.

Previous studies have demonstrated that the unilateral labyrinthectomy on albino rats and triple semicircular canal occlusion (TSCO) in guinea pigs are generally accepted animal models in vestibular compensation.[5,6] Findings have sought to elucidate that vestibular compensation after unilateral labyrinthectomy appears to be relatively independent of any recovery in the deafferented vestibular nerve.[7] It is a process of the central compensation,

and vestibular nuclei is responsible for plasticity predominantly.[8] As the biggest vestibular nucleus, some electrophysiological data have indicated that the medial vestibular nucleus (MVN) may be one of the important areas in vestibular compensation after semicircular canal occlusion.[9,10] Serotonin, 5-hydroxytryptamine (5-HT) have been demonstrated to form a major modulatory network in the peripheral and central nervous systems, being stored in vesicles and released from nerve terminals in the MVN. Despite studies reporting that serotonin is important to the plasticity of central nerve, which modifies firing rate and resting activity of vestibular neurons,[11] and the concentration of serotonin in blood of patients with vestibular disturbances,[12] the changes of serotonin during the compensation in MVN remain to be determined.

In the this research, we established a model by using guinea pigs and performed unilateral horizontal semicircular canal occlusion which was a complete simulation of clinical surgery. We then monitored the change of 5-HT level in MVN with in vivo microdialysis coupled with high-performance liquid chromatography (HPLC) with electrochemical detector (ECD). We found that unilateral horizontal semicircular canal occlusion could increase the serotonin level in MVN. It indicates that 5-HT and central compensation may play significant roles in the process of compensation on residual vestibular function.

Experimental Section

Chemicals and Solutions

Heptanesulphonic acid sodium salt in HPLC grade was purchased from Shanghai Chemical Reagent (Shanghai, PR China). Serotonin was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Artificial cerebral spinal fluid (aCSF) was prepared by mixing NaCl (126 mM), KCl (2.4 mM), $CaCl_2$ (1.1 mM), $MgCl_2$ (0.85 mM), $NaHCO_3$ (27.5 mM), Na_2SO_4 (0.5 mM) and KH_2PO_4 (0.5 mM) into doubly distilled water, the solution pH was adjusted pH 7.4.

Animal and Surgery

Adult male guinea pigs (*n*=12), each weighing 0.3-0.4 kg, were born and reared at Department of Laboratory Animal Science of Peking University Health Science Center. The experimental protocol followed in this study was conformed to the "Guide for Care and Use of Laboratory Animals" and approved by the Animal Care and Use Committee of Peking University Health Science Center. They were randomly divided into two groups, i.e., USSCO group and control group, and were housed on a 12:12 h light–dark schedule with food and water *ad libitum*. All animals were anaesthetized by intraperitoneal injection of 1% pentobarbital sodium (35 mg/kg), and were then secured in a stereotaxic frame with an incisor bar at 3.3 mm below the interaural line. The guinea pigs in USSCO group (n = 6) had a surgery of unilateral horizontal semicircular canal occlusion at the left ear. A diamond bit (Nakanishi Inc. Japan) was used to open the bone of external semicircular canal distal from the ampulla. The local muscle tissue was filled into the window of the canal, and the wound was closed by interrupted sutures. While those in the control group (n = 6) underwent the operation just to expose the lateral semicircular canal without occlusion. A microdialysis probe was guided through a cannula that was placed into the medial vestibular nucleus during the surgery (AP = 1.8 mm, ML = 1 mm, and DV = 9 mm). [13] The probe was secured

in the guinea pig's head with three skull screws and dental acrylic cement. During the whole surgery and recovery from the anesthesia, body temperature of the animals was maintained at 37 °C with a heating pad. After surgery, the animals were housed individually.

