Age-dependent decrease of Nurr1 protein expression in the gerbil hippocampus

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Abstract. Nuclear receptor related-1 protein (Nurr1) serves important roles in hippocampal-dependent cognitive process. In the present study, the protein expression of Nurr1 was compared in the hippocampi of young [postnatal month 3 (PM 3)], adult (PM 12) and aged (PM 24) gerbils using western blot analysis and immunohistochemistry. Results indicated that the protein level of Nurr1 was significantly and gradually decreased in the gerbil hippocampus with increasing age. In addition, strong Nurr1 immunoreactivity was primarily observed in pyramidal neurons and granule cells of the hippocampus in the young group, which was determined to be reduced in the adult group and to a greater extent in the aged group. Collectively the data demonstrated that Nurr1 immunoreactivity was gradually and markedly decreased during normal aging. These results indicate that gradual decrease of Nurr1 expression in the hippocampus may be associated with the normal aging process and a decline in hippocampus-dependent cognitive function.

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Introduction

As part of the normal aging process, neuroanatomical and neurophysiological changes occur in the central nervous system (CNS) (1,2), and our group previously reported age-dependent increase in antioxidant-like protein-1 expression in the gerbil hippocampus (3). In the brain, normal aging affects the functions of N-methyl-D-aspartate receptors, which may serve important roles in the initiation of long-term potentiation and be associated with age-related decline in memory (4,5). Critical for learning and memory, the hippocampus is considered to be one of the brain regions most sensitive to changes induced by the normal aging process (6,7).

Nuclear receptor related-1 protein (Nurr1), also known as nuclear receptor subfamily 4 group a member 2, is a member of the inducible nuclear receptor superfamily of transcription factors (8). Nurr1 mRNA expression has been reported in several regions of the CNS, including parts of the cortex, hippocampal formation and substantia nigra, in developing and adult mice and rats (9). It has been documented that Nurr1 is associated with differentiation, maturation, function and survival of midbrain dopaminergic neurons and that reduction of Nurr1 expression or polymorphisms in the Nurr1 gene may be associated with Parkinson's disease etiology (10-15). Nurr1 may protect against inflammation-induced dopaminergic neuronal death by inhibiting expression of pro-inflammatory mediators in microglia and astrocytes (16). Furthermore, a recent study demonstrated that Nurr1 participated in the regulation of adult hippocampal neurogenesis, and that activation of Nurr1 using amodiaquine increased hippocampal neurogenesis by stimulating neural stem cells (17). In addition, Nurr1 has been observed to be under the regulation of neural activity in cultured hippocampal neurons, in that basal expression of Nurr1 was reduced when neuronal activities were blocked (18).

A number of studies on Nurr1 have been performed in dopaminergic neurons, and significant decrease in Nurr1 expression has been reported in dopaminergic neurons of the substantia nigra in aged humans and rats (19,20). However, whether there

is age-related change of Nurr1 expression in the hippocampus, a region which has been associated with neurogenesis and neural activity (21), is yet to be fully elucidated. Therefore, the objective of the present study was to investigate age-dependent change of Nurr1 protein expression in the hippocampi of young, adult and aged gerbils, as a suitable model for research on aging (22), using western blot analysis and immunohistochemistry. Overall this aimed to provide novel insight into the association of Nurr1 with the decline of hippocampus-dependent cognitive function during the normal aging process.

Materials and methods

Experimental animals. The present study used male Mongolian gerbils (*Meriones unguiculatus*) at postnatal month 3 (PM 3; 40-50 g) as a young-group, PM 12 (65-75 g) as an adult-group and PM 24 (85-95 g) as an aged-group (total, n=42; n=14/group). This model was selected due to its suitability for research on aging (22). The gerbils were obtained from the Experimental Animal Center of Kangwon National University (Chuncheon, Republic of Korea). The animals were housed under conventional conditions at an ambient temperature (23±3˚C) and relative humidity (55±5%) under a 12-h light/dark cycle and were allowed free access to food and water. The study was conducted to minimize the number of gerbils. The procedures for animal handling and care adhered to guidelines in compliance with the current international laws and policies (Guide for the Care and Use of Laboratory Animals, The National Academies Press, 8th ed., 2011) (23). All experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee of Kangwon National University (approval no. KW-160802-2).

