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A novel treatment for vestibular disorder with FGLM-NH_2 plus SSSR

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HIGHLIGHTS

- The FGLM-NH_2 plus SSSR mixture was administered in the vestibular disorder model.
- Spontaneous nystagmus decreased after medication administration.
- Gain ratios were also statistically higher after medication administration.
- More synaptic ribbons were stained after medication administration.

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ABSTRACT

Topical FGLM-NH_2 (Phenylalanine–Glycine–Leucine–Methionine–Amide) plus SSSR (Serine–Serine–Serine–Arginine) facilitates recovery from vestibular disorders induced by (±)-α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA) in guinea pigs and might offer a treatment strategy for patients with peripheral vestibular disorders.

The tetrapeptide FGLM-NH_2 derived from substance P (SP) can be used to treat corneal disorders when combined with SSSR, which is a tetrapeptide derived from insulin-like growth factor-1 (IGF-1). We examined the influence of FGLM-NH_2 plus SSSR when locally applied to the unilateral inner ear of guinea pigs with vestibular disorder induced by AMPA. A total of 18 Hartley white guinea pigs were assigned to groups receiving either FGLM-NH_2 plus SSSR, artificial perilymph, or no treatment at all. A hole was drilled adjacent to the round window, with AMPA then infused into the hole in order to induce the vestibular disorder. Thereafter, FGLM-NH_2 plus SSSR or artificial perilymph was delivered via an osmotic pump that was inserted into the hole. Sinusoidal rotation tests were used for observing spontaneous nystagmus and for measurements of the vestibulo-ocular reflexes (VOR). Two animals from each group were immunohistochemically examined at 24 h after the treatment. Spontaneous nystagmus decreased immediately after FGLM-NH_2 plus SSSR infusion. The recovery of the VOR gains was statistically faster than that seen in the control group at 3 and 7 days after treatment. Immunohistochemical examination revealed that many synaptic ribbons, which are markers of the synapse, were stained in the FGLM-NH_2 plus SSSR group compared with the untreated group. Topical application of FGLM-NH_2 plus SSSR accelerates functional recovery from AMPA-induced vestibular disorders by facilitating synaptic regeneration in guinea pigs.

1. Introduction

Substance P (SP) is an excitatory neurotransmitter that is extensively distributed throughout the central and peripheral nervous systems. In the 1980s and 1990s, high levels of SP in the peripheral vestibular apparatus were reported [2,4,11,14,16,17,18,22,25,27]. Although the role of SP in peripheral vestibular function has yet to be completely clarified, it has been shown that direct administration of SP into the unilateral inner ear resulted in excitation of the peripheral vestibule [15]. On the other hand, cellular insulin-like growth factor-1 (IGF-1) affects sensory hair cell protection in the inner ear. In fact, IGF-1 has been clinically combined with gelatin hydrogel on the round window membrane in order to treat acute sensorineural hearing loss [13]. The tetrapeptide FGLM-NH_2 derived from SP can cure corneal disorders when combined with SSSR (which is a tetrapeptide derived from IGF-1) [3]. A mixture of FGLM-NH_2 and SSSR has been previously clinically applied. We hypothesized that treatment with FGLM-NH_2 plus SSSR would be useful in facilitating recovery of peripheral vestibular function. It is well known that glutamate induces excitotoxicity in the neuronal

Abbreviations: SP, substance P; NK-1, neurokinin-1; FGLM-NH_2, Phenylalanine–Glycine–Leucine–Methionine–Amide; SSSR, Serine–Serine–Serine–Arginine; IGF-1, insulin-like growth factor; AMPA, (±)-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; VOR gain, vestibulo-ocular reflex gain.

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cells in brain ischemia [10]. Additionally, topical application therapy has been found to be useful for treating inner ear diseases. The benefit of using this therapy is that it makes it possible to administer adequate doses of various kinds of drugs, even those that normally cannot pass the blood–inner ear barrier, all while avoiding systemic side effects. In the current study, we used a previously developed model that induced partial vestibular dysfunction by topical application of (±)-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) [19]. Our study directly administered FGLM-NH_2 plus SSSR into the inner ears of guinea pigs with AMPA-induced vestibular disorders in order to evaluate its effect on the vestibular function. This model has been shown to be suitable for studying the pharmacological effects of candidate drugs for topical application therapy.

