

# Age-dependent decreases in insulin-like growth factor-I and its receptor expressions in the gerbil olfactory bulb

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**Abstract.** Insulin-like growth factor-I (IGF-I) is a multi-functional protein present in the central nervous system. A number of previous studies have revealed alterations in IGF-I and its receptor (IGF-IR) expression in various regions of the brain. However, there are few reports on age-dependent alterations in IGF-I and IGF-IR expressions in the olfactory bulb, which contains the secondary neurons of the olfactory system. The present study examined the cellular morphology in the olfactory bulb by using cresyl violet (CV) staining at postnatal month (PM) 3 in the young group, PM 6 in the adult group and PM 24 in the aged group in gerbils. In addition, detailed examinations were performed of the protein levels and immunoreactivities of IGF-I and IGF-IR in the olfactory bulb in each group. There were no significant changes in the cellular morphology between the three groups. The protein levels and immunoreactivities of the IGF-I and IGF-IR were

the highest in the young group and they decreased with age. The protein levels and immunoreactivities of the IGF-I and IGF-IR were the lowest in the aged group. In brief, our results indicate that IGF-I and IGF-IR expressions are strong in young olfactory bulbs and significantly reduced in aged olfactory bulbs. In conclusion, subsequent decreases in IGF-I and IGF-IR expression with age may be associated with olfactory decline. Further studies are required to investigate the roles of IGF-I and IGF-IR in disorders of the olfactory system.

## Introduction

It has been studied that instances of instinctive behavior and emotion are controlled by smelling in many animals (1,2). The olfactory bulb is the first relay of the olfactory system, and its synaptic, phylogenetic and cortical structures are well conserved compared with other cortical structures of the brain (3,4). The olfactory bulb is one of the special substructure as seen in the brain, because it connects to the limbic system such as the hippocampus and amygdala (5,6). Mitral cells in the olfactory bulb play a key role in the olfactory system, because they receive olfactory information from olfactory sensory neurons, and axons of the mitral cells transfer the information to the olfactory cortex (7-9).

The case of aging brings physiological changes in the central nervous system (CNS) (10,11). Studies show that alterations have been also discovered in aged olfactory bulb (12,13). In particular, it has been reported that growth factors are changed in the brain during normal aging (14,15). For example, insulin-like growth factor-I (IGF-I) is well known to be a multifunctional protein in the CNS, because in that case it acts as a controller of brain development and neural plasticity (16). In addition, IGF-I promotes neuronal survival and protection against neuronal damage induced by brain damage, including

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ischemia-reperfusion injury (17). Furthermore, we have noted that some studies have shown that exogenous IGF-I promotes neurogenesis in the case of an aged brain (18,19).

To the best of our knowledge, however, in this case we have noted that there are few reports on age-induced alterations of IGF-I and its receptor (IGF-IR) in the olfactory bulb. Therefore, in the present study, we investigated expression patterns of IGF-I and IGF-IR at postnatal month (PM) 3 as a young group, PM 6 as an adult group, and PM 24 as an aged group, in the olfactory bulb of the gerbil, which shows physiologically inherent attributes in auditory processes, reproductive and nervous systems (12,20,21).

## Materials and methods

**Experimental animals.** Gerbils were obtained from the Experimental Animal Center, Kangwon National University, Chuncheon, Republic of Korea. The gerbils were divided into 3 groups (n=14, in each group): i) Young group at PM 3 group; ii) adult group at PM 12; and iii) aged group at PM 24. The gerbils were housed in standard conditions under suitable temperature (23°C) and humidity (60%), control with a 12 h light and dark cycle per day and provided with freely accessible feed and water. Experimental protocol was approved (approval no. KW-160802-2) by Institutional Animal Care and Use Committee (IACUC) at Kangwon National University and adhered to guidelines that are in compliance with the current international laws and policies (Guide for the Care and Use of Laboratory Animals, The National Academies Press, 8th edition, 2011).