Western blot analysis. Changes in Nurr1 protein levels during the normal aging process were examined in the hippocampi of gerbils (n=7/group). Western blot analysis was performed according to the method described in our previous studies (24,25). In brief, following euthanasia of the animals, hippocampi were removed. The hippocampi were homogenized and centrifuged, and the supernatants were subjected to western blot analysis. The tissues were homogenized in 50 mM phosphate-buffered saline (PBS; pH 7.4) containing 0.1 mM ethylene glycol-bis(2 -aminoethylether)-N,N,N',N'-tetraacetic acid (pH 8.0), 0.2% Nonidet P-40, 10 mM ethylendiamine tetraacetic acid (pH 8.0), 15 mM sodium pyrophosphate, 100 mM β-glycerophosphate, 50 mM NaF, 150 mM NaCl, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl floride and 1 mM dithiothreitol (DTT; all from Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Following centrifugation at 16,000 x g for 20 min at 4˚C, the protein level of Nurr1 in the supernatants was determined using a micro bicinchoninic acid protein assay kit (Sigma-Aldrich; Merck KGaA), with bovine serum albumin as the standard (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Aliquots containing 20 μg 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. Following electrophoresis, the gels were transferred onto nitrocellulose transfer membranes. To reduce background staining, the membranes were incubated with 5% non-fat dry milk in PBS containing 0.1% Tween-20 for 45 min at room temperature. Following three washes with PBS with Tween-20 (PBST; each for 5 min), the membranes were incubated with rabbit anti-Nurr1 (PA5-13416; 1:500; Invitrogen; Thermo Fisher Scientific, Inc.) overnight at 4˚C. Following another three washes with PBST (each for 10 min), the membranes were incubated with peroxidase-conjugated donkey anti-rabbit immunoglobulin G (IgG; sc-2305; 1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 1 h at room temperature, followed by ECL reagents (Pierce; Thermo Fisher Scientific, Inc.). The resulting protein bands were scanned, and densitometric analysis for quantification of the bands was performed using Image J 1.59 software (National Institutes of Health, Bethesda, MD, USA), which was used to calculate relative optical density (ROD). The protein level of Nurr1 was normalized to that of β-actin (A5316; 1:5,000; Sigma-Aldrich; Merck KGaA). A ratio of the ROD was calibrated as a percentage, with the young-group designated as 100%.

Immunohistochemistry. The gerbils (n=7/group) were anesthetized with pentobarbital sodium (40 mg/kg, intraperitoneal injection; JW Pharmaceutical Corporation, Seoul, Republic of Korea) and perfused transcardially with 0.1 M PBS (pH 7.4) followed by 4% paraformaldehyde in 0.1 M PBS (pH 7.4). The brains were removed and postfixed with the same fixative for 7 h at room temperature. The brain tissue including the hippocampi was sectioned at 30-μm thickness with a cryostat.

To examine age-related changes in Nurr1 immunoreactivity in young, adult and aged hippocampi, immunohistochemical staining was performed according to the method described in our previous studies (24,25). Immunohistochemical staining for Nurr1 was performed using the rabbit anti-Nurr1 antibody (1:100) as the primary antibody overnight at 4˚C. Following three washes with PBS (each for 10 min), the brain tissues were incubated with biotinylated goat anti-rabbit IgG (BA-1000; 1:200; Vector Laboratories, Burlingame, CA, USA) for 2 h at room temperature, and then streptavidin peroxidase complex (SA-5004; 1:200; Vector Laboratories) for 45 min at room temperature. To establish the specificity of the immunostaining, a negative control test without primary antibody was performed, which resulted in the absence of immunoreactivity in all structures.

A total of six sections at 120-μm intervals per animal were selected to quantitatively analyze Nurr1 immunoreactivity. Digital images of Nurr1 immunoreactive structures of the hippocampal regions were observed and captured with an Axio Imager 2 light microscope (Carl Zeiss AG, Oberkochen, Germany) equipped with a digital camera (Axiocam; Carl Zeiss). According to the method of previous studies (24,26), semi-quantification of the immunostaining intensity of Nurr1 was evaluated and analyzed using Image J 1.59. The mean intensity of Nurr1 immunoreactivity in the immunoreactive structures was measured using a 0-255 gray scale system (white to dark signal corresponding to 255 to 0). Based on this approach, the background density was subtracted, and the level of immunoreactivity was scaled as -, ±, +, ++ or +++, representing no staining (gray scale value, ≥200), weakly positive (gray scale value, 150-199), moderate (gray scale value, 100-149), strong (gray scale value, 50-99) or very strong (gray scale value, ≤49), respectively.