Microdialysis In Vivo Coupled with HPLC–ECD

The guinea pigs were allowed to recover from the surgery at least 24 h before *in vivo* microdialysis sampling. The metal core of the guide cannula was carefully extracted, and the microdialysis probe (BAS; dialysis length, 4 mm; diameter, 0.24 mm) was inserted into the cannula with the inlet connecting to a microinjection pump. Guinea pigs were placed in a rotating disc device and were continuously perfused at the rate of 1 μL min^{-1} with aCSF. In order to eliminate the damage to the brain tissue resulted from inserting the probe, dialysate samples were collected after the period equilibration of at least 90 min.[11] Subsequently, 20μl samples were immediately injected into a high performance liquid chromatography with electrochemical detector (HPLC-ECD) system for the assay of 5-HT. [12]

The HPLC-ECD system consisted of a liquid chromatograph (model LC-10AT, Shimadzu, Japan), a 5 μm particle size C^{18}-Nucleosil reversed phase column (4.6 mm i.d.×150 mm) proceeded by a C^{18} precolumn, a sample injector (20 μl valve), and an electrochemical detector (CHI832A: Shanghai, China). For the analysis of the serotonin, the mobile phase was prepared with 30 mM $Na_2HPO_4 \cdot 2H_2O$, 0.1 M EDTA-Na_2 (pH 4.0) and 10% (v/v) methanol. Elution was performed at a flow rate of 1μL min^{-1} and working electrode potential of ECD was set at +800 mV for the measurements. Prior to the injection of dialysate, chromatographic column was flushed for 2 hours with the mobile phase at the constant velocity of 1μL min^{-1}, in order to stabilize the column pressure and smooth the detection baseline.

Calibration of Microdialysis Probes

Implanted probes were calibrated in vivo by delivery experiment with 50 μM standard solution of 5-HT after microdialysis.[13] Probe extraction efficiency (EE) was calculated as $EE=(C_p-C_d)/C_p$. C_p is the concentration of the analyte in the perfusate and C_d is the concentration of the analyte in the dialysate. All concentrations were corrected for the probe extraction efficiency.

5-HT Standard Curve

5-HT standard solutions were prepared at a mass-volume concentration of 0.2μM, 0.5μM, 1μM, 2μM and 5μM with doubly distilled water. 20μl samples of each solution were injected into HPLC-ECD systems, and eluted at a flow rate of 1μL min^{-1}. The peak areas and concentrations of 5-HT were disposed by Linear Regression Analyse.

Electronystagmography (ENG) Examination

Each guinea pig was placed in a special fixator that fixed the head position with non-limited eye movements. Guinea pigs were placed in a customized fixator that tilted the head forward at 48°. Thus the horizontal canal was in a vertical position of gravity, and the sinusoidal oscillation evoked nystagmus would reflect the function of the horizontal semicircular canal. Spontaneous nystagmus was recorded in the chamber for 60 seconds, fixed oscillating frequency of 0.16 Hz for four cycles with the different peak rates of 60° / s, 90° / s, 120° / s, 150° / s, and 180° / s.

Histological Ascertainment

At the end of the experiments, animals were sacrificed with an overdose injection of chloral hydrate. The brains and left otocysts were removed surgically and placed in 4% paraformaldehyde solution for fixation. After a 7-day decalcification and soaking in 20% sucrose solution for 24 hours, the otocysts were embedded by OCT embedding agent (ZSGB-BIO, China), and cut into 6 μm sections using a cryomicrotome (CM 1900, Leica, Germany). The brains were cut into 4 μm sections. The correct location of the occlusion of

left horizontal semicircular canal and the microdialysis probe were visually verified with HE staining.

Statistical Data Analysis

The concentration of microdialysate serotonin was represented as mean ± S.E. One-way ANOVA followed by Tukey's HSD post-hoc comparison was carried out to determine whether there was significant difference in the effect of surgery between observing times,, and the results were applied comparative *t*-test to perform statistical analysis between the two groups. Values of *P* less than 0.05 were considered statistically significant. All statistical procedures employed the IBM SPSS version 19.0 software.