2. Materials and methods

The study protocol was reviewed by the Yamaguchi University School of Medicine's Committee for Ethics in Animal Experiments. A total of 18 male Hartley white guinea pigs with normal tympanic membranes and normal Preyer reflexes were randomly assigned to receive FGLM-NH_2 (2.14 mM) plus SSSR (130 nM) ($n=8$), artificial perilymph (113.5 mM NaCl, 5.4 mM KCl, 2.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 10.0 mM glucose, and 10.0 mM N-2-hydroxyethylpiperazine N′-2-ethanesulfonic acid) ($n=8$, and which served as the controls), or no treatment at all ($n=2$).

Anesthesia was induced with a combination of xylazine (16 mg/kg, i.p.) and ketamine (16 mg/kg, i.p.), with 1.5 mL of lidocaine hydrochloride injected into the right postauricular region for local anesthesia. Body temperature was maintained at 37 °C. The mastoid bulla was opened by a postauricular incision to allow visualization of the round window using a surgical microscope (Carl Zeiss, Oberkochen, Germany). A hole was drilled adjacent to the round window using a perforating burr (Proxxon, 0.5 mm diameter; Kiso Power Tools, Osaka, Japan). A polyethylene catheter (0.2 mm inner diameter, 0.5 mm outer diameter; Natsume Co., Ltd., Tokyo, Japan) containing 10 mM AMPA (Sigma–Aldrich, St. Louis, MO, USA) was connected to a syringe that was placed in a syringe pump (SP-70; Nipro Co., Osaka, Japan). After insertion of the catheter into the hole, AMPA was infused at 0.6 mL/h for 5 min. Thereafter, the polyethylene catheter containing the artificial perilymph was connected to an osmotic pump (Model 2002, Alza Corporation, Palo Alto, CA, USA) and inserted into the hole. The length of the polyethylene catheter was adjusted to deliver artificial perilymph over a period of 12 h. After the pump was positioned under the skin on the back of the animal, the wound was washed with saline. After closure, a small amount of piperacillin sodium (PIPC) (40 mg/kg) was injected intramuscularly, and oxytetracycline HCl ointment was applied to the wound. The body temperature of the animals was maintained at 37 °C throughout the procedure and for 6 h thereafter, with each animal warmed by an electric blanket (Sanyo, Osaka, Japan). Subsequently, the pump infused drugs (FGLM-NH_2 plus SSSR, or artificial perilymph) for 12 h after the initial AMPA infusion.

Spontaneous nystagmus was observed throughout the experimental procedure. To measure the vestibulo-ocular reflexes (VOR), sinusoidal rotation tests were performed before and at 3, 7, and 14 days after treatment. VOR gains were calculated using an in-house analysis system [24]. A cage designed to immobilize the guinea pigs during experiments was mounted on top of a turntable apparatus (Daiichi Medical, Tokyo, Japan). The head of each animal was firmly fixed with both auricles held between sponge-covered plates. In this position, both acoustic meati were horizontally situated so that the midpoint of a straight line joining the lateral semicircular canals was aligned with the rotation axis of the turntable. An infrared charge-coupled device CCD camera (Nagashima Medical, Tokyo, Japan) was positioned perpendicular to the sagittal plane of each guinea pig's head and parallel to the rotational plane of the turntable apparatus. By opening an aperture on the left side of the head cage, eye movements could be videotaped in the dark using the infrared CCD camera (mini DV format, Canon, Tokyo, Japan). Video images were stored on a Power Mac G5 (Apple Computer, Cupertino, CA, USA) and converted to image files using QuickTime (Apple Computer). A macro was created for use with the Image J analysis software in order to automatically analyze the guinea pig eye movements. Unnecessary portions were removed from the images of the eye movements captured by the macro, with thresholds then set to ensure clear outlines of the pupils. The X–Y center of each pupil was analyzed and horizontal and vertical components of the eye movements were calculated. Slow-phase velocities were calculated and the maximum slow-phase velocity was identified. The horizontal VOR gain was calculated by dividing the maximum slow-phase velocity by the peak angular velocity. Rotation testing was performed at 0.1 Hz, with a peak angular velocity of 60°/s. To evaluate vestibular function, the gain ratio was defined as the ratio between the VOR gain on the treated side after treatment and the VOR gain on the treated side before treatment. Differences in the gain ratios between the groups on each examination day were evaluated using the Friedman test. The significance level was set at $P<0.05$. All data are shown as means ± S.E.