**Western blotting.** In order to examine alterations of IGF-I and IGF-IR levels in the olfactory bulb, western blot analyses were carried out as we previously described (12). In short, 21 gerbils (7 gerbils in each group) were sacrificed, and their brains were removed. Their olfactory bulbs were dissected with a surgical blade and homogenized in 50 mM PBS (pH 7.4) containing EGTA (pH 8.0), 0.2% NP-40, 10 mM EDTA (pH 8.0), 15 mM sodium pyrophosphate, 100 mM  $\beta$ -glycerophosphate, 50 mM NaF, 150 mM NaCl, 2 mM sodium orthovanadate, 1 mM PMSF and 1 mM DTT. After centrifugation, each protein level in the supernatant was determined using a Micro BCA protein assay kit with bovine serum albumin as a standard (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Aliquots containing 20  $\mu$ g of total protein were boiled in loading buffer containing 150 mM Tris (pH 6.8), 3 mM DTT, 6% SDS, 0.3% bromophenol blue and 30% glycerol. The aliquots were then loaded onto a 10% polyacrylamide gel. After electrophoresis, the gel was transferred to nitrocellulose transfer membrane (Pall Corp, East Hills, NY, USA). Rabbit anti-IGF-I (1:1,000, cat. no. sc-9013; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), rabbit anti-IGF-IR (1:1,000, cat. no. sc-712; Santa Cruz Biotechnology, Inc.) or mouse anti- $\beta$ -actin (1:5,000, cat. no. A5441; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany, ) were used as primary antibodies. The primary antibodies were incubated overnight at 4°C. Peroxidase-conjugated donkey anti-rabbit IgG (1:1,000, cat. no. sc-2305; Santa Cruz Biotechnology, Inc.) or goat anti-mouse IgG (1:1,000, cat. no. sc-2031; Santa Cruz Biotechnology, Inc.) were used as secondary antibodies (2 h

at room temperature). Antibody binding was detected with an enhanced luminol-based chemiluminescent (ECL) kit (cat. no. 32106; Pierce; Thermo Fisher Scientific, Inc.).

**Tissue preparation for histology.** For histochemical and immunohistochemical analyses, 21 gerbils (7 gerbils in each group) were anesthetized with pentobarbital sodium (30 mg/kg, i.p.; JW Pharmaceutical Co., Ltd., Seoul, Korea) and perfused transcardially with 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in 0.1 M phosphate-buffer (PB, pH 7.4). Their olfactory bulbs were removed and post-fixed in the same fixative for 6 h, infiltrated by 30% sucrose solution (in 0.1 M PB, pH 7.4) for cryoprotection, and serially sectioned into 30  $\mu$ m thickness in a cryostat (Leica Microsystems GmbH, Wetzlar, Germany).

**Cresyl violet (CV) histochemistry.** To examine change of cellular morphology in the olfactory bulb with age, CV histochemistry was carried out according to our published protocol (22). In short, the sections were mounted on gelatin-coated microscopy slides. CV acetate (Sigma-Aldrich; Merck KGaA) was dissolved at 1.0% (w/v) in distilled water, and glacial acetic acid (Sigma-Aldrich; Merck KGaA) was added to this solution. The sections were stained by CV solution and dehydrated. Finally, the stained sections were mounted Canada balsam (Kanto, Tokyo, Japan).

**Immunohistochemistry for IGF-I and IGF-IR.** To compare neuronal distribution and change in the olfactory bulb between the 3 groups, IGF-I and IGF-IR immunohistochemistry was performed according to our published method (23). Briefly, the sections were incubated with diluted rabbit anti-IGF-I (1:200, cat. no. sc-9013; Santa Cruz Biotechnology, Inc.) and rabbit anti-IGF-IR (1:200, cat. no. sc-712; Santa Cruz Biotechnology, Inc.) and exposed to biotinylated goat anti-rabbit IgG and avidin-biotin complex subsequently (1:200; Vector Laboratories, Burlingame, CA, USA). Finally, they were visualized by staining with 3,3'-diaminobenzidine (Sigma-Aldrich, Merck KGaA).