Results and Discussion

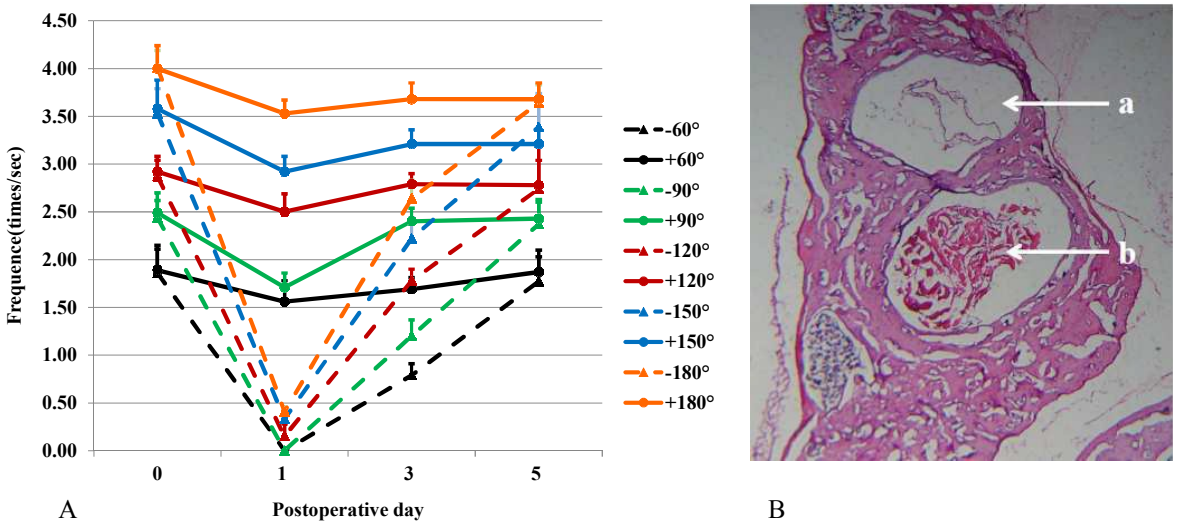
Changes of behaviour and ENG after unilateral horizontal semicircular canal occlusion.

There was no vestibular dysfunction symptoms identified from the daily behavior of all the 12 guinea pigs before the surgery. However, obvious difficulties of direction were found after surgery in cases of USSCO group. Head tilts to the left, head shakes to the left, forced circular movements and limb abduction were observed immediately on the first day after left side horizontal semicircular canal occlusion. These symptoms started diminishing from the second day and disappeared after 3 to 5 days. While the same behavioural imbalance symptoms were absent in the control group.

All 12 guinea pigs exhibited symmetric rotation-induced nystagmus before the operation. Fig.1 illustrated the afunction of left horizontal semicircular canal. The frequency of nystagmus was significantly different between left and right ($F = 38.09$, $P = 0.000004$; Fig. 1 A), and absence of the left nystagmus was observed during the counter-clockwise rotation on the 1st day after the USSCO operation. Finally, rotation-induced nystagmus had become symmetric in 5 days.

The above results suggest, in the first postoperative day, the horizontal semicircular canal on the left side lost function, which was gradually compensated for 3 to 5 days after surgery. At the end of the experiments, the slices of otocysts were observed under microscope. The left horizontal semicircular canals of all 6 animals in USSCO group were still totally blocked with muscles totally, and the structure of ampullae, superior semicircular canal and posterior semicircular canal remained normal (Fig. 1 B) . It favored the idea that the rebalance of vestibular function was not relevant to the recovery of semicircular canal, but rather, the rebalance was highly determined by central compensation. Therefore, unilateral horizontal semicircular canal occlusion should be a successful model to investigate central vestibular

compensation.



A B

Fig. 1 (A) Rotation-induced nystagmus exhibited symmetric before the operation in USSCO group, while the guinea pigs demonstrated the absence of nystagmus to the left while normal response was observed on the right, one day after unilateral horizontal semicircular canal occlusion. Three days after the operation, nystagmus to the left was observed, and become symmetric in 5 days. (B) The slices of otocysts showed that left horizontal semicircular canals were totally blocked (10×10, HE) with muscles (b) and the structure of ampullae remained normal (a).