Two animals from each group were deeply anesthetized with diethylether and then immediately decapitated 24 h after treatment in order to obtain samples for immunohistochemical assessment. After dissecting the temporal bone, the ampulla of the lateral semicircular canal was excised, and then all specimens were placed in 4% paraformaldehyde in 0.1 M phosphate buffer for 1 h. The specimens were decalcified in 10% ethylenediamine-tetraacetic acid for 1 h, and then rinsed in 0.01 M phosphate-buffered saline (PBS). Nonspecific binding was blocked by incubating the specimens in blocking solution (1% bovine serum albumin, 0.4% normal goat serum, 0.4% normal horse serum, 0.4% Triton X-100 in PBS) at 4 °C for 12 h. To detect synaptic ribbons (which are markers of the synapses), the specimens were then incubated in a 1:200 dilution of anti-c-terminal binding protein antibody (BD Biosciences, San Jose, CA, USA), followed by a 1:200 dilution of anti-heavy neurofilament antibody (CHEMICON, Temecula, CA, USA) at 4 °C for 12 h in order to detect the nerve fibers. After a PBS rinse, the specimens were placed in a 1:100 dilution of Alexa Fluor® 594-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR, USA) for synaptic ribbons at 4 °C for 8 h, followed by a 1:200 dilution of fluorochrome NorthernLights® 493-conjugated goat anti-chicken IgY (R&D Systems, Minneapolis, MN, USA) for Neurofilament at 4 °C for 12 h. Subsequently, specimens were rinsed in PBS, embedded in a semi-water-soluble resin (Immuno-Bed®; Polysciences, Inc., Warrington, PA, USA) and then cut into 3-μm-thick sections that were counterstained with 4′,6-diamino-2-phenylindole (DAPI) (Vectashield®; Vector Laboratories, Inc., Burlingame, CA, USA). Immunolabeled proteins were visualized under bright field illumination using a fluorescence microscope (BZ-8100, Keyence, Osaka, Japan).

3. Results

Spontaneous nystagmus decreased just after infusion (12 h after treatment) in the FGLM-NH_2 plus SSSR-treated group, with a significant difference observed from the control group at 15 h after treatment (Fig. 1).

Gain ratios were also significantly higher at 3 and 7 days after FGLM-NH_2 plus SSSR administration when compared to the control group (Fig. 2).

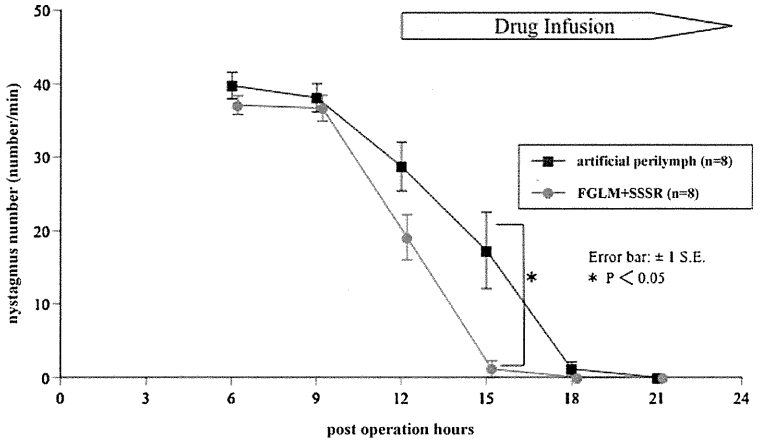

Fig. 1. Time course of the frequency of the spontaneous nystagmus. The FGLM-NH_2 plus SSSR mixture-treated group showed a tendency for a decrease in the frequency of nystagmus beginning immediately after the start of the medication. *$P < 0.05$. Error bar indicates mean ± S.E.

An immunohistochemical analysis of the ampulla of the lateral semicircular canal removed at 24 h after AMPA infusion showed there were fewer synaptic ribbons in the control group than in the untreated group (Fig. 3). On the other hand, comparatively more synaptic ribbons were stained in the group administered FGLM-NH_2 plus SSSR. We counted the numbers of synaptic ribbons in ten back-and-forth cuts from the center of the ampulla on 3-μM serial sections and found a larger number in the FGLM-NH_2 plus SSSR group as compared to the control group (Fig. 4).

