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Research Report

The systemic application of diazepam facilitates the reacquisition of a well-balanced vestibular function in a unilateral vestibular re-input model with intracochlear tetrodotoxin infusion using an osmotic pump

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TTX, tetrodotoxin
VOR, vestibuloocular reflex
DP, directional preponderance of the nystagmus
MVN, medial vestibular nucleus
GABA, γ-aminobutyric acid

ABSTRACT

Diazepam is a popular medicine used in the treatment of acute vertigo. In the past, many studies investigating the effect of diazepam in peripheral vestibular destruction have been reported. However, no previous study has yet investigated the effect of diazepam on a model with a transient and reversible vestibular function similar to recurrent vertigo as seen in Meniere's disease. We thus made a peripheral vestibular re-input model by the unilateral intracochlear administration of tetrodotoxin (TTX) using an osmotic pump and then examined the influence of diazepam on the vestibular system in this model. Hartley white guinea pigs were intracochlearly administered with TTX on the right side for 3 days by an osmotic pump. Animals were divided into three groups, TTX alone (control group (n = 7)), TTX and an intraperitoneal diazepam injection once a day for 3 days (diazepam group (n = 6)) and vehicle injection (vehicle group (n = 6)). A caloric response and vestibuloocular reflex (VOR) were observed at 7 and 14 days after completing 3 days of TTX administration. Seven days after vestibular re-input, a directional preponderance of the nystagmus (DP) to the TTX-treated side was observed in the control and vehicle groups on VOR examination. DP was not observed in the diazepam group on any examined day. The R/L time ratio of caloric response showed no statistical difference between three groups on any examined day. These results suggest that diazepam may thus be useful for patients in an acute stage of peripheral vestibular vertigo by decreasing their vertiginous symptoms.

1. Introduction

Following peripheral vestibular destruction, body trunk deviation to the lesioned side and spontaneous nystagmus to the intact side are observed, however, these symptoms tend to gradually decrease over time. This phenomenon is well known as "vestibular compensation", namely the plasticity of the central nervous system. Many studies on vestibular compensation have been previously reported (Smith and Darlington, 1991; Curthoys and Halmagyi, 1995; Dieringer, 1995). However,

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peripheral vestibular disorder tends to be clinically transient rather than permanent (Brandt, 1999).

In the past, several studies have reported using TTX on the inner ear for the transient blockage of the peripheral vestibular or cochlear input (Weisleder and Rubel, 1990; Brown et al., 1993; Praetorius et al., 2001; Saxon, 2003). The aim of the present study was thus to make a transient peripheral vestibular input blockage model by tetrodotoxin (TTX) administration directly to the inner ear by an osmotic pump and to investigate the effect of diazepam which was commonly used for acute vertiginous patients in clinical (Brandt, 1999; Cesarani et al., 2004) using this model.

2. Results

2.1. VOR gain

2.1.1. Control group

At 3 days after operation, vestibuloocular reflex (VOR) was observed to have almost vanished. The VOR gains on first, second and third rotation to the TTX-treated side were 0.045 ± 0.061, 0.023 ± 0.060, 0.017 ± 0.045, respectively. The VOR gain on each rotation to the intact side decreased, and a statistical difference between preoperation and 3 days after operation was observed (preoperation vs. 3 days after operation; 0.476 ± 0.108 vs. 0.164 ± 0.105; $P = 0.0027$, 0.485 ± 0.081 vs. 0.184 ± 0.121; $P = 0.0027$, 0.452 ± 0.090 vs. 0.174 ± 0.134; $P = 0.0040$) (Fig. 1).

At 7 days after the vestibular re-input, there was no statistical difference in the VOR gains between right and left at first rotation (0.320 ± 0.144 vs. 0.416 ± 0.146; $P = 0.2248$). However, a significant difference was seen in the VOR gains at second and third rotation between the right and left side (0.242 ± 0.136 vs. 0.497 ± 0.164; $P = 0.0127$ and 0.171 ± 0.108 vs. 0.556 ± 0.123; $P = 0.0017$) (Fig. 1). The third rotation showed a greater discrepancy than the second rotation. In addition, the postrotatory nystagmus toward the lesioned side was observed (Fig. 3).