As demonstrated in early studies, [6,1-15] the surgery of triple semicircular canal occlusion (TSCO) and unilateral vestibular labyrinthectomy were effective for eliminating the response of semicircular canals to rotation and caloric stimulation, and was used in ears with vestibular compensation following with vestibular lesion. However, USSCO showed a remarkable advantage over TSCO and labyrinthectomy in terms of the speed and completeness of compensation.[6] The recovery of behavior and balanced rotatory nystagmus

were quicker and more complete in our animal models after USSCO than the two early approaches. Recovery from TSCO took about 1 month and recovery from labyrinthectomy took 1 to 3 months, while recovery from USSCO took place in just 5 days. After TSCO surgery, the directional difference of guinea pigs did not totally disappear until Day 30 and nystagmus was generally not significant except at days 45 and 75 for rotations of 90 and 120 degrees.[6] One of the possible explanations was that the three approaches imposed different degrees of damage on vestibular organ. The labyrinthectomy totally destroyed the vestibular peripheral system, including the sensory organ, and TSCO blocked the fluid movement in three canals and caused more transaction of the membranous labyrinth than USSCO. Both of them may be more difficult to compensate than implementing dysfunctioning in one canal by USSCO. Animal studies showed that varied hearing impairments have been documented after TSCO, while single semicircular canal occlusion has been recognized as an efficient method of eliminating vertigo without causing a significant hearing impairment.[16]

Meanwhile, in our animal models, the symptoms and compensatory period after USSCO were all similar to what have been reported in patients of intractable BPPV (benign paroxysmal positional vertigo) after surgery, which could improve imbalance symptoms in 94% of patients.[2] The model used in this experiment was proved to be simple and stable and could thus be used for the studies of the mechanisms of vestibular central compensation and acute unilateral peripheral vestibular lesion.

Change of 5-HT concentration in the MVN microdialysate

In this experiment, in vivo microdialysis sampling technique was applied to extract small molecules from the extracellular fluid. The working principle of in vivo microdialysis is that the semi-permeable membrane tip of the probe is placed into the target tissue and that the hydraulic fluid is pumped through the probe at a constant rate. Hydraulic fluid could diffuse

bi-directionally through the semi-permeable membrane, and the substance to be determined could also diffuse into the probe in accordance with its concentration difference. Therefore, the molecules to be determined are obtained by collecting the hydraulic fluid at the outlet of the probe. In order to have a high recovery for 5-HT, a low perfusion rate of 1μl/min was applied in the experiments. The HPLC–ECD detection method used in this experiment demonstrated excellent specificity and stability, and showed a good response to 5-HT (data not shown). After testing with standard solutions, the current response was linear with 5-HT concentration within a concentration range from 0.2-5 μM. It was described by the following equation: $A_{5\text{-HT (nA*s)}} = 0.15C_{5\text{-HT (μM)}} - 6.23$ ($R^2 = 0.998$), where $A_{5\text{-HT (nA*s)}}$ was the peak area of the 5-HT in the perfusate and $C_{5\text{-HT (μM)}}$ was the concentration of the 5-HT in the microdialysate. The recovery ratio of the probes ranged from 13% to 21%. The actual concentration of the 5-HT could then be calculated using the empirically derived linear equation and the recovery ratio of the probe. A typical chromatogram of a 20 μL microdialysate sample was shown in Fig. 3. The retention time of 5-HT was 387-396 sec under optimal conditions.