**Data analysis.** Western blot analysis was done according to our published method (12). In short, the bands were scanned, and the quantification of the western blotting was done using Scion Image software (Scion Corp., Frederick, MD, USA), which was used to count relative optical density (ROD). The ROD was presented in graphs as percentage of the young group.

To analyze immunoreactivity of IGF-I and IGF-IR, respectively, 7 sections per animal in each group were selected and analyzed according to our published method (24). In brief, digital images were taken through a light microscope (BX53, Olympus, Germany) equipped with digital camera (DP72, Olympus) connected to a PC monitor. The images were calibrated into an array of 512 x 512 pixels corresponding to a tissue area of 140 x 140  $\mu$ m (x40 primary magnification). Each immunoreactivity was measured by a 0-255 gray scale system, and the background density was subtracted. A ratio of the relative immunoreactivity (RI) for each antibody was calibrated as % using Adobe Photoshop version 8.0 and then analyzed using NIH Image 1.59 software. A ratio of the RI was calibrated as percentage of the young group.

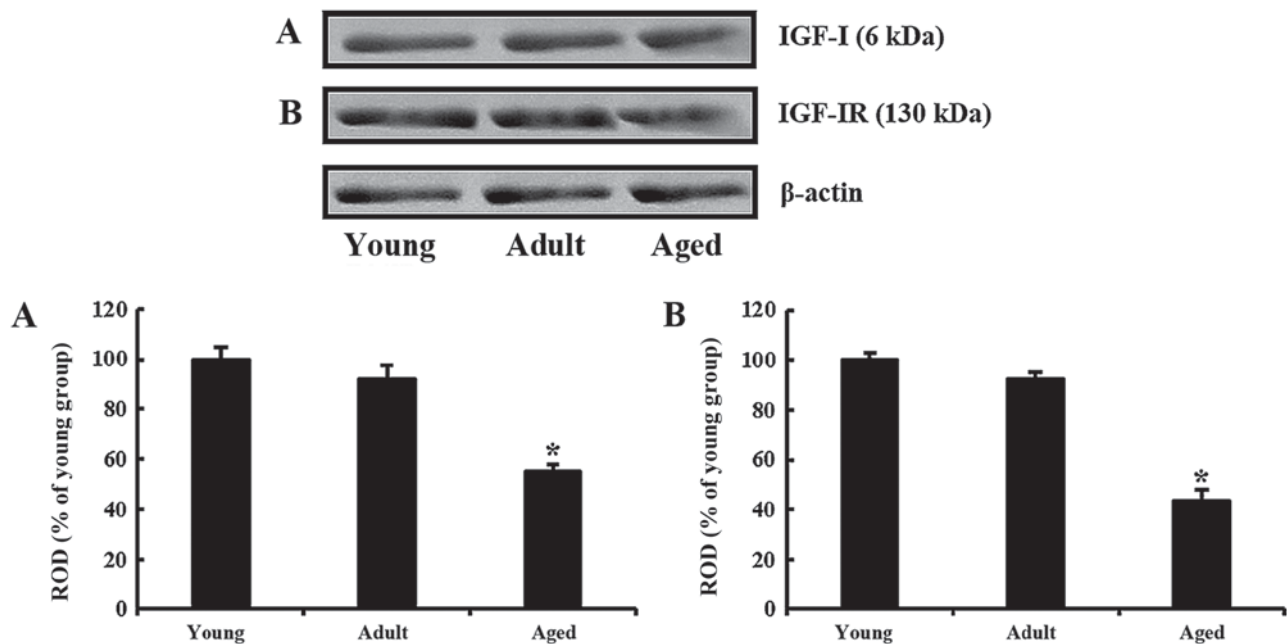


Figure 1. Western blot analysis of (A) IGF-I and (B) IGF-IR in the olfactory bulb of the young, adult and aged groups. The protein level of IGF-I decreased with age. In the aged group the IGF-I level was significantly compared with the young group. The level of IGF-IR was also significantly reduced in the aged group compared with the young group. ROD is represented as the mean percentage value of the immunoblot bands. (n=7 per group). \* $P<0.05$  vs. the young group. The bars indicate the means  $\pm$  standard error of mean. ROD, relative optical density; IGF-I, insulin-like growth factor I; IGF-IR, insulin like growth factor 1 receptor.

**Statistical analysis.** Data were expressed as the mean  $\pm$  standard error of mean (SEM). The data were evaluated by one-way analysis of variance (ANOVA) with a post hoc Bonferroni's multiple comparison test to express differences among the 3 groups. Data was analysed using SPSS software (version 12.0; SPSS, Inc., Chicago, IL, USA).  $P<0.05$  was considered to indicate a statistically significant difference.

## Results

**IGF-I and IGF-IR levels.