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研究課題名（和文）New strategies for cochlear afferent dendrite and synaptic regeneration.

研究課題名（英文）New strategies for cochlear afferent dendrite and synaptic regeneration.

研究代表者
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研究成果の概要（和文）：これまでの動物モデルによる研究と臨床研究によって、インスリン様成長因子-1（IGF-1）の急性難聴における聴力改善効果が示された。今回我々は、IGF-1の聴力改善効果のメカニズムを検討するために、内有毛細胞（IHC）とらせん神経節ニューロン（SGN）のシナプス再生に焦点を当てた。蝸牛から得た組織、抽出物によるex vivoモデルを用いて細胞傷害モデルを作成し、IGF-1を作用させた。結果、IGF-1は、ホスホイノチド-3-キナーゼ-Aktシグナル伝達経路を介し、IHC-SGN間シナプスの再生を促進した。

研究成果の概要（英文）：Previous studies in animals and humans have demonstrated the efficacy of insulin-like growth factor-1 (IGF-1) for hearing recovery in acute hearing loss. In the present study, we focus on regeneration of the synapse between inner hair cells (IHCs) and spiral ganglion neurons (SGNs) as a mechanism of hearing recovery following IGF-1 application. To test the capability of IGF-1 for promoting regeneration of IHC-SGN synapses, an ex vivo model of excitotoxicity was employed. In cochlear explants, IGF-1 promoted IHC-SGN regeneration after excitotoxic insult via the phosphoinositide 3-kinase-Akt signaling pathway. These results demonstrated that IGF-1 promoted regeneration of IHC-SGN synapses, which may be involved in mechanisms of hearing recovery following IGF-1 treatment.

研究分野：　耳科学

キーワード：　らせん神経　IGF-1

1. 研究開始当初の背景

Insulin-like growth factor-1 (IGF-1) is known to play crucial roles in the development and maintenance of the cochlea. Genetic deletion of IGF-1 or its receptor causes the abnormal development of cochleae, leading to SNHL. We have focused on IGF-1 as a novel therapeutic candidate for the treatment of SNHL. Studies using cochlear explant cultures of mouse pups demonstrated an involvement of both phosphoinositide 3-kinase (PI3K)-Akt and MEK-ERK signaling pathways in IGF-1-induced protection of both inner (IHCs) and outer HCs (OHCs) against aminoglycoside ototoxicity. During this process, netrin-1 and growth-associated protein 43 were identified as downstream molecules of IGF-1 signaling in cochlear explants. These findings strongly suggested the potential of IGF-1 as a protectant for HCs in clinical settings.

2. 研究の目的

In the present study, we hypothesize that IGF-1 has the potential to promote regeneration of synaptic contacts between inner IHCs and SGNs. To test this hypothesis, we evaluated the effects of IGF-1 on regeneration of IHC–SGN synapses using an ex vivo excitotoxicity model to induce degeneration of IHC-SGN synapses. The number of IHC ribbon structures was significantly preserved after IGF-1 treatment. Then two major IGF-1 pathways, PI3K-Akt and MEK-ERK, were investigated to identify role for regeneration of IHC–SGN synapses.

3. 研究の方法

(1) *Ex vivo cochlear explants culture*

Cochlear explants dissected from postnatal day 2 mice were used. To evaluate the capacity of IHC–SGN synapses for regeneration, we established a model for selective degeneration of IHC–SGN synapses. For this purpose, we optimized the concentration and exposure time of excitatory amino acids to NMDA (0.5 mM) and kainate (0.5 mM), 8-h in cochlear explant cultures. To test IGF-1 effects on IHC–SGN synapses, we excluded growth factors and serum from culture medium. Control specimens were cultured without exposure to NMDA and kainate.

To investigate the effects of IGF-1 on regeneration of IHC–SGN synapses, IGF-1 was applied to culture media for 48 h at concentrations of 0.1 or 1.0 μg/ml after 8 h of exposure to NMDA and kainate (Figure 2A). To confirm IGF-1 effects on SGN afferent dendrites and ribbon synapses, we performed pharmacological inhibition of IGF-1 receptors. An IGF-1 receptor inhibitor, picropodophylline (PPP) was added to culture medium containing 1.0 μg/ml IGF-1 at a final concentration of 1 μM.