At 14 days after vestibular re-input, the VOR gain on each rotation recovered to the preoperative value, and no statistical difference was seen in the VOR gains between preoperation and 14 days after vestibular re-input or between the right and left side on each rotation (preoperation vs. 14 days after vestibular re-input; 0.507 ± 0.055 vs. 0.526 ± 0.093; $P = 0.5672$, 0.480 ± 0.065 vs. 0.509 ± 0.144; $P = 0.7494$, 0.475 ± 0.078 vs. 0.491 ± 0.117; $P = 0.8480$ on the TTX-treated side; 0.476 ± 0.108 vs. 0.472 ± 0.123; $P = 0.7494$, 0.485 ± 0.081 vs. 0.450 ± 0.121; $P = 0.5653$, 0.452 ± 0.090 vs. 0.402 ± 0.124; $P = 0.3379$ on the intact side) (Fig. 1). In addition, no postrotatory nystagmus was observed.

2.1.2. Diazepam and vehicle group

In the vehicle group, no statistical difference was seen in the VOR gains of all time courses between the control and the vehicle group.

In the diazepam group, at 3 days after operation, the VOR gains on each rotation to both sides were greater than in the vehicle group, but no statistical difference was seen in the VOR gains between the right and left side on each rotation (vehicle vs. diazepam group; 0.000 ± 0.000 vs. 0.104 ± 0.121; $P = 0.0614$, 0.015 ± 0.037 vs. 0.102 ± 0.097; $P = 0.0688$, 0.018 ± 0.044 vs. 0.078 ± 0.115; $P = 0.2589$ on the TTX-treated side; 0.200 ± 0.081 vs. 0.221 ± 0.081; $P = 0.6642$, 0.198 ± 0.050 vs. 0.227 ± 0.100; $P = 0.5376$, 0.165 ± 0.056 vs. 0.215 ± 0.120; $P = 0.3788$ on the intact side) (Fig. 2).

At 7 days after vestibular re-input, no statistical difference was seen in the VOR gains between the right and left side on each rotation (0.476 ± 0.205 vs. 0.441 ± 0.242; $P = 0.6310$, 0.538 ± 0.181 vs. 0.401 ± 0.193; $P = 0.3358$, 0.493 ± 0.147 vs. 0.369 ± 0.151; $P = 0.2002$) (Fig. 2). These results were different from those of the other groups. In addition, the number of postrotatory nystagmus was statically smaller than that of the vehicle group (3.167 ± 2.137 vs. 0.667 ± 1.211; $P = 0.0318$) (Fig. 3).