Cresyl violet (CV) staining. CV staining was performed to investigate cellular distribution and morphology. In brief, according to the method of our previous study (27), the sections of the hippocampal regions were mounted on gelatin-coated microscopy slides. Cresyl violet acetate (Sigma-Aldrich; Merck KGaA) was dissolved at 1.0% (w/v) in distilled water, and glacial acetic acid (0.25% v/v) was added to this solution. The sections were stained with CV and dehydrated by immersing in serial ethanol baths. Finally, the stained sections were mounted with Canada balsam (Kanto Chemical, Co., Inc., Tokyo, Japan). A total of six sections at 120-μm intervals per animal were selected to identify the distribution of Nurr1 immunoreactivity in the hippocampus. Digital images of CV stained structures were observed and captured with the Axio Imager 2 microscope and digital camera.

Statistical analysis. Data are expressed as the mean ± standard error of the mean. Differences in the mean ROD among the groups were statistically analyzed using one-way analysis of variance followed by post hoc Bonferroni's multiple comparison tests using GraphPad InStat (version 3.05; GraphPad Software, Inc., La Jolla, CA, USA). Statistical significance was considered at $P<0.05$.

Results

Age-related changes in Nurr1 protein level. Results from western blot analysis indicated an age-related change of Nurr1 protein level in the gerbil hippocampus (Fig. 1). The protein expression of Nurr1 in the adult hippocampus was significantly decreased compared with that in the young hippocampus ($P<0.05$). In addition, the expression of Nurr1 in the aged hippocampus was significantly decreased compared with that in the adult and young hippocampi ($P<0.05$). Notably, the protein level of Nurr1 in the aged hippocampus was reduced by 73.6% compared with that in the young hippocampus.

Age-related change in Nurr1 immunoreactivity. Age-related changes in Nurr1 immunoreactivity (Table I and Fig. 2) were identified in the hippocampus proper (CA1-3 regions). In the young group, very strong Nurr1 immunoreactivity was primarily observed in pyramidal neurons of the stratum pyramidale (SP; Fig. 2A and D). In the adult group, Nurr1 immunoreactivity in pyramidal neurons was decreased compared with that in the young group (Table I and Fig. 2B and E). Furthermore, a marked reduction of Nurr1 immunoreactivity in the SP was identified in the aged group, compared with that in the adult group (Table I and Fig. 2C and F).

In the dentate gyrus (DG), Nurr1 immunoreactivity was primarily observed in granule cells of the granule cell layer (GCL; Fig. 2G, H and I). Similar to the change of Nurr1 immunoreactivity in the hippocampus proper, Nurr1 immunoreactivity in the DG gradually and markedly decreased during normal aging (Table I and Fig. 2G, H and I). Therefore, Nurr1 immunoreactivity decreased in the hippocampal regions in an age-dependent manner.

CV positive cells. In the young, adult and aged groups, CV positive cells were well distributed, mainly in the SP of the hippocampal CA1-3 regions, and the granular cell layer (GCL) and polymorphic layer (PL) of the DG in the hippocampus (Fig. 3A-I). The distribution of Nurr1 immunoreactivity was concentrated to pyramidal neurons of the SP in the hippocampus proper and to granule cells of the GCL in the DG when comparing with the results of CV staining (Fig. 3).

Table I. Mean intensity of Nurr1 immunoreactivity in principal cells of the gerbil hippocampus during normal aging.

	Postnatal month		
	Young	Adult	Aged
Pyramidal neurons of hippocampus proper	+++	++	+
Granule cells of dentate gyrus	+++	++	+

The level of Nurr1 immunoreactivity was defined by five grades: Negative (-), weakly positive (±), moderate (+), strong (++) and very strong (+++). Nurr1, nuclear receptor related-1 protein.

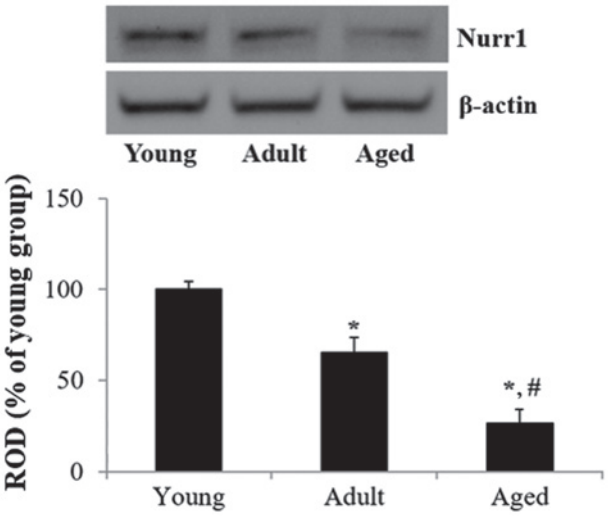

Figure 1. Western blot analysis of Nurr1 protein expression in hippocampi derived from young, adult and aged gerbils. The RODs of immunoblot bands are shown as percentage values. Data are presented as the means ± standard error of the mean. *P<0.05 vs. young group; #P<0.05 vs. adult group. Nurr1, nuclear receptor related-1 protein; ROD, relative optical density.