Two major intracellular signaling pathways, PI3K-Akt and MEK-ERK, are known for IGF-1 receptor-mediated activity. To investigate intracellular signaling in IGF-1 receptor-mediated regeneration of SGN afferent dendrites and synaptic ribbons, we evaluated the effects of pharmacological inhibition of the PI3K-Akt or MEK-ERK pathway. A PI3K-Akt inhibitor, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002) was added to medium containing 1.0 μg/ml IGF-1 to a final concentration of 10 μM, and 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene (U0126) was used as a MEK-ERK inhibitor at a final concentration of 10 μM.

(2) Immunohistochemistry for IHCs, SGNs and IHC–SGN synapses

Immunostaining was performed in whole mounts. Primary antibodies used were rabbit anti-myosin VIIa to label HCs, chicken anti-neurofilament-H to label the soma and afferent dendrite of SGNs, mouse monoclonal IgG1 anti-CtBP2 to label presynaptic ribbons and mouse monoclonal IgG2a anti-PSD95 to label postsynaptic densities. All specimens were viewed with a TCS-SP2 laser-scanning confocal microscope.

(3) Western bolt analysis

To confirm the involvement of these signaling pathways in IGF-1 effects on IHC–SGN synapses, Western blotting for phosphorylated Akt (pAkt) and phosphorylated ERK (pERK) was performed. The expression levels of pAkt and pERK were evaluated in cochlear explants cultured for 30 min with or without 1.0 μg/ml IGF-1 or with 1.0 μg/ml IGF-1 supplemented with PPP, LY294002, or U0126 following exposure to NMDA and kainite. For each culture condition, eight cochlear explants were used for Western blotting, and experiments were performed six times.

4. 研究成果

(1) Ex vivo excitotoxicity model

Immediately after 8-h exposure to NMDA and kainate, immunostaining for neurofilaments or CtBP2 and PSD95 revealed significant loss of SGN afferent dendrites attaching to IHCs (Figure 1G–I) and ribbon synapses (Figure 1M–O), which were identified as puncta expressing both CtBP2 and PSD95. No significant HC loss was found in specimens exposed to NMDA and kainate in comparison with control specimens (Figure 1A–C). In addition, there was no significant loss of SGN somata between specimens exposed to NMDA and kainate and controls (Figure 1D–F). After an additional 48 h of culture, no further loss of SGN afferent dendrites attaching to IHCs was observed (Figure 2B). On the other hand, we identified some degree of spontaneous regeneration of ribbon synapses following the additional 48 h of

culture. The numbers of ribbon synapses (Figure 2F) slightly increased in comparison with those immediately after exposure to NMDA and kainate.

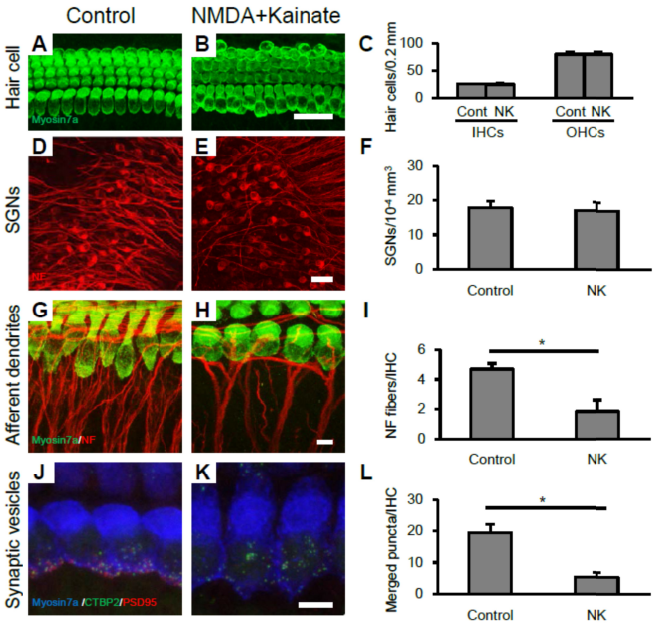

Figure 1

Immediate damage effect after 8 h exposure of 0.5 mM NMDA and Kainate. Student's t-test, *p < 0.05. n = 4-5 explants per condition. Scale bars in B and E: 25 mm apply to A-E. Scale bars in H and K: 5 mm apply to G-K.