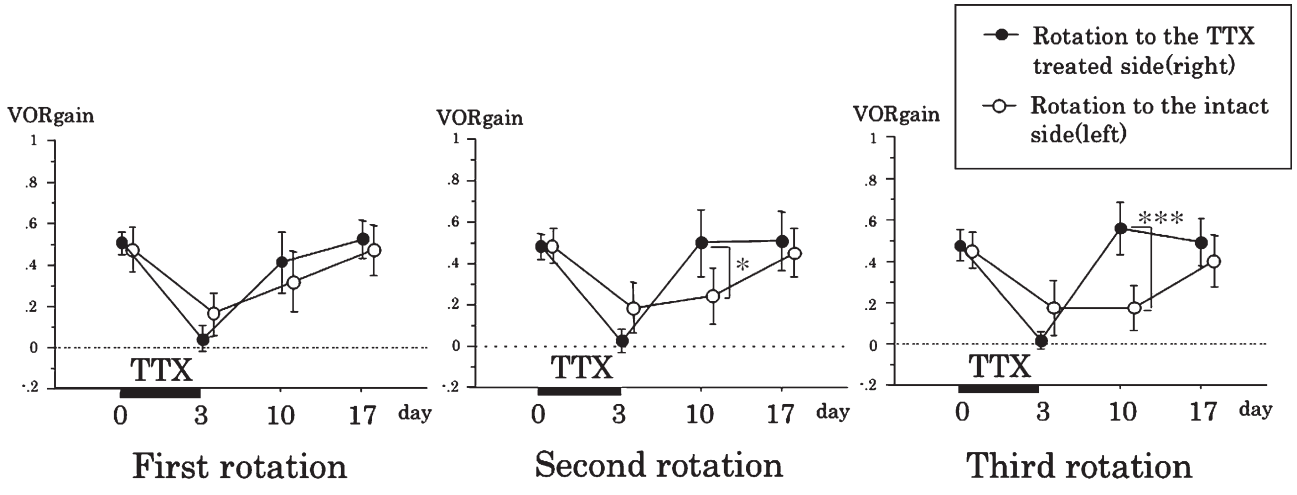

Fig. 1 – The VOR gain of each rotation in the control group. The VOR gain mean ± SD is plotted. At 7 days after vestibular re-input, there was no statistical difference in the VOR gains between right and left at first rotation. But there were significant differences in the VOR gains at second and third rotations between right and left. Third rotation showed greater discrepancy than second rotation. *$P < 0.05$, ***$P < 0.005$. TTX: tetrodotoxin, VOR: vestibuloocular reflex.

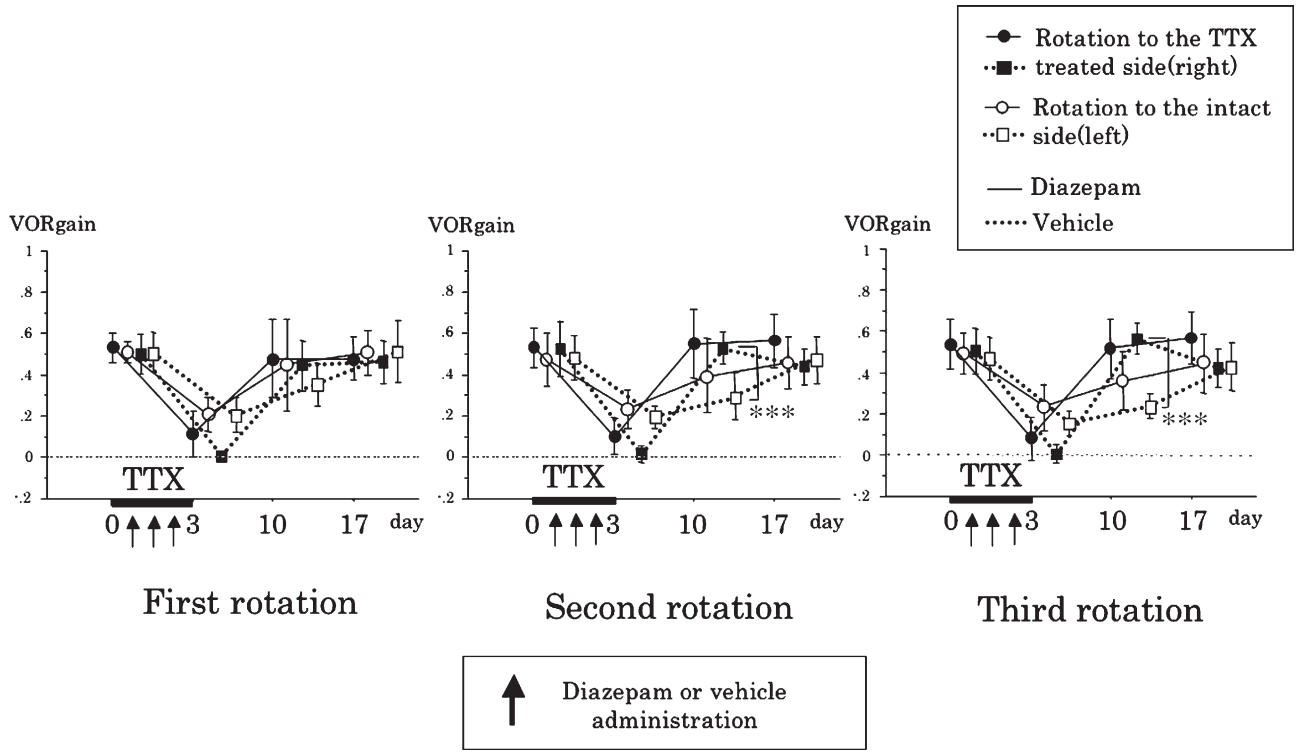

Fig. 2 – The VOR gains of each rotation in the diazepam and vehicle group. The VOR gain mean ± SD is plotted. In vehicle group, at 7 days after vestibular re-input, as well as the control group, there were significant differences in the VOR gains at second and third rotations between right and left. Third rotation showed greater discrepancy than second rotation. In the diazepam group, at 7 days after vestibular re-input, there was no statistical difference in the VOR gains between right and left on each rotation. ***$P < 0.005$. TTX: tetrodotoxin, VOR: vestibuloocular reflex.