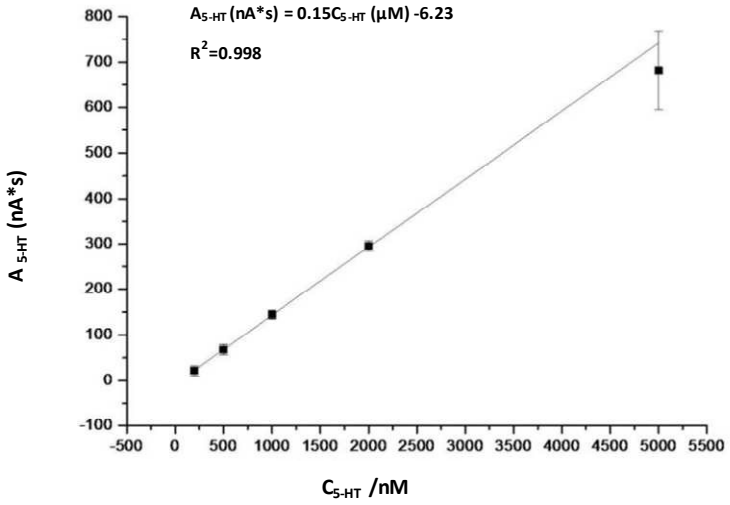


Fig. 2 The current response was linear with level of 5-HT within a concentration range

from 0.2 μM to 5 μM on-line amperometric response to standards.

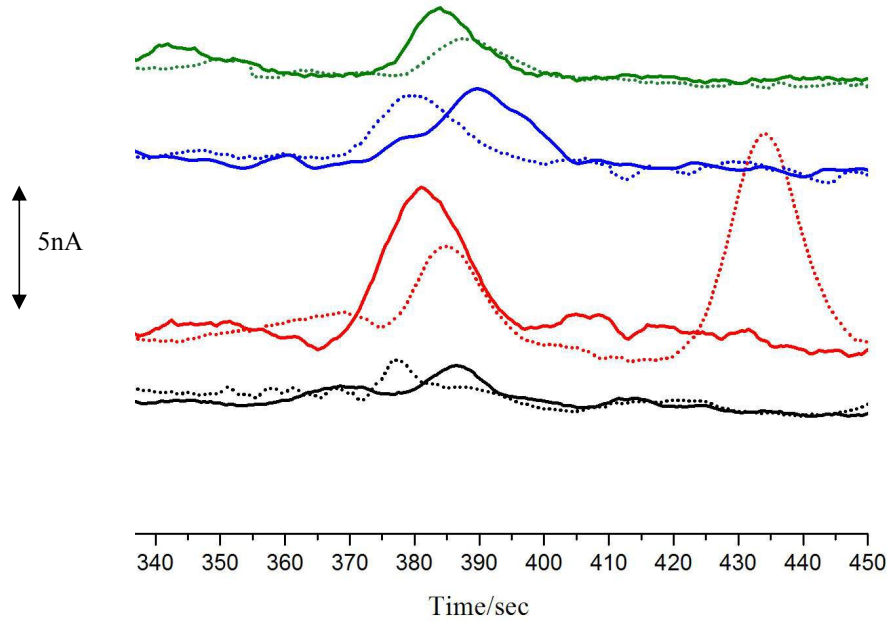
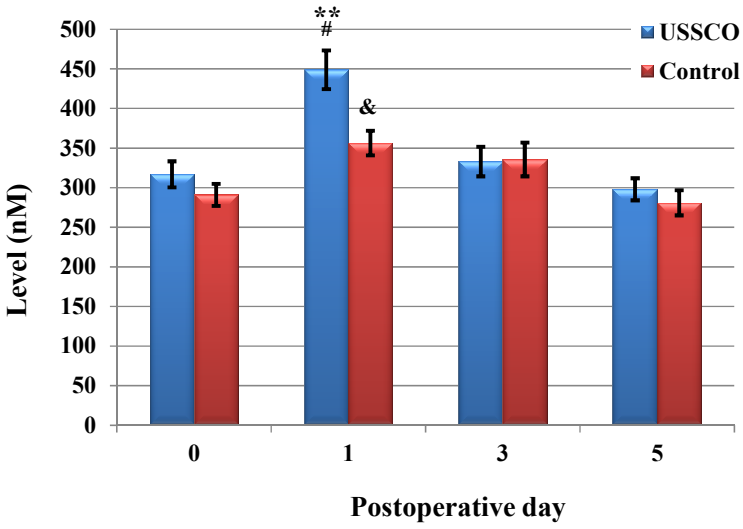