** Age-dependent changes in IGF-I and IGF-IR levels in the olfactory bulb were identified by western blot analyses (Fig. 1). IGF-I level in the adult group was decreased to approximately 91% of the young group and very low (approximately 54% of the young group) in the aged group (Fig. 1A). Similarly, as the animals were getting older, IGF-IR levels were continuously decreased. The level in the adult was approximately 92% of the young group and approximately 43% of the young group in the aged (Fig. 1B).

**CV positive cells.** To investigate alteration of cellular morphology in the olfactory bulb, CV staining was performed in each group. Regardless of age, CV positive cells in the olfactory bulb were clearly observed. As the animals were getting older, significant change in cellular morphology was not found in the olfactory bulb (Fig. 2A-C). In addition, among the three groups, significant differences in cellular distribution were not found (Fig. 2D-F).

**IGF-I immunoreactivity.** To examine changes in IGF-I immunoreactivity in the olfactory bulb, immunohistochemistry for IGF-I was carried out in each age group (Fig. 3). In the young group, IGF-I immunoreactivity was the strongest in cells of the

mitral cell layer (MCL) (Fig. 3A). As the animals were getting older, IGF-I immunoreactivity in the MCL was continuously decreased. RI in the MCL in the adult was approximately 90% of the young group (Fig. 3B and D), and approximately 62% of the young group in the aged group (Fig. 3C and D).

**IGF-IR immunoreactivity.** IGF-IR immunoreactivity in the young group was found in all layers of the olfactory bulb, and the immunoreactivity was strong (Fig. 4A). IGF-IR immunoreactivity was decreased with age; the immunoreactivity in the adult group was approximately 71% of the young group (Fig. 4B), and approximately 38% of the young group in the aged group (Fig. 4C and D).

## Discussion

As a matter of course, aging gives rise to morphological and physiological changes in the various regions of the brain. In this study, we had focused on the characterization of the mitral cells, which display important roles in the synaptic circuit of the olfactory system (7). Axons of mitral cells transfer olfactory information to diverse subregions in the brain including the olfactory cortex (8,9). In this study, we found that cells in the olfactory bulb were not significantly changed in microscopic morphology during the incidence of normal aging. Up to date, researchers have studied age-dependent alterations in aged olfactory bulbs. For example, it is noted that a hindrance to delivering olfactory information is induced by a selective disruption of synaptic circuits (13).