At 14 days after vestibular re-input, the VOR gains of each rotation recovered to the preoperative value as well as the other groups, and no statistical difference was seen in the VOR gains between the right and left side on each rotation (0.492 ± 0.108 vs. 0.492 ± 0.110; $P = 0.9999$, 0.545 ± 0.128 vs. 0.456 ± 0.132; $P = 0.3776$, 0.541 ± 0.115 vs. 0.460 ± 0.142; $P = 0.2623$) (Fig. 2).

2.2. *Caloric test*

2.2.1. *Control group*

At 3 days after operation, canal paralysis (CP) on the TTX-treated side was observed. In addition, the duration time of the nystagmus on the intact side was shorter than that of preoperation.

At 7 days after vestibular re-input, the caloric response showed a gradual recovery, but statistical difference of the R/L ratio remained between the preoperative findings and those at 7 days after vestibular re-input (1.013 ± 0.089 vs. 0.481 ± 0.202; $P = 0.0026$) (Fig. 4).

At 14 days after vestibular re-input, the R/L ratio improved remarkably, and no statistical difference was seen between the preoperative levels and those at 14 days after vestibular re-input (1.013 ± 0.089 vs. 0.918 ± 0.299; $P = 0.0845$) (Fig. 4).

2.2.2. *Diazepam and vehicle group*

In the vehicle group, no statistical difference was seen in the R/L ratio at any time courses between the control and the vehicle group.

In the diazepam group, at 3 days after operation, CP on the TTX-treated side was observed as well as in the other groups. However, the duration time of the nystagmus on the intact side tended to be longer than that of the control.

At 7 days after vestibular re-input, the caloric response showed a gradual recovery the same as in the other groups (vehicle vs. diazepam, 0.387 ± 0.091 vs. 0.569 ± 0.235; $P = 0.1062$) (Fig. 4), but a statistical difference in the R/L ratio remained between the preoperative levels and those at 7 days after vestibular re-input (0.993 ± 0.046 vs. 0.569 ± 0.235; $P = 0.0129$) (Fig. 4).

At 14 days after vestibular re-input, the R/L ratio improved remarkably the same as in the control group. As a result, no statistical difference was seen between the preoperative levels and those at 14 days after vestibular re-input (0.993 ± 0.046 vs. 0.953 ± 0.306; $P = 0.5218$) (Fig. 4). In addition, no statistical difference was seen between the vehicle group and the diazepam group (1.097 ± 0.327 vs. 0.953 ± 0.306; $P = 0.4509$) (Fig. 4).

3. Discussion

TTX is a voltage-dependent sodium channel blocker which is able to block the action potential of neurons without causing any histological damage. In the past, several studies have reported using TTX on the inner ear for the transient blockage of peripheral vestibular or cochlear input (Weisleder and Rubel, 1990; Brown et al., 1993; Praetorius et al., 2001; Saxon, 2003). We previously reported that, according to our method,

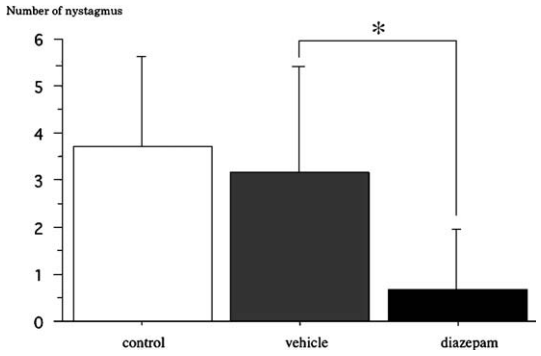

Fig. 3 – The postrotatory nystagmus of each group after 7 days from vestibular re-input. Number of nystagmus beats as the mean ± SD is plotted. No statistical difference showed the number of postrotatory nystagmus between the vehicle group and the control group. However, the number of postrotatory nystagmus in the diazepam group was statistically smaller than that in the diazepam group. *P < 0.05.

no functional or histological damage occurred in the inner ear by the direct intracochlear administration of drugs using an osmotic pump in guinea pigs (Shimogori et al., 1999; Shimogori and Yamashita, 2000a,b, 2001). In the present study, we used the TTX characteristics and our osmotic pump implantation technique to study vestibular compensation on the process of the functional recovery of the vestibular periphery.