*(2) Effects of IGF-1 on IHC–*SGN *synapses*

IGF-1 application resulted in significant effects on the numbers of both SGN afferent dendrites and synaptic ribbons. IGF-1 induced significant recovery in the numbers of SGN afferent dendrites at either 0.1 or 1.0 μg/ml (Figure 2B–E). The numbers of ribbon synapses were also significantly increased in specimens treated with IGF-1 compared with those in control specimens cultured in the absence of IGF-1 (Figure 2F–I). These findings demonstrated that IGF-1 induced reinnervation of SGN afferent dendrites and promoted recovery of synaptic ribbon numbers. In regeneration of ribbon synapses, IGF-1 exhibited additional effects to spontaneous recovery.

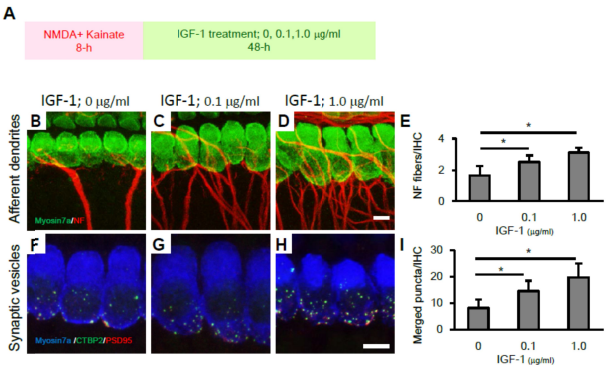

Figure 2

IGF-1 showed dose-dependent effects on an increase of numbers of afferent dendrites attaching to IHCs and synaptic contacts. ANOVAs, *p < 0.05. n = 5-6 explants per condition. Scale bars: 5 mm apply to all panels.

Following 48 h of culture, PPP significantly inhibited the effects of IGF-1 on both SGN afferent dendrites and synaptic ribbons (Figure 3). The numbers of SGN afferent dendrites and ribbon synapses in specimens cultured with IGF-1 and PPP were almost identical to those in control specimens cultured without IGF-1, indicating that PPP completely blocked IGF-1-induced regeneration in IHC–SGN synapses. These findings demonstrated that regeneration of SGN afferent dendrites and ribbon

synapses was induced by IGF-1 receptor-mediated actions. In all specimens treated with PPP, abnormal morphology of nerve fibers was observed. Nerve fibers adjacent IHCs formed circles (Figure 3). Such degenerative changes in nerve fibers may be caused by the toxicity of PPP.

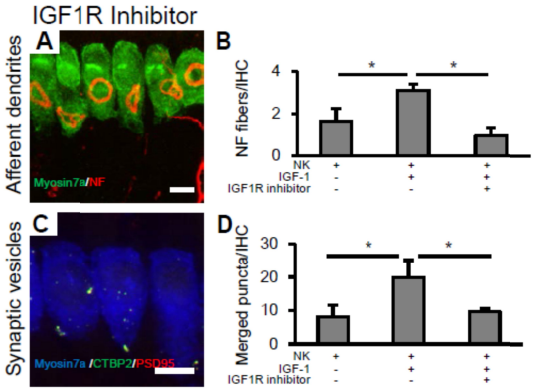

Figure 3

IGF1R inhibitor effect on SGN afferent dendrites and synapses. Student's t-test, *p < 0.05. n = 4-6 explants per condition. Scale bars: 5 mm apply to all panels.

(3) Involvement of PI3k-Akt signaling in IGF-1 effects

Pharmacological inhibition by LY294002 or U0126 showed reduction of IGF-1 effects on SGN afferent dendrites and ribbon synapses (Figure 4). The numbers of SGN afferent dendrites and ribbon synapses in specimens cultured with LY294002 were significantly lower than those in IGF-1-treated specimens. U0126 also significantly reduced the numbers of SGN afferent dendrites and ribbon synapses. These results suggested that both PI3K-Akt and MEK-ERK pathways were involved in IGF-1 effects on regeneration of IHC–SGN synapses.