4. Discussion

The striking new finding for the present study was that topical applications of FGLM-NH_2 plus SSSR facilitated vestibular functional recovery from AMPA-induced toxicity. Since it has been shown that SP is expressed in the small cells of the vestibular ganglion [7] and that these are abundantly present in the peripheral vestibular organ, particularly in the basal portions of the crista ampullaris [25], this suggests that SP is released in the synaptic cleft between the hair cells and vestibular nerve endings. It has been previously reported that the AMPA receptor is also present on the presynaptic or postsynaptic membrane of the hair cells [5,12]. In our current study, we also found that the first region that tended to be disrupted was the AMPA-applied experimental area. Our results clearly demonstrated that FGLM-NH_2 facilitates functional recovery from AMPA-induced vestibular damage. FGLM-NH_2 is the tetrapeptide derived from SP. Although the localization of neurokinin-1 (NK-1) receptor, which possesses a high affinity for SP, has yet to be determined in the vestibular apparatus, it is inferred that SP and FGLM-NH_2 act on the postsynaptic membrane of the hair cells via the putative NK-1 receptor, as main targets. In the present study, the number of synaptic ribbons at 24 h after treatment was decreased in the control group, while it was significantly recovered in the group given FGLM-NH_2 plus SSSR. This indicates that our AMPA model represents a reversible synaptic disorder and that FGLM-NH_2 plus SSSR facilitates synaptic recovery. We have previously reported that SP alone exerts a similar effect on AMPA-induced vestibulotoxicity in guinea pigs [21]. These data indicate that the main factor contributing to synaptic recovery is FGLM-NH_2, as although IGF-1 acts as a trophic factor on the inner ear hair cells, the concentration in the present study was too low to be able to exert any trophic effects [8].

Numerous reports have focused on the electrophysiological or neurotrophic effects of SP [1,6,7,9,26]. It has been shown that SP acts as an excitatory neurotransmitter or as a facilitatory neuromodulator on the vestibular ganglion cells [6,7]. Furthermore, it has been

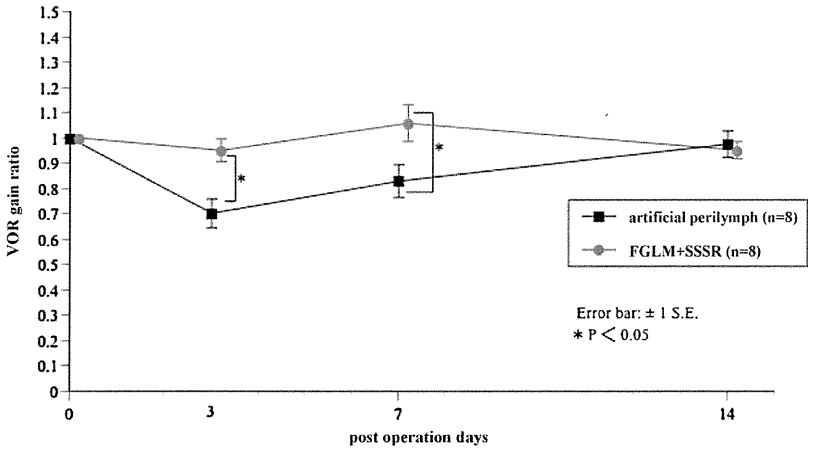

Fig. 2. Changes in the VOR gain ratio (postoperative VOR gain divided by preoperative VOR gain). In the FGLM-NH_2 plus SSSR mixture-treated group, the reduction in VOR gain ratio was significantly suppressed 3 days after the surgery. *$P < 0.05$. Error bar indicates mean ± S.E. VOR, vestibulo-ocular reflex.