In a guinea pig, at 48 h after a unilateral labyrinthectomy, the sensitivity of the contralesional vestibular neurons to horizontal rotation stimulation decreased to a half of control (Ris and Godaux, 1998). We confirmed that no spontaneous nystagmus was observed in any animals from each group within 60 h from the operation. This result is assumed to be one of the vestibular compensation processes that is caused by cerebellar inhibition to the intact side. Our results thus suggested the process of vestibular compensation between TTX administration to unilateral inner ear and unilateral labyrinthectomy to be similar.

At 14 days after vestibular re-input, the vestibular function recovered to preoperative levels, and no statistical difference was seen between the right and left side, thus indicating that our model was a reversible peripheral vestibular damage model. The VOR asymmetry by a unilateral labyrinthectomy has been shown to be permanent (Maioli and Precht, 1985; Fetter and Zee, 1988). It is a characteristic of our model that VOR asymmetry, which once occurred, later vanished.

Concerning the inhibitory system to the vestibular nucleus, two pathways are well known, namely the commissural and cerebellum. Both pathways have been said to be GABAergic (Prechet et al., 1973; Kitahara et al., 1997). Several previous studies have suggested that an adaptive regulation of the functional efficacy of GABA receptor could play a role as an important mechanism in the control of excitability of the medial vestibular nucleus (MVN) neurons (Eleore et al., 2005). Based on another study, following a unilateral labyrinthectomy in the rat, down-regulation of GABA receptor in the ipsilesional medial vestibular nucleus (MVN) neurons and up-regulation of GABA receptor in the contralesional MVN neurons were observed (Yamanaka et al., 2000). These results could be due to changes in the affinity and/or efficacy rather than changes in total protein expression (Gliddon et al., 2005). Moreover, the cerebellar inhibition to the contralesional MVN neurons continued for 1 week and then disappeared at 2 weeks (Kitahara et al., 1995, 1997).

In this study, the results at 7 days after vestibular re-input suggested that the cerebellar inhibition to the MVN neurons on the intact side might remain. The accumulation of sinusoidal rotation stimulation might cause an increase in the inhibition to the intact side of MVN neurons and a decrease in the inhibition to the TTX-treated side of MVN neurons. As a result, the directional preponderance of nystagmus (DP) to the TTX-treated side may thus be formed.

We often clinically use the head-shaking test to observe the presence of any after shaking nystagmus as semicircular canal stimulus was done as one of the vestibular function tests (Takahashi et al., 1990; Katsarkas et al., 2000; Guidetti et al., 2002; Pérez Vazquez et al., 2004). When vestibular function asymmetry existed, semicircular canal stimulus by shaking the head accumulated on the vestibular commissure (Holstein et al., 1999). Head-shaking nystagmus, namely nystagmus toward the contralesional side, was observed after shaking the head by discharging the accumulated velocity that is known to be a velocity storage mechanism (Raphan et al., 1979; Hain et al., 1987). The nystagmus toward the lesioned side which occurs after the head-shaking test was observed in the recovery period of peripheral vestibular disorder (Matsuzaki and Kamei, 1995). The direction of head-shaking nystagmus correlated with that of postrotatory nystagmus (Takahashi et al., 1990). In this model, the DP observed during vestibular recovery may be related to the velocity storage mechanism.

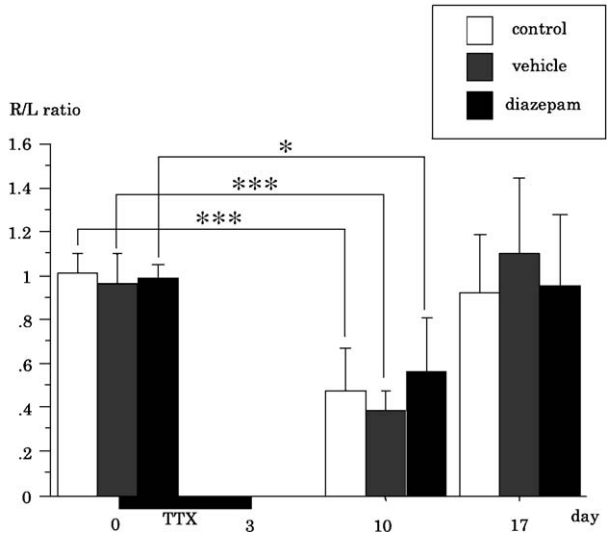

Fig. 4 – The R/L ratios of each group in caloric test. The R/L ratio mean ± SD is plotted. At 7 days after vestibular re-input, caloric response showed a gradual recovery on all groups. Statistical difference of the R/L ratio remained between preoperation and at 7 days after vestibular re-input on all groups. At 14 days after vestibular re-input, the R/L ratio improved remarkably in both groups. No statistical difference was observed between all groups on any examined day. *P < 0.05, ***P < 0.005. TTX: tetrodotoxin.