Discussion

In the present study, Nurr1 immunoreactivity was primarily observed in pyramidal neurons and granule cells, which are well established as principal neurons of the hippocampus (28), in young, adult and aged gerbil hippocampi. The results of Nurr1 immunoreactivity in the hippocampi were generally consistent with those of a previous study in C57BL/6 mice, which demonstrated that clear and specific Nurr1 immunoreactivity occurred in the hippocampus (29).

Chu *et al* (19) reported that Nurr1 protein was significantly reduced in the substantia nigra of elderly human subjects. Furthermore, they identified a significant age-dependent decline in the number of Nurr1-immunoreactive neurons

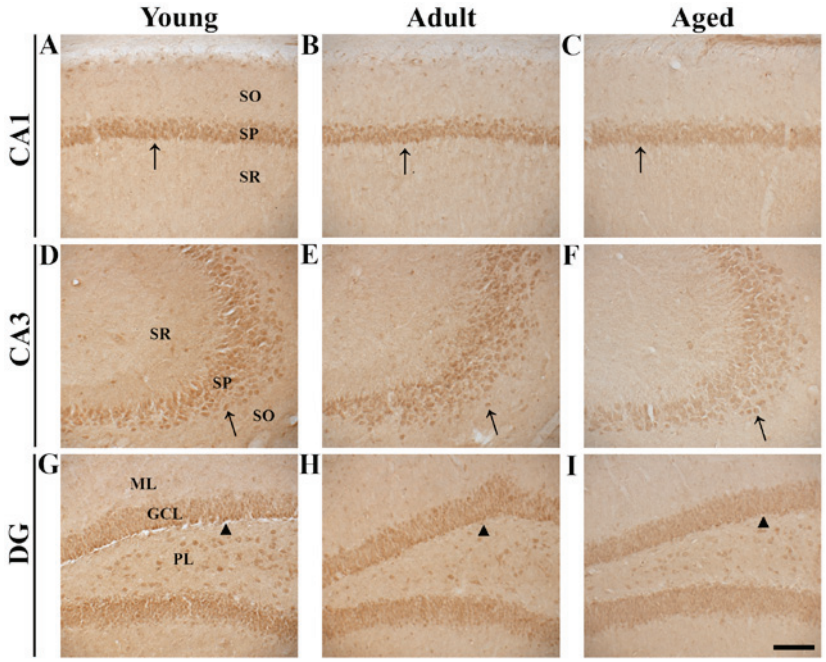

Figure 2. Nurr1 immunohistochemistry in hippocampal regions of young, adult and aged gerbils. Nurr1 expression was detected in the (A-C) CA1 and (D-F) CA3 regions and (G-I) DG. In the young group, strong Nurr1 immunoreactivity was detected in pyramidal neurons of the SP in the CA1 and 3 regions (arrows) and in granule cells of the GCL in the DG (arrowheads). Nurr1 immunoreactivity in the SP and GCL gradually decreased in the adult and aged groups. Magnification, x20; scale bar, 100 μm. Nurr1, nuclear receptor related-1 protein; DG, dentate gyrus; SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum; ML, molecular layer; GCL, granule cell layer; PL, polymorphic layer.