It has been studying that neuroactive substances in various regions of the brain are continuously altered during normal aging. For example, dynamin 1, which is known as a regulator of presynaptic endocytosis in the hippocampus, is known to be



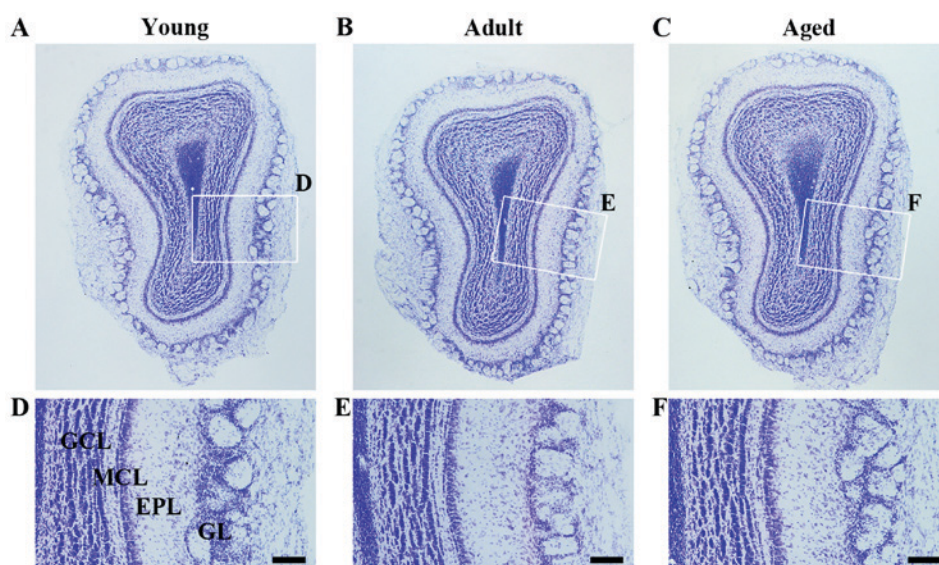


Figure 2. CV staining in the olfactory bulb of the (A and D) young, (B and E) adult and (C and F) aged groups (n=7 per group). CV positive cells were clearly observed in each layer. No notable change in their cellular morphology was observed among the groups. (A-C) Scale bar, 400  $\mu$ m. (D-F) Scale bar, 200  $\mu$ m. EPL, external plexiform layer; GCL, granule cell layer; GL, glomerular layer; MCL, mitral cell layer; CV, cresyl violet.