In the past, there have been many studies about the effect of diazepam for vestibular compensation in the peripheral vestibular destruction model. These studies insisted that diazepam reduced the static symptoms in the acute stage of peripheral vestibular damage, but it did not impair vestibular compensation process (Martin et al., 1996).

In vestibular research, Hutchinson et al. examined the neuronal activity in the MVN neuron using diazepam 2 mg/kg i.p. injection in the guinea pig (Hutchinson et al., 1995). In another study, once daily 6 mg/kg i.p. injection of diazepam for 5 days did not produce any significant tolerance (Smith and Darlington, 1994). We referred to these studies, so we used a daily 2 mg/kg i.p. injection of diazepam for 3 days.

It is known that diazepam inhibits neuronal activity in the MVN neuron by conjugating $GABA_A$ receptors in the MVN neurons. At acute stage of peripheral unilateral vestibular disorder, diazepam decreases vestibular asymmetry. Our results suggested that diazepam reduces the inhibition to the intact side MVN neurons induced by a vestibular compensation mechanism when vestibular re-input was going on.

A caloric test is an effective clinical examination of peripheral vestibular function (Saadat et al., 1995; Allum and Ledin, 1999; Enticott et al., 2003). Observations of the DP by rotation stimulus have also been reported to be effective for investigating the progress of rehabilitation in chronic vertiginous patients (Szturm et al., 1994; Black et al., 2001; Enticott et al., 2005).

At 7 days after vestibular re-input, DP to the TTX-treated side was observed in the sinusoidal rotation, but the duration time in the caloric response on the TTX-treated side statistically decreased in comparison to that observed preoperatively. These results look conflicting.

In our model, in the process of the vestibular function recovery, it was suggested that the central nervous system affected the VOR gain strongly rather than the caloric response. In the findings of the caloric test, at 7 days after vestibular re-input, the diazepam group showed less asymmetry of the caloric response than the vehicle group, but no statistical difference was seen between the two groups. Thus, indicating that, when we try to evaluate vestibular asymmetry, the duration time in the caloric stimulus may thus be less sensitive than the VOR gain in the sinusoidal rotation stimulus.

Inhibiting the asymmetry of the VOR at an acute stage of peripheral vestibular disorder leads to a decrease in the vertiginous symptoms of the dynamic equilibrium.

Our results show direct evidence that diazepam facilitates the reacquisition of a well-balanced vestibular function in the acute stage of a peripheral vestibular disorder, thus indicating the diazepam administration commonly used for patients with acute peripheral vestibular disorders to be clinically effective.

4. Experimental procedures

4.1. *Animals*

Nineteen male Hartley guinea pigs with normal Preyer's reflexes and tympanic membranes were used in this study. The animals were divided into three groups. Seven of the nineteen animals received TTX alone (control group). Six of the nineteen animals received diazepam 2 mg/kg (0.4 ml/kg) i.p. once a day during TTX administration (diazepam group). Six of the remaining animals received vehicle (0.4 ml/kg) i.p. once a day during TTX administration (vehicle group). The experimental protocol was approved by the Committee for Ethics for Animal Experiments of the Yamaguchi University School of Medicine. All experiments were carried out under the Guidelines for Animal Experiments of the Yamaguchi University School of Medicine and the Law and Notification of the Government of Japan.

4.2. *Medicine*

Diazepam (Cercine, Takeda pharma, Osaka, Japan) was administered i.p. to all animals. The vehicle consisted in 48.5% deionized water, 40% propylene glycol, 5% sodium benzoate, 5% benzoic acid and 1.5% benzyl alcohol.

4.3. *Osmotic pump implantation*

Before osmotic pump implantation, we observed the VOR and caloric response and used animals without a right and left discrepancy of the vestibular function for this study.