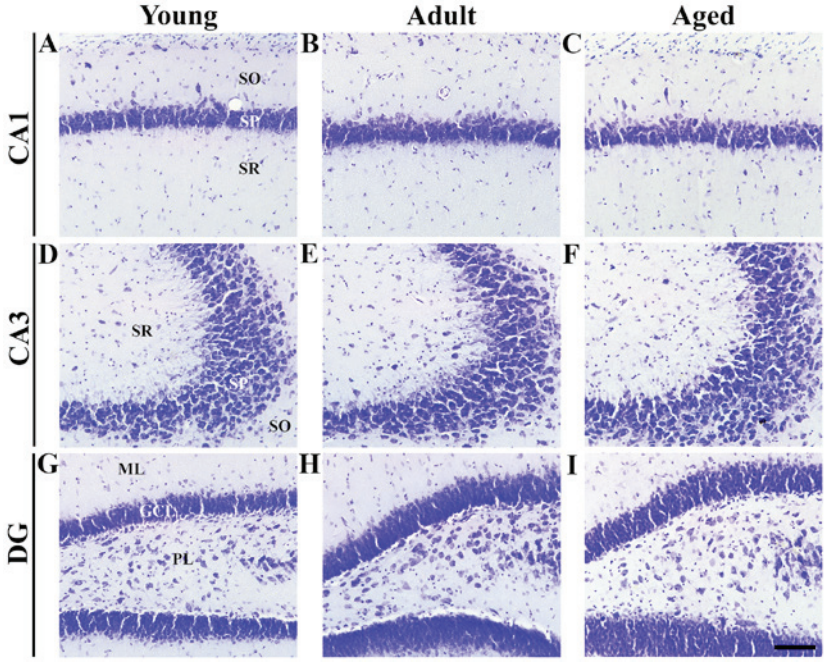

Figure 3. CV staining in hippocampal regions of young, adult and aged gerbils. Neuronal morphology and distribution was observed by CV staining in the (A-C) CA1 and (D-F) CA3 regions and (G-I) DG. In all groups, pyramidal neurons were identified in the SP in the CA1 and 3 regions, and granule cells were identified in the GCL in the DG. Magnification, x20; scale bar, 100 μm. CV, cresyl violet; DG, dentate gyrus; SP, stratum pyramidale; SO, stratum oriens; SR, stratum radiatum; ML, molecular layer; GCL, granule cell layer; PL, polymorphic layer.

in the substantia nigra of middle-aged and aged individuals compared with in young subjects (19). In aged rats, Nurr1 gene expression has been reported to be significantly decreased by 33% in dopaminergic neurons of the substantia nigra (20); as such, it was suggested that age-dependent decrease of Nurr1 in dopaminergic neurons may be associated with impairment of nigrostriatal signaling and compromised motor function with age (20). In addition, it has been reported that heterozygous Nurr1 knockout mice exhibit accelerated age-dependent reduction in the number of dopaminergic neurons and impaired dopamine signaling compared with wild-type littermate controls (30).

In the current study, the protein level of Nurr1 in the hippocampus was significantly decreased during the normal aging process. In addition, Nurr1 immunoreactivity, predominantly identified in the principal neurons (pyramidal and granule cells) of the hippocampus, was also decreased during the normal aging process. To the best of our knowledge, this is the first study to demonstrate age-dependent decrease of Nurr1 protein expression in the hippocampus. However, it is difficult to conclude the implications of this marked reduction in hippocampal Nurr1 due to aging. Nurr1 has been considered to be among the key target genes controlled by acetylation during long-term memory formation (31), and has also been implicated to serve critical roles in the formation of long-term memory, as Nurr1 expression is increased during memory acquisition and consolidation (31-34). In addition, knockdown of Nurr1 or blocking Nurr1 activity in the hippocampus may lead to impairment of long-lasting cognitive function (35). Principal neurons are serially or multi-directionally connected within trisynaptic hippocampal circuits for information processing (36). Pyramidal cells are involved in encoding spatial, contextual and emotional information to form cognitive memory (37), and granule cells form major structures involved in pattern separation (the ability to discriminate among similar events) (38). Therefore, it has been suggested that Nurr1, expressed in principal cells, serves critical roles in hippocampal-dependent cognitive processes (35). In addition, it has been identified that cognitive impairment begins around PM 12 and major cognitive decline occurs around PM 24 in mice (39-41). This is somewhat consistent with the present results demonstrating that Nurr1 protein level and immunoreactivity in the hippocampus gradually decreased with age. Therefore, it may be postulated that a decrease of Nurr1 protein expression in the hippocampus with increasing age may be associated with age-dependent cognitive impairment. However, limitations of the study include the lack of data on age-related changes in Nurr1 mRNA expression, which should be obtained in future studies.

In conclusion, the present results indicate that Nurr1 protein expression age-dependently decreases in the hippocampus. Overall, the findings suggest that age-dependent decrease in Nurr1 expression may be associated with a decline of cognitive function in the hippocampus.

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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

JHA, JSL, JHC, JHP, TKL, MS, HK and SHK analyzed and interpreted all data, and CHL and MHW made substantial contributions to conception and design of this study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee of Kangwon National University, Chuncheon, Republic of Korea (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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