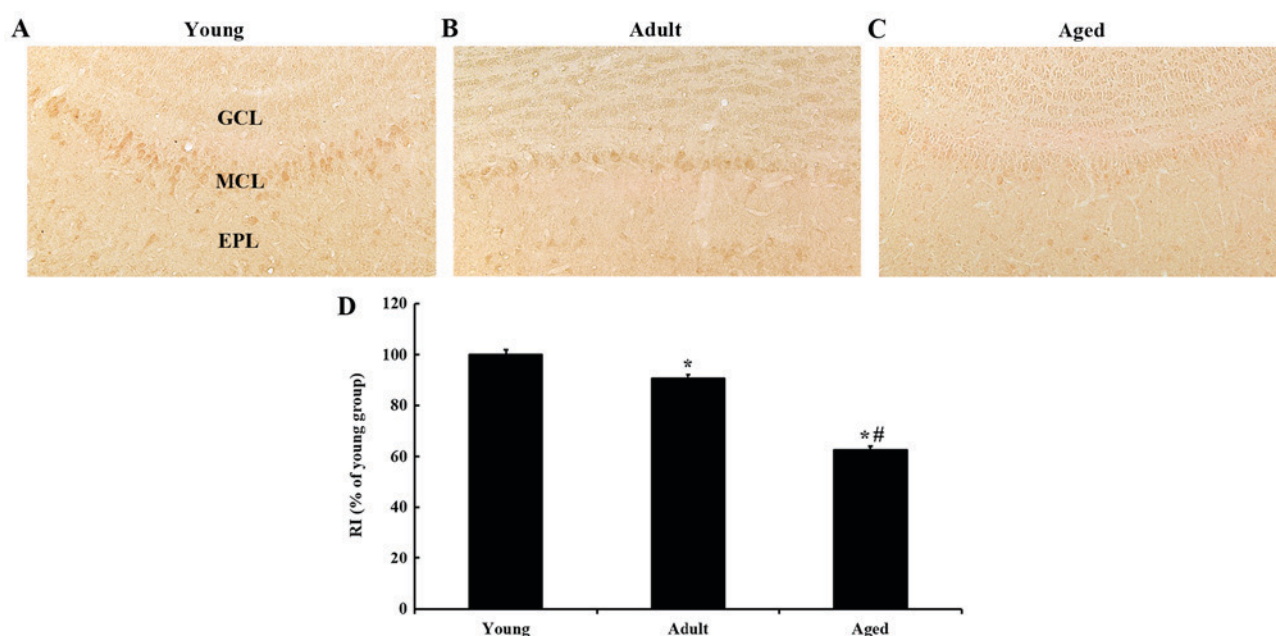


Figure 3. Immunohistochemical staining for IGF-I in the (A) young, (B) adult and (C) aged groups. In the young group clear IGF-I immunoreactivity was observed in cells of the MCL. IGF-I immunoreactivity in the MCL is slightly decreased in the MCL of the adult group and significantly reduced in the MCL of the aged group compared with the young group. Scale bar, 100  $\mu$ m. (D) RI as the percentage of IGF-I immunoreactivity in the MCL (n=7 per group). \*P<0.05 vs. the young group. #P<0.05 vs. the adult group. The bars indicate the means  $\pm$  standard error of mean. MCL, mitral cell layer; EPL, external plexiform layer; GCL, granule cell layer; RI, relative immunoreactivity; IGF-I, insulin-like growth factor 1.

decreased with age (25). In addition, calcium binding proteins are differently changed according to the proteins in the somatosensory cortex with age (26). In this case, some studies have reported that IGF-I and IGF-IR play key roles in the olfactory system. For example, IGF-I and IGF-IR are involved in the transmission of olfactory information and development of olfactory axons (27,28). In this respect, in this study, we had meticulously examined IGF-I and IGF-IR expressions in the gerbil olfactory bulb during normal aging, and we found that IGF-I and IGF-IR expressions were in deed strong in young

olfactory bulbs, and noted as significantly reduced in aged ones.

To the best of our knowledge, our literature review and studies regarding alterations of IGF-I and IGF-IR in the olfactory bulb with aging have been poorly produced. In this regard, we report that IGF-I and IGF-IR were strongly expressed in mitral cells of young gerbils, and they were significantly reduced in the mitral cells of aged gerbils. It has been reported that there are instances of IGF-I modulating synaptic formation and secretions of neurotransmitters in the brain (29,30). In the