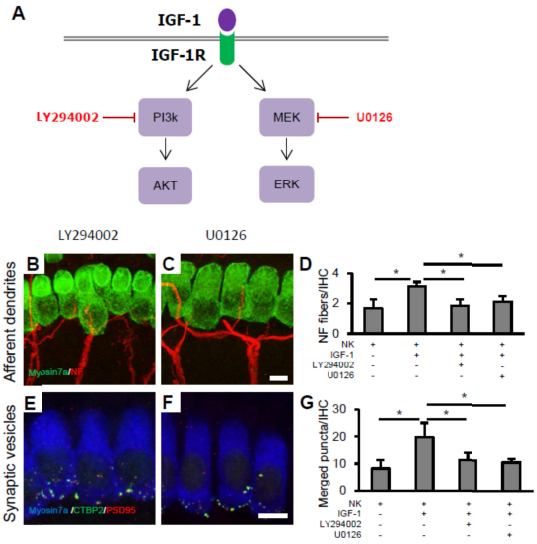

Figure 4

IGF-1 signaling pathway and two major signaling cascades inhibitors. Student's t-test, *p < 0.05. n = 5-6 explants per condition. Scale bars: 5 mm apply to all panels.

Western blotting showed upregulation of pAkt in cochlear specimens treated with IGF-1 in comparison with control specimens cultured without IGF-1 (Figure 5A, B). The expression level of pAkt was reduced by the addition of the IGF-1 receptor inhibitor PPP, although it was not statistically significant (Figure 5A, B). The PI3K-Akt inhibitor LY294002 significantly reduced the expression level of pAkt (Figure 5A, B). The MEK-ERK inhibitor U0126 induced an increase in the expression level of pAkt. These findings

supported our hypothesis that the PI3K-Akt pathway was activated by IGF-1 receptor-mediated actions. On the other hand, no apparent differences in the expression level of pERK were found between controls and IGF-1-treated samples (Figure 5A, C). The IGF-1 receptor inhibitor PPP also showed no effects on the expression level of pERK (Figure 5A, C). These findings indicated that the MEK-ERK pathway did not play a role in IGF-1 receptor-mediated actions, although application of the MEK-ERK signaling inhibitor U0126 apparently reduced pERK levels (Figure 5A, C). Thus, MEK-ERK signaling may play a certain role in regeneration or maintenance of IHC–SGN synapses besides IGF-1 receptor-mediated actions.

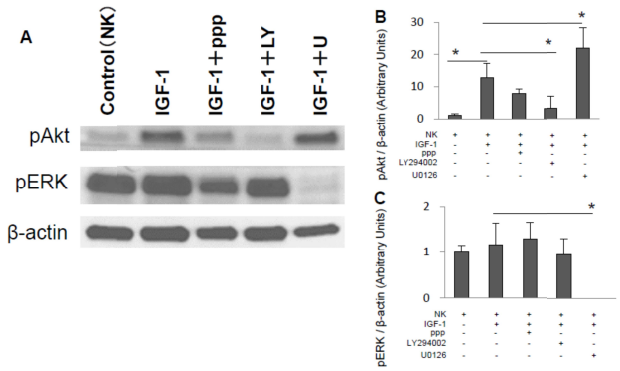

Figure 5

Western blot analysis and quantitative bolt analysis of IGF-1 signaling of IHC-SGN dendrites and synapses regeneration. ANOVA with Tukey–Kramer test, *p < 0.05.

5. **主な発表論文等**

〔学会発表〕(計 3 件)

① **浅香　ナカリン**、Effect of Netrin-1 for spiral ganglion afferent dendrite and synapse regeneration、Inner Ear Biology in Kyoto、2014 **年** 11 **月** 2-4 **日**、Kyoto Japan

② **浅香　ナカリン**、Effect of IGF-1 for spiral ganglion afferent dendrite and synapse regeneration、50th Inner Ear Biology Workshop、2013 **年** 9 **月** 10-13 **日**、Acala Spain

③ **浅香　ナカリン**、The role of IGF-1 for re-innervation and synapse formation by spiral ganglion neurons on inner hair cells after excitotoxic traumaincochlear slice cultures、9th Molecular Biology of Hearing and Deafness Conference、2013 **年** 6 **月** 22-25 **日**、Stanford USA

6. **研究組織**

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