Fig. 3 Chromatograms of 5-HT in 20 μL microdialysate of MVN in USSCO group (solid line), in control group (dot line) before surgery (black), and postoperative one day(red),three day(blue), five day(green). Chromatographic conditions: mobile phase 10% (v/v) methanol–30 mM phosphate buffer, pH 4.0; flow rate 1 μL min^{-1}

Changes in extracellular levels of 5-HT in the left MVN were determined by *in vivo* microdialysis combined with HPLC–ECD following unilateral horizontal semicircular canal occlusion. The basal level of 5-HT in the dialysates was 316.78 ± 16.62 nM (n=6) in USSCO group, and 290.92 ± 14.06 nM (n=6) in control group. As shown in Fig.3, extracellular levels of 5-HT were elevated following operation. The levels of 5-HT reached 448.85 ± 24.56 nM in USSCO group at 24 h, and 356.37 ± 15.69 nM in control group. The changes in concentrations were significantly different (USSCO: F=13.000, P=0.00006; Control:

F=4.494, P=0.014) as compared with its respective basal level in both groups. This increase in concentrations was gradually abolished and returned to basal levels in 3 to 5 days. Meanwhile, at the postoperative day one, the increase of 5-HT was found to be statistically significant in USSCO group than that in control group (F=10.072, P=0.010).



B

Fig. 4 Changes of 5-HT levels were assessed by in vivo microdialysis combined with HPLC–ECD in the left MVN following USSCO. Each point is the mean± S.E. of 6 guinea pigs. **P<0.01 as compared with basal level before the surgery in USSCO group, $^{\&}$P<0.05 as compared with basal level before the surgery in control group, and $^{\#}$P<0.05 means significant difference between two groups at postoperative day 1 with t test.

At least one study has demonstrated that 5-HT increased significantly in the MVN depolarization following infusion of high concentrations of K^+, but was inhibited by perfusion of a Ca^{2+}-free Ringer's solution.[12,17-20] 5-HT has been stored in neurons vesicles as one kind of the most important neurochemicals. Some key neurons are activated in ipsilateral MVN after acute injury of vestibular. 5-HT will release from nerve terminals in the MVN, following infusion of K^+.Once receives excited signals, they will inhibit the excitement of

contralateral MVN by vestibular -cerebellum pathway. So the synchrony between the vestibular nucleus neurons activities is modulated.

In addition, previous electrophysiological findings suggest that 5-HT plays an important role in the perception and processing of painful stimuli and is particularly important in the physiological processes.[21] These findings implicated the released large amounts of 5-HT in the control group on the first day after surgery.

Moreover, we found that the levels of 5-HT in MVN increased significantly on the first day after unilateral semicircular canal occlusion surgery, which was extremely higher than that of the control group. This suggests that the significant increase of 5-HT in MVN is resulted from unilateral vestibular dysfunction. Further, it can be envisioned that 5-HT plays an important role in the process of vestibular decompensation, and possibly be an important neuromodulator in the process of vestibular compensation.

Conclusions

The changes of 5-HT in ipsilateral MVN of guinea pigs during vestibular compensation were detected by in vivo microdialysis combined with HPLC–ECD detection. The results suggested that unilateral horizontal semicircular canal occlusion could induce an increase of 5-HT in MVN, and that 5-HT and central compensation might play significant roles in the process of compensation on residual vestibular function. In addition, we have successfully performed the animal model of vestibular compensation by unilateral horizontal semicircular canal occlusion. It would be useful for the investigation on chemical essences in vestibular compensation.

Acknowledgments

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