Under ketamine hydrochloride (16 mg/kg, i.p.)–xylazine (16 mg/kg, i.p.) anesthesia, 1.5 ml of lidocaine HCl was injected into the right postauricular region of each guinea pig, and the mastoid bulla was opened by a postauricular incision to allow for the visualization of the round window under a surgical microscopy. A tiny hole was made adjacent to the round window with an ultrasonic mini cutter (USW-335, Honda Electronics co., Aichi, Japan). A catheter filled with TTX (0.03 μM, dissolved in water, Sigma Chemical Co., St. Louis, MO, USA) and connected to an osmotic pump (Model 2002, Alza, Palo Alto, CA, USA) was then inserted. The pump was placed under the skin on the back. After the wound was washed with saline, a small amount of piperacillin sodium was introduced. After wound closure, piperacillin sodium at a dose of 50 mg/kg was injected i.m. and oxytetracycline HCl ointment was applied to the wound. During the operation and for 3 h following the operation, each animal was kept warm using electric blanket. In our previous study, the vestibular function after the implantation of an osmotic pump and the infusion of saline was within the preoperative range (Shimogori et al., 1999; Shimogori and Yamashita, 2000a,b, 2001).

4.4. *Evaluation of the vestibular function*

At 3 days after operation, after determining the caloric response and VOR observations, the TTX administration was stopped by clamping the catheter. The caloric response and VOR were observed at 7 and 14 days after the 3-day TTX administration was finished (7 and 14 days after vestibular re-input).

VOR was observed using our method (Horiike et al., 2003). In brief, for the purpose of immobilizing the guinea pig, a cage designed to hold the animal still during experiments was mounted on top of a turntable apparatus (Daiichi Medical, Tokyo, Japan). The animal's head was fixed firmly with both

auricles held between sponge-covered plates that held both acoustic meati horizontally such that the midpoint of a straight line joining the lateral semicircular canals was located on the rotation axis of the turntable. We set up an infrared CCD camera (Nagashima Medical, Tokyo, Japan) perpendicular to the sagittal plane of the guinea pig's head and in a plane parallel to the rotational plane of the turntable apparatus. By opening an aperture on the left side of the head cage, eye movements of guinea pigs were videotaped (mini DV format, Canon, Tokyo, Japan) in the dark with the infrared CCD camera. We stored the video images on a computer (Power Mac G4, Apple Computer, CA, USA). Each image was converted to an image file using QuickTime 4.0 optional (Apple Computer). For the automatic analysis of guinea pig eye movement, we created a macro for use with the National Institutes of Health (NIH) Image analysis software (http://rsb.info.nih.gov/nih-image/). Our macro is available at http://www.cc.yamaguchi-u.ac.jp/~ent/gankyu3d/ikeda.html. After capturing the eye movement on the computer with this macro, we removed any unnecessary portions from the images and set the threshold to provide for clear outlines of the pupil. The X–Y center of the pupil was analyzed, and the horizontal and vertical components of eye movements were calculated. We calculated the slow-phase velocities, found the maximum slow-phase velocity and calculated the horizontal vestibuloocular reflex gain by dividing the maximum slow-phase velocity by the peak angular velocity.

We measured the gains with sinusoidal rotation at 0.1 Hz and a peak angular velocity of 60°/s for three rotations. We calculated the VOR gain of first, second and three rotation by dividing the maximal slow-phase velocity of each direction by the peak angular velocity (60°/s).

A caloric test was done with our method (Shimogori and Yamashita, 2004). In brief, the caloric test was performed by irrigation of the external auditory meatus with 5 ml of ice water for 10 s in the dark. Nystagmus was recorded on videotape using an infrared CCD camera, and the caloric response time was measured. We calculated the R/L ratio as the TTX-treated side response time (right) to the untreated side response time (left).

4.5. *Statistical analysis*

Data are expressed as the mean ± SD. The differences in the VOR gain and the L/R ratio and the number of postrotatory nystagmus were analyzed using the StatView version 4.5 J software package for Macintosh (Abacus Concepts, Berkley, CA, USA). The differences in these values among the groups with the same time course were assayed using the unpaired *t* test, and the differences in these values among differential time course in same group were assayed using the paired *t* test. A value of $P < 0.05$ was considered to be as statistically significant.

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