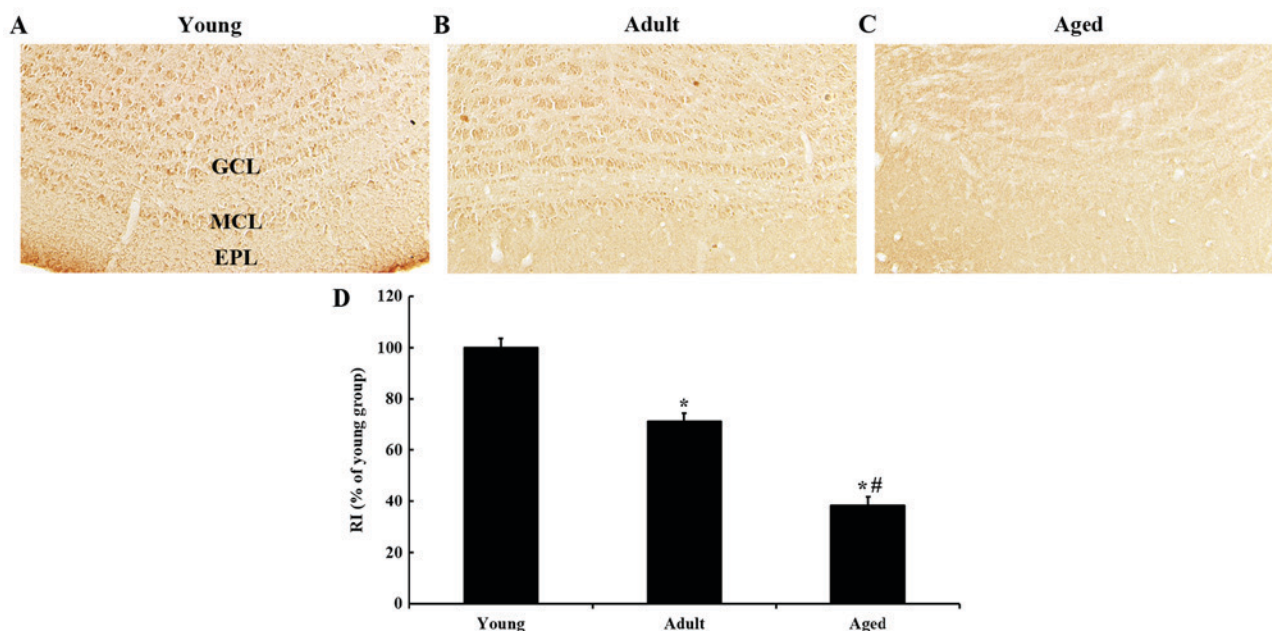


Figure 4. Immunohistochemical staining for IGF-IR in the (A) young, (B) adult and (C) aged groups. In the young group clear IGF-IR immunoreactivity was observed in all layers. IGF-IR immunoreactivity was decreased in all layers with age and significantly reduced IGF-IR immunoreactivity was observed in the aged group compared with the young group. Scale bar, 100  $\mu$ m. (D) RI as the percentage of IGF-I immunoreactivity in the MCL (n=7 per group). \*P<0.05 vs. the young group. \*\*P<0.05 vs. the adult group. The bars indicate the means  $\pm$  standard error of mean. EPL, external plexiform layer; GCL, granule cell layer; MCL, mitral cell layer; RI, relative immunoreactivity; IGF-IR, insulin-like growth factor 1 receptor.

hippocampus, IGF-I plays an important role in synaptogenesis in the dentate gyrus (31).

It is noted that some researchers have reported that an age-induced decrease of IGF-I expression in the brain is related to an identified cognitive impairment (32-35). On the other hand, there is a split in age-dependent alteration of IGF-IR expression in this case. Some papers have shown that the expression of IGF-IR is decreased or increased or sustained in aged hippocampi (35-38). We have recently reported that expressions of IGF-I and IGF-IR are decreased in aged mouse hippocampus and somatosensory cortex (33).

Although, in this study, we found that the morphology and distribution pattern of olfactory cells was not decidedly different regardless of age, IGF-I and IGF-IR were strongly expressed in mitral cells of young gerbils, and the expressions were significantly decreased in the mitral cells with time. Therefore, based on our present and precedent researches, we suggest that IGF-I and IGF-IR must be a key factor in olfactory transmission through mitral cells. In this regard, noted subsequent decreases of IGF-I and IGF-IR expressions with age may be related with olfactory decline. Further studies must be needed to study characteristic roles of IGF-I and IGF-IR in disorders of the olfactory system.

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#### Availability of data and materials

All data generated or analyzed during this study are included in this published article.

#### Authors' contributions

SYC and MHW were responsible for the experimental design, data, collection, data analysis and manuscript writing. TKL, JCL, JHA, JHP and MCS performed the experiments. BHC, JHC, HAL, JHC, IKH and IJK performed data analysis and critical comments on the whole process of this study. All authors have read and approved the final version of manuscript.

#### Ethics approval and consent to participate

The experimental protocol was approved by Institutional Animal Care and Use Committee at Kangwon National University (approval no. KW-160802-2).

#### Consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests.



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