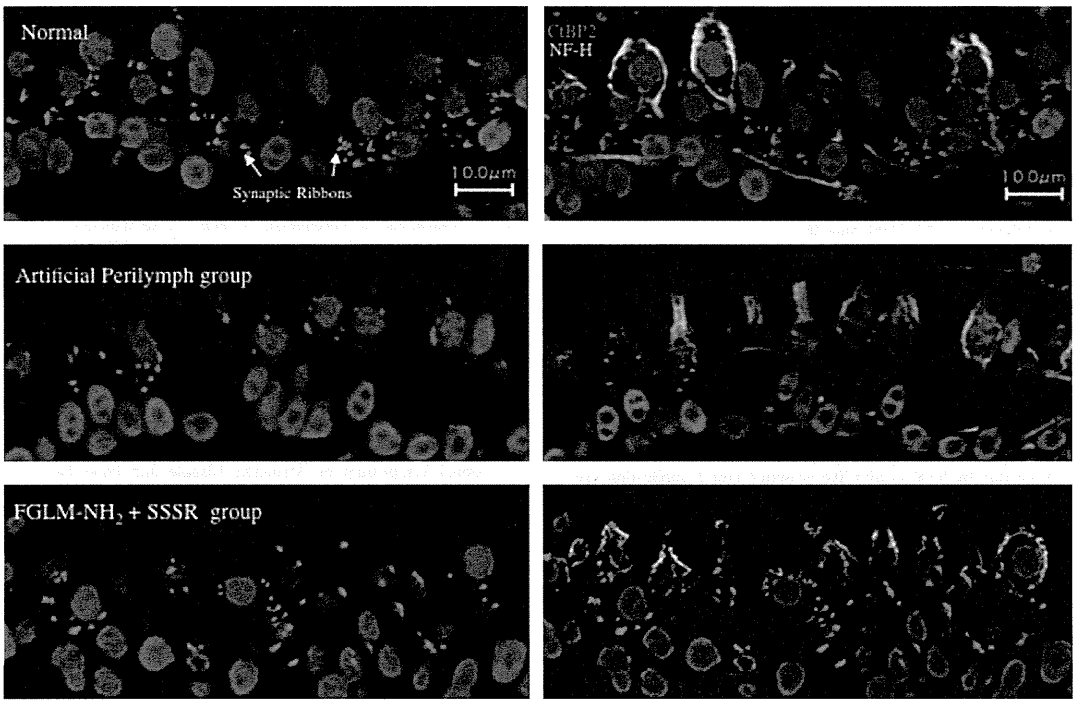

Fig. 3. An immunohistochemical analysis of the ampulla of the lateral semicircular canal 24 h after AMPA infusion. In the FGLM-NH_2 plus SSSR mixture-treated group, many synaptic ribbons were observed. CtBP2, C-terminal binding protein-2; NF-H, neurofilament heavy.

reported that intratympanic administration of gentamicin induced an increase in the SP-like immunoreactivity in the vestibular ganglion cells, which suggests the possibility that SP protein synthesis was upregulated [9]. SP may act as a neurotrophic factor in damaged vestibular ganglion cells. AMPA is known to have an excitatory neurotoxic effect on neurons. It therefore seems probable that SP and FGLM-NH_2 exert neurotrophic effects against AMPA-induced disorders.

A previous study showed that intracochlear infusion of AMPA induced partial vestibular dysfunction predominantly through the activation of the AMPA receptors [19] that were localized in the postsynaptic region of the vestibular hair cells [6]. When an excess of AMPA is applied topically, AMPA receptor activation induces excitotoxicity. When the damage is localized within the synapse, morphological and functional recovery can occur within 7 days [16]. When the damage is more severe, hair cells themselves may be involved, leading to cell death. The AMPA model presented here represents a reversible synaptic disorder, and for which the data suggest that SP facilitates synaptic recovery.

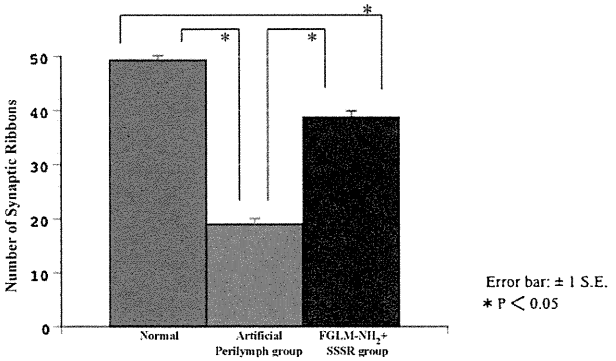

Fig. 4. Statistical analysis of the number of synaptic ribbons in each group. In the FGLM-NH_2 plus SSSR mixture-treated group, the number of synaptic ribbons was significantly higher than that in the artificial perilymph group.

In the current surgical procedures, particularly cochlear fenestration for drug delivery, it was demonstrated that the vestibular function recovered within 2 weeks in all animals, even in the artificial-perilymph-treated group, which is in line with the results of other previously reported studies [20,23]. Therefore, our current infusion method causes no critical inner ear damage (i.e., due to pressure injury), nor does it require an effluent hole.

Several AMPA concentrations were evaluated with the infusion method described here. In our current study, we used a relatively higher AMPA concentration than was used in a previous study [16]. Concentrations <10 mM caused no obvious static symptoms such as spontaneous nystagmus [19], possibly because of the features of the infusion method. Since there was not a tight fit between the catheter and the cochlear hole, there was leaking of the perilymph at the catheter insertion point after 2 min of infusion, which caused the AMPA concentration in the perilymphatic space to be <10 mM.

This study clinically applied a mixture of FGLM-NH_2 plus SSSR and established its safety in humans. Moreover, the ability to easily transfer FGLM-NH_2 plus SSSR to the inner ear appears to be due to a lower molecular weight (466.6 for FGLM-NH_2 and 435 for SSSR) as compared to that of SP (1348.7).

These findings suggest that topical FGLM-NH_2 plus SSSR administration may be useful for treating patients with peripheral vestibular disorders.

Disclosure of competing interest

None.

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